

Antiangiogenic Agents in Cancer Therapy

SECOND EDITION

Edited by

Beverly A. Teicher
Lee M. Ellis



Humana Press

ANTIANGIOGENIC AGENTS IN CANCER THERAPY

CANCER DRUG DISCOVERY AND DEVELOPMENT

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PREFACE

Angiogenesis as a therapeutic target for malignant disease has evolved from a pioneering idea outside of the mainstream of therapeutic development to an FDA-approved therapy widely used in patients with metastatic disease. The success in achieving such rapid progress in realizing the importance of angiogenesis in tumor growth its value as a therapeutic target, as well as reflects the impact of vocal pioneers in the field and the dedication, creativity, and insight of scientific investigators in oncology over the past 35 years.

This second edition of *Antiangiogenic Agents in Cancer Therapy* is intended to give a current perspective on the state of the art of angiogenesis and therapy directed at this process. Part I reflects the enormous progress in understanding the cell types, the growth factors, the environmental influences, and the genetic and physiologic abnormalities that mediate angiogenesis and its role in progression of malignant disease. Part II is a tribute to the intellect and creativity of those who developed working models of tumor angiogenesis. These scientists have developed *in vivo* systems and mechanical and computational tools to examine the structure and function of vessels in malignant tissues and their response to therapeutics in the preclinical setting. Part III is devoted to the role of angiogenesis inhibition in the therapy of malignant disease in humans. Clinical trial design for elucidating the activity of treatment agents and the vasculature and methods for imaging these effects are addressed. Selected malignant diseases are treated in each of several chapters with overviews of angiogenesis in those diseases and the impact of antiangiogenic agents in treatment and on therapeutic outcomes. In addition, clinical investigators provide a background on current directions of the use of these agents in clinical practice and ongoing trials. Antiangiogenesis remains a dynamic and evolving field in oncology. New therapeutic targets continue to emerge followed by the rapid development of new therapeutic agents to be investigated in clinical trials. Optimizing the therapeutic potential of antiangiogenic agents in combination with the other therapies in the armamentarium to fight cancer will be an ongoing challenge. *Antiangiogenic Agents in Cancer Therapy, Second Edition* represents a compendium of scientific findings and approaches to the study of angiogenesis in cancer that will be useful for many years.

Beverly A. Teicher, PhD
Lee M. Ellis, MD

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- Color Plate 26 *Fig. 5, Chapter 25:* Improvement in progression-free survival for sunitinib compared with placebo in patients with previously untreated renal cell carcinoma. Reproduced with permission (30). (See discussion on p. 434.)

1

Vascular Endothelial Growth Factor Family and Its Receptors

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SUMMARY

The vascular endothelial growth factors (VEGFs) are key regulators of blood and lymphatic vessel development during embryogenesis and in promoting new vascular growth during physiological and pathological processes in the adult. The VEGF family of ligands in mammals includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PIGF). These ligands bind to and activate three receptor tyrosine kinases, designated VEGFR-1, VEGFR-2, and VEGFR-3. VEGF ligands bind to these receptors with overlapping ligand-receptor specificities, and activation may be further modulated through interaction with coreceptors such as the neuropilins (NRP-1 and NRP-2), integrins, or Vascular endothelial-cadherin (VE-cadherin). Ligand activation of VEGFRs triggers a network of distinct downstream-signaling pathways in a cell-type-specific manner that promotes vascular permeability, endothelial cell growth, migration, and survival. VEGF is an important survival factor for hematopoietic stem cells (HSCs) and stimulates the mobilization of endothelial progenitor cells (EPC) from the bone marrow to distant sites of neovascularization. A large body of experimental evidence has established VEGF as an essential molecule in promoting angiogenesis during tumor growth. These findings have led to the development of therapeutic agents that selectively target various VEGF ligands and their receptors. This chapter reviews the biology of VEGF and its receptors, emphasizing their important role for cancerous growth.

Key Words: Angiogenesis; cancer; growth factor; ligand; neuropilin; receptor; VEGF.

1. INTRODUCTION

Tumor growth and metastasis are dependent on the formation of new blood vessels from preexisting vasculature (angiogenesis) (1,2). Angiogenesis supports tumor growth by providing a source of oxygen, nutrients, growth factors, proteolytic enzymes, and coagulation and fibrinolytic factors. Tumor angiogenesis is a complex process that is regulated by several proangiogenic and antiangiogenic molecules that maintain normal homeostasis and initiate the angiogenic process during pathological conditions (3).

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One of the major pathways involved in the process of tumor angiogenesis and lymphangiogenesis is the vascular endothelial growth factor (VEGF) family of ligands and receptors (4, 5). Overexpression of VEGF has been associated with tumor progression and poor prognosis in several human malignancies including carcinomas of the breast, colon, kidney, liver, lung, pancreas and prostate, and stomach (reviewed in refs 6, 7). During cancerous growth, activation of the VEGF/VEGFR axis triggers multiple signaling networks that result in increased vascular permeability, endothelial cell mitogenesis, migration, survival, and mobilization of various progenitor cell populations from the bone marrow to sites of tumor growth and metastasis (5, 6, 8). A large body of experimental evidence has subsequently shown that interfering with VEGF or VEGFR function can potently inhibit tumor growth and angiogenesis (6, 9, 10). Owing to its central role in tumor angiogenesis, the VEGF/VEGFR pathway continues to be a major focus of cancer research and in the development of new therapies for this disease.

2. VEGF FAMILY OF LIGANDS AND RECEPTORS

In mammals, the VEGF gene family of angiogenic and lymphangiogenic growth factors consists of five glycoproteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PIGF) (5, 11, 12). A homolog of VEGF, referred to as VEGF-E, has been identified in the genome of the parapoxvirus Orf virus and shown to have VEGF-like activities (13). Recently, another VEGF homolog, referred to as VEGF-F, was identified from snake venom (14). The VEGF ligands bind to and activate three structurally similar type III receptor tyrosine kinases, designated VEGFR-1, VEGFR-2, and VEGFR-3 (Fig. 1). The assortment of VEGF ligands has distinctive-binding specificities for each of these receptors, which contribute to their diversity of function. VEGF-A binds to both VEGFR-1 and VEGFR-2 (15). VEGF-B and PIGF bind exclusively to VEGFR-1 (16, 17). Heterodimers of VEGF-A and PIGF have been identified, which can bind to and activate VEGFR-2 (18, 19). The VEGFR-3 is a specific receptor for VEGF-C and VEGF-D (20, 21). VEGF-C and VEGF-D can be proteolytically processed that allow binding to VEGFR-2 as well. VEGF-E binds specifically to VEGFR-2, whereas VEGF-F can bind both VEGFR-1 and VEGFR-2. The neuropilins NRP-1 and NRP-2 (22) can also act as coreceptors for certain VEGF–VEGFR complexes and along with other molecules such as integrins (23) and Vascular endothelial-cadherin (VE-cadherin) (24), can modulate VEGF–VEGFR activation and signaling.

Gene targeting studies have shown that VEGFs and VEGFRs are essential during vasculogenesis during development (25–28). In the adult, VEGFs play a role in physiological processes such as wound healing, endochondral bone formation, and follicular growth and development of the corpus luteum during menstrual cycling. VEGF ligands and their receptors also have important roles in pathological conditions such as age-related macular degeneration (AMD), various inflammatory diseases, polycystic ovary syndrome, endometriosis, rheumatoid arthritis, and psoriasis. For a review of the VEGF biology in normal and pathological angiogenesis, see ref. (29).

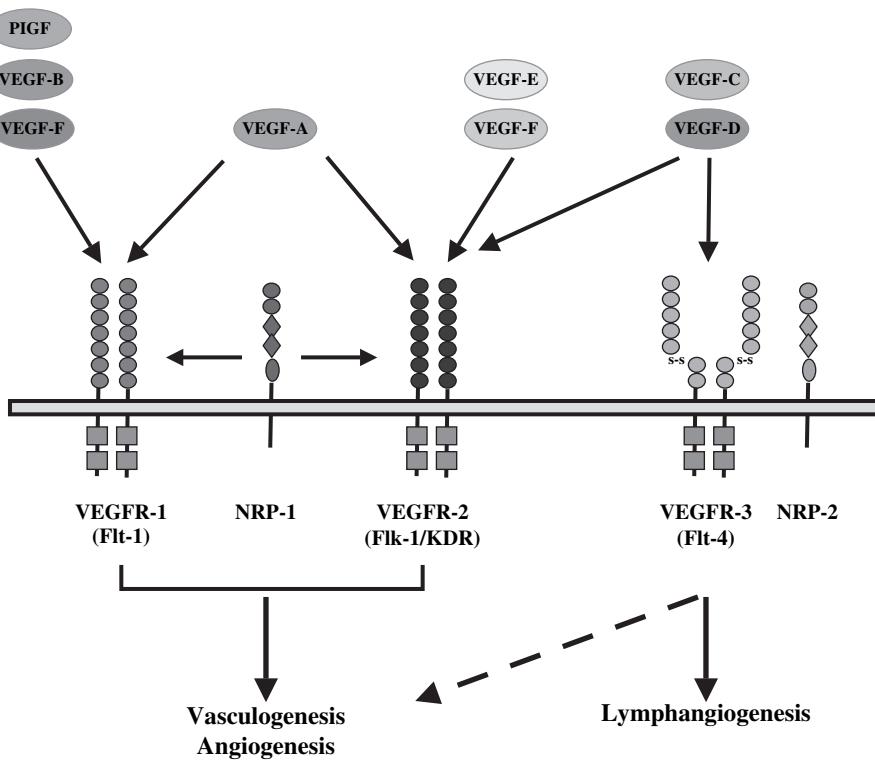


Fig. 1. Binding specificity of VEGF ligands and their receptors. The VEGF family consists of seven ligands: VEGF-A, -B, -C, -D, -E, and PIGF. VEGF ligands have specific binding affinities to VEGFR-1, -2 and -3 as shown. NRP-1 and -2 are co-receptors for specific isoforms of VEGF family members as shown and increase binding affinity of these ligands to their respective receptors. (Please see color insert.)

2.1. VEGF-A

The VEGF-A gene is located on chromosome-6 and is encoded by eight exons (30). The VEGF-A gene undergoes alternative splicing to yield mature isoforms of 121, 145, 165, 183, 189, and 206 amino acids (11, 12, 31). The VEGF₁₂₁ isoform is a secreted diffusible ligand. VEGF₁₆₅ is the predominant isoform and exists in both a soluble and an extracellular matrix (ECM)-bound form (32, 33). VEGF₁₆₅ (and VEGF₁₈₉ and VEGF₂₀₆) can be released from the ECM as a diffusible form by plasmin cleavage generating a bioactive fragment. Alternatively, VEGF can be released from the ECM by matrix metalloproteinase 9 (MMP9) to liberate soluble ligand and initiate angiogenesis (34). VEGF-A is essential for vasculogenesis during development. Homozygous or heterozygous deletion of the VEGF gene in mice is embryonically lethal resulting in defects in vasculogenesis and cardiovascular abnormalities (25, 35). The expression patterns of VEGF-A isoforms are tissue specific, implying that these isoforms have defined functions during vasculogenesis and angiogenesis (36).

VEGF-A (primarily VEGF₁₆₅) is commonly overexpressed in several human solid tumors and hematologic malignancies (4, 6, 7, 9, 32, 37). VEGF-A expression is upregulated in tumor cells, surrounding stromal cells including endothelial cells, smooth muscle cells, and fibroblasts, and also expressed by various infiltrating bone

marrow-derived cell populations. Selective gene targeting studies in mice have shown that VEGF-A is essential for efficient tumor angiogenesis (38). The important role of VEGF-A in tumor angiogenesis has been further established in studies showing that various anti-VEGF inhibitors can potently inhibit angiogenesis and tumor growth in preclinical models (6, 9). One of the first studies used a neutralizing murine anti-VEGF monoclonal antibody that inhibited angiogenesis and growth of human tumor xenografts (39). A number of subsequent studies using neutralizing antibodies to VEGF, soluble VEGF receptors/receptor hybrids, or VEGF antisense approaches have shown similar results (6, 40–42).

2.2. *VEGF-B and PlGF*

The VEGF-B gene is located on chromosome-11 and contains eight exons (43). Two isoforms of VEGF-B have been identified, referred to as VEGF-B₁₆₇ and VEGF-B₁₈₆. VEGF-B₁₆₇ binds heparin sulfate and is sequestered in the ECM, whereas VEGF-B₁₈₆ does not bind heparin and is found as a soluble, diffusible molecule. VEGF-B binds specifically to VEGFR-1 and the coreceptor NRP-1. The role of VEGF-B during development and in postnatal angiogenesis is not completely understood. VEGF-B-deficient mice are healthy and fertile but develop hearts with reduced size (44, 45). VEGF-B-deficient mice also display vascular dysfunction after coronary occlusion and impaired recovery from experimentally induced myocardial or cerebral ischemia. Recent experimental evidence advocating a role for VEGFR-1 in pathological angiogenesis, including cancer (described later), raises the possibility that VEGF-B may be important in certain diseases requiring angiogenesis. However, there is currently no evidence to support this role for VEGF-B.

The PlGF gene has been mapped to chromosome-14 and is encoded by seven exons (30). Four isoforms of PlGF have been identified—PlGF-1, PlGF-2, PlGF-3, and PlGF-4. PlGF-1 and PlGF-3 are non-heparin binding, whereas PlGF-2 and PlGF-4 contain heparin-binding regions (46). All PlGF isoforms bind exclusively to VEGFR-1. PlGF expression was first identified in the placenta, but it is also known to be expressed in the heart and lungs (47). The precise role of PlGF in angiogenesis is unclear at present. PlGF also appears to play a prominent role in the process of arteriogenesis (48). Studies have shown that PlGF can indirectly promote endothelial cell survival and angiogenesis through upregulation of VEGF-A (49). PlGF null mice are viable, but its loss results in impaired angiogenesis and tumor growth, collateral growth during ischemia, inflammation, and wound healing suggesting a role for PlGF in pathological states in the adult (51). Overexpression of PlGF in various tissues, or by tumor cells, results in stimulation of angiogenesis that can be blocked by VEGFR-1 inhibition (51).

2.3. *VEGF-C and VEGF-D*

The VEGF-C and VEGF-D genes are located on chromosomes 4 and X, respectively (43, 52). The VEGF-C and VEGF-D gene products are produced as precursor molecules that are proteolytically processed at the cell surface (53). The VEGF homologs, VEGF-C and VEGF-D, play key roles during embryonic and postnatal lymphangiogenesis (54). Homozygous deletion of the VEGF-C gene in mice is embryonically lethal, and heterozygous deletion results in postnatal defects associated with

defective lymphatic development (55). Interestingly, VEGF-D null mice lack profound lymphatic vessel defects (56), suggesting that this ligand does not play an essential role during development or that a compensatory mechanism for lymphatic development exists. Transgene expression of VEGF-C or VEGF-D induces lymphangiogenesis in mouse models (57, 58).

VEGF-C and VEGF-D are proposed to play a role in tumor growth by inducing the formation of lymphatic vessels, which in turn is hypothesized to promote lymph node metastasis (59–61). VEGF-C and VEGF-D do not appear to influence the growth of primary tumors although their role in primary tumor growth and angiogenesis require further study. Several correlative studies have shown an association between tumor expression of VEGF-C or VEGF-D and lymph node metastasis in human malignancies (62). As VEGF-C and VEGF-D can signal through VEGFR-2, these ligands may also play a role in new blood vessel growth during tumor growth. Specific blockade of VEGF-C-induced tumor lymphangiogenesis and metastasis was achieved in preclinical models using soluble VEGFR-3 inhibitors (63–65). In addition, inhibition of tumor cell VEGF-C expression by a VEGF-C RNAi approach suppressed lymphangiogenesis and metastasis in a murine breast cancer model (66). A blocking antibody to VEGF-D inhibited tumor lymphangiogenesis and lymphatic metastasis of VEGF-D-dependent mouse tumors (60).

2.4. VEGF-E and VEGF-F

VEGF-E is a viral protein encoded by the parapoxvirus Orf virus that infects sheep and goats (13). The VEGF-E gene product shares approximately 22% sequence identity to VEGF-A and does not contain a heparin-binding domain. VEGF-E preferentially binds to VEGFR-2 and NRP-1 and potently stimulates endothelial cell proliferation and vascular permeability. Another VEGF-like molecule, referred to as VEGF-F, was recently identified in the venom of the viper snake (14). VEGF-F consists of two VEGF-like proteins designated vammin and VR-1. These two proteins share 50% sequence homology to VEGF-A and, like VEGF-E, bind selectively to VEGFR-2. However, distinct from VEGF-E, the VEGF-F molecule contains a heparin-binding region.

2.5. The VEGF Receptors

VEGF ligands mediate their biological effects through selective binding and activation of three different type III receptor tyrosine kinases—VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-1 (also referred to as fms-like tyrosine kinase 1, Flt-1) (67) and VEGFR-2 (also referred to as kinase-insert-domain-containing receptor, KDR (68), and the murine homologue, fetal liver kinase-1, Flk-1) (69) were originally identified on endothelial cells. VEGFR-1 and VEGFR-2 are also expressed on various hematopoietic cell lineages in the adult. These two receptors share 44% homology and possess a characteristic structure consisting of seven extracellular immunoglobulin (Ig)-like domains, a single transmembrane domain, and a consensus tyrosine kinase domain interrupted by a kinase insert domain. VEGFR-3 (also referred to as fms-like tyrosine kinase 4, Flt4) (70) was cloned from human leukemia cells and has been found to be primarily associated with lymphangiogenesis (71, 72). VEGFR-3 is distinct from VEGFR-1 and VEGFR-2 in that it is proteolytically processed at the fifth Ig domain yielding two subunits that are held together by a disulfide bond. Activation of the

VEGFRs triggers a network of distinct downstream-signaling pathways involved in proliferation, migration, and survival. For recent reviews on VEGFR signaling, see refs 73, 74.

2.5.1. VEGFR-1

VEGFR-1 is a receptor for all VEGF-A isoforms and a specific receptor for VEGF-B and PIGF. VEGFR-1 is expressed on endothelial, hematopoietic, and smooth muscle cells. VEGFR-1 is critical for developmental vasculogenesis. VEGFR-1 null mice die in utero between 8.5 and 9.5 because of excessive hemangioblast proliferation and poor organization of vascular structures (26). Despite its important role in development, the precise function of VEGFR-1 in the process of angiogenesis, as well as other processes such as hematopoiesis, is still under investigation. VEGFR-1 was initially thought to be a negative regulator of VEGF activity either by acting as a decoy receptor for VEGF or by downregulating VEGFR-2-mediated signaling (75, 76). VEGF-mediated stimulation of VEGFR-1 autophosphorylation and signaling in endothelial cells is weak when compared to signaling through VEGFR-2 (77). A repressor motif has been identified in the juxtamembrane region of VEGFR-1 that impairs PI-3-kinase signaling and endothelial cell migration in response to VEGF stimulation (78, 79). However, other studies have indicated that VEGFR-1 has a positive, functional role in certain cell types—participating in monocyte migration (80, 81), recruitment of endothelial cell progenitors (82), increasing the adhesive properties of natural killer cells (83), and inducing growth factors from liver sinusoidal endothelial cells (84).

Activation of VEGFR-1 by PIGF results in transphosphorylation of VEGFR-2 in endothelial cells coexpressing these receptors (85). Furthermore, VEGF/PIGF heterodimers were capable of activating intramolecular VEGFR cross-talk through formation of VEGFR-1/VEGFR-2 heterodimers. Other studies have shown that during pathological conditions, such as tumorigenesis, VEGFR-1 is a potent, positive regulator of angiogenesis (50, 51, 86). Hence, current evidence now suggests that the function of VEGFR-1 differs with stages of development, various states of physiological and pathological conditions, and the cell type in which it is expressed.

2.5.2. VEGFR-2

VEGFR-2 is considered the principle mediator of VEGF-A-stimulated function in vasculogenesis and angiogenesis. VEGFR-1 is expressed on endothelial cells, hematopoietic cells, and neurons. Hetero- and homozygous VEGFR-2 knockout mice die in utero of defects in blood island formation and vascular development demonstrating the critical dependence of this receptor during the process of vasculogenesis (27). VEGFR-2 is also the principle VEGF-A-signaling receptor for microvascular permeability, endothelial cell proliferation, invasion, migration, and survival during angiogenic processes (32, 79, 87). VEGFR-2-mediated proliferation of endothelial cells involves activation of a phospholipase-C-gamma-Raf-MAP kinase-signaling pathway (88), whereas survival and migration are believed to involve phosphatidylinositol 3-kinase (PI3K) and focal adhesion kinase (FAK), respectively (89). Specific activation of VEGFR-2 with VEGF-E has demonstrated potent endothelial cell activity *in vitro* and *in vivo* strongly supporting the notion that activation of VEGFR-2 alone can efficiently stimulate angiogenesis. As described above,

coexpression and activation of VEGFR-1 can negatively or positively influence the activation and signaling of VEGFR-2.

Studies with neutralizing anti-VEGFR-2 antibodies, or VEGFR-2-selective tyrosine kinase inhibitors, have shown that these approaches are capable of potently inhibiting tumor angiogenesis and primary and metastatic tumor growth in a variety of preclinical models (90–97). A neutralizing anti-Flk-1 mAb (DC101) suppressed the growth and metastasis of human tumor xenografts in mice, and this antitumor effect was associated with decreased microvessel density, tumor cell apoptosis, decreased tumor cell proliferation, and tumor necrosis (90, 93, 94). Similar effects have been shown with small molecule VEGFR-2-selective tyrosine kinase inhibitors (91, 92). Anti-VEGFR-2 treatment in various tumor models has been combined with cytotoxic, metronomic, or radiation therapy, resulting in improved antitumor effects (96–98).

2.5.3. VEGFR-3

VEGFR-3 is a receptor tyrosine kinase originally cloned from a human leukemia cell line and human placenta (71, 72, 99). VEGFR-3 preferentially binds VEGF-C and VEGF-D. VEGFR-3 expression in the adult is limited to lymphatic endothelial cells. Homozygous deletion of the VEGFR-3 gene in mice leads to embryonic death at day 10–12.5, with an underdeveloped yolk sac, poor perineural vasculature, and pericardial fluid accumulation (28). Hereditary functional mutations of the VEGFR-3 tyrosine kinase domain have been identified in human kindreds with lymphedema. In adult tissues, VEGFR-3 expression has been correlated with transient lymphangiogenesis in wound healing (100). Thus, VEGFR-3 has critical and diverse functions, assisting in cardiovascular development and remodeling of primary vascular networks during embryogenesis and facilitating postnatal lymphangiogenesis. Moreover, some evidence supports a continuing role of VEGFR-3 in the vasculature and suggests that it modulates VEGFR-2 signaling to maintain vascular integrity (101).

VEGFR-3 activation and upregulation of its ligands have been observed in several human cancers with elevated levels of VEGF-C or VEGF-D associated with lymph node metastasis in patients (61, 101–104). Of interest, it appears that in addition to lymphatics, some tumor-associated blood vessels may also express VEGFR-3 (102). Overexpression of VEGF-C or VEGF-D and activation of VEGFR-3 in preclinical models of human breast tumor xenografts, or genetic models of pancreatic islet cell carcinoma, were shown to enhance tumor-associated lymphangiogenesis and dissemination of tumor cells to regional lymph nodes (60, 105).

A number of recent studies have evaluated VEGFR-3-specific inhibitors in preclinical tumor models. VEGFR-3 blockade using a neutralizing monoclonal antibody reduced the incidence of lymph node and organ metastasis in a VEGF-C-overexpressing breast tumor model (106). In another study, treatment with VEGFR-3 antibody in a mouse tumor model reduced lymphatic hyperplasia, inhibited transit of tumor cells to draining lymph nodes, and consequently suppressed lymph node metastasis (107). However, growth of tumor cells already seeded in lymph nodes was unaffected by VEGFR-3 therapy in this model.

2.5.4. NEUROPILINS, INTEGRINS, AND VE-CADHERIN

A number of molecules, most notably neuropilins, integrins, and VE-cadherin, have been identified as coreceptors and/or modulators of VEGF-binding specificity

and signaling. These molecules bind VEGF ligands (neuropilins), can interact with certain VEGFRs (neuropilins, integrins, and VE-cadherin), and modulate VEGF ligand-stimulated signaling (neuropilins, integrins, and VE-cadherin).

NRP-1 and NRP-2 are cell-surface glycoproteins that serve as receptors for the semaphorin/collapsins, a large family of secreted and transmembrane proteins that serve as repulsive guidance signals in axonal and neuronal development (108). NRP-1 and NRP-2 also serve as coreceptors for VEGF, suggesting a potential role in angiogenesis (22, 109). NRP-1 does not have an intracellular signaling domain, and it acts as a coreceptor for VEGFR-1 and VEGFR-2 by enhancing the binding affinity of VEGF ligands to the receptors (110). The binding of VEGF-A isoforms and other VEGF ligands to NRP-1 is highly specific. For example, VEGF-A isoforms 165, 189, 189, and 203 and PIGF-2 bind NRP-1, but VEGF₁₂₁ does not bind NRP-1 (110, 111). NRP-2 binds to VEGF₁₆₅ and VEGF-C and interacts with VEGFR-2 and VEGFR-3 (112, 113).

Although the specific functions of NRP in vessel development and angiogenesis are not fully known, *in vitro* inhibition of VEGF₁₆₅ binding to NRP-1 on endothelial cells decreased its binding to VEGFR-2 and subsequent mitogenic activity (114), whereas cotransfection of NRP-1 into VEGFR-2-expressing endothelial cells enhanced the binding of VEGF₁₆₅ to VEGFR-2 and subsequent mitogenic and chemotactic activity. NRP-1 null mice have lethal defects in vascular and neuronal development establishing the important role for this receptor in vasculogenesis (114). Similar defects in vascular development were shown in NRP-2 null mice with heterozygous expression of NRP-1 or with knockouts of NRP-1 and heterozygous expression of NRP-2 (115). Homozygous NRP-2 mutants are viable although their small lymphatic vessels and capillaries are reduced in number or absent, consistent with NRP-2's interaction with VEGFR-3.

3. INTEGRINS

Integrins are a family of ECM receptors that directly or indirectly regulate endothelial cell function by positively or negatively modulating cell-cell and intracellular signaling (116). Integrins containing the $\beta 3$ subunit specifically bind to VEGFR-2 and augment receptor signaling (117–119). Mice lacking integrin subunits $\beta 3$ or $\beta 5$ have increased VEGFR-2 activation and vascularization of tumors, which can be abrogated using a VEGFR-2-neutralizing antibody (120, 121). In addition, lymphangiogenesis during tissue repair appears to be modulated by interaction of the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ with VEGFR-2 (122).

4. VE-CADHERIN

Vascular endothelial-cadherin (VE-cadherin) is an endothelial cell-specific adhesion molecule that is responsible for the formation of adheren junctions (24). VE-cadherin-mediated adhesion is crucial for proper assembly of vascular structures during angiogenesis, as well as for maintenance of a normal vascular integrity. Several studies have shown that VE-cadherin interacts with VEGFR-2, and this binding is regulated by β -catenin (123–128). First observations of this interaction showed that targeted inactivation or truncation of the β -catenin-binding domain of VE-cadherin gene reduced complex formation with VEGFR-2, resulting in endothelial apoptosis (123). Subsequent studies have shown that VE-cadherin limits cell proliferation by retaining

VEGFR-2 at the membrane and preventing its internalization into signaling compartments. In addition, VE-cadherin attenuates tyrosine phosphorylation of VEGFR-2 through associated phosphatases resulting in reduced downstream signaling (125). Recent studies have shown that one of the mechanisms responsible for VEGFs induction of vascular permeability is through VEGFR-2 modulation of VE-cadherin (128). VEGF promotes endothelial permeability through β -arrestin-2-dependent endocytosis of VE-cadherin. This process is initiated by the activation of the GTPase Rac by VEGFR-2 through Src-dependent phosphorylation of Vav2.

5. EXPRESSION OF VEGFRS ON TUMOR CELLS

Evidence is emerging that various VEGFRs are expressed on tumor cells (reviewed in ref. (6)). Several studies have reported the presence of VEGFRs on liquid and solid tumor cells including carcinomas of the breast, colon, hematologic tumors, lung, melanoma, pancreas, and prostate. Thus far, VEGFR-1 appears to be the most broadly expressed VEGFR on tumor cells. Specifically, VEGFR-1 expression has been found on solid tumors of the breast (129–131), colon (132), pancreas (133, 134), prostate (135), melanoma (136) and several hematologic malignancies (37, 137). VEGFR-2 expression has been identified on hematologic tumors (37, 137, 138) and in melanoma (136). VEGFR-3 is expressed on leukemia cells (137, 139), and one recent study has shown that this receptor may be broadly expressed on several human solid tumors (140).

The role of VEGFR expression on tumor cells is still under investigation. However, it could be hypothesized that various VEGF ligands support tumor growth not only by inducing angiogenesis but also through a paracrine or autocrine manner by directly activating VEGFRs expressed on tumor cells. Consistent with this hypothesis, studies have shown that functional VEGF/VEGFR-2 autocrine loops are present in subsets of human leukemias and support *in vivo* leukemic cell survival and migration (138, 141). Treatment of mice bearing human leukemia xenografts with a function-blocking VEGFR-2 antibody led to a decrease in tumor growth and prolonged survival. Stimulation of VEGFR-1-positive tumor cells with VEGF-A or VEGF-B leads to activation of the MAPK or AKT pathways in tumor cells and induces cell migration and invasion *in vitro*, suggesting a possible autocrine pathway leading to increased tumorigenesis (129–132). Treatment of mice with a function-blocking antibody specific for human VEGFR-1 inhibited growth of human breast tumor xenografts (130). Cotreatment with antibodies targeting human VEGFR-1 on tumor cells and murine VEGFR-1 on vasculature led to more potent growth inhibition of breast tumor xenografts. Thus, in certain human cancers, VEGF ligands may promote growth by directly activating various VEGFRs on tumor cells, as well as promoting angiogenic functions.

6. REGULATORS OF VEGF EXPRESSION

VEGF ligands and receptors are expressed by endothelial cells, various stromal cell populations, and hematopoietic cells. Expression of some VEGF ligands and receptors is restricted to certain cell types, whereas others are expressed in an overlapping manner leading to a highly complex and regulated biology for this family of growth

factors. In tumors, coordinate expression of different VEGF ligands and receptors may be expressed by tumor and stromal cells.

The tumor vasculature is abnormal with leaky, disorganized vessels and excessive branching leading to poor function (142). Lack of adequate blood supply often leads to regions of hypoxia in tumors. In response to a hypoxic environment, tumors upregulate the expression of factors that stimulate new blood vessel growth to restore normoxia. The hypoxia-inducible factor-1 (HIF-1) is the key mediator of this hypoxic response (143). HIF-1 α is rapidly degraded under normoxic conditions by the ubiquitin–proteosome pathway—a process that is controlled by the von Hippel–Landau (vHL) tumor suppressor gene product. In tumors with mutant or deficient VHL protein, such as renal cell carcinoma, HIF-1 α is constitutively active resulting in overexpression of hypoxia-inducible genes. During states of hypoxia in tissue, HIF-1 α dimerizes with HIF-1 β followed by translocation of this complex to the nucleus. The active HIF-1 α/β binds to hypoxia response elements (HREs) in the promoter of hypoxia-inducible genes leading to increased transcription. Hypoxia has been shown to play an important role in the regulation of VEGF-A gene expression (144). Interestingly, the VEGF-B promoter lacks HIF-1-binding elements and consequently, is not inducible by hypoxia; which may explain why this ligand does not play an apparent role in tumor angiogenesis. The role of hypoxia in the regulation of VEGF-C and VEGF-D is unclear. Some studies have shown that VEGF-C transcripts are not upregulated in response to hypoxia (144). In contrast, other studies showed that hypoxia increased transcripts encoding VEGF-C (and VEGF-D), but this hypoxia-inducible upregulation of VEGF-C was cell-type specific and restricted to vein endothelial cells (145).

Several studies have demonstrated that growth factors and cytokines, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF1), transforming growth factor beta (TGF- β), hepatocyte growth factor (HGF), interleukin-1-beta (IL-1 β), and IL-6, can upregulate VEGF expression in tumor or stromal cells and stimulate angiogenesis (reviewed in refs 5, 6). The EGF–EGFR pathway is an important regulator of VEGF expression and angiogenesis in a number of human cancers (146–148). Stimulation of EGFR or HER-2 signaling in tumors upregulates VEGF expression and stimulates tumor-associated angiogenesis. Inhibition of EGFR or HER-2 signaling in preclinical models of human tumors downregulates VEGF expression and indirectly suppresses tumor angiogenesis (146, 149–151). Similarly, activation of the IGF–IR pathway is an important regulator of VEGF expression (152, 153). IGF1/IGF1R signaling upregulates VEGF expression in several human cancers including those of the colon, head and neck, pancreas, and thyroid (154–159). HGF can induce VEGF expression in normal and tumor cells through activation of mesenchymal epithelial transition (cMet) receptor (160). PDGFs modulate angiogenesis *in vivo* by regulating endothelial cell survival and pericyte/smooth muscle cell recruitment as well as by inducing VEGF in several model systems (161–163). PDGF-AA expressed by tumor cells is responsible for the recruitment of VEGF-producing, tumor-associated fibroblasts (164). Moreover, blockade of ligand-induced PDGFR- α signaling between tumor cells and stromal fibroblasts suppresses angiogenesis and tumor growth.

Several oncogenes play a role in tumor angiogenesis because of their ability to induce proangiogenic growth factors such as VEGF (165, 166). The induction of VEGF expression by mutant H- or K-ras oncogenes has been reported in various types of cells

such as pancreatic cancer, colon cancer, and non-small cell lung cancer (167–170). In addition, genetic disruption of the mutant K-ras allele in human colon carcinoma cells is associated with a reduction in VEGF activity (171). The proto-oncogene c-Src regulates VEGF expression and promotes neovascularization of growing tumors (172, 173). Antisense suppression of src in tumor cells results in decreased VEGF expression and reduced tumor growth and angiogenesis in preclinical models. Similarly, transfection of the breakpoint cluster region-abelson (BCR-ABL) oncogene human megakaryocyte cells results in enhanced VEGF expression (174). Several studies have shown that the tumor suppressor p53 has an important role in the regulation of VEGF in malignant tumors (175–177). Direct interaction of the p53 protein with the transcription factor Sp1 prevents transcriptional activation of the VEGF promoter in breast cancer cells and inhibits the hypoxic induction of Src kinase (178). In studies with colon and endometrial carcinoma, stable transfection of wild-type p53 resulted in decreased VEGF expression (176, 179). Several studies have also shown that genetic alterations of tumor suppressor genes such as phosphatase and tension homolog (PTEN) (180) and VHL (181) can induce HIF-1 activity in tumor tissues leading to increases in VEGF.

7. FUNCTIONS OF VEGF

7.1. Permeability

VEGF was originally discovered as a secreted factor capable of rendering venules and small veins hyperpermeable to circulating macromolecules and was, therefore, given the name vascular permeability factor (VPF) (182). VEGF elicits a number of functions important for tumor growth and angiogenesis by activating VEGFRs expressed by a variety of cell types in the tumor microenvironment (Fig. 2). VEGF is one of the most potent inducers of vascular permeability known. This ability to enhance microvascular permeability remains one of VEGF's most important properties, especially with regards to the hyperpermeability of tumor vessels that is thought to be largely attributable to tumor cell expression of VEGF. Increased vascular permeability results in the leakage of several plasma proteins, including fibrinogen and other clotting proteins (183). This can lead to the deposition of fibrin in the extravascular space, which subsequently retards the clearance of edema fluid and transforms the normally antiangiogenic stroma of normal tissues into a proangiogenic environment. VEGF increases permeability in a variety of vascular beds including in those of the skin, peritoneal wall, mesentery, and diaphragm, and can lead to pathologic conditions such as malignant ascites (184) and malignant pleural effusions (185). In fact, there is evidence that inhibition of VEGF can lead to decreased formation of pleural effusions and that antibodies directed against VEGF or VEGFR-2 can lead to decreases in tumor vessel permeability and ascites formation (184–186).

The precise mechanisms by which VEGF increases microvascular permeability are not entirely clear (for review, see ref. 187). However, recent studies are providing evidence for several potential mechanisms. Early work showed that VEGF-induced transit of macromolecules cross the endothelium by means of a transendothelial cell pathway involving vesicovascular organelles (VVOs) (188). Others investigators have proposed that VEGF induces endothelial fenestrations that provide an additional transcellular pathway for solute extravasations or that VEGF leads to increases in an interendothelial cell pathway by opening of junctions between adjacent endothelial

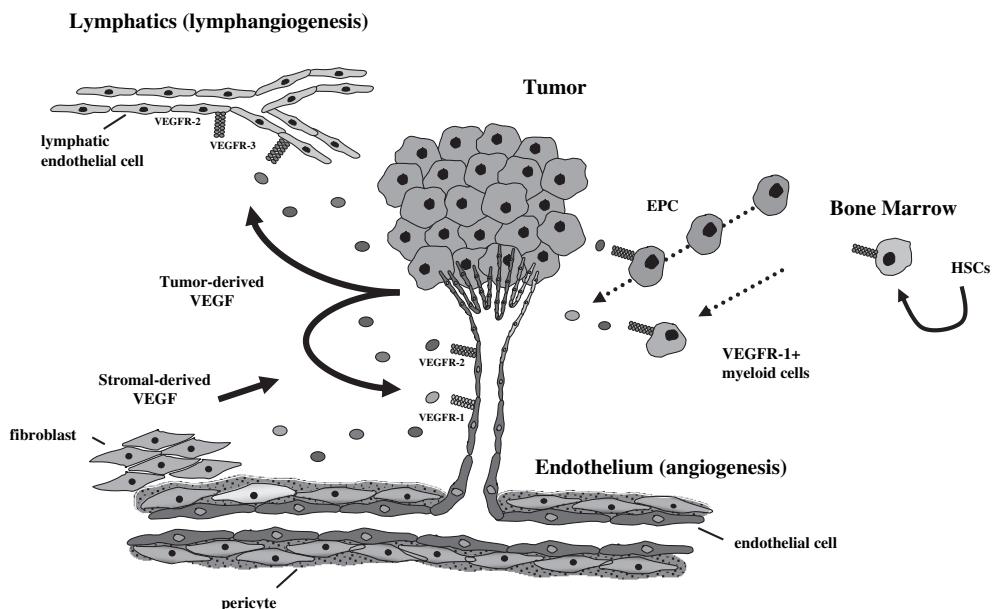


Fig. 2. Role of VEGF ligands and receptors in tumor angiogenesis. VEGF ligands expressed by tumor cells or host stromal cells stimulate VEGFR-1, -2 or -3 expressed by a variety of cell types in the tumor microenvironment including endothelial, lymphendothelial, hematopoietic and tumor cells. VEGF stimulation of VEGFR-1+ and -2+ endothelial cells activates proliferation, migration survival and vascular permeability. VEGF can act through paracrine or autocrine stimulation of VEGFR-1 on tumor cells to promote tumor cell migration and survival. VEGF may also stimulate mobilization and recruitment of endothelial progenitor cells (EPC) and VEGFR-1+ myeloid cells in the bone marrow to sites of tumor angiogenesis. VEGF-C and -D stimulate VEGFR-3+ lymphatic endothelial cells and lymphangiogenesis. (Please see color insert.)

cells (189, 190). Additional evidence suggests that VEGF-induced permeability may be mediated through a calcium-dependent pathway that involves nitric oxide production and activation of the Akt pathway and increases in cGMP (191). Other work has shown that VEGF-induced vascular permeability specifically depends on the Src or Yes kinase signaling (192). As described earlier, recent evidence has shown that VEGF promotes endothelial permeability through β -arrestin-2-dependent endocytosis of VE-cadherin (128).

7.2. Proliferation

VEGF is an important mitogen for endothelial cells and was originally characterized based on this activity (4). However, VEGF is a weak endothelial cell mitogen compared with other growth factors such as FGF. VEGF-stimulated endothelial cell proliferation is mediated by signaling through VEGFR-2 and downstream activation of the phospholipase-C-gamma-MAP kinase pathway (4, 73).

7.3. Invasion and Migration

Degradation of the basement membrane is necessary for endothelial cell migration and invasion and is an important early step in the initiation of angiogenesis.

VEGF induces a variety of enzymes and proteins important in the degradation process including matrix-degrading MMPs, MMP interstitial collagenase, and serine proteases such as urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (TTPA) (4, 5). Activation of these various compounds leads to a prodegradative environment that facilitates migration and sprouting of endothelial cells.

Other studies have shown that VEGF promotes expression of the uPA receptor (uPAR) on vascular endothelial cells (4). Considering that the PA-plasmin system, in particular, the interaction of uPA with uPAR, is an important element in the chain of cellular processes that mediate cellular invasion including proteolysis and tissue remodeling, these findings are consistent with the proangiogenic activities of VEGF. Furthermore, it has been shown that uPA itself leads to increased production of a variety of different angiogenic factors including VEGF suggesting that an autocrine regulatory loop may exist.

The intracellular mechanisms by which VEGF leads to increased endothelial cell migration involve PI-3 kinase, Akt, and FAK-associated signaling leading to focal adhesion turnover and actin filament organization, as well as P38 MAPK-induced actin reorganization (73). NO has been implicated in podokinesis of endothelial cells, and Akt-dependent activation of eNOS has been shown to be required for VEGF-induced cell migration. NO has also been reported to regulate focal adhesion integrity and FAK tyrosine phosphorylation, suggesting signaling cross-talk between FAK and NO in the regulation of migration (193).

7.4. Survival

VEGF is a potent survival factor for endothelial cells. *in vitro*, VEGF has been shown to inhibit apoptosis by activating the PI3K–Akt pathway and upregulating antiapoptotic proteins such as bcl-2 and A1 (194, 195). These events activate upstream caspases and upregulate members of the inhibitors of apoptosis (IAP) family including survivin and XIAP. As described earlier, VEGF has also been shown to activate focal FAK and associated proteins that have been shown to maintain survival signals in endothelial cells.

7.5. Mobilization and Recruitment of Bone Marrow-Derived Progenitor Cells

Bone marrow contains numerous VEGF-responsive cells including endothelial cells, HSCs, osteoblasts, and osteoclasts (8). VEGF has been shown to be an important survival factor for HSCs in mice (196). Conditional knockout of the VEGF gene in mice results in reduced colony formation and *in vivo* repopulation rates of HSCs. VEGF-dependent HSC survival appears to be VEGFR-1 mediated through an internal autocrine loop mechanism.

Bone marrow-derived EPC originate from HSCs and have similar features to that of embryonic angioblasts (8). EPC migrate from the bone marrow to the circulation where they are referred to as circulating EPC (CEP). By definition, CEPs originate from EPC within the bone marrow and differ from mature endothelial cells that enter circulation as a result of vascular injury. Accumulating evidence suggests that VEGF plays a major role in mobilization and recruitment of bone marrow-derived

EPC to sites of neovascularization in the adult and contribute to the process of angiogenesis (8, 197). In an angiogenesis-deficient *Id1+/- Id3-/-* mouse model, transplantation of VEGF-mobilized, VEGFR-1-expressing bone marrow cells restored tumor growth angiogenesis (82). Donor-derived VEGFR-1+ CEPs were detected throughout the neovessels of tumors. Furthermore, treatment of recipient *Id1+/- Id3-/-* mice with a VEGFR-1-blocking antibody reduced the number of EPC recruited to the tumor vasculature suggesting that VEGF/PIGF-mediated stimulation of VEGFR-1 is important for EPC mobilization or recruitment. In supporting studies, neovascularization in PIGF-null mice, which have deficiencies in various pathological angiogenesis settings, could be rescued by transplantation of VEGF-mobilized EPC from wild-type mice (51). Repopulation experiments in these mice conducted with wild-type marrow cells restored tumor angiogenesis and growth, which was found to be associated with the recruitment of VEGFR-1+ myeloid cells in the neovessels. Additional supporting evidence for the role of bone marrow-derived EPC in tumor vascularization have been provided by other transplantation studies (197), demonstrating that EPC isolated from donor mice were incorporated into the tumor vasculature and contributed to tumor angiogenesis. Lastly, a recent study has shown that VEGF-1-positive bone marrow progenitors home to sites of tumor metastasis prior to the arrival of tumor cells, and thereby orchestrate organ-specific metastatic dissemination (198). Collectively, these studies suggest that a population of VEGFR+ EPC contributes to new blood vessel formation during states of pathological angiogenesis.

8. CONCLUSION

In this chapter, we have reviewed the important role of VEGF ligands and their receptors in the formation of new blood and lymphatic vessels during tumor growth. Early recognition of the importance of VEGF/VEGFR family in angiogenesis and lymphangiogenesis led to several years of intensive research into defining the role of this growth factor family in cancer. These studies have clearly established that tumor-associated angiogenesis is dependent on VEGF/VEGFR function. Moreover, these studies have shown that various approaches to block VEGF or VEGFR activity result in potent inhibition of tumor growth and angiogenesis in preclinical models, providing strong validation for targeting this pathway in cancer. The most convincing experimental evidence to date has been generated using agents that block VEGF-A or VEGFR-2 and has led to the development of several VEGF/VEGFR-2-targeted therapeutics. A number of these VEGF/VEGFR-2-targeted agents have recently demonstrated clinical benefit in patients and have become part of standard treatment in oncology.

Recognizing the established role of VEGF and VEGFR-2 in tumor angiogenesis, future research will likely focus on understanding the importance of other VEGF/VEGFR family members in this process. For example, PIGF has been shown to stimulate angiogenesis under experimental conditions, and inhibition of VEGFR-1 function can inhibit tumor growth and angiogenesis. However, the function of PIGF and VEGFR-1 in tumor angiogenesis is not completely understood. Similarly, the role of VEGF-C, VEGF-D, and VEGFR-3 in lymphatic vessel development is well established. Nevertheless, research on the contribution of the VEGF-C/VEGFR-3 axis during tumor growth and metastasis is still early, and studies with VEGFR-3 inhibitors

to help address this question are just emerging. Another important question that will be the subject of future investigation is the role of VEGFRs on tumor cells. The recent evidence that VEGFR-1 or VEGFR-3 is expressed by certain cancers suggests that these receptors may play a direct role in growth and survival of tumor cells through autocrine or paracrine stimulation by VEGF ligands.

Given the complexity of VEGF/VEGFR biology and interplay among family members, it seems reasonable to argue that one should consider blockade of multiple family members when considering anticancer treatments that target the VEGF/VEGFR axis. Data are still emerging to address the question of whether the contribution of multiple VEGF/VEGFRs are crucial for various stages of malignant growth and whether a more robust clinical benefit may be obtained by blocking multiple VEGFR pathways simultaneously.

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2

The Cycle Between Angiogenesis, Perfusion, and Hypoxia in Tumors

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SUMMARY

This chapter will present a pathophysiologic paradigm that occurs in solid tumors that is characterized by a self-propagating cycle of abnormally regulated angiogenesis, instability in perfusion, and hypoxia. Interactions between tumor and endothelial cells occur during tumor growth and in response to therapy. These interactions are of central importance in establishing codependence that contributes to promotion of tumor and endothelial cell survival, treatment resistance, enhanced invasion, and metastasis. Results indicate that concurrent targeting of both tumor and endothelial cells may be of central importance in improving treatment responses to both radiation and chemotherapy.

Key Words: angiogenesis; perfusion; hypoxia; hypoxia-inducible factor.

1. INTRODUCTION

The objective of this chapter is to examine the interrelationships between tumor hypoxia, angiogenesis, and perfusion in tumors. These three features of tumor growth are inextricably tied together and collectively contribute to maintaining a microenvironment typified by unstable oxygenation, hypoxia, and acidosis, promoting treatment resistance and increased propensity for invasion and metastasis. This chapter will emphasize the process of vascular angiogenesis. Lymphangiogenesis is also important in tumor growth but will not be discussed in this chapter. Readers are referred to other excellent reviews on this subject.

2. THE ANGIOGENIC SWITCH

Angiogenesis is the process by which new vascular segments are added to an existing vascular system. This process is largely quiescent in the normal adult, with the exception of processes such as the menstrual cycle and exercise adaptation (1). On the

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other hand, angiogenesis is a prominent feature of pathologic conditions, such as wound healing, chronic inflammation, diabetic retinopathy (2), and cancer. The initiation of angiogenesis in a nascent tumor is often referred to as the “angiogenic switch,” a term initially coined by Folkman (3). There are at least two stimuli that can be involved in triggering the angiogenic switch: hypoxia (4) and/or alterations in oncogene or tumor suppressor gene function (5). Although there are numerous molecular signals that mediate this switch (Table 1), there are some master regulators that play predominant roles, hypoxia-inducible factor-1 (HIF-1) being the best-studied example. HIF-1 is activated directly by hypoxia as well as by hyperactivity in certain signaling pathways. HIF-1 is a transcriptional activator that serves to enhance the expression of dozens of genes, including those for a number of important proangiogenic cytokines (4).

The regulatory mechanisms controlling HIF-1 stability are important in this context (Fig. 1). The protein is a heterodimer consisting of an alpha and a beta subunit; these are constitutively expressed in nearly all cells. However, in aerobic conditions HIF-1 α is constantly targeted for degradation through ubiquitylation (6). This process depends on modification of HIF-1 α ’s oxygen-dependent degradation (ODD) domain by a family of hydroxylases, using elemental oxygen as a cofactor, rendering it recognizable by the ubiquitin ligase von Hippel–Lindau (VHL) complex (6). Therefore, when a cell is normally oxygenated, the heterodimer does not form. Although the most powerful inducer of HIF-1 stabilization is hypoxia, there are circumstances wherein the heterodimer can form under normoxic conditions. For example, overexpression of oncogenes such as HER2 can lead to increased HIF-1 α synthesis, which can

Table 1
Direct and Indirect Acting Angiogenic Factors Upregulated by Hypoxia

| <i>Direct-acting factors</i> | <i>Indirect-acting factors</i> |
|--|---|
| ^a Vascular endothelial growth factor (VEGF) | Hypoxia-inducible factor-1 (HIF-1) |
| ^a Basic fibroblast growth factor (bFGF) | Nuclear factor- κ B (NF- κ B) |
| ^a Angiopoietin 2 (Ang-2) | AP-1 |
| Platelet-derived growth factor (PDGF) | ^a Pyruvate |
| Placental growth factor (PIGF) | ^a Lactate |
| ^a Transforming growth factors (TGFs) | |
| ^a Plasminogen activator inhibitor-1 (PAI-1) | |
| Thrombospondins (TSPs) | |
| Matrix metalloproteinases (MMPs) | |
| Endothelins (ETs) | |
| Adrenomedullin (ADM) | |
| Angiogenin (ANG) | |
| ^a Endoglin (ENG) | |
| placental growth factor (PGF) | |
| Fractalkine (C-X3-C) | |
| Connective tissue growth factor (CTGF) | |
| Interleukin-8 (IL-8) | |
| Macrophage migration inhibitory factor (MIF) | |
| ^a Leptin (LEP) | |

^aFactors directly or indirectly influenced by HIF-1 activity.

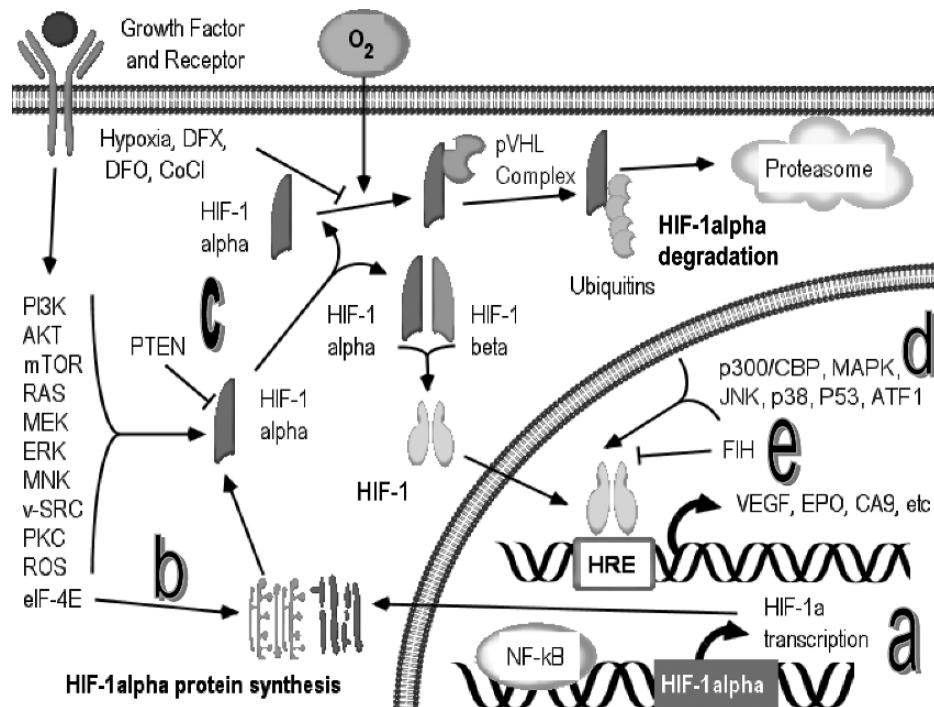


Fig. 1. Different regulatory points of hypoxia-inducible factor 1 (HIF-1) signaling. HIF-1 promoter activity is regulated in at least five ways: (a) Transcription of HIF-1 α : nuclear facto- κ B (NF- κ B) can upregulate the transcription of HIF-1 α . (b) Translation of HIF-1 α : both the phosphatidylinositol 3-kinase (PI3K)-AKT-mTOR pathway and RAS-MEK-ERK pathway can upregulate eIF-4E-mediated HIF-1 α protein synthesis. (c) Post-translational modification: protein kinase C (PKC), PTEN, and potentially other suppressor genes can control post-translational modification of HIF-1 α , which is important for its heterodimerization with HIF-1 β . (d) Degradation of HIF-1 α : oxygen controls the binding of HIF-1 α to pVHL protein complex, which is responsible for HIF-1 α degradation. (e) Transcriptional activity of HIF-1 by other transcriptional regulators or cofactors: MAPK family members such as p38 and p42/p44 can phosphorylate HIF-1 α . FIH can hydroxylate HIF-1 α . Those modifications directly affect the binding of HIF-1 to other transcriptional coactivators such as p300/CBP. P53 controls the degradation of HIF-1 and might affect the binding of HIF-1 and p300.

outpace the degradation machinery (7). Also, mutations in tumor suppressor genes, such as phosphatase and tensin homolog deleted on chromosome Ten (PTEN), can block the degradation of HIF-1 α by von Hippel-Lindau protein (pVHL) (8). Once the heterodimer is formed, there are other points of regulation including cofactors, such as p300/CBP, that influence binding to DNA (9). It has also been reported that reactive oxygen and nitrogen species may also prevent the degradation of HIF-1 α (10, 11). As tumors tend to have elevated levels of reactive oxygen/nitrogen species (12), this may serve as another source of proangiogenic stimulus in tumors. Reactive oxygen species formation may in fact be stimulated by hypoxia-reoxygenation injury, which may occur in tumors as a result of instabilities in perfusion (13, 14).

A large group of the downstream genes transactivated by HIF-1 α are proangiogenic factors (15). Thus, it is not surprising that tumor hypoxia or HIF-1 activation stimulate angiogenesis by upregulating key proangiogenic factors. For example, overexpression

of HIF-1 in HCT116 tumor cells with a vector encoding HIF-1 α markedly promoted vascular endothelial growth factor (VEGF) expression and angiogenesis (16). Peptide blockade of HIF-1 α degradation is also effective in stimulating angiogenesis (17). However, it has been unclear whether hypoxia is a required factor for angiogenesis initiation in early-stage tumor growth. Recently, we successfully revealed the relationship between incipient tumor angiogenesis and the initiation of hypoxia in mouse dorsal skin-fold window chamber tumor models (18). We established HCT116 human colon carcinoma cells and 4T1 mouse mammary carcinoma cells with constitutively expressed red fluorescence protein as a tumor marker and green fluorescence protein (GFP) as a reporter for hypoxia and HIF-1 activation. Serial observation of tumor growth, angiogenesis, and expression of GFP in murine skin-fold window chambers revealed that incipient angiogenesis preceded a detectable level of hypoxia and HIF-1 activation

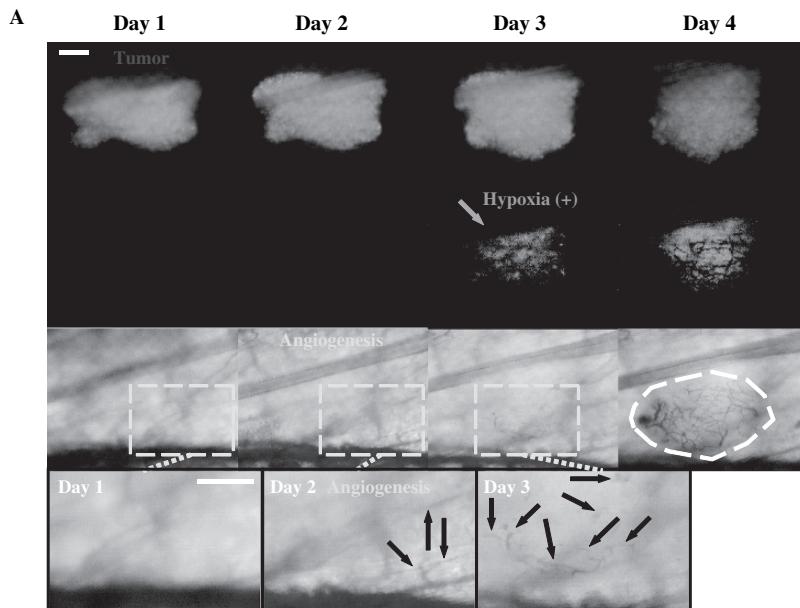


Fig. 2. Suppression of hypoxic response by selectively killing hypoxic cells does not delay incipient tumor angiogenesis. **(A)** Representative window chamber images of a saline-treated HCT116 tumor reveal incipient angiogenesis (day 2, black arrows) before detectable green fluorescence protein (GFP)-hypoxia-inducible factor 1 (HIF-1) reporter-hypoxic response (day 3, green arrow). Endothelial cords and sprouts surrounding hypoxic region (day 3, black arrows) develop into a vascular plexus (day 4, white dashed circle). Bar, 0.3 mm. **(B)** Representative window chamber images of a tirapazamine (hypoxic cytotoxin)-treated HCT116 tumor. Incipient angiogenesis began on day 2 (black arrows) followed by vascular plexus development on day 10 in the absence of detectable levels of HIF-1 reporter gene expression. Bar, 0.3 mm. **(C)** Kaplan-Meier plot of the time to develop HIF1 reporter gene expression in tirapazamine-treated versus saline treated HCT116 window chamber tumors. Tirapazamine significantly delayed detection of HIF-1-mediated GFP production (median time: 9.5 days in the tirapazamine (TPZ) -treated group vs. 3.5 days in the saline-treated group, $n = 8$, log-rank test, $p < 0.001$). **(D)** Kaplan-Meier plot of time required for onset of incipient angiogenesis in tirapazamine-treated versus saline-treated HCT116 window chamber tumors revealed no difference between these groups ($n = 8$, log-rank test, $p = 0.33$). Reproduced with permission from (18).

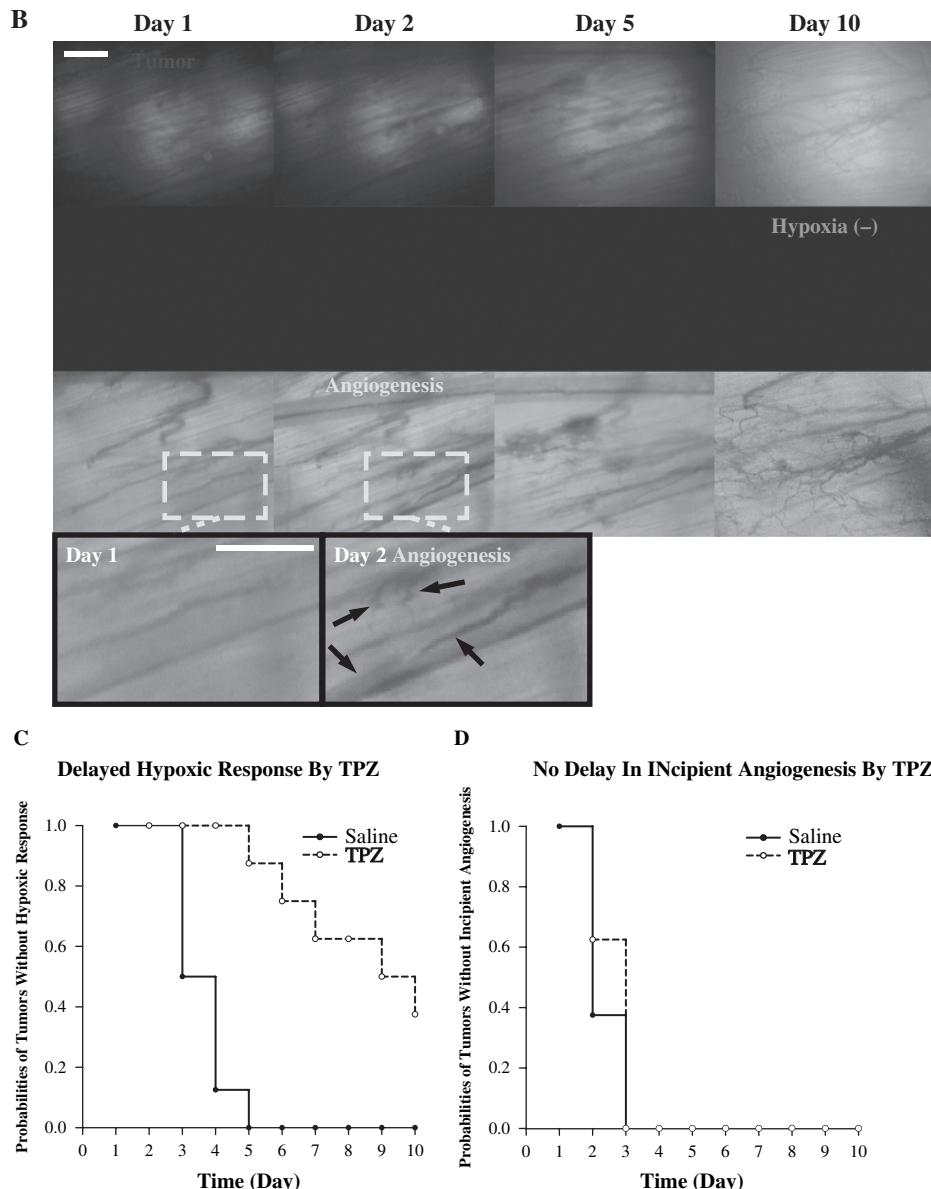


Fig. 2. Continued.

in both tumor lines. However, HIF-1 activation was spatially and temporally related to more intensive secondary angiogenesis following the initiation of angiogenesis. Selective killing of hypoxic cells by the hypoxia-specific cytotoxin, tirapazamine, delayed the appearance of hypoxic cells, but it did not delay the onset of incipient angiogenesis (Fig. 2). These findings provided the first direct experimental evidence that incipient tumor angiogenesis may not require hypoxia or HIF-1 activation. Alternatively, hypoxia appeared to accelerate the process of angiogenesis once it was initiated. Although there could be other non-hypoxia-regulated proangiogenic

factors contributing to incipient tumor angiogenesis, our findings suggest that VEGF expression induced by genetic mutations or other non-HIF-signaling pathways is crucial for the initiation of tumor angiogenesis that is independent of hypoxia or HIF-1 activation. This is consistent with the findings by several other groups showing that VEGF is upregulated in tumor cells with mutations in oncogenes (ras, Her-2, etc.) and suppressor genes (VHL, etc.) even in the absence of hypoxia (5, 7, 19). Consistent with this concept is our previous observation that neutralizing VEGF by a soluble VEGF receptor efficiently delays incipient angiogenesis in 4T1 window chamber tumors (20).

Clarification of the interaction between incipient tumor angiogenesis and initial hypoxia/HIF-1 activation also provides new insights into clinical application of hypoxia/HIF-targeting therapies. Therapeutic reagents targeting tumor hypoxia and HIF-1 are currently actively pursued with the hope that these drugs may convert hypoxia from therapeutic obstacles into tumor-targeting advantages (21). One key rationale behind this therapeutic strategy is that inhibition of tumor hypoxia or HIF-1 activation may significantly inhibit tumor angiogenesis (4, 22). Our study shows that suppression of the hypoxia response only may not be sufficient to prevent or delay the initiation of tumor angiogenesis—a key step for the establishment of metastatic tumor sites. Although hypoxia/HIF-targeting therapies still have great promise as anti-angiogenic agents, it will be intriguing to explore whether additive or synergetic therapeutic benefits could be achieved by the inhibition of incipient angiogenesis in the adjuvant setting where a proportion of metastatic sites may be pre-angiogenic.

Angiogenesis occurs through two physically different pathways: sprouting and intussusception (23, 24). Sprouting is mediated primarily by VEGF and begins with vasodilation of existing vessels (25). The hypoxic trigger for HIF-1-mediated VEGF upregulation is thought to be caused by limitations in oxygen diffusion into the interior of a tumor as it grows *in situ* or collapse of pre-existing coopted host vessels, leading to a hypoxic crisis (26). Once angiogenesis has been established, however, hypoxia persists as a result of aberrancies in tumor microvascular geometry and function as well as imbalances between oxygen consumption rates and supply (Fig. 3) (27, 28). The resultant persistent hypoxia maintains a constant proangiogenic stimulus as the tumor continues to grow. Clinically, hypoxia is a prominent pathophysiological feature of solid tumors. It has been observed in nearly all solid-tumor histologies in which it has been examined. It is important to note, however, that some human tumors appear to rely exclusively on cooption of pre-existing host vasculature for growth, as opposed to stimulation of angiogenesis. This phenotype has been observed in early stage gliomas (29), in primary non-small cell lung cancers (30), and in metastatic breast cancer of the liver (31). Some information is emerging about how tumors mediate this type of growth.

Specific blockade of VEGF receptor-2 (VEGFR-2) with antibody (32), VEGF Trap (33), or antibody to VEGF (34) has been reported to reduce intratumoral microvessel density and inhibit tumor growth. Interestingly, however, use of a VEGFR-2 antibody has been reported to effectively inhibit angiogenesis in primary tumors of an intracerebral glioma model, yet it exacerbates vascular cooption, leading to increased formation of satellite tumor recurrences removed from the primary site (32). Both angiopoietin 2 (Ang-2) and VEGF are upregulated at the margin of these tumors, suggesting that they play a role in the cooption process (35). However, these same factors are involved in regulation of angiogenesis as well. Thus, the underlying mechanisms that regulate angiogenesis versus vessel cooption remain undefined.

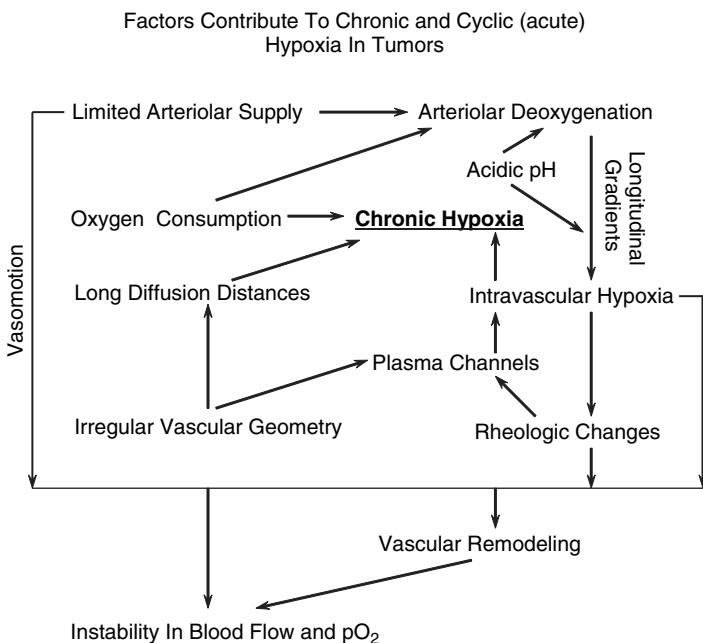


Fig. 3. Factors that contribute to chronic and cyclic hypoxia in tumors. Multiple factors influence oxygen delivery to tumors, including oxygen consumption, vascular geometry (including inadequate vascular density), limited number and orientation of feeding arterioles, longitudinal oxygen gradients that result from inadequate arteriolar input, and rheologic changes that occur in microvessels as a result of intravascular hypoxia and acidosis. Superimposed on the basic limitations of oxygen delivery is instability in microvessel red cell flux (perfusion). The underlying cause for this well-described phenomenon is not defined currently but could be related to arteriolar vasomotion, vascular remodeling, and angiogenesis as well as rheological effects that influence the distribution of red cells at bifurcation points..

3. REGULATION OF NEW BLOOD VESSEL GROWTH (ANGIOGENESIS)

Angiogenesis is initiated by a combination of molecular and environmental signals. To initiate the process, VEGF increases vascular permeability, partly through stimulating endothelial cell production of nitric oxide (NO) (36). The VEGF receptor, VEGFR2, is also upregulated in response to hypoxia, which increases vascular responsiveness to VEGF within the tumor (37). The resulting hyperpermeability permits extravasation of plasma proteins into the extravascular space. One of these proteins, fibrinogen, is rapidly converted to fibrin and cross-linked through the actions of thrombin and tissue transglutaminase (38). The fibrin matrix promotes angiogenesis by providing scaffolding for endothelial cell migration and proliferation (38). Transglutaminase upregulation has been observed in breast and pancreatic cancer (39, 40) and is affiliated with poor prognosis in breast cancer and may be associated with poorer overall prognosis (41). Transforming growth factor- β (TGF- β) and basic fibroblast growth factor (bFGF), which are also HIF-1-mediated proangiogenic factors, work with tissue transglutaminase and VEGF to promote angiogenesis (38).

In order for angiogenesis to be fully activated, appropriate signaling through the Tie2 receptor is required. Tie2 is an endothelial cell-specific tyrosine kinase receptor that is regulated by two primary ligands, Ang-1 and Ang-2 (42, 43). Angiopoietin 1 is constitutively expressed and activates the receptor, promoting stable intercellular junctions and tight association with basement membrane and vascular-supporting cells, such as pericytes and smooth muscle cells (44). Vessels that show high levels of Ang-1 binding to Tie 2 are relatively refractory to VEGF signaling (45). Ang-2, on the other hand, competes with Ang-1 for binding to Tie2, promoting disassociation of endothelial cells from basement membrane and pericytes and priming vessels to respond to VEGF and promote angiogenesis (Fig. 4) (46). Hypoxia plays an angiogenesis-stimulating role in this pathway as well, downregulating Ang-1 (47) and upregulating Ang-2 (48, 49). The effect of Ang-2 in vessel remodeling depends upon the context in which it is expressed. Ang-2 upregulation leads to vessel remodeling in the presence of VEGF. In contrast, Ang-2 acts as a destabilizing factor and results in vessel regression in the absence of VEGF (50). These results suggest that the relative ratio of VEGF to Ang-2 could determine whether these factors contribute to either vessel remodeling or regression.

The actual process of angiogenesis involves migration and proliferation of endothelial cells in cords, which join other cords and then form a lumen (38). Under normal circumstances, this process is tightly regulated. As new vessels are formed, normal pO₂ is restored, leading to a reduction in hypoxia-mediated proangiogenic cytokines and re-establishment of a mature vasculature, as Ang-1 once again dominates binding to Tie2. It has been demonstrated experimentally and theoretically in a corneal pocket angiogenesis model that the nature of this vascular bed (in terms of vessel

Pericytes Of Normal Capillary and Tumor Capillary

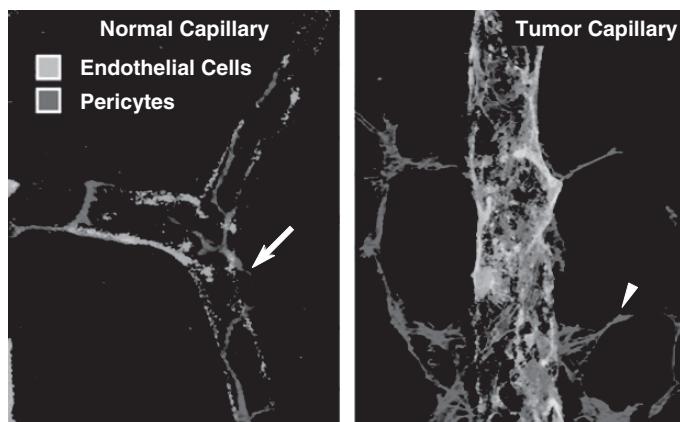


Fig. 4. Morphologic differences in pericyte-endothelial contacts: normal versus tumor tissues. Pericytes of normal capillaries have skeletal shapes and are closely attached to endothelial cells. In contrast, pericytes in a tumor model (MCA-IV) show irregular shapes and are loosely attached to endothelial cells. Many projections are observed from the pericyte into the interstitial space. Arrow: pericytes of normal capillary. Arrowhead: pericytes of tumor capillary. Reproduced with permission from (46).

lengths, branching patterns and overall density that is formed) is highly dependent upon the relative concentration of VEGF at the tips of the vascular sprouts (51).

4. ANGIOGENESIS IN METASTASES—EVIDENCE FOR PARACRINE SIGNALS BETWEEN TUMOR CELLS, HOST VASCULATURE, AND CANCER STROMA

It is generally believed that host vessels tend to be coopted by tumor cells prior to the onset of overt angiogenesis. It has been shown that this process involves selective invasion and proliferation of tumor cells toward host vessels, followed by formation of tumor cell cuffs around such vessels. It has been suggested that vascular collapse leads to a hypoxic catastrophe following vascular cuff formation, and this event triggers new vessel formation (26). We have evidence against this theory, at least for metastatic tumors. Using tumor cell lines stably transduced with GFP, we serially monitored tumor cell behavior and growth following transplantation into a window chamber model (52, 53). Both of the tumor types studied expressed VEGF at baseline in the absence of hypoxia—a scenario that would often be typical of a metastatic tumor. The 4T1 tumor line, a mammary carcinoma, underwent the epithelial–mesenchymal cell transition, typified by a change in shape to a fibroblastic-appearing cell. This adaptation has been linked to hypoxia-regulated expression of cell surface receptors such as autocrine motility factor, metalloproteinase, and keratin subtype expression that facilitates cell fluidity (54). Perivascular cuffs formed, but we saw no evidence for vascular shut down prior to the onset of new vessel formation.

Interestingly, when VEGF signaling was blocked in this model, the epithelial–mesenchymal cell transition failed to materialize. Instead, the tumor cells underwent apoptosis and failed engraftment prior to the onset of angiogenesis. This observation suggested the existence of a paracrine relationship between tumor and host microvessels. Recently, we have shown that this putative paracrine relationship is modulated by bFGF (promotes better tumor cell survival) and Tie2 function (blockade of Tie2 tends to reduce tumor cell survival) (53). It has not been reported whether hypoxia plays a role in this pre-angiogenic behavior, but we have observed that tumor cells farthest removed from host vasculature tend not to make the epithelial–mesenchymal cell transition and instead undergo apoptosis.

The metastatic behavior of lung metastases is not consistent with this paradigm. These metastases have been reported to adhere to and proliferate inside lung vasculature until they break down the vessel wall, allowing the tumor cells to escape and grow in the interstitial space (55, 56). It is not known whether paracrine relationships exist between host vasculature and tumor cells in primary sites or in this model of pulmonary metastasis.

We recently found that stromal cells in A549 human lung carcinoma xenografts are an important source of VEGF, which in turn affects tumor angiogenesis, vascular permeability, interstitial fluid pressure, and hypoxia (57). Platelet-derived growth factor receptor (PDGFR)- β appears to play a regulatory role in this scenario. Phosphorylated PDGFR- β participates in the recruitment of pericyte and smooth muscle cells for angiogenesis (58), which are required for angiogenesis and vessel stabilization (59). PDGFR- β also increases interstitial fluid pressure by stimulating stromal proliferation (60, 61). Imatinib (Gleevec) is a PDGFR- β -specific small molecule tyrosine

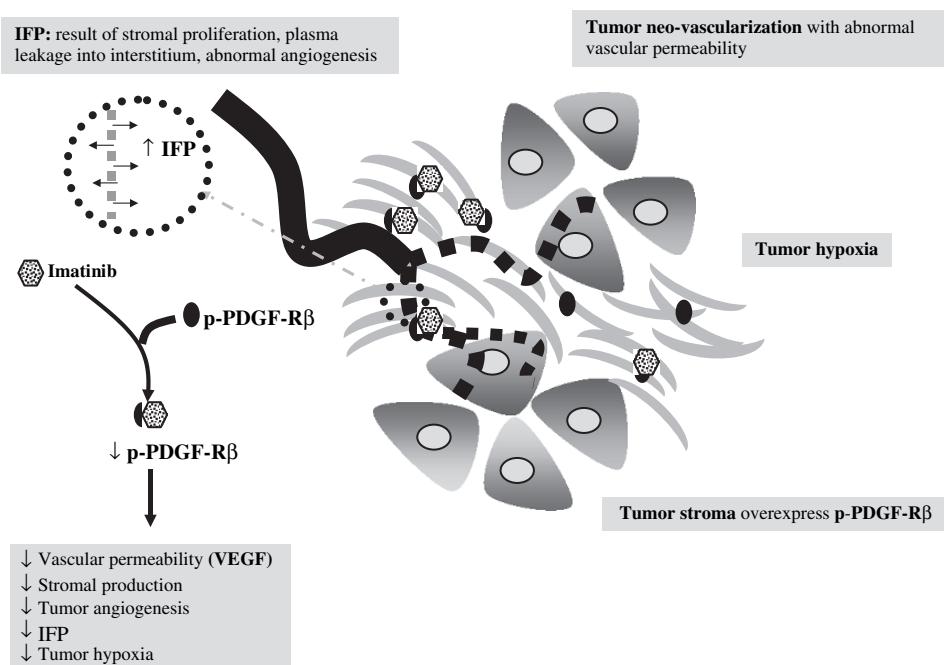


Fig. 5. Schematic diagram illustrating the proposed mechanism of action by imatinib. Phosphorylated platelet-derived growth factor receptor- β inhibition and vascular endothelial growth factor (VEGF) downregulation decreases stromal production, vascular permeability, tumor hypoxia, and IFP. Reproduced with permission from (57).

kinase inhibitor. Imatinib treatment efficiently inhibits phosphorylation of PDGFR- β in A549 mouse xenografts. Imatinib treatment decreased tumor hypoxia and lowered interstitial fluid pressure by inhibiting VEGF expression and angiogenesis. This tumor vessel normalization in response to imatinib treatment facilitated oxygen transport and enhanced drug delivery. Therefore, the paracrine relationships between tumor cells, endothelial cells, and stromal cells determine multiple tumor microenvironmental factors and directly affect the efficiency of radiotherapy and chemotherapy (Fig. 5).

5. VASCULAR REMODELING—INTUSSUSCEPTION AND PRUNING

Intussusception is the formation of new vessels by insertion of transcapillary tissue posts into an existing vessel, using a mechanism that does not involve sprouting or endothelial cell proliferation. The first step in intussusceptive growth is creation of a contact point in the lumen between endothelial cells from opposing capillary walls. The intercellular junctions of the endothelial layer are then reorganized to create central perforations, and an interstitial pillar core is formed from invading endothelial support cells. These pillars then enlarge, stabilized by the migration of pericytes and the laying down of interstitial matrix proteins to form a thicker wall between the vessels (62). Although intussusception has been reported to occur in tumors (23, 63), it has been most extensively studied in the chorioallantoic membrane and in a variety of developing organs (64). At least three different types of intussusception occur:

intussusceptive microvascular growth (IMG), which expands the capillary plexus; intussusceptive arborization (IAR), which develops arterial and venous feeding vessels; and intussusceptive branching remodeling (IBR), which alters arterial and venous bifurcations (62, 65) (Fig. 6). Intussusception does not require VEGF, and in fact in one tumor line the process was most active in tumor regions devoid of VEGF expression (63). However, studies in the chick chorioallantoic membrane have indicated that VEGF can stimulate this process, depending upon how it is presented to the tissue (acute vs. chronic exposure) (24). In addition to creating new segments through sprouting and intussusception, vessels also eliminate unnecessary segments of vasculature through vessel pruning(65).

The mechanisms regulating intussusceptive angiogenesis are much less well characterized than those for sprouting angiogenesis. There is clear evidence that shear stress is involved. If shear stress is acutely modified, ion channels within endothelial cells are activated, resulting in rearrangements in cytoskeleton and gap junctions within minutes to hours (62). Mature (stable) formation of intussusceptive angiogenesis involves interactions between endothelial cells and pericytes, which leads to the hypothesis that Tie2 and the angiopoietins may be involved (50,62). The relative lack of pericytes in tumors could be influential in the stability of this process in tumors. Currently, it is not known whether hypoxia alters vascular intussusception. However, it has been hypothesized that pruning can be regulated by vessel shear stress as well as hypoxia (50). Pries et al. have modeled structural responses of microcirculatory networks to small changes in demand and have compared the predictions to experimental observations. Their conclusion was that the primary mode of control was through shear stress, as compared with transmural wall pressure and oxygenation (66). The molecular-signaling processes that govern intussusception are not well understood, but it is speculated that many of the ligands and receptors involved in sprouting angiogenesis may play a role (Table 2). Additionally, theoretical analyses based on experimental observations indicate that the initiation of vascular adaptation may involve information transfer up and down

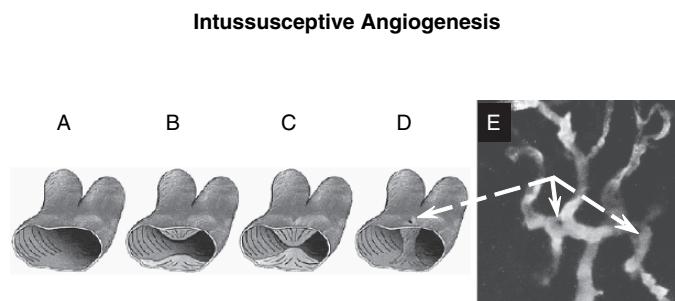


Fig. 6. Intussusceptive angiogenesis—the alternative to capillary sprouting. Three-dimensional representation of discrete steps in intussusceptive angiogenesis: (A) a capillary before intussusceptive angiogenesis. (B) Endothelial cells opposite of each other in the capillary wall protrude into the lumen and form a pillar. (C) Direct contact of the protruded endothelial cells. (D) Perforation of the endothelial pillar forms a cylindrical bridge extending across the capillary lumen. (E) A confocal microscopic image of intussusceptive angiogenesis. Arrows: cylindrical endothelial bridges during intussusceptive angiogenesis. Fig. 6A–D reproduced with permission from (24). Panel E (Unpublished data from Dr. Dewhirst and Dr. Matthew Dreher).

Table 2
Factors Regulating Intussusceptive Angiogenesis

| <i>Category</i> | <i>Regulating Factors</i> | <i>Effects</i> |
|------------------------------|---|---|
| Physical factors | Hemodynamic forces, shear stress (in endothelial cells), and wall stress (in smooth muscle cells) | Activation of ion channel, rearrangements of cytoskeletal system, and changes in gap-junction complex |
| Environmental factors | Hypoxia, normoxia, and hyperoxia | Induce multiple growth factors involved in vessel destabilization, angiogenesis, and remodeling and adjust VEGF expression to pro-angiogenic, maintenance and sub-maintenance levels to cooperate with other growth factors |
| Growth factors | Angiopoietin-1, angiopoietin-2, Tie-receptors, PDGF- β , monocyte chemotactic protein 1, ephrins, and Eph-B receptors | Recruitment of pericytes in type I and type IV pillars and stabilize intussusceptive endothelial meshes. |

PDGF- β , platelet-derived growth factor beta and VEGF, vascular endothelial growth factor.

the vascular network by as yet clearly defined mechanisms. In a region where acute changes in shear stress occur, it is speculated that information transfer occurs through transmission up and down the vascular network or through metabolic changes. For example, if a particular segment experiences a change in diameter, then the resultant shear stress change will alter the flow properties of the contiguous segments up- and down-stream, leading to vascular responses.

In tumors, hypoxia may influence intussusception in an indirect way. One of the hallmarks of tumor microvasculature is the presence of microvascular hypoxia (67,68). Although some have speculated that this is due to temporary flow stasis, we have shown that it is due to (i) longitudinal tissue oxygen gradients that result from inadequate arteriolar supply (69), (ii) relatively low vascular density with disorganized vascular geometry (70), and (iii) oxygen demand that is out of balance with supply (71). Importantly, hypoxia occurs in microvessels that are actively perfused. The combination of low pO_2 and acidosis decreases the deformability of red cells by causing them to shrink, thereby losing optimal volume to surface area ratio. The crenation of these cells increases red cell suspension viscosity, leading to increased flow resistance and rouleaux formation (72). The increase in blood viscosity alters shear stress, thereby creating a scenario that is primed for stimulation of vascular adaptation (Fig. 3).

6. TUMORS ARE “WOUNDS THAT DO NOT HEAL”

Wound healing presents a unique paradigm regarding angiogenesis as a mechanism to re-establish homeostasis. It is well established that neovasculature is present only to facilitate the closure of the wound. Once that occurs, the neovasculature regresses, leaving in its wake an avascular scar (73). We serially studied punch biopsy wounds of

rats to monitor angiogenesis, growth factor expression, and hypoxia. Surprisingly, the initial surge of VEGF, bFGF, and TGF- β , occurring 24 h after the wound was created, was not associated with hypoxia; it likely came from tissue stores of these cytokines as well as from platelets. The greatest level of hypoxia was observed at a point in time where the wound surface had re-epithelialized and there was active proliferation in many cells of the maturing wound. The hypoxia at this time point was ubiquitous, involving endothelial cells, macrophages, and fibroblasts. It was hypothesized that the hypoxia was induced as a result of high oxygen consumption by the granulating wound. Concomitant with the hypoxia, there was widespread apoptosis of endothelial cells (73). In the following days, the vasculature continued to regress, eventually leaving a fibrous, avascular scar. It is interesting to speculate that the signal for wound vessel regression may have been hypoxia, as upregulation of factors such as P53 in response to hypoxia could lead to apoptosis (74).

Tumor microvasculature can demonstrate a similar behavior, exemplified by the onset of new vessel formation followed by vascular regression and/or pruning (Fig. 7). The difference with tumors, of course, is that the signals for new vessel formation do not cease, leading to the paradigm coined by Dvorak, “tumors are wounds that do not heal” (75). Thus, when vessels regress, there remains a stimulus for a new wave of angiogenesis. Whether or not vessels undergo regression is also dependent upon the maturity of the vessel and the balance of factors that favor survival versus apoptosis. In this regard, VEGF is believed to be an important survival signal for immature vasculature, whereas Ang-1 is believed to be an important signal for maintenance of mature vessels (50).

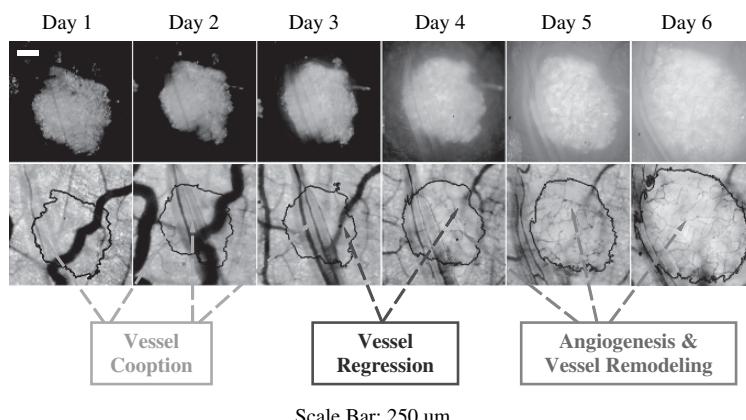


Fig. 7. Tumor vessel cooption, regression, and angiogenesis. Human colon cancer cells (HCT116 with a constitutively expressed red fluorescence protein gene) were inoculated into a nude mouse dorsal skin-fold window chambers on day 0. Cooption of host vessels occurred first. With continued tumor growth, pre-existing vessels destabilized. Vessel regression, angiogenesis, and vessel remodeling reveal dynamic day-to-day changes in this developing tumor vasculature (black closed curves). Scale bar, 250 μ m (Unpublished data of Y.C. and M.W.D.).

7. EFFECTS OF CANCER THERAPY ON ANGIOGENESIS

Teicher was the first investigator to show that the combination of angiogenesis inhibition with either chemotherapy or radiation therapy yielded superior anti-tumor effects compared with either treatment alone (76). This occurred while tumor oxygenation was improved, and it was speculated that the improvement in oxygenation favored increased radiosensitivity (77). This result was surprising to many, who speculated that use of anti-angiogenic therapies would lead to reduction in vascular density and increased tumor hypoxia. However, it put important emphasis on the role of the endothelial cell in controlling treatment response. This result, along with the suggestion that selective killing of endothelial cells would be a very efficient means for killing tumor cells as a result of ischemia, led to the development of therapies that selectively target tumor vascular endothelium (78).

It has been speculated that the key target cell for radiotherapy is the endothelial cell. Garcia-Barros and coworkers (79) studied the role of the endothelial cell in tumor response to radiotherapy by using a sphingomyelinase-deficient knockout mouse. Endothelial cells of this mouse are resistant to radiation-induced apoptosis because of the deficiency in this enzyme. Identical tumor lines transplanted into wild-type versus the knockout strain showed remarkable resistance to radiation treatment in the latter. Although this chapter stimulated significant controversy (80), there are other emerging evidence supporting the importance of the endothelial cell in governing treatment response.

Forty years ago, Rubin and Cassarett (81) described a “supervascularized” state after radiotherapy, using a microangiographic technique in a murine tumor model. In fact, this phenomenon was thought for many years to be responsible for the process of tumor “reoxygenation,” which provides logic for using fractionated radiotherapy to take advantage of improved oxygenation in subsequent treatments. Since that time, others have reported on this same type of phenomenon using a variety of pre-clinical models (82, 83).

We have recently reported, however, that tumor reoxygenation may have negative consequences for treatment efficacy (84). Using a fluorescent reporter of HIF-1 activity, we found that HIF-1 signaling increased twofold after radiotherapy, peaking 48 h after the last treatment fraction (Fig. 8A). This activation was associated with increased HIF-1 protein levels, as well as increased expression of several downstream proteins that are important for stabilizing tumor endothelium, such as VEGF and bFGF. Therefore, it was reasoned that radiation-induced HIF-1 activation might contribute to treatment resistance by minimizing radiation damage to the tumor vasculature. This hypothesis was proven correct in experiments using RNA interference and YC-1, a drug recently found to inhibit HIF-1, which were both able to significantly interfere with the ability of tumors to protect endothelial cells from radiation damage. The HIF-1 pathway, then, may serve as a critical “node” for radiation resistance whose targeting could significantly improve radiotherapy.

Mechanistically, radiation-induced HIF-1 hyperactivity was found to be attributable to two separate events: (i) HIF-1 α stabilization in aerobic tumor regions through production of free radicals and (ii) dissolution of hypoxia-induced stress granules during reoxygenation. We demonstrated the relative importance of free radicals in stabilizing HIF-1 in several ways. First, we showed that free radicals were produced in tumors after radiation treatment and that scavenging of these free radicals with a small molecule superoxide dismutase (SOD) mimetic blocked both the upregulation

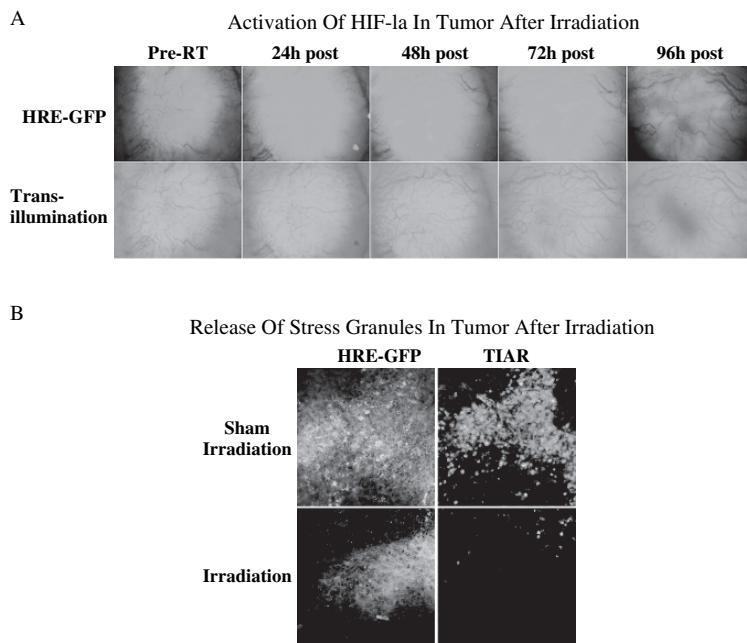


Fig. 8. Increased hypoxia-inducible factor 1 (HIF-1) activity and release of stress granules post-irradiation. **(A)** A representative time course of HIF-1-driven green fluorescence protein (GFP) reporter activity following radiation. 4T1 mouse mammary carcinomas, stably expressing HIF-1 green fluorescence protein reporter construct, were grown in dorsal skin-fold window chambers implanted onto Balb/C mice. Tumors were irradiated (2×5 Gy) and monitored with serial intravital fluorescence microscopy to determine relative HIF-1 activity levels. HIF-1 signaling typically peaked 48 h after treatment. **(B)** 4T1 mouse mammary carcinoma tumor sections were stained with an anti-TIAR antibody to visualize stress granules. In sham-irradiated tumors, these granules demonstrated tight colocalization with hypoxia, as marked by an endogenous HIF-1-driven GFP reporter. In irradiated tumors, examined 48 h after treatment, stress granules were much less abundant. Data from the work of M.W.D., Y.C., and Benjamin Moeller.

of HIF-1 protein levels and signaling activity after radiation treatment. Importantly, we demonstrated that when the SOD mimetic was given after radiotherapy, it led to significant vascular regression, supporting the theory that HIF-1 is a critical targetable molecule regulating vascular radiosensitivity.

Stress granules are a recently recognized defense mechanism identified in a wide variety of eukaryotic cells (85, 86). They are composed of several mRNA-binding proteins and stress-responsive proteins that coalesce in the cytoplasm and sequester transcripts so that they cannot enter the endoplasmic reticulum to be translated to protein. They assemble when the cell is exposed to a stressor (e.g., heat shock and osmotic shock) and disassemble when the stress is alleviated. Teleologically, stress granules are believed to function to prevent cells from expending crucial energy unnecessarily during potentially lethal stress conditions. We found that hypoxia is amongst the stressors that can stimulate stress granule polymerization and that stress granules are abundant in hypoxic regions of tumor tissue (Fig. 8B). Moreover, HIF-1-regulated transcripts, in particular, appear to associate with stress granules during hypoxia. Disrupting stress granule polymerization, by expressing a mutant form of a stress

granule scaffolding protein, significantly increased the ability of tumor cells to upregulate downstream HIF-1 targets during hypoxia. When tumors reoxygenate, as occurs during treatment, these stress granules depolymerize and allow their previously sequestered hypoxia-induced transcripts, including those stimulated by HIF-1 activity, to be translated.

These two mechanisms contributed, therefore, to a HIF-1-dependent proangiogenic stimulus after radiotherapy that, in turn, protected tumors from radiation damage to their vasculature. This mechanism is likely to occur following any treatment that leads to tumor cell apoptosis and reoxygenation, but it is predicated on a pre-existing condition of hypoxia (*in vitro* we observed stress granule formation after a few hours at 0.5% O₂). For example, Taxol has been reported to induce apoptosis and increase tumor oxygenation (87). Hyperthermia treatment has also been reported to cause reoxygenation in pre-clinical models and in clinical trials (88, 89). As we have previously shown that VEGF is important for tumor cell survival post transplant by a yet to be defined paracrine mechanism, one can conclude that therapies that cause reoxygenation will favor endothelial cell survival. It is also important to note that the instability in tumor oxygenation at baseline, discussed above, could also contribute to stabilization of HIF-1-mediated transcripts, through the same mechanisms described for irradiated tumors.

More recently, we have shown that HIF-1 upregulation after radiation treatment participates in radiosensitization of tumor cells as assessed by apoptosis induction and by clonogenic assay. The sensitization of tumor cells by HIF-1 appears to be related to P53, as a tumor cell line that was P53 null did not show this phenotype. These radiosensitizing effects of HIF-1 are opposite to the radioprotective effects seen with vascular endothelium. Importantly, however, it was demonstrated that proper

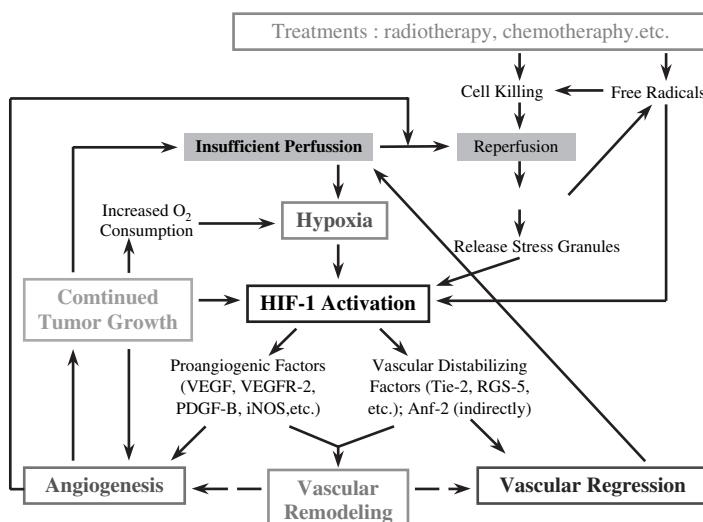


Fig. 9. Cycle between angiogenesis, perfusion, and hypoxia in tumors. The network of interactions between tumor growth, perfusion, angiogenesis, and hypoxia. The process of tumor reoxygenation, occurring as a result of instabilities in perfusion (causes depicted in more detail in Fig. 2) and/or as a result of therapeutic interventions, serves to initiate HIF-1 promoter activity, promoting angiogenesis and tumor cell survival.

sequencing of radiation and HIF-1 inhibition can lead to enhanced anti-tumor effect. In particular, inhibition of HIF-1 after radiation exposure was the most effective sequence. In a separate report, it was shown that a HIF-1 knockout tumor line is more sensitive to radiotherapy than a wild-type line (90). These results provide strong rationale for developing methods to inhibit HIF-1 as an adjuvant to radiotherapy.

8. CONCLUSIONS

In this brief review, we have emphasized the dynamic nature of tumor angiogenesis, which interplays with the fundamental limitations of oxygen delivery to create a tumor microenvironment that is typified by cycles of hypoxia and reoxygenation. This type of injury leads to increased concentrations of free radicals, which in turn contribute to upregulation of HIF-1, propagation of angiogenesis, and alterations in other cellular functions that promote survival of both tumor and endothelial cells (Fig. 9). Tumor therapies that cause reoxygenation can further exacerbate this prosurvival interdependency between tumor and endothelial cells. The results suggest that successful therapies should selectively target HIF-1 and/or its downstream target genes, such as VEGF, to break this cycle of interdependency.

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3

The Role of Integrins in Tumor Angiogenesis

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SUMMARY

An understanding of the mechanisms regulating endothelial cell roles in angiogenesis has led to the development of novel anti-tumor agents directed against members of the integrin family of cell adhesion proteins. Several integrins play important roles in promoting endothelial cell migration and survival during angiogenesis. Antagonists of these integrins either suppress cell migration and invasion of endothelial cells, suppress intercellular adhesion of endothelial cells and pericytes, or induce apoptosis of endothelial cells. Integrin antagonists also block tumor angiogenesis and metastasis. Currently, humanized or chimeric antibody antagonists of integrins $\alpha 5\beta 1$, $\alpha v\beta 3$ and $\alpha v\beta 5$ and peptide inhibitors of these integrins are in clinical trials as angiogenesis-inhibiting agents for cancer therapy.

Key Words: Integrin $\alpha 5\beta 1$; integrin $\alpha 4\beta 1$; integrin $\alpha v\beta 1$; integrin $\alpha v\beta 3$; angiogenesis; apoptosis; endothelial cell; pericyte.

1. INTRODUCTION

The integrin family of adhesion proteins is an extensive group of cell structurally related receptors for extracellular matrix (ECM) proteins and immunoglobulin superfamily molecules. Integrins promote cell attachment and migration on the surrounding ECM and also mediate interactions with neighboring cells during embryonic development, tissue repair, inflammation, angiogenesis, and lymphangiogenesis (1, 2). Tumors typically arise from acquired mutations in cellular proliferation and survival pathways. Like all cells, tumor cells require a supply of nutrients and oxygen for survival; therefore, tumor growth depends on the development of vascular network (3, 4). In addition to providing nutrition, vascular networks can promote dissemination of the tumor cells to distant sites by a process called metastasis (3, 5).

In recent years, many of the mechanisms that promote tumor angiogenesis have been delineated. Tumor cell expression of proangiogenic growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), leads to

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endothelial cell activation and cell proliferation (3). *De novo* expression of integrins such as integrin $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha v\beta 3$ then promotes the survival and migration of endothelial cells. Each of these events is critical for development of a functional vasculature; antagonists of both growth factors and these integrins have been shown to block tumor angiogenesis and metastasis (6).

In this article, we will review the structure and function of integrins in general as well as the roles of integrins in angiogenesis during normal development and tumorigenesis. We will also discuss clinical studies of integrin antagonists as anti-angiogenic agents in cancer therapy.

2. INTEGRIN STRUCTURE AND LIGAND SPECIFICITY

Integrins are heterodimeric membrane glycoproteins comprised non-covalently associated α - and β -subunits (1). Eighteen α - and eight β -subunits have been identified in mammals; these can associate to form 24 different integrin heterodimers (7). Each integrin subunit consists of an extracellular domain, a single transmembrane region, and a short (~30–40 amino acids) cytoplasmic region (1, 8).

2.1. Structure of the α -Subunit

The N-terminus of the α -chain consists of a β -propeller domain that is formed by seven repeats of 60 amino acids each (9, 10). The β -propeller domain is linked to the transmembrane domain by three regions that have been named the Thigh, Calf-1, and Calf-2 domains (Fig. 1A). In addition, a highly flexible region, termed the Knee, is present between the Thigh and Calf-1 domains (10). Half of all α -chains have an additional 200 amino acid inserted domain between repeats two and three of the β -propeller (the I-domain), which is homologous to the A domain of von Willebrand factor (Fig. 1A) (11). The I-domain functions as the major ligand-binding site in those integrins with this domain, whereas the β -propeller serves as the ligand binding in integrins without I-domains (12). Cytoplasmic tail domains of individual α -subunits are well-conserved across species boundaries (13, 14).

2.2. Structure of the β -Subunit

The N-terminal region of the β -subunit consists of a cysteine-rich region termed the plexin-semaphorin-integrin (PSI) domain. C-terminal to this domain is an evolutionarily conserved I-like domain flanked on either side by immunoglobulin folds called hybrid domains. The membrane proximal region of the β -subunit contains four EGF-like repeats. The β -subunit also has a flexible “knee” region, which is formed by the hybrid domain and the first two EGF-like repeats (Fig. 1B) (12). The intracellular regions of the β -subunits are more conserved between subunits than are the α -subunit cytoplasmic tails (14, 15). These beta chain cytoplasmic tails play significant roles in regulating integrin activity (13). The $\beta 4$ subunit differs from the other β -subunits by the presence of a long intracellular domain (around 1000 amino acids) that comprises four fibronectin type III-like repeats (Fig. 1C) (8, 16, 17).

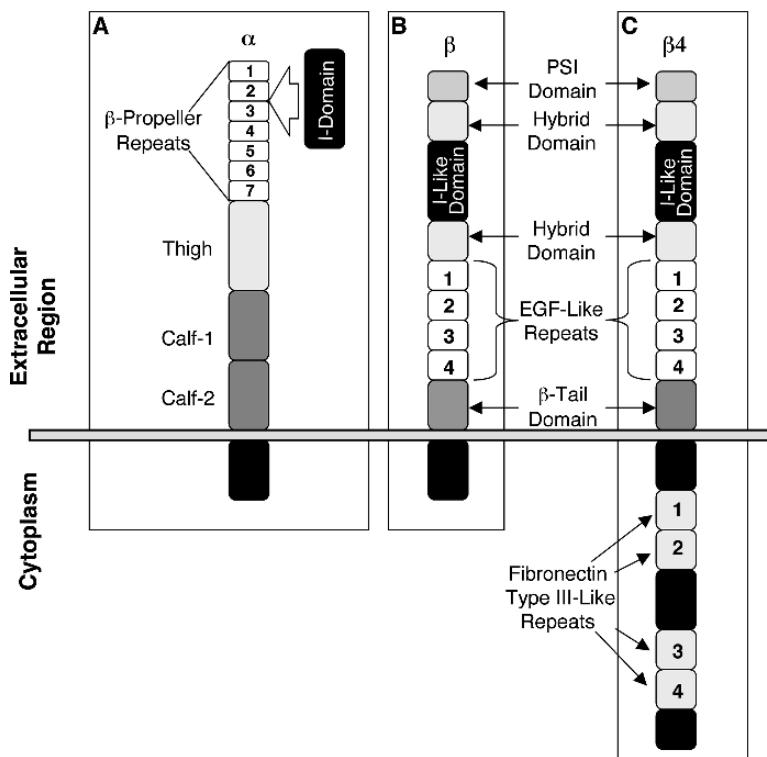


Fig. 1. Integrin structure. (A) Primary structure of integrin α -subunits. Half of the β -subunits also have an I-domain inserted between β -propeller repeats 2 and 3. (B) Primary structure of integrin β -subunits. (C) The 1000 amino acid β_4 -subunit cytoplasmic tail differs from that of other β -subunits.

2.3. Ligand Specificity of Integrins

Each α -chain combines with a β -chain to form a unique heterodimer with selectivity for ECM proteins, cell surface molecules, plasma proteins, or microorganisms (18–20). Integrins bind to their ligands in a divalent cation-dependent fashion (21, 22). Although some integrins recognize primarily a single ECM protein ligand (e.g., $\alpha_5\beta_1$ recognizes primarily fibronectin), others can bind several ligands (e.g., integrin $\alpha v\beta 3$ binds vitronectin, fibronectin, fibrinogen, denatured or proteolyzed collagen, and other matrix proteins). Many integrins recognize the tripeptide Arg–Gly–Asp (RGD) (e.g., $\alpha v\beta 3$, $\alpha 5\beta 1$, $\alpha IIb\beta 3$, $\alpha v\beta 6$, and $\alpha 3\beta 1$), but sequences flanking the RGD peptide are also important for selectivity (18, 19). Other integrins recognize alternative short peptide sequences (e.g., integrin $\alpha 4\beta 1$ recognizes Glu Ile Leu Asp Val (EILDV) and Arg Glu Asp Val (REDV) in alternatively spliced CS-1 fibronectin and $\alpha IIb\beta 3$ binds KQAGDV in the fibrinogen γ chain) (1, 6). In addition, some integrins can also bind cell surface receptors to induce cell–cell adhesion. For example, integrin $\alpha 4\beta 1$ can bind VCAM-1 and MadCAM-1 on neighboring cells, in addition to binding ECM proteins (19, 20). The ligands bound by common integrins and their recognition sites are shown in Table 1.

Table 1
Integrin Ligands and Recognition Sequences

| <i>Integrin</i> | <i>Ligands (recognition sequences)</i> |
|---------------------|--|
| $\alpha 1\beta 1$ | Collagen (GFOGER, fibrillar collagen domain) and laminin |
| $\alpha 2\beta 1$ | Collagen (GFOGER, fibrillar collagen domain), laminin, and $\alpha 3\beta 1$ |
| $\alpha 3\beta 1$ | Fibronectin (RGD), collagen, laminin, epiligrin, entactin, and $\alpha 2\beta 1$ |
| $\alpha 4\beta 1$ | CS-1 fibronectin (EILDV) and VCAM-1 (QIDS) |
| $\alpha 5\beta 1$ | Fibronectin (RGD), fibrinogen (RGD), and L1-CAM |
| $\alpha 6\beta 1$ | Laminin (several sites), merosin, and kalinin |
| $\alpha 7\beta 1$ | Laminin |
| $\alpha 8\beta 1$ | Fibronectin (RGD) and tenascin |
| $\alpha 9\beta 1$ | Tenascin (AEIDGIEL), collagen, and laminin |
| $\alpha 10\beta 1$ | Collagen |
| $\alpha 11\beta 1$ | Collagen |
| $\alpha L\beta 2$ | ICAM-1, ICAM-2, and ICAM-3 (ICAM peptides) |
| $\alpha M\beta 2$ | iC3b, fibrinogen (P1 and P2 peptide in γ -chain), factor X, and ICAM-1 (ICAM peptides) |
| $\alpha X\beta 2$ | iC3b and fibrinogen (GPR in α -chain) |
| $\alpha D\beta 2$ | ICAM-3 and VCAM-1 |
| $\alpha v\beta 1$ | Fibronectin (RGD) and vitronectin (RGD) Fibronectin (RGD), vitronectin (RGD), von Willebrand Factor (RGD dependent), |
| $\alpha v\beta 3$ | thrombospondin (Cryptic RGD site), tenascin (RGD), Del-1 (RGD), osteopontin (RGD), MMP2 (PEX domain), and bFGF (DGR) |
| $\alpha v\beta 5$ | Vitronectin (RGD and KKQRFRHRNRKG), osteopontin, and Del-1 (RGD) |
| $\alpha v\beta 6$ | Fibronectin (RGD) and tenascin (DLXXL) |
| $\alpha v\beta 8$ | Collagen, laminin, and fibronectin |
| $\alpha IIb\beta 3$ | Fibronectin (RGD), Fibrinogen (KQAGDV), vitronectin (RGD), and von Willebrand factor (RGD) |
| $\alpha 6\beta 4$ | Laminin (several sites) |
| $\alpha 4\beta 7$ | CS-1 fibronectin, VCAM-1, and MAdCAM-1 (LDT) |
| $\alpha E\beta 7$ | E-Cadherin |

This table lists the integrin subunits by family ($\beta 1$, $\beta 3$, αv , and other) and indicates major ligands to which each integrin binds.

2.4. Integrin Crystallography

Electron microscopy (EM) studies have shown that the integrin complex resembles two stalks (“legs”) with a globular “head” region at the extracellular N-terminus (23, 24). The two subunits interact in the “head” region to generate binding sites that recognize specific ligands (18). Crystallography of tail-less integrin $\alpha v\beta 3$ complexes revealed that the major contact between the subunits occurs between the α chain β -propeller and the I-like domain of the β -chain (10). Furthermore, these studies also revealed that the globular head region was bent toward the plasma membrane at the knee region (Fig. 2A). Crystal structures of $\alpha v\beta 3$ integrin bound to cyclic RGD ligand peptide revealed that the ligand-binding region was located between the α -chain β -propeller domain and the β -subunit I-like domain (25). NMR and EM analyses have shown that in its low affinity state, when the integrin is not bound to its ligand,

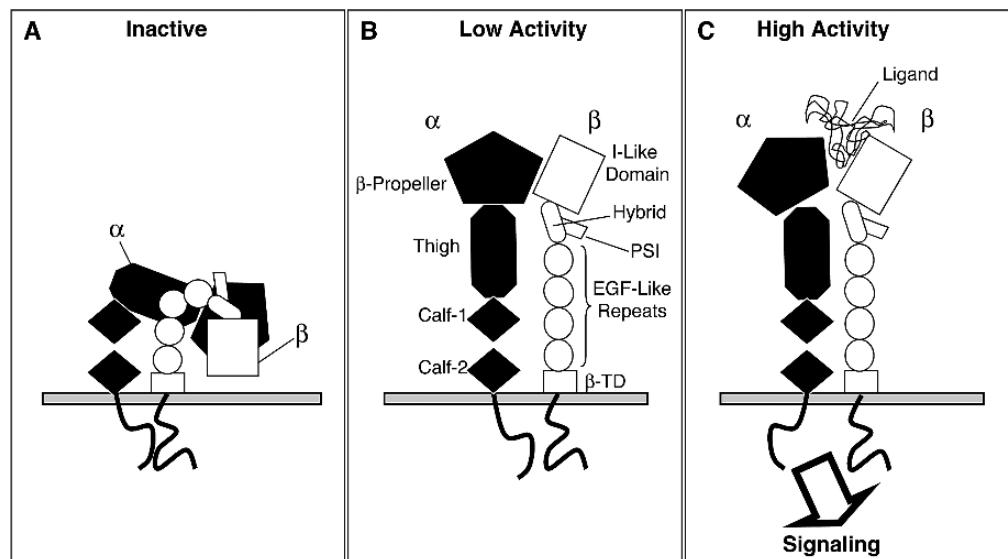


Fig. 2. Conformational changes during integrin activation. In the inactive state, integrins are bent toward the plasma membrane and the cytoplasmic domains are closely associated (A). On activation, conformational changes allow the extracellular region of the integrin to elongate and the cytoplasmic regions begin to separate (B). In the highly active state, the head region of the integrins is in an open conformation, which allows ligand binding. The cytoplasmic domains are well separated, permitting interactions with cytoplasmic proteins and intracellular signaling (C).

the headpiece is bent toward the membrane, and the cytoplasmic regions of the two subunits are closely associated with one another (26, 27). EM studies on different activation states of integrins have shown that activation is associated with an unbending and elongation of the dimer (27, 28) (Fig. 2B). Activation also requires separation of the cytoplasmic domains (27–29), which allows the tail region to bind cytoplasmic proteins and initiate signaling events (13) (Fig. 2C).

3. INTEGRIN ACTIVATION INITIATES MULTIPLE SIGNALING PATHWAYS

Binding of polyvalent or crosslinked ECM ligands by integrins leads to integrin clustering nucleating the formation of focal adhesions at the sites of attachment to the substratum. Focal adhesions comprises integrins, as well as protein kinases, adaptor proteins, signaling intermediates, and actin-binding cytoskeletal proteins such as talin, α -actinin, paxillin, tensin, and vinculin. Focal adhesions provide a scaffold for the initiation of integrin and growth factor-mediated signaling cascades (Fig. 3A) (30). Unlike growth factor receptors, integrins have no intrinsic enzymatic activity (31, 32) but activate signaling pathways by coclustering with kinases and adaptor proteins in focal adhesion complexes.

Extensive studies have shown that the β -subunit cytoplasmic tail interacts directly with several cytoskeletal proteins such as talin (33), α -actinin (34–36), and paxillin, as well as non-receptor kinases, such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK) (31). Several α -chain-binding proteins have also been identified.

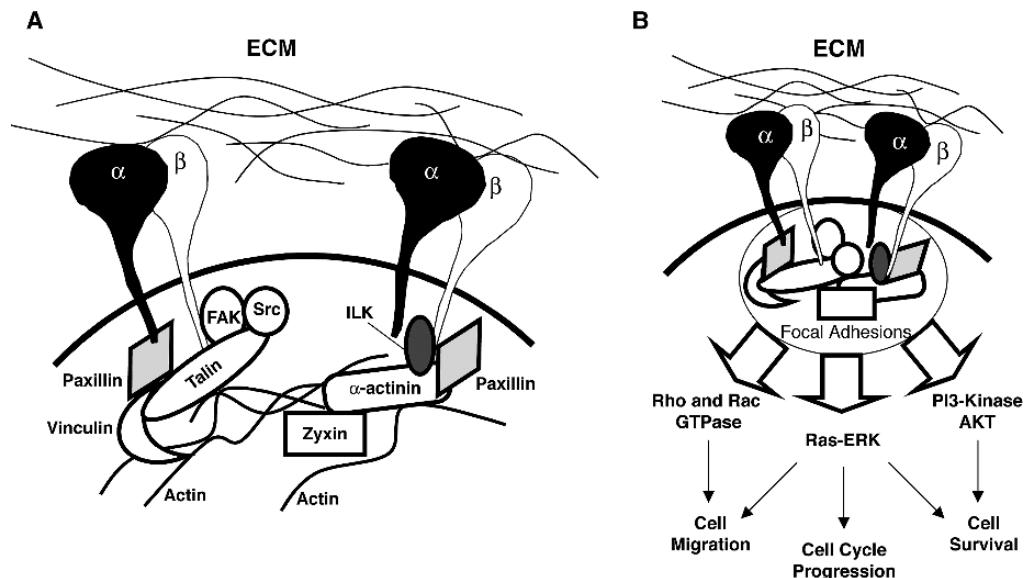


Fig. 3. Focal adhesion formation and downstream signaling. Binding of integrins to ligands leads to conformational changes in integrins that result in recruitment of various proteins to the cytoplasmic domains of the integrins to form focal adhesions (A). These focal adhesions are sites of active signaling, and the signaling cascades initiated here regulate several critical cellular processes such as migration, survival, and proliferation (B).

Cytoplasmic domains of α -chains are very diverse and contain a single membrane proximal conserved domain that binds calreticulin, which is required for normal integrin-mediated cell adhesion (31). Another molecule, paxillin, has been shown to bind to the $\alpha 4$ chain and to thereby induce cell migration (31, 37). However, much remains to be discovered regarding α -chain roles in integrin function.

The signaling cascades initiated in focal adhesions control a variety of physiological process, such as cell adhesion, migration, proliferation, and even survival. For example, integrins stimulate cell migration by activating Rho and Rac GTPases (38) and by anchoring actin filaments to the membrane. Integrin signaling also induces cell migration through the Ras–Erk pathway (32, 39). Integrin-induced signaling through the Ras–Erk pathway promotes cell-cycle progression by inducing cyclin D1 expression. In addition, integrins also synergize with growth factors to promote cell proliferation (40). In most normal cells, cell proliferation is dependent on cell adhesion. Loss of adhesion leads to a block in the cell-cycle machinery and eventually to cell death (anoikis). Integrin signaling promotes cell survival through Akt- and Raf-signaling cascades, which can block the mitochondrial apoptotic pathway. In addition, the Ras–Raf–Erk pathway can also modulate death receptor-mediated cell death (41). The various signaling cascades originating from the focal adhesions are shown in Fig. 3B. Interestingly, unligated integrins can initiate apoptosis in a stress response and death receptor-independent manner termed “integrin-mediated death” or IMD (Fig. 4) (41, 42).

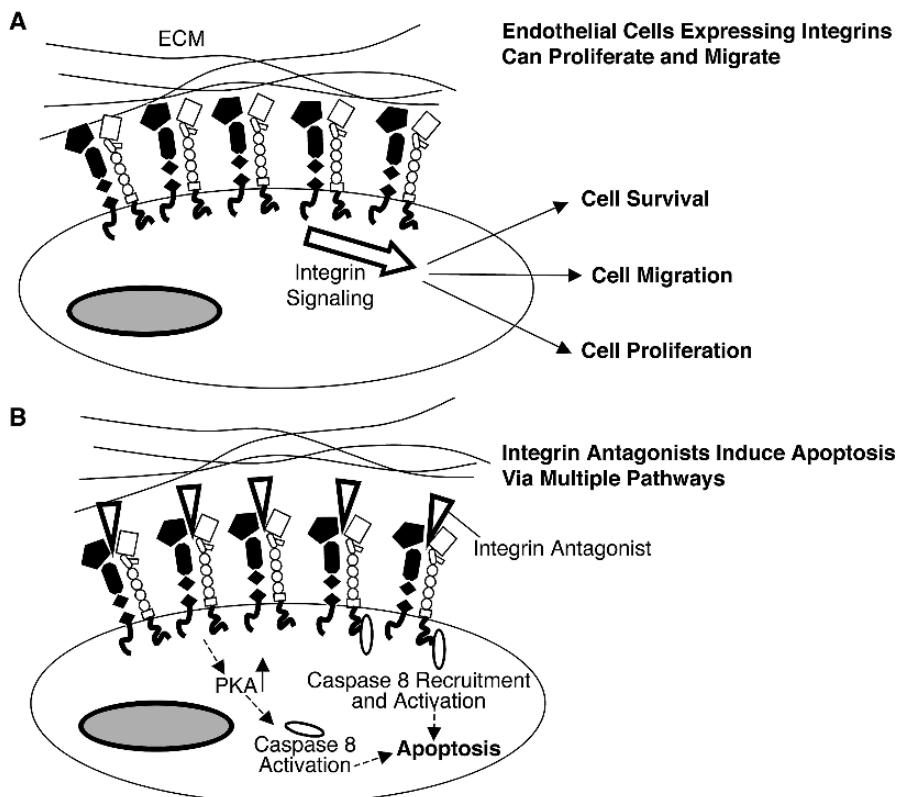


Fig. 4. Integrin antagonists induce endothelial cell apoptosis. In the presence of the ligands, integrins on endothelial cells signal to promote cell survival, proliferation, and cell migration (A). However, in the absence of the appropriate ligand or in the presence of antagonists that prevent binding to ligand (B), integrins induce apoptosis by activating PKA, which in turn activates caspase-8. Alternatively, integrins may directly recruit and activate caspase-8.

4. REGULATION OF INTEGRIN SIGNALING

4.1. Outside-In and Inside-Out Signaling

Integrins expressed on the cell surface exist in active or inactive states. When inactivated, integrins bind ligands and initiate signaling cascades, a process that is termed “outside-in” signaling (Fig. 3B). However, in circulating cells, integrins are generally inactive until these circulating cells are stimulated by external signals. For example, integrin α IIb β 3 is inactive in resting platelets. This integrin becomes activated from within (inside-out signaling) by an external stimulus such as thrombin or epinephrine. These platelet activators induce largely uncharacterized signaling events that stimulate changes to integrin cytoplasmic domains that, in turn, lead to conformational changes in the extracellular domain. Integrin α IIb β 3 then binds its ligand (fibrinogen), leading to ligand binding, platelet aggregation, and outside-in signaling (1, 43) (Fig. 5). Similarly, integrin α 4 β 1 and β 2 activation on leukocytes may be regulated by chemokine signaling.

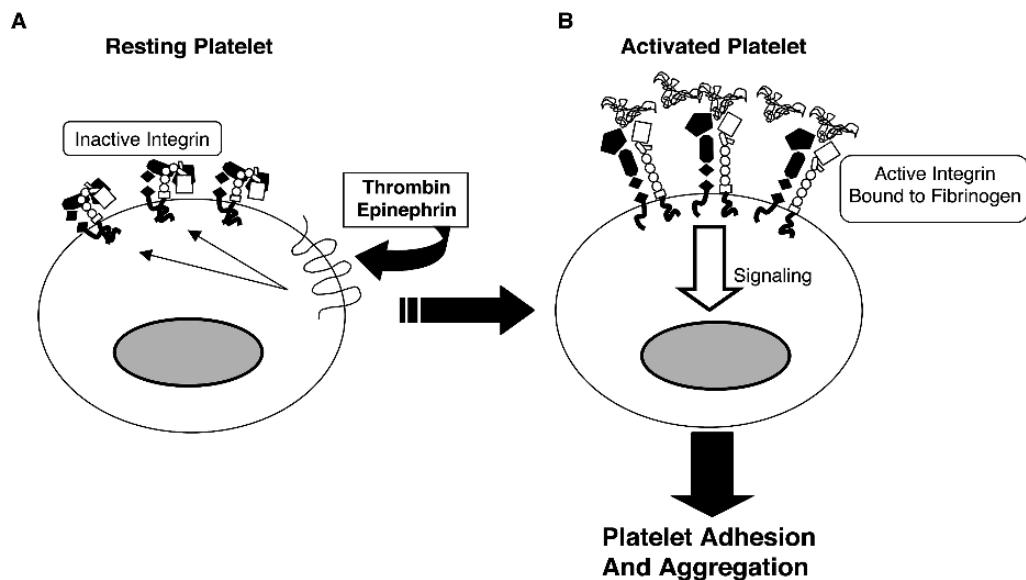


Fig. 5. Platelet activation by inside-out signaling. Integrin $\alpha IIb\beta 3$ on resting platelets is in an inactive state (A). On activation by thrombin or other stimuli, the C-terminal regions of integrins undergo conformational changes, which are relayed to the extracellular domain and lead to elongation and fibrinogen binding. This event promotes platelet aggregation (B).

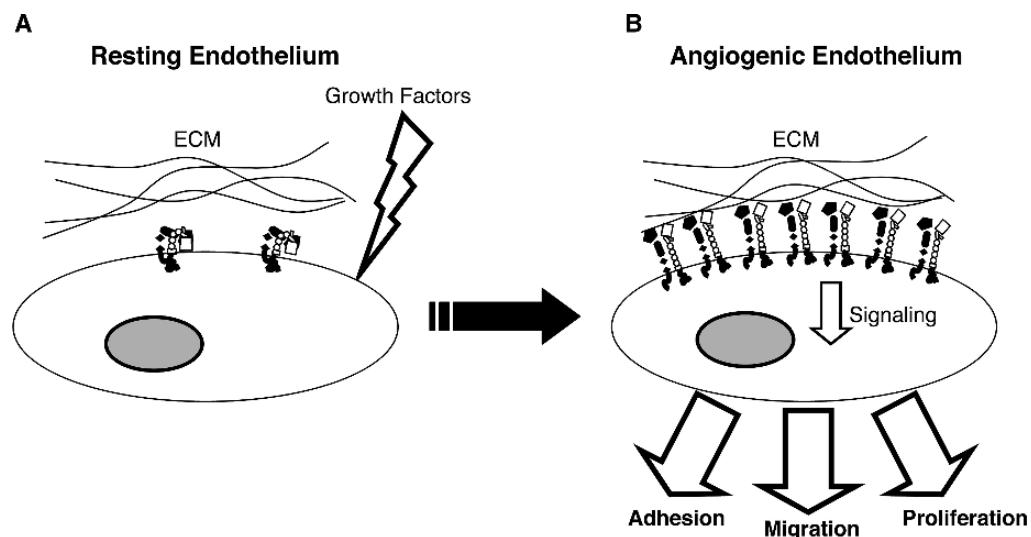


Fig. 6. Integrin activation by gene expression in endothelial cells. Quiescent endothelial cell usually expresses very low levels of integrins in an inactive state (A). Several integrins are upregulated in response to angiogenic growth factors and are expressed on the cell surface in an active conformation. Binding of ligands by these integrins then leads to outside-in signaling and induction of processes that support angiogenesis (B).

4.2. Integrin Expression

Integrin-mediated biological roles are often controlled by integrin expression on the cell surface. For example, quiescent endothelial cells express integrins $\alpha 4\beta 1$, $\alpha v\beta 3$, and $\alpha 5\beta 1$ at low levels. However, stimulation by growth factors like bFGF or VEGF leads to upregulation of the expression of these integrins like $\alpha 4\beta 1$, $\alpha v\beta 3$, and $\alpha 5\beta 1$, thereby promoting integrin signaling and participation in angiogenesis and other processes (44) (Fig. 6).

4.3. Post-Translational Modification (*Phosphorylation and N-Glycosylation*)

Some studies have shown that integrin α - and β -subunits can be regulated by phosphorylation. These modifications promote the interaction of integrins with other cell surface receptors and signaling molecules (14). Other studies suggest that integrin function may be affected by glycosylation (45).

5. INTEGRINS REGULATE DEVELOPMENTAL AND TUMOR ANGIOGENESIS

Neovascularization is the process by which new blood vessels develop in tissues. Angiogenesis is the process whereby new vessels form from pre-existing vessels. The growth of new blood vessels promotes embryonic development, wound healing, the female reproductive cycle, and also plays a key role in the pathological development of solid tumor cancers, hemangiomas, diabetic retinopathy, age-related macular degeneration, psoriasis, gingivitis, rheumatoid arthritis, and possibly osteoarthritis and inflammatory bowel disease (46).

Neovascularization promotes the growth and spread of tumors. New blood vessels in tumors can grow by sprouting from pre-existing vessels (46) or by recruitment of circulating bone marrow-derived endothelial progenitor cells (47, 48). Several cell types within tumors, including tumor cells, monocytes, and fibroblasts, secrete growth factors, such as VEGF that induce blood vessel growth into tumors (49). Studies have shown that angiogenesis plays a major role in tumor growth and that inhibiting angiogenesis can inhibit tumor progression and metastasis. Although growth factors and their receptors play key roles in angiogenic sprouting, adhesion to the ECM also regulates angiogenesis. Formation of new vasculature requires endothelial cell attachment and migration on ECM proteins. One ECM protein, fibronectin is associated with vascular proliferation (50). Mice lacking fibronectin die early in development from a collection of defects including abnormal vasculature (51, 52). As integrins are critical for the cell to bind ECM, many integrins play crucial roles in regulating vascular growth, both during embryonic development and in various pathologies. Proliferating endothelial cells express several integrins that are not expressed on quiescent blood vessels. Studies on mutant mice and in experimental angiogenesis models have shown crucial roles for several integrins in regulating angiogenesis. To date, at least eight heterodimeric integrins ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, $\alpha 5\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$) have been identified in endothelial cells. Roles for these integrins in developmental and tumor angiogenesis are discussed below. Fig. 7 depicts the function of integrins in tumor angiogenesis.

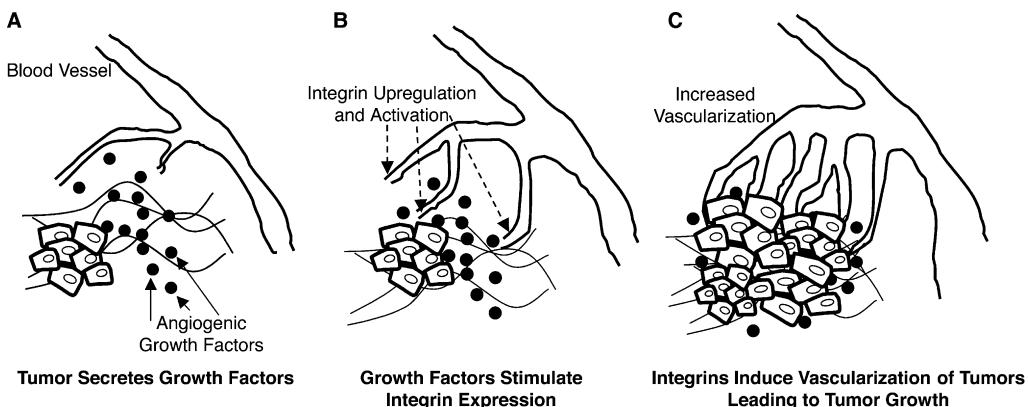


Fig. 7. Integrins regulate tumor vascularization and growth. Nascent tumors secrete growth factors (A), which induce integrin upregulation on neighboring endothelial cells and thereby promotes angiogenesis (B). Increased vascularization of tumors promotes the growth and spread of the cancer (C).

5.1. Integrin $\alpha 5 \beta 1$

Embryonic deletion of integrin $\alpha 5\beta 1$ induces early mesenchymal abnormalities, leading to lethality of $\alpha 5$ -null embryos. Embryos lacking integrin $\alpha 5$ have a truncated posterior and lack posterior somites. These embryos also present with abnormal organization of the emerging extraembryonic and embryonic vasculature and reduced complexity of the emerging vasculature (53, 54). Further studies using $\alpha 5$ null Embryonic Stem cells (ES) cells to grow teratocarcinomas showed decreased proliferation, increased apoptosis, and decreased vascularization in teratocarcinomas derived from $\alpha 5$ -null ES cells as compared with controls (55). *in vitro* growth of embryoid bodies lacking $\alpha 5$ integrins showed a delay and reduction in the formation of the early vascular plexus and formation of complex vascular structures. Together, these data indicate a possible role for $\alpha 5\beta 1$ in early vasculogenesis and angiogenesis.

Our laboratory found that integrin $\alpha 5\beta 1$ is significantly upregulated in tumor angiogenesis in both mice and humans but is not expressed on quiescent endothelium (50). Studies in chick embryos showed that integrin $\alpha 5\beta 1$ is required for the angiogenic response to several growth factors but not VEGF. Antagonists of $\alpha 5\beta 1$ also inhibited tumor angiogenesis in chicks and mice, thus leading to tumor regression (50). Our studies have begun to elucidate the mechanisms by which integrin $\alpha 5\beta 1$ regulates vascular growth. Integrin $\alpha 5\beta 1$ -mediated adhesion promotes endothelial cell survival *in vivo* and *in vitro* (42). Our recent studies showed that integrin $\alpha 5\beta 1$ promotes the survival of endothelial cells by suppressing the activity of protein kinase A (PKA). Integrin $\alpha 5\beta 1$ antagonists activate both PKA and caspase-8, thereby inducing apoptosis and inhibiting angiogenesis (56). Although inhibition of integrin ligation can prevent cell attachment to the ECM, recent studies show that integrin $\alpha 5\beta 1$ antagonists also actively suppress signal transduction that leads to cell survival. For example, the inhibition of integrin $\alpha 5\beta 1$ negatively regulates endothelial cell survival, even when other integrin receptors for provisional matrix proteins remain activated. Antagonists of $\alpha 5\beta 1$ suppress cell migration and survival on vitronectin, but not cell attachment to vitronectin, indicating that these antagonists affect the migration and survival machinery rather than integrin receptors for vitronectin (57). In fact, $\alpha 5\beta 1$ antagonists

activate PKA, which then inhibits cell migration in part by disrupting the formation of stress fibers (57). These data put together suggest a mechanism by which angiogenesis might be regulated *in vivo* by manipulating the downstream effectors of integrins.

5.2. Integrin $\alpha 4\beta 1$

Integrin $\alpha 4\beta 1$ can bind both CS-1 fibronectin and VCAM-1, a member of the immunoglobulin superfamily. Loss of integrin $\alpha 4$ during development leads to defects in placenta and heart development, causing lethality between E10.0 and E12.0 (58).

Integrin $\alpha 4\beta 1$ is known as a lymphocyte integrin involved in adhesion and extravasation of lymphocytes by binding to VCAM expressed on inflamed endothelial cells (59). Our recent studies showed that integrin $\alpha 4\beta 1$ is expressed on neovessels in murine and human tumors and promotes close association of endothelium with VCAM-1 expressing vascular smooth muscle during blood vessel formation. We found that the cell-cell attachment mediated by these two molecules is essential for the survival of both cell types. Blocking this interaction with antibody inhibitors of the integrin reduced tumor neovascularization by inducing cell death of both endothelial cells and pericytes and in turn decreased tumor growth (60).

Circulating bone marrow progenitor cells have been shown to home to sites of neovascularization (48). Some studies suggest that these cells can contribute to approximately 15% of the neovasculation (61). Little has been known about the mechanisms by which these cells home to sites of neovascularization. We recently found that integrin $\alpha 4\beta 1$ regulates monocyte and progenitor cell homing to sites of neovascularization (62). We found that progenitor cells expressing $\alpha 4\beta 1$ homed to sites of tumor neovascularization that express VCAM and fibronectin. These cells did not home to normal tissue. Antagonists of $\alpha 4\beta 1$, but not other integrins, blocked adhesion of the progenitor cells to endothelium both *in vivo* and *in vitro*, as well as their homing and differentiation into endothelium (62). Thus, antagonists of $\alpha 4\beta 1$ could be beneficial in cancer therapy as they would inhibit multiple mechanisms by which $\alpha 4\beta 1$ regulates tumor neovascularization.

5.3. Integrin $\alpha v\beta 3/\alpha v\beta 5$

The αv integrin subunit can combine with several different beta subunits ($\beta 1$, $\beta 3$, $\beta 5$, $\beta 6$, and $\beta 8$). Integrin $\alpha v\beta 3$ is expressed on angiogenic blood vessels (63) but not on resting vessels. Inhibitors of $\alpha v\beta 3$ antibody block angiogenesis in a variety of animal models. In addition, inhibition of $\alpha v\beta 3$ function in quail embryos affected vasculogenesis by blocking lumen formation and disruption of vascular patterning (64). These data indicate a key role for $\alpha v\beta 3$ in vasculogenesis and angiogenesis. Peptide and antibody antagonists of $\alpha v\beta 3$ also block tumor angiogenesis and growth. Further analysis showed that these antagonists induce apoptosis in the vasculature leading to tumor regression (65). Use of the anti- $\alpha v\beta 3$ monoclonal antibody revealed that $\alpha v\beta 3$ is a marker of human breast tumor-associated blood vessels (66) that is expressed on a majority of vessels in several human colon, pancreatic, and lung carcinomas (67).

Different members of the integrin αv subfamily transduce angiogenic signals by different growth factors. *in vivo* angiogenesis assays showed that bFGF or TNF- α depend on $\alpha v\beta 3$ to initiate angiogenesis, whereas $\alpha v\beta 5$ is required for TGF- α - and

VEGF-mediated angiogenesis (44). These data taken together have established a role for $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins in angiogenesis and as important therapeutic targets.

Embryonic deletion of the αv subunit is lethal at E9.5 for 80% of null mice. The remaining 20% of these null mice survive until birth, dying within hours after birth with significant defects in brain development, including failure of blood vessels to form properly (68). Recent studies of mice with αv integrins conditionally deleted in endothelium revealed the effect observed in complete αv -null animals is due to a deficiency in the neuroepithelium rather than endothelium (69). Interestingly, individual loss of the $\beta 3$ (70) or $\beta 5$ (71) subunit during embryogenesis does not cause noticeable defects in the formation of the cardiovascular system. These animals undergo normal developmental angiogenesis. Furthermore, $\beta 3$ -null mice show normal postnatal retinal neovascularization and $\beta 5$ -null mice do not show any defects in wound healing. In fact, one study showed that animals lacking $\beta 3$ or $\beta 3$ and $\beta 5$ subunits displayed increased tumor angiogenesis (72). This led to the controversial conclusion that $\alpha v\beta 3/\alpha v\beta 5$ integrins might actually be involved in suppressing angiogenesis. However, given the ability of unligated integrins to induce apoptosis in endothelial cells (42), it is likely that the increased vascularization in $\beta 3$ - and $\beta 5$ -deficient tumors is due to absence of this apoptotic mechanism, which probably controls tumor vascular growth. Together, these studies indicate that in normal animals, αv integrins are critical for angiogenesis and tumor growth.

5.4. Integrin $\alpha 3\beta 1$

Integrin $\alpha 3\beta 1$ binds laminin, type IV collagen, thrombospondin 1 (TSP1), and invasin (73). This integrin $\alpha 3\beta 1$ is expressed in the endothelium of many tissues, including the brain, where its expression is restricted to small blood vessels (74). In cultured cells, $\alpha 3\beta 1$ is localized in cell–cell junctions (75). This integrin plays a role in the chemotaxis, adhesion, and proliferation of endothelial cells in response to TSP1. Some studies indicate that integrin $\alpha 3\beta 1$ may exist in an inactive state in normal endothelium *in vivo*, where the endothelial cells maintain close contact with one another (76). However, angiogenic and tumor endothelium are marked by loss of endothelial cell–cell integrity leading to activation of $\alpha 3\beta 1$.

5.5. Integrin $\alpha 6\beta 1$ and $\alpha 6\beta 4$

Integrin $\alpha 6\beta 1$ is expressed on a variety of cell types; this integrin binds laminin-1, laminin-8, invasin, tumstatin, TSP-1, and TSP-2 (73). The ligand-binding ability of $\alpha 6\beta 1$ may depend upon its activation state (77). Integrin $\alpha 6\beta 1$ is expressed at high levels in capillary endothelial cells *in vivo*. Endothelial cells form tube-like structures *in vitro* and an anti- $\alpha 6$ antibody was shown to block tube formation, suggesting a role for $\alpha 6\beta 1$ in the angiogenic process (78–80).

Another integrin, $\alpha 6\beta 4$, has also been implicated in tumor angiogenesis. Interestingly, this integrin is not expressed during developmental angiogenesis (81). Mice with a targeted deletion of the $\beta 4$ subunit cytoplasmic tail did not show any vascular defects during development. However, these mice have a highly reduced angiogenic response to bFGF and VEGF. *in vitro* studies showed that $\alpha 6\beta 4$ did not affect proliferation of endothelial cells but was required for normal adhesion and migration. Tumor growth

in these animals was suppressed, as was tumor angiogenesis (82). Thus, integrin $\alpha 6\beta 4$ is a novel target for anti-angiogenic therapies.

5.6. Integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$

Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are receptors for laminin and collagen that may also play roles in regulating blood vessel formation. VEGF upregulates $\alpha 1\beta 1$ and $\alpha 2\beta 1$ expression by stimulating mRNA expression of the alpha subunits (83). Integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$ function-blocking antibodies reduced VEGF-induced angiogenesis *in vivo* in a matrigel assay and reduced tumor growth by suppressing angiogenesis (84).

6. INTEGRINS AND LYMPHANGIOGENESIS

Lymphatic vessels form a network that drains fluids and cells (lymph) from tissues; these vessels are required for tissue homeostasis. Lymphatic capillaries are lined by loosely associated endothelial cells without pericytes or smooth muscle (85). This structure allows ready passage of immune cells and possibly tumor cells into the lymphatic system. Indeed, lymph nodes are typically the first organ to exhibit tumor metastasis, and sentinel node monitoring is used extensively to detect metastases (5).

Analysis of the role of lymphatics in tumor growth and metastasis had been hindered until recently by the absence of lymphatic markers. Recent identification of specific lymphatic markers, such as the transcription factor Prox-1 and the CD44 homolog lymphatic vessel hyaluronan receptor-1 (LYVE-1), has made it possible to study mechanisms regulating lymphangiogenesis (86–88). These vessels arise from the venous vessel network during embryogenesis and differ structurally from vascular endothelium. The growth factors VEGF-C and VEGF-D, which can be expressed by tumor cells or macrophages in tumors, promote growth of the lymphatic vessel network by activating the lymphatic endothelial cell receptor VEGFR-3 (89–93). VEGF-C expression in the tumor periphery induces lymphangiogenesis and promotes tumor metastasis (94–97).

Little is known about the integrins that promote tumor lymphangiogenesis, but recent studies have shown that integrin $\alpha 9\beta 1$ is expressed in quiescent lymphatic endothelial cells. Mice deficient in $\alpha 9\beta 1$ die 6–12 days after birth due to chylothoraces, an accumulation of lymph in the pleural cavity, suggesting a role for $\alpha 9\beta 1$ in developmental lymphangiogenesis (98, 99). Other studies have shown that VEGF-A induces $\alpha 1$ and $\alpha 2$ expression in lymphatic endothelial cells in healing wounds. Inhibition of these integrins blocked lymphangiogenesis in these wounds (100). However, the role of these integrins in tumor lymphangiogenesis remains to be determined. These studies have therefore opened up an exciting new field and further studies of the adhesion molecules that regulate lymphangiogenesis in tumor should provide novel methods to suppress tumor spread through the lymphatics.

7. INTEGRIN-BASED DIAGNOSTIC APPROACHES

Endothelial cell integrins make excellent targets for imaging and targeting of drugs to angiogenic vessels in tumors. These integrins are not expressed on quiescent vessels but are expressed on angiogenic endothelium. They are localized to both the abluminal and luminal side of vessels and can therefore be directly exposed to the circulating bloodstream (101). These molecules are also internalized regularly (102–104).

Thus, integrin-targeted agents are likely to detect only angiogenic vessels, thereby limiting side effects of drugs.

A number of diagnostic strategies directed at detecting integrin $\alpha v\beta 3$ have shown that integrin $\alpha v\beta 3$ is expressed at greater levels on tumor vasculature than normal vasculature. Furthermore, $\alpha v\beta 3$ expression has been shown to correlate strongly with tumor stage and outcome (105–107).

7.1. Positron Emission Tomography

A cyclic RGD peptide (cyclic RGDDYK) labeled with (¹⁸F) was recently used to image brain tumors in an orthotopic U251 model. Micro-positron emission tomography (PET) analysis showed significant accumulation in tumors with very low uptake in normal brain (108, 109). These results indicated a potential for the use of this labeled tracer for imaging tumors. To minimize liver uptake, the ¹⁸F-cRGDDYK was PEGylated (110). Similarly, a dimeric cyclic RGD peptide conjugated to (¹⁸F) also exhibited increased tumor retention (111). In addition, ⁶⁴Cu-labeled dimeric RGD peptides were used to image xenografts of human breast tumors with a high signal to background ratio and significant tumor retention. PEGylation of this peptide tracer reduced non-specific binding of the tracer and allowed identification of the primary tumor as well as metastases (112). Most recently, these imaging approaches were able to identify tumors as small as 1.5 mm in diameter in a glioblastoma xenograft model (113).

7.2. Magnetic Resonance Imaging

In a seminal study, Sipkins and colleagues used paramagnetic polymerized liposomes conjugated to an anti- $\alpha v\beta 3$ antibody, LM609, using an avidin–biotin linkage, to visualize tumors in a rabbit squamous cell carcinoma model (114). Their results showed that integrin-targeted agents can be used to detect neovasculature in tumors. Winter and colleagues (115) used paramagnetic nanoparticles that were covalently linked to integrin $\alpha v\beta 3$ -selective RGD-peptidomimetics to detect angiogenesis in rabbit tumors. In both studies, the magnetic resonance imaging (MRI) result was confirmed by immunohistochemistry, demonstrating that integrin-targeted molecular imaging systems have the potential to identify angiogenesis in tumors, as well as metastases.

7.3. Ultrasound

Another non-invasive approach to detect tumor growth and angiogenesis includes the use of contrast-enhanced ultrasound. Microbubbles targeted to αv integrins by conjugation to echistatin or to an antibody against mouse αv integrins attach to neovasculature (116). When injected into animals, these $\alpha v\beta 3$ -targeted microbubbles preferentially bind tumor neovasculature; binding was observed primarily in the tumor periphery (117). Further studies have shown that these targeted contrast agents can detect angiogenesis using conventional ultrasound techniques (118).

8. INTEGRIN INHIBITORS AS ANTI-CANCER AGENTS

Like bevacizumab (Avastin), a humanized antibody inhibitor of VEGF approved by the FDA for the treatment of metastatic colon cancer, integrin-based anti-angiogenesis therapies are under development as cancer therapeutics. Several integrin inhibitors are in clinical trials as therapeutics for cancer. Antibody and peptide inhibitors of integrins

$\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ are being tested for the inhibition of tumor angiogenesis, and other promising integrin-blocking peptides with anti-angiogenesis and anti-metastasis activities are in pre-clinical development (Table 2 and Fig. 8).

8.1. Antibody Inhibitors of Integrins

Integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ have been implicated in tumor angiogenesis. Antibody inhibitors of $\alpha v\beta 3$ and $\alpha 5\beta 1$ are in clinical trials for the inhibition of angiogenesis in cancer. Vitaxin is a humanized version of the anti-integrin $\alpha v\beta 3$ monoclonal antibody LM609, which has been shown to block tumor angiogenesis by inducing apoptosis in newly formed endothelial cells. A phase I study showed that Vitaxin has very low toxicity and is well tolerated (119). When tested on patients with metastatic cancer who had failed other treatments, Vitaxin again led to disease stabilization without toxicity (120).

Table 2
Integrin Antagonists in Clinical and Pre-Clinical Trials

| Drug name | Target | Drug type | Trial | Tumor type | Manufacturer |
|-----------------------------|-------------------|----------------|-------------|----------------------------|---------------------|
| MEDI-522 (Vitaxin) | $\alpha v\beta 3$ | Antibody | Phase II | Metastatic melanoma | MedImmune |
| | | | Phase II | Metastatic prostate cancer | |
| | | | Phase II | Metastatic melanoma | |
| M200 (Volociximab) | $\alpha 5\beta 1$ | Antibody | Phase II | Renal cell carcinoma | Protein Design Labs |
| | | | Phase II | Non-small cell lung cancer | |
| EMD 121974 (Cilengitide) | $\alpha v\beta 3$ | Peptide | Phase I | Childhood brain tumors | Merck KgaA |
| | $\alpha v\beta 5$ | | Phase I | Advanced solid tumors | |
| | | | Phase II | Metastatic melanoma | |
| | | | Phase II | Metastatic prostate cancer | |
| | | | Phase II | Pancreatic cancer | |
| | | | Phase II | Non-small cell lung cancer | |
| | | | Phase II | Glioblastoma multiforme | |
| S247 | $\alpha v\beta 3$ | Peptidomimetic | Preclinical | | Glaxo-Smith Kline |
| ATN-161 | $\alpha 5\beta 1$ | Peptide | Preclinical | | Attenuon and LLC |

This table lists integrin antagonists that are currently in clinical development for cancer therapy.

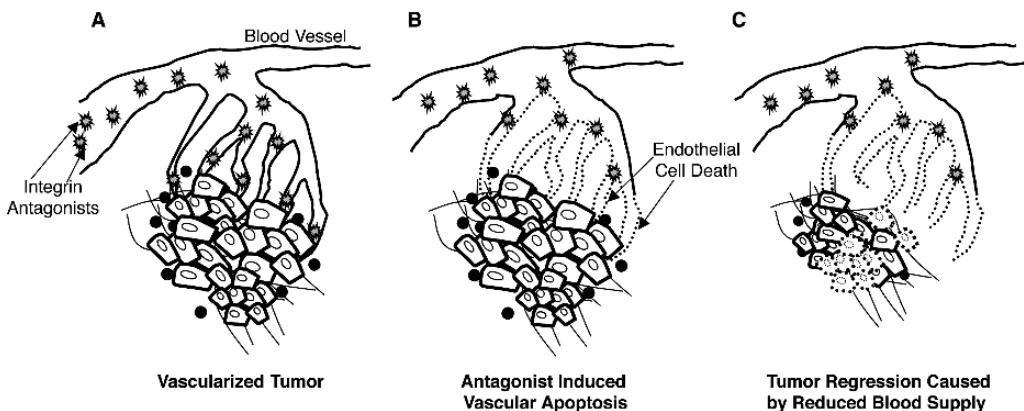


Fig. 8. Integrin antagonists induce endothelial cell death and tumor regression. Intravascular administration of integrin $\alpha v\beta 3$ or $\alpha 5\beta 1$ antagonists results in specific binding of antagonists to tumor vasculature, which expresses these integrins. These antagonists do not bind to normal blood vessels, which do not express integrins $\alpha v\beta 3$ or $\alpha 5\beta 1$ (A). These integrin antagonists induce cell death in the tumor endothelium (B), leading to decreased vascularization and tumor regression (C).

However, use of Vitaxin on patients with leiomyosarcoma did not lead to anti-tumor activity (121). In 2001, MedImmune began clinical trials using Vitaxin and in 2003 initiated phase II trials in patients with advanced metastatic melanoma and in patients with metastatic prostate cancer. As reported at ASCO in May 2005, a phase II study on metastatic melanoma showed that 53% patients treated with Vitaxin survived greater than 1 year as compared with 27% of patients receiving standard therapy.

A human monoclonal antibody directed against both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, CNTO 95, was developed by Centocor. CNTO 95 reduced angiogenesis and tumor growth in melanoma xenografts in nude mouse and rats of human melanoma (122). Preclinical safety studies on cynomolgus macaques showed no toxicity (123). This antibody is currently in phase I safety trials.

A humanized anti- $\alpha 5\beta 1$ antibody, M200 (volociximab), developed by Protein Design Lab, has shown low toxicity and is currently in phase II trials for metastatic melanoma, renal cell carcinoma, and non-small cell lung cancer.

Through novel discovery strategies, several new antibodies directed against αv integrins have been developed for cancer therapy. Screening of combinatorial antibody libraries from cancer patients has led to the identification of antibodies with integrin-binding RGD motifs in the complementarity-determining regions (CDR). These antibodies are selective for the activated form of the integrin $\alpha v\beta 3$ and suppress breast carcinoma metastases in a mouse model (124). In addition, a novel β -diketone derivative of RGD peptides conjugated to a monoclonal anti-alcohol dehydrogenase antibody has inhibited tumor growth and angiogenesis in nude mice by specifically targeting integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ (125).

8.2. Peptide Inhibitors of Integrins

Another group of integrin antagonists with potent anti-angiogenic function includes peptide inhibitors. The cyclic RGD-peptide cilengitide (EMD 121974) is an $\alpha v\beta 3/\alpha v\beta 5$ -specific antagonist. Phase I clinical trials have shown a favorable safety

profile and no dose-limiting toxicities (126). This drug is currently in phase II trials for glioblastoma, non-small cell lung cancer, and pancreatic cancer (127). ATN-161, a peptide inhibitor of $\alpha 5\beta 1$, suppressed tumor angiogenesis. When combined with chemotherapy, ATN-161 reduced metastases and improved survival in the colon cancer model (128). ATN-161 is in phase II clinical trials for multiple myeloma and other tumors.

Cyclic RGD peptides conjugated to doxorubicin or paclitaxel inhibit tumor growth as well. Doxorubicin–RGD conjugates suppressed growth of human breast tumors in nude mice at much lower concentrations and was also less toxic than doxorubicin alone (129). A dimeric cRGDyK–paclitaxel conjugate also suppressed tumor growth (130). Targeted chemotherapy thus reduces toxicity and increases drug specificity.

A new integrin $\alpha v\beta 3/\alpha v\beta 5$ antagonist was identified by screening a library of bicyclic lactam RGD-containing pseudopeptides. This high-affinity ligand for $\alpha v\beta 3/\alpha v\beta 5$, ST1646, blocked angiogenesis in the CAM assay and reduced growth and vascularization in a mouse xenograft model (131). A screen of a hexapeptide library using a microarray protein chip identified novel peptides with high affinity for integrin $\alpha v\beta 3$. These peptides were shown to have anti-angiogenic functions *in vivo* and *in vitro* (132).

8.3. Nanoparticles

Nanoparticles are lipid, carbon, or silicon-based delivery vehicles that are 100 nm or less in diameter. A lipid-based cationic nanoparticle targeted to $\alpha v\beta 3$ -expressing vasculature was effective in suppressing tumor growth (133). These nanoparticles were coupled to a synthetic organic $\alpha v\beta 3$ -binding ligand and incorporated plasmid DNA encoding a mutant Raf gene that causes endothelial cell apoptosis. Studies showed a specific uptake of the DNA by tumor endothelial cells. These nanoparticles lead to endothelial and tumor cell death, resulting in angiogenesis inhibition and tumor regression (133). Another group reported a similar effect by targeted radiotherapy. They also used an $\alpha v\beta 3$ antagonist to target Y90-labeled nanoparticles to the tumor vasculature in mouse models of colon adenocarcinoma and melanoma. This group demonstrated a significant delay in tumor growth, lower vascular density, and increased apoptosis in tumors (134). Expression of integrins $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha v\beta 3$ on tumor vasculature especially makes it possible to target genes or drugs for safer anti-tumor agent delivery.

8.4. Small Molecule Inhibitors

A number of small molecule inhibitors of integrins have been developed. Although small molecule inhibitors of integrin $\alpha 4\beta 1$ are in clinical trials for asthma, no small molecule inhibitors of integrins are yet in clinical trials for cancer. However, S247 (Glaxo Smith Kline), a small molecule inhibitor of $\alpha v\beta 3$, is being tested pre-clinically. S247, a peptidomimetic $\alpha v\beta 3$ integrin antagonist, reduced colon cancer metastasis and increased survival in a mouse model. Treatment with S247 also reduced tumor angiogenesis by inducing apoptosis and reduced pericyte coverage (135).

8.5. Combination Therapies

Although monotherapies using anti-integrin agents have some efficacy, studies have shown greater efficacy of these antagonists when combined with other therapies. A study on a mouse xenograft model of breast cancer showed that combining Cilengitide

administration with radioimmunotherapy significantly reduced tumor size and caused increased apoptosis in tumor and endothelial cells (136). Another study has reported an individual case of a heavily pre-treated patient with a 15-cm tumor (a fourth relapse of squamous cell carcinoma) that was treated with Cilengitide at 600 mg/m² plus gemcitabine for 5 months. The patient achieved partial remission and was stable for 12 months while on Cilengitide maintenance therapy (137). This case indicates a potential for combination therapies in inhibiting the growth of such tumors that have been refractive to other therapies.

Tumors treated with S247, a RGD-peptidomimetic antagonist of $\alpha v\beta 3$, in combination with radiotherapy were smaller and showed decreased angiogenesis than tumors treated with single agents (138). In another study, radiation-induced antigens included integrin $\alpha IIb\beta 3$; the use of fibrinogen-conjugated nanoparticles or liposomes suppressed tumor blood flow and delayed growth of irradiated tumors (139).

9. CONCLUSION

Recent studies indicate that integrins promote cellular migration, proliferation, and survival in primary endothelial cells. Expression of select integrins on activated or proliferating endothelial cells serve as markers as well as targets for anti-angiogenesis strategies. Antagonists of integrins $\alpha v\beta 3$, $\alpha 5\beta 1$, $\alpha v\beta 5$, and $\alpha 6\beta 4$ show great potential as inhibitors of tumor growth and metastasis as well as tumor angiogenesis. Clinical trials are currently underway to evaluate inhibitors of integrin $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ for their usefulness in cancer treatment. Furthermore, studies are in progress to develop integrin-targeted nanoparticles for cancer therapy and diagnosis and to determine whether integrin expression in tumors can predict tumor outcome. Research in recent years has helped uncover the complex mechanisms underlying integrin signaling and function. However, further studies on the mechanisms regulating integrin function and the multiple signaling cascades activated by integrin ligation are essential to identify new targets for integrin-based anti-angiogenic cancer therapies.

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4

Tumor Endothelial Cell Abnormalities

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SUMMARY

In the 1970's, anti-angiogenic therapy was realized as a potential strategy to shrink solid tumors. Since that time, many aspects of vascular biology have been investigated in attempts at understanding the mechanisms that regulate how blood vessels develop in health and disease. In the tumor microenvironment, factors such as hypoxia and acidosis impinge on normal endothelial cell (EC) function. Accordingly, the vascular structure in tumors is highly disorganized, vessels are of variable diameter, and there is vessel leakiness. Although these morphological changes in the tumor vasculature have been known for some time, it was only recently shown that tumor-associated EC are indeed distinct from normal EC at the molecular level. This chapter will discuss the abnormalities observed in the tumor endothelium.

Key Words: Tumor; endothelial cells; angiogenesis; chromosomal abnormalities; drug resistance; tumor stroma; p53.

1. INTRODUCTION: THE ENDOTHELIAL CELL

A continuous layer of endothelial cell (EC) lines the heart, walls of the arterioles, capillaries, venules, connective tissue, neural tissue, lungs, and mesentery. Owing to its expansiveness and dynamic nature, the collective endothelium can even be thought of as a functioning organ (1).

EC are heterogeneous and differ dramatically from tissue to tissue (2). The great range of EC heterogeneity parallels the broad range of biomechanical and biochemical cues they must endure, that is, shear stress, extremes in oxygenation, direct interaction with blood born pathogens, and daily fluctuations in hormones, cytokines, growth factors, and nutrients such as glucose. Thus, the spatial and temporal dynamics of the surrounding microenvironment requires that EC have enough plasticity to adapt to local conditions. Overall, EC are uniquely tailored to perform several diverse functions

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including maintenance of a barrier between blood and tissues and processes related to regulation of vascular tone, inflammation, homeostasis, fibrinolysis, wound healing, and angiogenesis.

2. MORPHOLOGICAL CHARACTERISTICS OF NORMAL AND TUMOR VASCULATURE

2.1. *Normal Vasculature*

Cutting edge microscopy techniques have allowed for often stunning images of the mammalian vasculature. One common method for viewing large vessels and capillaries in laboratory mice is perfusion with Fluorescein Isothiocyanate (FITC)-conjugated lectins (*Bandeira simplicifolia* or *Lycopersicon esculentum*) or FITC-dextran followed by *in situ* formaldehyde fixation—this technique effectively prevents tiny blood vessels from collapsing post mortem and thus presents a more accurate image of vascular structures (3). Using fluorescently labeled antibodies against matrix proteins or other proteins present in smooth muscle cells (α -smooth muscle actin) and EC (CD31 and CD34), a complete three-dimensional picture of a blood vessel *in situ* is possible. In the normal microvasculature, there is a single layer of EC surrounded by microvascular smooth muscle cells called pericytes which function to maintain vessel structure and regulate the endothelium through release of paracrine factors (4). Enveloping the two cell types is a complex array of proteoglycans, glycoproteins, and structural proteins such as laminin, collagen, and fibronectin which constitute the basement membrane. Viewed by scanning electron microscopy (SEM), the luminal surface of a normal blood vessel is smooth with tight endothelial junctions (5). At the lateral side, smooth muscle actin-positive pericytes tightly envelop the EC tubes. Depending on the tissue, the ratio of pericytes to EC can vary dramatically. For example, in the brain, pericytes far outnumber their endothelial counterparts, whereas in the retina, the ratio of EC to pericytes is roughly one to one.

2.2. *Tumor Vasculature*

By comparison, tumor vasculature is strikingly different from normal vasculature. Abnormalities encompass all components of the tumor vessel including the EC, pericytes, and basement membrane (6). For example, tumor-specific pericytes are loosely associated, irregularly shaped, and display unusual cellular processes. The basement membrane in tumor vessels often shows redundant layers of type IV collagen and laminin, gaps, and structural abnormalities (7). Overall, SEM of polymer casts of tumor vessels shows absence of the ordinary hierarchical organization of arterioles, capillaries, and venules. Instead, there is random branching and chaotic, tortuous growth (5). Within the vessels, there are often gaps between adjacent EC or the EC are missing altogether. Where there are gaps in the vessel wall, tumor cells may mimic EC by filling in these empty spaces; however, the contribution of tumor cells to the blood vessel wall is still being debated (8–11). Some EC in the tumor vessels lack typical markers such as CD31 and CD105 that might account for focal alterations in EC permeability by disrupting cell-to-cell contact (12). Loss of stability in the vessel wall can lead to the extravasation and pooling of erythrocytes and the potential for intravasation of tumor cells which give rise to metastasis (6).

A hallmark of tumor angiogenesis is the increased leakage of fluorescein dye when injected systemically (13). Leaky sites in tumors are non-uniform, and pore cutoff sizes are tumor type and location dependent (14). Many of the vascular malformations in solid tumors including leakage, irregular diameter, and high interstitial pressures could be due to physical compression or distortion by the associated mass of tumor cells. In addition, the imbalance of pro- and anti-angiogenic growth factors in the tumor microenvironment disrupts the vascular remodeling that occurs during active tumor angiogenesis (15). For example, breakdown and reconstitution of basement membranes, cooption and incorporation of EC from adjacent tissue and bone marrow, and pericyte recruitment requires a balance between factors such as vascular endothelial growth factor (VEGF), placenta growth factor (PIGF), and PDGFB. Over-expressed VEGF can directly contribute to tumor vessel leakiness because of its vasodilatory effect. Failure to recruit pericytes or perturbed endothelial–pericyte interactions can result in vessel permeability because of a loss in vessel stability (16).

2.2.1. NORMALIZATION OF TUMOR VESSELS

The fact that tumor blood vessels are leaky or variable in their permeability may affect adequate delivery of therapeutics from the bloodstream to the tumor cells. One idea that has emerged in recent years is that some anti-angiogenic therapies can reverse the structural and functional abnormalities of tumor blood vessels (17). This idea, termed normalization, is based on the principle that improved drug delivery can be achieved by making tumor vessels of more uniform shape and size, by augmenting pericyte-to-EC contact, and by improving endothelial barrier function and blood flow. Indeed, treatment with the VEGF-specific antibody bevacizumab (Avastin) prunes abnormal tumor vessels and increases the fraction of pericyte-covered vessels in rectal carcinoma (18). Normalization of tumor vessels enhances the delivery of blood to the tumor which may underlie the combined benefit of both anti-angiogenic and cytotoxic therapies (19).

3. MOLECULAR CHARACTERIZATION OF TUMOR-ASSOCIATED EC BY SERIAL ANALYSIS OF GENE EXPRESSION

The use of antibodies specific for EC receptors coupled with immunomagnetic separation has allowed for the isolation of pure populations of EC from normal tissue and tumors of various origins. Negative selection of epithelial and hematopoietic cells can significantly enrich the pure EC fraction. In a seminal study, this purification technique was used followed by serial analysis of gene expression (SAGE), a quantitative method to measure mRNA transcripts, to identify changes in gene expression patterns in human tumor EC from normal versus malignant colorectal tissue (20). The isolated EC were never cultured, so artifacts due to cell culture including loss of EC markers and phenotypic plasticity could be excluded. This report yielded a wealth of new knowledge about the differences in tumor EC and demonstrated, for the first time, that normal and tumor EC were indeed distinct at the molecular level. Seventy nine transcripts were found to be differentially expressed in tumor endothelium. At least 46 transcripts were specifically elevated in tumor endothelium, many of these encoding matrix proteins, but most of unknown function. Nine novel cell surface markers, the tumor endothelial markers or TEMs (TEM1–TEM9), were also identified. TEM8, for example, was unique in that its expression was confined to tumor endothelium and

not developing corpus luteum, whereas TEM7, now known to bind to cortactin (21) or nidogen (22), has subsequently been shown to be highly expressed in the endothelium of breast, lung, and brain tumors. Many of the same genes expressed during tumor angiogenesis were also expressed in the developing corpus luteum and during wound healing, reiterating the fact that both physiological and pathological angiogenesis are inherently linked.

3.1. Characterization of Cultured Tumor EC

To date, only a handful of laboratories have reported successful isolation and subsequent culture of tumor EC (see Table 1 for summary), which may be due to the difficulties of replicating the tumor microenvironment *in vitro* or due to inherent abnormalities of tumor EC. A temporal and spatial release of paracrine factors by tumor cells, variations in blood flow and oxygen tension, nutrient deprivation, exposure to potentially toxic waste products, and a physical compression of immature vessels by tumor cells are all characteristics of the tumor microenvironment. Taken together, it

Table 1
A Time-line of Tumor Endothelial Cell (EC) Isolation and Characterization

| <i>Tumor EC Abnormalities</i> | <i>Study</i> |
|--|-------------------------------|
| Tumor EC express high levels of VEGF receptors and respond to FGFs. | Alessandri et al. (1999) |
| Forty six transcripts were elevated in human colon tumor EC by SAGE. Identification of the TEMs. | St. Croix et al. (2000) |
| Brain tumor EC express lower levels Factor-VIII-related antigen. | Unger et al. (2002) |
| Tumor EC express the progenitor marker SCA-1 and have unusual cellular distribution of CD31 and CD106. Oncofetal fibronectin was required to maintain phenotype. | Allport and Weissleder (2003) |
| Renal carcinoma tumor EC up-regulate VEGF-D, ANG-1, and Akt and survive without serum. | Bussolati et al. (2003) |
| Melanoma and liposarcoma-derived EC have multiple centromes and are aneuploid. | Hida et al. (2004) |
| B-cell lymphoma-derived EC harbor lymphoma-specific genetic alterations. | Streubel et al. (2004) |
| Renal tumor EC overexpress NCAM. | Bussolati et al. (2006) |
| Brain tumor EC proliferated more slowly than normal EC. Tumor EC produced higher levels of VEGF and ET-1. | Charalambous et al. (2005) |
| High-grade glioma-derived EC proliferate more rapidly than low-grade glioma EC. | Miebach et al. (2005) |
| Tumor EC express EGFR, ErbB2 and ErbB4 and proliferate in response to EGF. | Amin et al. (2006) |
| Breast tumor-derived EC are resistant to vincristine and doxorubicine. | Grange et al. (2006) |
| GSTP1 and RAR β 2 are hypermethylated in prostate tumor EC. | Grover et al. (2006) |

is likely that tumor EC in culture might have unique requirements including growth supplements or attachment factors that normal EC would not necessarily need. Alternatively, tumor EC may be able to thrive under conditions in which normal EC would not. As described below, alterations in growth factor receptors, cellular survival pathways, surface adhesion molecules, and passage-related changes in EC markers are all consistent with molecular perturbations in tumor EC.

3.1.1. GROWTH FACTOR RECEPTORS

A good example that highlights the underlying differences between tumor and normal EC is a study demonstrating that growth factor receptors and responses to their ligands are different in tumor compared with normal EC *in vitro* (23). For example, ErbB1 (EGFR), ErbB2, and ErbB4 are expressed in isolated tumor EC, whereas normal dermal EC do not express ErbB1. As a consequence of gained ErbB1 expression, tumor EC are responsive to EGF ligands and proliferate, whereas normal EC are unaffected. The expression of ErbB1 also sensitizes tumor EC to ErbB1 kinase inhibitors such as Iressa, a compound that does not affect normal EC. In contrast, normal EC are growth inhibited by neuregulin, which is a ligand for ErbB3 and ErbB4, whereas tumor EC are not affected.

Higher levels of VEGF receptors have also been detected on tumor EC compared with normal EC. *In vivo*, VEGFR1 and VEGFR2 are elevated in developing tumor blood vessels (24). Over-expression of VEGFR2 is also reported in cultured breast tumor and renal carcinoma-derived EC (25, 26). The up-regulation of chemokine receptors, CXCR1/CXCR2, and VEGF receptors on brain tumor EC is associated with increased migration (27). Taken together, up-regulation of growth factor receptors in tumor EC may enhance proliferation and migration—two integral steps contributing to tumor angiogenesis.

3.1.2. SURVIVAL PATHWAYS

The up-regulation of transcription factors and other signaling molecules in tumor EC may also account for their increased proliferative and migratory abilities. Comparing normal and tumor EC isolated from human renal carcinoma, tumor EC were found to up-regulate angiopoietin-1, phospho-Akt, and VEGF-D (28). Pax2 is over-expressed in renal carcinoma EC that is associated with tumor suppressor PTEN down-regulation and augmented cell survival (29). Tumor EC isolated from glioblastoma multiforme produce higher levels of VEGF and endothelin-1 and have increased migratory ability compared with normal brain EC (30).

Interestingly, tumor grade seems to affect the growth of tumor EC *in vitro* (31). For example, EC isolated from high-grade glioma proliferate more rapidly in culture compared with EC from low-grade glioma (31). Differences in tumor angiogenesis because of tumor grade might be dependent on the variable incorporation of bone marrow-derived EC precursors. Id1 and Id3, for example, are required for the recruitment of bone marrow progenitors and are expressed in the tumor vasculature of poorly differentiated but not well-differentiated prostate adenocarcinoma (32).

3.1.3. ADHESION MOLECULES

Cultured tumor EC show atypical expression of cell surface molecules, which is consistent with that observed *in vivo* (12). EC isolated from Lewis lung carcinoma

xenografts in C57BL/6 mice show that, compared with normal heart or lung EC, the cellular distribution of the common EC surface markers CD31 and CD106 are punctate throughout the cytoplasm rather than at the cell periphery (25). Loss of cell-adhesion molecules such as VE-cadherin could contribute to leakiness in tumor blood vessels and may be indicative of de-differentiation (30). Increased surface expression of inducible adhesion molecules such as ICAM-1 may account for the recruitment of inflammatory cells or bone marrow-derived progenitor cells to the tumor vasculature (25). Similarly, constitutive E-selectin and VCAM expression on tumor vessels is indicative of an inflammatory response (25, 33). The neural cell adhesion molecule (NCAM), which is widely expressed during embryogenesis, is expressed by tumor but not normal EC and is associated with tumor EC organization into tube-like structures (34). The expression of smooth muscle actin, a cytoskeletal protein usually associated with mural cells, might also play a role in the increased ability of tumor EC to migrate and form tubes (30).

3.1.4. PASSAGE-RELATED CHANGES IN CULTURED TUMOR EC

Routine passaging of cultured tumor EC results in a rapid loss of endothelial-specific markers. For example, von Willebrand factor (VWF), CD31, and VE-cadherin are lost as early as passage four in human glioma-derived EC (31). Similarly, factor VIII-related antigen (VWF) is lower in brain tumor EC compared with normal EC though VWF expression can vary between normal EC (35). Tumor EC also have distinct morphologies including a spindle-like or fusiform shape and absence of a cobblestone appearance. Large senescent cells are present in early passaged tumor EC, which usually occurs in normal EC only after serial propagation (26). A switch to a progenitor phenotype once tumor EC are placed *in vitro* has been hypothesized (25). Therefore, it has been proposed that the oncofetal forms of fibronectin are necessary as an attachment factor to maintain the phenotype of cultured tumor EC (25).

4. TUMOR STROMAL CELL CYTOGENETIC ABNORMALITIES

Tumor infiltrating stromal cells can carry selective genetic alterations suggesting a “bystander” effect of the tumor microenvironment on otherwise genetically normal cells (36). For example, stromal fibroblasts show a loss of heterozygosity for tumor suppressor genes, such as p53, probably as a consequence of a selection pressure within the tumor microenvironment (37). Epigenetic changes including alterations in DNA methylation also occur in the stromal compartment of breast tumors (38, 39) and in tumor EC isolated by laser capture microdissection (40). These results indicate that cytogenetic changes in tumor stroma (including EC, smooth muscle cells, fibroblasts, myoepithelial cells, and lymphocytes) could enable or even induce tumorigenesis (41). Indeed, when tumor-associated fibroblasts are coinjected with tumor cells, there is a significant augmentation of tumor growth (42).

4.1. Human Lymphoma

The finding that in some solid tumors the tumor cells themselves could fill in gaps in the walls of the infiltrating blood vessels suggests a close relationship between tumor cells and the endothelial compartment. Confirming this close relationship, lymphoma-specific genetic aberrations in 15–85% of invading microvascular EC in B-cell lymphoma have been reported. Notably, the presence of both primary and

secondary translocation mutations in the EC were identical to those commonly found in follicular lymphoma (43). Although the mechanisms are unclear, there is the potential of gene transfer between tumor cells and the invading stroma or cell fusion of lymphoma cells and EC. Alternatively, these results may suggest a common bone marrow-derived hemangioblastic cell shared between lymphoma cells and EC.

4.1.1. HUMAN-TO-MOUSE XENOGRAFTS OF MELANOMA AND LIPOSARCOMA

Using tumor EC isolated from human melanoma or liposarcoma xenografted into nude mice, it was noticed early on that the nuclei of freshly isolated tumor EC were of larger size compared with their normal EC counterparts from skin or adipose tissue (44). The presence of large nuclei in tumor EC suggested a change in DNA content. Karyotype analysis and fluorescence *in situ* hybridization (FISH) confirmed that cultured tumor EC and tumor EC in frozen sections *in situ* are indeed cytogenetically abnormal. Further cytogenetic studies including SKY-paint determined that about 16–35% of cultured tumor EC were aneuploid with heterogeneous translocations, missing chromosomes, and double minutes. Individual tumor EC had different cytogenetic profiles indicating non-clonality. No incorporation of human genetic material was evident in the mouse karyotype that argues against a cell-fusion mechanism for the acquisition of aneuploidy.

Centrosomes constitute the microtubule organizing centers of the cell and establish cell polarity. Most tumor cells have multiple centrosomes (>2) and are aneuploid, but it is not clear whether centrosome amplification precedes aneuploidy and genomic instability or whether the opposite is true (45). Tumor EC were found to have between one and five centrosomes per cell, which is in good accord with the observed aneuploidy, but the mechanism of centrosome amplification is still unclear (46). There is definitive evidence supporting a relationship between loss of p53 and centrosome amplification in non-EC (47–49). Whether or not tumor EC, like other cells in the stromal compartment, may undergo alterations in p53 that contributes to centrosome amplification remains to be determined.

5. TUMOR EC DRUG RESISTANCE

Since the identification of cytogenetic abnormalities in tumor EC, there has been speculation as to whether acquired drug resistance might be a feature of the tumor endothelium. Thus, the conventional wisdom that tumor EC remain genetically stable and do not develop drug resistance is at present uncertain.

It is interesting that tumor EC are suggested to mediate the tumor's response to radiation (50) and chemotherapy (51, 52). Moreover, recent studies indicate that breast tumor EC, compared with normal EC, are less sensitive to vincristine and doxorubicine *in vitro* (53). Similarly, renal tumor EC were resistant to vincristine-induced apoptosis and proliferated despite serum withdrawal (28). *in vivo* EC resistance to epirubicin has been observed in nasopharyngeal carcinoma in mice (54). In human patients, up-regulation of the P-glycoprotein drug exporter was noted in renal tumor endothelium and was associated with an unfavorable prognosis (55).

Drug resistance is typically a property of the tumor cells themselves and probably arises due to a combination of chromosomal instability and selection pressure within the tumor microenvironment (56). Factors such as hypoxia and variability in blood flow have been hypothesized to create selective barriers that favor the propagation of better

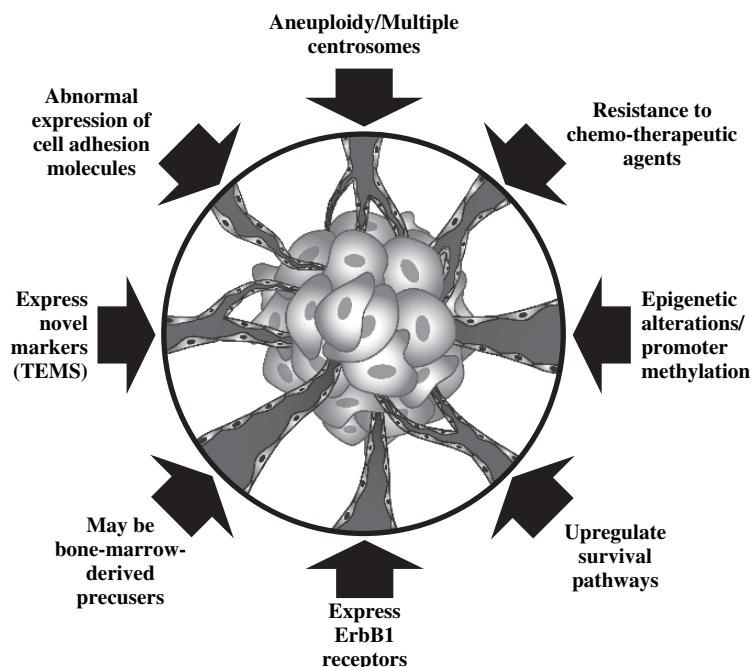


Fig. 1. Tumor endothelial cell Abnormalities. (Please see color insert.)

adapted cells (57). Given that tumor infiltrating stromal cells would be subjected to the same selection pressure as the tumor cells, it might be expected that a growth advantage could be gained by loss of a tumor suppressor in the stromal compartment (37). It is not yet fully understood how genetic or epigenetic alterations in tumor-associated stromal cells such as fibroblasts might contribute to tumorigenesis. However, because tumor EC contribute to the growth of the tumor by providing a conduit for the delivery of oxygen and nutrients, drug resistance due to genetic alterations in the tumor EC population could directly facilitate tumorigenesis.

6. CONCLUSIONS

Improved techniques for the isolation and expansion of tumor EC have allowed for characterization of their biological properties *in vitro* (see summary in Fig. 1). As a whole, and compared to normal EC, tumor EC express growth factor receptors such as EGFR, they may express VE-cadherin and CD31 in the cytoplasm, rather than at the cell periphery, they show increased proliferative, tube forming, and migratory abilities, they are cytogenetically abnormal including the acquisition of multiple centrosomes, they express markers indicative of a hematopoietic or progenitor origin, and they are more resistant to chemotherapeutic drugs such as vincristine and doxorubicin.

7. PERSPECTIVES

While the successful isolation, culture, and characterization of tumor EC is still an emerging field, important differences exist between tumor and normal EC. One example of this with direct clinical applications is the finding that tumor, but not

normal EC, expresses EGF receptors. Therefore, it appears that EGF receptor-targeting drugs, such as Erbitux, Tarceva, Iressa, or PKI 166, which were originally designed to target epithelial cells might have a combined anti-tumor and anti-angiogenic effect (58).

The expression of other novel tumor EC markers such as the TEMs may lead to the development of new, specific tumor EC targets. TEMs have proved valuable as biomarkers in human cancer as their increased levels are associated with nodal involvement and disease progression (59). It is expected that high throughput analyses such as microarray and phage display will identify other novel tumor EC-specific targets in addition to the TEMs that can one day translate into the clinic.

The mechanism of acquired cytogenetic abnormalities in tumor EC has not been determined. It is possible that selection pressure in the chaotic microenvironment of the tumor might play a role by preventing survival of genetically stable cells in the stromal compartment. These microenvironmental bottlenecks are well known to play a role in tumors by selecting for genetically unstable cells with a growth advantage. Loss of or mutations in tumor suppressors such as p53 in tumor EC are good candidates as p53 plays a definitive role in genome stability and is reportedly lost in tumor stromal fibroblasts (60).

The possibility that resistance to anti-angiogenic therapies could arise in tumor EC remains to be addressed. In neoplasms, genetic instability and heterogeneity are the principle reasons for their resistance to conventional chemotherapy. Targeting the genetically stable vasculature has been proposed, because it was thought that somatic mutations would never arise in the tumor stroma. It is now known that the tumor microenvironment can affect the genetic or epigenetic composition of the host cells including the infiltrating fibroblasts and tumor-associated EC. It cannot be ignored that many of the anti-angiogenic agents in clinical trials today have been met with mixed results, but it remains to be formally proven in a clinical setting whether tumor EC may acquire resistance to conventional chemotherapeutic or anti-angiogenic therapies.

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5

The Extracellular Matrix and VEGF Processing

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SUMMARY

Tumor neovascularization requires the activation of a subset of endothelial cells from normal vascular beds, the digestion of the underlying basement membrane, and the directional migration of these cells toward an avascular site. The contribution of vascular endothelial growth factor (VEGF) to each one of these steps has received large experimental support, and it has been demonstrated that pharmacological and/or genetic inactivation of this growth factor can impact the angiogenic response and consequently suppress tumor growth. Thus, understanding the mechanisms that control VEGF levels has become an important focus of investigation. Today, we have a fairly comprehensive understanding of the mechanisms that regulate VEGF transcriptional rate and half-life. In contrast, little emphasis has been placed on the regulation of VEGF biology post-secretion. In this chapter, we focus our attention on the question of how VEGF becomes released from the extracellular environment and contributes to tumor neovascularization. We discuss this point in the larger context of matrix interaction with growth factors and their modulation by matrix metalloproteinases (MMPs).

Key Words: Matrix metalloproteinases; growth factors; angiogenesis; tumor microenvironment; endothelial cells; capillaries; neovascularization.

1. INTRODUCTION

It is generally accepted that the tissue/tumor microenvironment plays an active role in regulating the angiogenic response. Matrix molecules serve as substrate for migration of endothelial cells, as well as provide differentiation cues for the maturation and stabilization of new vascular beds (1–3). Thus, the nature and physical features of the extracellular environment can provide differential permissive signals for angiogenesis progression and stabilization.

More pertinent to tumor angiogenesis, a cohort of extracellular matrix (ECM) fragments resulting from the cleavage-specific matrix proteins has been shown to be effective in the inhibition of vascular growth. These fragments, named “endogenous

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inhibitors of angiogenesis,” were identified *in vivo* and were shown to modulate growth of neovessels in pathological conditions. Moreover, loss and gain of function studies have supported their biological relevance in the regulation of capillary density and in the modulation of pathological angiogenesis (4–6). Thus, it has become critical to explore the dynamics of matrix degradation in the context of an angiogenic response to fully appreciate its impact on vessel growth.

Matrix remodeling is accomplished by a cohort of extracellular enzymes that include matrix metalloproteinases (MMPs), cathepsins, serine proteases, and aminopeptidases (7). In the past, these molecules were considered to participate in the digestion and complete degradation of the matrix needed to repair and remodel tissues. Clearly, proteases are required for the digestion of ECM to allow cell migration. Specifically during angiogenesis, proteolysis is needed to remove the basement membrane of differentiated vessels and to detach pericytes enabling the migration of endothelial cells. More recently, however, the contribution of extracellular proteases in the fine modulation of multiple biological responses has gained a deeper appreciation.

During neovascularization, matrix proteases are required for the release of ECM-bound angiogenic growth factors. Angiogenic growth factors interact with multiple matrix proteins. It has been demonstrated both *in vivo* and *in vitro* that the extracellular milieu functions as a reservoir for growth factors that can be released by specific proteolysis. Thus, the activity of ECM enzymes can significantly enhance the angiogenic response through this property. In addition, matrix proteases are able to expose cryptic integrin-binding sites that would not be accessible in the absence of selective proteolysis. These fragments can facilitate migration and adhesion of endothelial cells during the neovascular response and can enhance or inhibit angiogenesis depending on their relative abundance and presentation to migrating endothelial cells. Finally, proteases are also required for the disruption of endothelial cell–cell adhesions, a process that is essential to the initiation of the neovascular response. For example, MMPs have been shown to cleave the ectodomain of VE-cadherin and thereby releasing endothelial cells from pre-existent blood vessels (8).

Another pertinent aspect to this discussion is that growth factors themselves can be substrates for extracellular proteases. Indeed, extracellular proteolysis is a requirement for activation of some members of the vascular endothelial growth factor (VEGF) family, specifically VEGF-B and VEGF-C. Recently, we found that VEGF-A can also be processed extracellularly by a subset of MMPs and that this processing alters the type of vasculature induced by this growth factor (9).

In this chapter, we will summarize our current understanding of growth factor interaction with matrix and the relevance of MMPs, as a prototype of extracellular proteases, in the modulation of growth factor function. Although much of the discussion will bring examples of several growth factors, our focus will be VEGF because of its prominent position in the angiogenic cascade.

2. INTERACTION OF GROWTH FACTORS WITH THE ECM

The ECM accounts for over 50% of the dry weight of the vasculature, and it is largely deposited toward the end of development (10). As previously discussed, matrix proteins affect fundamental aspects of endothelial cell/vascular biology. These processes are complex and involve both external structural support and regulation of

multiple signaling pathways within the cell. ECM proteins provide a scaffold essential for maintaining the organization of vascular endothelial cells into blood vessels through adhesive interactions with integrins on the endothelial cell surface. In addition, the ECM modulates most if not all aspects of neovascularization such as endothelial cell proliferation, migration, morphogenesis, survival, control cytoskeleton and cell shape, and ultimately blood vessel stabilization through endothelial cell adhesion to the substrate.

The ECM is composed of complex arrangement of fibrous proteins and associated glycoproteins and proteoglycans (11). The diversity of ECM components in the endothelial cell microenvironment provides an intricate level of complexity sufficient to exert significant and precise control over many aspects of neovascularization (3). For example, basement membrane (a specialized form of ECM) of quiescent blood vessel contains different ECM components that when exposed out of context can both inhibit and promote angiogenesis. The basement membrane of normally quiescent endothelial cells is composed of type IV collagen, laminin, heparan-sulfate proteoglycans, perlecan, nidogen/entactin, SPARC/BM-40/osteopontin, type XV collagen, type XVIII collagen, and other molecules (12–14). In a quiescent vessel, the basement membrane is highly cross-linked, and only certain domains of various constituents are exposed to (and can interact with) endothelial cells. In contrast, ECM undergoing remodeling (i.e., during tumor angiogenesis) exposes different constituents that can interact with endothelial cells, including collagen cryptic domains. Depending on the nature of the proteolysis (enzymes that participate in the process), the products of basement membrane digestion can be pro-migratory or anti-angiogenic (1, 15).

As mentioned previously, the ECM is also a reservoir of cell-binding proteins and growth factors such as VEGF, basic fibroblast growth factor (bFGF), and transforming growth factor beta (TGF- β) (16). Most angiogenic cytokines have affinity for heparin and heparan sulfate proteoglycan and directly bind to ECM scaffolds such as collagen type I and fibrin/fibronectin matrices (16–18).

VEGF binds to heparin with high affinity, and both heparin and heparin sulfate can compete for binding of VEGF to the ECM. Heparinase I or II can induce the release of VEGF, providing further support for the notion that heparin-containing proteoglycans are binding sites for this growth factor (19). In addition, VEGF has been shown to bind to fibrinogen, fibrin, and fibronectin. More importantly, these studies showed that bound VEGF retains functional activity *in vitro* (20, 21). However, the nature of the interaction with a specific matrix molecule is likely to alter receptor usage and biological response.

The FGF family comprises 20 members of structurally homologous, functionally distinct small polypeptides with a central core of 140 amino acids. Among all, FGF1 (acidic FGF) and especially FGF2 (basic FGF) are most preferentially implicated in angiogenesis (22, 23). FGF1 and FGF2 are about 18-kDa, single-chain, non-glycosylated proteins, share about 55% sequence homology with similar biological activities (24). One characteristic shared by these two molecules is that they both strongly interact with heparin-like molecules and heparan sulfate proteoglycans of the ECM (25, 26).

TGF- β s are cytokines with multiple key roles in modulating cell proliferation, differentiation, apoptosis, immune responses, tissue repair, and the ECM formation (27). TGF- β s are produced as large latent complexes that are linked to one of four latent

TGF- β -binding proteins (LTBP) through disulfide bonds (28). Although overall *in vivo* roles of LTBPs remain unidentified, they bind the latent TGF- β complex to ECM, likely through covalent linkage of LTBPs to ECM components such as fibrillin 1, decorin, biglycan, and beta glycan (29, 30). Therefore, in addition to the various intracellular, cell surface, and extracellular inhibitory proteins, TGF- β -mediated signaling is further controlled by the ECM-binding property of TGF- β s. It has been shown that binding of TGF- β 1 to thrombospondin can activate the growth factor (31). Thus, in this case, interaction with a matrix protein alone can be critical in the regulation of growth factor activity.

As mentioned previously, many growth factors can also become anchored to heparan sulfate proteoglycans (i.e., syndecans, perlecan, and versican) either on the surface of endothelial cells or within the surrounding ECM by binding to the heparan sulfate. Syndecans are a family of transmembrane core proteins carrying with attachment sites for three to five heparan sulfate or chondroitin sulfate chains (32). By virtue of the presence of heparan sulfate, syndecan interacts with a large number of heparin-binding growth factors such as FGF, VEGF, TGF- β , and platelet-derived growth factor (PDGF). Subsequently, the heparan sulfate chains of syndecans facilitate interactions between heparin-binding growth factors with various ECM molecules, including fibronectin (32). Overexpression of syndecan-1 or shedding of its ectodomain inhibits FGF-2-induced cell proliferation (33).

The consequence of the ECM binding to the growth factors is broad. For PDGFB, its binding to the ECM is critical to the recruitment of tumor pericytes and their integration into the vascular wall (34). ECM can also mask growth factors, for example binding of decorin, biglycan and beta glycan to TGF- β competes for receptor activation and thereby diminish TGF- β signaling (35). Growth factor immobilization may also provide important guidance cues for directional growth and morphogenesis. In fact, only the heparin-binding VEGF isoforms (VEGF164 and VEGF188) generate extracellular gradients that are required for directional migration of endothelial cells (18, 36).

Therefore, a concrete understanding of binding portfolio of each growth factor is critical to gain insights into complex biological processes, such as angiogenesis. Along the same lines, the interface between this growth factor-rich matrix and its body of degrading enzymes offers multiple opportunities for therapeutic intervention.

3. MMPs: DEGRADATION AND PROCESSING OF THE EXTRACELLULAR ENVIRONMENT

Pericellular proteases play an important role in angiogenesis and vasculogenesis. They comprise MMPs, serine proteases, cysteine cathepsins, and membrane-bound aminopeptidases (37). We will center our comments to MMPs.

MMPs are a family of over 20 zinc-containing endopeptidases that can degrade/process various components of the ECM (1). Although each member has its own substrate specificity, redundancies exist, and MMPs in concert are capable of degrading a wide spectrum of matrix proteins, and therefore they are considered to be the major proteases involved in the remodeling of the endothelial basement membrane and interstitial matrix (37). Quiescent endothelial cells produce little or no MMPs, whereas the activated (i.e., angiogenic, in wound healing, and in inflammation) endothelial cells strongly up-regulate the expression of several MMPs *in vitro*.

Recently, it has become clear that MMPs' role in angiogenesis is more complex than simply degrading the ECM to facilitate invasiveness of endothelial cells. For example, MMPs have been shown to generate both pro- and anti-angiogenic molecules by proteolytic cleavage of ECM components. MMPs cleave type IV collagen to expose cryptic $\alpha v\beta 3$ -binding sites. Following cleavage, type IV collagen loses its binding to integrin $\alpha 1\beta 1$ but binds to $\alpha v\beta 3$ integrin with higher affinity and this promotes angiogenesis (15). This cleavage is associated with increased MMP-2 expression and activation. Exposure of these cryptic sites occurs within the endothelial basement membranes in angiogenic and tumor blood vessels but not in quiescent vessels. Relevant to anti-angiogenic activities of MMPs, the C-terminal non-collagenous I (NC1) domain of several collagen chains, generated by proteolytic cleavage, shows anti-angiogenic activity (16). These include endostatin (the NC1 domain of type XVIII collagen $\alpha 1$ chain), tumstatin (type IV collagen $\alpha 3$ chain NC1), and arrestin (type IV collagen $\alpha 1$ chain NC1). Endostatin can be released by MMP-3, MMP-9, MMP-12, MMP-13, and MMP-20, as well as by several cathepsins (38). Endostatin binds to cell surface proteoglycans, to VEGFR-2, and to integrin $\alpha 5\beta 1$ to inhibit VEGF- and bFGF-induced endothelial cell migration and to induce apoptosis (39). In addition, endostatin blocks the activation and activity of MMP-2, MMP-9, MMP-13, and MT1-MMP (40, 41). Tumstatin, which can be released by MMP-9, inhibits endothelial cell proliferation and promotes apoptosis. Decreased levels of tumstatin in MMP-9 null mice were shown to be responsible for increased tumor growth (39, 42). With respect to other ECM molecules proteolyzed by MMPs, fibronectin is concentrated at the pericyte–endothelial cell interstitium, and its degradation by MMPs gives rise to biologically active fragments (43). Among these, 145-kDa fibronectin fragment inhibits endothelial cell proliferation and stimulates pericytes and VSMC proliferation, suggesting a role for this fragment in vessel maturation (44).

In addition to degrading ECM components and activating other enzymes, MMPs can enhance the availability/bioactivity of growth factors and cytokines. Degradation of ECM releases ECM/basement membrane-sequestered angiogenic factors such as VEGF, bFGF, and TGF- β . In tumor angiogenesis, MMP-2 and MMP-9 have been shown to be critical for the “angiogenic switch” when tumors first become vascularized by the selective release of VEGF (45). Similarly, overexpression of MMP-9 in human breast cancer MCF-7 cells resulted in increased tumor angiogenesis, tumor growth, and VEGF/VEGFR-2 complex formation suggesting that MMP-9 regulates the release of VEGF from the ECM (46).

MMPs can target many non-ECM proteins, including growth factors, growth factor receptors, cell-associated molecules, and cytokines. MMPs release active growth factors by cleavage of growth factor precursor or growth factor-binding proteins. For example, MMP-3 and MMP-7 have been shown to cleave the membrane-bound precursor of heparin-binding EGF (HB-EGF), releasing active HB-EGF, whereas tumor necrosis factor- α (TNF- α) is released from the cell surface by MMP-1, MMP-3, and MMP-7 (47). MMP-1, MMP-3, MMP-7, or MMP-13 releases active VEGF165 from connective tissue growth factor (CTGF)/VEGF complex by direct cleavage of CTGF (48). MMP-2 and MMP-9 proteolytically activate latent TGF- $\beta 1$ and TGF- $\beta 2$ (47, 49). Also recently, it was shown that bone morphogenetic protein 1 (BMP1)-like MMP cleaved latent TGF- $\beta 1$ -binding protein LTBP_s at two specific sites, thus liberating a large latent complex of TGF- β from ECM and resulting in subsequent activation of TGF- $\beta 1$ *in vitro* (50).

Relevant to angiogenesis, MMP-9 cleaves the pro-inflammatory, pro-angiogenic cytokine IL-8 increasing its activity by tenfold (51). On the other hand, MMP-2 cleaves the FGF receptor 1 (FGFR1) releasing the soluble ectodomain of FGFR1 that can still bind FGFs, but lacks signaling capacity (52). Also, MMPs cleave the ectodomain of VE-cadherin, a major cell–cell adhesion molecule in endothelial cells (8). MT1-MMP processes the $\alpha\beta\beta$ integrin into two disulfide-linked fragments that retain RGD-ligand binding, and this processing enhances integrin signaling through focal adhesion kinase, contributing to enhanced adhesion and cell migration *in vitro*. MMP-2, MMP-7, MMP-9, and MMP-12 have the capacity to hydrolyze plasminogen to form the potent angiogenesis inhibitor, angiostatin (53–55).

4. VEGF PROCESSING: A NEW ROLE FOR MMPS IN ANGIOGENESIS

VEGF signaling is essential for specification, morphogenesis, differentiation, and homeostasis of vessels during development and in the adult (56–60). Furthermore, this signaling pathway is an integral component of pathological angiogenesis during tumor expansion (61). In fact, decreased levels of VEGF result in suppression of vascular expansion and concomitant reduction of tumor growth and metastasis (62,63). Therefore, it is not surprising that VEGF levels are under exquisite transcriptional and translational control, and slight alterations in expression levels can have devastating effects during development. In fact, unlike most mammalian genes, inactivation of only one allele results in embryonic lethality at mid-gestation due to severe cardiovascular defects (64,65). Interestingly, a twofold increase of VEGF can also lead to lethality (66). Thus, both decrease and increase in VEGF levels translate into significant pathological effects to the vasculature and to the organism as a whole. Therefore, regulation of VEGF synthesis, secretion, and availability carries important implications in the modulation of the angiogenic response.

VEGF-A (also known as simply as VEGF) exists as five different isoforms termed according to the number of amino acids, they are 121, 145, 165, 189, and 206 in humans (in mouse each isoform lacks one amino acid). These forms are generated by alternative splicing of a single pre-mRNA and differ in their ability to bind to heparan sulfate and to ECM molecules. The gene encoding VEGF-A comprises 14 kb and contains eight exons. Exons 2–5 code for the receptor-binding sites, whereas exons 6a, 6b, and 7 code for residues that bind to matrix proteins. These last three exons can be selectively spliced generating the different protein isoforms. The last exon (exon 8) is present in all isoforms. VEGF121 lacks exons 6a, 6b, and 7 and is the only highly soluble form. All other variants bind to ECM proteins restricting access of the growth factor to receptors on target cells. The affinity for matrix proteins is thought to be proportional to the length of the carboxy-terminal end (Fig. 1).

How can matrix-bound VEGF become free from its interactions with the ECM? A favored hypothesis has been that MMPs mediate its release by degradation of ECM proteins. Although this possibility is likely correct, we have been interested in testing an alternative (yet not exclusive) possibility, namely that VEGF-A could be cleaved intramolecularly to specifically mediate release. We showed that VEGF could indeed be cleaved by several MMPs, releasing a dimeric fragment of 32 kDa able to phosphorylate VEGFR2 and induce angiogenesis *in vivo* (9) (Fig. 2). The location of the cleavage site (113 amino acid) indicates that all VEGF forms (including VEGF120) are susceptible to this event.

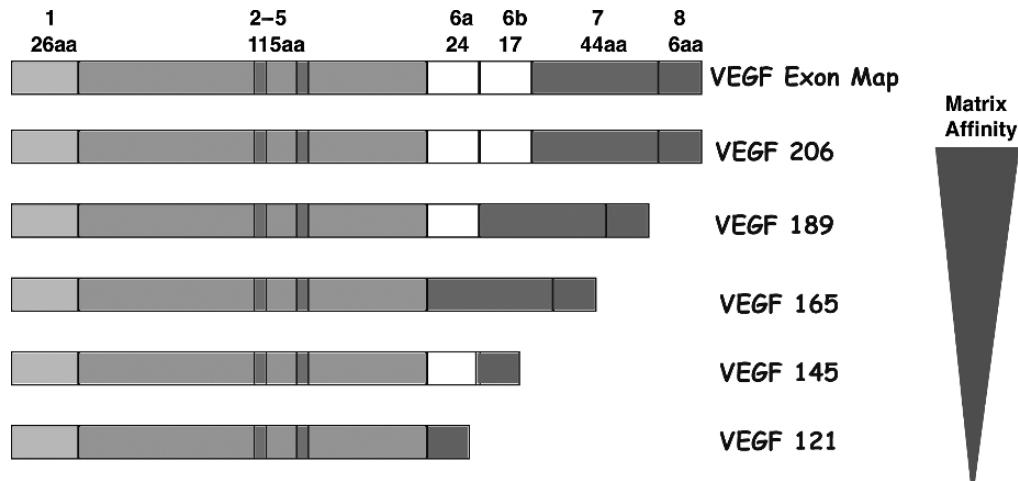


Fig. 1. Structure of vascular endothelial growth factor (VEGF) isoforms and matrix affinity. VEGF is coded by eight exons. Exons 2–5 code for the receptor-binding region, whereas exons 6a, 6b, and 7 code for amino acids involved in binding to the extracellular matrix (ECM). These can be alternatively spliced to give rise to multiple isoforms.

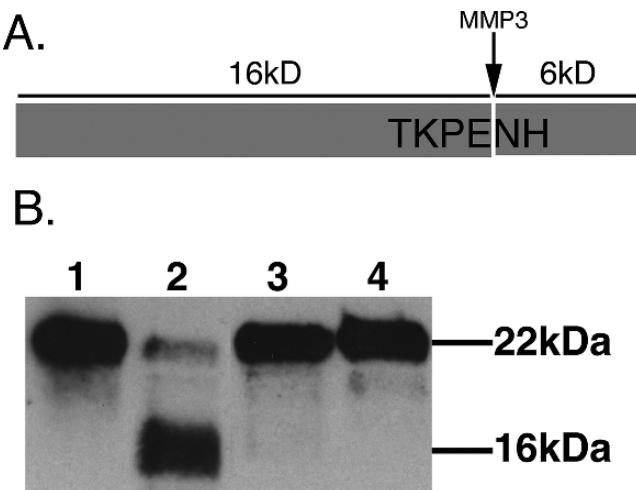


Fig. 2. Vascular endothelial growth factor (VEGF) cleavage by matrix metalloproteinase-3 (MMP-3). (A) Schematic representation of VEGF monomer and the site for MMP cleavage. (B) Biotinylated VEGF165 was incubated with MMP-3 in the presence of specific inhibitors indicated below. The digestion products were resolved in tricine gels and were detected by avidin-HRP. Lanes 1, VEGF; 2, VEGF + MMP-3; 3, VEGF + MMP-3 + EDTA; 4, VEGF + MMP3 + BB94. EDTA blocks MMPs function and BB94 is a pan-MMP inhibitor. 22-kDa, glycosylated mVEGF165 monomer and 16-kDa, cleaved fragment.

In that same study, we determined the significance of VEGF processing to tumor angiogenesis and compared the effects of MMP-cleaved VEGF and an MMP-resistant VEGF to wild-type VEGF. Our findings demonstrated that bound (MMP resistant) and soluble VEGF (MMP cleaved) are able to activate VEGFR2 equivalently *in vitro*, yet each form elicits distinct modes of vascular expansion *in vivo*. Signaling initiated by matrix-bound VEGF resulted in the formation of a highly branched vasculature, in contrast, signaling initiated by soluble (MMP cleaved) VEGF leads to vascular hyperplasia and hypertrophy with poor sprouting response (Fig. 3) (9).

To gain further insight onto the cellular effects mediated by soluble and bound VEGF, we evaluated their effects *in vitro*. Purified growth factors at identical concentrations were included in fibrinogen/fibronectin gels before polymerization, and the behavior of endothelial cells bound to sepharose beads was examined. Our data showed that MMP-resistant VEGF induced capillary morphogenesis, whereas cleaved VEGF (VEGF113) induced the proliferation of endothelial cells in sheets (9). These results indicate that local and discrete VEGF164 cleavage is likely to occur as endothelial cells migrate and grow as sheets, whereas in the absence of such digestion, VEGF mediates the organization of cord-like structures. Although further mechanistic exploration of these findings is in progress, it is likely that specific recruitment of distinct downstream signaling targets is a key molecular initiator of these morphogenetic events.

VEGF is known to signal through two receptor tyrosine kinases: VEGFR1 (flt-1 or FLT-1) and VEGFR2 (flk-1 or KDR) (67). In addition, a non-tyrosine kinase receptor, neuropilin-1, is known to bind to VEGF and modulate the responses to VEGFR2 (68). The two tyrosine kinase receptors share 44% homology and consist of seven extracellular immunoglobulin-like domains, a single transmembrane region, a split tyrosine kinase domain that is interrupted by a 70 amino acid kinase insert, and a C-terminal tail.

VEGFR1 exists in both transmembrane and soluble forms, and although it displays high affinity, it negatively regulates vasculogenesis and angiogenesis during early development (69). Genetic inactivation of VEGFR1 results in increased hemangioblast commitment toward the endothelial fate, an outcome that leads to profuse vascular disorganization due to endothelial cell overgrowth (69). These data, as well as additional information from several laboratories, indicate that VEGFR1 functions as a decoy

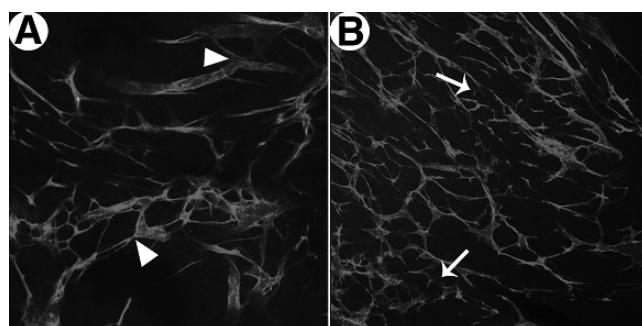


Fig. 3. Distinct tumor vessel phenotype by different vascular endothelial growth factor (VEGF) forms. Platelet/endothelial cell adhesion molecule (PECAM) staining of tumor sections expressing cleaved form of VEGF (**A**) and MMP-resistant form of VEGF (**B**). Arrowheads point to dilated and fused vessels. Arrows point to excess vessel branching. Note the difference in vessel density between **A** and **B**. (Please see color insert.)

receptor and regulator of VEGFR2 function by controlling the levels of VEGF at the cell surface (70). Furthermore, VEGFR1 has been shown to display pro-angiogenic properties during inflammatory responses. Its contribution appears to be relevant in rheumatoid arthritis although it is unclear whether this activation is mediated by VEGF-A, VEGF-B, placental growth factor (PLGF), or a combination thereof (71).

Our current understanding indicates that the sum of pro-angiogenic responses initiated by VEGF originates primarily through activation of VEGFR2 (67). VEGFR2 has been shown to induce proliferation, migration, survival, and permeability (67). However, much remains to be learned about how this single receptor regulates downstream signals to specify each of these possible responses. Six auto-phosphorylation sites have been identified on the intracellular domain of VEGFR2: 951, 996, 1054, and 1059 are located in the kinase domain, and 1175 and 1214 are located within the C-terminal tail (67). Using a combination of genetic deletion and cell biological approaches, tyrosine 1175 has been identified as a VEGFA-dependent auto-phosphorylation site essential to developmental angiogenesis (72). Other individual, and some combinations of, tyrosine sites have been thought to play different roles in signal transduction pathways that affect neovascularization of tumors and permeability events.

In terms of specific intracellular signal transduction pathways, it is well accepted that VEGFR2 activates PLC gamma and phosphatidylinositol 3-kinase (PI3K) through binding to phosphorylated VEGFR2_{Tyr1175} (73). Activation of PI3K pathway through Tyr1175 leads to signaling through AKT/PKB and regulation of endothelial cell survival (74, 75) and in binding to the adaptor protein Sck/ShcB (76). In addition, Shb also binds to phosphorylated Tyr1175 although the biological significance of this binding is yet to be determined (77).

Tyrosine 951 also has important downstream-signaling events. Once phosphorylated, this residue binds to TSAd [T-cell-specific adaptor; also known as VEGFR-associated protein (VRAP)] (78). The phosphorylated Tyr-951-TSAd complex regulates cell migration and has been implicated in neovascularization of tumors (78, 79). In addition, VEGF-A induces the formation of a complex between TSAd and Src, which indicates that TSAd might regulate Src activation and vascular permeability downstream of VEGFR2 (78, 80).

Targeted mutation of Tyr1212 (corresponding to human Tyr1214) to phenylalanine (Tyr1212Phe) results in mice that are viable and fertile (72). However, phosphorylation of Tyr1214 has been implicated in VEGF-induced actin remodeling through the sequential activation of cdc42 and p38MAPK (81). Inhibition of the p38MAPK augments VEGF-induced angiogenesis in the chorioallantoic membrane (CAM) (82, 83) without an accompanying increase in vascular permeability (82). In addition, p38MAPK induces the phosphorylation of the heat-shock protein-27, a molecular chaperone that positively regulates VEGF-induced actin reorganization and migration (84, 85). In light of these findings, it is unclear how to reconcile the outcome of the mutant mouse (Tyr1212Phe) (72). Either additional sites are involved or compensatory mechanisms might overcome the mutation. Perhaps, a detailed evaluation of the p38MAPK activation in the mutant mice could shed light on this paradox.

An integration between the selective activation of tyrosine residues and their specific downstream targets together and the soluble versus bound VEGF-induced activation of VEGFR2 will likely shed light on the molecular orchestration that leads to the morphogenesis of differential vascular beds.

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6

Endothelial Precursor Cells

Rebecca G. Bagley, MS

SUMMARY

The recognition that blood vessel growth is a critical process in developing tumors has led to the increased study of tumor vasculature. Originally, it was considered that only nearby host vasculature provided the necessary cellular components that comprise blood vessels, endothelial cells (EC), and pericytes. Data gathered in recent years have supported the notion that endothelial precursor cells (EPC) exist postnatal and that EPC can also contribute to both physiological and pathological angiogenic processes including wound healing and cancer. EPC possess or can acquire many of the characteristics of mature, fully differentiation EC such as the ability to form tubes, to incorporate into developing vasculature, and to express many EC markers such as vascular endothelial growth factor 2 (VEGFR2) or von Willebrand factor (vWF). The most primitive EPC exhibit properties of progenitor cells that are denoted by expression of the hematopoietic stem cell marker CD34 and typically co-expressing CD133. Experiments in genetically engineered mice have demonstrated recruitment of cells from bone marrow to tumor vasculature. Studies in humans have also proven the existence of EPC. Although EPC represent a small percentage of the EC population, these progenitor cells offer new insights into tumor biology and may reveal novel targets for drug development.

Key Words: Endothelial; precursor; progenitor; vasculature; tumor; angiogenesis; CD133.

1. INTRODUCTION

The identification of endothelial precursor cells (EPC) in postnatal neovascularization came to the forefront of scientific research following efforts in the areas of wound healing and cardiovascular disease (1–6). The leap to investigating the role of EPC in tumor angiogenesis was a logical next step as targeting tumor vasculature became a focus of new drug endeavors following the recognition that blood vessels play a vital role in driving tumor growth towards malignancy (7–9). In more recent years, the burst of stem cell research has also increased interest in identifying progenitor cells for tissue renewal or in recognizing their contribution to pathological processes. The need for progressive therapies to target tumors has expanded beyond attacking the cancer cells themselves and toward disrupting the supporting vasculature. A distinct

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population of immature EPC represents a new source of targets for anti-angiogenic therapy and may have value as a surrogate biomarker.

2. DERIVATION AND CHARACTERIZATION OF EPC

CD133, earlier reported as AC133, has been one of the more widely associated markers distinguishing EPC. The CD133 antigen possesses five transmembrane domains and is also associated with hematopoietic stem cells (10, 11). CD133 is detected at the earliest stages of EPC development but becomes downregulated as the cells respond and mature to angiogenic stimuli (12). CD133 is typically co-expressed with the marker CD34 on a subpopulation of hematopoietic stem cells that denote the most primitive and immature form of EPC (13).

Stimulation of CD133+/CD34+ progenitors in culture that were isolated from a variety of tissue sources has yielded populations of cells that phenotypically resemble endothelial cells (EC) *in vitro*. CD133+ bone marrow cells stimulated with vascular endothelial growth factor (VEGF) differentiated into cells expressing von Willebrand factor (vWF) and VEGFR2 and acquired properties associated with EC, such as uptake of acetylated low density lipoprotein (LDL) or binding of ulex europaeus lectin (UAE)-1 lectin (14, 15). CD34+/CD133+ cells that differentiated along an EC lineage were capable of forming capillary-like networks on Matrigel (Fig. 1) (16). A similar progenitor population was obtained from peripheral blood of healthy volunteers that, when exposed to VEGF, began to express CD31, a key marker that frequently defines an endothelial phenotype (17). CD34+ cells obtained from human fetal livers expressed CD133 and also co-expressed VEGFR-3 (18) in addition to presenting other traits associated with EC. Establishment of EPC in culture is not limited to bone marrow, peripheral adult blood, or fetal liver, and EPC may also be derived from cord blood (19, 20).

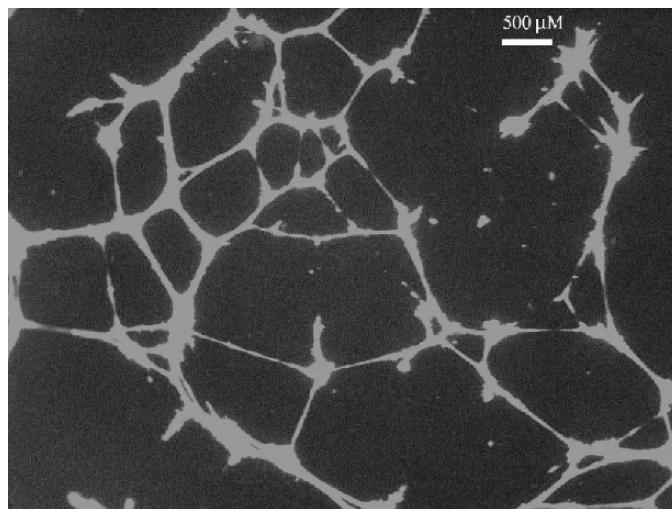


Fig. 1. CD34+/CD133+ cells from human bone marrow were stimulated in culture with bFGF, VEGF, and heparin on fibronectin-coated flasks. The newly adherent, differentiated EPC were capable of forming tube networks on Matrigel after an overnight incubation. EPC were stained with calcein prior to imaging. (Please see color insert.)

The defining factors that identify EPC revolve around the expression of molecular markers. There is some variation among reports of EPC as to which markers are expressed and most representative of this cell type. The expression of markers is likely to vary depending upon the stage of maturity of the EPC under investigation. The multistage process begins with the most primitive form of EPC that then differentiates and matures into a phenotype that eventually resembles mature EC. In addition, the stimulus of EPC mobilization may also influence the presentation of antigens. Molecular markers that are expressed under a condition such as ischemic injury could be very different than what may be presented in response to tumorigenic growth factors.

CD133 expression may not be limited to EPC but could denote a stem cell population with additional capabilities (21, 22). Many of the markers associated with EPC may also be expressed on other cell types, CD34 on hematopoietic precursors for example. Co-expression of a panel of markers is now *de riguer* in identification of EPC, particularly when analyzed by flow cytometry. Therefore, in addition to CD133, VEGFR2 is widely considered to be a key molecular marker for EPC that denotes an endothelial phenotype (12, 23, 24). EPC mobilization can be induced by VEGF in mice resulting in an increase in EPC levels in circulation, enhanced corneal neovascularization, and more efficient repair of ischemic tissues (25, 26). VEGF has been shown to promote EPC recruitment in human patients with critical limb ischemia that received intramuscular VEGF gene transfer (27). Elevated VEGF levels in plasma correlated to an increase in circulating EPC (cEPC). This change was detectable at 1 week after gene transfer and up to 4 weeks thereafter. In those experiments, EPC were defined by flow cytometry detection for VEGFR2, VE-cadherin, CD34, AlphavBeta3, and E-selectin.

The distribution and frequency of CD133-positive cells were investigated in clinical samples through immunohistochemical (IHC) analysis of a panel of 79 non-small cell lung cancers (NSCLC) (28). Normal, adjacent lung tissue from most patients was also available for comparison. In addition to CD133, CD31 and VEGFR2 staining was also performed to identify vasculature and to determine microvessel density (MVD). Approximately two-thirds of tumor specimens had increased numbers of CD133-positive cells compared to healthy tissue. In some cases, CD133 expression was detected in newly forming capillaries. Increased CD133 expression correlated with increased VEGFR2 expression but not with proliferation. MVD data based on CD31 immunostaining was also enhanced in about half the tumor samples indicating active tumor vascularization. The detection of CD133-expressing cells in the vasculature of NSCLC tumors in a majority of the biopsies analyzed suggests that EPC can contribute to malignant blood vessels. The results also further support the claims that tumor vasculature can develop in part from EPC recruited from the bone marrow or other parts of the body and does not have to be derived exclusively from the extension of nearby host vasculature.

Serial analysis of gene expression (SAGE™) was performed on EPC derived from CD34+/CD133+ bone marrow cells stimulated with VEGF and bFGF (16). The gene expression profile was compared with fully differentiated EC, HMVEC, and EC isolated from a total of seven human brain, breast, and colon tumors. Genes overexpressed in tumor EC versus normal, adjacent tissue were identified. EPC and HMVEC libraries were probed for tags highly expressed in tumor endothelium at various levels of stringency (Fig. 2). EPC expressed more of the genes in tumor EC compared to

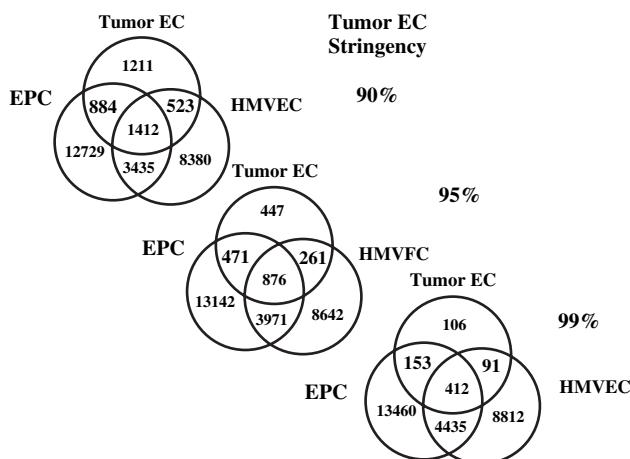


Fig. 2. Schematic showing overlaps in gene expression determined by SAGE analysis for tumor endothelial cells derived from human surgical specimens of three breast tumors, three brain tumors, and one colon tumor, and EPC and HMVEC grown in cell culture. The SAGE gene expression data from the seven tumor endothelial cell libraries were pooled and genes expressed at higher levels in the tumor endothelium were compared to normal endothelial cells derived from one normal breast, two normal brain, and one normal colon specimen at ≥ 99 , ≥ 95 , and $\geq 90\%$ confidence levels by chi square analysis.

HMVEC, indicating that EPC may offer novel targets in an immature state that are subsequently found in tumor vasculature. There was also a similar overlap in gene expression between EPC and HMVEC indicating that the EPC in this study represent an intermediary between a primitive stem cell and a mature EC.

Bone marrow and peripheral blood are common sources of origin for obtaining EPC with a large body of data identifying CD34+/CD133+-expressing cells as the relevant population. However, the close relationship between hematopoietic cells and EPC led to additional reports suggesting that monocytes could likewise yield EPC populations upon stimulation under similar conditions, that is, exposure to VEGF and bFGF on fibronectin-coated plates. CD34-/CD14+ monocytes were isolated from human peripheral blood, stimulated in culture, and within 2 weeks began to express the EC marker vWF and acquired the ability to form tubes on Matrigel (29). Similar data were obtained with human peripheral blood mononuclear cells that resulted in an EPC population that responded to VEGF by proliferation and vWF induction, was capable of acetylated LDL uptake, and yet also expressed markers associated with macrophages and monocytes (30). Human CD14+ monocytes were also capable of binding to UEA-1 lectin and expressed CD31 (31). Most of the EPC in these studies did not strongly express VEGFR2; however, the close lineage between hematopoietic cells, EC, and their precursors suggest that more than one subpopulation of cells are capable of adopting an EC phenotype and contributing toward tumor vasculature.

3. EPC CONTRIBUTION TO TUMOR VASCULATURE

The ability of EPC to home from the bone marrow to areas of tumor growth or to angiogenic growth factors *in vivo* was demonstrated in mice lacking the *Id* gene (32). *Id*-mutant mice are characterized as tumor resistant with impaired angiogenic potential.

However, following transplant with wild-type (WT) bone marrow, the ability to support tumor growth was restored. Donor bone marrow expressing β -galactosidase from Rosa-26 mice was detected in tumor vasculature, indicating that bone marrow is a source of endothelial precursors. Inhibitors against VEGFR1 and VEGFR2 were able to diminish the effect of EPC, thereby identifying EPC as candidates for anti-angiogenic therapy. In addition to influencing tumor growth, angiogenic activity in a VEGF-driven Matrigel assay was also restored in *Id*-mutant mice that received WT bone marrow.

Subsequent studies were performed in *Pten*^{+/−} tumors that investigated the effect of anti-angiogenic stress on spontaneous lymph hyperplasias and uterine carcinomas in animals crossed with an *Id* mutant phenotype (33). The contribution of bone marrow-derived endothelial precursors varied among the tumors that developed. When EPC were incorporated into malignant vasculature, tumors were more viable with reduced areas of necrosis and hemorrhagic events. These results indicate that EPC can contribute toward enhancing vasculature function. Although EPC contributed to 16% of neovessels in the uterine tumors, there was no apparent contribution to lymph hyperplasias. This data are in contrast to xenograft tumors in *Id* mutant mice where EPC were detected in nearly 100% of the tumors, indicating that the processes that drive the formation of neovasculature in tumors can differ between traditional xenograft models and tumors that arise spontaneously and therefore may be more indicative of the clinical phenotype.

In human cancers, EPC have been implicated in multiple myeloma (34). Results obtained from 31 patients indicate that there are higher levels of circulating endothelial cells (cEC) and cEPC compared with healthy controls. EPC were distinguished from EC by co-expression of CD133 and VEGFR2. *Ex vivo*, cEPC were capable of late colony formation outgrowth and capillary-like tube formation on Matrigel. Treatment of myeloma patients with the anti-angiogenic agent thalidomide not only resulted in a reduction of cEPC and cEC but also inhibited cEPC function *in vitro*. Reduced levels of cEPC also correlated with levels of serum M protein and β 2-microglobulin that are indicative of disease activity in multiple myeloma. These clinical observations suggest that in some cancers, cEPC may serve as a surrogate biomarker that will be further discussed in this chapter.

In solid tumors, EPC have been identified in circulation of patients with NSCLC (35). EPC were detected from peripheral blood by flow cytometry with antibodies against CD34, CD133, and VEGFR2. Although incorporation in the tumor tissue itself was a rare event (9 of 22 cases), cEPC were significantly increased in patients prior to treatment versus healthy controls with higher numbers of cEPC correlating with poor overall survival. Levels of cEPC were significantly lower in those patients that responded to treatment.

cEPC have also been identified in a small population of patients with cervical cancer compared to healthy volunteers (36). Intratumoral oxygen tension was assessed also as an indication of the degree of hypoxia in the tumors. Although the total number of cEPC was unchanged, the fraction of cEPC compared with the number of hematopoietic stem cells (HSC) was elevated. This observation was noted to a reduction of HSC rather than an increase of cEPC. A significantly inverse correlation between oxygen tension and cEPC was found with the conclusion that the fraction of cEPC versus HSC could be used as an indication of tumor tissue oxygenation.

As evidence supporting the existence of EPC in tumor vasculature was generated, some controversy arose as to the significance of endogenous EPC contribution in the tumor microenvironment. Some studies involving murine tumor models have also demonstrated a minimal contribution of EPC to tumor vasculature and that tumor endothelium is derived solely from existing vasculature (37,38). In humans, the degree of bone marrow-derived EPC incorporation into tumor vasculature was investigated in a quantitative analysis performed on tumor specimens arising from patients who had previously received gender-mismatched bone marrow transplants and whose cancer had relapsed (39). The gender mismatch enabled the detection of bone marrow contribution to endothelium versus that derived from the host vasculature. Fluorescent *in situ* hybridization (FISH) analysis was performed with sex chromosome-specific probes in conjunction with fluorescent antibody staining for vWF to identify endothelium. The six cancer studies represented lymphomas, sarcomas, and carcinomas that developed 15 months to 5 years after bone marrow transplantation. Positive staining confirmed that bone marrow-derived cells indeed incorporated into the tumor endothelium, albeit at low levels. The percentage of bone marrow-derived EC ranged from 1 to 12, with a 4.9 average. These results support the role of bone marrow-derived endothelium in malignant tissues, yet also suggest that they are not a driving force, at least in the specimens that were evaluated.

Tumor development is a dynamic process that results in heterogeneous vasculature and variable gene expression within the various cell populations comprising the tumor microenvironment. Transgenic mice that develop spontaneous tumors are an ideal model system for study as the engraftment of donor bone marrow cells to tumor vasculature can be more extensively investigated. The contribution of bone marrow-derived EPC to tumor vasculature was assessed in prostate tumors of various grades arising in *Id*-deficient TRAMP mice (40). Rosa-26 mouse bone marrow cells expressing the β -galactosidase reporter gene were delivered to lethally irradiated recipient mice. Prostate tumors were graded as either poorly differentiated or well differentiated. In the well-differentiated prostate adenocarcinomas, there was little evidence of bone marrow-derived EC incorporation with only about 2.6% of EC being LacZ+. By comparison, in poorly differentiated prostate tumors, bone marrow EC comprised 14.2% of vasculature. Therefore, the extent to which EPC incorporate into tumor vasculature varied depending upon tumor grade. The numbers obtained in the analysis (2.6–14.2%) are in a similar range to figures reported in the bone marrow, gender-mismatched study (39), indicating that across mouse and human species, EPC involvement in tumor vasculature is real but represents a minor proportion of contributing cells. The contribution of bone marrow-derived cells to tumor endothelium has also been demonstrated in conventional mouse models that involved TC71 human Ewing's sarcoma cells where donor-positive vessels accounted for approximately 10% of the tumor vasculature (41).

The studies that quantified the degree of EPC recruitment in tumors were gathered from specimens that represent a snapshot in time. In addition to bone marrow engraftments, transgenic mouse models allow for the analysis of tumor progression throughout the multistage process as tumors develop from a premalignant stage to end-stage tumor that arises in the relevant tissue of origin. RIP1-Tag5 mice spontaneously develop cancer of the pancreas from hyperplastic and angiogenic islets that grow into highly vascularized insulinomas (42). AlbTag transgenic mice are another model of *de novo* carcinogenesis whereby mice develop hyperplasia, dysplasia, and eventually

hepatocellular carcinoma (43). RIP1-Tag5 mice or AlbTag mice were lethally irradiated followed by reconstitution of bone marrow from enhanced green fluorescent protein (GFP) reporter mice, generating a model whereby any contribution of bone marrow cells to tumor endothelium could be traced back to the bone marrow (44).

Bone marrow-derived EC were quantified at various stages of tumor progression in these murine tumor models. In the RIP1-Tag5 mice, the smaller, microscopically detectable tumors displayed 15% GFP+ cells in blood vessels compared with about 38% of vessels in late-stage tumors (week 20 vs. week 32). Similarly, in AlbTag mice, GFP+ cells comprised approximately 5.8% of EC in tumors collected at 12 weeks versus ~26.8% of tumor EC at 16 weeks. The identification of these cells further indicated a rather homogenous distribution, as opposed to being localized to tumor “hotspots.” The same study also concluded that bone marrow-derived EPC integrated into tumor vasculature synchronized with elevated VEGF serum levels that directly correlated to tumor size. Results from this study indicate that in the mouse models employed, EPC recruitment and involvement occur at a later stage of tumor development when vasculature becomes more heterogeneous (44, 45).

Allogeneic bone marrow transplant studies have been instrumental in tracking cells from the bone marrow to sites of tumor growth. Genetically engineered mice that express GFP or LacZ allow for the distinction between EC derived from bone marrow and those from host vasculature. This strategy was employed in an orthotopic glioma model where irradiated mice received GFP bone marrow cells (46). GFP+/CD34+ cells preferentially migrated to the developing tumor seeded from RT-2/RAG rat glioma cells compared to healthy brain tissue. In addition, expression of angiopoietin-2 in the tumor often co-localized with GFP+/CD34+ detection; some co-localization of bone marrow cells was observed with angiopoietin-1, but this effect was less pronounced. Further analysis revealed that up to 8% of EC in tumor vasculature was derived from the bone marrow. This percentage is consistent with other reports (40, 41, 44).

An additional study involving irradiated T-cell knockout mice (RAG/KO5.2) receiving GFP+ bone marrow cells found that these cells could be detected in over 50% of orthotopic RT2 glioblastoma tumors (47). These cells could express VEGFR2 and CD34 were more commonly found at the loops and branch points of vessels. The bone marrow-derived cells typically represented approximately 4% of all EC in the tumor vasculature at day 12. The percentage of tumor vessels that contained GFP+ cells increased with tumor size and vascular density. These results support the hypothesis that a population of cells from the bone marrow can be recruited and contribute to vascular development at the stage when there is a burst of angiogenic activity.

By contrast, another study involving an orthotopic murine glioma model found little contribution of EPC to tumors despite the utilisation of GL261 glioma cells that were genetically engineered to overexpress VEGF and resulted in highly vascularized tumors (48). Recipient mice were engrafted with bone marrow cells from transgenic mice constitutively expressing the β-galactosidase or GFP reporter genes. Co-staining for the EC markers CD105 and CD31 identified vasculature in both subcutaneous and orthotopic tumors. In subcutaneous tumors, LacZ+ cells were present but did not co-stain with CD105 or CD31 and were primarily found in areas of necrosis. In intracerebral tumors, LacZ+ cells were found in the tumor stroma or in peripheral vessels that did co-localize with EC markers. However, in both subcutaneous and intracranial tumors, less than 1% of the vessels were LacZ+.

The differences in the degree of EPC incorporation in murine gliomas indicate that not only the stage or grade of the tumors will influence EPC involvement but suggest that differences at the molecular level may be involved. Differential gene expression amongst gliomas may dictate which pathways will be initiated in driving vascular development. Alternatively, the animal model systems employed to investigate EPC may also influence results. The recruitment of EPC in response to angiogenic stimuli was investigated across multiple inbred mouse strains (49). Circulating EC or progenitors in unstimulated mice were detected in peripheral blood, albeit at various levels depending upon the strain. For example, mice of the 129/SvlmJ or Balb/c/J background had significantly higher levels of cEPC than C57BL/6J or C3H/Hej. The ability of the mice to then respond to angiogenic stimuli and promote EPC mobilization *in vivo* was assessed in several settings. In the corneal neovascular micropocket assay, a correlation was found between the angiogenic response and number of EC or EPC in circulation. The strains of mice identified as having higher endogenous levels of circulating EC or EPC (129/SvlmJ) were also the ones that generated greater vascularization in a Matrigel plug perfusion assay. Tumor-bearing mice also possessed greater numbers of cEPC compared with non-tumor-bearing mice, indicating that under certain conditions, tumor development can promote EPC recruitment. Just as some mice are predisposed to EPC involvement in angiogenesis, the same may be true for humans with further selection defined through the genes expressed by malignant cells.

The study of EPC in tumor models often utilizes bone marrow transplants that then necessitate the recruitment of EPC out of the marrow, into circulation, and subsequent homing to the developing tumors. When human-derived EPC were directly co-injected with MDA-MB-231 human breast carcinoma cells into the subcutaneous space of immunodeficient mice, the resulting tumor reached a great volume compared to tumors arising from cancer cells alone (50). IHC staining for CD31-positive vessels indicated that there was no increase in MVD. However, quantitative

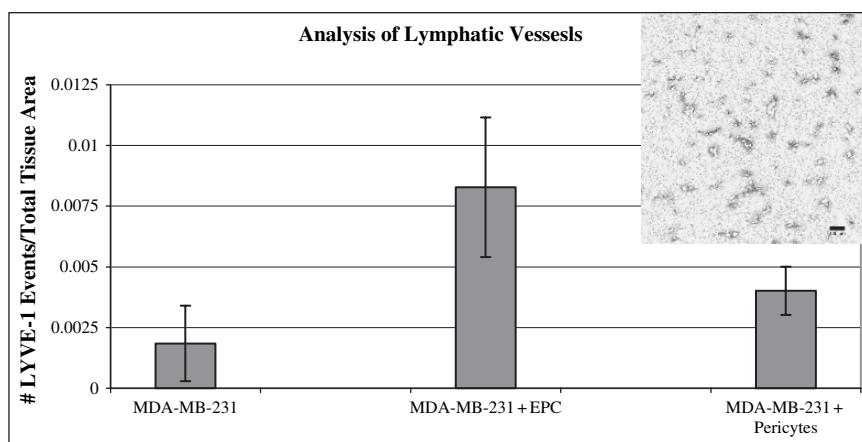


Fig. 3. Lymphatic vessels were identified with an antibody against murine LYVE-1. The degree of lymphatic vessels that developed was increased by the incorporation of EPC ($p < 0.05$, student's *t*-test). The effect of pericytes on lymphatic development was not as significant. Inset: immunohistochemical staining for LYVE-1 in a tumor generated from the co-injection of EPC and MDA-MB-231 cancer cells. Scale bar represents 100 microns.

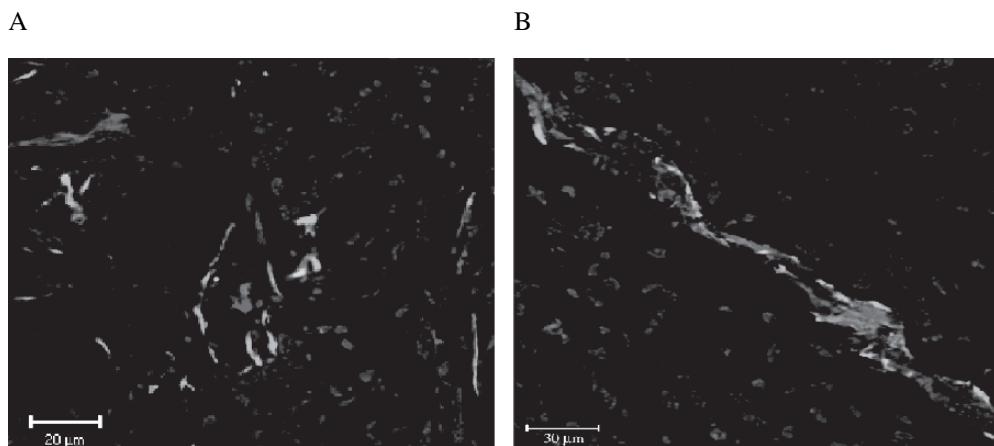


Fig. 4. Vasculature of tumors was analyzed by immunohistochemistry with antibodies against CD31 (red) and α -SMA (green). Subcutaneous tumors were derived from a co-injection of human EPC and MDA-MB-231 breast cancer cells or breast cancer cells alone. **(A)** Blood vessels in control tumors are disorganized with little association between endothelium and perivascular cells. **(B)** Blood vessel of a tumor arising from the co-injection of MDA-MB-231 cells and EPC; direct contact is evident between CD31+ and α -SMA+ cells. (Please see color insert.)

analysis of lymphatic vessels for the antigen LYVE-1 indicated a threefold increase in the presence of lymphatic vessels (Fig. 3). This effect was more pronounced when EPC were included versus pericytes, another cellular component of vasculature. Also, dual staining for CD31 (EC) and for α -smooth muscle actin (SMA) (pericytes) indicated that vessels in the tumors arising from the co-injection of EPC and cancer cells were better organized and possessed a more “normalized” appearance compared to control tumors (Fig. 4). These data suggest that EPC contribution to tumor development may not be limited to direct incorporation into the EC layer of blood vessels but could expand to other cellular functions that support the delivery of growth factors and nutrients such as lymphatic vessel development that may establish a microenvironment where metastasis are more likely to occur.

In the aforementioned xenograft study, EPC resulted in more lymphatic vessel development detected by IHC for LYVE-1. In humans, the expression of the lymphatic antigen LYVE-1 is associated with a poor prognosis in human breast cancer patients (51). Research into six distinct murine breast cancer models showed elevated levels of EPC in bone marrow, peripheral blood, and tumors, further implicating a role for EPC in breast cancer (52). The degree of EPC involvement was particularly pronounced in an inflammatory breast cancer (IBC) xenograft model that displays hypervasculature structures. In a parallel study, human breast cancer specimens were analyzed by IHC methods for the detection of tumor-infiltrating cells (53). IBC biopsies revealed a significantly higher population of infiltrating EC or EPC in the stroma compared to non-IBC samples. IBC is associated with a less favorable prognosis compared to non-IBC. Thus, the recruitment of EPC to tumor development is likely to vary depending upon the properties of the malignant cells and in some cases can be linked to a greater severity of the disease.

4. INHIBITION OF EPC ACTIVITY

EPC can mobilize in response to signals released by malignant cells or stroma. The ability to demonstrate that EPC function can be inhibited would offer a new strategy for anti-angiogenic intervention. The effects of angiostatin, a product of cleaved plasminogen and one of the earlier proteins investigated for preventing blood vessel growth in tumors, were tested *in vitro* on EPC derived from human volunteers (54). Angiostatin inhibited EPC colony formation as well as proliferation. Interestingly, human umbilical vein endothelial cells (HUVEC) proliferation was not affected. Human umbilical vein endothelial cells (HUVEC) represents a fully differentiated, mature EC. The selective inhibition of EPC but not normal EC by angiostatin indicates that EPC represent a unique niche of cells with novel targets that may be more sensitive to progressive therapeutics directed against tumor vasculature.

Endostatin is a fragment of collagen XXVIII and has also been evaluated for anti-angiogenic properties against EC and precursors. In a mouse model of lymphoma, endostatin was either continuously infused or given by bolus injection. Cells collected from peripheral blood were analyzed by flow cytometry with co-expression of CD34 and CD133 to identify an EPC population. Quantification indicated that circulating EPC are present at a concentration of approximately 4 cells/ μ l of blood. The level of EPC was reduced by half following intraperitoneal delivery of endostatin, whereas the continuous infusion of endostatin resulted in a further reduction of EPC. Sustained delivery of endostatin also prevented growth of both Namalwa and Granta 519 tumors (55). Endostatin further inhibited the levels of circulating EC recruited into circulation by VEGF in non-tumor-bearing mice (56).

Impairment of EPC mobilization was further demonstrated *in vivo* in immunodeficient mice bearing human lymphoma cells (57). Metronomic dosing of cyclophosphamide resulted in a consistent decrease in EPC numbers in circulation and retarded tumor growth. By contrast, less frequent dosing of cyclophosphamide at the maximum-tolerated dose caused an enhancement of EPC mobilization, and the tumors derived from Namalwa cells subsequently became drug resistant. The results from this study have important implications for the design of dosing regimens in the clinic. The data suggest that bolus delivery of chemotherapeutics or anti-angiogenic agents such as endostatin are less effective than metronomic dosing and possibly even detrimental in the long term if it serves as a physiological insult thereby driving EPC recruitment as one might expect in a wound healing setting.

Indeed, vascular trauma has been proven to stimulate EPC recruitment (4). Further evidence that therapies can negatively promote EPC involvement was more recently obtained with vascular-disrupting agents (VDA) that occlude existing blood vessels rather than prevent the growth of neovessels (58). Mice bearing subcutaneous Lewis lung tumors received transplant of GFP-labeled bone marrow cells from donor mice. In mice that were treated with VDA, GFP+ cells were localized to the rim or periphery of the tumor, an area that remains difficult to target and often leads to re-growth. By comparison, tumors from untreated mice showed a lesser degree of incorporated GFP+ bone marrow cells. A spike in the levels of circulating EPC was also detected in response to VDA treatment. These data indicate the need not only for a combinatorial approach to treat cancer but also the determination of the appropriate timing and scheduled delivery of agents that target different biological processes that drive tumor development.

Additional studies also indicate that clinical treatments that promote stem cell mobilization may yield undesirable effects. In a traditional xenograft model, CD34+ hematopoietic stem cells were evaluated for their ability to influence the growth of a subcutaneous tumor (59). Daudi cells, a B lymphoblast cell line, were injected in NOD/SCID mice that subsequently received intravenous delivery of human CD34+ cells from peripheral blood of multiple myeloma patients undergoing blood stem cell mobilization with G-CSF and high-dose cyclophosphamide. Tumor growth was enhanced two-fold, and VEGFR-2 transcripts were found only in mice receiving the hematopoietic stem cells. These results further support the hypothesis that precursor cells expressing the antigen CD34 can augment tumor growth and have significant implications for clinical protocols that promote the recruitment of these cells in cancer patients.

In a clinical study, levels of circulating EC and EPC were measured in breast cancer patients that received anthracycline and/or taxane-based neoadjuvant chemotherapy with subsequent surgery (60). Serum levels of proangiogenic factors were also investigated. Although cEC levels were elevated 4.4 times compared with controls prior to treatment, they subsequently decreased following therapy. By contrast, the levels of cEPC and other progenitors increased by 5.7-fold in patients who began treatment with lower levels of these cells initially. The precursor cells that were mobilized following chemotherapy were characterized by the expression of CD34 and VEGFR2, CD34 and CD133, or CD34. In addition, serum levels of VEGF, angiopoietin-2, and erythropoietin were also elevated. These clinical findings illustrate the need to combine anti-angiogenic therapy with chemotherapy in certain indications.

5. EPC AS SURROGATE MARKERS OF ANGIOGENESIS

The development of anti-angiogenic therapies has sparked a field of interest in utilizing circulating EC as a surrogate marker for evaluating efficacy (61–64). The search for EPC as a surrogate for monitoring progression or predicting outcome of disease was pursued in a panel of women with infiltrating breast carcinoma or with earlier stage disease, ductal carcinoma *in situ* (DCIS) (65). Healthy volunteers or those with benign breast disease served as controls for comparison. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of RNA isolated from peripheral blood quantified expression levels of VEGFR2, Tie-2, VE-cadherin, and CD133 as markers distinguishing EPC. In the patient population profiled, there was only a clear correlation between severity of disease and Tie-2 levels. By comparison, levels of CD133 and VEGFR-2 were marginally increased. The lack of increase in CD133 mRNA levels in patients with tumor progression may be an indication of the stage of development of the EPC as they are mobilized from the bone marrow from the most immature state and subsequently begin to differentiate into a more mature phenotype in circulation. In the case of breast cancer, Tie-2 may be the more relevant marker to monitor EPC recruitment into circulation or to track EC populations.

In a similar investigation, the RT–PCR approach was used to measure the same panel of markers as above (65) in healthy controls, pregnant women, or those with newly diagnosed or relapsed cancer (66). The cancer patients represented a variety of cancer types including breast cancer, ovarian cancer, leukemia, B-cell lymphoproliferative disease, hepatocellular carcinoma, neuroendocrine cancer, and lung cancer. In this study, only circulating VE-cadherin levels were significantly increased compared

to Tie-2, VEGFR-2, and CD133 levels. Furthermore, the increase in VE-cadherin levels was found in cancer patients that also presented hematological malignancies and in pregnant women. Those with hematological disease also had increased levels of circulating VEGF and EC. Although the limitations of RT-PCR prevent the conclusive determination of the cell population responsible for the increased VE-cadherin levels, the correlation with serum VEGF levels is suggestive of active angiogenesis in these patients. The ability to profile patients for surrogate markers of angiogenesis in peripheral blood would identify those that would be most likely to respond to anti-angiogenic agents that could target EPC or other vascular cells expressing the same targets.

6. EPC IN SDF-1/CXCR4 PATHWAY

Chemokines play an important role in angiogenesis and several such as CCL2, CCL3, and CCL5 have been implicated in the recruitment of EPC to late-stage tumors in particular (44). The identification of the chemokine receptor CXCR4 as a co-receptor for HIV entry into cells led the way for the subsequent detection of CXCR4 overexpression in many tumor types including those of the breast, renal, brain, pancreas, and lung (67–71). CXCR4 expression can also be detected on mature EC exposed to VEGF and bFGF, or under other activating conditions, and EC function can be stimulated by SDF-1, the ligand for CXCR4 (72–74). EPC, as progenitor cells representing an intermediary between fully differentiated EC and the most immature stem cell, were examined in several studies on the SDF-1/CXCR4 pathway.

EPC derived from human peripheral blood and stimulated in culture with growth factors (VEGF, FGF, EGF, and IGF-1) expressed CXCR4 after 1 week of culture (75). Exposure of CXCR4+ EPC to SDF-1 resulted in an increase in migration *in vitro*. Subsequent delivery of human EPC to nude mice with ischemic limb injury resulted in greater neovascularization of muscle and capillary density when mice also received SDF-1 compared with controls. The recruitment of CXCR4+ progenitor cells to sites of ischemia was further elucidated in experiments demonstrating that HIF-1 induces upregulation of SDF-1 (76).

In an *in vivo* model of cancer, SDF-1 secreted by cancer-associated fibroblasts (CAF) from human breast tumors increased migration of EPC implicating a role for EPC in developing tumor vasculature (77). Antagonists against the SDF-1/CXCR4 pathway demonstrated that angiogenesis could be inhibited (78). CXCR4-neutralizing antibodies delivered to mice bearing Colon38 or PancO2 tumors resulted in a marked reduction in CD31-positive capillaries and a 35% decrease in blood flow.

The expression of CXCR4 on EPC can also be driven by stimulation of PAR-1, a thrombin receptor (79). EPC derived from human CD34+ cord blood cells express PAR-1 following culture with EC growth factors. Stimulation of PAR-1 with a hexapeptide (SFLLRN) led to an increase in mRNA expression of both CXCR4 and the ligand SDF-1 within 4 h. Additional activities that increased upon PAR-1 activation include actin cytoskeleton reorganization and enhanced migration. EPC tube formation that was induced by PAR-1 activation could then be inhibited with monoclonal antibodies against either SDF-1 or CXCR4. Co-expression of SDF-1 and CXCR4 by EPC indicates that the pathway functions through an autocrine loop. As the focus on the overexpression of CXCR4 on human cancers increases, the participation of EPC and other progenitor cells in this pathway remains an understudied area that could yield new insights.

7. CONCLUSIONS

The identification of EPC has led to a rapid flurry of activity in the field that has produced many noteworthy reviews (80–88). The interest in EPC continues to move forward as the field of oncology perseveres to discover new targets that will lead to the development of more effective therapies. The investigation of the biological pathways and processes underlying the malignant transformation of tissues supported by progenitor and stem cells is an exciting area of research that will surely prove to be rewarding in the years to come.

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7

Role of Pericytes in Angiogenesis

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SUMMARY

Pericytes are morphologically, biochemically, and physiologically heterogeneous and play an essential role in angiogenesis. Pericytes interact with endothelial cells, stabilize the newly formed endothelial tubes, modulate blood flow and vascular permeability, and regulate endothelial proliferation, differentiation, migration and survival. This chapter will review the biology, physiology and pathology of pericytes, as well as their role in angiogenesis. We will also discuss the potential relevance of pericytes in anti-angiogenic therapeutic approaches. Dual targeting of both endothelial and pericytes may provide more efficacious anti-angiogenic approaches for cancer therapy.

Key Words: Pericyte; endothelial cell; angiogenesis; PDGF-B; PDGFR- β .

1. INTRODUCTION

A blood vessel consists of at least two cell types: endothelial cells and pericytes. In the process of blood vessel maturation, endothelial cells first form tubes, then recruit pericytes for external coating. The physical and chemical communication between endothelial cells and pericytes is essential for normal blood vessel development. Although endothelial cells have been extensively studied, much less is known about the pericytes. Pericytes were first noted more than 100 years ago as perivascular cells that wrap around endothelial cells to form small blood vessels and over the years have been called several names including Rouget cells, adventitial cells, deep cells, Ito cells, and perivascular cells (1, 2). In 1923, the term “pericyte” was first introduced by Zimmerman and now is the widely accepted name (2, 3). Pericytes are important components of the microvasculature and play a critical role in stabilization of blood vessels. This chapter will focus on the biology, physiology, and pathology of pericytes, as well as their role in angiogenesis. We will also discuss the potential relevance of pericytes in anti-angiogenic therapeutic approaches.

2. PERICYTE BIOLOGY, PHYSIOLOGY, AND PATHOLOGY

The origin of pericytes is not clearly known but they can develop from various cells depending on their location: for example, mesenchymal cells surrounding the

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dorsal aorta (4); the neural crest, as in the forebrain and cardiac outflow tract (5); and epicardial cells, as in the coronary vessels of the heart (6). It was suggested that endothelial cells are derived from angioblasts, which give rise only to endothelium, or from hemangioblasts, which are precursors of both endothelial cells and blood cells (7). Recent studies using embryonic stem cells described the existence of Flk-1-positive precursors, which can serve as a progenitor for both vascular smooth muscle cells (VSMCs) and endothelial cells in vitro and in vivo (8).

It has been demonstrated that pericytes can differentiate into various types of mesenchymal cells. For example, pericytes are suggested to give rise to VSMC and vice versa during vessel enlargement or remodeling (9). In addition, pericytes may also give rise to other types of mesenchymal cells including fibroblasts, osteoblasts, chondrocytes, and adipocytes (10). It has been reported that pericytes can detach from the vessel wall and differentiate into collagen type I producing fibroblast-like cells during wound healing and inflammatory processes (11). Several molecular markers have been used to identify pericytes, including intracellular proteins such as smooth muscle actin (SMA) (12), desmin (13), and the regulator of G-protein signaling-5 (RGS5) (14, 15). Cell-surface proteins such as high molecular weight melanoma antigen (HMW-MMA) (called NG2 in the mouse) (16), 3G5 ganglioside (17), and platelet-derived growth factor receptor beta (PDGFR- β) have also been used (18). Some studies have utilized the promoter trap transgene XlacZ4 (19), aminopeptidase N (20), and Slug (21) as pericyte markers. Although a number of pericyte markers have been identified, none is a pan-pericyte marker or absolutely specific for pericytes. These markers are dynamic and vary according to species, tissue type, and developmental stage. For example, SMA is expressed in pericytes of brain tissue in chicken embryos but not in mice or rats (22, 23). Slug is expressed in pericytes of chicken embryos but not in mouse embryos (21). RGS5 expression is upregulated during tumor and physiological angiogenesis and coincides with active vessel remodeling (24). Because of heterogeneous morphology and marker expression, use of a single marker may lead to misinterpretation (pericytes may be present but may not express a particular single marker). Therefore, the use of multiple markers and high-resolution confocal imaging is a better approach for studying pericyte presence and architecture. In ovarian tissues (normal and cancer), the authors tested several markers including desmin, SMA, and NG2 and found desmin to be the best marker for pericyte staining (Fig. 1). However, as discussed above, the optimal marker is likely to vary depending on the type of tissue being analyzed.

Pericytes are polymorphic, elongated, multi-branched periendothelial cells covered by the same basement membrane as endothelial cells. Based on location and histological characteristics, there are at least three types of pericytes: pre-endothelial cell capillary, capillary, and post-capillary venule (25). Electron microscopy studies reveal that pericytes are embedded within the basement membrane of microvessels with a prominent nucleus, a small amount of cytoplasm, and several long processes embracing the abluminal endothelial wall (26). Pericytes are morphologically heterogeneous in different organs. For example, in the central nervous system (CNS), pericytes are flattened or elongated and have a stellate-shaped solitary cell with multiple cytoplasmic processes encircling the capillary endothelium and contact a large abluminal vessel area. In contrast, pericytes in the renal glomerulus are rounded, compact, and contact a minimal abluminal vessel area, making only focal attachments to the basement membrane (27,20). On a subcellular level, pericytes are also morphologically distinct.

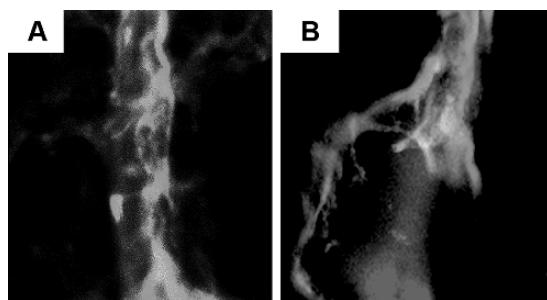


Fig. 1. Pericyte coverage on the microvasculature in (A) normal and (B) ovarian cancer tissues. Dual immunofluorescence staining for endothelial cells (CD31, red) and pericytes (desmin, green) was performed to demonstrate the physical relationships between these cell types in normal and cancer tissues. (Please see color insert.)

For example, pericytes may be “granular” because cytoplasmic lysosomes are abundant (such as human cerebral pericytes) or “agranular” because cytoplasmic lysosomes are sparse (1, 28).

Pericytes interact with endothelial cells by direct physical contact and by paracrine signaling pathways. Pericytes directly interact with endothelial cells in a “peg-and-socket” fashion, which reflects the indentation of endothelial cells by pericytes and vice versa. Such contact enables the two cell types to penetrate through discontinuities in the vessel basement membrane and directly communicate with each other (29). These heterologous “peg-and-socket” contacts contain tight and gap junctions (30), N-cadherin- and β -catenin-based adherens junctions (23), and are rich in fibronectin deposition. Moreover, these contact points are thought to be capable of supporting transmission of the mechanical contractile forces from the pericytes to the endothelium (1, 3). In vitro studies have demonstrated that the gap junctions between pericytes and endothelial cells are capable of ion and small molecule exchange (31).

The relationship between pericytes and endothelial cells is distinct based on tissue type, species, and developmental stages. The reason for the wide range of distribution is not known but may reflect specific functional features of the microvasculature in different organs or relate to the organ metabolic demand and specialized cellular functions. For example, in rat capillaries, the extent of pericyte coverage of microvessels is 11% in cardiac muscle, 21% in skeletal muscle, 22–32% in the cerebrum, and 41% in the retina (28). The greatest pericyte density has been noted in the retina, which may be needed due to the particularly high metabolic activity that requires meticulously regulated blood flow. In addition, the distribution of pericytes in the vessel wall of any specific capillary bed is not random. Theoretically, the greater the pericyte number and coverage, the higher the capillary and venular blood pressure and the better the microvascular barrier. For example, blood vessels in the retina and brain have the highest pericyte coverage, the highest capillary blood pressure, and the best microvascular barrier function followed by lung, skeletal muscle, and adrenal gland (25).

Pericytes have been shown to have contractile properties both in vitro and in vivo. Ultrastructural and immunohistochemical analyses show that pericytes contain the myofilament guanosine monophosphate (GMP)-dependent protein kinase, which can regulate VSMC contraction (32). In vitro studies with retinal pericytes have shown

that they are able to dedifferentiate to a smooth muscle-like phenotype, which would be expected to show both contractility and expression of related proteins (9, 33). In response to vasoactive agents, pericyte contraction has also been observed in skeletal muscle and in fresh whole retinal mounts *in vivo* (34).

As mentioned earlier, pericyte density differs depending on the function of vessels and organs. It is not clear how pericytes choose their exact location; however, they seem to play specific roles in different organs (Table 1). The highest density of pericytes in the body is found in neural tissues, such as in the brain and the retina. Pericytes play an essential role in the structural integrity of vessels and the blood-brain barrier. This barrier is formed when endothelial cells in the brain interact with astrocytic pedicles and with numerous pericytes to protect brain cells from potentially toxic blood-derived factors (35, 36). Pericytes can protect hypoxia-induced disruption of the blood-brain barrier *in vitro* and induce endothelial cell maturation and a tighter barrier function (37). Pericytes also have phagocytic activity and provide immunological defense mechanisms (38). For example, like macrophages, pericytes can take up small and soluble molecules by pinocytosis, thus cleaning the extracellular fluid.

Liver pericytes, also known as hepatic stellate cells, have close contact with endothelial cells through incomplete basement-membrane components and interstitial collagen fibers. Hepatic stellate cells regulate the remodeling of the extracellular matrix (ECM) by producing both ECM components and matrix metalloproteinases (39). Hepatic stellate cells contain more than 80% of the total vitamin A in the body and play a role in vitamin A metabolism (39). In addition, hepatic stellate cells promote recruitment of inflammatory cells during hepatic tissue repair and in fibrotic responses to liver diseases (40).

Pericytes of the glomerular capillaries in the kidney, also called mesangial cells, account for approximately 30% of the glomerular cells. These cells form a continuous tree-like core, around which capillary loops are arranged in specific high-density tufts. Lack of mesangial cells can result in functional defects—for example, the complex tufts are exchanged for simpler structures or even single-dilated capillary loops, thus causing defective kidney glomeruli (41). This reflects a critical function for the glomerular pericytes in forming the tissue pillars involved in capillary splitting. Mesangial cells also increase capillary surface area for blood ultrafiltration.

Table 1
Pericyte Function in Specific Tissues

| <i>Organ</i> | <i>Role</i> |
|--------------|---|
| Brain | <ul style="list-style-type: none"> • Blood-brain barrier • Immunological defense • Phagocytic activity |
| Liver | <ul style="list-style-type: none"> • Vitamin A metabolism • Tissue repair |
| Kidney | <ul style="list-style-type: none"> • Glomerular vascular function |
| Eye | <ul style="list-style-type: none"> • Retinal vascular flow and function |

As pericytes play an important role in the maintenance of microvascular homeostasis, pericyte abundance, loss, or dysfunction is involved in pathogenesis of various disorders. For example, the number of pericytes in brain microvessels can be increased up to four times in the spontaneously hypertensive rats (42). In addition, the relationship between endothelial cells and pericytes is also different in these animals—pericytes encircle the endothelial cells in hypertensive rats, whereas in normotensive conditions, pericytes and endothelial cells are not associated. Furthermore, actin and stress fiber distribution are also different between the two cultures—pericytes from normotensive rats contained well-differentiated fibers, whereas pericytes from hypertensive rats were essentially free of organized stress fibers. It is known that retinal capillaries have a greater coverage of pericytes than brain capillaries. As pericytes are contractile and control blood flow, they may play a role in the pathogenesis of hypertension.

In diabetic patients, pericyte loss has also been reported in microvessels in skeletal muscle and skin but not in the cerebral cortex or optic nerve (43,44). The pathogenesis of pericyte damage is not clearly understood. Selective pericyte loss was first observed in eyes removed at autopsy of diabetic patients (45), and a similar phenomenon was demonstrated in several animal models of diabetic retinopathy (46). Remarkably, the earliest histopathological feature of diabetic retinopathy is loss of pericytes. The ratio of endothelial cells to pericytes in the retinal capillaries is decreased several fold from 1:1 in normal to 1:10 in diabetic retinopathy (47). As a consequence of pericyte loss, thickening of the basement membrane, hyperpermeability, and formation of microaneurysms occurs and causes microvascular occlusion in the retina. These changes eventually progress to proliferative changes associated with neovascularization (48). Studies using mice with a single functional allele of PDGF-B demonstrated that retinal capillary pericyte coverage is crucial for the survival of endothelial cells and that pericyte deficiency leads to reduced inhibition of endothelial cell proliferation, thus promoting angiogenesis in the retinopathy of prematurity model (49).

3. ROLE OF PERICYTES IN ANGIOGENESIS

Pericytes play a critical role in the stabilization and hemodynamic processes of blood vessels. Pericytes can respond to angiogenic stimuli, guide sprouting tubes, provide endothelial survival signals, and have macrophage-like activities.

3.1. Role of Pericytes in Physiological Angiogenesis

Angiogenesis includes endothelial intussusception and cell bridging, vessel sprouting, or a combination of these processes. In vessel sprouting, angiogenic factors (e.g., VEGF) stimulate endothelial cells to degrade the vessel basement membrane, thus allowing endothelial cells to invade the surrounding ECM and form a migration column. Studies of the corpus luteum have observed that pericytes are also capable of guiding sprouting processes by migrating toward endothelial cells and expressing VEGF (50). Newly formed sprouts cease proliferation behind this migration zone and form a new, lumen-containing vessel. Endothelial cells then secrete growth factors, recruit pericytes that envelop the vessel wall, and promote vessel maturation. Using NG2 and PDGFR- β as markers, activated pericytes were found to be associated with angiogenic sprouts during the early phases of neovascularization in both normal retinal tissues and tumors (50). Pericytes alone can invade tissues in the absence of endothelial cells and form

functional, endothelium-free tubes (50). These studies provide evidence that pericytes play a role in early phases of angiogenic sprout formation during neovascularization.

Newly formed endothelial tubes are unstable and subsequently become stable through the formation of a perivascular matrix and the recruitment of pericytes. It is proposed that pericytes mainly influence vessel stability by matrix deposition and/or by the release and activation of signals that promote endothelial cell differentiation and quiescence (27). Although the molecular mechanisms by which pericytes mediate vessel stability are not fully known, several ligand receptor systems have been suggested to play a role (see sections 3.2 and 3.3 for additional details).

Pericytes also play a role in blood vessel morphogenesis by interacting with endothelial cells and regulating their proliferation and differentiation and by depositing ECM. PDGF-B retention motif-deficient mice show decreased numbers of pericytes with abnormal detachment of the abluminal endothelial surface (51). The PDGF-B or PDGFR- β knockout mice show primarily a lack of pericytes and consequently a series of abnormal features including endothelial hyperplasia, hypervariable diameter and tortuosity, abnormal endothelial junctions, signs of increased vesicular transport, excessive luminal membrane folds, and increased leakage of plasma and erythrocytes. These defects combined with vascular dysfunction may cause a compensatory VEGF-A upregulation and, in turn, promote further abnormalities, including vascular leakage and hemorrhage, finally causing perinatal death (18, 52).

Functionally, pericytes can produce vasoconstriction and vasodilation within capillary beds to regulate vascular diameter and capillary blood flow (53). Similar to smooth muscle cells, pericytes express contractile proteins such as α -SMA, tropomyosin, and myosin. Several molecules that regulate pericyte contractile tone have been identified as well. For example, pericytes possess cholinergic and adrenergic (α -2 and β -2) receptors. The β -adrenergic response in pericytes leads to relaxation, whereas the α -2 response is antagonistic and produces contraction (53). In addition, there is evidence that endothelial cells and pericytes interact in the regulation of blood flow (53). Studies with mouse cerebral cortex have shown that endothelial cells induce the expression of endothelin-1, nitric oxide, and angiotensin II, which regulate pericyte contraction and relaxation. Moreover, oxygen levels also regulate pericyte contraction. In vitro experiments show that hyperoxia increases pericyte contraction, whereas elevated levels of carbon dioxide induce relaxation (26). Genetic inhibition studies with PDGF-B and PDGFR- β knockout mice show that angiogenic sprouting in the embryonic brain proceeds relatively normally in the absence of pericytes. However, the diameter of the pericyte-deficient vessels is abnormal. The capillary diameter varies extensively, with both increased and decreased diameters (52).

3.2. Role of Pericytes in Tumor Angiogenesis

Although the role of pericytes in tumor angiogenesis is not fully understood, they appear to play a significant role in the microvascular stability and function. As shown in Figs 1 and 2, pericytes are abundant on tumor blood vessels but appear abnormal in shape and have an abnormal association with endothelial cells. Most pericytes are loosely attached to endothelial cells and paradoxically extend cytoplasmic processes away from the vessel wall, which may cause weakening of the vessel wall and thus increase the risk of hemorrhage (54). It is known that tumor blood vessels eventually become mature and quiescent and that pericytes are involved in these processes.

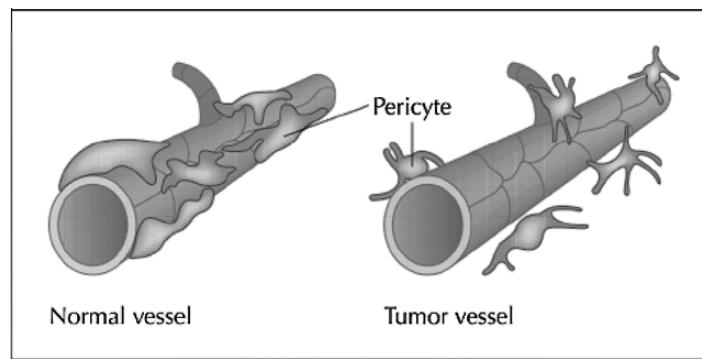


Fig. 2. Structural alterations in tumor vasculature are depicted. Mature blood vessels have endothelial cells with tight gap junctions and uniform pericyte coverage (left). Tumor blood vessels are leaky (right). Although pericytes are present, they are poorly attached to the endothelial cells and have processes projecting toward the abluminal surface or into the tumor stroma. Model of pericyte coverage of vessels in normal versus cancer tissues. Reproduced with permission from Kamat AA and Sood Ak, *Current Oncol Rep* 2005, 7:444–450.

In tumor tissue, the amount of pericyte coverage in different tumors ranges from extensive to little or none (55, 56). As measured by colocalization of an endothelial cell marker and α -SMA-positive cells, the pericyte coverage varied considerably from 10–20% in human glioblastoma and renal cell carcinomas, 30–40% in prostate and lung carcinomas, and to 70% in mammary and colon carcinomas (56). In other studies, pericyte coverage has been noted in up to 97% of blood vessels in spontaneous tumors developed in mice.

The function of pericytes noted in physiological angiogenesis also seems to be relevant in the development and maintenance of tumor microvasculature. First, pericytes modulate endothelial cell function by producing survival factors and inhibiting their proliferation. Work by our group and others has demonstrated that pericytes produce VEGF (in response to PDGF-BB), which is a known survival factor for endothelial cells (57). It is possible that pericytes may serve as a local source of VEGF for the adjacent endothelial cells. Tumor vessels lacking pericytes appear to be more dependent on VEGF for their survival than vessels invested by pericytes (55). Thus, if pericytes were absent or could not produce VEGF, the endothelium would theoretically become more vulnerable to VEGF blockade. Therefore, tyrosine kinase inhibitors affecting multiple receptors may exert their anti-tumor activity in part by reducing pericyte density in the tumor vessels, thereby sensitizing the endothelial cells to anti-angiogenic therapies (58). Second, pericytes appear to play a role in the stabilization of nascent cancer microvessels. It is known that during angiogenesis, multiple endothelial cell sprouts form immature vessels that lack pericytes initially. Subsequently, pericyte recruitment around these sprouts reduces endothelial cell proliferation and sprouting, thus leading to larger perfused microvessel formation. In neuroblastoma and melanoma models, inhibition of MMPs reduces pericyte recruitment and decreases tumor vessel perfusion (59, 60). Third, pericytes can control blood flow due to their contractile activity. These functions allow pericytes and VSMCs to modulate the blood flow into normal blood microvessels and regulate oxygen, metabolites, and drug delivery into the

tumor tissue (61). In addition, cellular interactions between pericytes and endothelial precursor cells may also contribute to tumor angiogenesis (13).

3.3. Mechanisms of Pericyte Recruitment During Angiogenesis

The exact mechanisms of pericyte recruitment around vascular endothelial cells during angiogenesis is not clearly known. It is proposed that the following four key molecular signaling pathways may be involved: PDGF-B/PDGF receptor- β (PDGFR- β), angiopoietin (Ang1)/Tie2 (a receptor tyrosine kinase with immunoglobulin and epidermal growth factor (EGF) homology domains-2), sphingosine 1-phosphate (S1P)/endothelial differentiation gene-1 (Edg-1), and tumor growth factor (TGF)- β 1/activin-like kinase receptor (Alk5) (27, 62). In addition, MMPs may also be involved in pericyte recruitment and play a role during tumor angiogenesis (62).

Pericyte homeostasis is regulated in significant part by signaling through the PDGF ligand/receptor system (51, 63). As shown in Fig. 3, PDGF is a potent mitogen for mesenchymal cells and fibroblasts and is composed of A, B, C, and D polypeptide chains that form homodimers PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD and heterodimer PDGF-AB. Its biological activities are linked to two tyrosine kinase receptors, PDGFR- α and PDGFR- β (64). PDGFR- α binds to PDGF-AA, PDGF-BB, PDGF-AB, and PDGF-CC, whereas PDGFR- β interacts with BB and DD (65). The PDGF-B/PDGFR- β pathway plays an important role in the recruitment of pericytes to newly formed vessels. During angiogenesis, sprouting endothelial cells secrete PDGF-BB, which binds to PDGFR- β expressed on VSMCs and pericytes and leads to pericyte proliferation and migration. The knockout of PDGF-B or PDGFR- β in mouse models is known to cause perinatal death due to vascular abnormalities resulting from lack of pericytes (18, 52, 66). Using Cre-lox techniques, it was demonstrated that genetic ablation of PDGF-B in endothelial cells leads to impaired recruitment

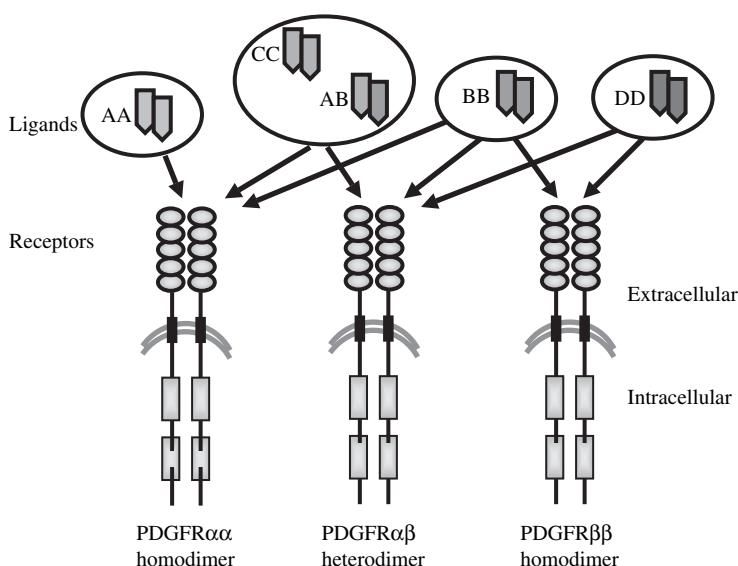


Fig. 3. Platelet-derived growth factor receptor (PDGF)/PDGF-R-binding interactions.

of pericytes, resulting in glomerular, cardiac, and placental abnormalities (67). Using a PDGFR- β tyrosine kinase inhibitor, SU6668, a functional role for PDGF-R β has also been implicated in pericyte recruitment in mouse insulinomas (68). Interestingly, the tyrosine kinase inhibitors had no effect on normal tissues with regard to pericyte detachment, but they disrupted the association of pericytes in the tumors (68). Apte and colleagues (69) have previously demonstrated that PDGF-AA and PDGF-BB ligands are expressed in most ovarian cancer samples, and tumor cells were positive for PDGF-R β in about 40% of the samples. PDGF-BB produced by tumor endothelium has been shown to be required for recruitment of adequate numbers of pericytes and for proper integration of pericytes in the vascular wall. PDGF-B expression from the tumor cells further enhanced tumor pericyte recruitment (70). Other growth factors that may affect pericyte function include endothelin-1 (produced by endothelial cells and stimulates pericyte proliferation), IGF-1 (increases proliferation of pericytes), interleukin-2 (increases localization of pericytes to endothelial cell junctions), and TGF β (produced by pericytes, may inhibit endothelial cell proliferation).

Ang1, mainly expressed by VSMCs and pericytes (71, 72), provides a pericyte-derived paracrine signal for the endothelium. Ang1 binds to the receptor Tie2, which is expressed on the endothelial cells and is generally held as being endothelial specific (73, 74). Ang1/Tie2 signaling maintains and stabilizes mature vessels by promoting interactions between endothelial cells and pericytes, mediating cell–matrix interactions in vessel morphogenesis, and upregulating the expression of endothelial mitogen and chemotactic heparin-binding (HB) EGF, which promotes VSMC migration by binding to the epidermal growth factor receptors (EGFRs) ErbB1 and ErbB2 (75, 76). Genetic studies show that Ang1- or Tie2-null mice die at midgestation from cardiovascular failure. These embryos show defective angiogenesis with reduced coverage and detachment of pericytes and poorly organized basal membrane in blood vessels (71, 73). Conversely, overexpression of Ang1 leads to an expanded and stabilized, leakage-resistant vasculature (77, 78), and recombinant Ang1 can partially rescue the vascular defects in the retina attributable to pericyte loss (79). Ang1 expression varies based on the type of tumor. It is overexpressed in some types of human tumors including glioblastoma, neuroblastoma, and lung cancer but is minimally expressed in others (80). Ang1 may either promote tumor angiogenesis or reduce tumor growth depending on the model. For example, in a human glioma xenograft model, Ang1 promoted pericyte recruitment and increased tumor growth (81). However, in a colon cancer model, Ang1 enhanced pericyte recruitment and inhibited tumor growth and angiogenesis (82). S1P is a secreted lipid, which is generated by phosphorylation of sphingosine by sphingosine kinase (SphK) and degraded by S1P phosphatases and S1P lyases (83). Most of serum S1P is secreted by mast cells, monocytes, and activated platelets (83). S1P triggers cytoskeletal, adhesive, and junctional changes, affecting cell migration, proliferation, and survival (84). Beside intracellular functions, S1P interacts with its G-protein-coupled receptor called Edg-1 or S1P₁. Edg-1 is expressed widely in cultured cells, including endothelial cells and mesenchymal cells. Activation of Edg-1 on endothelial cells increases the production of ECM proteins that promote the recruitment of pericytes (83). Endothelial-specific knockout of S1P₁ (Edg1) recapitulated the mural sheath defects observed in the full S1P₁ knockouts, whereas S1P₁ knockout in VSMCs, on the other hand, had no adverse effects. These findings suggest that pericyte coverage is directed by the activity of S1P₁ in the endothelium (84). *Edg1(−/−)* mice showed

defective pericyte coverage of vessels, which leads to vascular abnormalities and mid/late-gestational lethality (85). During tumor angiogenesis, Edg-1 expression is induced in endothelial cells and pericytes, which can promote pericyte recruitment. In the Lewis lung carcinoma xenograft model, inhibition of Edg-1 expression in endothelial cells strongly reduced pericyte coverage (86).

TGF- β 1 is expressed by various cell types, including endothelial cells and pericytes. Depending on the concentration and context, TGF- β 1 inhibits or promotes angiogenesis (87). TGF- β 1 is secreted in a latent form that needs to be activated for binding to TGF-type II receptors (88) and then recruits and phosphorylates type I receptors, such as activin-like kinase (ALK) receptors, that transduce the signal to the nucleus through a phosphorylation cascade involving Smad proteins (62). The juxtaposition and collaboration of endothelial cells and pericytes appears to be involved in the activation of latent TGF- β 1 (62). The context-dependent angiogenic effects of TGF- β 1 are mediated by two type I receptors, Alk1 and Alk5. The TGF- β 1/Alk1-signaling pathway stimulates endothelial cell proliferation and migration as Alk1 is mainly expressed by endothelial cells. Although Alk5 is expressed by pericytes, TGF- β 1/Alk5 signaling inhibits cell proliferation and migration, stimulates the differentiation of pericytes, and promotes the expression of fibronectin and plasminogen activator inhibitor 1 (PAI-1) (62). TGF- β 1 is required for differentiation of the pericyte-like 10T1/2 cells to a SMC-like phenotype (89) and plays an important role in the development of SMCs in embryonic stem cells (24). Various components of the TGF- β 1 signaling machinery play critical roles in vascular development and function. Genetic inactivation in mice of *TGF β 1* and genes encoding its receptors, *Alk1*, *Alk5*, TGF- β receptor II (*T β rII*), *endoglin* (type III TGF- β receptor), and its downstream effector Smad5, all lead to similar cardiovascular defects and embryonic lethality (27, 90–93). In humans, mutations in *endoglin* and *Alk1* cause hereditary hemorrhagic telangiectasia (HHT) type 1 and 2, respectively (94, 95). TGF- β 1 expression has been associated with increased tumor vascularization in tumors, such as human breast and liver cancer (96). In a human prostate cancer xenograft model, inhibition of TGF- β 1 activity caused fewer but larger and immature vessels (97).

Recent studies suggest that MMPs play a role in pericyte recruitment in tumor angiogenesis. The expression of MMP-9 by pericytes was reported in human glioma and breast cancer (98, 99). In a human neuroblastoma xenograft model, pericyte coverage was decreased by half in tumors engrafted to MMP-9-deficient mice; however, the transplantation with MMP-9-expressing bone marrow cells restored the formation of mature tumor vessels (59, 87). TIMP-3, the inhibitor of MMPs, results in decreased pericyte recruitment in neuroblastoma and melanoma (60). The regulation of pericyte recruitment by MMPs may occur through the following mechanisms: MMPs mediate ECM degradation, thus promoting pericyte invasion; ECM-cell interactions stimulate pericyte proliferation and/or decrease pericyte apoptosis; release of angiogenic growth factors such as VEGF and TGF- β 1 to the ECM; transactivation of cell-surface receptors such as EGFR; and function as a cofactor for PDGF-B/PDGFR- β signaling (62).

4. PERICYTE AS A THERAPEUTIC TARGET

The progressive growth of primary tumor and metastases is dependent on angiogenesis. Inhibition of tumor angiogenesis may provide an efficient strategy to block tumor growth. Studies of anti-angiogenic strategies have been largely focused on

endothelial cells and progenitors and less on pericytes. VEGF plays a pivotal role in developmental, physiological, and pathological neovascularization and mediates not only endothelial cell proliferation but also endothelial cell survival (100, 101). A recent study comparing several anti-angiogenesis strategies concluded that anti-VEGF signaling approaches were the most efficacious (102). However, VEGF targeting alone is not sufficient to regress bulky tumors. Pericytes covering vessels may limit the effectiveness of anti-angiogenic therapy by providing local survival signals for endothelial cells. Work from our group and others has demonstrated that PDGF-BB secretion from tumor and endothelial cells stimulates VEGF production by pericytes, which in turn functions as a survival factor for endothelial cells (57). Although anti-VEGF therapy reduced microvessel density in pre-clinical models, it also increased the percentage of pericyte-coated vessels, suggesting that pericytes may protect these vessels (103). Therefore, combination of anti-endothelial and anti-pericyte agents might have additive or synergistic activity in anti-angiogenic therapy.

Pericyte homeostasis is regulated in significant part by signaling through the PDGF ligand/receptor system (51, 63). Therefore, inhibition of the PDGF ligand/receptor signaling pathway may represent an appealing approach to target pericytes. There are several available approaches to block PDGF-B/PDGFR- β -signaling pathways—for example, tyrosine kinase inhibitors of PDGFR (such as Gleevec or STI571 and SU6668) and PDGF-B aptamer (a modified DNA-based aptamer to PDGF-B chain that blocks binding of PDGF to its cell-surface receptor). Currently, the most commonly used agents are tyrosine kinase inhibitors, which have been shown to be safe and therapeutically active in selected populations of cancer patients and animal models. Although Gleevec alone has anti-tumor activity in some tumors such as human gastrointestinal stromal tumors (GIST) due to c-kit targeting (104), it has some activity in epithelial tumors in combination with chemotherapy due to its anti-PDGFR- β activity (69, 105). However, several studies with various tumor models recently have shown that dual-targeting of endothelial cells (with agents such as AEE 788 or SU5416) and pericytes (with STI571 or SU6668) is more efficacious than targeting either cell type alone, even in established or drug-resistant tumors (68, 103, 106). Furthermore, using *in vivo* bioluminescence imaging, we have observed that targeting tumor cells, endothelial cells, and pericytes (using AEE788 and STI571 in combination with chemotherapy) was effective in regressing large ovarian tumors in orthotopic models (103). Similarly, dual targeting of endothelial cells and pericytes has been reported to cause regression of pancreatic tumors in RIP1 Tag 2 mice (68). These findings may have clinical relevance because many cancer patients have bulky tumor at relapse.

It is possible that the anti-angiogenic activity of Gleevec is not only through blocking PDGF signaling but also through blocking other pathways, such as BCR-ABL and c-kit. Studies of more specific PDGF-signaling blockers such as PDGF-aptamer will be instructive in future studies. Following the combination concept, several new inhibitors (such as SU11248 and SU14813) have been developed to target VEGFR, PDGFR, and other kinases such as stem-cell factor and Fms-like tyrosine kinase receptor 3 (FLT3) receptor tyrosine kinase. Some of these inhibitors show anti-angiogenic and anti-tumor effects in acute myeloid leukemia, imatinib-resistant GIST, and renal cell cancer (107). Additional clinical and pre-clinical studies are ongoing with these inhibitors. In addition to PDGF-B/PDGFR- β , other pathways such as Ang1/Tie2, S1P/Edg-1,

TGF- β 1/Alk5, and MMPs, as well as molecular markers of pericytes may also offer additional opportunities for therapeutic targeting.

5. CONCLUSIONS

In conclusion, pericytes are morphologically, biochemically, and physiologically heterogeneous and play a critical role in angiogenesis. Pericytes interact with endothelial cells, stabilize the newly formed endothelial tubes, modulate blood flow and vascular permeability, and regulate endothelial proliferation, differentiation, migration, and survival. As key players in angiogenesis, pericytes represent an additional target for treatments designed either to increase (e.g., in ischemic disorders) or decrease (e.g., in cancer) vascularization. Therefore, dual targeting of endothelial and pericytes may provide a more efficacious anti-angiogenic approach for cancer therapy.

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8

Newer Vascular Targets

Beverly A. Teicher, PhD

SUMMARY

The identification of cell-surface markers expressed selectively by tumor vasculature is challenging. To get as close to the human disease as possible, investigators have isolated endothelial cells from fresh human tumor specimens and subjected them to RNA-based gene-expression analysis. The data indicate that there are few proteins that distinguish tumor vasculature from normal vasculature and re-enforce the notion that the endothelium is a tissue specialized cell type. Endosialin and tumor endothelial marker 7 (TEM 7) were identified as a cell-surface TEMs. The selective expression of endosialin and TEM 7 by tumor vasculature and stroma has been confirmed. Although the function of endosialin and TEM 7 remains to be elucidated, the expression pattern for this protein may be favorable for cancer therapy. PRL-3 was also identified by SAGE (serial analysis of gene expression) as a TEM. PRL-3 is an intracellular phosphatase that is expressed not only in tumor vasculature but in aggressive disease. SAGE analysis of subpopulations of tumors has provided useful leads for new vascular targets. It remains to the basic scientists to elucidate the function of these proteins and to the “drug hunters” to determine whether these targets can be used in therapeutically meaningful ways.

Key Words: SAGE; endosialin; TEM; antiangiogenesis; vascular targets; gene-expression analysis.

The field of antiangiogenic therapies has moved very quickly from laboratory discoveries into the clinic. As with other areas of science, the rapidity of the development of the antiangiogenic field was fueled by the availability of models and the identification of therapeutic targets. The field was also fueled by the early hypothesis which held that angiogenesis was the same no matter where it occurred. Therefore, angiogenesis during embryo development or wound healing was the same as angiogenesis during the growth of malignant disease (Fig. 1) (1–4). The corollary to this hypothesis was that models of normal embryo development and models working with mature well-differentiated endothelial cells in culture would be sufficient and satisfactory models for tumor endothelial cells. This hypothesis also held that because endothelial cells involved in malignant disease were normal, these cells would be less susceptible to developing drug resistance because they were genetically stable (5, 6).

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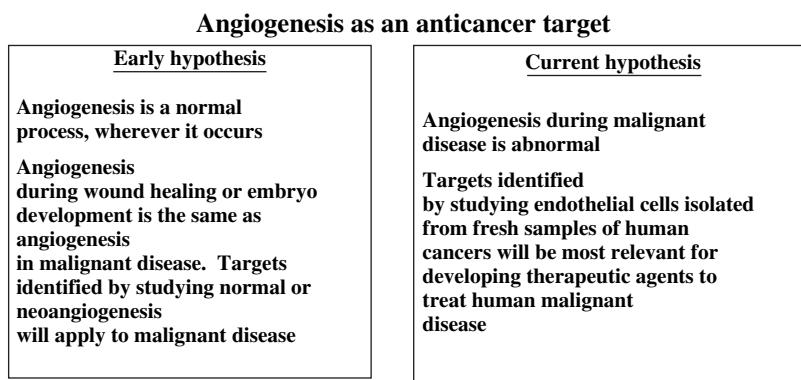


Fig. 1. Hypotheses supporting angiogenesis as a target for cancer therapy are shown.

The current hypothesis is that angiogenesis occurring during malignant disease is abnormal and that therapeutic targets identified by studying endothelial cells isolated from fresh samples of human cancers will be most relevant for developing therapeutic agents to treat human malignant disease (7–10).

1. NEW TARGET DISCOVERY

Early studies of gene expression were carried out primarily with cell lines. As the importance of the tissue microenvironment and the easy plasticity with which cells alter gene expression in response to the microenvironment became evident, the severe limitations, indeed, inaccuracies in disease representation by monolayer cell culture, were recognized. “Drug-target hunters” realized the need to get as close to the human disease as possible to identify disease critical molecular targets. To accomplish this, fresh samples of human malignant tumors and corresponding normal tissues were used as starting materials (11–25). Gene-expression profiling techniques such as microarray analysis (11–20) and SAGE (serial analysis of gene expression) (21–25) have provided global views of the levels of mRNAs in malignant tissues compared with normal tissues and allowed identification of genes and pathways involved in the malignant process. Specific diseases including ovarian cancer, breast cancer, gastric cancer, multiple myeloma, lung adenocarcinoma, Wilm’s tumor, and neuroblastoma have been analyzed for diagnostic and prognostic gene-expression characteristics and for identification of potential drug targets (14–20). Chief among the issues being faced by these studies is developing data analysis methods that allow investigators to draw biologically meaningful conclusions from very large datasets (12, 13).

The one of the challenges for gene-expression studies is to translate research findings of multigene-expression signature classifiers/genomic signatures of disease into applications in diagnostics and therapeutics (26–30). Integrative computational and analytical data analysis approaches including meta-analysis, functional enrichment analysis, interactome analysis, transcriptional network analysis, and integrative model system analysis are being applied to gene-expression data. Some studies focus on the expression of mRNAs that code for enzymes as potential drug targets, some search for functional regulators driving large-scale transcriptional signatures, and others focus on epigenetic alterations that regulate gene expression (27–32).

2. SAGE ANALYSIS OF TUMOR SUBPOPULATIONS

SAGE is a gene-expression profiling method that allows global unbiased, quantitative determination of the transcriptome of the sample at the time of RNA collection (21–26). SAGE expression profiling depends upon the notions that a short (10–27 base-pair sequence) fragment of mRNA cut by a restriction enzyme is sufficient to uniquely identify a transcript and that concatemerization of these fragments (tags) increases the efficiency of sequence-based transcriptome analyses (21). Approximately 90% of genes are represented by SAGE tags (S. Madden, personal communication). Because SAGE does not depend upon *a priori* knowledge of the genes of interest, it can identify novel, un-named, and unexpected transcripts. For these reasons, SAGE methodology has been selected as the method of choice to examine gene expression from subpopulations of cells isolated from fresh clinical specimens (22–26).

Fresh specimens of colon carcinoma, normal colon mucosa, breast carcinoma, normal breast tissue, brain tumors, and normal brain were obtained for analysis of cellular subpopulations by SAGE analysis (Fig. 2) (25, 26, 33, 34). The tissues were disaggregated, and the endothelial cells were isolated using selection with an antibody to P1H12 linked to a magnetic bead (33–36). The RNA from the endothelial cells isolated from tumor and normal tissues was collected and subjected to SAGE analysis. This methodology allows elucidation of the RNA transcripts in the cells at the time of RNA isolation, providing the identity of the transcript and the relative abundance of each transcript. Thus far, SAGE-derived transcript libraries have been generated for endothelial cells isolated from seven fresh human tumor specimens and five specimens of corresponding normal tissues.

The first bioinformatics analysis was to compare the genes/mRNA expressed in each of the three tumor types, with the genes/mRNA expressed in each corresponding normal tissue. In each case, a similar pattern emerged. The vast majority of the genes/mRNA expressed by the tumor endothelial cells was very similar to the genes/mRNA expressed by the endothelial cells from the corresponding normal tissue. However, there was a small subpopulation of genes/mRNA that was expressed at much higher levels by the tumor endothelial cells than by normal endothelial cells and a different small

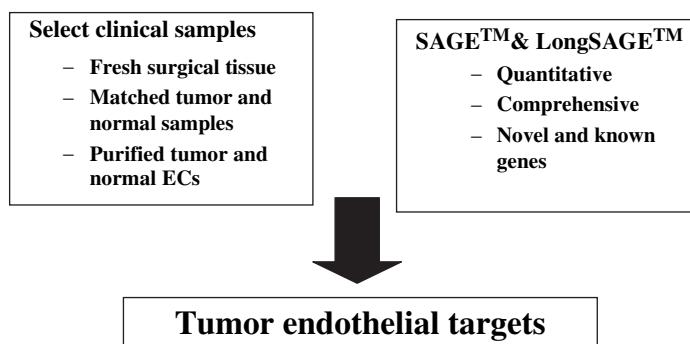


Fig. 2. Schema for isolation of tumor endothelial cells from fresh samples of human tumors and normal tissues and subsequent preparation of tumor endothelial cell RNA for SAGE analysis is depicted. Because the tumor endothelial cells were never placed into culture, tissues samples of at least 2 g were required to obtain sufficient numbers of cells for expression analysis.

subpopulation of genes/mRNA that was expressed at much higher levels by the normal endothelial cells than by the tumor endothelial cells. Generally, the tumor endothelial cells appeared to be expressing at least a partial “malignant phenotype.” The tumor endothelial cells appeared to be relatively de-differentiated or immature relative to the corresponding normal endothelial cells.

The second bioinformatics analysis was to compare the genes/mRNA that were expressed at high levels by the tumor endothelial cells from the colon carcinoma, breast cancer, and brain tumors with each other. Venn diagrams were developed for the subpopulations of genes that by the Chi-square test had >99% confidence of being overexpressed in the tumor endothelial cells compared with the corresponding normal endothelial cells (Fig. 3). The genes/mRNA that fulfilled these criteria included 280 genes from the colon carcinoma, 109 genes from the breast carcinomas, and 111 genes from the brain tumors. The number of genes that were overexpressed in endothelial cells from both breast cancer and brain cancers was 22, from brain cancers and colon cancer was 24, and from breast cancer and colon cancer was 30. Thus, there is a high degree of organ/tissue specificity in the endothelium and there is a high degree of heterogeneity among tumor endothelium. When the highly overexpressed genes from the endothelial cell libraries for each of the three tumor types were compared, there were only 12 genes that were highly overexpressed in all three tumor types. Based on these findings, it may be less likely that therapeutic antiangiogenesis targets can be identified that are universally applicable. It may be more likely that antiangiogenic therapeutic targets can be found that will apply to major tumor categories.

Hierarchical clustering analysis using GeneSpring™ software was applied to the SAGE data from the normal and tumor brain and breast endothelial cell libraries. Each SAGE library included 30,000–40,000 SAGE tags. When the complete gene-expression libraries were analyzed, the libraries formed two sub-clusters based on the tissue of origin of the endothelial cells, that is the normal and tumor breast endothelial cells

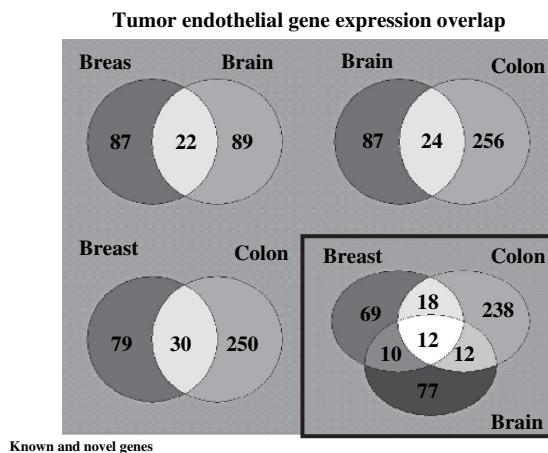


Fig. 3. Venn diagrams depicting the overlap in the number of genes expressed at higher levels in tumor endothelial cells derived from breast, brain, and colon tumors compared with endothelial cells from the corresponding normal tissues. The selected genes were overexpressed in tumor endothelial cells with >99% confidence by Chi-square analysis. The data include known and un-named genes. (Please see color insert.)

clustered apart from the normal and tumor brain endothelial cells. Therefore, genes that distinguish tumor from normal endothelial cells did not dictate the general gene-expression profiles. Statistical confidence filtering was then applied to all the libraries to isolate genes upregulated with 90, 95, and 99% confidence. When hierarchical clustering was applied to the gene population upregulated in these libraries with 90 and 95% confidence, tumor endothelial cell libraries formed a distinctive sub-cluster from the normal endothelial cell libraries. Thus, a group of genes could be identified that were involved in the switch from normal tissue endothelium to malignant disease tissue endothelium without tissue type distinction (Fig. 4). Interestingly, when hierarchical clustering analysis was performed with genes upregulated at the 99% confidence level, the libraries from different tissues formed distinctive sub-clusters. Thus, at this high level of statistical stringency, genes expressed by the endothelial cells were dominated by the tissue of origin of the cells and not by the normalcy or malignancy of tissue.

The final bioinformatics analysis was to examine the expression of the genes/mRNA that were highly upregulated in tumor endothelial cells, with genes/mRNA expressed in cells commonly used as a model systems in the angiogenesis and antiangiogenesis fields. The cells whose gene/mRNA expression was examined included HUVEC (human umbilical vein endothelial cells), HMVEC (human microvascular endothelial cells), and EPC (human endothelial precursor cells) (37). SAGE libraries were available

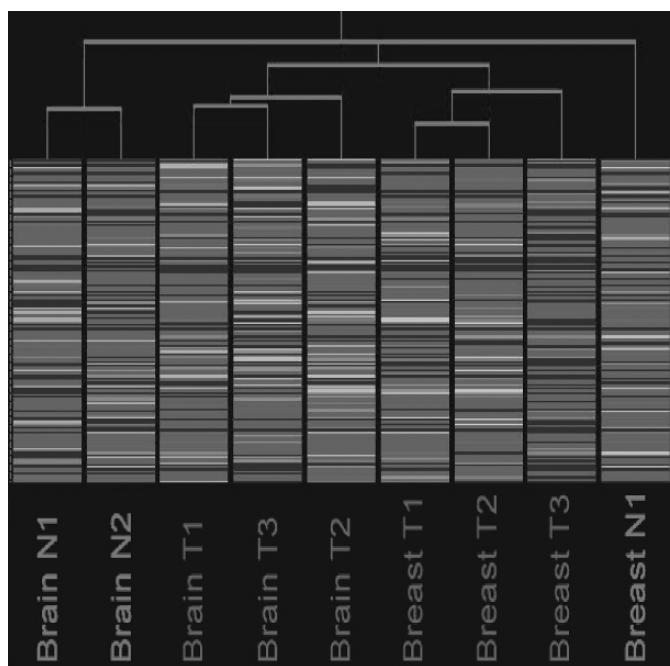


Fig. 4. Hierarchical clustering of tumor endothelial cell and normal tissue endothelial cells SAGE libraries by GeneSpring™ is shown for breast tumor and normal breast and brain tumors and normal brain. SAGE tags from statistical confidence filtering (90% confidence) were used. At the 90% confidence level shown, the tumor endothelial cells libraries formed distinctive sub-clusters from the normal endothelial cells libraries, indicating that there is a group of genes expressed by endothelial cells involved in the switch from normal to tumor independent of tissue of origin. (Please see color insert.)

for several cell-culture conditions including cells stimulated with VEGF and unstimulated cells. Many of the genes/mRNA expressed at high levels in the tumor endothelial cells isolated from fresh human tumor specimens either were not expressed or were expressed at very low levels in HUVEC and HMVEC under stimulated and unstimulated conditions.

3. ENDOTHELIAL PRECURSOR CELLS, PERICYTES, AND MESENCHYMAL STEM CELLS

Growth of blood vessels normally occurs during embryo development and wound healing and abnormally as a component of tumor and inflammatory disease processes (38). The abnormality of tumor vasculature and the value of working with fresh endothelial cells isolated from solid tumors were recognized by cancer researchers, and the role of endothelial precursor cells from bone marrow was recognized by developmental biologists (39, 40). Asahara et al. (41–43) isolated putative endothelial precursor cells (angioblasts) from human peripheral blood by magnetic bead selection and described a role for these cells in postnatal vasculogenesis and pathological neovascularization. Studies in allogeneic bone marrow transplant recipients confirmed that circulating endothelial precursor cells in peripheral blood originated from the bone marrow (44). CD34+/AC133+ progenitor cells from bone marrow can differentiate into endothelial cells in culture (37, 38). Several studies have tied circulating endothelial precursor cells to the development of tumor vasculature (45–49).

In culture with VEGF, AC133+ multipotent human bone marrow progenitor cells differentiate into CD34+/VE-cadherin+/VEGFR2+ cells (37). Upon maintenance in cell culture, these cells continue to differentiate toward a more mature endothelial phenotype.

Most research directed toward the development of antiangiogenic anticancer agents has utilized HUVEC and HMVEC as the cell-based models of the tumor endothelium (50). As determined by gene-expression profiling using SAGE, the endothelial precursor cell developed by driving AC133+/CD34+ human bone marrow progenitor cells toward endothelial cell differentiation in cell culture was a better model for tumor endothelial cells than were HUVEC and HMVEC (37). Analysis of several cell-surface markers by flow cytometry showed that endothelial precursor cells, HUVEC and HMVEC, have similar expression of P1H12, VEGFR2, and endoglin but that endothelial precursor cells have much lower expression of ICAM1, ICAM2, VCAM1, and thrombomodulin than do HUVEC and HMVEC. The endothelial precursor cells generated can form tubes/networks on Matrigel™, migrate through porous membranes, and invade through thin layers of Matrigel™ similar to HUVEC and HMVEC. However, in a co-culture assay using human SKOV3 ovarian cancer cell clusters in collagen as a stimulus for invasion through Matrigel™, endothelial precursor cells were able to invade into the malignant cell cluster while HMVEC were not able to invade the malignant cell cluster. *In vivo*, a Matrigel™ plug assay where human endothelial precursor cells were suspended in the Matrigel™ allowed tube/network formation by human endothelial precursor cells to be carried out in a murine host.

Endothelial precursor cells appear to represent a more immature endothelial cell or a more de-differentiated endothelial cell than do HUVEC and HMVEC and thus provide a more accurate mimic of tumor endothelial cells. These cells function well

in cell-based assays including proliferation, tube formation, migration, and invasion. Endothelial precursor cells from several donors express targets identified by studying tumor endothelial cells and thus may represent an improved or second-generation model cell system that can be used to study and screen potential antiangiogenic therapeutics (37).

In the search for tumor vascular targets, it became evident that some potential therapeutic target proteins were expressed by tumor endothelial cells and by tumor-associated pericytes (34, 51). Pericytes are key cells in vascular development, stabilization, maturation, and remodeling and are intimately associated with endothelial cells (52–55). In normal tissue, the pericyte/endothelial interface reflects the vessel function. In tumors, however, endothelial cells of tumor vessels do not form a tight barrier, and pericytes are loosely attached (52). Pericytes express several cell-surface markers including smooth-muscle α -actin (α SMA), desmin, NG-2, platelet-derived growth factor receptor (PDGFR)- β , aminopeptidase A and N, and RGS5; however, none of these cell-surface proteins are exclusive to pericytes. Several secreted factors including transforming growth factor β , angiopoietins 1 and 2, platelet-derived growth factors, sphingosine-1-phosphate, and Notch ligands are involved in the intercellular communication between endothelial cells and pericytes (55). Pericytes are likely of mesenchymal origin, although other possibilities include trans-differentiation of endothelial cells into pericytes and derivation for bone marrow progenitor cells (56). Pericyte progenitor cells may move from the bone marrow to differentiate into fibroblast-like cells and contribute to extracellular matrix formation during wound healing, to chronic inflammation, and to tumor stroma. Tumor vessels are heterogeneous in their pericyte coverage. It appears that antiangiogenic therapies directed toward endothelial targets can produce an ablation of naked endothelial tubes and that pericyte-covered endothelium is less susceptible to damage. Thus, pericytes may be a valid target for anticancer therapeutics. Using the RIP/Tag2 mouse, a single transgenic that is a model of islet cell carcinogenesis, Bergers et al. (57) found that a combination of tyrosine kinase inhibitors directed toward endothelial and pericyte targets was a superior therapy compared with each molecule administered alone. As primary cells in culture, pericytes and endothelial precursor cells share many properties such as tube/network formation and response to kinase inhibitors selective for angiogenic pathways. Expression of cell-surface proteins including PDGFR, VCAM, ICAM, endoglin, desmin, and NG2 was similar between pericytes and endothelial precursor cells, while expression of P1H12 and LFA-1 clearly differentiates the cell types (51).

Mesenchymal stem cells are multipotent bone marrow-derived cells (58). All mesenchymal tissues develop from mesenchymal stem cells. The remarkable plasticity of mesenchymal stem cells allows them under different conditions to differentiate into the tissues and organs which they form in the body including bone, cartilage, muscle, ligament, tendon, adipose, and stroma (58). Mesenchymal stem cells can readily be isolated from bone marrow and grown in culture (59). These cells have been shown to be immunologically neutral and to home to sites of tissue injury. Therefore, mesenchymal stem cells have been proposed for use in several cellular therapy applications including induction of vascular network formation following ischemic injury and targeting tumors with gene therapy to activate prodrugs or deliver anticancer protein therapeutics and/or imaging agents (60, 61).

4. NEWER VASCULAR TARGETS: ENDOSIALIN

In 2000, St. Croix et al. (33) reported results of a SAGE study using RNA prepared from endothelial cell samples from the colon carcinoma and normal colon mucosa of a human patient. The SAGE tag for TEM 1/endosialin was found at a level of 0 tags in the normal endothelial cells and 28 tags in the tumor endothelial cells per 100,000 tags sequenced. Earlier, Rettig et al. (62) recognized that cells of the reactive tumor stroma differed from corresponding cells in normal tissues in proliferative and invasive behavior and raised an antibody against cultured fetal fibroblasts and identified the tumor vascular endothelial antigen, endosialin. In cell culture, several human fibroblast cell lines and human neuroblastoma cell lines (some of these are now classified as Ewing's sarcoma) were positive for endosialin protein using the FB5 antibody, while melanoma, glioma, sarcoma, carcinoma, and leukemia cell lines and both growth factor-stimulated HUVEC and unstimulated HUVEC were negative for endosialin. Most normal human tissues were immunohistochemically negative for endosialin, while 41/61 sarcomas, 26/37 carcinomas, 18/25 neuroectodermal tumors, and 0/5 lymphomas were positive for endosialin by FB5 staining. Later, the full-length cDNA for endosialin was cloned and found to encode a type I membrane protein (757aa) that corresponds to TEM 1 described by St. Croix et al. (63). Endosialin is a C-type lectin-like protein with a signal leader peptide, five globular extracellular domains (a C-lectin domain, one domain similar to the Sushi/ccp/scr pattern and three EGF repeats), followed by a mucin-like region, a transmembrane segment, and a short cytoplasmic tail (63). The core protein is abundantly sialylated with O-linked oligosaccharides and is sensitive to *O*-sialoglycoprotein endopeptidase and is therefore in the group of sialomucin-like molecules. The N-terminal (360aa) shows homology to thrombomodulin, a receptor involved in regulating blood coagulation and to complement receptor C1qRp (64–66). This overall protein structure indicates that endosialin may be a receptor (63).

The murine homolog of TEM 1 was found to be expressed abundantly in the vasculature of the developing embryo but only in very limited adult vasculature by Carson-Walter et al. (36). Opavsky et al. (67) found 77.5% identity between human and murine endosialin and 100% identity between the transmembrane portion of the human and murine proteins. Using Rapid-Scan panel (Ori-Gene) for mouse mRNA, Opavsky et al. were able to detect endosialin message in all tissues, with highest expression in heart, kidney, stomach, skin, pancreas, uterus embryo, and virgin breast. In the human gene panel, the highest expression of endosialin message was found in placenta, ovary, heart, skeletal muscle, small intestine, and the cardiovascular system. Mouse cell lines from embryonic fibroblasts, pre-adipocytes, and immortalized endothelial cells expressed endosialin; however, human HUVEC cells were negative for endosialin message.

An endosialin knockout (KO) mouse was fertile and appeared to develop normally in body weight, vasculature, and wound healing (68). However, when human HCT116 colon carcinoma was implanted orthotopically on the cecum of nude endosialin KO mice, the take rate was about 33% compared with 90% take rate in normal nude mice. The HCT116 tumors that grew in the endosialin KO animals were slower growing than the tumors in the normal nude mice. There were significantly fewer HCT116 liver metastases in the endosialin KO mice. The tumors in the endosialin KO mice had a larger number of very small vessels than did the tumors in the normal nude mice.

Several recent reports have detected endosialin mRNA and/or endosialin protein in various tumor settings. Davies et al. (69) examined the levels of expression for tumor endothelial markers (TEMs) in human breast cancer. Breast cancer tissues ($n = 120$) and normal breast tissues ($n = 33$) were obtained after surgery. RNA was extracted from frozen sections for gene amplification. The expression of TEMs was assessed using RT-PCR, and the quantity of the transcripts was determined using real-time quantitative PCR (Q-RT-PCR). After a median follow-up of 72.2 months, it was found that patients who had recurrent disease and/or who had died from breast cancer had a significantly ($p < 0.05$) elevated levels of endosialin compared to those patients who were disease free. Patients who had developed nodal involvement exhibited significantly ($p < 0.05$) higher levels of endosialin compared to patients who were node negative. The data indicated that elevated levels of endosialin associated with either nodal involvement or disease progression and may have a prognostic value in breast cancer.

Kaposi's sarcoma is a multifocal, vascular, proliferative disease made up of clusters of spindle-shaped cells, slit-like vessels, and a variable inflammatory infiltrate (70). Wang et al. (71) showed by gene-expression microarrays that neoplastic cells of Kaposi's sarcoma are closely related to lymphatic endothelial cells and that Kaposi's sarcoma herpesvirus infects both lymphatic endothelial cells and blood vascular endothelial cells in vitro. Oligonucleotide microarrays were used to compare the gene-expression profiles of Kaposi's sarcoma and normal skin. Nodular Kaposi's sarcoma biopsy samples with >80% spindle cells were used to minimize the dermal and epidermal components. A subset of the global expression profile provided a "Kaposi sarcoma expression signature." The signature contained 1,482 genes that differentiate Kaposi's sarcoma from normal skin ($p \leq 0.05$). Endosialin was among the genes highly upregulated in Kaposi's sarcoma. Recent studies using standardized high-throughput RNA detection with microarray chips allowing for electronic Northern blot analysis of marker genes and laser capture microdissection on antibody-stained tissue sections for collection of RNA confirmed that endosialin was a tumor stromal marker and a tumor vascular marker (72–75). These studies also showed that endosialin was expressed on malignant cells of mesenchymal origin including malignant fibrous histiocytoma, liposarcoma, and other sarcomas.

Conejo-Garcia et al. (76) analyzed tumor-infiltrating host cells from ten consecutive stage III ovarian carcinomas for their expression of leukocyte marker CD45 or endothelial marker VE-cadherin by flow cytometry. A subpopulation of cells that co-express CD45 and VE-cadherin were identified and termed vascular leukocytes. RT-PCR of sorted human tumor endothelial cells, vascular leukocytes, HUVEC, and normal human spleen indicated similar levels of CD31 mRNA in the vascular leukocytes and HUVEC and higher levels in tumor endothelial cells. The CD45 mRNA expression was high in vascular leukocytes and spleen cells and very low in tumor endothelial cells and HUVEC. Endosialin was expressed by both tumor endothelial cells and vascular leukocytes. Thus, a new cell type was defined as CD45+VE-cadherin+P1H12+CD34+CD31+TEM 1+TEM 7+ that can form functional blood vessels (76).

When Madden et al. (25) performed SAGE analysis on endothelial cells derived from fresh surgical samples of normal temporal lobe cortex (two patients) or glioma (three patients); 16 genes were highly upregulated in tumor endothelial cells compared with normal endothelial cells, and endosialin was amongst these genes. Brady et al. (77) investigated the expression pattern of endosialin in human brain tumors and

brain metastasis. A rabbit polyclonal antibody to endosialin was generated and used to study 30 human brain tumor specimens by immunoblotting and immunohistochemistry. Twenty of 30 tumors expressed endosialin protein. The largest proportion of endosialin-expressing tumors was highly invasive glioblastoma multiforme (6/6), anaplastic astrocytomas (2/3), and carcinoma brain metastasis (4/7). Endosialin was expressed by melanoma (1/1), oligodendrogloma (2/2), astrocytomas (2/5), meningioma (2/5), and ependymoma (2/7). Endosialin localized to small and large vessels and was also expressed by Thy-1+ fibroblast-like cells in some vessels. Endosialin expression was associated with high-grade primary and metastatic tumors and was absent in normal blood vessels (77). Rettig et al. reported that radiolabeled FB5–endosialin complex was rapidly internalized into endothelial cells (62).

In a recent study, MacFayden et al. (78) immunized Balb/C mice with human AG1523 foreskin diploid fibroblasts and isolated four mouse monoclonal antibodies that bind to endosialin/CD248. In several tissues, endosialin was a cell-surface glycoprotein expressed predominately by fibroblasts and pericytes associated with tumor vasculature. Using phage display technology, Marty et al. (79) isolated a single-chain antibody fragment directed toward the endosialin extracellular domain. The single-chain antibody fragment was used to guide a liposome-encapsulated cytotoxic agent to tumor vasculature.

The function of endosialin remains undetermined. Endosialin belongs to the superfamily of proteins containing C-type lectin domains and specifically to Group XIV along with CETM, thrombomodulin, and C1qRP (80,81). These proteins are involved in several biological processes including coagulation, inflammation, and recognition of self versus non-self. Lectin carbohydrate recognition domains play a major role in pathogen detection (82–85). Pathogens can use carbohydrates specifically targeting C-type lectin domains to escape immune surveillance (83). Autoimmune peripheral vascular impairment in antiphospholipid syndrome may in part represent a breakdown in this system (84). By analogy to other Group XIV members, endosialin may have roles in cell–cell interaction and maintenance of immune system recognition of the proliferating tumor vasculature as self (86).

The message for endosialin was amongst those identified as TEMs by SAGE. Endosialin is expressed by endothelial precursor cells, pericytes, mesenchymal stem cells, a subset of T cells (vascular leukocytes), and some malignant cells of mesenchymal origin (sarcomas). The protein structure of endosialin places it in the Group XIV thrombomodulin-like family of C-lectin domain proteins. However, the absence of expression of endosialin in mature endothelium differentiates it from thrombomodulin. While the function of endosialin has yet to be elucidated, the expression pattern of this protein may make it a favorable target for cancer therapy with an antibody or antibody-toxin conjugate.

5. NEWER VASCULAR TARGETS: TEM 7

In 2000, St. Croix et al. (33) reported the results of a study designed to determine whether tumor-specific endothelial markers exist. Forty-six SAGE tags corresponding to 40 genes were described as expressed at greater than tenfold higher levels in the tumor endothelial cells compared with the normal endothelial cells. There are two SAGE tags for TEM 7. In the St. Croix et al. (33) report of a SAGE study using RNA

prepared from endothelial cell samples from the colon carcinoma and normal colon mucosa of a human patient, the SAGE tags for TEM 7 were found at a level of 74/18 in the RNA of tumor endothelial cells and at a level of 4/1 in the normal endothelial cells per 100,000 tags sequenced. Bioinformatics evaluation of TEM 7 indicated that it is a Type I membrane protein with a signal leader peptide, an N-terminal region similar to G1 domain of nidogen, followed by a 100aa region with homology to plexins, a transmembrane segment, and a short cytoplasmic tail. The 100aa region with homology to plexins contains a short cysteine-rich motif. Semaphorins, the plexin family of semaphorin receptors, and scatter factor receptors share these evolutionarily conserved protein modules. These proteins have in common a role in mediating cell guidance cues. Plexin C1/VESPR, a divergent member of the plexin family known to bind to GPI-linked semaphorin, shows close similarity to the TEM 7 plexin-like region. This overall protein structure indicated that TEM 7 may be a receptor.

The murine homolog of TEM 7 was found to have 81% amino acid identity with the human protein and was found to be expressed in the Purkinje cells of the mouse cerebellum and in some neuronal cells but no where else in adult mouse tissues (36). Similarly, in rat brain *in situ* hybridization study, TEM 7 mRNA localized to cerebellar Purkinje cells, the cerebral cortex layer IV and V, hippocampal pyramidal cells, and hypothalamic magnocellular nuclei (87).

Unexpectedly, Carson-Walters et al. were unable to detect significant levels of mTEM 7 by *in situ* hybridization in the blood vessels of murine B16 melanoma or human HCT116 colon carcinoma xenografts growing subcutaneously C57Bl or nude mice, respectively (36). However, exposure of mouse aortic endothelial cells to phorbol ester (PMA) or to a fibrin/fibrinogen-based three-dimensional culture to induce capillary morphogenesis produced upregulation of TEM 7 expression (88). St. Croix et al. (33) examined the expression of TEM 7 mRNA in human tissue specimens by *in situ* hybridization in a liver metastasis from colorectal cancer, a primary sarcoma, and in primary cancers of the lung, pancreas, breast, and brain. In each case, the TEM 7 mRNA was expressed specifically in the tumor endothelium. Conejo-Garcia et al. (76) was also able to detect TEM 7 mRNA in vascular leukocytes.

The nidogen domain of TEM 7 indicates that this protein may have a role in cellular migration *in vivo* (89). Expression of nidogen promotes cell migration (90, 91). Nidogen can bind collagen type IV, fibulins, laminin, and perlecan (92, 93). However, during development, nidogens may have additional roles in homeostasis and hematopoiesis (94). Using a recombinant TEM 7 ectodomain as a probe, Lee et al. identified a saturable interaction between nidogen and TEM 7 *in vitro* and an interaction between nidogen and TEM 7 expressed on the cell surface (95). The interaction between nidogen and TEM 7 resulted in increased spreading of TEM 7-transfected 293T cells lending support to the notion that nodogen may be a ligand for TEM 7.

The plexin domain of TEM 7 may be involved in semaphorin–plexin signaling. Plexins are receptors for multiple and perhaps all classes of semaphorins, either alone or in combination with neuropilins (96–99). Semaphorin–plexin signaling plays a role in a number of clinically important processes including cancer metastasis, angiogenesis, and regulation of the immune system (96–100). Recently, Gu et al. (101) found that signaling by semaphoring 3E and its receptor plexin-D1 controls endothelial cell positioning and the patterning of developing vasculature in the mouse. Semaphorin 3A is highly expressed in developing somites, where it acts as a repulsive cue for

plexin-D1-expressing endothelial cells of adjacent intersomitic vessels in a process that does not require neuropilins.

Serini et al. (102) showed that during vascular development and experimental angiogenesis, endothelial cells generate autocrine chemorepulsive signals of class 3 semaphorins (Sema3 proteins) and that these repulsive cues might regulate vascular morphogenesis by modulating integrin activation. Among the plexins, TEM 7 has highest homology with the human gene VESPR (103, 104). The plexin-C subfamily is defined by VESPR (now plexin-C1). Plexin-C1 (VESPR) binds with Sema7A, a GPI membrane-linked semaphoring (class 7). The cytoplasmic domains of plexins often associate with tyrosine kinase activity. For example, neuropilin-1, a semaphorin receptor, binds an isoform of vascular endothelial growth factor VEGF165 as well as several VEGF homologs, strongly suggesting a role for these systems in angiogenesis (105–107). In addition, semaphorin 4D binding to its receptor, plexin 1B, triggers invasive growth (108). The receptor plexin 1B extracellular domain has similarity to Met, the scatter factor/hepatocyte growth factor receptor.

6. NEWER VASCULAR TARGETS: PRL-3

Phosphatases involved in critical aspects of malignant disease could be interesting drug targets. The family of protein phosphatases of regenerating liver (PRL) comprises three members known as PRL-1, -2, and -3. Rat PRL-1 was originally identified as an immediate early gene in regenerating liver (109). Murine PRL-2 and PRL-3 were subsequently discovered by amino acid sequence homology and display 87 and 76% sequence identity to murine PRL-1 (110). All three PRL proteins contain a C-terminal prenylation motif (110). The human PRL-1, -2, and -3 have been elucidated more recently, beginning with the description of human PRL-3 as a human muscle-specific tyrosine phosphatase (111–113).

Evidence is accumulating that these proteins may be associated with oncogenic states (114). The first link between PRL expression and cancer was from tissue distribution showing widespread PRL-1 expression in embryonic tissues. In the rat embryo, PRL-1 is expressed in brain, intestine, liver, and esophageal epithelia. In the murine embryo, PRL-1 is expressed in the nervous and skeletal systems (115–118). The hypothesis is that neoplastic cells are de-differentiated abnormal cells that have in part reverted to embryonic plasticity (119–124). PRL-1 is frequently elevated in breast, ovarian, colon, prostate, and pancreatic cancers (125, 126).

Additionally, PRL-1 was isolated from regenerating liver, a proliferative physiological process that, if deregulated, may contribute to primary liver cancers (109, 127). NIH3T3 cells transfected with PRL-1 display abnormal morphology and enhanced growth rate and fibroblasts upregulate PRL-1 mRNA expression upon serum stimulation (109, 128). These findings suggest a role for PRL-1 in cell-cycle regulation which is supported by the finding that that endogenous PRL-1 subcellular localization varies with PRL-1 located in the endoplasmic reticulum of non-mitotic cells and at the centrosomes and spindle apparatus of mitotic cells (129, 130).

All three PRL proteins are implicated directly in cancer progression. Flag-tagged human PRL-1 protein expression in D27 hamster pancreatic ductal epithelial cells led to loss of contact inhibition in culture and tumor growth in nude mice (131). Human PRL-1 mRNA was upregulated in benign prostatic fibroblast cells after stimulation

with human prostate tumor cell line LNCaP-conditioned medium and in prostatic tumor fibroblast cells (130). PRL-1 mRNA was expressed in several tumor epithelial cell lines including HeLa cervical adenocarcinoma cell line, HepG2 hepatoblastoma cell line, and a Burkitt's lymphoma cell line (109). PRL-2 mRNA overexpression was detected in prostate cancer cell lines and prostate tumor tissues (129). PRL-1 and PRL-3 protein increased cell motility and invasion in vitro (132–139).

PRL-3 is a TEM, expressed at higher levels in endothelial cells from a fresh human colon cancer specimen than in normal colon mucosa endothelial cells (33). PRL-3 mRNA was expressed at higher levels in metastases of colorectal cancers compared to nonmetastatic tumors and normal colorectal epithelium (140). PRL-3 mRNA was not detected in normal human colon tissue, in non-metastatic colorectal carcinoma, or in lung and liver metastases of non-colorectal cancer origin. PRL-3 mRNA was detected in 4/4 colorectal cancer metastases to lymph nodes, 10/11 colorectal metastases to the liver, 6/7 colorectal metastases to the lung, 4/4 colorectal metastases to the brain, and 3/3 colorectal metastases to the ovary (141). Thus, PRL-3 was selectively expressed by colorectal cancer metastases.

Using *in situ* hybridization, Kato et al. investigated the significance of PRL-3 expression in the progression and development of colorectal cancer in both primary (177 cases) and metastatic (92 cases) human colorectal cancers and elucidated the relationships with clinicopathological parameters including the incidence of metachronous liver and/or lung metastasis after curative surgery for primary tumor (142). In human primary colorectal cancers, PRL-3 expression was upregulated in cases with liver (84.4%) or lung (88.9%) metastasis and was statistically higher than in cases without either type of metastasis (liver, 35.9%; lung, 43.2%). In metastatic colorectal cancer lesions, high PRL-3 expression was frequently detected (liver, 91.3%; lung, 100%). Metachronous metastasis was observed more frequently in cases with high PRL-3 expression ($p < 0.0001$), indicating that PRL-3 expression in colorectal cancers may contribute to liver metastasis and may be a useful biomarker to identify patients at high risk for distant metastasis (142).

In metastatic colorectal cancer lesions, high PRL-3 expression was found in 21/23 (91.3%) cases of liver metastasis and in 6/6 (100%) cases of lung metastasis (142). However, in lymph node metastasis and peritoneal dissemination, high PRL-3 expression was observed in only 47.5 and 50.0% of cases, respectively. In liver and lung metastatic lesions, almost all of the cancer cells homogeneously demonstrated high PRL-3 expression. In 10 cases, serial analyses of both primary and metastatic tumors were performed, and all of specimens had high PRL-3 expression of (142).

A prognostic study was conducted to determine whether PRL-3 expression was a useful biomolecular marker to monitor metachronous metastasis risk after curative colorectal cancer surgery. Postoperative development of occult liver and/or lung metastasis appeared in 14/104 cases (13.5%). Kaplan–Meier analysis showed that cases with high PRL-3 expression had greater risk of metachronous metastasis development than those with low PRL-3 expression ($p < 0.0001$). The incidence of metachronous metastasis was not statically related to conventional indicators such as primary tumor size, angiolympathic invasion, or the presence of lymph node metastasis (142, 143).

Parker et al. found PRL-3 highly induced in RNA derived from breast cancer endothelial cells compared with normal breast endothelial cells by SAGE analysis (26). PRL-3 expression was elevated in tumor endothelium relative to normal endothelial

cells, bulk tissue samples, and breast tumor cell lines supporting preferential PRL-3 expression in breast tumor vasculature relative to epithelial cells. By *in situ* hybridization, there was an absence of PRL-3 in the normal tissues and endothelial specific expression in the blood vessels of invasive carcinoma (26).

Zeng et al. and Wu et al. (110, 135) developed genetically engineered systems that in culture and in mice demonstrated effects of PRL-3 overexpression on tumor growth and invasion. Chinese hamster ovary cells and murine B16 melanoma cells transfected to overexpress PRL-3 were more aggressive and invasive than the parental cells.

PRL-3 mRNA expression was highly variable among primary tumor-derived colorectal cancer cell lines and suggests that advanced primary tumors may express higher PRL-3 mRNA levels than lower grade tumors (132). Cell lines such as HT29, SW480, and LST174T colorectal adenocarcinomas, with moderate PRL-3 mRNA, or high PRL-3 mRNA, such as SW837 rectal adenocarcinoma and HCT116 colorectal carcinoma, might have been derived from primary tumors that had developed a metastatic phenotype. Indeed, Leibovitz et al. (144) stated that SW480 line was derived from a Duke's class B primary tumor, indicating that the primary tumor had invaded the muscularis propria of the bowel wall and that cancer recurred a year later with widespread metastasis in that patient. The SW837 line was derived from a Duke's class C rectal tumor, indicating that the tumor had invaded the regional lymph node (144). In addition, HCT116 line, a high expresser of PRL-3 mRNA, is a poorly differentiated and aggressive cell line, a characteristic consistent with advanced cancer stage.

In non-colorectal cancer cell lines, high PRL-3 mRNA expression was observed in cells derived from primary tumors, such as Hep3B hepatocellular carcinoma line (145), and metastases, such as SK-NAS neuroblastoma line, derived from a bone marrow metastasis (146), and MCF-7 mammary carcinoma line, derived from a pleural effusion (147). Thus, PRL-3 may play a role in cancer broadly. Low level or absent PRL-3 mRNA expression in melanoma lines suggests that PRL-3 might not be a factor in skin cancers (132).

PRL-3 is upregulated by exposure of endothelial cells to PMA and can be directly implicated in increased tube formation by PRL-3 adenovirus-infected HMVEC and in invasion by stably transfected DLD-1 subclones and endogenously expressing tumor cell lines (132). Furthermore, the invasive behavior of these cells can be reversed by intracellular expression of PRL-3 siRNA. PRL-3 represents both an antiangiogenic and antitumor target in malignant disease (148). Recently, integrin α1 was identified as a PRL-3 interacting protein and it was found that PRL-3 can downregulate the tyrosine phosphorylation of integrin β1 while increasing phosphorylation of Erk1/2, thus elucidating, in part, the molecular processes by which PRL-3 activity promotes the malignant phenotype (149).

7. CONCLUSIONS

SAGE analysis of subpopulations of tumors has provided useful leads for new vascular targets. It remains to the basic scientists to elucidate the function of these proteins and to the "drug hunters" to determine whether these targets can be used in therapeutically meaningful ways.

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9

Chemokines in Angiogenesis

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SUMMARY

Angiogenesis is the process of new blood vessel growth and is a critical biological process under both physiological and pathological conditions. Angiogenesis can occur under physiological conditions that include embryogenesis and the ovarian/menstrual cycle. In contrast, pathological angiogenesis is associated with chronic inflammation/chronic fibroproliferative disorders and tumorigenesis of cancer. Net angiogenesis is determined by a balance in the expression of angiogenic, as compared with angiostatic factors. CXC chemokines are heparin-binding proteins that display unique disparate roles in the regulation of angiogenesis. Based on their structure, CXC chemokines can be divided into two groups that either promote or inhibit angiogenesis, and they are therefore uniquely placed to regulate net angiogenesis in both physiological and pathological conditions including cancer growth.

Key Words: Chemokine; chemokine receptor; angiogenesis; cancer.

1. INTRODUCTION

Angiogenesis can occur under physiological conditions including normal wound repair and embryogenesis. In contrast, pathological angiogenesis is associated with chronic inflammation/chronic fibroproliferative disorders and growth of tumors.

A variety of factors have been described that either promote or inhibit angiogenesis. In the local microenvironment, net angiogenesis is determined by a balance in the expression of angiogenic, as compared with angiostatic factors. The family has four highly conserved cysteine amino acid residues, with the first two cysteines separated by a non-conserved amino acid residue (1, 2). A second structural domain dictates their functional activity. The NH₂-terminus of several CXC chemokines contains three amino acid residues (Glu-Leu-Arg; "ELR" motif), which immediately precedes the first

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Table 1
The CXC Chemokines that Display Disparate Angiogenic Activity

| | |
|---|--|
| <i>Angiogenic CXC chemokines containing the ELR motif</i> | |
| CXCL1 | Growth-related oncogene alpha (GRO- α) |
| CXCL2 | Growth-related oncogene beta (GRO- β) |
| CXCL3 | Growth-related oncogene gamma (GRO- γ) |
| CXCL5 | Epithelial neutrophil-activating protein-78 (ENA-78) |
| CXCL6 | Granulocyte chemotactic protein-2 (GCP-2) |
| CXCL7 | Neutrophil-activating protein-2 (NAP-2) |
| CXCL8 | Interleukin-8 (IL-8) |
| <i>Angiostatic CXC chemokines that lack the ELR motif</i> | |
| CXCL4 | Platelet factor-4 (PF-4) |
| CXCL9 | Monokine induced by interferon- γ (MIG) |
| CXCL10 | Interferon- γ -inducible protein (IP-10) |
| CXCL11 | Interferon inducible T-cell alpha chemoattractant (ITAC) |
| CXCL12 | Stromal cell-derived factor-1 (SDF-1) |
| CXCL14 | Breast- and kidney-expressed chemokine (BRAK) |

Table 2
CXC Chemokine Ligands and Receptors that have Been Implicated in Angiogenesis

| <i>Receptor</i> | <i>Ligand</i> |
|-----------------|---|
| CXCR2 | CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8 |
| CXCR4 | CXCL12 |

Table 3
CXC Chemokine Ligands and Receptors that have been Implicated in Angiostasis

| <i>Receptor</i> | <i>Ligand</i> |
|-------------------------------|------------------------------|
| CXCR3B | CXCL4, CXCL9, CXCL10, CXCL11 |
| Unknown/non-receptor mediated | CXCL4, CXCL14 |

CXCL4 may act through CXCR3B or non-receptor mediated mechanisms.

cysteine amino acid residue (1, 2). The CXC chemokines with the “ELR” motif (ELR $^{+}$) promote angiogenesis (Tables 1 and 2) (1). In contrast, CXC chemokines that are, in general, interferon (IFN) inducible and lack the ELR motif (ELR $^{-}$) inhibit angiogenesis (Tables 1 and 3) (1). The dissimilarity in structure dictates the use of different CXC chemokine receptors on endothelial cells, which ultimately leads to signal coupling and either promotion or inhibition of angiogenesis. CXCR2 mediates the angiogenic signals of the ELR $^{+}$ CXC chemokines, whereas the ELR $^{-}$ CXC chemokines mediate their angiostatic actions through CXCR3. Furthermore, it has recently been suggested that angiostatic signals are specifically mediated through CXCR3B, whereas CXCR3A may mediate angiogenic signals (3, 4).

2. THE CXC CHEMOKINES

The CXC chemokines can be divided into two groups on the basis of a structure/function domain consisting of the presence or absence of three amino acid residues (Glu-Leu-Arg; “ELR” motif) that precedes the first cysteine amino acid residue in the primary structure of these cytokines. The ELR⁺ CXC chemokines are chemoattractants for neutrophils and act as potent angiogenic factors (5). In contrast, the ELR⁻ CXC chemokines are chemoattractants for mononuclear cells and are potent inhibitors of angiogenesis (Table 1) (5).

Based on the structural/functional difference, the members of the CXC chemokine family are unique cytokines in their ability to behave in a disparate manner in the regulation of angiogenesis. The angiogenic members include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8. CXCL1, CXCL2, and CXCL3 are closely related CXC chemokines, with CXCL1 originally described for its melanoma growth-stimulatory activity (Table 1). CXCL5, CXCL6, and CXCL8 were all initially identified on the basis of neutrophil activation and chemotaxis. The angiostatic (ELR⁻) members of the CXC chemokine family include CXCL4, which was originally described for its ability to bind heparin and inactivate heparin’s anticoagulation function. Other angiostatic ELR⁻ CXC chemokines include CXCL9, CXCL10, CXCL11, and CXCL14 (Table 1).

2.1. CXCR2 is the Receptor for Angiogenic ELR⁺ CXC Chemokine-Mediated Angiogenesis

The fact that all ELR⁺ CXC chemokines mediate angiogenesis highlights the importance of identifying a common receptor that mediates their biological function in promoting angiogenesis. While the candidate CXC chemokine receptors are CXCR1 and/or CXCR2, only CXCL8 and CXCL6 specifically bind to CXCR1, whereas all ELR⁺ CXC chemokines bind to CXCR2 (6). The ability of all ELR⁺ CXC chemokine ligands to bind to CXCR2 supports the notion that this receptor mediates the angiogenic activity of ELR⁺ CXC chemokines.

While CXCR1 and CXCR2 are detected in endothelial cells (6–8), the expression of CXCR2, not CXCR1, has been found to be the primary functional chemokine receptor in mediating endothelial cell chemotaxis (6, 9). Heidemann and associates (10) have further confirmed the importance of CXCR2 in mediating the effects of angiogenesis in human microvascular endothelial cells. They found that endothelial cells respond to CXCL8 with rapid stress fiber assembly, chemotaxis, enhanced proliferation, and phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK 1/2) related to activation of CXCR2 (10). Blocking the function of CXCR2 by either specific neutralizing antibodies or inhibiting downstream signaling using specific inhibitors of ERK1/2 and PI3kinase impaired CXCL8-induced stress fiber assembly, chemotaxis, and endothelial tube formation in endothelial cells (10).

CXCR2 activation **leads** to receptor internalization, **leads to** recycling of the receptor back to the cell membrane, or targets CXCR2 for degradation. ELR⁺ CXC chemokine activation of CXCR2 under conditions in which the receptor is transiently exposed or stimulated with less than saturable concentrations results in movement of CXCR2 into clathrin-coated pits, movement into the early endosome, the sorting endosome, and on to the recycling endosome with trafficking back to the plasma membrane

compartment and re-expression on the cell surface (11). However, if CXCR2 is exposed to prolonged saturating concentrations of ELR⁺ CXC chemokines, a significant proportion of CXCR2 will move into the late endosome and on to the lysosome for degradation (11). Interestingly, CXCR2 internalization is necessary for generating a chemotactic response. Mutation of the CXCR2, which impairs receptor internalization by altering the binding of adaptor proteins AP-2 or beta arrestin to the receptor results in a marked reduction in the chemotactic response (11).

The importance of CXCR2 in mediating ELR⁺ CXC chemokine-induced angiogenesis has been shown *in vivo* using the cornea micropocket assay of angiogenesis in CXCR2^{+/+} and CXCR2^{-/-} animals. ELR⁺ CXC chemokine-mediated angiogenesis was inhibited in the corneas of CXCR2^{-/-} mice, and in the presence of neutralizing antibodies to CXCR2 in the rat corneal micropocket assay (6). These studies have been further substantiated using CXCR2^{-/-} mice in a wound repair model system (12). Devalaraja and associates (12) have examined the significance of CXC chemokines in wound healing. In this study, full excisional wounds were created on CXCR2^{+/+}, heterozygous +/-, or CXCR2^{-/-} mice. Significant delays in wound healing parameters were found in CXCR2^{-/-} mice, including decreased neovascularization (12).

2.2. *CXCR3 is the Major Receptor for CXC Chemokines that Inhibit Angiogenesis*

The major receptor that has been identified for angiostatic CXC chemokines is CXCR3 which is involved in mediating recruitment of T-helper 1 cells and acts as the receptor for inhibition of angiogenesis (13–17). Endothelial expression of CXCR3 was originally identified on murine endothelial cells (18); subsequent studies demonstrated that CXCR3 ligands could block both human microvascular endothelial cell migration and proliferation in response to a variety of angiogenic factors (8, 19). Further clarification of the role of CXCR3 in mediating angiostatic activity has come from the discovery that CXCR3 exists as two alternative splice forms (3). These variants have been termed CXCR3A and CXCR3B (3). CXCR3A mediates the CXCR3 ligand-dependent chemotactic activity of mononuclear cells (3). CXCR3B mediates the angiostatic activity of CXCL4, CXCL9, CXCL10, and CXCL11 on human microvascular endothelial cells (3). Moreover, specific antibodies to CXCR3B immunolocalize to endothelial cells within neoplastic tissues (3). This supports the notion that if CXCR3 ligands can be spatially expressed within the tumor, then CXCR3B activation can inhibit tumor-associated angiogenesis (3). To add to the complexity of CXCR3 biology, a variant of human CXCR3 has been recently identified, which is generated by post-transcriptional exon skipping referred to as CXCR3-alt (17). This receptor is expressed and responds to CXCL11>>CXCL9 and CXCL10 (17). These findings support the notion that augmenting CXCR3/CXCR3 ligand biology will be a therapeutic strategy to enhance angiostasis within the tumor.

While the above studies have supported that CXCR3 is the receptor for CXCL4, CXCL9, CXCL10, and CXCL11, it has remained unclear *in vivo* whether these CXCR3 ligands use CXCR3 on endothelium to mediate their angiostatic effect. Yang and Richmond (20) have recently demonstrated that CXCL10 mediates its angiostatic activity *in vivo* by binding to CXCR3, and not via binding to glycosaminoglycans (GAGs). To clarify this issue, they created expression constructs for mutants of CXCL10 that exhibit partial or total loss of binding to CXCR3 or loss of binding to

GAGs. They transfected a human melanoma cell line with these expression vectors, and stable clones were selected and inoculated into immunodeficient mice (20). Tumor cells expressing wildtype CXCL10 showed remarkable reduction in tumor growth compared to control vector-transfected tumor cells. Surprisingly, mutation of CXCL10 resulting in partial loss of receptor binding (IP-10C), or loss of GAG binding (IP-10H), did not significantly alter the ability to inhibit tumor growth. The reduction in tumor growth was associated with a reduction in tumor-associated angiogenesis, leading to the observed increase in both tumor cell apoptosis and necrosis (20). In contrast, expression of the CXCL10 mutant that fails to bind to CXCR3 failed to inhibit tumor growth (20). These data suggest that CXCR3 receptor binding, but not GAG binding, is essential for the tumor angiostatic activity of CXCR3 ligands.

2.3. Chemokines and Angiogenesis in Various Tumor Models

CXC chemokine-mediated angiogenesis has been shown to have an important role in tumor growth in a variety of tumors, including melanoma, lung cancer, pancreatic cancer, ovarian cancer, brain tumors and gastric carcinoma, breast, and head and neck cancer (21–26).

2.3.1. MELANOMA

The ELR⁺ CXC chemokines are important mediators of tumorigenesis related to their angiogenic properties. Studies using melanoma tumor models support the concept that CXCL1, CXCL2, and CXCL3 play a significant role in mediating tumorigenesis related to both their mitogenic and angiogenic activities (27). For example, CXCL1, CXCL2, and CXCL3 have all been found to be highly expressed in human melanoma (27). When human CXCL1, CXCL2, and CXCL3 genes were transfected into immortalized murine melanocytes that otherwise by themselves do not form tumors, these cells transformed their phenotype to one with anchorage-independent growth in vitro and the ability to form tumors in vivo in immunoincompetent mice (27, 28). The tumors were highly vascular and similar to the vascularity of B16 melanoma controls (27, 28). When tumors were depleted of CXCL1, CXCL2, or CXCL3, there was a marked reduction of tumor-derived angiogenesis directly related to inhibition of tumor growth (27, 28). These findings support the notion that the ELR⁺ CXC chemokines have the ability to act both as autocrine growth factors for melanoma and as potent paracrine mediators of angiogenesis to promote tumorigenesis and metastases.

2.3.2. OVARIAN CANCER

The progression and growth of ovarian carcinoma is also dependent on successful angiogenesis, and CXCL8 has been determined to play a significant role in mediating human ovarian carcinoma-derived angiogenesis and tumorigenesis (29). The expression of CXCL8, bFGF, and VEGF was examined in five different human ovarian carcinoma cell lines (29). All cell lines in vitro expressed similar levels of bFGF; however, these cells expressed either high or low levels of CXCL8 or VEGF. When implanted into the peritoneum of immunoincompetent mice, the high-expressing CXCL8 tumors were associated with all animals dying at <51 days (29). The expression of CXCL8 was directly correlated with neovascularization and inversely correlated with survival, whereas VEGF expression was only correlated with production of ascites (29). No correlation was found for bFGF with either tumor neovascularization or survival (29).

This study has been substantiated in patients with ovarian cancer, where ascites fluid demonstrates angiogenic activity directly correlated to CXCL8 (30). These findings support the notion that antigenic ELR⁺ CXC chemokines play a greater role than bFGF and VEGF in mediating angiogenesis associated with ovarian cancer.

2.3.3. LUNG CANCER

CXCL8 is markedly elevated and contributes to the overall angiogenic activity of non-small cell lung cancer (NSCLC) (31). Extending these studies to an *in vivo* model system of human tumorigenesis (i.e., human NSCLC/severe combined immunodeficiency (SCID) mouse chimera) (32), tumor-derived CXCL8 was found to be directly correlated with tumorigenesis (32). Tumor-bearing animals depleted of interleukin-8 (IL-8)/CXCL8 demonstrated a >40% reduction in tumor growth and a reduction in spontaneous metastases which correlated to reduced angiogenesis (32). These findings have been further corroborated using several human NSCLC cell lines grown in immunoincompetent mice. NSCLC cell lines that constitutively express CXCL8 display greater tumorigenicity that is directly correlated to angiogenesis (33).

While CXCL8 was the first angiogenic ELR⁺ CXC chemokine to be discovered in NSCLC, CXCL5 has now been determined to have a higher degree of correlation with NSCLC-derived angiogenesis (34). Surgical specimens of NSCLC tumors demonstrate a direct correlation of CXCL5 with tumor angiogenesis. These studies were extended to a SCID mouse model of human NSCLC tumorigenesis. CXCL5 expression was directly correlated with tumor growth (34). Moreover, when NSCLC tumor-bearing animals were depleted of CXCL5, both tumor growth and spontaneous metastases were markedly attenuated (34). The reduction of angiogenesis was also accompanied by an increase in tumor cell apoptosis, consistent with the previous observation that inhibition of tumor-derived angiogenesis is associated with increased tumor cell apoptosis (34). Similarly, *in vivo* and *in vitro* proliferation of NSCLC cells was unaffected by the presence of CXCL5. While a significant correlation of CXCL5 exists with tumor-derived angiogenesis, tumor growth, and metastases, CXCL5 depletion does not completely inhibit tumor growth (34). This reflects that the angiogenic activity of NSCLC tumors is related to many overlapping and potentially redundant factors acting in a parallel or serial manner. Furthermore, when all ELR⁺ CXC chemokines are evaluated in human NSCLC, it appears that they correlate with patient mortality (35,36).

These studies have been further extended to a lung cancer syngeneic tumor model system in CXCR2^{-/-}, as compared with CXCR2^{+/+} mice. Lung cancer in CXCR2^{-/-} mice demonstrate reduced growth, increased tumor-associated necrosis, inhibited tumor-associated angiogenesis, and metastatic potential (37). These *in vitro* and *in vivo* studies establish that CXCR2 is an important receptor that mediates ELR⁺ CXC chemokine-dependent angiogenic activity.

2.3.4. PROSTATE CANCER

Prostate cancer tumorigenesis and metastases are dependent on angiogenesis (38,39). Serum levels of CXCL8 have been found to be markedly elevated in patients with prostate cancer. These levels are highly correlated with the stage of the disease and have been determined to be an independent variable from the ratio of free/total prostate-specific antigen (PSA) (39). In fact, the combined use of free/total PSA and IL-8/CXCL8 levels were more effective in distinguishing prostate cancer from benign

prostatic hypertrophy (39). This suggests that ELR⁺ CXC chemokines may be playing an important role in mediating prostate cancer-derived angiogenesis in support of tumorigenesis and metastases. This observation in patients has been substantiated in human/SCID mice chimeras of human prostate cancer tumorigenesis (40). Three human prostate cancer cell lines were examined for constitutive production of angiogenic ELR⁺ CXC chemokines (40). Tumorigenesis of the human prostate cancer cell line, PC-3, was shown to be attributable, in part, to the production of the angiogenic CXC chemokine, CXCL8. Depletion of endogenous CXCL8 inhibited PC-3 tumor growth in SCID mice, which was entirely attributable to inhibition of PC-3 tumor-derived angiogenesis (40). In contrast, the human prostate cancer cell line, Du145, was found to utilize a different angiogenic CXC chemokine, CXCL1, to mediate tumor-derived angiogenesis (40). Depletion of endogenous CXCL1, but not CXCL8, reduced tumor growth that was directly related to attenuated angiogenic activity (40). Other studies have confirmed this observation in prostate cancer models (41). Similarly Shen et al. (42) have shown that there is progressive dysregulation of nuclear factor-kappa B (NF-κB) and signal transducers and activators of transcription (STAT1) in prostate cancer cells that leads to production of angiogenic CXC chemokines. Thus, prostate cancer cell lines can utilize distinct CXC chemokines to mediate their tumorigenic potential.

2.3.5. BRAIN TUMORS

Glioblastoma are highly aggressive brain tumors, with mortality approaching 80% in the first year post-diagnosis (43). The hallmark of these tumors is the marked presence of angiogenesis (44), which suggests that it is a biomarker necessary for malignant progression of this tumor. Garkavtsev and associates (44) have recently identified a candidate tumor-suppressor gene, ING4, is involved in regulating glioblastoma tumor growth and angiogenesis. In this study, the expression of ING4 was found to be significantly reduced in glioblastomas, as compared with normal human brain tissue, and the extent of reduction correlated with the progression from lower to higher grade of tumor (44). Human glioblastomas that exhibited decreased expression of ING4 when engrafted into immunoincompetent mice grew markedly faster and displayed greater angiogenesis than control tumors (44). The mechanism for increased tumorigenicity in glioblastomas that expressed lower levels of ING4 was related to ING4's physical ability to bind the p65 (RelA) subunit of NF-κB, impair its nuclear translocation, and subsequently inhibit transactivation of NF-κB-dependent genes (44). In fact, the mechanism for the angiogenic activity of glioblastomas that expressed low levels of ING4 was CXCL8 dependent, as inhibition of CXCL8 in vivo markedly reduced their tumor growth and tumor-associated angiogenesis (44). These findings link a tumor-suppressor gene to function and control of the expression of angiogenic ELR⁺ CXC chemokines in human tumors and provides the unique opportunity to consider targeting ELR⁺CXC chemokine-mediated angiogenesis.

2.3.6. PANCREATIC CANCER

Human pancreatic cancer cell lines have been shown to secrete the angiogenic chemokines CXCL1 and CXCL8 (45). Similarly, of three human pancreatic cancer cell lines tested, BxPC-3 cells produced the highest levels of CXC chemokines, whereas MIA PaCa-2 cells revealed expression to a lesser extent and the PANC-1 cells showed very low expression of the tested angiogenic chemokines (46). Corneal

vascular response was markedly positive in BxPC-3, and this could be attenuated with neutralizing CXCR2 antibody (46). This complete inhibition of angiogenic activity by a neutralizing antibody to CXCR2 underscores the importance of the ELR⁺ chemokine/CXCR2 axis in stimulating angiogenesis in the BxPC-3 cell line. In contrast, in the cell lines with lower CXC chemokine expression, MIA PaCa-2 and Panc-1, the effect of inducing neovascularization samples could not be affected by blocking CXCR2 (46).

2.4. Non-ELR⁺ CXC Chemokines Attenuate Angiogenesis Associated with Tumorigenesis

Non-ELR⁺ CXC chemokines have been shown to inhibit angiogenesis in several model systems, for example, Burkitt's lymphoma cell lines form tumors in immunoincompetent mice (47). Angiogenesis is essential for tumorigenesis of these lymphomas, analogous to carcinomas. The expression of CXCL9 and CXCL10 was found to be higher in tumors that demonstrated spontaneous regression and was directly related to impaired angiogenesis (48). To determine whether this effect was attributable to CXCL9 or CXCL10, more virulent Burkitt's lymphoma cell lines were grown in immunodeficient mice and subjected to intra-tumor inoculation with either CXCL9 or CXCL10. Both conditions resulted in marked reduction in tumor-associated angiogenesis (48–51). Although these CXCR3 ligands have been shown to bind to CXCR3 on mononuclear cells (13, 16, 52–56), the ability of these non-ELR⁺ CXC chemokines to inhibit angiogenesis and induce lymphoma regression in immunodeficient mice supports the notion that these chemokines can mediate their effects in a T-cell independent manner.

To examine the role of CXCL10 in the regulation of angiogenesis in carcinoma, the level of CXCL10 from human surgical NSCLC tumor specimens was examined and was found to be significantly higher in the tumor specimens than in normal adjacent lung tissue (57). The increase in CXCL10 from human NSCLC tissue was entirely attributable to the higher levels of CXCL10 present in squamous cell carcinoma (SCCA), as compared with adenocarcinoma (57). Moreover, depletion of CXCL10 from SCCA surgical specimens resulted in augmented angiogenic activity (57). The marked difference in the levels and bioactivity of CXCL10 in SCCA and adenocarcinoma is clinically and pathophysiologically relevant and represents a possible mechanism for the biologic differences of these two cell types of NSCLC. Patient survival is lower, metastatic potential is higher, and evidence of angiogenesis is greater for adenocarcinoma, as compared with SCCA of the lung (58–60). These studies were extended to a SCID mouse system to examine the effect of CXCL10 on human NSCLC cell line tumor growth in a T- and B-cell independent manner. SCID mice were inoculated with either adenocarcinoma or SCCA cell lines (57). The production of CXCL10 from adenocarcinoma and SCCA tumors was inversely correlated with tumor growth (57). However, CXCL10 levels were significantly higher in the SCCA, as compared with adenocarcinoma tumors (57). The appearance of spontaneous lung metastases in SCID mice bearing adenocarcinoma tumors occurred after CXCL10 levels from either the primary tumor or plasma had reached a nadir (57). In subsequent experiments, SCID mice bearing SCCA tumors were depleted of CXCL10, whereas, animals bearing adenocarcinoma tumors were treated with intra-tumor CXCL10 (57).

Depletion of CXCL10 in SCCA tumors resulted in an increase in their size (57). In contrast, reconstitution of intra-tumor CXCL10 in adenocarcinoma tumors reduced both their size and metastatic potential, which was unrelated to infiltrating neutrophils or mononuclear cells (i.e., macrophages or NK cells) and directly attributable to a reduction in tumor-associated angiogenesis (57). Similar strategies have been found for CXCL10 in melanoma using a gene therapeutic strategy (61).

Similar to CXCL10, CXCL9 also plays a significant role in regulating angiogenesis of NSCLC. CXCL9 levels in human specimens of NSCLC were not significantly different from that found in normal lung tissue (62). However, these results suggested that the increased expression of ELR⁺ CXC chemokines and other angiogenic factors found in these tumors were not counter-regulated by a concomitant increase in the expression of the angiostatic CXC chemokine, CXCL9. Thus, this imbalance could promote a microenvironment that promotes angiogenesis. To alter this imbalance, studies were performed to overexpress CXCL9 by three different strategies including gene transfer (62). These experiments resulted in the inhibition of NSCLC tumor growth and metastasis via a decrease in tumor-associated angiogenesis (62). These findings support the importance of the IFN-inducible non-ELR⁺ CXC chemokines in inhibiting NSCLC tumor growth by attenuation of tumor-derived angiogenesis. In addition, the above study demonstrates the potential efficacy of gene therapy as an alternative means to deliver and overexpress a potent angiostatic CXC chemokine.

3. DARC AND TUMOR ANGIOGENESIS

The Duffy antigen receptor for chemokines (DARC) is known to be a promiscuous chemokine receptor that binds chemokines in the absence of any detectable signal transduction events (63). Within the ELR⁺ CXC chemokines, DARC binds the angiogenic CXC chemokines including CXCL1, CXCL5, and CXCL8, all of which have previously been shown to be important for promoting tumor growth in a variety of tumors, including non-small cell lung carcinoma (NSCLC) tumor growth (34, 40, 64). Addison and colleagues (63) demonstrated that stable transfection and overexpression of DARC in an NSCLC tumor cell line resulted in the binding of the angiogenic ELR⁺ CXC chemokines by the tumor cells. The binding of tumor cell-derived ELR⁺ CXC chemokines to the tumor cells themselves interfered with the local tumor paracrine microenvironment of tumor cell interaction with host responding endothelial cells and prevented the ability of these angiogenic factors to stimulate endothelial cells and promote tumor-associated angiogenesis (63). NSCLC tumor cells that constitutively expressed DARC in vitro were similar in their growth characteristics as compared with control-transfected cells. However, they found that tumors derived from DARC-expressing cells were significantly larger in size than tumors derived from control-transfected cells. Interestingly, upon histological examination, DARC-expressing tumors had significantly more necrosis and decreased tumor cellularity, as compared with control tumors. Expression of DARC by NSCLC cells was also associated with a marked decrease in tumor-associated vasculature and a reduction in metastatic potential. Similarly, in a murine model of prostate cancer, DARC^{-/-} mice had increased tumor growth, intra-tumor levels of CXC chemokines, and increased intra-tumor vessel density, indicating an important role for DARC in inhibiting the biologic effects of CXC chemokines in tumor growth (65). The findings of this

study suggested that competitive binding of ELR⁺ CXC chemokines by tumor cells expressing a decoy receptor could prevent paracrine activation of endothelial cells in the tumor microenvironment and reduce tumor-associated angiogenesis.

3.1. The Role of CXCR4 in Angiogenesis

CXCL12 has been shown to have an important role in metastases of cancer (66). However, CXCL12 is also a non-ELR⁺ CXC chemokine that via CXCR4 has been implicated in promoting angiogenesis (67–70). This has lead to the speculation that the predominant function of this ligand/receptor pair in tumorigenesis is due to its angiogenic effect, not necessarily due to its potential of mediating organ-specific metastases. However, in order for the biological axis of CXCL12/CXCR4 to mediate tumor-associated angiogenesis, both the ligand and receptor should be temporally and spatially present within the tumor. Schrader and colleagues (71), demonstrated in both renal cell carcinoma cell lines and patient specimens that CXCR4 is expressed predominately by the tumor cells, and its ligand CXCL12 is essentially absent within the tumor. These findings have been further substantiated in human breast cancer and NSCLC tumor specimens, in which CXCR4 was found to be expressed on the tumor cells and does not mediate tumor-associated angiogenesis *in vivo* (66, 72). The studies demonstrated that when animals bearing breast or NSCLC tumors were treated with either neutralizing anti-CXCL12 or anti-CXCR4 antibodies, there was no change in the size of the primary tumor nor was there any evidence for a decline in primary tumor-associated angiogenesis (66); however, there was a marked attenuation of tumor metastases in an organ-specific manner (66, 72). These studies support the notion that CXCL12/CXCR4 biology mediates metastases of the tumor cells in an angiogenesis-independent manner.

An explanation for the disparity of the tumor studies *in vivo* from *in vitro* studies of CXCL12/CXCR4-mediated angiogenesis is that tumor cells expressing CXCR4 are themselves able to “out compete” endothelial cells for CXCL12 if present. In support of this contention, classical angiogenic factors are elevated in human tumors, whereas CXCL12 is not (57, 64, 66, 71, 73, 74). Moreover, the depletion of classical angiogenic factors *in vivo* results in a net reduction of angiogenesis, and a consequent reduction in primary tumor size and metastatic potential (57, 64, 73, 74). These findings suggest a dichotomy in the function for CXCL12 versus classical angiogenic factors, such that angiogenic factors promote metastasis through their effect in mediating angiogenesis, whereas CXCL12 promotes metastasis in an angiogenesis-independent manner via CXCR4-dependent tumor cell migration.

4. POSSIBLE NON-RECEPTOR-MEDIATED INHIBITION OF ANGIOGENESIS

Platelet factor-4 (PF-4)/CXCL4 was the first chemokine described to inhibit neovascularization (75). Although this angiostatic chemokine was the subject of extensive research as a candidate anti-cancer drug (76), its non-allelic gene variant

PF-4_{alt}/PF-4_{var1}/SCYB4V1* has not been previously investigated (77,78). The product of the non-allelic variant gene of CXCL4, PF-4var1/PF-4alt, designated CXCL4L1, was recently isolated from thrombin-stimulated human platelets and purified to homogeneity (79). Although secreted CXCL4 and CXCL4L1 differ in only three amino acid residues, CXCL4L1 is more potent for inhibiting angiogenesis in response to angiogenic factors in both *in vitro* and *in vivo* models of angiogenesis (79).

The molecular mechanism for CXCL4 angiostatic function is still a matter of debate. Petersen et al. suggested that CXCL4 is a unique chemokine that does not bind to a G protein-coupled receptor (GPCR); however, it activates cells (i.e., neutrophils) through binding to cell-surface GAGs (80). However, it is not clear whether CXCL4 binding to GAG sites alone is both necessary and sufficient to trigger endothelial cell signaling. For instance, CXCL4 is reported to prevent activation of the extracellular signal-regulated kinase by bFGF and to inhibit downregulation of the cyclin-dependent kinase inhibitor p21 (81,82). Furthermore, CXCL4 function is not abrogated in heparan sulfate-deficient cells, and CXCL4 mutants or peptides lacking heparin affinity are capable of inhibiting angiogenesis (76,83). Recently, Lasagni et al. (3) has identified a splice variant of CXCR3, designated CXCR3B, and has found that this GPCR binds CXCL4 and mediates its angiostatic activity. Finally, other studies have reported that the inhibitory effect of CXCL4 is mediated through complex formation with bFGF or CXCL8 (83,84). These findings suggest that the mechanisms involved in CXCL4L1-mediated attenuation of angiogenesis are complex. Furthermore, the important discovery of a variant of CXCL4 that is more efficacious for inhibiting angiogenesis than authentic CXCL4 has significant implications for the use of this angiostatic factor as a therapeutic tool to inhibit aberrant angiogenesis in a variety of diseases.

Breast- and kidney-expressed chemokine (BRAK)/CXCL14 is another non-ELR⁺ CXC chemokine, which has been recently identified to inhibit angiogenesis (85). CXCL14 was first identified by differential display of normal oral epithelial cells and head and neck squamous cell carcinoma (86). CXCL14 was downregulated in tumor specimens, as compared with normal adjacent tissue (86). The biological significance of the absence of CXCL14 in these tumors remained to be elucidated until Shellenberger and associates (85) discovered that CXCL14 inhibited microvascular endothelial cell chemotaxis *in vitro* in response to CXCL8, bFGF, and VEGF and inhibited neovascularization *in vivo* in response to the same angiogenic agonists. Schwarze and associates (87) have found that CXCL14 expression is observed in normal and tumor prostate epithelium and focally in stromal cells adjacent to prostate cancer. Interestingly, CXCL14 was found to be significantly upregulated in localized prostate cancer and positively correlated with Gleason score (87). In contrast, CXCL14 levels were unchanged in benign prostate hypertrophy (BPH) specimens (87). Using a model of human prostate cancer in immunodeficient mice, prostate cancer cells transfected with CXCL14 were found to have a 43% reduction tumor growth, as compared with controls (87). The above studies support the notion that the loss or inadequate expression of CXCL14 is associated with the transformation of normal epithelial cells to cancer and the promotion of a pro-angiogenic microenvironment suitable for tumor growth. The receptor that mediates the actions of CXCL14 remains to be determined.

*Accession numbers P10720 and M26167 at Swiss-Prot and Genbank databases, for the CXCL4L1 protein and gene, respectively.

5. CONCLUSION

Although CXC chemokine biology was originally felt to be restricted to recruitment of subpopulations of leukocytes, it has become increasingly clear that these cytokines can display pleiotropic effects in mediating biology that goes beyond their originally described function. CXC chemokines are a unique cytokine family that exhibit on the basis of structure/function and receptor binding/activation either angiogenic or angiostatic biological activity in the regulation of angiogenesis. CXC chemokines appear to be important in the regulation of angiogenesis associated with tumorigenesis relevant to cancer. These findings support the notion that therapy directed at either inhibition of angiogenic or augmentation of angiostatic CXC chemokines may be a novel approach in the treatment of a variety of cancers. Similarly, the different profiles of chemokines produced by different tumors may aid in targeted therapies toward specific tumors.

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10

Angiopoietin/Tie2 Signaling Regulates Tumor Angiogenesis

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SUMMARY

Tie2 was identified as a receptor tyrosine kinase (RTK) expressed principally on vascular endothelium. Disrupting Tie2 function in mice resulted in embryonic lethality with defects in embryonic vasculature. Multiple ligands for Tie2, named angiopoietin (Ang), have been identified. Ang1, an agonist, stimulates Tie2 phosphorylation in endothelial cells (EC). Ang2 has been considered to be an antagonist of Tie2 that blocks Tie2 activation induced by Ang1 in EC. Disrupting the function of Ang1 or overproduction of Ang2 yielded a phenotype similar to the Tie2 knockout, confirming the importance of the Ang/Tie2 pathway during embryonic vascular development. The genetic evidence suggests that the VEGF pathway and the Tie2 pathway seem to work in a complementary and coordinated fashion during vascular development. Many clinical and animal studies show critical roles of Ang/Tie2 pathway in tumor angiogenesis. The interaction of the Ang/Tie2 pathway with the VEGF pathway has also been demonstrated. Elevated VEGF and Ang2 expressions are associated with increased tumor angiogenesis. Ang2 corresponds positively with tumor development and progression as well as with metastasis and correlates negatively with patient survival in many studies. Blocking the Ang/Tie2 pathway has been shown to inhibit tumor angiogenesis, growth, and metastasis, demonstrating the potential of future clinical therapies based on this pathway. As anti-angiogenic therapies move from the bench to a clinical setting, an understanding of the role of the Ang/Tie2 pathway is vital.

Key Words: Angiopoietin; Tie; angiogenesis; VEGF; cancer; metastasis.

1. INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing vessels, plays essential roles in tumor growth, progression, and metastases; thus, focusing on the tumor endothelium rather than the genetically unstable tumor cells themselves remains the subjects of intense investigation. A significant clinical milestone was reported in

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2003 at American Society of Clinical Oncology (ASCO) annual meeting and published in 2004 (1). This led to the approval of the first anti-angiogenic agent, bevacizumab (a humanized anti-VEGF-A antibody), for metastatic colorectal cancer treatment in combination with chemotherapy. This outcome further validated the importance of anti-angiogenic therapy for cancer treatment. Here, we focus on the role of angiopoietin (Ang)/Tie2 in tumor angiogenesis and tumor progression.

2. TIE2 AND ANGIOPOIETINS

Of the molecular mechanisms identified to date, activation of endothelial RTKs by polypeptide growth factors appears to play the most pivotal role in blood vessel growth and differentiation. Importantly, RTKs for two families of angiogenic growth factors, the vascular endothelial growth factor receptor (VEGFR) and the Tie receptor, are expressed predominantly on vascular EC, making them attractive targets for anti-angiogenic therapy.

Tie receptors, including Tie1 and Tie2, were originally described as members of an orphan RTK subfamily expressed predominantly in the embryonic endothelium (2–8). Tie1, the original member of the subfamily, and Tie2 share an identical domain structure with an unusual N-terminal ligand-binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain (Fig. 1). Studies indicate that the N-terminal two immunoglobulin-like domains of Tie2 harbor the Ang-binding site. It was shown that the extracellular domain of Tie receptors consists of a globular head domain and a short rod-like stalk that probably forms a spacer between the cell surface and the Ang-binding site. Mutational analysis demonstrated that the head domain consists of the three immunoglobulin-like domains and the three epidermal growth factor-like modules and that the stalk is formed by the three fibronectin type III repeats (9). Tie2 was found to be highly conserved across vertebrate species, predicting the importance of its biological function. In fact, the domain structure

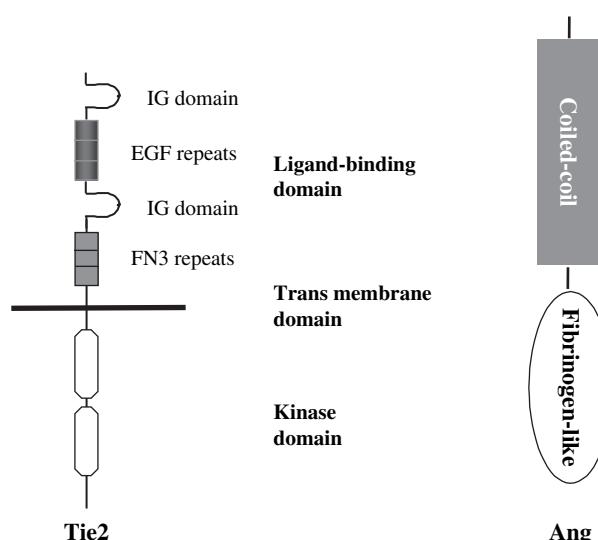


Fig. 1. Schematic diagram of Tie2 and angiopoietin.

of Tie2 is highly conserved from zebrafish to human, with the greatest amino acid homology occurring in the kinase domain (10). Disruption of the function of Tie2 in transgenic mice results in early embryonic lethality secondary to vascular abnormalities (11, 12). Tie2^{-/-} embryos show a decreased number of EC and decreased contact between EC and the underlying perivascular cells [pericytes and smooth muscle cells (SMCs)], suggesting a role in the maturation and stabilization of the embryonic vasculature.

Tie2 was no longer an orphan receptor with the discovery of the Angs, Ang1 and Ang2 (13). Structurally, Ang mainly contains two domains, coiled-coil domain involved in ligand oligomerization and fibrinogen-like domain involved in receptor binding (Fig. 1). As is the case with other RTKs, Ang1 binding stimulated autophosphorylation of the kinase domain of Tie2. However, unlike activation of most growth factor RTKs, Ang1 activation of Tie2 did not stimulate mitogenesis, suggesting a novel role in endothelial biology. Ang1 stimulates endothelial migration, whereas Ang2, in most cases, blocks actions of Ang1 in EC (14–16). Consistent with this finding, mice lacking functional Ang1 expression and mice overexpressing Ang2 both displayed a phenotype similar to Tie2^{-/-} mice (14, 17). Recently, we demonstrated that Ang1 regulates SMC recruitment through induction of hepatocyte growth factor (HGF) expression in EC and that Ang2 inhibits Ang1-induced HGF expression leading to decreased SMC recruitment (18). These findings establish an intriguing and delicate regulation mechanism of Angs in SMC recruitment during vascular formation and remodeling (Fig. 2).

Biochemical studies on Ang2 have yielded controversial results (15, 19–21). Ang2 blocks Ang1-induced Tie2 activation in EC but induces Tie2 phosphorylation when Tie2 is genetically introduced into NIH3T3 fibroblast cells (14). Studies show that high levels of Ang2 stimulation activates Tie2 in vascular EC (20) and induces vascular tube formation (22), revealing the complexity of Ang2 in angiogenesis. Recently, we

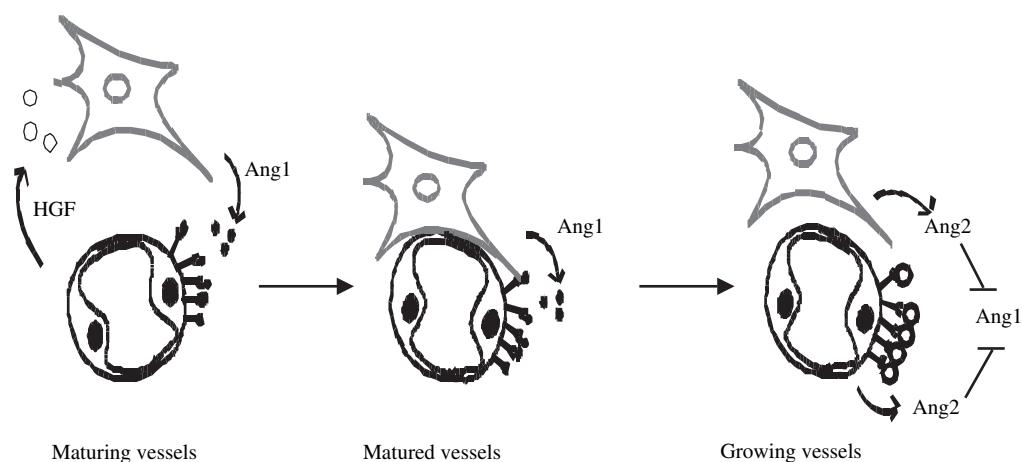


Fig. 2. Potential mechanisms of angiopoietin (Ang)-mediated vascular maturation. Ang1 induces hepatocyte growth factor (HGF) production in endothelial cells (EC) that recruit smooth muscle cells (SMCs) to form stable vessels. Ang2 antagonizes Ang1 function and causes dissociation of SMCs from EC during angiogenesis.

observed that long-term stimulation of EC with Ang2 induced a delayed activation of Akt and protected EC from TNF α -induced cell death (23).

In addition to the blood vascular network, the lymphatic system plays a vital role in maintaining homeostasis, returning interstitial fluid escaping from tissue capillary beds to the circulatory system. The lymphatic system is a distinctive vasculature similar to the blood vasculature. In tumors, the lymphatics have received a great amount of attention because of its association with metastasis. Although the mechanisms controlling angiogenesis are relatively well characterized, the molecular mechanisms regulating lymphangiogenesis are only starting to be elucidated. The involvement of Ang/Tie2 signaling in lymphangiogenesis was first noticed in the Ang2-knockout mice. Unexpectedly, mice lacking Ang2 exhibit major lymphatic vessel defects. Genetic rescue with Ang1 corrects the lymphatic, but not the angiogenesis defects, suggesting that Ang2 acts as a Tie2 agonist in the former setting, but as an antagonist in the latter setting (19). This observation was confirmed by various *in vitro* and *in vivo* studies. Ang1 enhanced lymphatic EC colony formation *in vitro* (24), lymphatic EC proliferation, and lymphangiogenesis *in vivo* (24, 25).

While Ang1 is mainly expressed by vascular accessory cells, such as pericytes and SMCs, Ang2 is predominantly expressed in EC. Ang2 is stored in the Weible–Palade body of these cells mutually exclusive of P-selectin (26). Stored Ang-2 has a long half-life and can be secreted within minutes of stimulation [e.g., by phorbol 12-myristate-13-acetate (PMA), thrombin, and histamine], suggesting functions of the Ang/Tie2 system beyond the established roles during angiogenesis. Ang2 is likely to be involved in rapid vascular homeostatic reactions such as inflammation and coagulation (26). Indeed, a study found that mice deficient in Ang2 cannot elicit an inflammatory response, and recombinant Ang2 restores the inflammation defect in the null mice. Cellular experiments showed that Ang2 enhances TNF- α -induced vascular inflammation, indicating that Ang2 acts as a switch of vascular responsiveness, exerting a permissive role for the activities of proinflammatory cytokines (27). An alternatively spliced Ang2, Ang2₄₄₃, named so because it contains 443 amino acids, has a yet-to-be-identified function. While Ang2₄₄₃ binds to Tie2 receptors, it does not induce its phosphorylation (28).

Two more Tie2 ligands have also been identified, mouse Ang3 and human Ang4 (29). Ang4 has been shown to increase EC migration and tube formation like Ang1 (30). On the other hand, Ang3 is believed to compete with Ang1 and act as antagonist (29). Ang3 needs to be tethered on the cell surface via heparin sulfate proteoglycans (HSPG) in order to be functional (31), and HSPG protects Ang3 from proteolytic cleavage and increases the half-life of the molecule. Taken together, the Ang/Tie2 pathway is an independent pathway. It is finely regulated and plays crucial roles in the remodeling and maturation/stabilization of blood vessels, lymphangiogenesis, as well as vascular inflammation.

3. CLINICAL OBSERVATIONS

3.1. *Elevated Expression of Ang and Tie2 in Cancer Tissues*

The potential role of Tie2/Ang signaling in cancer was first reported by Peters et al. (32), in which the authors found a significant elevation of Tie2-positive vessels in human breast tumor tissues compared to benign or normal breast tissues (Fig. 3).

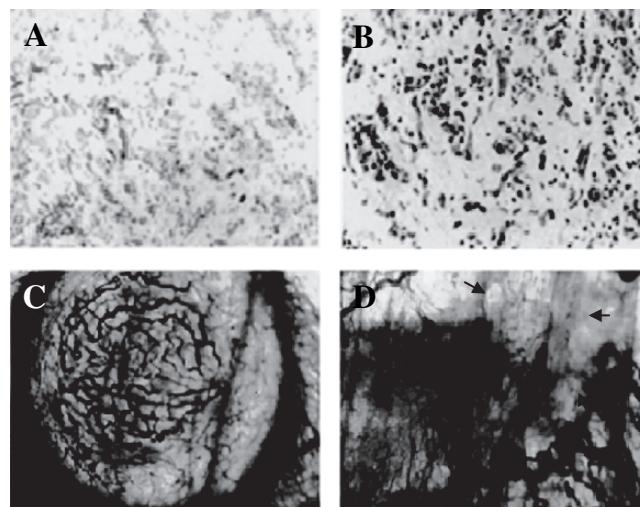


Fig. 3. Tie2 is elevated in human breast cancer tissues, and Tie2 signaling plays a role in tumor angiogenesis. Tie2 expression was examined in human breast tumor samples (B) and normal breast tissues (A) by immunohistochemistry using a specific antibody against Tie2. A significant elevation of Tie2 expression was observed in tumor samples compared to normal tissues. Blocking Tie2 activity using a soluble Tie2 receptor, ExTek, significantly inhibited mammary tumor angiogenesis and tumor growth (D) compared to control-treated tumors (C) using a vascular window model. (Please see color insert.)

Moreover, Tie2 expression was concentrated in “vascular hot spots” at the leading edge of invasive tumors. Since then, many clinical data confirmed a role of Ang and Tie2 in various types of cancers (Table 1). Studies have shown that Ang2 levels are elevated in cancer tissues compared to normal or adjacent noncancerous tissues, which include stomach (33–35), breast (36, 37), colon (38, 39), liver (40–42), kidney (43), pancreas (44), brain (45, 46), lung (47), and leukemia (48). Ang2 expression correlates with malignancy of human brain cancer (49). Ang2 was suggested as an early marker of tumor-induced neovascularization of glioma (50). The same was suggested in skin cancer using human squamous cell carcinoma (SCC) xenografts. In that study, Ang2 was induced in angiogenic tumor vessels in early stages of carcinogenesis (51).

Together with upregulation of Ang2, elevated expression of Tie2 and/or Ang1 was also reported in colonic carcinoma (38), Kaposi’s sarcoma and cutaneous angiosarcomas (52), and hemangiomas (53). In addition, a correlation between Ang2 and Tie2 was observed in papillary thyroid carcinoma (54). Tie2 expression in hepatocellular carcinomas (HCCs) was correlated with cell dedifferentiation and tumor size (55). Tie2 was also upregulated in brain tumor blood vessels. Small capillaries with few periendothelial support cells showed strong expression of Ang2, whereas larger glioblastoma vessels with many periendothelial support cells showed little or no expression (56), which is consistent to the observation that Ang2 regulates vascular destabilization (14). In addition, elevation of Ang1 and Tie2 was also indicated in lymphangiogenesis in inflammatory breast cancer (57).

Although large bodies of data reveal an elevation of Ang/Tie2 in cancers, opposite findings were also reported. No differences in Ang2 and Tie2 expression level between cancerous tissues and normal tissues was found in ovarian cancer (58). Whereas, a

Table 1
Differential Expression of Angiopoietins and Ties in Clinical Samples

| <i>Site of tumor</i> | <i>Ang1</i> | <i>Ang2</i> | <i>Ang4</i> | <i>Tie1</i> | <i>Tie2</i> | <i>Reference</i> |
|---------------------------|-------------|-------------|-------------|-------------|-------------|----------------------|
| Stomach | Up | Up | | | Up | (33–35) |
| Breast | Up | Up | | | Up | (36, 37) |
| Breast | Down | Down | Down | | Down | (59) |
| Prostate | | | | | Up | (36) |
| Colon | Up | Up | Up | Up | Up | (38,39) |
| Liver | Up | Up | | | Up | (40–42) |
| Kidney | | Up | | | Down | (43) |
| Pancreas | | Up | | | | (44) |
| Brain | Up | Up | | | Up | (45, 46, 49, 50, 56) |
| Lung | Up | Up | | | Up | (47, 61) |
| Lung | Down | | | | Down | (60) |
| Leukemia | Up | Up | | | Up | (48, 62) |
| KS, angiosarcoma | | Up | | Up | Up | (52) |
| EC (infantile hemangioma) | | Up | | Up | Up | (53) |
| Ovary | | Down | | | | (58) |

significant reduction of Ang1, Ang2, Ang4, and Tie2 mRNA was found in some breast cancer tissues compared to normal tissues (59). These conflicting data indicate a complex and dynamic nature of Ang/Tie2 signaling in tumor angiogenesis. This discrepancy may arise from different tumor types and tumors at different progression stages.

Clinical data regarding elevated Ang2 and Tie2 expression in cancer appear to be more consistent; however, Ang1 expression in cancer is more confusing. Conflicting results were reported in same type of cancers as well as different type of cancers. Reduced Ang1 expression in cancer tissues was found in NSCLC (60) and in ovarian cancer tissues (58). In an HCC study, Ang1 level is lower in well-differentiated HCC compared to normal liver tissue; however, it is elevated in moderately and poorly differentiated HCC (40). However, in another NSCLC study, Ang1, Tie2, and VEGF mRNAs were found to be higher in cancer tissues than in adjacent noncancerous tissues with a positive correlation between Ang1 and Tie2 (61). The levels of Ang1, Ang2, and phosphorylated Tie2 were higher in glioblastoma multiforme than normal brain or low-grade astrocytoma specimens (45). In acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) patients, Ang1 and soluble Tie2 (sTie2) were significantly elevated compared to those of healthy subjects (62). Since these angiogenic factors possess multiple functions in vessel formation, stabilization, and maintenance, its expression varies with angiogenic status and tumor types. Future clinical interventions should consider these variations.

In addition to the tumor samples, abnormal levels of Angs and sTie2 were also reported in cancer patient blood samples (36). In breast cancer and prostate cancer, serum sTie2 and VEGF were elevated in cancer patients compared to healthy controls, though Ang1 and Ang2 were elevated only in the breast cancer patients. Interestingly, one study with renal cancer patients found that an increase of sTie2 following an anti-angiogenic treatment

with razoxane correlated with progressive disease and a decrease of sTie2 correlated with stable disease and improved survival. Assessment of efficacy of anti-angiogenic drugs is hampered by the lack of good endpoints. This finding raises the possibility of using serum levels of these angiogenic factors as surrogate markers for cancer therapy (63).

3.2. Correlation of Angs and VEGF in Cancer

Angiogenesis is a multistep process that requires sequential activation of different growth factor receptors. Genetic evidence suggests that Ang and VEGF collaboratively and coordinately regulate vascular formation and maturation (64–66). Our recent data illustrated a delicate “Yin-Yang” regulation mechanism of Ang1 and Ang2 in regulating vascular maturation through induction of HGF (18). It has been suggested that Ang2 might exert different effects in angiogenesis depending on the status of VEGF, vessel destabilization and angiogenic response in the presence of VEGF, and vessel regression in the absence of VEGF (14). It is not surprising that a correlation of Ang and VEGF expression was reported in a variety of human cancer samples (Table 2). Ang2 expression was correlated with VEGF expression in invasive ductal breast carcinoma (67), colon cancer (39), papillary thyroid carcinoma (54), brain cancer (68), and ovarian cancer (69). Elevated VEGF and Ang2 expression often correlates with an increased microvascular density (MVD). In NSCLC, patients with Ang2(+)/VEGF(high) had higher intratumoral MVD than those with Ang2(+)/VEGF(low), Ang2(−)/VEGF(high), or Ang2(low)/VEGF(−) (70). In invasive ductal breast carcinoma, Ang2 and VEGF expression was correlated with MVD in tumor tissues (67). Although the VEGF level was not examined, a positive correlation between Ang2 and MVD was also observed in HCC (41,71,72), in lung cancer (47), in prostate cancer (73), and in gastric cancer (34).

Besides tumor tissues, the plasma levels of Ang1, Ang2, and sTie2 were also correlated with the level of VEGF in breast cancer patients (36), and Ang2 and Tie2 were correlated with VEGF in prostate cancer patients (36). Further analysis identified a direct link between Ang2 and VEGF. Studies also found that VEGF and VEGF-overexpressing tumors upregulate Ang2 expression in tumor endothelium and in ovarian cancer cells (69). Since high expression of Ang2 accompanied with elevated expression of VEGF was often observed in clinical tumor samples and these two mediators work together in angiogenesis, future studies should consider a combination approach to examine and target both VEGF and Ang signaling. This combination approach may provide more effective regime for cancer diagnosis and treatment than focusing on VEGF alone.

3.3. Ang/Tie2 Expression with Tumor Progression and Patient Survival

Tumor angiogenesis is essential for tumor growth and progression. Thus, angiogenic factor expression often correlates with tumor development (Table 2). A large body of published data have shown a positive correlation between Ang2 levels and tumor progression. For example, higher Ang2 expression was seen in more advanced stages of gastric cancer (33,34), colon cancer (74), and neuroblastoma (75). Besides the elevated Ang2, Tie2 level increases with HCC dedifferentiation (40). In addition, Tie1, Tie2, and Ang4 expression was correlated with Duke’s classification in colon cancer (38).

Since tumors disseminate through the vascular systems to distant organs, the expression of angiogenic factors such as Ang and Tie2 is associated with tumor invasion

Table 2
Correlation Between Angiopoietins and Ties with Clinical Pathological Factors

| Site of tumor | VEGF | Microvascular Survival rate | Stage | Metastasis | Invasion | Tumor size | Other | Reference |
|-------------------|---|-----------------------------------|-----------------------------------|---|-----------------------|--|------------------------------------|----------------------|
| Stomach | | Ang2 (-) | Ang2 (+) | Ang2 (+) | Ang1 (+), Ang2 (+) | | | (33-36, 76) |
| Breast | Ang1(+), Ang2(+), Tie2 (+), Ang2 (+) | Ang2 (+) | Ang2 (-) | Ang2 (+) | | Tie2 with CD31 (+) | | (32, 36, 67, 77) |
| Colon | | VEGF & Ang2 (+) | | Tie1 (+), Tie2 (+), Ang2 (+), Ang4 (+) | Ang2 (+) | Tie1 (+), Tie2 (+), Ang1 (+), Ang2 (+), Ang4 (+) | Ang2 (+) | (38, 39, 74) |
| Prostate Liver | Ang2 (+), Tie2 (+) Ang2 (+) | Ang2 (+) Ang2 (+), Tie2 (+) | Ang2 (-) Ang2 (-) | Ang2 (+) | Ang2 (+) | | | (36, 73) |
| Ovary | Ang2 (+) | | Ang1/ Tie2 (+) | Ang1/ Ang2 (+) | Ang2 (+) | | | (40, 41, 55, 71, 72) |
| Lung | | | High Ang2 and high VEGF (+) | Ang2 (+) | Ang2 (+) | Tie2 (+) | Dedifferentiation with Tie2 (+) | (69, 78) |
| Lung | | Ang2 (+) | Ang2 (+) | Ang2 (+) | Ang2 (+) | Tie2 (+) | | (70) |
| Brain Thyroid | | Ang2 (+) | | Ang2 (+) | Ang2 (+) | | Tie2 with Ang1 (+) | (47, 61) |
| Leukemia | | | | High Ang2/ low VEGF-C (+) | | | Tie2 with Ang2 (+) | (68, 75) (54) |
| Kidney | | | | | | | Tie2 with Ang2 (+) | 48 |
| | | | | | | | | 43 |

(+): positive correlation and (-): negative correlation.

and metastasis. For example, expression of Ang2 and/or Ang1 is correlated with tumor venous invasion in gastric adenocarcinoma (76) and portal invasion in HCC (71). A high Ang2/Ang1 mRNA ratio was closely associated with tumor portal vein invasion in HCC (41). The expression level of Tie1, Tie2, and Ang1 is positively correlated with lymphatic invasion in colon cancer (38).

Ang2 expression was also correlated with lymph node metastasis as well as with distant metastasis in colon cancer (39) and gastric cancer (33). In the gastric cancer study, the Ang2 isoform, Ang2₄₄₃, was elevated as well, though its function is still unclear (33). Increased levels of Ang2 in metastatic lymph nodes compared to non-metastatic lymph nodes were observed in gastric cancer patients (35).

Consistent with the positive correlation of Ang2 expression and tumor metastasis, a negative correlation between the level of Ang2 and overall survival was observed in various cancers, which include gastric cancer (34, 35), lung cancer (47, 70), and breast cancer (77). Patients with a strongly positive VEGF and Ang2 had worse disease-free survival in ductal breast carcinoma (67). In ovarian cancer, the Ang1/Ang2 ratio was positively correlated with survival (78). Collectively, these data suggest that levels of these angiogenic factors could be further developed for cancer diagnosis and prognosis.

An opposite observation was made in AML patients. Among the angiogenic factors tested (Tie2, VEGF-A, VEGF-C, Ang1, and Ang2), only Ang2 was found to be an independent prognostic factor in AML. Furthermore, patients with a high level of Ang2 and with a low level of VEGF-C expression had a significantly higher survival rate (48). In this situation, treatment of AML with recombinant Ang2 and VEGF inhibitors may lead to a better clinical response (48).

4. PRECLINICAL STUDIES OF ANG/TIE2 SIGNALING IN CANCER PROGRESSION

4.1. Tie2

A direct role of the Tie2 pathway in tumor angiogenesis *in vivo* was tested by using a soluble Tie2 receptor (ExTek) that neutralizes endogenous cell-surface Tie2 activity. Blocking Tie2 by ExTek significantly inhibited tumor angiogenesis and tumor growth in a tumor vascular window model (79) (Fig. 3). Systemic delivery of ExTek using an adenoviral vector inhibited the growth of both well-established primary tumors and tumor metastases (80). Interestingly, ExTek also inhibited corneal angiogenesis induced by tumor-conditioned medium in which VEGF is also present. Blocking either the Tie2 or VEGF pathway for the mammary tumor significantly inhibited tumor angiogenesis and tumor growth (79–81). These results suggest that Tie2 and VEGF are two independent mechanisms essential for mammary tumor angiogenesis. This notion was confirmed by another study (82), in which A375v human melanoma cells, which express both VEGF and Ang1, were stably transfected to overexpress the extracellular ligand-binding domains of VEGFR and Tie2, respectively. Nude mouse xenografts revealed that interference with either the VEGF receptor pathway or the Tie2 pathway resulted in a significant inhibition of tumor growth and tumor angiogenesis. The inhibition of the VEGF receptor pathway cannot be compensated by the Tie2 pathway, or vice versa.

In brain tumors, Tie2 receptor levels and receptor activity are positively correlated to malignancy of astrocytoma in patients and explants of human astrocytoma growing in

mice. As expected, inhibition of Tie2 activation using ExTek protein attenuated tumor growth in both subcutaneous xenografts and orthotopic intracranial tumor models (83). Similar findings were also reported in HCC (55). These data further confirmed a role of Tie2 activity in tumor angiogenesis and tumor progression.

Angiogenic factor expression profiling in human primary breast cancer samples and murine breast cancer cell lines grown in nude mice shows that human tumors expressed VEGFR2 and Tie2 but varied considerably in VEGF, Ang1, and Ang2 expression. Similar heterogeneity of angiogenic profiles were also observed in the murine tumor model (84). Functional testing using these tumor lines, which differs in their angiogenic expression profile, showed that tumors that were positive for VEGFR2 responded well to anti-VEGF treatment. Tumors that are positive for both VEGFR and Tie2 responded well to either inhibitor *in vivo* (84). These findings indicate that for adequate application of angiogenic inhibitors in cancer patients, analysis of prevailing angiogenesis pathways may be a prerequisite. It also suggests that a significant additive effect of VEGF and Tie2 pathway inhibitors may exist in some type of tumors, but not in others.

4.2. *Ang1*

Ang1 was shown to be anti-angiogenic and inhibit tumor growth in several animal studies. Overexpression of Ang1 in tumor cells caused growth retardation of breast tumor and skin cancer in mice (51, 85), as well as reduction of metastatic growth in colorectal cancer cells (86, 87). Histological analysis of these tumor samples suggested that elevated Ang1 resulted in reduced vessel counts and reduced tumor cell proliferation.

Ang1 seems to play a pro-angiogenic role, however, in tumors of the brain. Ang1 was shown to be elevated in transgenic astrocytoma mouse model (45), and in human glioma samples (45, 46). Ang1 secreted by these brain tumor cells enhanced angiogenesis *in vitro* (46). One of the hallmarks of astrocytomas is the formation of glomeruloid bodies which is characterized by microvascular proliferation and piling of EC around the vessel lumen. A role of Ang1 in the formation of glomeruloid bodies was explored using Tet-Off Ang1-inducible tumor cell lines. Induction of Ang1 expression in glioma U87-MG cells led to faster tumor growth and poorer survival compared to control cells in mice. Moreover, those tumor vessels had a highly serpentine structure and piling of EC similar to glomeruloid bodies. Additionally, reducing Ang1 expression using doxycycline or neutralizing Ang1 function using ExTek impaired the formation of these abnormal vascular structures (88). This finding suggests that elevated Ang1 in brain tumors may contribute to the formation of glomeruloid bodies and brain tumor progression.

Currently, it is unclear from where this discrepancy of Ang1 in tumor progression and inhibition arise. Besides its angiogenic function, Ang1 is also implicated in endothelial progenitor cell differentiation and immune suppression. Ang1 derived from Lewis lung carcinoma (LLC)-conditioned media increased the expression of EC marker expression on cell surface, increased the differentiation of CD34⁺ progenitor cells and recruitment of these cells to tumor sites (89). Conversely, blocking Tie2 function using soluble Tie2 inhibited cell differentiation and tumor recruitment (90). Furthermore, LLC-conditioned media skewed the differentiation of CD34⁺ cells toward EC from dendritic cells and concomitantly reduced the production of IL-12 (90). The reduction of mature dendritic cell number could result in immune suppression as seen in many cancer patients.

4.3. *Ang2*

In contrast to Ang1, Ang2 has been shown consistently in tumor promotion by different groups using different tumor models. Overexpression of Ang2 in gastric cancer cells, colon cancer cells, and HCC led to an increased tumor growth and tumor metastasis with increased tumor vascular density and proliferative indexes (34, 91, 92), and dramatically shortened the survival of HCC tumor-bearing animals (93). In brain tumors, Ang2 expression correlates with the expression of matrix metalloprotease-2 (MMP-2) and tumor invasion. Consistent with these features, overexpression of Ang2 in brain tumor cells led to an increase of MMP2 production, tumor angiogenesis, and increased tumor invasiveness. Conversely, MMP inhibitors suppressed Ang2-stimulated activation of MMP-2 and Ang2-induced cell invasion. These results suggest that Ang2 plays a critical role in inducing tumor cell infiltration and that this invasive phenotype is caused by activation of MMP-2 (94).

Consistent with these data, blocking Ang2 activity using inhibitors, peptide-Fc fusion proteins or an antibody that block the interaction of Ang2 and Tie2, resulted in tumor stasis, followed by elimination of all measurable tumors in a subset of animals. These effects were accompanied by reduced EC proliferation, consistent with an anti-angiogenic therapeutic mechanism. Anti-Ang2 therapy also prevented VEGF-stimulated neovascularization in a rat corneal model of angiogenesis (95). This finding implies that anti-Ang2 could be an effective method for cancer therapy. In agreement with the tumor-promoting role of Ang2, treatments with chemotherapeutic agent and anti-angiogenic agent decreased Ang2 levels in tumor samples. It has been shown that cannabinoid treatment resulted in a reduction of Ang2 expression and impairment of angiogenesis as well as an increased apoptosis in non-melanoma skin cancer (96). Endostatin, an anti-angiogenic agent, inhibited tumor growth in the C3(1)/Tag mammary cancer transgenic mice, and this was accompanied with the decrease in Ang2 and Tie1 expression (97).

Despite mounting evidence of Ang2 in tumor progression, there is at least one study that reports anti-tumor effects of Ang2. Ang2 overexpression in LLC and TA3 mammary carcinoma cells inhibited their ability to form metastatic tumors and prolonged the survival (98).

4.4. *Ang3 and Ang4*

Ang3 is believed to compete with Ang1 and acts as a Tie2 antagonist (29). However, different from Ang2, another antagonist of Tie2, overexpression of Ang3 in tumor cells inhibited metastasis of LLC and TA3 mammary carcinoma. The inhibition of metastasis and tumor growth was accompanied with the inhibition of tumor angiogenesis and the promotion of apoptosis of the tumor cells. Ang3 also inhibits EC proliferation and survival and blocks Ang1- and VEGF-induced Akt and Erk1/2 activation (99).

Ang4 is thought to be an agonist ligand for Tie2. Similar to Ang1, Ang4 increases EC migration and tube formation (30). Ang4 expression can be induced by hypoxia and overexpression of HIF-1 α in normal kidney proximal tubule epithelial cells (RPTE). Ang4 is also induced in von Hippel-Lindau (VHL)-deficient cells. Under normoxic conditions, Ang4 is higher in renal clear cell carcinoma than RPTE. Stable transfection of VHL in cancer cells suppressed VEGF and Ang4 and restored the hypoxic responses (100), whereas Ang2 expression was downregulated by hypoxia in VHL wildtype

RCC786-0 and VHL-transfected RCC4 (43). The study regarding Ang3 and Ang4 is very limited, and it is an area expected to expand in the future.

5. THERAPEUTIC MODULATION OF TIE2 ACTIVATION IN CANCER PATIENTS

Published data implicate a role of Ang/Tie2 signaling in tumor angiogenesis and suggest that therapeutic modulation of Tie2 activation may be beneficial. Based on a broad range of data, it appears that therapeutic manipulation of angiogenesis will likely be an important clinical application of Tie2 biology. Most of the data today suggest that activation of Ang/Tie2 signaling plays an important role in blood vessel assembly and that inhibiting the Tie2 pathway will inhibit pathological angiogenesis. Although the therapeutic utility of soluble receptors has been shown for other ligand/receptor systems, further proof of concept of Tie2 inhibition for anti-angiogenesis would be greatly facilitated by the advent of potent and selective small molecule inhibitors of the Tie2 and its ligands. Small targeting ligand for Tie2 such as recently developed GA3 peptide (101) and anti-Ang2-specific neutralizing antibody (95) could greatly facilitate our understanding of Tie2 biology as well as therapeutic application in cancer treatment.

Although a tempting therapeutic target, modulation of Tie2 signaling in the adult vasculature should be approached with caution. Published results indicate a role of Tie2 in maintenance of the adult vasculature, perhaps mediating endothelial survival. Our studies with ExTek, however, suggest that at least short-term inhibition of Tie2 signaling will be well tolerated. In addition, chronic expression of Ang1 and Ang2 in murine skin enhanced dermal vascularity without apparent adverse effects. Studies in animal models of ischemia indicate that short-term enhancement of Tie2 signaling is sufficient to improve collateral circulation without evidence of untoward effects. Further development of potent and selective Tie2 inhibitors will be necessary to determine the effects of chronic blockade of the Tie2 pathway in adult vasculature. Taken together, these data strongly suggest that although caution is warranted, therapeutic modulation of the Tie2 pathway to inhibit or enhance blood vessel growth and remodeling will have a favorable therapeutic index. An understanding of Tie2 biology and the mechanism underlying the differential effects of Ang1 and Ang2 on Tie2 activation will enhance our ability to develop more specific and effective inhibitor for cancer therapy.

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11

Imaging Angiogenesis

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SUMMARY

Imaging of angiogenesis is clinically relevant for tissue characterization, early detection of tumor and assessing tumor response to targeted therapies. Current imaging techniques with CT and MRI provide opportunity to assess tumor blood flow, blood volume and permeability. The kinetic models employed for estimation of these parameters are not yet standardized, however, the methods were found to be reliable and reproducible. Imaging techniques such as contrast-enhanced ultrasound, PET and optical imaging are still under research. This chapter provides a review of various methods for imaging the angiogenesis, their advantages, disadvantages and potential role in the current practice.

Key Words: Perfusion CT; Perfusion MRI; Contrast-enhanced Ultrasound; Angiogenesis;

1. INTRODUCTION

Angiogenesis plays an important role in pathophysiology of tumors, chronic inflammatory diseases, macular degeneration, and reversal of ischemic heart and limb disease (1). In a clinical management perspective, it is worth evaluating angiogenesis in these clinical settings. Currently, imaging evaluation of tumor angiogenesis is of worldwide interest due to the emergence of anti-angiogenic drugs that specifically target angiogenesis, the crucial process in tumor growth and metastases (2, 3). Clinical response in the form of tumor size shrinkage is slow to occur with anti-angiogenic therapy (2). However, changes in hemodynamic parameters due to angiogenesis inhibition occur soon after the start of anti-angiogenic therapy. Hence evaluation of angiogenesis plays a key role in monitoring response to therapy. Furthermore, the prognostic potential of measurements of angiogenic activity holds great promise. Indeed, Karademir et al. (4) has reported that measurement of angiogenic activity in the form of vascular surface density for pancreatic carcinoma could serve as independent prognostic factor for survival.

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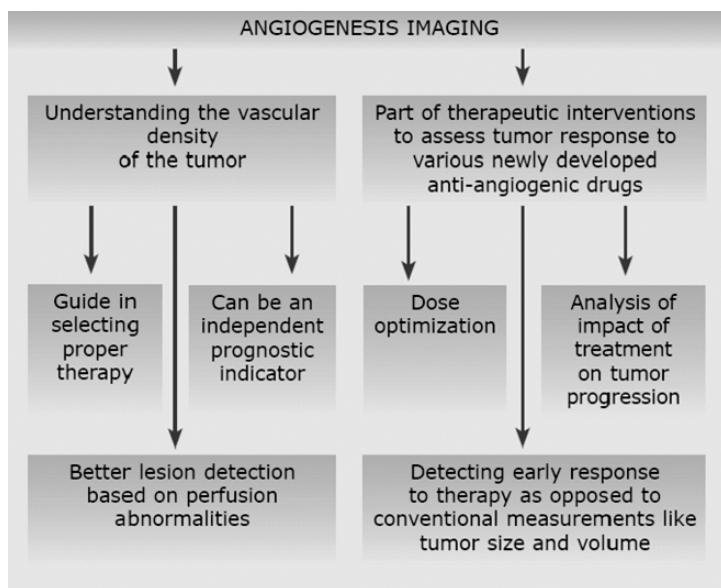


Fig. 1. Clinical significance of angiogenesis imaging.

Other potential role of imaging angiogenesis could be for tissue characterization (benign vs. malignant or low-grade vs. high-grade malignancy). Tumor tissue perfusion is generally higher in malignancy than their benign counterpart. Similar hypothesis can be applied in the high-grade and low-grade tumors. Since tissue hemodynamic alterations occur prior to the advent of morphologic changes from tumor, perfusion imaging could potentially detect occult malignancy. Figure 1 illustrates the clinical significance of imaging tumor angiogenesis.

The direct method of quantifying angiogenesis is measurement of microvessel density (MVD, maximal number of blood vessels per unit area of section), an index of minimal inter-capillary distance measured on histologic tissue sections (1). With the rapidly emerging role of anti-angiogenic therapy in oncology, monitoring response would invariably require obtaining multiple biopsies for MVD assessment. This approach is not only invasive, but its reliability is dependent on the accuracy of tissue sampling. Heterogenous distribution of angiogenic pattern within the tumor tissue (2) often introduces sampling errors.

2. ANGIOGENESIS IMAGING TECHNIQUES

Table 1 illustrates the comparison of the various imaging techniques available for imaging of angiogenesis. Imaging techniques for angiogenesis may be classified based on the imaging modality [computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound (US)] or based on the requirement to administer a suitable contrast material or tracer materials intravenously. Contrast material-enhanced studies include dynamic contrast-enhanced CT, MR, and US, and tracer-based studies include position emission tomography (PET). Molecular imaging studies utilize monoclonal antibodies as tracers, whereas the optical imaging studies utilize fluorescent dyes.

Table 1
Comparison of Techniques for Imaging Angiogenesis

| | <i>Contrast-enhanced ultrasound</i> | <i>Contrast-enhanced CT</i> | <i>Contrast-enhanced MR</i> | <i>O-15 PET</i> |
|--|-------------------------------------|-----------------------------|-----------------------------|-----------------|
| Signal in relation to enhancement of tumor | Linear | Linear | Complex | Linear |
| Temporal resolution of less than 1 s | Possible | Possible | Not possible | Possible |
| Area covered | 1 slice | Up to 4 cm | Large area | Large area |
| Type of study | First pass | First pass and permeability | First pass and permeability | First pass |
| Respiratory misregistration | Affects study | Affects study | Affects study | Affects study |
| Repeatability (more number of studies within 24 h) | Yes | No | No | Yes |

Techniques that do not involve intravenous administration of contrast material or other tracers include the blood oxygen level-dependent (BOLD) MR, arterial spin-labeled MR, Doppler US perfusion study, and absorption band optical imaging.

3. PHYSIOLOGICAL BASIS OF CONTRAST ENHANCEMENT

The physiological principle of tumor enhancement with contrast material is illustrated in Fig. 2. The basic principle of interrogation of perfusion parameters using intravenous contrast material is to measure the temporal changes in the contrast material concentration in the tumor tissue by acquiring sequential images of the tumor by CT or MR. The dynamics of tissue enhancement depend on the shape of the arterial input function at the entry of the tissue, the kinetic distribution of the blood containing the contrast material into the capillary bed, leakage of the contrast material across the capillary walls into the tumor interstitial (extravascular and extracellular) space, and the volume of the interstitial space where the contrast material can diffuse (5). The tumor tissue time-attenuation curves thus obtained from dynamic imaging studies are analyzed by applying pharmacokinetic modeling to estimate the physiological perfusion parameters. This principle can be extrapolated to dynamic CT studies using iodinated intravenous contrast material. However, using this principle for dynamic MR is more complex as the tumor tissue signal does not correlate directly with the absolute concentration of gadolinium in the tumor tissue unlike in dynamic CT studies. For example, at 120 kVp for dynamic CT studies, an attenuation increase of 25 HU is equivalent to an iodine concentration of 1 mg I/mL of tissue (6). However, unlike dynamic CT, tumor signal does not increase linearly with gadolinium concentration in dynamic MR studies.

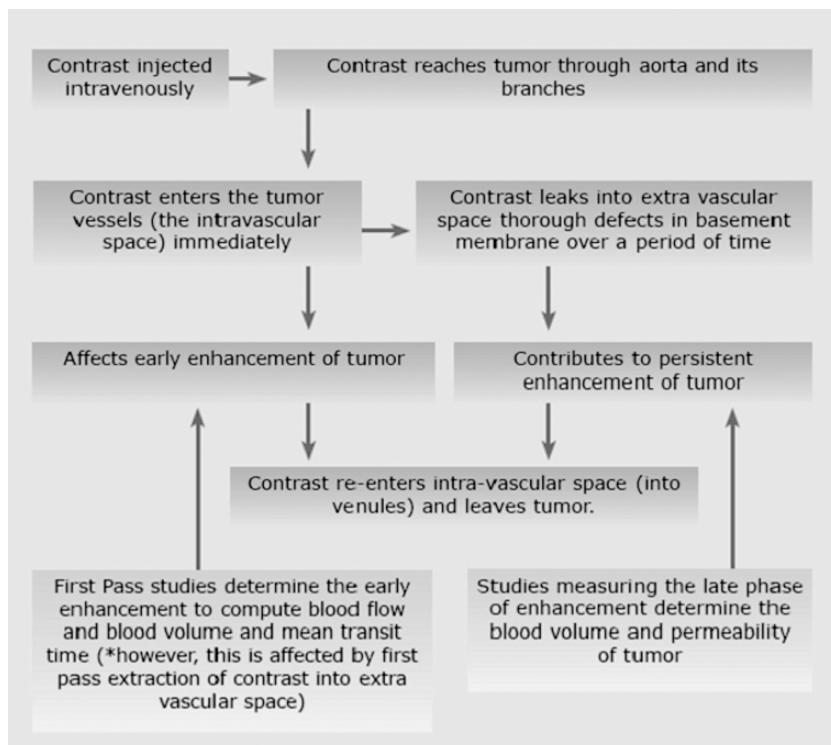


Fig. 2. Physiological basis of tumor tissue-contrast enhancement.

4. MODELS USED FOR CONTRAST-ENHANCED IMAGING

Pharmacokinetics of intravenous contrast materials is governed by their distribution in both the intravascular and extravascular spaces. There is minimal intracellular uptake of contrast material, and excretion is primarily by glomerular filtration (6). This has allowed three basic perfusion data analysis paradigms to emerge: (a) model-independent approaches such as deconvolution analysis, (b) compartmental modeling such as Patlak model, and (c) modeling that accounts for convective transport (perfusion) and diffusional exchange (capillary permeability) by means of a distributed parameter model as proposed by Johnson and Wilson (7).

Deconvolution method uses arterial and tissue time-attenuation curves to calculate the impulse residue function (IRF) for the tissue of interest. IRF is a theoretical tissue curve that would be obtained from instantaneous arterial input. IRF curve comprises of a plateau followed by a single exponential decay, wherein the height of the curve represents tissue perfusion and area under the curve gives the blood volume.

Compartment analysis may be based on single- or two-compartment models. Compartment analysis for first-pass studies is based on Fick principle, which considers the intravascular and extravascular spaces as a single compartment. Blood flow is calculated from maximal slope of the tissue concentration-time curve or from its peak height, normalized to arterial input function. Two-compartment model assumes

that back flux of contrast material from extravascular space to intravascular space is negligible for the first 1–2 min (Patlak analysis).

A distributed parameter (Johnson and Wilson) model enables permeability measurement (6). Johnson and Wilson model incorporates a concentration gradient within the intravascular space from the arterial inlet to the venous outlet, while the extravascular space is modeled as a compartment. This model allows estimation of blood flow (BF), blood volume (BV), mean transit time (MTT), and permeability surface area product (PS) from a single CT or MR study. This method is extremely versatile due to the fact it can estimate a wide range of PS values for any given BF value for a particular tumor.

Currently, a few manufacturers provide software programs to calculate the perfusion parameters. Such software programs are based on various models described above. However, it should be emphasized that perfusion values may not be reproduced across different softwares. Hence, the perfusion values measured are specific to the software used and may not be comparable to other softwares due to different mathematic models used. For example, slope method shows consistently lower perfusion values than deconvolution method, due to use of vessel exclusion algorithm in slope method (8).

5. PERFUSION CT STUDIES

5.1. Technical Considerations

Various CT techniques have evolved for performing perfusion CT studies. In essence, perfusion CT comprises of acquisition of unenhanced images followed by a series of post-contrast dynamic images in axial cine mode with static table position. The unenhanced images are obtained to select the region of interest for dynamic imaging to study the tumor perfusion. Current CT techniques (4- to 64-slice Multi-Detector Computed Tomography (MDCT) allow only 2–4 cm of tumor tissue to be scanned. Hence, careful selection of region of interest of scanning through the tumor is crucial. The images are reconstructed at 5 mm slice thickness. The number of reconstructed slices could be 4 to 8 depending on the type of CT scanner used. Dynamic imaging in cine mode is started after a scanning delay of 5–10 s from the start of injection of contrast bolus, depending on the circulation time to organ of interest. Breath-hold is crucial for perfusion CT study of upper abdominal viscera that are more susceptible to breathing artifacts. Duration of scanning would be 30 s to 2 min from arrival of contrast depending on the clinical need, organ of interest, and analytic method.

Duration of scanning should always be considered in conjunction to time of arrival of contrast as this may vary depending on the location of the tumor. For example, contrast would arrive early in a lung mass when compared to a mass in the foot. Earlier reports recommended longer duration of scanning for least 2 min when permeability measurement was required (9). However, current recommendations advocate for scanning durations shorter than this. A recent study reported that 65-s scan duration is adequate for measurement of permeability of colorectal carcinoma (10). Radiation dose, breath-hold, and patient motion invariably limit the maximum duration of scanning. There is always a trade-off between radiation dose and temporal resolution.

The shape of the intravenous contrast bolus is crucial for perfusion CT. Deconvolution analysis can tolerate slightly slower injection rates and in our experience 5–7

mL/second is optimum. Compartment analysis requires a short sharp bolus because the validity of the method requires that peak arterial enhancement occurs prior to peak tissue enhancement. Typically, the contrast material is administered as a small bolus of 40–50 cc at a rate of 5–10 cc/s (8).

CT acquisition technique should be tailored to the analytic method used to allow optimization of radiation dose. For example, slope method requires fewer images, but it is sensitive to noise. Although, higher radiation dose may be required to offer less noisy images, overall radiation dose is not substantially increased as the number of images acquired is less. On the contrary, the deconvolution method requires more images but is not sensitive to noise. Although the deconvolution method can tolerate low-radiation dose data, overall the radiation dose of the study may be comparable to the slope method, due to higher number of images in the deconvolution method (8).

5.2. Validation and Reproducibility

Methods for mathematic analysis of perfusion data have been validated against either microsphere methods in animal studies or stable xenon washout methods in humans (8). Deconvolution method has been validated by rabbit cerebral blood flow evaluation using microspheres and human cerebral blood flow using stable xenon (11, 12). Typical variability of perfusion measurements using deconvolution analysis is 13% (8). Excellent correlation ($r = 0.9$, $p < 0.01$) of CT perfusion parameters of hepatocellular carcinoma (HCC) with repeat CT perfusion studies within 24 h has been reported (Holalkere et al., Annual Meeting of Radiological Society of North America 2005). Slope method is reported to have an interoperator variability of 8% when splenic perfusion was evaluated (8). Mullani-Gould formulation, an optional add-on to slope method, has been validated against microsphere measurements of cerebral perfusion in dogs (13).

5.3. Clinical Studies

Potential role of perfusion CT had been reported for evaluation of liver tumors such as HCC and metastases, and pancreatic, prostatic, colorectal, cervical, and head and neck carcinoma and lymphoma. Furthermore, studies have established that micrometastases alter liver perfusion despite the absence of morphologic changes in liver on CT (6, 14). Hence, it is conceivable that in selected patients, this technique might help detection of micrometastases by mapping the areas of altered liver perfusion. In addition, Miles et al. (15) reported that increased peritumoral arterial perfusion and increased global liver perfusion indicate a more favorable outcome following chemotherapy.

In our institution, we have investigated the role of perfusion CT before and after treatment in patients with rectal carcinoma to monitor treatment response. We found that rectal carcinoma showed substantially higher BF and shorter MTT when compared to normal rectal wall (16). Following chemoradiation, rectal carcinoma showed considerable reduction in BF and increase in MTT (16). Likewise, early anti-angiogenic treatment effects can also be monitored with this technique for tumors such as rectal carcinoma, HCC, and sarcoma (Figs 3 and 4). Willet et al. (17, 18) have reported drop in tumor perfusion in patients with rectal carcinoma 2 weeks after initiation of

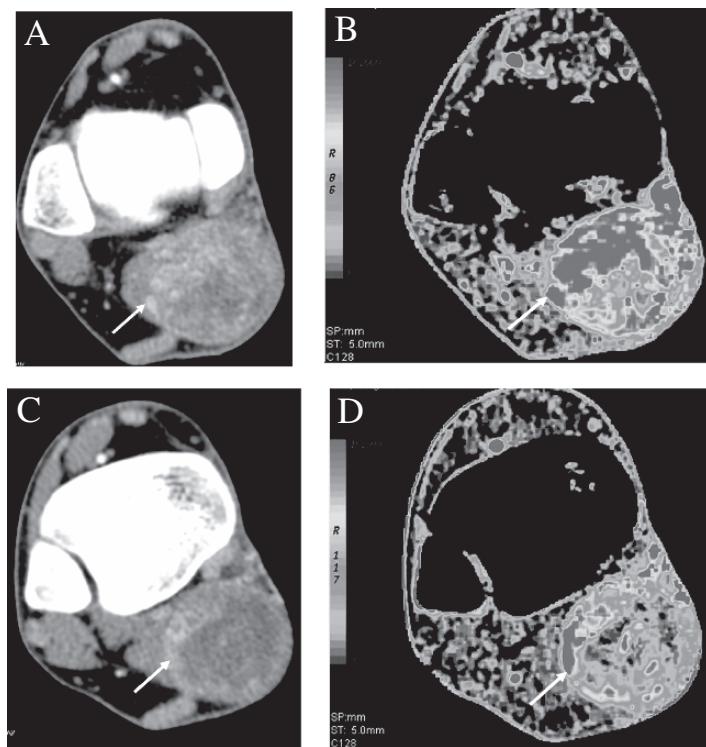


Fig. 3. Thirty-one-year-old man with leiomyosarcoma of right ankle, undergoing treatment bevacizumab (anti-angiogenic drug). Serial contrast-enhanced CT (A = baseline, C = post-anti-angiogenic therapy) and corresponding blood volume maps (B, D, respectively) are shown. (A) Baseline contrast-enhanced CT demonstrates a heterogeneously enhancing mass (arrow) in the posterior aspect of right ankle with high tumor blood volume (arrow) seen on perfusion map (B). Follow-up imaging (C, D) performed after 2 weeks of initiation of bevacizumab therapy shows reduction in tumor tissue enhancement (C, arrow), with about 38% reduction in tumor blood volume (D, arrow). (Please see color insert.)

bevacizumab therapy, further supporting the role of perfusion CT in tumor angiogenesis assessment.

As angiogenesis is not a predominant feature of lymphomas, perfusion imaging has not drawn the attention of researchers for tumor angiogenesis assessment. Dugdale et al. (19) evaluated 39 patients with lymphoma and found that median perfusion values of lymph nodes were higher in active disease and intermediate/high-grade lymphoma, when compared to inactive disease and low-grade lymphoma, respectively. In addition, the blood flow substantially decreased when nodal disease became inactive. However, there was no correlation of permeability with disease activity or grades of lymphoma.

5.4. Limitations

It is important to emphasize that none of the perfusion CT acquisition or analytic techniques are standardized for implementation in routine clinical practice for oncologic body imaging, and all the perfusion parameters calculated by the commercial softwares are only estimates and not absolute values. One of the principal limitations of CT

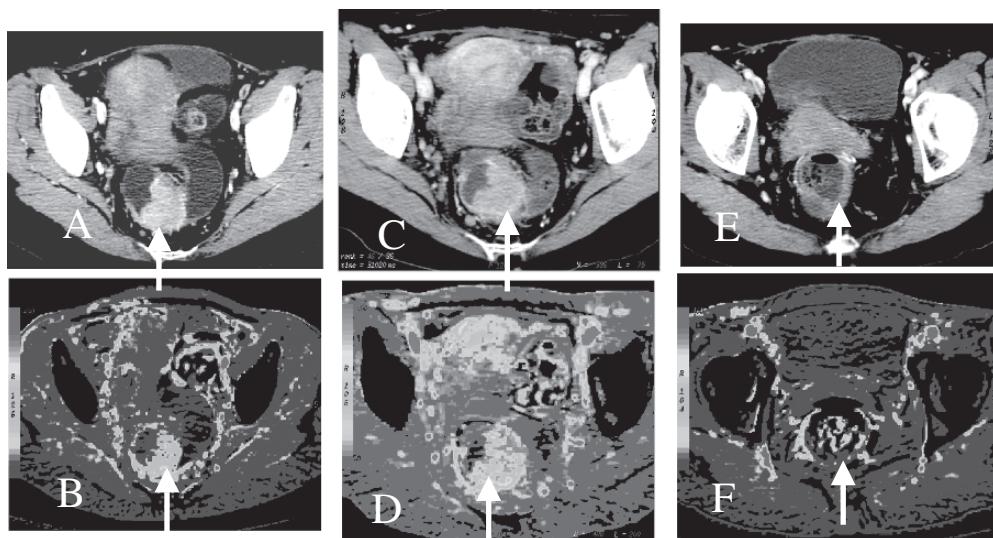


Fig. 4. Fifty-year-old woman with rectal adenocarcinoma undergoing treatment with neoadjuvant chemoradiation that composed of initial bevacizumab (anti-angiogenic drug) followed by chemotherapy and radiotherapy. Serial contrast-enhanced CT (A = baseline, C = post-anti-angiogenic therapy, E = post-chemoradiation) and corresponding blood flow maps (B, D, F, respectively) are shown. (A) Baseline contrast-enhanced CT demonstrates a polypoidal enhancing mass (arrow) in the rectum with high tumor blood flow (arrow) seen on perfusion map (B). Follow-up imaging (C, D) performed after 2 weeks of initiation of bevacizumab therapy shows no change in tumor size (C, arrow), with about 45% reduction in tumor blood flow (D, arrow). Images from scan done at 6 weeks post-chemoradiation show almost complete resolution of the tumor (E, arrow) with normalization of blood flow (F, arrow). (Please see color insert.)

perfusion is the limited scanning volume. Current MDCT scanners (4–16 slice) allow 2-cm tissue coverage for dynamic CT acquisition. Even with a 64-slice MDCT scanner, the maximum scan coverage is only 4 cm. Hence, only a portion of the tumor/organ can be sampled for perfusion measurements. Hence, scan volume selection is a crucial aspect of scanning technique.

Patient motion or movement of the tissue of interest (such as bowel) during acquisition of the data leads to problems in perfusion calculations. Patient motion within the image plane may be corrected by registration. However, patient motion out of image plane causes data loss and errors in perfusion values. Respiratory gating may improve the motion problems at the expense of temporal resolution (8). Adequate patient instruction for breath-hold with immobilization straps over the abdomen may help. Beam hardening artifacts from metallic stents, prostheses, and surgical implants can also result in variation in the perfusion values. Hence, careful selection of region of interest for scanning is important. Likewise, adequate distension of hollow viscus such as colorectum or stomach with saline/water or any neutral contrast is crucial for optimum perfusion measurements. In our institution, we use 250–300 mL saline to distend the rectum before doing perfusion CT studies. Radiation dose is an important concern in implementing perfusion CT in routine clinical practice.

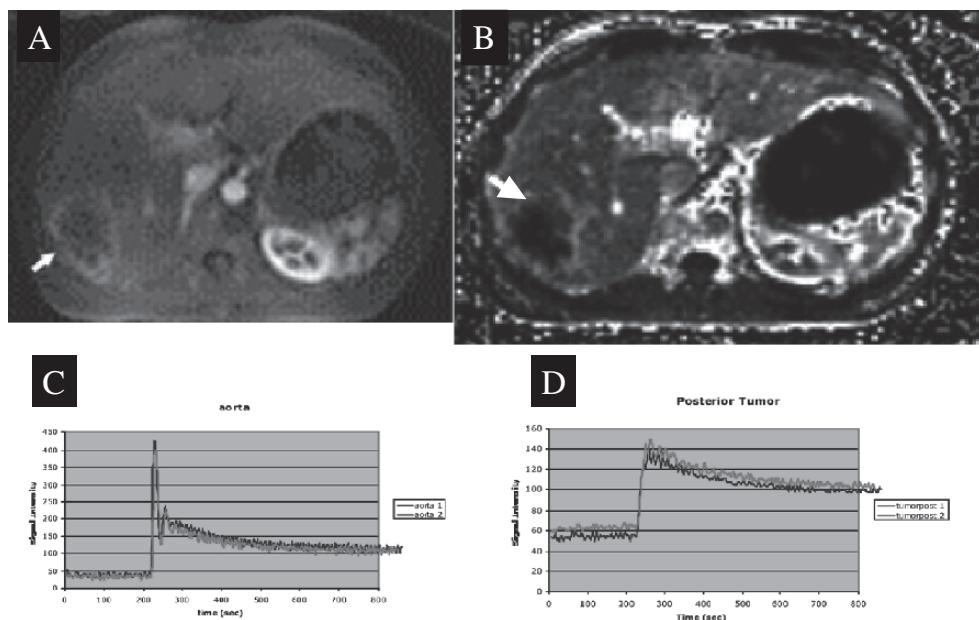


Fig. 5. (A) MR perfusion study of liver metastasis from colonic adenocarcinoma. MR perfusion study demonstrates higher blood flow (A) and permeability (B) in the periphery of the metastatic deposit in liver. The time-signal intensity curves for aorta (C) and periphery of tumor (D) are shown.

6. MR IMAGING OF ANGIOGENESIS

MR imaging for assessing tumor angiogenesis offers many advantages over CT. MR has the capability to perform dynamic scanning of the whole tumor without the risk of radiation exposure and adverse reactions to iodinated contrast material. MR imaging techniques either with or without intravenous contrast material are available for assessment of tumor angiogenesis (Fig. 5).

6.1. Contrast-Enhanced Dynamic MR Imaging

6.1.1. SMALL MOLECULAR CONTRAST MEDIA (SMCM)

SMCM (<1 kDa) are extracellular agents such as gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) that get quickly equilibrated between blood and most compartments of the extracellular fluid (ECF). They are used for rapid first-pass imaging with T2*-weighted sequences to estimate perfusion and blood volume and with T1-weighted sequences for estimating permeability (20). Degree of signal enhancement on T1-weighted images is dependent on tissue perfusion, capillary permeability, volume of extracellular leakage space, native T1 relaxation time of the tissue, dose of contrast agent, imaging sequence and parameters, and machine-scaling factors (21). Objective analysis of T1-weighted contrast-enhanced MR images can be performed by measuring the changes in signal intensity, or by fitting pharmacokinetic models to the tissue contrast medium concentration-time curves, or both. Quantitative analysis with

pharmacokinetic models can provide estimates of physiologic parameters such as PS, volume of extravascular extracellular space per unit volume of tissue, and rate constant (20). Color-coded pixel maps of quantitative analysis can be superimposed on anatomic gray-scale images. Advantages of pixel-mapping techniques include improved appreciation of the heterogeneity of tissue enhancement and removal of the need for selective placement of user-defined regions of interest (ROI) (20). Pixel-mapping techniques also enable improved visualization of the anatomic location for changes in the tumor microvascular function in response to treatment (22).

In malignant tumors of breast with no substantial vascular endothelial growth factor (VEGF) expression, PS measured on MR perfusion studies had shown significant correlation with MVD (20). However, once tumor tissue VEGF expression becomes prominent, PS increased rapidly and independently of MVD. Furthermore, Hawighorst et al. (23) reported that enhancement pattern on dynamic MR can predict patient survival. Tumors with fast initial rate of enhancement were more likely to carry a poor prognosis.

6.1.2. MACROMOLECULAR CONTRAST MEDIA (MMCM)

The widely used Gd-DTPA agents are not suited for evaluation of tumor microvasculature as they show free trans-endothelial permeability in even normal tissue. Hence, the SMCM are complexed with proteins to render them macromolecular. MMCM (>30 kDa)-enhanced dynamic MR has the advantages of tumor tissue characterization, as malignant tumors unlike benign lesions show macromolecular hyperpermeability and accurate measurement of fractional plasma volume (fPV) as they stay longer in intravascular compartment. One of the first macromolecular formulations to be evaluated for tumor characterization was albumin-Gd-DTPA in rat fibrosarcoma model (24). In these rats, tumor tissue had significantly greater permeability to MMCM than normal tissue. In another study using MMCM, van Dijke et al. (25) reported significant correlation of PS and fPV of adenocarcinoma of breast with tumor grade and MVD. Furthermore, treatment response to anti-angiogenic therapy can also be assessed using MMCM. Pham et al. (26) reported that after administration of anti-VEGF antibodies to rats with implanted human breast adenocarcinomas, the MR imaging-assayed MMCM permeability declined by 71%.

6.2. *Intrinsic Contrast MR Imaging Techniques*

Tumor vascular bed can be depicted at MR imaging without administration of exogenous contrast media by using pulse sequences that are sensitive either to the distortion of magnetic field homogeneity by deoxyhemoglobin which serves as an intrinsic oxygen-sensitive paramagnetic marker or to the motion of water in the vascular bed.

6.2.1. BOLD CONTRAST FUNCTIONAL MRI

BOLD contrast relates to the endogenous change of paramagnetic deoxyhemoglobin that is translated in variation of MR signals. BOLD contrast MR offers a non-invasive and clinically applicable tool to detect changes in tumor oxygenation due to angiogenesis, without the need for intravenous contrast (27). On BOLD contrast, gradient echo-planar sequence functional MR imaging, a decrease in T2*-weighted signal indicates increase in deoxyhemoglobin and thus, tumor tissue hypoxia. Landuyt et al. (27) reported that BOLD contrast functional MR can be used to assess the degree of

oxygenation of rhabdomyosarcoma in rats. Disadvantages of this technique include low spatial resolution, sensitivity to susceptibility artifacts, and signal averaging within each voxel (27).

6.2.2. MAGNETIZATION TRANSFER METHODS

Relatively long T1-weighted relaxation in tissues provides the option of tagging the magnetization by using positional selective inversion or saturation radio-frequency pulses. Translocation of the labeled spins can be observed and used to calculate flow. Magnetization transfer methods were developed for mapping tissue perfusion. Changes in the apparent T1-weighted relaxation reflect the exchange of water between the vessels and tissue (20). Magnetization can be tagged either in the feeding artery (arterial spin labeling) or in the target tissue (flow-sensitive alternating inversion recovery or FAIR). Changes in the signal intensity of the target tissue reflect inflow of tagged spins in the former or of untagged spins in the latter. This method has been applied to the study of angiogenesis in the rat ovarian follicle (20).

6.3. Clinical Studies

The role of dynamic MR imaging is evolving for evaluation of angiogenesis in tumors of brain, liver, lung, and prostate (28–31). Relative cerebral blood volume (rCBV) correlates with tumor grade of brain tumor and MVD, and PS correlates with tumor grade. Tumors such as gliomas of brain show greater rCBV and lymphomas show lower rCBV (28). Likewise, atypical meningiomas show greater PS than typical meningiomas. MR perfusion study represents a promising technique for HCC surveillance and a high-temporal-resolution MR perfusion imaging may improve detection of small HCC (29). Fujimoto et al. (30) reported significant positive correlation of maximum enhancement ratio and slope value and negative correlation of time to maximum enhancement measured on dynamic MR study with microvessel count of small peripheral lung carcinomas. When compared to T2-weighted MR imaging, dynamic contrast-enhanced MR imaging improved the accuracy of localization of prostate carcinoma (31).

6.4. Limitations

Some pharmacokinetic models involve the assumption of an instantaneous injection, which is impractical and can introduce errors in quantification. In the presence of recirculation effects, extracting flow information from T2*-weighted MR imaging data can result in falsely low BV measurements. All quantitative analyses require specialized perfusion softwares which are time consuming and not clinically robust.

7. ULTRASOUND FOR IMAGING OF ANGIOGENESIS

7.1. Doppler Ultrasound

Duplex and power Doppler ultrasound offer cheap non-invasive means for measurement of blood flow velocity and volume of flow. Earlier reports indicated that Doppler signal correlated well with MVD when vessels were larger than 50 µm, but it correlated poorly with MVD when vessels were smaller than 50 µm in diameter (32). Traditional Doppler methods have been evaluated for detection of breast and prostate

cancers and to date, these studies have reported mixed results (32). This may be caused by the lack of sensitivity of Doppler techniques in detecting the small vessels and slow flow associated with tumor neovascularity.

7.2. Contrast-Enhanced Ultrasound

Administration of microbubble contrast material would improve the visualization of microvasculature on ultrasound. Thus, contrast-enhanced ultrasound with and without Doppler would be more effective than unenhanced Doppler studies in imaging of angiogenesis. Newer ultrasound contrast agents such as sonovue (BR1, Bracco) are effective at low mechanical index and prolong the total duration of enhancement. Lassau et al. (33) evaluated the response of gastrointestinal stromal tumors to imatinib therapy using contrast-enhanced ultrasound. They reported that initial contrast uptake on day 1 predicted the tumor response and there was strong correlation of decline in contrast uptake on days 7 and 14 with tumor response. Likewise, McCarville et al. (34) reported that quantification of intratumoral flow of ultrasound contrast agent can be used for monitoring anti-angiogenic therapy response in murine neuroblastoma models.

8. PET IMAGING FOR ANGIOGENESIS

Oxygen-15-based compounds such as H_2O and CO_2 are used for evaluation of angiogenesis using PET. The two methods used for this purpose are static equilibrium method and dynamic method. Dynamic method is better and gives accurate values as compared with static method. As these metabolites have very short half-life, the imaging can be performed repeatedly. PET studies determine BF, BF per unit of volume, and BV to the tumors. Permeability cannot be assessed by PET studies.

The clinical role of PET for imaging angiogenesis is still evolving as the relationship between glucose metabolism and blood flow in tumor tissue is not well understood. Literature results on correlation of metabolic activity of tumor with BF are controversial. Inverse correlation of metabolic activity with BF had been reported in patients with HCC and metastatic colon cancer and animal models with liver tumors (35, 36). However, other studies have reported direct correlation or a variable correlation between metabolic activity and tumor BF (37, 38). Further studies are needed to assess the potential role of PET in the imaging of angiogenesis.

9. MOLECULAR IMAGING OF ANGIOGENESIS

Molecular imaging is a novel non-invasive imaging approach for detection of indicative marker molecules of the tumor angiogenesis process that can improve the accuracy of the established imaging techniques for evaluation of angiogenesis. Molecular imaging techniques for interrogation of tumor angiogenesis still remain as research tools, and their clinical role is under investigation. For a targeted approach in imaging angiogenesis, existence of marker molecules specific for the tumor neovascularity that are present at high density sufficient enough to be detected is a prerequisite (39). To date, only a few target molecules fulfill these demands. One of them is the ED-B-domain of fibronectin, which is specifically located within the tumor stroma or surrounding angiogenic blood vessels (14). In preclinical animal models, excellent targeting properties of the anti ED-B-antibody L19 could be demonstrated (40), and the

first evidence of selective localization in tumor lesions of lung cancer and colorectal cancer in humans was recently shown by Santimaria et al. (39).

Radiolabeled single-chain antibodies to specific tumor angiogenesis targets such as ED-B fibronectin, tenascin-C, and integrin $\alpha_v\beta_3$ can be used for scintigraphic evaluation of angiogenesis (14). Besides imaging detection of angiogenesis, these specific radiolabeled antibodies may be used to provide targeted radiotherapy for the tumors.

Several molecular targets such as endothelial receptors (integrin $\alpha_v\beta_3$ and intercellular adhesion molecule (ICAM)-1) or structural proteins (extradomain β -fibronectin and matrix fibrin) are being studied in animal models for delivery of MR-contrast agents specifically to tumors with increased angiogenesis (14). The practical consideration in using molecular targeting for MR would be the minuscule concentration of the molecular targets (picomolar to low micromolar) which would result in excessive dilution of contrast agent far below the detection limit of MR. This translates to higher doses for MR unlike scintigraphic evaluation. Hence, MR-contrast agents with increased relaxivity would improve use of molecular targets in MR imaging.

Target-specific ultrasound-contrast agents under research consist of shell (e.g., lipids, proteins, or polymers)-stabilized microbubbles as signaling moiety and shell surface-bound ligands as binding moiety (14). Such microbubbles can only bind to the tumor endothelial cells and cannot leave the vascular system. This hypothesis has already been supported by pioneering experiments demonstrating the active targeting of endothelial cell receptors such as α_v integrins in angiogenesis model or adhesion molecules such as P-selectin or ICAM-1 (14).

10. OPTICAL IMAGING OF ANGIOGENESIS

Measurement of absorption bands of oxy- and deoxyhemoglobin using optical imaging may help estimate BF and BV (41). However, this can be applied only to superficial tumors such as breast carcinoma. Optical imaging can combine conventional tissue display (diffuse optical tomography) with the capability of molecular-based diagnosis due to the high sensitivity of photon detection (e.g., direct surface imaging in endoscopy). Fluorescence emission generated either from intrinsic fluorophores or from exogenously applied dyes is repeatedly excitable at any desired time and is not limited by a fast decay process, as with diagnostically relevant radioactive isotopes (i.e., ^{99m}Tc) (42). The major limitation is limited penetration of light into tissue, so that this modality has primarily been directed toward near-surface diseases that are accessible by light (42).

A way to impart molecular specificity into optimal imaging agents is to synthesize reactive dye derivatives and conjugate them to target-specific vehicles such as peptides, antibodies, or antibody fragments (42). Target-specific cyanine dye conjugates with single-chain antibodies directed against the angiogenesis-specific target protein ED-B-fibronectin were reported by Neri et al. (43). Molecular targeting of a matrix protein has been successfully extended to photodynamic therapy by the use of photosensitizer antibody conjugates. A probe design approach involving injectable, fluorescence-quenched polymers to image protein function (enzymatic activity) was reported by Weissleder et al. (44). After injection of cyanine-labeled substrate polymers into animals, enzyme activity correlated with the extent of recovered fluorescence and signal appearance in fluorescence images.

11. SUMMARY

Various imaging techniques are under investigation for assessment of angiogenesis, fueled by the increasing demands due to emerging treatment paradigm in oncology. Currently, CT and MR remains the most widely studied and used imaging tool for evaluation of tumor angiogenesis, and their clinical role as a routine in oncologic patient management is rapidly evolving. However, standardization of imaging protocols, analytical methods, and reproducibility of these techniques continue to be a challenge. The ongoing research work worldwide should solve the technical issues in all these imaging techniques for angiogenesis and standardize them for routine clinical use, so that these imaging techniques may be implemented in routine oncology practice.

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12

Tumor Blood Vessels

Structure, Function and Classification

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SUMMARY

The tumor vasculature is largely induced by secreted VEGF-A and consists of a heterogeneous mixture of highly abnormal blood vessels. Recently, it has been possible to replicate many of these vessel types by introducing an adenovirus expressing VEGF-A¹⁶⁴ (Ad-VEGF-A¹⁶⁴) into mouse tissues. At least five different microvessels form in sequence from preexisting venules, each with distinctly different structural and functional properties. Mother vessels (MV) from first and evolve into several types of daughter vessels: bridged MV, capillaries, glomeruloid microvascular proliferations (GMP), and vascular malformations (VM). In addition to this angiogenic response, feeder arteries (FA) and draining veins (DV) develop from preexisting arteries and veins, respectively, to supply and drain the tumor microvasculature. This classification has helped to elucidate the steps and mechanisms by which tumors induce new blood vessels and hopefully will lead to the identification of new therapeutic targets that can improve anti-angiogenic tumor therapy.

Key Words: VEGF-A; angiogenesis; tumor blood vessels; arteriogenesis; venogenesis.

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1. INTRODUCTION

It has been known for more than a century that tumors have their own blood supply and for the better part of that time that the tumor vasculature is highly abnormal, differing from that of normal tissues with respect to organization, structure, and function. At one time, it was believed that the tumor vasculature was more abundant than that of normal tissues; this misconception arose because tumor vessels are often of large size and were therefore more obvious to the naked eye than the smaller but more numerous and functionally superior vessels supplying normal tissues. By the early 1970s, however, it was clear that tumor blood flow was unevenly distributed and, overall, significantly lower than that of normal tissues. It was also clear that tumor vessels were hyperpermeable to plasma and plasma proteins. Further, it was known that tumor vessels were induced by tumor-secreted products, though the tumor angiogenic factor(s) responsible had not as yet been identified. In the years that followed, much was learned about the molecular basis of angiogenesis and particularly about the central importance of one cytokine/growth factor, vascular permeability factor/vascular endothelial growth factor (VPF/VEGF, VEGF-A). More recent work has elucidated the steps and mechanisms by which VEGF-A induces tumor blood vessels and has demonstrated convincingly that tumor blood vessels are not of a single type but rather exhibit extensive heterogeneity. Further, it has become clear that, in addition to angiogenesis (generation of microvessels), both arteriogenesis and venogenesis contribute importantly to the tumor vasculature. Taken together, tumor blood vessels can now be classified in a manner that has clinical and therapeutic significance. Further, recent successes with agents that block VEGF-A or its receptors provide proof of principle that antiangiogenesis can provide a valuable new adjunct to traditional tumor therapy. This chapter reviews the properties of tumor blood vessels, their differences from normal vessels, and the steps and mechanisms by which they form.

2. THE NORMAL MICROVASCULATURE

Before discussing the tumor vasculature, it will be helpful to review briefly the structure of normal blood vessels as a standard of comparison. In most normal tissues, arterial blood enters arterioles, and, thereafter, capillaries, post-capillary venules, and veins. In some tissues (e.g., skin), blood can bypass capillaries by way of arteriovenous shunts. Though part of a continuum with some degree of overlap, each type of vessel has a characteristic structure and function. Arteries are large vessels lined by endothelium and coated with varying amounts of elastic tissue and several layers of smooth muscle cells. Arterioles have a structure similar to muscular arteries but are smaller in size, typically 10–20 µm in diameter. Arteriolar tone is regulated by autonomic, generally sympathetic, nerves that modulate vascular smooth muscle cell contraction and in this way regulate blood pressure and flow. Smooth muscle relaxation is modulated in part by endothelial cell-secreted nitric oxide.

Capillaries are small vessels, typically 4–9 µm in diameter, which are lined by a thin, flattened but, in most tissues, continuous endothelium and are enveloped by basement membrane and a variable coating of pericytes. In some tissues (e.g., kidneys and endocrine glands), the endothelium is not continuous but fenestrated. Fenestrae are 50–150 nm zones of extreme endothelial cell thinning that in most tissues are closed by diaphragms. Capillaries are normally spaced at intervals of approximately 100–200 µm,

i.e., distances corresponding to the diffusion range of oxygen. This is important because capillaries are the principal “exchange vessels” responsible for tissue nutrition and waste disposal. Low-molecular-weight plasma constituents (i.e., oxygen, glucose, and salts) pass freely out of proximal capillaries by convection and diffusion. Distally, these same processes lead to reabsorption of plasma solvent and metabolic waste products such as carbon dioxide. Capillary endothelial cells are the principle barrier to molecular exchange, and two pathways across them have been identified: paracellular (involving inter-endothelial cell junctions) and transcellular (caveolae). Small molecules such as gases and simple sugars can make use of the paracellular pathway, but plasma proteins are too large to pass through endothelial cell junctions and have been thought to extravasate from capillaries by means of caveolae (1). Caveolae are (~70 nm in diameter) membrane-bound vesicles that are thought to shuttle across endothelial cell cytoplasm from the vascular lumen to the albumen where they discharge their cargo of plasma protein-rich solute into the tissues. The importance of caveolae for exchange of large molecules has recently been challenged by the finding that the capillaries of caveolin-1-null mice, which lack caveolae, are actually more permeable to plasma albumin than their wildtype counterparts (2). At present, it is unclear whether this paradox can be explained by increases in paracellular capillary permeability in caveolin-1-null mice or, alternatively, by compensatory increased transcellular permeability across venules by vesiculo-vacuolar organelles (VVOs) (see below) which are normal in number and structure in caveolin-1-null mice.

Venules are larger vessels than capillaries, typically ~20 µm, and are lined by cuboidal endothelium, basement membrane, and pericytes. They are thought to play a lesser role in metabolite transport but are the key segment of the microvasculature that is activated in both humoral and cellular inflammation. Many years ago, Majno (3) demonstrated that venules are the primary site of solute and plasma protein leakage that is induced by inflammatory mediators such as histamine, serotonin, and so on. He also proposed a mechanism for venular hyperpermeability in inflammation, namely, that inflammatory mediators caused venular endothelial cells to contract and pull apart, generating inter-endothelial cell gaps through which fluid and macromolecules could extravasate (4). However, more recent studies have provided convincing evidence that many of the openings across endothelial cells induced by inflammatory mediators in endothelium are transcellular, not intercellular (5–7). Also, a structure has been identified in venular endothelium, the VVO, which contributes importantly to the increased transendothelial flux of plasma that occurs in response to mediators such as VEGF-A or histamine (Fig. 1a–c) (6). VVOs are grape-like clusters of hundreds of uncoated, largely parajunctional cytoplasmic vesicles and vacuoles that together form an entity that traverses venular endothelial cytoplasm from lumen to albumen (6,8). The smallest vesicles that comprise VVOs closely resemble caveolae, and larger vacuoles were found to have volumes that were multiples of those of caveolae; however, VVOs are distinct from caveolae in that they stain irregularly with antibodies to caveolin-1 and remain intact in caveolin-1-null mice (unpublished data). The individual vesicles and vacuoles that comprise VVOs are linked to each other and to the luminal and abluminal plasma membranes by stomata that are normally closed by thin diaphragms that resemble those closing fenestrae. VEGF-A and other inflammatory mediators are thought to cause these diaphragms to open, providing a transcellular pathway for plasma and plasma protein extravasation. It is possible that mechanical forces of the

type envisioned by Majno are responsible for opening VVOs. In any event, we find that inter-endothelial cell junctions remain tightly closed in tumor vessels and do not admit macromolecular tracers of the size of plasma proteins. Transendothelial openings have been observed in tumor vessels and have been claimed to represent inter-endothelial cell gaps. However, intercellular gaps cannot be distinguished from transcellular holes except by demonstration of junction-specific proteins in the lining plasma membrane, and this has never been accomplished.

Leukocytes also leave the vasculature by traversing venules. As with the passage of fluid, it was originally thought that in inflammation leukocytes crossed venules through opened inter-endothelial cell junctions. However, more recent structural studies, making use of serial sections and three-dimensional reconstructions, have shown that granulocytes and monocytes often pass through endothelial cells, possibly through VVOs, independent of intercellular junctions (9, 10). More work will be required to sort out the relative importance of paracellular versus transcellular pathways for both fluid and inflammatory cell extravasation. The problem is a difficult one because inter-endothelial cell junctions are complicated, interdigitating structures (Fig. 1c). Also, VVOs are concentrated parajunctionally, and individual VVO vesicles may open to the intercellular cleft above and below specialized junctions, as well as to the luminal and abluminal surfaces (Fig. 1c). The types of blood vessels from which inflammatory cells extravasate in tumors have not as yet been established.

3. TUMOR BLOOD VESSELS

3.1. *Organization and Distribution of Tumor Blood Vessels*

Whereas normal microvessels are arranged in a hierarchy of evenly spaced, well-differentiated arterioles, capillaries, and venules, tumor microvessels follow a chaotic pattern and are hierarchically disorganized (11). A characteristic feature is *spatial heterogeneity* (uneven distribution), and the frequency of localized zones of increased microvascular density ("hot spots") has been used as a predictor of clinical outcome (12). One possibility is that these microvascular hot spots are sites that allow cancer cells to enter the blood and thereby favor metastasis; by contrast, tumor cells only rarely invade muscle-coated arteries or arterioles.

Tumor vessels often exhibit a serpentine course, branch irregularly, and form arteriovenous shunts. Typically, vessels are most abundant at the host interface where they may form a prominent, circumferential mantle enveloping tumors. Internal portions of tumors are typically less well vascularized. Blood flow through the tumor vasculature does not follow a consistent, unidirectional path. Rather, the tumor vasculature consists of a maze of interconnected vessels through which blood flows haphazardly and irregularly. Not all open vessels are perfused continuously, and, over intervals of even a few minutes, blood flow may follow different paths and actually reverse direction (13).

The tumor vasculature is also heterogeneous over longer periods of time (11). Morphometric studies have shown that vascular volume, length, and surface area all increase during early tumor growth. As a result, small tumors tend to be relatively well vascularized. As tumors grow, however, vascular growth slows and the tumor blood supply becomes progressively deficient, leading to central ischemia and necrosis. Thus, tumors are said to outgrow their blood supply or, put in other words, the developing vasculature and other stromal elements fail to keep pace with tumor cell growth.

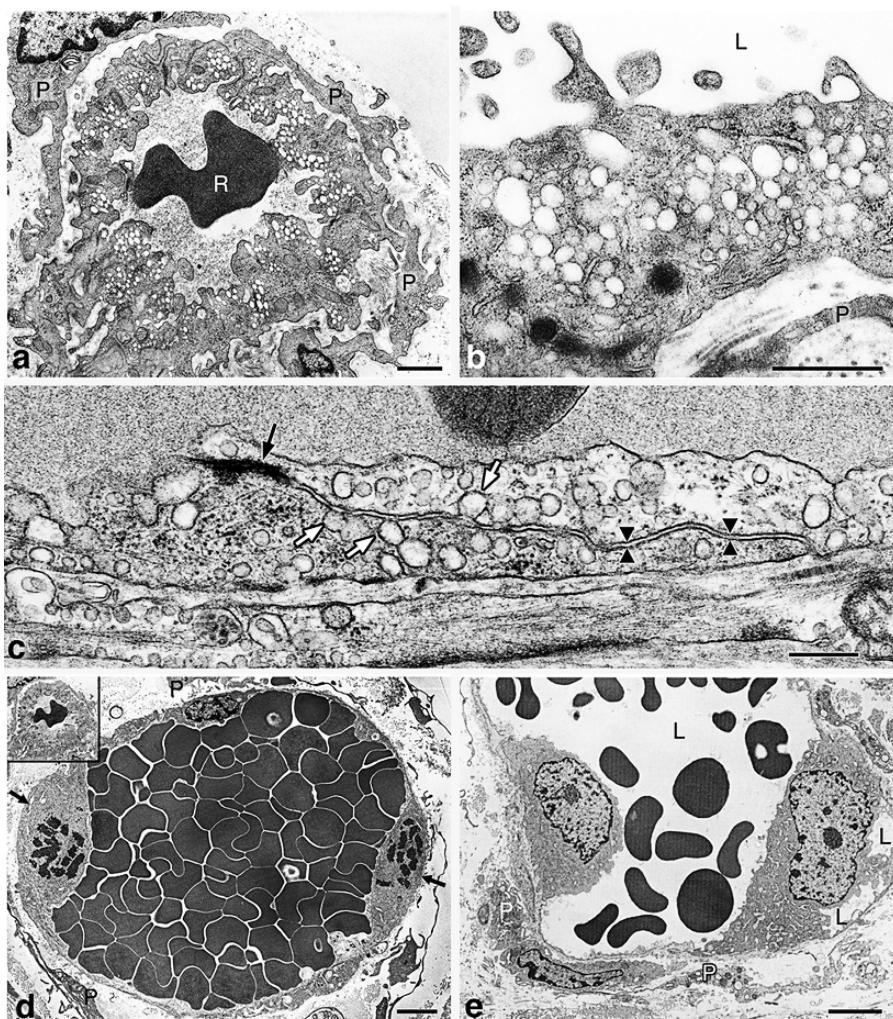


Fig. 1. Transmission electron micrographs of control ear venules (**a–c**) and of mother vessels (MV) (**d, e**) 3 days after local injection of Ad-VEGF-A¹⁶⁴. **(a, b)** Typical normal venules lined by cuboidal endothelium. The cytoplasm contains prominent vesiculo-vacuolar organelles (VVOs) and is enveloped by a complete coating of pericytes (P). R, red blood cell. **(c)** Enlarged segment of a normal venule illustrating the typical long intercellular interface between adjacent endothelial cells. Black arrow indicates specialized adherens junction, arrow heads indicate the long intercellular cleft, and white arrows indicate VVO vesicles fusing with the intercellular cleft. **(d, e)** Typical MV are greatly enlarged vascular structures, characterized by extensive endothelial cell thinning, striking reduction in VVOs and other cytoplasmic vesicles, prominent nuclei that project into the vascular lumen, frequent mitotic figures (arrows, **d**), endothelial cell bridging with the formation of multiple lumens (L, **e**), and decreased pericyte (P) coverage. Note that the MV lumen (**d**) is packed with red blood cells, indicative of extensive plasma extravasation. Inset. The normal venule depicted in **(a)** is reproduced in **(d)** at the same magnification as the MV to illustrate the dramatic differences in relative size of normal venules and MV. Scale bars: **(a–c)**, 1 μ m; **(d, e)**, 5 μ m. **(a, b, d, e)** (republished from 44).

3.2. Tumor Blood Vessel Structure

Surprisingly little has been written about the structure of tumor blood vessels, though it has long been recognized that they are a caricature of their normal counterparts. They do not conform to the hierarchical pattern of normal vascular beds and until quite recently have resisted categorization. Writing in the late 1970s, Warren (11) described at least eight distinct types of tumor vessels, not all of which were found in any one tumor. He concluded that the main difference between the tumor and normal vasculature was that in the former, capillaries and veins became tortuous and dilated. In fact, however, the notion that tumor vessels are “dilated” is a misconception. Dilatation is correctly understood as vessel enlargement that results from the relaxation of vascular smooth muscle cells, as, for example, in the response of normal arterioles to nitric oxide. Many tumor vessels exhibit a paucity of smooth muscle cells, and their enlarged size reflects abnormalities in their generation, not muscle relaxation (see below). In recent years, studies with adenoviral vectors have worked out the steps and mechanisms by which VEGF-A induces new blood vessels, and the same types of vessels have been found in mouse and human tumors (see below).

3.3. Tumor Blood Flow and its Consequences

As noted above, it was thought at one time that tumors had a more extensive blood supply than normal tissues (14). However, in now classic studies, Gullino (15) demonstrated that blood flow in animal tumors was generally much lower than in normal tissues. Moreover, as tumors grew in size, their average perfusion rate decreased further as the blood supply became increasingly inadequate (14). Subsequent studies by many investigators have confirmed and generalized these observations, though, as with other properties of tumor vessels, there is extensive heterogeneity.

What accounts for the relatively reduced blood flow found in tumors? Although the reasons responsible are not as yet fully understood, measurements by many investigators have shown that all of the variables affecting blood flow are altered in experimental tumors (reviewed in 16–18). Blood flow is proportional to the *drop in blood pressure* across a vascular bed and inversely proportional to blood *viscosity* and *extrinsic geometric resistance*, a complex function of vascular morphology dependent on vessel number and types, their branching pattern, diameter, and length. Whereas pressures in the arteries supplying normal and tumor vessels are quite similar, microvascular pressures within tumors are actually elevated due to venous compression, whereas pressures in draining veins (DV) are significantly reduced. Also, tumor blood vessels exhibit greater resistance to flow than do the vessels supplying normal tissues. Because of their serpentine course, thin walls, exposure to increased interstitial pressure, and other local factors, the extrinsic geometric resistance may be increased in experimental tumors by more than an order of magnitude. Finally, the viscosity of the blood within tumor vessels is increased because vascular hyperpermeability leads to plasma leakage with resulting increased hematocrit, rouleaux formation, and resulting increase in shear rate.

Attempts have been made to increase or decrease tumor blood flow with vasoactive drugs, but the response has been inconsistent (14). In general, the vascular beds of most tumors behave as rigid tubes that are in a state of near-maximal diameter; thus, there is little capacity to increase flow in response to higher vascular pressure. One reason for this is that many tumor vessels lack normal coatings of smooth muscle

cells. Another reason may be that, unlike normal vessels, tumor blood vessels lack innervation, and therefore their smooth muscle cells are unable to relax. The result is low maximum perfusion capacity (i.e., high vascular resistance) compared with that of most normal tissues.

Not unexpectedly, a combination of abnormal tumor vessel organization, structure, and reduced blood flow leads to poor tumor vessel function. The uneven distribution of vessels results in uneven delivery of oxygen and nutrients and uneven clearance of waste products; together, these lead to zones of metabolic insufficiency, ischemia, and necrosis. Also, the increased average diameter of tumor vessels results in an altered surface area to volume ratio that further impairs tissue nutrition. As a result, and also because of arteriovenous shunts, nutrients are not taken up efficiently by tumors as is manifest by the higher than normal oxygen content of the venous blood draining tumors. Poor clearance of carbon dioxide and other metabolites, coupled with high tumor cell glycolytic activity, results in a tumor microenvironment that is acidic as compared with that of normal tissues ($\text{pH} \sim 7.2$ vs. $\text{pH} \sim 7.4$) (19).

3.4. Tumor Vessel Hyperpermeability

Another general property of the tumor vasculature is abnormally increased permeability to plasma and plasma proteins. As early as 1959, investigators reported increased clearance of plasma proteins in tumors (reviewed in 20). As already noted, Gullino (15) found that, as compared with normal tissues, tumor interstitial fluid was increased both in quantity and in plasma protein content, with a resulting increase in colloid osmotic pressure. He and others suggested that vascular hyperpermeability, coupled with a lack of functional lymphatics, accounted for the accumulation of protein-rich interstitial fluid in tumors.

In tumor vessels as in their normal counterparts, permeability is regulated at the level of the endothelial cell; that is, the vascular endothelium is the ultimate barrier to the passage of solutes. However, a number of variables affect vascular permeability, whether in normal tissues or in tumors (21–24). These are reviewed more extensively elsewhere (21, 25) but include the properties of the particular solute being measured (i.e., its molecular size, shape, and charge), microvascular surface area, concentration of solute in plasma relative to tissues, the net filtration rate, the ratio of convective to diffusive flux, the solute permeability coefficient, and the solute reflection coefficient. Obviously, therefore, increased solute extravasation can result from changes in a number of variables other than intrinsic properties of the lining endothelium.

What accounts for the hyperpermeability of tumor blood vessels? The increased volume, high protein content, and increased pressure of tumor interstitial fluid would be expected to retard extravasation of molecules of all sizes, as would the relative increase in vessel diameter (resulting in a decreased ratio of vascular surface area to plasma volume) and reduced blood flow. Nonetheless, plasma and plasma proteins extravasate from tumor blood vessels to a much greater extent than from normal vessels, and this results largely, if not entirely, from changes in the intrinsic properties of the vascular endothelial cells lining tumor blood vessels that lead to an increased extravasation of plasma and plasma proteins. These changes are the more impressive in that they involve only a subset of tumor blood vessels (see below).

3.5. Proliferation of Tumor Blood Vessel Endothelium

One property shared almost universally by tumor vascular endothelium is an increased rate of proliferation. In a comprehensive literature review, Denekamp and Hobson (26) reported that the labeling index (LI) of tumor vascular endothelium was significantly higher than that of normal tissues in which endothelial cells rarely divide. However, as with other properties of tumor vessels, there was great variability; studies of 131 individual tumors demonstrated an endothelial cell LI that varied from 3.6 to 32.3%. Endothelial cell LI did not correlate closely with tumor growth rate but was generally significantly lower than that of the tumor cells (range of tumor cell LI: 7.1–60.5%). The rates of tumor cell and endothelial cell proliferation were not directly related in different tumors.

4. VEGF-A AND TUMOR ANGIOGENESIS

Very early in their growth, and, interestingly, also at very late stages of malignant progression, tumors may satisfy their nutritional and waste removal needs by co-opting the normal vasculature. In some cases, this is dramatically manifest as tumor cells grow in cuffs around preexisting normal blood vessels (27, 28). However, for the most part, tumors must induce the formation of new blood vessels if they are to grow beyond minimal size. While many factors can stimulate endothelial cell growth *in vitro* and *in vivo* (29–33), it is now generally agreed that VPF/VEGF (VEGF-A), and particularly its 164/5 isoform, is the primary factor responsible for inducing tumor angiogenesis. VEGF-A is overexpressed by nearly all malignant tumors, and its expression is often upregulated in premalignancy. For example, VEGF-A expression levels are already elevated in pre-invasive low-grade squamous intraepithelial lesions of the cervix and increase further with malignant progression (34). As another example, overexpression of VEGF-A and angiogenesis characterize ductal carcinoma *in situ* of the breast prior to invasion (35).

There are many other reasons for regarding VEGF-A as the predominant tumor angiogenic factor. VEGF-A receptors are consistently upregulated in tumor vascular endothelium; many other angiogenic factors, as well as oncogenes and inactivation of tumor-suppressor genes, serve to upregulate VEGF-A expression; blocking VEGF-A or its receptors can effectively inhibit the growth of animal tumors and, more recently, human tumors (36–41); and, finally, as will be discussed below in detail, VEGF-A^{164/5} can induce many, and perhaps all, of the different types of blood vessels found in tumors.

5. INDUCTION OF TUMOR SURROGATE BLOOD VESSELS WITH VEGF-A^{164/5}

If VEGF-A^{164/5} is necessary and sufficient for tumor angiogenesis, it should be possible to induce the various types of blood vessels found in tumors by overexpressing this cytokine in normal tissues. In fact, this has been accomplished. Local overexpression of VEGF-A^{164/5} by a variety of methods (implants of VEGF-A^{164/5} protein in Matrigel or collagen, various genetic manipulations, and adenoviral vectors) leads to the formation of abnormal vessels that closely resemble those found in tumors (33, 42–44). However, adenoviral vectors engineered to express VEGF-A have been

particularly useful for this purpose and have contributed importantly to elucidating the sequential steps and mechanisms by which the different types of tumor vessel form. When injected into normal mouse tissues, such vectors infect host cells that then serve as local factories that synthesize and secrete VEGF-A^{164/5} (Fig. 2). The result is an angiogenic response that closely mimics that found in many tumors (33, 42).

Adenoviral vectors expressing VEGF-A offer many advantages for generating tumor surrogate blood vessels. They allow angiogenesis to be induced in any tissue accessible to vector injection; cytokine expression levels can be varied by adjusting viral dose; different cytokines can be combined, together or in sequence; and no foreign matrix need be introduced. Finally, because adenoviral vectors are not integrated into the cell DNA, their encoded proteins are expressed for only a limited period of time (several weeks); as a result, it is possible to determine the consequences that cessation of exogenous cytokine expression has on the various types of new blood vessels that have been generated. One caution regarding the use of adenoviral vectors is that they are highly immunogenic, and therefore experiments lasting more than a few days must be performed in immunodeficient animals such as nude or SCID mice.

Using adenoviral vectors engineered to express VEGF-A¹⁶⁴, we have induced the formation of at least five different types of angiogenic blood vessels (Fig. 3): mother vessels (MV), bridged MV, capillaries, glomeruloid microvascular proliferations (GMP), and vascular malformations (VM) (Figs. 1d,e, 4–9). All five of these vessel types are commonly found in animal and human tumors. Like tumor vessels, which show only limited tissue specificity, the new blood vessels induced by Ad-VEGF-A¹⁶⁴ are largely independent of the tissues in which they arise, i.e., similar vessel types form in response to VEGF-A¹⁶⁴ and with similar kinetics in a wide variety of normal mouse and rat tissues, including skin, subcutaneous space, fat, skeletal and heart muscle, and brain (33, 42, 45). In addition to promoting angiogenesis, Ad-VEGF-A¹⁶⁴ causes adjacent arteries and veins to enlarge and remodel, resulting in the additional processes of arteriogenesis and venogenesis (Fig. 10). Finally, Ad-VEGF-A¹⁶⁴ also induces the formation of abnormal, enlarged lymphatics (lymphangiogenesis) (46, 47). The extent to which new lymphatics are induced in tumors and their importance for tumor metastasis is the subject of considerable current debate but is beyond the scope of this chapter (48–50).

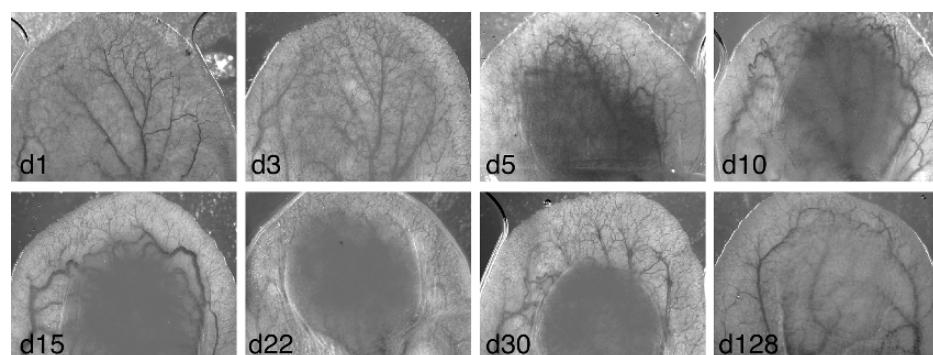


Fig. 2. The angiogenic response induced by Ad-VEGF-A¹⁶⁴ in the ears of nude mice from day 1 to day 128 (modified from 44). (Please see color insert.)

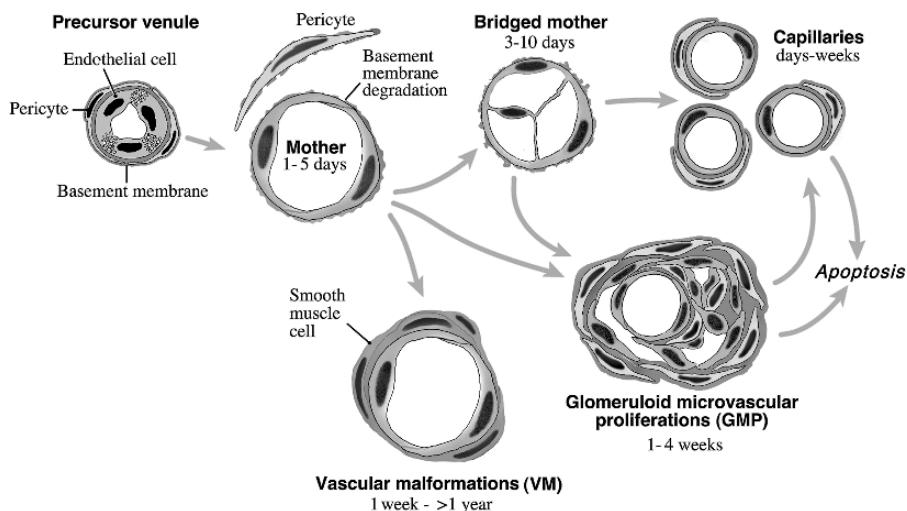


Fig. 3. Schematic diagram of mother vessel formation and subsequent evolution into daughter capillaries, glomeruloid microvascular proliferations, and vascular malformations (modified from 42). (Please see color insert.)

The different types of new blood vessels induced by VEGF-A and their properties will now be discussed in greater detail.

5.1. Mother Vessels

The term “mother” vessel was coined by Paku and Paweletz (51) to refer to the first type of new blood vessel to form in experimental tumors. MV are also the first type of new blood vessel to develop in response to VEGF-A¹⁶⁴; the other types of angiogenic vessels evolve from MV and thus may be properly regarded as “daughter” vessels (Fig. 3) (33,42,44,46). MV are enlarged, thin-walled, lightly fenestrated, pericyte-poor sinusoids that, as a consequence of their hyperpermeability and consequent loss of plasma, are commonly engorged with red blood cells (Figs. 1d, 42, 44). MV arise from preexisting venules by a three-step process of basement membrane degradation, pericyte detachment, and extensive enlargement. This process begins within hours of Ad-VEGF-A¹⁶⁴ administration, and, at least for the first 2 days, proceeds without significant endothelial cell division. Basement membrane degradation is an essential early step because vascular basement membranes are non-compliant (non-elastic) structures that do not allow microvessels to expand their cross-sectional area by more than approximately 30% (52), i.e., far less than the three- to fivefold enlargement in cross-section that characterizes the development of MV from normal venules. The specific proteases responsible for basement membrane degradation have not as yet been identified. Whether pericytes detach from MV by an active process or simply fall off as the result of basement membrane degradation is not known. Rapid vascular enlargement requires an increase in plasma membrane, and this is accommodated in part by membrane stored in VVOs. VVOs provide an abundant intracellular membrane store, one that corresponds to more than twice that of the plasma membrane of normal venular endothelium (8,20,44). As MV develop, the venular endothelial cell cytoplasm thins and VVOs decrease in both number and complexity as they contribute their

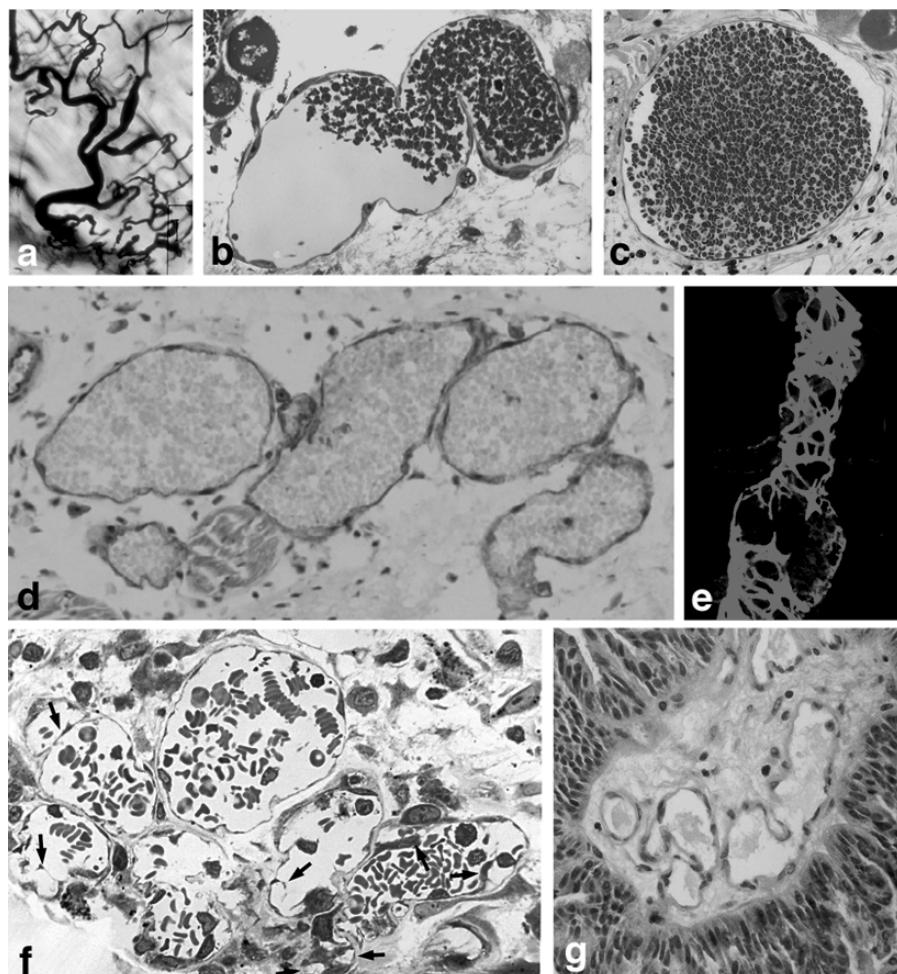


Fig. 4. (a–e) Mother vessels (MV) induced by Ad-VEGF-A¹⁶⁴ in mouse tissues. (a) Whole mount of colloidal carbon-perfused vascular bed. MV appear as enlarged segments of much smaller, normal venules. (b–d) MV are greatly enlarged, tortuous vessels with few pericytes that overexpress VEGFR-2 (d). (e) is a confocal microscopic image of a MV stained for pericytes with an antibody to (α -smooth muscle actin. Note incomplete pericyte covering, especially over segments of greatest vessel enlargement. (f, g) MV are the predominant type of blood vessel in many mouse tumors (here mouse MOT tumor (f)) and are also common in human tumors (here a human papillary carcinoma of the ovary (g)). Arrows in (f) indicate bridging (a, e, republished from 33; f, republished from 53). (Please see color insert.)

membrane to the greatly expanded plasma membrane (Figs. 1d,e, 6). In addition, as the endothelium thins, the intercellular cleft is shortened, and that portion of the intercellular cleft that is not comprised of specialized junctions (Fig. 1c) contributes to the luminal and abluminal plasma membrane.

MV are highly permeable to circulating macromolecules such as plasma proteins and fluoresceinated macromolecular dextrans and account to a large extent for the overall net hyperpermeability of the tumor vasculature (Fig. 5). As in normal venules exposed to VEGF-A and other permeabilizing mediators, circulating macromolecules

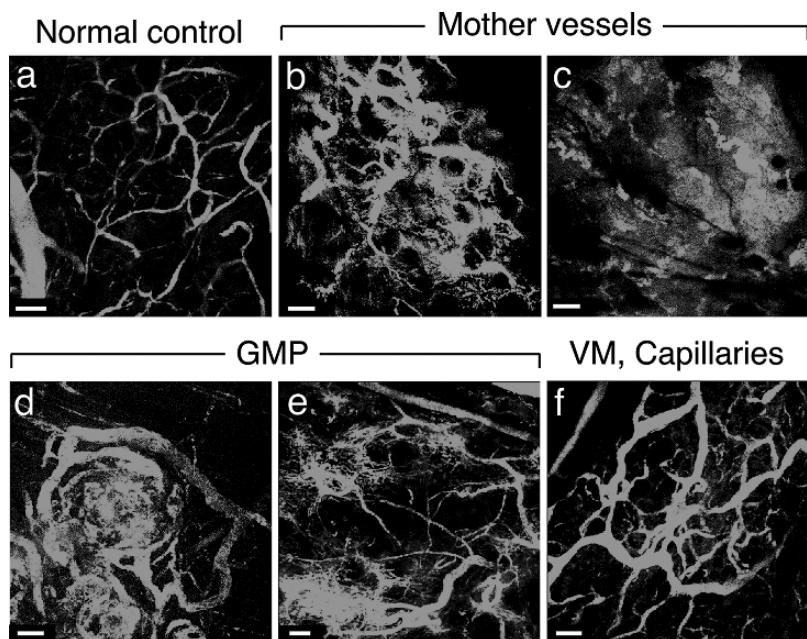


Fig. 5. Confocal microscopy 30 min after i.v. injection of macromolecular FITC-D at various times following injection of Ad-VEGF-A¹⁶⁴. (a) Normal, uninjected control ear vessels did not exhibit vascular leakage. (b, c) Mother vessels exhibited focal leakage on day 1 (b) and extensive leakage at 4 days (c) after Ad-VEGF-A¹⁶⁴ injection. (d, e) GMP with moderate leakage of FITC-D, days 21 and 28 respectively. (f) Vascular malformations (VM, large vessels) and capillaries (smallest vessels) did not leak FITC-D. Bars = 100 µm (republished from 44). (Please see color insert.)

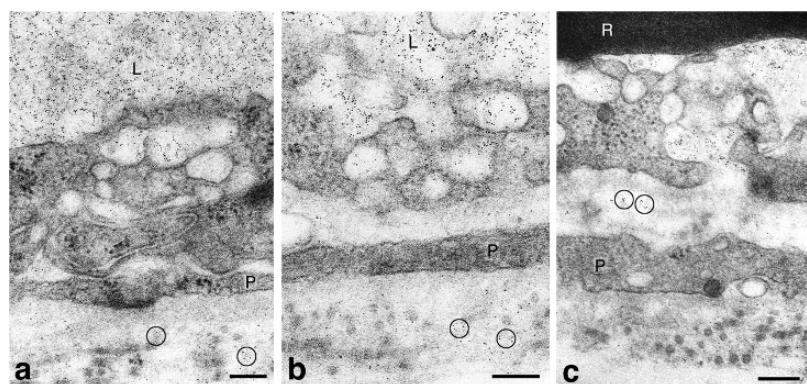


Fig. 6. Electron micrographs of mother vessel endothelial cells at 1–5 days after local injection of Ad-VEGF-A¹⁶⁴ and 30 min after i.v. injection of ferritin, a plasma protein and macromolecular tracer. Thinned endothelium shows ferritin (dark black particles) in vascular lumens (L) and in vesiculo-vacuolar organelle (VVO) vesicles/vacuoles and extravasated into the extravascular space. To facilitate viewing, some clusters of extravasated ferritin particles are encircled. L, lumen; P, pericytes; R, red blood cells. Scale bars: 200 nm (republished from 44).

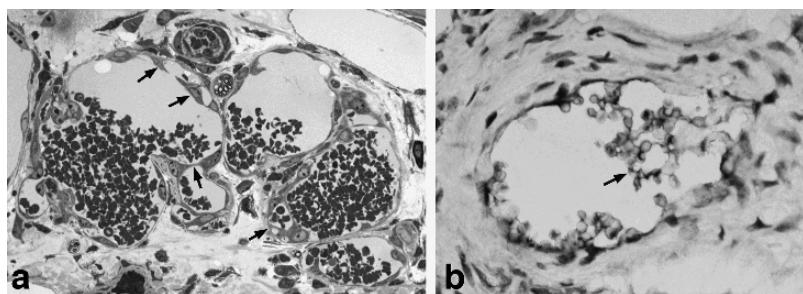


Fig. 7. Mother vessels undergoing bridging (arrows) in ear skin injected with Ad-VEGF-A¹⁶⁴ (**a**) and in a human renal cell carcinoma (**b**). (Please see color insert.)

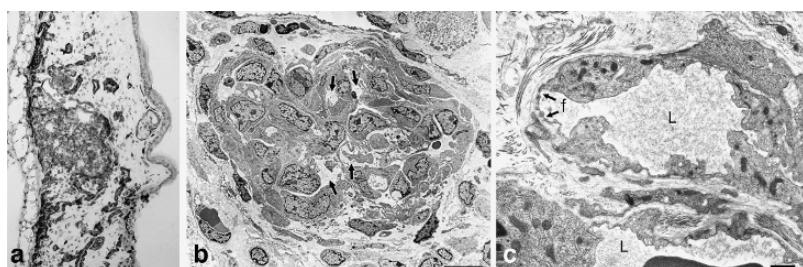


Fig. 8. Glomeruloid microvascular proliferations (GMP) induced by Ad-VEGF-A¹⁶⁴. (**a**) Immunoperoxidase staining for type IV collagen to illustrate extensive basal lamina reduplication. (**b**) Overview electron micrograph. Arrows indicate reduplicated basal lamina. (**c**) Higher magnification illustrates two small vascular lumens (L); note irregular endothelial cell thinning and fenestrations (**c**, arrows). Scale bars: **b**, 10 μ m; **c**, 1 μ m. (Please see color insert.)

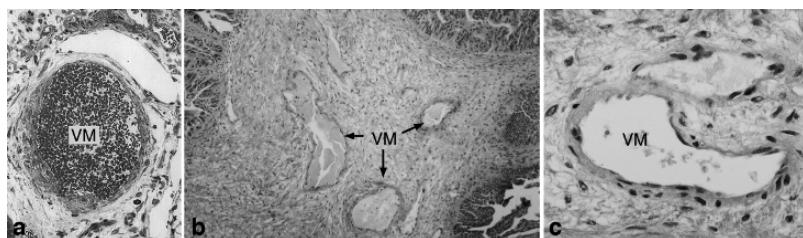


Fig. 9. Vascular malformations (VM) induced by Ad-VEGF-A¹⁶⁴ (**a**) and found in a human papillary ovarian carcinoma removed at surgery (**b**, **c**). (Please see color insert.)

such as ferritin extravasate through VVOs (Fig. 6). This may seem paradoxical in that mother vessel endothelial cells have fewer VVOs than normal venular endothelium. The answer to this conundrum apparently lies in the fact that mother vessel endothelium is greatly thinned and, although fewer in number, the VVOs remaining are less complex (have fewer vesicles and vacuoles); therefore, the transcellular pathway is greatly shortened and solutes need to pass through many fewer VVO vesicles and vacuoles to reach the extracellular space. Macromolecules also extravasate from MV by way

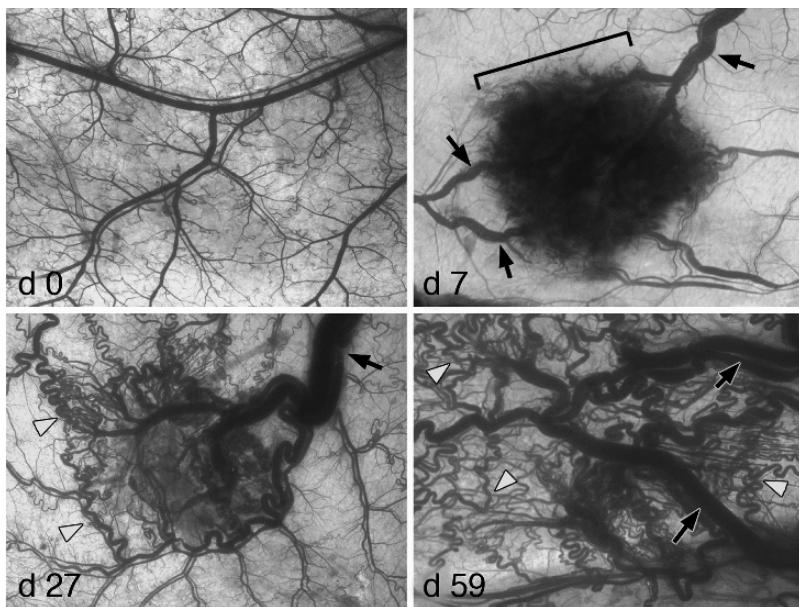


Fig. 10. Feeder arteries (FA) and draining veins (DV) induced in mouse flank skin at days indicated after injection of Ad-VEGF-A¹⁶⁴ are indicated by black arrows. Bracketed area at day 7 demarcates zone of angiogenesis. Over time, as VEGF-A¹⁶⁴ levels fall, MV and GMP undergo apoptosis; however, VM (yellow arrowheads), FA, and DV persist indefinitely, indicating that they are not dependent on exogenous VEGF-A¹⁶⁴. (Please see color insert.)

of fenestrae, though these cover <1% of the endothelial surface, and from openings in the endothelium that may be transcellular or intercellular (44).

MV are common in both animal and human tumors (Figs. 4f,g) (53). They are transitional forms, however, and require the continuing presence of exogenous VEGF-A for their maintenance; therefore, they are likely to be especially susceptible to anti-VEGF-A therapy. Over time, MV evolve into the several different types of daughter vessels (Fig. 3). The steps involved are fairly well understood, but the molecular events have not been elucidated nor is it known why different MV evolve into different types of daughter vessels.

5.2. Bridged MV and Capillaries

One mechanism by which MV evolve into capillaries involves intraluminal bridging, a process that was originally discovered in tumor vessels (54). A similar process was subsequently found in healing myocardial infarcts (55) and in skeletal muscle exposed to chronic vascular dilators (56). Endothelial cells extend cytoplasmic processes into and across mother vessel lumens, forming transluminal bridges that divide blood flow into multiple smaller sized channels (Figs. 1e, 4f, 7). This finding was unexpected in as much as mammalian cells migrate on surface matrices and would not be expected to extend processes into a rapidly flowing stream of blood. In fact, they are probably not doing so. First, because they are highly permeable to plasma, MV have little retained plasma, their lumens are packed with red blood cells (Figs. 1d, 4, 7a), viscosity is increased, and blood flow is greatly slowed. Second, intravascular clotting is favored

both by sluggish blood flow and by virtue of the fact that VEGF-A upregulates endothelial cell expression of tissue factor (57). For these reasons, MV induced by Ad-VEGF-A¹⁶⁴ and by tumors commonly undergo thrombosis. Thus, in bridge formation, endothelial cells are likely migrating on an intraluminal fibrin matrix; that is, bridging may be analogous to the well-known process by which thrombosed vessels undergo recanalization. Whatever the mechanism, the smaller channels separate from each other to form individual, smaller-caliber capillaries that are, as far as is known, normal in structure.

5.3. Glomeruloid Microvascular Proliferations

GMP, also referred to as glomeruloid bodies, are poorly organized vascular structures that resemble renal glomeruli (hence the name) (42, 58, 59) (Fig. 8). They are found in a wide variety of human tumors, particularly glioblastoma multiforme but also cancers of the stomach, breast, and so on. All of the human tumors known to form GMP express VEGF-A, and tumors such as glioblastoma multiforme that make unusually large amounts of VEGF-A are among those that most commonly induce GMP. GMP are permeable to plasma and plasma proteins but, because they are poorly perfused, account for much less plasma extravasation than MV. Recently, it has been reported that GMP correlate with poor prognosis in breast cancer (59, 60).

In response to Ad-VEGF-A¹⁶⁴, nascent GMP first appear as focal accumulations of large, poorly differentiated, CD31- and VEGFR-2-positive cells in the endothelial lining of MV (42, 58). The source of these cells, whether from local mother vessel endothelium or from circulating endothelial progenitor cells, is not known. Whatever their source, these cells proliferate rapidly, extending inwardly into mother vessel lumens, and also outwardly into the surrounding extravascular matrix. In this manner, they encroach upon and compress the MV from which they arose, eventually dividing single large mother vessel lumens into multiple, much smaller channels that barely admit the passage of red blood cells. For a time, the great majority of cells comprising GMP continue to express endothelial cell markers. However, as they grow in size, cells expressing pericyte markers and ultrastructural characteristics also appear (58). Macrophages may also accumulate peripherally. An additional prominent feature is deposition of an abundant abnormal multilayered basal lamina. GMP require the continued presence of exogenous VEGF-A¹⁶⁴ for their maintenance. As adenoviral vector-derived VEGF-A¹⁶⁴ expression declines, GMP, like MV, undergo apoptosis and progressively devolve into smaller, normal-appearing capillaries (58).

5.4. Vascular Malformations

Thin-walled MV lacking adequate pericyte and basement membrane support are in danger of thrombosis or collapse. While some MV avoid these fates by evolving into capillaries or GMP, others maintain their large size by acquiring an irregular supporting coat of smooth muscle cells (Fig. 9). Such stabilized MV are readily distinguished from normal arteries and veins by their inappropriately large size (for their location) and by their thinner and often asymmetric muscular coat. Vessels of this description closely resemble the non-malignant VM that occur, for example in skin, brain, and so on (61), suggesting a mechanism by which such malformations may form. As their structure implies, VM are not permeable to plasma proteins (Fig. 5f). Also unlike MV

and GMP, VM persist indefinitely, long after adenoviral vector-induced VEGF-A¹⁶⁴ expression has ceased. Thus, VM have attained independence from exogenous Ad-VEGF-A¹⁶⁴, though it is quite possible that they are supported by VEGF-A secreted by the smooth muscle cells that closely envelop them. This independence from exogenous, tumor-secreted VEGF-A has important implications because these vessels would not be expected to be susceptible to anti-VEGF-A antibodies such as Avastin.

5.5. Feeder Arteries (FA) and DV

It is now clear that the arteries that supply and the veins that drain Ad-VEGF-A¹⁶⁴ injection sites (Fig. 10) and mouse and human tumors (Fig. 11) are not normal as had been supposed (11). Rather, they are strikingly abnormal vessels, greatly enlarged in size and often tortuous. They are to a large extent located outside the tumor mass and result from the dual processes of arteriogenesis and venogenesis. Relatively few in number, as compared with the intra-tumor microvessels induced by angiogenesis, they would seem to offer an attractive therapeutic target, because their ablation would be expected to cut off the tumor blood supply more efficiently than would attacks on the much more numerous downstream angiogenic vessels that are the target of current antiangiogenesis therapy. FA and DV have long been appreciated by ophthalmologists,

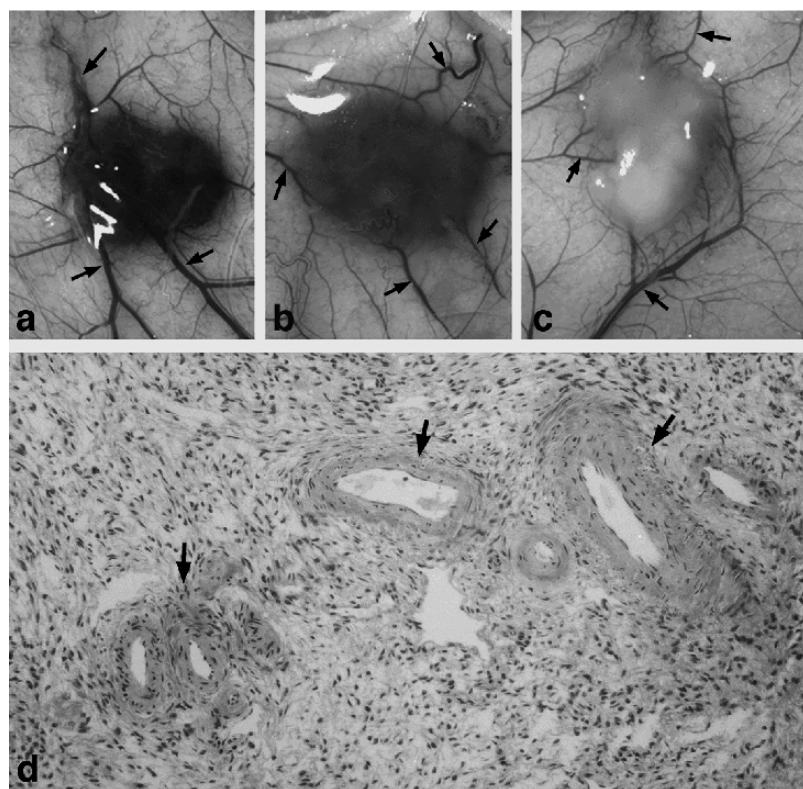


Fig. 11. Feeder arteries (FA) and draining veins (DV) induced by three different mouse tumors (**a**, B16 melanoma; **b**, TA3/St mammary carcinoma; and **c**, MOT ovarian cancer) and by a human bladder cancer (**d**, arrows). (Please see color insert.)

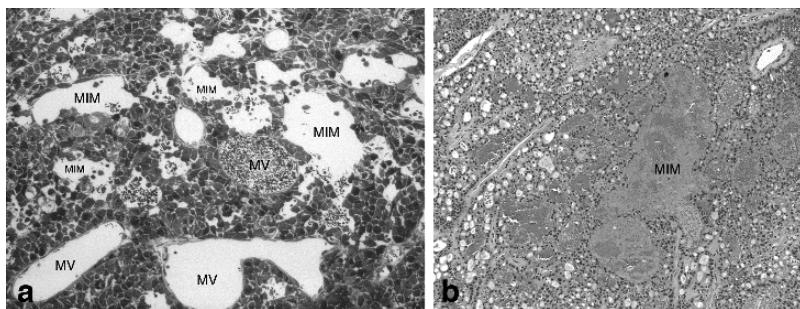


Fig. 12. Vascular mimicry (MIM) in B16 melanoma (a) and a human renal carcinoma removed at surgery (b). MV, typical mother vessels. (Please see color insert.)

because, as they supply and drain retinal tumors, they stand out strongly in comparison with the normal, much smaller arteries and veins of the surrounding normal retina (62–64). However, FA and DV are not exclusive to ocular tumors and occur in all tumors that have been imaged with vascular casts or by radiological techniques.

5.6. Vascular Mimicry and Mosaic Vessels

In some tumors, red blood cell-filled spaces appear that are apparently lined by tumor cells instead of endothelial cells (Fig. 12) (65–67). Such spaces have been referred to as examples of vascular mimicry, i.e., red blood cell-filled channels that resemble blood vessels but that are lined by tumor cells rather than endothelium. It has further been postulated that the lining tumor cells have acquired some of the structural and molecular properties of endothelial cells. That such spaces exist in certain tumors is not in question, but their significance is much debated (68). The issue hangs on whether these spaces are part of a functional vascular network or whether they simply reflect sites of hemorrhage into tumors from leaky or damaged but otherwise conventional blood vessels.

6. SUMMARY

In summary, the tumor vasculature is heterogeneous and highly abnormal with respect to organization, structure, and function. Physiological angiogenesis, though as yet poorly understood, results from the balanced secretion, in appropriate amounts and sequence, of many different cytokines and inhibitors including VEGF-A. By comparison, tumor angiogenesis is now fairly well understood and results from the unbalanced secretion of a small number of growth factors, particularly VEGF-A^{164/5}. Ad-VEGF-A¹⁶⁴ induces at least five distinctly different types of tumor surrogate microvessels and, either directly or indirectly, stimulates arteriogenesis and venogenesis to generate FA and DV. Tumor and surrogate tumor blood vessels induced by VEGF-A¹⁶⁴ develop according to a highly ordered and consistently reproducible sequence of events that is largely independent of the tissues in which they arise. All of these vessel types are found in human tumors, though not all in the same tumors. The hyperpermeability of two types of tumor blood vessels, MV and GMP, results in extravascular deposition of a fibrin provisional stroma that supports and further stimulates angiogenesis. Over time, this provisional stroma is replaced by vascular connective tissue

(33, 69–72). Some of the newly formed vessels (MV and GMP) depend for their survival on a continuing supply of exogenous VEGF-A, whereas others (VM, FA, and DV) survive indefinitely long after production of exogenous VEGF-A has ceased. This finding has important implications for tumor therapy because it predicts that MV and GMP will be susceptible to anti-VEGF-A therapies such as Avastin, whereas VM, FA, and DV will not be responsive.

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13

Lymphatic System in the Pathology of Cancer

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SUMMARY

Blood vessels in general and micro-vessels in particular, must be maintained in a highly dynamic state. The ability of the circulation to alter vessel permeability during inflammation or to repeatedly grow and regress during the female reproductive cycle are but two examples of such dynamism. One consequence that stems from above considerations is that the microcirculation cannot be both flexible and completely impermeable to plasma. Thus, certain amount of plasma escapes from blood vessels into the interstitial tissue under normal conditions and this leakage is greatly increased during pathological states. The lymphatic system represents a parallel vasculature whose primary function is to return excess interstitial fluid to the circulation. The lymphatic system also acquired a secondary function as a conduit of leukocytes from the periphery to lymph nodes thus facilitating immune response to pathogens. This function is frequently subverted by cancer cells that invade tumor-associated lymphatic vessels and migrate to the regional lymph nodes. The presence of tumor cells in lymph nodes is an important clinical predictor of disease severity and prognosis. In some cancers, lymphatic invasion also represents a route of tumor dissemination to distant organs.

Key Words: endothelial cells; Lymph; Lymph Nodes; Lymphatic Vessels; Lymphoid Tissues; Cancer; Development; Metastasis.

1. INTRODUCTION

The need to maintain fluid balance is the likely force behind the existence of lymphatic vasculature. However, the lymphatic system of mammals also includes lymphoid organs such as lymph nodes, tonsils, spleen, and thymus that play crucial roles in the trafficking, maturation, and function of white blood cells. It is probable that the lymphatic and immune systems co-evolved to utilize a common set of vessels and structures.

For most people, an enlarged lymph node is a sign of a normal immune response to an infectious agent. However, an enlarged lymph node may herald a much darker reality—the presence of cancer and the initiation of the process of metastasis, a word

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of Greek origin meaning “a change of place.” The complex set of events that constitute metastasis leads to the establishment of tumor foci in distant organs and, all too frequently, the eventual death of the cancer patient (reviewed in 1). Since lymphatic capillaries evolved to facilitate fluid and immune cell entry from the tissues, they are commonly the first structures invaded by the cancer cells that acquired a malignant phenotype. This chapter summarizes the structure and function of the lymphatic system, its involvement in the dissemination of cancer, and new approaches being developed to limit the progression of malignancies by targeting the growth of lymphatic vessels.

2. INTRODUCTION TO THE LYMPHATIC SYSTEM

The cardiovascular system is in essence a closed, pressurized fluidic system. Its mechanical equivalents such as the cooling system of a car can be designed to minimize the loss of fluid without sacrificing function. The vertebrate circulatory system, on the other hand, is of necessity a product of a compromise. Blood vessel walls must possess sufficient integrity to prevent loss of blood into tissues while maintaining adequate plasticity to permit embryonic development, regeneration of damaged vessels, and the ability to increase permeability in response to injury or infection. Furthermore, the endothelial lining of capillary walls must be sufficiently thin to allow passive transport of gases and dissolved minerals. This compromise dictates that vertebrate circulation must be able to accommodate a degree of normal, low-level leakage. The

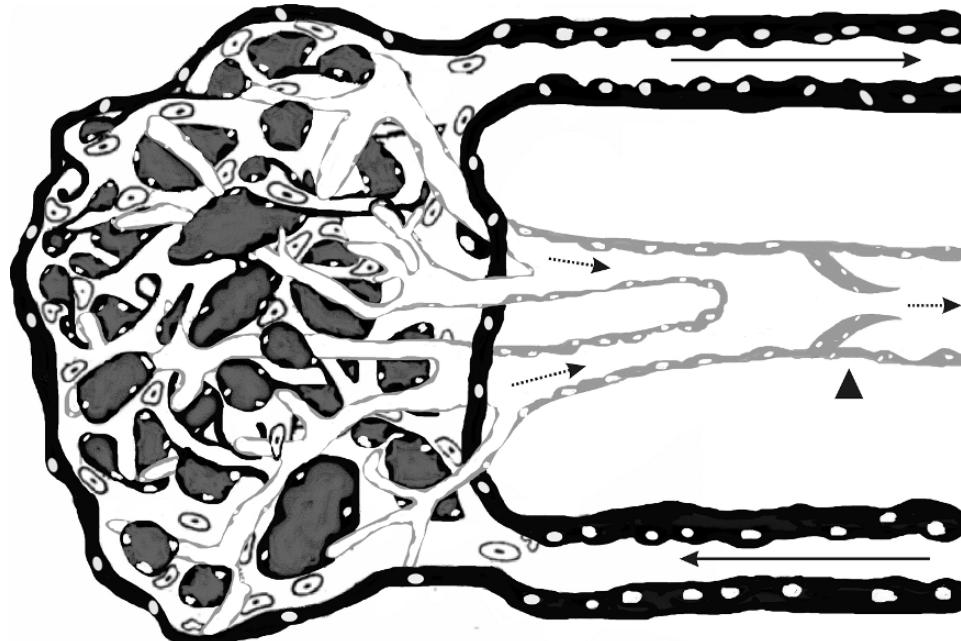


Fig. 1. Spatial relationship of the venous and lymphatic capillary plexuses. Venous circulation enters via an arteriole (arrow) and exits through a venule (dotted arrow). Blind-ended lymphatic capillaries are shown in gray and the unidirectional flow of lymph is shown with dashed arrows. The lymphatic capillaries are characterized by thinner walls and larger lumens than venous capillaries. A bicuspid valve (arrowhead) in a collecting lymphatic vessel assures unidirectional flow of lymph.

fluid that consequently forms in the interstitial space is lymph, a protein-rich exudate that excludes the cellular components of blood. To deal with the need to channel lymph back to the circulation, vertebrates evolved a parallel network of vessels, called the lymphatic system, which directs a unidirectional flow of lymph from blind-ended capillaries within tissues into a coalescing network of larger collecting vessels (Fig. 1). The terminal and largest of these lymphatic vessels, the thoracic duct, returns the lymph to the circulation at the inferior vena cava.

3. DEVELOPMENT AND STRUCTURAL FEATURES OF LYMPHATIC ENDOTHELIUM

The historical underpinning of our understanding of development of the lymphatic system has been described previously (2 and references therein). Briefly, the discovery of the lymphatic system by the Italian anatomist Gasparo Aselli in the seventeenth century coincided with the elucidation of blood circulation by William Harvey. During ontogeny, the lymphatic capillaries first sprout from primitive sacs that form on one side of the cardinal vein around embryonic days 9.5–10.5 in mice or approximate embryonic days 45–50 in humans.

The endothelial cells lining the blood and lymphatic vessels differ on the molecular and structural levels. The genetic basis for the commitment of the blood endothelial cells (BECs) of the cardinal vein to differentiation into phenotypically distinct lymphatic endothelial cells (LECs) is regulated by the transcription factor Prox1, a homolog of the *Drosophila* homeobox gene *prospero*. Embryos of Prox1-null mice are not viable and completely lack lymphatic vasculature, while ectopic expression of Prox1 reprograms BECs to adopt a lymphatic phenotype (3 and references therein).

Recent establishment of near-homogeneous cultures of BECs and LECs has allowed comparative microarray analyses of the genes expressed by these two subtypes of endothelial cells. As expected from their close developmental relationship, BECs and LECs express the majority of genes investigated at similar levels. These studies have also revealed a number of novel markers differentially expressed on LECs whose function in lymphatic vessels remains largely unknown (4).

The recent rapid growth of investigation into the biology of lymphatic vessels was initiated by the discovery of markers that allowed researchers to differentiate lymphatic and vascular capillaries. The most frequently used of these markers, the endothelial hyaluronan receptor-1 or LYVE-1 is expressed by LECs, macrophages, and a limited number of other tissues (5). The function of LYVE-1 is unclear since LYVE-1-deficient mice have no lymphatic abnormalities (G. Thurston, (6)). The transcription factor Prox-1 is the most specific lineage marker for lymphatic endothelium (2). Another important marker of LECs that is not expressed by BECs is a mucin-type transmembrane glycoprotein, podoplanin (7). Although its biological function is unknown, podoplanin-null mice are characterized by dilated lymphatic vessels, impaired lymphatic transport, and consequent lymphedema (8).

4. LYMPHATIC FLUID TRANSPORT

The physiology of lymphatic transport has been intensely investigated (9, 10 and references therein). A portion of extravasated fluid and macromolecules is reabsorbed by the venules, but the remaining fluid in the interstitial space is absorbed by lymphatic

capillaries. The rate of interstitial transport of fluid and macromolecules is controlled by the osmotic pressure exerted by the proteoglycan density and the charge within the extracellular matrix (ECM). The blind-ended initial lymphatic capillaries possess unique adaptations that facilitate the uptake of lymph (Fig. 1). These capillaries have overlapping cell–cell junctions that remain open against the force of interstitial fluid pressure by the tension of anchoring filaments that attach the LECs to the collagen fibers in the surrounding ECM and act to widen the lumens of these vessels. Larger collecting lymphatic vessels but not initial lymphatics are surrounded by a basement membrane and a layer of smooth muscle cells. The unidirectional movement of lymph from the capillaries into progressively larger collecting lymphatic vessels results from combined action of bicuspid luminal valves (Fig. 1) (11) and peristaltic contraction of smooth muscle cells in vessel walls. These forces, together with the pressure from surrounding skeletal muscles, propel lymph into the thoracic duct that exhibits an autonomous pumping motion.

It is important to briefly review the role of the vascular endothelial growth factor (VEGF) family of receptor tyrosine kinases (RTKs) in the regulation of lymphangiogenesis to facilitate the discussion of efforts toward therapeutic inhibition of these receptors (Fig. 2). The reader is referred to Chapter 1 of this book and to excellent recent reviews for detailed analysis of the complex interaction of signaling pathways that regulate the development and postnatal function of the lymphatic system (12, 13).

As seminal achievement in understanding the molecular control of lymphangiogenesis was the discovery of the RTK VEGFR-3 previously known as Flt-4 (14). VEGFR-3 is the only RTK whose expression in normal adult tissues is largely restricted to the lymphatic endothelium (15). The role of VEGFR-3 signaling in initiating lymphangiogenesis following activation by its ligands, VEGF-C and VEGF-D, is well established (16). In contrast, lymphangiogenic role of VEGFR-2, an RTK that is the principal regulator of blood angiogenesis, has been controversial. Analysis of the relative contribution of VEGFR-2 versus VEGFR-3 toward the activation of LECs by VEGF-C and VEGF-D has been complicated by two factors. First, the specificity and affinity of binding of VEGF-C and VEGF-D are influenced by the degree of proteolytic processing of these growth factors. Nascent VEGF-C and VEGF-D specifically bind to VEGFR-3. Proteolytic cleavage of the N- and C-terminal regions of these proteins liberate mature VEGF-C and VEGF-D which acquire increased affinity for VEGFR-3 as well as the ability to bind to and activate VEGFR-2 (17, 18). Second, VEGFR-2 and VEGFR-3 are capable of heterodimerization, and the pattern of tyrosine phosphorylation in the C-terminal region of VEGFR-3 differs in homodimers and heterodimers (19).

5. LYMPHATIC PATHOLOGY

Impairment of lymph transport, irrespective of its biological basis, results in lymphedema, usually a non-fatal but disfiguring and disabling accumulation of fluid in the interstitial space. In the context of this chapter, a brief discussion of lymphatic dysfunction is warranted since therapeutic approaches aimed at reducing pathological lymphangiogenesis have the potential of precipitating lymphedema.

Primary lymphedema refers to a rare group of hereditary or developmental disorders early onset, characterized by dilated lymphatic capillaries and interstitial accumulation of lymph fluid (10). Mutations in the genes coding for VEGFR-3 gene and the

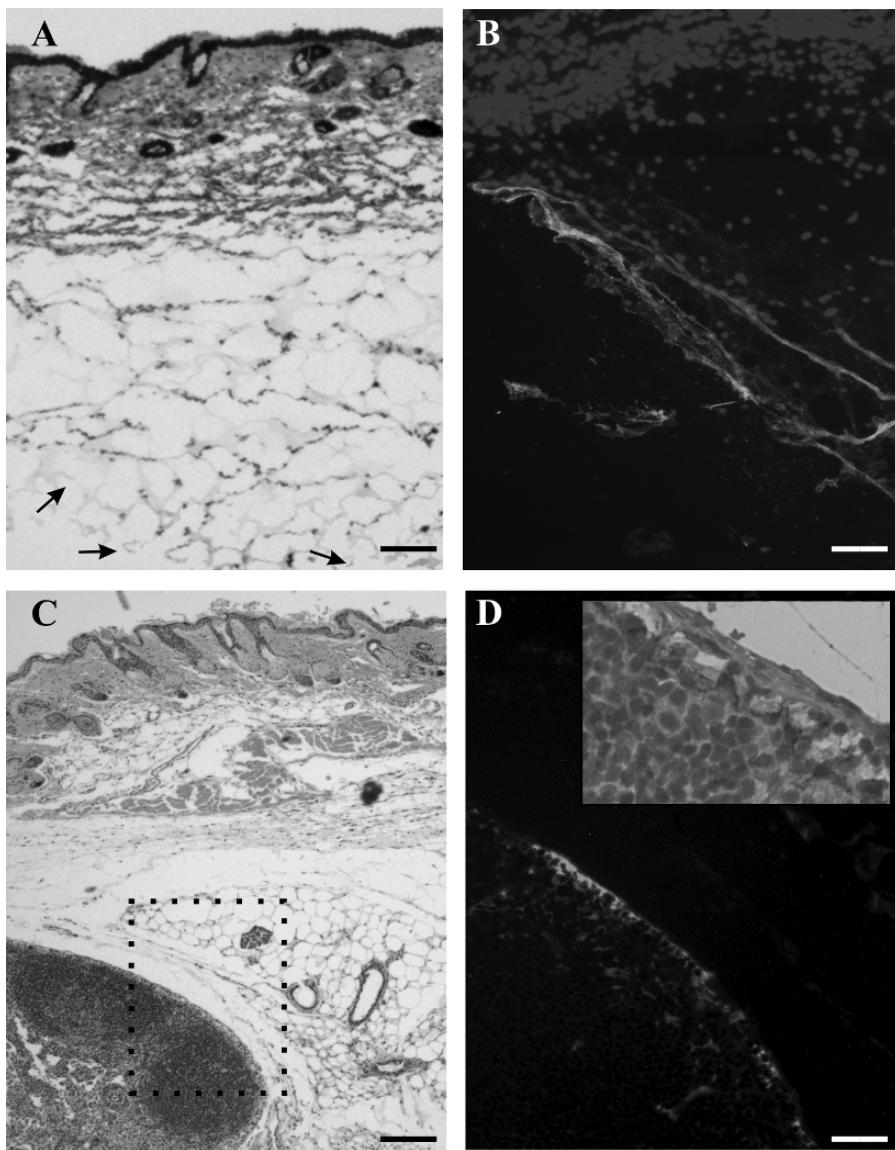


Fig. 2. Induction of lymphangiogenesis is illustrated by an experiment in which myeloma cells that produce high levels of VEGF-C were implanted subcutaneously into mice in Matrigel. Panels A and B: Serial sections, day 7. A. Lymphatic endothelial cells in the sub-dermal vessels respond by proliferating, invading the Matrigel and organizing into interconnected tubes. Blind-ended tips of lymphatic capillaries are shown by arrows. B. Functional state of the capillaries in A is shown by lymphangiography with a large-molecular weight fluorescent dextran injected into the center of the Matrigel plug. Newly formed lymphatic capillaries coalesce and drain the tracer towards the dermis. Panels C and D: serial sections, day 14. C: Tumor nodules within Matrigel. D. A high power view of a region corresponding to the area indicated by the rectangle in C. A pronounced ring of lymphatic capillaries surrounds the tumor nodule and contains the fluorescent tracer. Insert: Lymphatic endothelial cells at the tumor edge stain for the lymphatic marker podoplanin. Size bar: A, 100 μ m; B-D, 20 μ m. (Please see color insert.)

transcription factors FOXC2 and SOX18 have been identified as the initiating lesions for several primary lymphademas, but the genetic basis for majority of cases has not been established (13 and references therein).

Worldwide, the most common cause of secondary or acquired lymphadema results from the obstruction of lymphatic vessels with parasitic filarial worms (20), while in the developed world, secondary lymphadema is primarily a complication of surgery. Recently, sophisticated techniques to identify the sentinel lymph node (SLN), the first node draining the primary tumor in the regional basin, have become the standard of care and minimize the extent of resection of lymph nodes and the severity of regional lymphadema especially during breast cancer surgery (21). There is also an ongoing effort to develop gene therapy for human lymphedema which has not yet advanced beyond preclinical models (22 and references therein).

Rarely, developmental errors in lymphangiogenesis result in lymphangiodisplasia, an abnormal growth of lymphatic vessels with typically neonatal manifestation (10 and references therein). Malignancies of the lymphatic system are extremely uncommon. Lymphangiosarcoma (Stewart–Treves syndrome) is a rare but extremely lethal cancer that occurs exclusively in a setting of post-mastectomy upper extremity edema (23). Kaposi's sarcoma (KS) is considered a neoplasm of lymphatic endothelium that results from the infection of LECs with KS-associated herpesvirus (human herpesvirus-8, KSHV). Most frequently, KS manifests in patients infected with the human immunodeficiency virus (HIV). Several lines of evidence suggest potential lymphatic origin of the KS spindle cells. Infection of differentiated BECs with KSHV leads to their reprogramming to a LEC phenotype characterized by expression of the major lymphatic endothelial genes including Prox1, VEGFR-3, and podoplanin (24).

6. LYMPHATIC VASCULATURE AND CANCER

Carcinomas, by far the most common form of human cancer, show a strong predilection for dissemination to regional lymph nodes. The degree to which affected lymph nodes act as obligatory conduits to further tumor dissemination is controversial and most likely varies in different cancers (25). Nevertheless, it is well accepted that the presence of tumor in the lymph nodes is an adverse prognostic factor. The expression of pro-lymphangiogenic growth factors VEGF-C and VEGF-D and their receptor VEGFR-3 in clinical samples of tumors has been correlated with increased pathological scores of lymphatic vessel invasion (LVI) and lymphatic vessel density (LVD) (26 and references therein). Not surprisingly, increased LVI and LVD scores are predictive of the relative risk of lymph node metastasis in many types of human cancer (27 and references therein). More importantly, several studies showed that elevated expression of VEGF-C and VEGF-D is a poor prognostic factor for cancer patients. High expression of VEGF-C correlates with poor outcome in melanoma (28), ovarian (29), and cervical cancer (30). Similarly, elevated expression of VEGF-D is a marker of poor prognosis in ovarian carcinoma (31) and colorectal cancer (32). Overexpression of VEGF-D and VEGFR-3 are independent markers of poor outcome for patients with gastric adenocarcinoma (33).

These clinical observations have been recapitulated by experimental approaches in which tumor cells engineered to overexpress VEGF-C or VEGF-D were implanted into mice. The increase in intratumor and peritumor LVD was correlated with the extent of

tumor dissemination to regional lymph nodes and distant organs using human breast (34), melanoma (35), and embryonic kidney (36) cell lines. In RipTag2 mice that normally develop non-invasive β -cell insulinomas, concurrent expression of VEGF-C by the β -cells led to peritumor lymphangiogenesis and metastases to the draining lymph nodes (37).

6.1. Lymphangiogenic Growth Factors and Lymphatic Vessels as Targets for Anti-Cancer Therapy

The growing understanding of the molecular regulation of LEC biology and the availability of specific markers for these cells greatly facilitated efforts to develop and test anti-lymphangiogenic therapies in animal models (reviewed in 38). Some small-molecule tyrosine kinase inhibitors that are currently in clinical trials as anti-cancer agents have been shown to inhibit the VEGFR-3 kinase. Typically, such compounds also inhibit other closely related RTKs such as VEGFR-2 and the platelet-derived growth factor (PDGF) receptor, and it is impossible to determine what clinical benefits as well as toxicities seen in cancer patients treated with RTK inhibitors stem from anti-lymphangiogenic activity of these molecules (reviewed in 39).

Specific targeting of lymphatic vessels in cancer has been limited to date to preclinical studies in animals and involves the use of biological macromolecules such as soluble receptors and monoclonal antibodies (mAb) (Fig. 2). The first studies that attempted to inhibit the proliferation of lymphatic vessels in mouse tumor models were designed to reduce the effective concentration of the lymphangiogenic growth factors VEGF-C and VEGF-D in blood and tissues. The two approaches taken in these studies, the use of soluble receptors and mAbs to neutralize lymphangiogenic growth factors, have been validated in clinical use. For example, soluble version of the receptor for tumor necrosis factor (TNF) is used to treat rheumatoid arthritis (40) and a neutralizing mAb to VEGF-A is the first approved form of anti-angiogenic therapy for cancer (see Chapter 19).

Implantation of a variant of the breast cancer cell line MCF-7 engineered to express VEGF-C into the mammary fat pads of immunodeficient mice leads to enhanced peritumor lymphangiogenesis and metastasis to draining lymph nodes that were reversed by treatment with soluble VEGFR-3 (sVEGFR-3) (41). Likewise, lymphatic metastasis of the HEK 293 kidney cells expressing VEGF-D was prevented by a neutralizing mAb to this growth factor (36, 42).

It is important to note that the therapeutic effect of neutralization of VEGFR-3 ligands with sVEGFR-3 is not limited to situations where ligand overproduction is a consequence of experimental manipulation of the tumor cells. In several independent experiments, metastasis to the regional lymph nodes of highly metastatic tumor cell lines was significantly reduced by co-expression of sVEGFR-3. First, transfection of the human lung cancer cell line NCI-H460-LNM35 with an expression construct for sVEGFR-3 resulted in significant reduction of the number of intratumor lymphatic vessels in the tumor xenografts and in consequent reduction of metastasis to draining lymph nodes (43). Similar effects were obtained when expression cassettes for sVEGFR-3 were introduced into viral vectors and injected into tumor-bearing mice (43, 44). Second, rat MT-450 mammary tumor cells that expressed sVEGFR-3 exhibited lower metastasis formation in the draining lymph nodes as well as in the lungs

compared to parental tumor cells (45). Finally, Chen and colleagues (46) used small interfering RNA-mediated silencing of the VEGF-C gene in a mouse mammary tumor model to inhibit lymphangiogenesis as well as lymph node and lung metastasis.

Interpretation of animal models that depend on the neutralization of VEGF-C and VEGF-D is complicated by the unique manner in which the specificity of binding of these factors to VEGFR-2 and VEGFR-3 is regulated (see above). Neutralization of mature, proteolytically processed forms of these ligands might be expected to have anti-angiogenic activity by downmodulating signaling of VEGFR-2 as well as anti-lymphangiogenic activity by reducing signaling via VEGFR-3, VEGFR-2, or the heterodimers of these two RTKs.

An alternative experimental approach to the inhibition of lymphangiogenesis that promises to offer greater *in vivo* specificity involves the use of antagonist mAbs to block the activation of VEGFR-3 and/or VEGFR-2 by VEGF-C and VEGF-D. Clinical validation for this approach was provided by the introduction into clinical use in oncology of mAbs that target the EGF receptor (reviewed in 47).

Pioneering work on this approach was made possible by the generation of a first mAb to VEGFR-3 (AFL-4) with apparent antagonist properties (48). Histological studies of mouse tissues after embryonic day 17 demonstrated lymphatic specificity for AFL-4 in normal tissues. In this study, the primary effect of treatment of tumor-bearing mice with AFL-4 was a reduction in the rate of tumor growth via an anti-angiogenic mechanism (see below) (48). AFL-4 was also shown to inhibit lymphangiogenesis in the mouse cornea assay in which the production of VEGF-C was induced by implantation of the fibroblast growth factor-2 (FGF-2; 49). A more recent study confirmed the ability of AFL-4 to bind strongly to murine VEGFR-3 but failed to detect an antagonistic activity for this mAb (50). Thus, the *in vivo* activity of AFL-4 might be due to an indirect inhibitory mechanism such as an increased turnover of VEGFR-3.

We have recently reported the development of novel mAbs that bind to VEGFR-3 and block the binding of VEGF-C and VEGF-D. The mAb to murine VEGFR-3 (mF4-31C1) has been shown to potently inhibit the binding of VEGF-C to VEGFR-3 and to effectively antagonize VEGFR-3 activation (50). A fully human mAb to human VEGFR-3 (hF4-3C5) with similar inhibitory potency to mF4-31C1 has also been produced and represents a potential therapeutic for use in clinical trials in cancer patients (51). mF4-31C1 is a proof-of-concept mAb for use in preclinical studies in mice whose development was necessitated by the lack of cross-reactivity of mAb hF4-3C5 with murine VEGFR-3. *in vivo* activity of mF4-31C1 was first shown by its ability to block regeneration of adult lymphatic vessels (50) and to induce regression of lymphatic capillaries in neonatal mice (52).

The mAb hF4-31C1 has been increasingly used in preclinical studies of lymphatic metastasis. In an orthotopic model of breast cancer that utilized a highly metastatic tumor cell line engineered to overexpress VEGF-C, metastasis to regional lymph nodes and lungs was more potently inhibited by mF4-31C1 than by the mAb DC101 that targets murine VEGFR-2, although DC101 was much more potent in reducing the growth of primary tumors and lung metastases (53). A similar finding was made by treating mice implanted with HEK 293 kidney cells overexpressing VEGF-D with either mAbs mF4-31C1 or DC101 (S. Stacker and M. Achen, personal communication). Furthermore, systemic administration of mF4-31C1 was able to reduce the hyperplasia of collecting lymphatic vessels in the mouse ear induced by the implantation of tumors

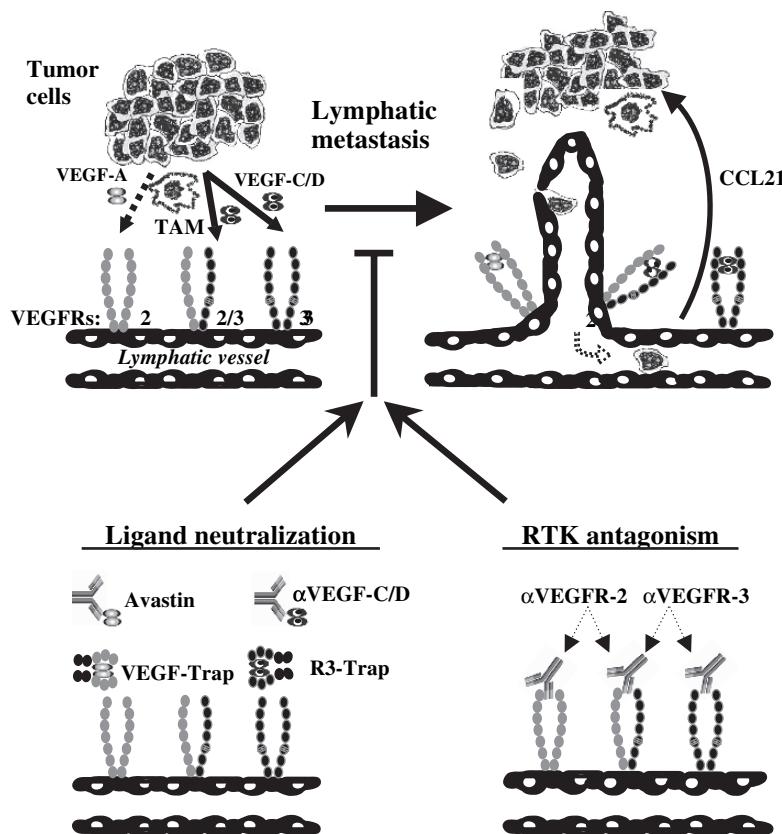


Fig. 3. Blockade of VEGFR-3 and/or VEGFR-2 activation on LECs reduces lymphatic tumor metastasis. Top: VEGF-A and VEGF-C and CEGF-D produced by either tumor cells or tumor - associated macrophages (TAMS) activate VEGFR-2, VEGFR-3, and VEGFR-2/3 heterodimers on LECs. Activated LECs proliferate, migrate, and sprout, facilitating entry of tumor cells into the lymphatic vessels. Tumor cells can be actively attracted to LECs by the chemokine CCL21. Bottom: Neutralization of growth factors (left) or blockade of VEGFR-3 and/or VEGFR-2 with antagonist mAbs and kinase inhibitors (right) prevents the lymphangiogenic response, reducing the extent of invasion of lymphatic vessels by the tumor cells and metastasis to draining lymph nodes. LECs, lymphatic endothelial cells; mAbs, monoclonal antibodies; VEGFR, Vascular endothelial growth factor receptor.

overexpressing VEGF-C at the tip (Fig. 4). The reduction in lymphatic vessel hypertrophy coincided with potent inhibition of the delivery of tumor cells shed from the primary tumor to the cervical lymph node at the base of the ear (54). These results are encouraging since tumor cell invasion of the lymphatic vessels has been correlated with the activation of LEC mediated by signaling of VEGFR-3 (55).

It warrants mentioning at this point that antagonist mAbs to VEGFR-3 might have the potential to also inhibit tumor angiogenesis by themselves or in conjunction with antibodies that either block VEGFR-2 or neutralize VEGF-A. VEGFR-3 expression has been detected on blood capillaries within tumors (56, 57). The relevance of these findings was demonstrated by a study in which mice bearing tumors showed dose-dependent inhibition of tumor growth after treatment with the VEGFR-3-specific

mAb AFL-4. This treatment resulted in the disruption of the lining of postcapillary venules within the tumors and the appearance of micro-hemorrhages (48). Similar anti-angiogenic effect of targeting VEGFR-3 was seen in an orthotopic model of breast cancer (53) and in our recent studies (B. Pytowski, unpublished data).

Recently, encouraging findings based on advances in experimental approaches to the study of metastasis in animals have offered rational basis for the eventual clinical trials of anti-lymphangiogenic approaches. First, experimental overexpression of VEGF-C in the skin of mice followed by chemical induction of skin carcinomas was shown to induce the expansion of lymphatic network within the draining lymph nodes even prior to the arrival of the tumor cells. The expansion of the intranodal lymphatic network was further enhanced after the dissemination of VEGF-C-expressing tumor cells into the lymph node. Furthermore, metastasis to distant organs was not detected in the absence of lymph node involvement (58). Second, in a highly metastatic murine model of Burkitt's lymphoma driven by forced expression of the c-Myc oncogene in B lymphocytes, expansion of the size and number of the lymphatic sinuses was observed in young (preneoplastic) transgenic mice (59). These observations suggest that lymph nodes may be valid targets of anti-lymphangiogenic therapy even after micrometastases have disseminated beyond the primary site prior to surgical intervention.

In addition, dye injection experiments have shown that lymph travels more efficiently toward and through draining lymph nodes in either the Burkitt's lymphoma model (59) or in the ear model of lymphatic metastasis induced by overexpression of VEGF-C (58). In the second model, the enhanced lymphatic flow in the draining channels correlated with VEGF-C-stimulated hypertrophy of the lymphatic vessels and with the rate of tumor dissemination to the lymph node, and both these parameters were significantly inhibited by concurrent systemic treatment with the anti-VEGFR-3 mAb mF4-31C1 (Fig 4). It is tempting to speculate that altering the dynamics of lymph flow might reduce the rate of tumor dissemination beyond the initial lymph nodes. However, these speculations must be balanced with caution since disruption of lymphatic drainage may precipitate the development of secondary lymphedema in the tissue containing the tumor mass (see above).

7. PROSPECTS FOR CLINICAL DEVELOPMENT OF ANTI-LYMPHANGIOGENIC THERAPY IN CANCER

Inhibition of tumor lymphangiogenesis represents, in principle, a novel approach to the treatment of cancer. There remains, however, a high level of skepticism regarding the relevance of lymphatic tumor spread to the eventual metastasis to distant organs (25). While it has been shown that the extent of local lymphatic involvement is a good predictor of distant organ metastasis (see above), it is not clear whether the dissemination of tumor cells to regional lymph nodes serves as a route of eventual entry into the circulation and spread to distant organs or if it primarily indicates that the tumor has acquired a metastatic phenotype. The resolution of this crucial question has been hampered by the fact that regional and distant metastasis in orthotopic models of cancer in mice poorly models the clinical course of most human malignancies. Management of patients with solid tumors typically involves surgical removal of the primary tumor and involved lymph nodes and is usually followed by months or years of tumor dormancy before a local recurrence or distant metastases become apparent.

In contrast, the implantation of tumor cells into orthotopic sites frequently results in rapid growth of the primary tumor that must be resected for ethical reasons before a long-term follow-up study of metastasis beyond the draining lymph nodes. This consideration makes such experiments performed on a statistically meaningful scale extremely challenging and expensive.

Thus, several critical factors combine to hinder the initiation of clinical trials of anti-lymphangiogenic therapies designed to reduce mortality from metastatic cancer: (i) the critical difference between current models of metastasis in rodents and the reality of human cancer imposes a formidable challenge in the design of preclinical experiments; (ii) disappointing results of earlier phase 3 trials targeting metastasis with inhibitors of matrix metalloproteases (60) dampen the enthusiasm of drug developers toward further anti-metastatic clinical trials; (iii) there exists a shortage of biomarkers that offer short-term clues that anti-metastatic therapy is effective, necessitating very long and thus expensive periods of treatment and follow-up; and (iv) there persists a view on the part of many oncologists and surgeons that the failure of surgery to completely eradicate a malignant neoplasm makes further therapy aimed at the metastatic process unrealistic. It is important that anti-metastatic therapy, as the term is used here, must not be confused with attempts to limit the growth of metastasized tumor which is the goal of much of oncological research.

One idea that has been discussed involves the use of anti-lymphangiogenic therapy either in conjunction with preoperative chemotherapy with neoadjuvant cytotoxic chemotherapy post-surgery (61 and references therein). Preoperative chemotherapy in patients with operable breast cancer or respectable esophageal cancer did not show statistically significant differences in survival or tumor recurrence. Whether addition of anti-lymphangiogenic treatment that can potentiate the effect of preoperative chemotherapy while reducing the risk of local lymphatic dissemination during the pre-treatment period can only be tested in clinical trials. Alternatively, anti-lymphangiogenic therapy could be used to enhance the effectiveness of neoadjuvant cytotoxic chemotherapy post-surgery. Successful use of such postoperative combinatorial use of chemotherapy with the mAb trastuzumab (Herceptin) has been reported (62).

I will conclude this chapter with a hypothetical example of a clinical niche setting that may offer an opportunity to objectively assess the benefits of anti-lymphangiogenic therapy within a reasonable time-frame and, consequently, may be considered for initial clinical trials. Inflammatory breast cancer (IBC) is a rare subtype of locally advanced epithelial breast cancer (LABC) (reviewed in 63) affecting between 2 and 6% of patients. IBC is the most aggressive form of this malignancy with the median overall survival of approximately 4 years compared to 12 years for other forms of LABC with the pathological classification of stage IIIA. Furthermore, IBC is diagnosed on the average 9 years earlier than non-IBC breast cancer so the disease strikes disproportionately at younger women. For these reasons, IBC has been classified as an urgent unmet therapeutic need by the National Cancer Institute.

The characteristic feature of IBC is the extensive lymphovascular invasion of the subdermal lymphatic capillaries with tumor cells organized into compact groups (Fig. 5). The lymphangiogenic nature of IBC is characterized by strong expression of VEGF-C, VEGF-D, and VEGFR-3 (64, 65). Of great interest, a recent study of IBC found significant correlation between elevated levels of nuclear factor-kappa B (NF- κ B)

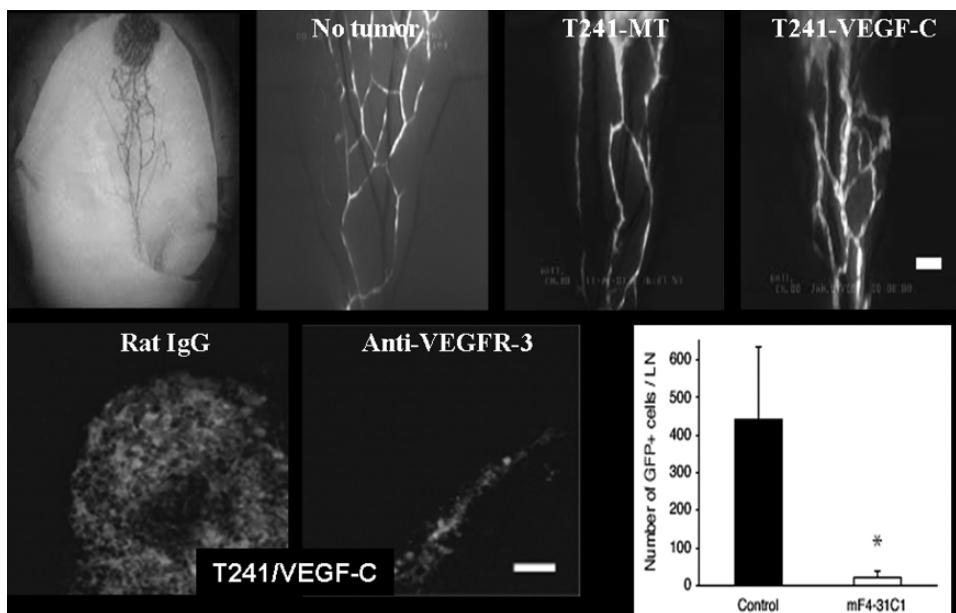


Fig. 4. VEGFR-3 blockade reduce hyperplasia of the collecting lymphatic vessels and delivery of T241 fibrosarcoma cells to lymph nodes. In this mouse ear model, the normal pattern of collecting lymphatic vessels draining the mouse ear to the cervical lymph node is visualized by injection of ink into the tip of the ear (A). The effect of tumor implantation and VEGF-C overexpression is shown by lymphangiography with a fluorescent tracer (B–D). B. Normal ear. C. Ear with a T241 fibrosarcoma tumor implanted at the tip of the ear. Note dilation of the vessels. D. Same as (C) but the tumor cells overexpress VEGF-C, leading to further hypertrophy of the draining lymphatic vessels and extensive metastasis of the tumor cells into the lymph node (E). E–F: Inhibition of metastasis of fibrosarcoma cell overexpressing VEGF-C (labeled with green fluorescent protein) into the cervical lymph node. E. Lymph node from an untreated mouse. F. Lymph node from a mouse treated with an antagonist monoclonal antibody to VEGFR-3. The number of GFP-positive cells in the lymph node is significantly reduced by anti-VEGFR-3 antibody treatment (G). Red, TMR-dextran. Hoshida et al., *Cancer Res.* 2006. (Please see color insert.)

detected by immunohistochemistry and the presence of transcriptionally active NF- κ B dimers. These investigators concluded that NF- κ B pathway is a likely contributing factor to the unique phenotype of IBC (66). Interestingly, the promoter region of the VEGF-C gene contains several NF- κ B-binding sites and interleukin-1 (IL-1) and TNF β , two potent inflammatory mediators that activate this signaling pathway increase expression of VEGF-C (67).

Acute and chronic inflammation is associated with lymphatic hyperplasia (reviewed in 68). Similarly, tumor growth within lymphatic vessels in IBC is characterized by large number of proliferating LECs (64). Thus, it seems reasonable to predict that the phenotype of IBC may be in part a result of a paracrine signaling loop. The individual components of such a loop are well characterized in multiple cancer types. VEGF-C produced by the tumor cells would produce lymphatic hypertrophy that might facilitate growth of intralymphatic tumor. Chemokines produced by LECs such as CCL21 (secondary lymphoid chemokine) would in turn act on CCR-7-expressing

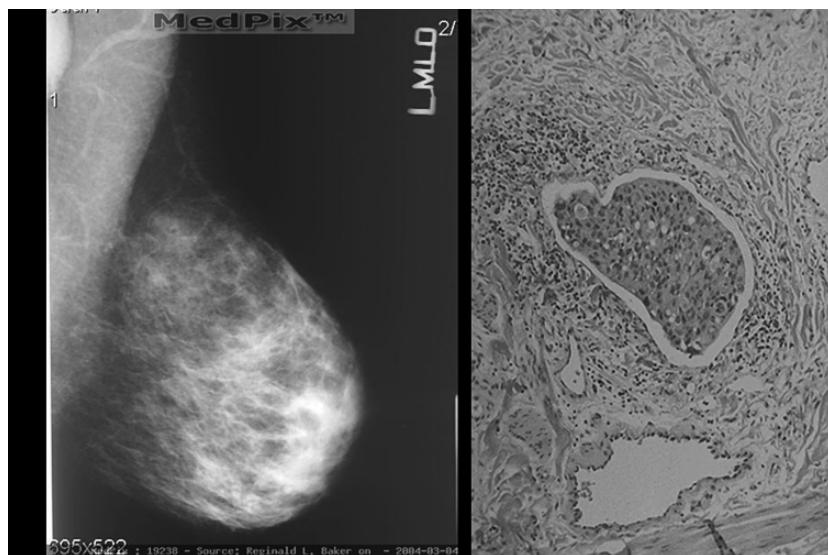


Fig. 5. Inflammatory breast carcinoma (IBC). Left: MRI image shows broad subdermal dissemination of the tumor with no discrete mass. Right: H&E staining of a punch biopsy specimen from a different patient (right) shows extensive leukocyte infiltration of tissue and invasive ductal carcinoma with involvement of the skin lymphatics. Radiography: Matthew J Hoffman, National Capital Consortium.

tumor cells enhancing migration within the lymphatic vessels and accelerating lymph node metastasis (69).

IBC is initially treated with an aggressive course chemotherapy and/or radiation to minimize the extent of tumor involvement prior to surgery (70). Anti-lymphangiogenic therapy could be combined with this standard of care in a cohort of patients. Typical initial and post-treatment evaluation includes radiological assessment and biopsy of the tumor and the axillary lymph nodes. Endpoint 1 of a hypothetical phase 1 trial would involve a comparison of pre-treatment and post-treatment radiographs and biopsy specimen to demonstrate a benefit of added anti-lymphangiogenic therapy in terms of overall tissue involvement, LVD, average size of intralymphatic tumor growths, and dissemination to axillary lymph nodes. Additional assessment could include immunohistochemical analysis of such molecules as CCR7 that have been proposed as novel biomarkers for metastatic dissemination in breast cancer (71). A secondary endpoint would be overall survival which, sadly, could be assessed fairly quickly due to the rapid course of this disease.

In conclusion, anti-lymphangiogenic therapy represents novel but extremely challenging approach to cancer treatment that would be used to supplement existing therapies. Although initial clinical testing would likely involve narrow clinical settings, the eventual use of such therapy could encompass all forms of cancer with documented extensive local involvement of the lymphatic system as well as post-operative anti-metastatic therapy for patients whose tumors are not amenable to complete resection.

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14

VEGF in the Adult

Implications for Anti-VEGF Therapies

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SUMMARY

Although the role of vascular endothelial growth factor (VEGF or VEGF-A) in angiogenesis is well studied, little is known about its role in the maintenance of quiescent vasculature. Recent observations from clinical trials using anti-VEGF therapy, as well as from the disease preeclampsia, point to a role for VEGF in the adult. This chapter explores both clinical and experimental VEGF neutralization studies and discusses the potential role of VEGF in the adult in maintaining endothelial cell (EC) survival and fenestration, as well as its action on non-ECs. Properties that may affect VEGF action *in vivo* are discussed. Finally, implications for use in anti- and pro-angiogenic therapy are proposed.

Key Words: VEGF; angiogenesis; preeclampsia; anti-angiogenic therapy; Bevacizumab; endothelial cells.

1. INTRODUCTION

Vascular endothelial growth factor (VEGF) belongs to a family of secreted glycoproteins of approximately 40 kD, which in mammals also includes VEGF-B, -C, and -D and placenta growth factor (PlGF). VEGF is a potent stimulator of angiogenesis, vasculogenesis, and permeability both *in vitro* and *in vivo*. During development, VEGF expression begins before gastrulation and continues throughout all stages of vascular development (1,2). Its importance in embryogenesis is highlighted by findings that mice with either a heterozygous or homozygous mutation of *VEGF* die during early development with severe vascular defects (3,4). Moreover, homozygous deletion of either of its receptors, VEGF receptor 1 (VEGFR1)/Flt1 (5) or VEGF receptor 2 (VEGFR2)/Flk1 (6), results in embryonic lethality. In addition to its role in developmental angiogenesis, VEGF is involved in physiological angiogenesis, including wound healing (7–9) and the female reproductive cycle (10, 11); pathological angiogenesis, including ocular neovascularization associated with macular degeneration (12, 13), diabetic retinopathy

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(14), and retinopathy of prematurity (15); as well as psoriasis (16), rheumatoid arthritis (17, 18), angiogenesis in atherosclerosis (19), and tumor angiogenesis (20, 21).

Although convincing evidence demonstrates a critical role for VEGF in developmental, physiological, and pathological angiogenesis, its role in the adult has not been well studied and remains controversial. Early reports of inducible deletion of *VEGF* in mice reported no obvious phenotype in the adult (4, 22). Similar findings were reported in tumor studies in mice using other neutralizing agents (23). *in vitro*, VEGF induces endothelial cell (EC) migration, proliferation and survival, invasion into the basement membrane and in formation of fenestrations. However, whether VEGF serves these roles in quiescent, adult vasculature is unclear.

Recent clinical and experimental observations suggest a role for VEGF in the adult. This chapter reviews the expression pattern of VEGF and its receptors in the adult, its association with fenestrations, its newly recognized effect on non-vascular cells, and finally, the implications of these observations for manipulating VEGF in disease treatment.

2. VEGF EXPRESSION IN THE ADULT

It is clear that VEGF remains important throughout adulthood during both the process of wound healing and in the female reproductive cycle, both of which require EC migration, proliferation and invasion, as well as recruitment of EC precursors. During wound healing, ovulation, endometrial thickening, and pregnancy, VEGF expression is upregulated, leading to neovascularization, similar to developmental angiogenesis. Therefore, the role of VEGF in these processes in the adult does not provide much insight into its role in quiescent vascular beds.

In addition to wound healing and female reproduction, a number of reports indicate that VEGF continues to be expressed in the adult in various tissues (24–30), albeit at relatively low levels. Systematic studies of VEGF expression reveal that it is constitutively expressed (31) in a cell-specific manner (32) in virtually all adult tissues (Fig. 1). For example, pericytes, which are mural cells derived from mesenchyme and are tightly associated with ECs, are recruited to immature blood vessels and are associated with vascular stability (33). *in vitro* studies indicate that concomitant with their differentiation, pericytes begin to synthesize VEGF, which has been suggested to mediate, at least in part, their observed stabilizing effect on newly formed capillaries (34). VEGF is expressed by a variety of other cell types in different tissues. The proximity of VEGF-expressing cells and the underlying vasculature suggests that VEGF acts in a paracrine manner to support the adjacent endothelium. In addition, fenestrated endothelium appears to be closely associated with VEGF-expressing cells, including VEGF expression by podocytes adjacent to the glomerular endothelium, by choroid plexus epithelium near the choroid plexus endothelium, by hepatocytes and β -islet cells, and by retinal pigment epithelium bordering the choriocapillaris (32, 35). This correlation implicates VEGF in the maintenance of fenestrae. VEGF is also highly expressed in tissues with high metabolic demands, including cardiac and skeletal muscle, and may have a role in maintaining a steady blood supply to these richly vascularized tissues.

For the most part, ECs themselves do not express VEGF (36). Since they express VEGF receptors, expression of the ligand could lead to an autocrine loop, resulting in uncontrolled proliferation such as that seen in tumorigenesis (37) and in hemangiomas (38). Interestingly, VEGF is expressed by aortic ECs but not by the endothelium

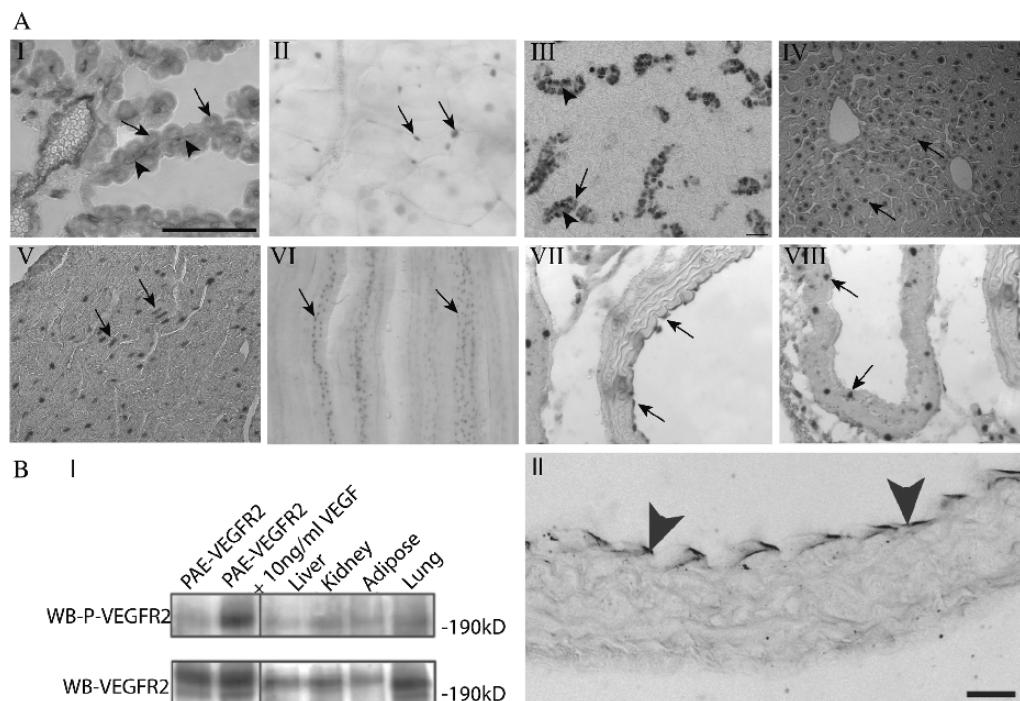


Fig. 1. Vascular endothelial growth factor (VEGF) and VEGFR2 expression in the adult. **A.** VEGF expression is shown in adult mice using mice in which a *lacZ* gene containing its own internal ribosomal entry site and nuclear localization signal was inserted in the 3' untranslated region of the *VEGF-A* gene (*I*) resulting in a nuclear localized β -galactosidase (β -gal) protein wherever VEGF is expressed. Blood vessels, where shown, were identified by immunohistochemistry using the pan-EC marker CD31 (arrowheads). VEGF expression in: (*I*) epithelial cells (arrows) of the choroid plexus, (*II*) adipocytes (arrows) in fat, (*III*) serous epithelial cells (black arrows), but not mucous epithelial cells (arrowheads) of the salivary gland, (*IV*) hepatocytes (arrows) in the liver, (*V*) cardiac myocytes (arrows) in the heart, (*VI*) skeletal myocytes (arrows) in striated muscle, (*VII*) endothelium of the aorta, and (*VIII*) media of inferior vena cava (arrows). **B.** VEGFR2 expression and activation in adult tissues as identified by (*I*) western blot for VEGFR2 of protein lysates from liver, kidney, adipose, and lung (bottom panel) and for phosphorylated VEGFR2 (top panel) and by (*II*) immunohistochemistry of aorta for phosphorylated VEGFR2 (arrows). Reprinted with permission (32) from the American Society of Investigative Pathology. (Please see color insert.)

of the inferior vena cava (32). Perhaps fluid dynamics and/or the cellular composition of the aorta lead to EC VEGF expression where it may act in an autocrine manner as a survival factor and/or as a hormone following release into the blood stream. Support for a possible hormonal role for VEGF comes from recent observations that circulating VEGF can mobilize endothelial precursor cells from bone marrow (39, 40).

VEGF is thought to act primarily through VEGFR2, a member of the receptor tyrosine kinase family [reviewed in (41)]. *In vitro*, VEGF-induced EC survival, proliferation and migration (42), and fenestrae formation (43–45) are mediated via VEGFR2 signaling. Recent evidence demonstrates that VEGFR2 is expressed (32, 46, 47) and activated (32) in tissues that express VEGF, suggesting that VEGF has a biologic role in these tissues.

3. OBSERVATIONS FROM VEGF NEUTRALIZATION IN CLINICAL SETTINGS

3.1. Bevacizumab Trials for Colorectal Cancer

Maintenance of the vascular system is paramount to cell survival, and because of the diffusion limitation of oxygen, cells, including tumor cells, need to be within 100 µm of a blood vessel. Early reports indicated that VEGF is important only during development and in physiologic and pathologic angiogenesis (4). This suggested that targeting VEGF for tumor treatment would lead to regression or growth inhibition of tumor blood vessels, which are reliant on VEGF, while sparing blood vessels in normal tissue. This belief led to the development of many anti-VEGF agents that are currently in clinical development [see (48) for a detailed list of agents]. However, evidence that VEGF may play a role in the adult became apparent with observations from Phase III clinical trials using Avastin™ (Bevacizumab, Genentech) for the treatment of colorectal cancer (49–51).

Avastin is a humanized neutralizing monoclonal antibody against VEGF. It is currently-FDA approved for the use in the treatment of metastatic colorectal cancer (50) and is being utilized in dozens of clinical trials for the treatment of cancers in virtually every organ system (52). Avastin treatment produced significant regression in tumor size and increased the median survival in the patients undergoing treatment for colorectal cancer. However, consistent side effects were noted including hypertension and proteinuria (50). More recently, an infrequent, but serious complication, reversible posterior leukoencephalopathy (RPLE) has been reported (53, 54) (Fig. 2). Although RPLE is very rare, it may lead to potentially life-threatening symptoms including cortical blindness, seizures, and even stroke.



Fig. 2. Reversible posterior leukoencephalopathy in a Bevacizumab-treated patient. This axial FLAIR image shows both frontal and posterior lesions (arrows). Reproduced with permission (54), Copyright © 2006 Massachusetts Medical Society.

3.2. Preeclampsia as a Human Model of VEGF Neutralization

Preeclampsia is a disease of pregnancy that affects 5–7% of women beginning in their second trimester. This disease is diagnosed by the findings of hypertension and proteinuria as well as edema. Left untreated, widespread endothelial damage can occur, resulting in pleural edema, ascites, thrombocytopenia, headaches, disseminated intravascular coagulation, and blindness. Progression to eclampsia is accompanied by the onset of seizures, and in severe cases even stroke.

That the insulting agent is primarily produced by the placenta is supported by the fact that delivery of the placenta results in almost immediate improvements and is currently the only way to completely reverse the disease. Of the agents found to be overexpressed in preeclamptic placentas, the serum/soluble fms-like tyrosine kinase 1 (sflt1/sVEGFR1) gene is highly upregulated, and the protein is markedly elevated in the plasma of the preeclamptic mother (55, 56). sflt1 is a natural splice variant of the VEGFR1 receptor and consists of a truncated protein, containing approximately 85% of the extracellular domain with 30 amino acids derived from the thirteenth intron (57). This soluble receptor has an affinity for VEGF that is tenfold higher than VEGFR2 and therefore acts as an effective, natural neutralizing agent.

Systemic administration of sflt1 to rats led to marked hypertension and proteinuria, and examination of kidney histology revealed glomerular endotheliosis (Fig. 3) (56), a biopsy finding that was previously used as the standard for the diagnosis of preeclampsia. This endothelial damage provided strong evidence that VEGF is required for maintenance of the glomerular endothelium. Hypertension may have occurred secondary to renal damage but is also likely to be a result of decreased vascular tone due to the neutralization of VEGF-induced nitric oxide (58).

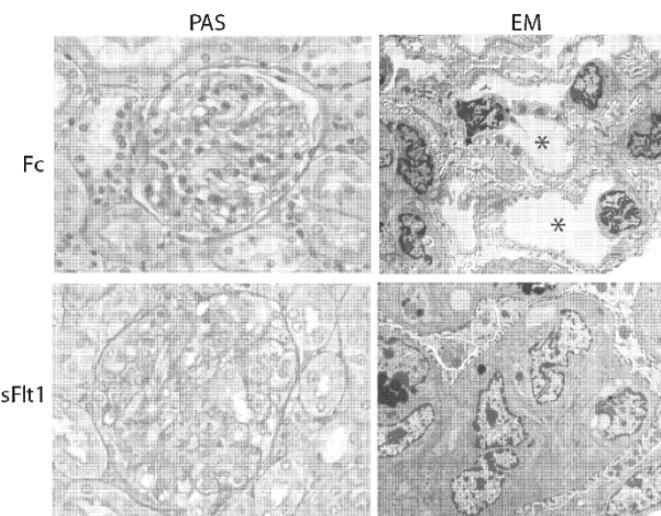


Fig. 3. Glomerular lesions in an experimental model of preeclampsia in the rat. Systemic administration of sflt1, a soluble VEGF receptor, to pregnant rats results in glomerular endotheliosis as observed by periodic acid schiff (PAS) staining (lower left panel). Electron microscopy (right panel) highlights the similarity in these lesions and those of human preeclamptic patients. Treatment with sflt1 results in obliteration of the vascular lumens (asterisks). Reproduced with permission (56). (Please see color insert.)

A severe, albeit more rare complication of both preeclampsia and Avastin treatment is RPLE. While this pathology is widely believed to be a secondary complication of hypertension, some evidence suggests that primary insult to brain endothelium may contribute to its pathogenesis (59). Although both Avastin treatment and preeclampsia patients are hypertensive, underlying primary damage to the central nervous system ECs may also be contributing to RPLE. It is not known whether RPLE is associated with other anti-VEGF treatments. Although it could be argued that the pathology is due to combination therapy, these symptoms were not observed in the irinotecan, fluorouracil, and leucovorin minus Avastin patients (50), suggesting that the Avastin was the cause of these symptoms.

4. VEGF AND VESSEL STABILITY

4.1. *Experimental VEGF Neutralization*

Emerging *in vivo* evidence indicates that VEGF neutralization leads to vascular defects, particularly at the level of the microvasculature. These alterations are accompanied by organ-specific functional deficits, including lung alveolar apoptosis with enlarged airspaces (60–62) (Fig. 4A), glomerular endotheliosis with associated proteinuria (56, 63) (Fig. 3), β -islet dysfunction (64, 65), tracheal vessel regression (64, 66), thyroid vessel destruction with impaired thyroid function (Fig. 4B), and loss of small intestinal villi capillaries (Fig. 4C) (64). These observations provide compelling support for the concept that VEGF has an important biologic function in the maintenance of the microvasculature in normal adult tissue.

The microvascular effects have been postulated to be the result of interference with the EC survival action of VEGF (66, 67). According to this model, VEGF neutralization

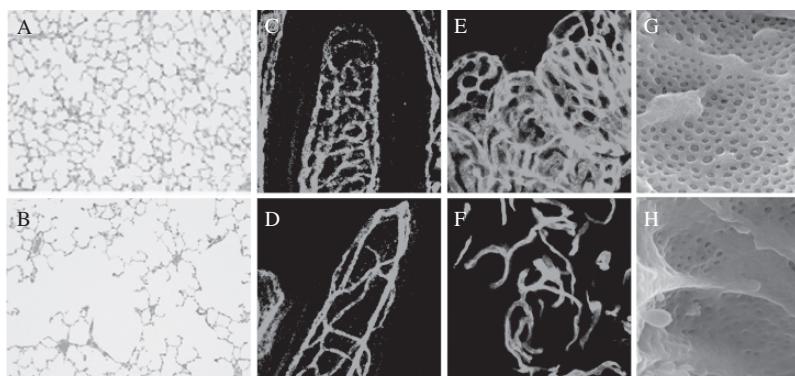


Fig. 4. Experimental inhibition of vascular endothelial growth factor (VEGF) *in vivo*. A. Administration of SU5416, a tyrosine kinase inhibitor with selective inhibition of VEGFR, to rats by bronchiolar delivery results in enlargement of air spaces, indicative of emphysema (B) compared to vehicle (A). Reproduced with permission (61). Adenoviral delivery of sVEGFR1 causes decreased vascular density in the small intestine and thyroid (D, F, respectively) compared to vehicle delivery (C, E) and decreased fenestrations in the kidney glomerular endothelium (H) compared to adenovirus only delivery in (G). Reproduced with permission (64) (Please see color insert.)

leads to endothelial dysfunction and the expression of coagulation factors, which causes capillaries to become obliterated by fibrin deposition. The cessation of blood flow and subsequent ischemia results in loss of EC with accompanying pericyte loss, leaving behind an empty basement membrane. If the neutralization is reversed within a certain time frame, a significant proportion of the remaining basement membrane “sleeves” can be repopulated by ECs, but prolonged neutralization results in basement membrane destruction as well (67).

This explanation assumes that VEGF neutralization primarily affects the vasculature, and although the evidence is consistent with such a model, there is also increasing evidence that VEGF can influence other cell types. Given that VEGF is upregulated by hypoxia, and is secreted by activated platelets (7, 68), one might predict that coagulation, and the resultant inflammation in response to injury, would result in compensatory angiogenesis. The fact that this does not occur suggests that VEGF neutralization may also influence other cell types including monocytes, neutrophils, and other inflammatory cells.

4.2. VEGF and Non-Vascular Cells

VEGF was so named because of its identification as an EC mitogen, and original reports indicated that VEGF receptors were specific to ECs. However, VEGF receptors have been identified on many other cell types including, megakaryocytes (69), hematopoietic stem cells/bone marrow-derived circulating cells (39, 40, 70), macrophages (71), mast cells (72), eosinophils (73), dendritic cells (74), lymphocytes (75), type II alveolar epithelial cells (76), lens epithelium (77), ventricular ependymal cells (46), and neural stem cells (78). VEGF acts on these cells to influence their survival (lymphocytes, neural cells, and hematopoietic stem cells), maturation (dendritic cells and megakaryocytes), and mobilization (bone marrow precursor cells). Some of these effects might also contribute to angiogenesis such as in the recruitment of bone marrow-derived circulating cells to sites of angiogenesis. Furthermore, the action of VEGF on inflammatory cells is central to wound healing. Wound healing [reviewed in (9)] is composed of four phases including acute inflammation, epithelialization, formation of granulation tissue, and finally tissue remodeling. Because these events involve an interplay between stromal and inflammatory cells, inhibition of VEGF signaling may interfere with these processes. Observations of thrombocytopenia, neutropenia, and gastroperforation due to impaired wound healing associated with Avastin treatment (48, 50) suggest that production of both white blood cells and platelets is affected by VEGF neutralization. In addition, neuropathy noted with Avastin is consistent with a neuro-protective role of VEGF.

Additionally, VEGF appears to be important for lymphangiogenesis (79, 80). A role for VEGF in lymphangiogenesis, and perhaps lymphatic vessel maintenance, may account for the common finding of edema in patients with preeclampsia (81). Although the primary cause of edema in these patients is likely to be decreased serum albumin due to proteinuria, and/or decreased liver production, it is possible that VEGF neutralization leads to lymphatic dysfunction, resulting in ineffective fluid resorption and edema. Future studies will elucidate functions of VEGF on other non-EC types, further underscoring its importance as a multifunctional growth factor.

4.3. VEGF and Fenestrations

In addition to its role as an angiogenic factor, VEGF was also initially identified as a potent permeability factor (82). VEGF promotes endothelial permeability by inducing the formation of vesiculovacuolar organelles, intercellular gaps, and fenestrations (83). Fenestrae, EC plasma membrane specializations, appear as circular discontinuities of approximately 60 nm in diameter. Most endocrine organs have fenestrated microvessels (45), which facilitate the delivery of hormones into the circulation. Fenestrae are also characteristic of the microvasculature of the kidney glomeruli, gastrointestinal tract, choroid plexus of the brain, choroid of the eye, as well as tumor vessels. An involvement of VEGF with fenestration maintenance is supported by the observation that VEGF is expressed in cells in close juxtaposition with fenestrated endothelium (32) (Fig. 3A).

Although VEGF has been reported to mediate the induction of fenestrae (43–45, 84), the mechanisms by which fenestrae are formed and maintained in the adult are poorly understood. Recent studies have provided one link between VEGF and fenestrae, demonstrating that the plasmalemmal vesicle-associated protein gene, which encodes for the caveolar protein PV-1, a component of diaphragmed fenestrae (85), is regulated by VEGF (86). However, the fact that PV-1 is associated only with diaphragmed and stomatal fenestrae implies that VEGF may regulate other components of fenestrae as well.

Although VEGF appears to be involved in the formation of fenestrations, not all tissues that express VEGF have fenestrated endothelium. Thus, there may be tissue-specific factors or conditions that modulate the ability of VEGF to induce fenestrae, and/or the level of VEGF may also be important in determining whether vessels are fenestrated. Candidates for factors that may modulate the formation of fenestrae include leptin, a growth factor secreted by adipose tissue as well as other tissues. In addition to promoting angiogenesis, leptin has been shown to act synergistically with VEGF to induce fenestrations (87).

It has recently been reported that VEGF neutralization leads to a loss of fenestrations on tumor vasculature (67), and in the normal vasculature in tissues including the thyroid (64), pancreas (65), and glomerulus (56, 63, 64) (Fig. 4). The reduction of vessel permeability by VEGF inhibition may have therapeutic advantages for the treatment of cancer, as it has been proposed that normalization of the tumor vasculature would allow more efficient delivery of chemotherapy (48, 88). That said, it is not yet clear whether therapeutic VEGF neutralization affects all fenestrated vascular beds, and whether this might have deleterious effects on the functions of these tissues.

4.4. Biochemical, Molecular and Cellular Factors Affecting VEGF Signaling *in vivo*

Given that VEGF is a potent angiogenic factor, it is intriguing that its expression in the adult does not result in widespread continued angiogenesis. It has become clear, however, that the angiogenic status of a tissue is the net result of the balance between pro-angiogenic factors (17) and endogenous inhibitors (89). VEGF has been shown to signal via multiple pathways including PLC, Ras, and PI3-K pathways (90). Thus, whether VEGF signaling leads to proliferation or survival is likely be determined by the context in which VEGF is acting, including the action of other factors.

The bioavailability of VEGF may also be important in determining the balance between angiogenesis and survival. Whereas VEGF is highly upregulated in tumors and in other pathologic states (91–93), its level in quiescent tissues is much lower. VEGF upregulation in tumors is mediated, at least in part, by the transcription factor hypoxia-inducible factor-1 alpha (HIF-1 α) (37, 94). HIF-1 α is a master regulator of oxygen-responsive genes; it is rapidly degraded during normoxic conditions, but it is stabilized upon oxidative stress and hypoxia. In addition to hypoxia, VEGF expression is induced by a variety of other growth factors and cytokines including tumor necrosis factor (TNF)- α , transforming growth factor (TGF)- β , FGF-4, PDGF, IL-1 α , IL-6, and insulin-like growth factor (IGF)-1 (95, 96), several of which act via HIF-1 α . Analysis of the VEGF promoter reveals a large number of transcription factor-responsive elements (94) that act independently of HIF-1 α , including, for example, an ErbB2-responsive element (97).

The bioavailability of VEGF is also dependent on the specific VEGF isoforms expressed. VEGF mRNA is alternatively spliced, resulting in five major isoforms (VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆) in humans and three in mice (VEGF₁₂₀, VEGF₁₆₄, and VEGF₁₈₈). The various isoforms display different biochemical properties including differences in molecular mass, receptor binding (VEGF₁₆₄ and VEGF₁₈₈ to neuropilins), and the ability to bind to heparan sulfate (VEGF₁₆₄ and VEGF₁₈₈); differences in affinity to heparan sulfate binding also results in different diffusion patterns, making VEGF₁₂₀ the most freely diffusible, whereas VEGF₁₈₈ is mostly cell bound (98). VEGF isoforms are differentially expressed, with different tissues displaying specific patterns of isoform expression (31). The isoforms appear to have non-overlapping roles in vascular development, as mice expressing single VEGF isoforms appear to have tissue-specific vascular defects (99). The distinct effects of the various isoforms may be due to their differential localization and receptor binding. In addition, although a systematic analysis of signaling has not been reported, it is possible that the VEGF isoforms may induce different downstream effects.

Differential expression of VEGFR1 and VEGFR2 on the target endothelium may also determine the outcome of EC signaling. VEGFR1 has a tenfold higher affinity for VEGF than does VEGFR2 (57, 100). Interestingly, though VEGFR1-null mice are embryonic lethal (5), the tyrosine kinase activity of VEGFR1 does not appear to be important for vascular development (101). Instead, it has been suggested that VEGFR1 may modulate the action of VEGF either by competing for VEGF binding or by recruiting VEGF to the cell membrane. Recent studies have demonstrated that there is cross-talk between VEGFR1 and VEGFR2 at the cell membrane (102), and that VEGFR1 is required for VEGF recruitment whereas VEGFR2 mediates VEGF signaling (103). It is therefore possible that a ratio of VEGFR1 to VEGFR2 receptors may also influence downstream signaling in response to VEGF.

5. PROSPECTS FOR THERAPEUTIC VEGF NEUTRALIZATION

As discussed, VEGF blockade has been implicated in the pathogenesis of preeclampsia. In addition, the side effects observed with anti-VEGF therapy appear to be due, in large part, to the effects of VEGF neutralization on normal tissues. Though limited research has been done to understand the role of VEGF in quiescent

tissues, these clinical findings, along with data demonstrating expression in the adult, constitutive activation of VEGF receptors, and effects on non-ECs, suggest that VEGF is important in the adult. Thus, the original concept that only growing vessels are dependent on VEGF must be reconsidered and presents a challenge for the use of both anti- or pro-angiogenic therapy.

Once the function of VEGF in major tissues is elucidated, steps can be taken to minimize morbidity during treatment. For example, recognizing that anti-VEGF treatment leads to decreased vascular tone with resulting hypertension (56) can be compensated with anti-hypertensive drugs. A thorough understanding of the function of VEGF in the adult is essential if maximal effects are to be obtained with anti-VEGF therapies while minimizing side effects.

In addition to being able to anticipate and treat side effects of anti-VEGF therapies, knowing specific, consistent effects of VEGF neutralization may provide useful surrogate markers of the effectiveness of anti-VEGF therapies. Examples of markers currently being investigated include skin wound healing time (104), VEGF levels in plasma (105) and urine (106), and circulating endothelial progenitor cells (107, 108). Ability to monitor anti-VEGF treatment will allow an assessment of treatment effectiveness and may also permit treatment regimens to be better tailored for individual patients. Furthermore, understanding potential side effects could lead to drug delivery to a specific target tissue without affecting multiple tissue vascular beds.

It is equally important to consider the potential implications for pro-angiogenic VEGF therapies, which may be useful in myocardial ischemia, limb ischemia, stroke, vascular dementia, Alzheimer's disease, and ulcers, as well as wound and fracture healing. VEGF therapy in the treatment of myocardial disease has been extensively evaluated (109, 110) and has been reported in some trials to result in increases in exercise time, improved perfusion, improved ventricular function, and decreased angina. However, there is a reasonable concern that systemic delivery of VEGF (111) may shift the balance between endogenous angiogenesis inhibitors and stimulators, thus leading to aberrant vessel growth and/or exacerbation of existing diseases. For instance, experimental overexpression of VEGF in the skin results in increased inflammation (112) and a psoriatic condition. Similarly, overexpression of VEGF in mouse podocytes leads to a collapsing glomerulopathy (63). Side effects that have been noted in early pro-angiogenic trials are edema and hypotension (113), perhaps attributable to increased vascular permeability. As in the case with anti-VEGF treatment, understanding which tissues might be sensitive to excess VEGF would be useful in determining the specificity necessary to reduce side effects.

Given the pleiotropic role of VEGF in development and in the adult as well as in normal and pathologic angiogenesis, a thorough understanding of these functions is essential to permit safe and effective manipulation of VEGF without substantial negative effects.

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15

Normalization of Tumor Vasculature and Microenvironment

*A Potential Mechanism of Action
of Antiangiogenic Therapies*

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SUMMARY

Solid tumors require blood vessels for growth, and many new cancer therapies are targeted against the tumor vasculature. The widely held view is that these antiangiogenic therapies destroy the tumor vasculature, thereby depriving the tumor of oxygen and nutrients. Indeed that is the ultimate goal of antiangiogenic therapies. However, emerging preclinical and clinical evidence support an alternative hypothesis, that judicious application of agents that block angiogenesis directly (e.g., bevacizumab and cediranib) and indirectly (e.g., trastuzumab) can also transiently “normalize” the abnormal structure and function of tumor vasculature. In addition to being more efficient for oxygen and drug delivery, the normalized vessels are fortified with pericytes, which can hinder intravasation of cancer cells, a necessary step in hematogenous metastasis. Drugs that induce vascular normalization can also normalize the tumor microenvironment—reduce hypoxia and interstitial fluid pressure—and thus increase the efficacy of many conventional therapies if both are carefully scheduled. Reduced interstitial fluid pressure can decrease tumor-associated edema as well as the probability of lymphatic dissemination. Independent of these effects, alleviation of hypoxia can decrease the selection pressure for a more malignant phenotype. Finally, the increase in proliferation of cancer cells during the “vascular normalization window” can potentially sensitize tumors to cytotoxic agents. Results from our recent phase II clinical trial of cediranib, an oral, pan-vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor (TKI) in glioblastoma

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patients, show that the normalization window—identified using advanced magnetic resonance imaging (MRI) techniques—can last 1–4 months, and the resulting changes in tumor vasculature correlate with blood circulating molecular and cellular biomarkers in these patients. Antiangiogenic therapies may provide benefit for cancer patients by working through different mechanisms at different points in time. Normalization may be an early consequence of antiangiogenic therapy and offers an opportunity for optimizing delivery and facilitating the cytotoxic effects of chemotherapy and radiation. However, additional consequences of antiangiogenic therapies may include vessel “pruning” and nutrient deprivation of tumors.

Key Words: Angiogenesis; biomarker; MRI; normalization; VEGF; tumor

1. INTRODUCTION

After nearly four decades of basic research and clinical development of antiangiogenic therapy for cancer, two anti-vascular endothelial growth factor (VEGF) approaches have yielded survival benefit in patients with metastatic cancer in randomized phase III trials (1). In one approach, the addition of bevacizumab, a VEGF-specific antibody (Genentech Inc., South San Francisco, CA), to standard therapy improved overall survival (OS) in colorectal and non-small cell lung cancer patients and progression-free survival (PFS) in breast cancer and renal cell cancer patients (2, 3). In the second approach, multi-targeted tyrosine kinase inhibitors (TKIs) that block not only VEGF receptor kinases but also other kinases in both endothelial and cancer cells demonstrated a survival benefit in gastrointestinal stromal tumor and renal cell cancer patients (sorafenib; Bayer AG, Leverkusen, Germany and Onyx Pharmaceuticals; Emeryville, CA, and sunitinib, Pfizer, New York, NY). By contrast, bevacizumab failed to increase survival with chemotherapy in patients with previously treated and refractory metastatic breast cancer or pancreatic cancer. Furthermore, the addition of vatalanib (Novartis International AG, Basel, Switzerland), a multi-targeted TKI developed as a VEGF receptor-selective agent, to conventional cytotoxic therapy did not produce a survival benefit in metastatic colorectal cancer patients. Finally, several agents—that target oncogenic signaling pathways (such as HER2 by trastuzumab; Genentech Inc.)—may indirectly inhibit angiogenesis and have yielded increased OS with chemotherapy in clinical trials.

These contrasting responses raise critical questions about how these agents work in patients and how to combine them optimally. There are multiple potential mechanisms of action of antiangiogenic agents, but the focus of this chapter is on normalization of tumor vasculature for improved delivery and efficacy of therapeutics (1, 4). After summarizing preclinical evidence in support of vascular normalization, clinical evidence from two trials, treatment of rectal carcinoma patients receiving bevacizumab (5, 6) and recurrent glioblastoma patients receiving cediranib (7), will be reviewed. A discussion of potential biomarkers of anti-VEGF agent efficacy in humans—molecular and cellular parameters obtained from tissue biopsies, interstitial fluid pressure, blood circulating endothelial cells (CECs), protein levels in bodily fluids and physiological parameters—measured with various imaging techniques will be highlighted followed by comments on the potential avenues of further investigation.

2. THE VASCULAR NORMALIZATION HYPOTHESIS

Beginning with the seminal work of Teicher, several preclinical and clinical studies have demonstrated that antiangiogenic therapy improves the efficacy of cytotoxic therapies (1, 8). This is paradoxical. One would expect that destroying the vasculature would severely compromise the delivery of oxygen and therapeutics to the solid tumor, producing hypoxia that would render many chemotherapeutics, as well as radiation, less effective. To resolve this paradox, our laboratory hypothesized in 2001 that the judicious application of antiangiogenic agents can “normalize” the abnormal tumor vasculature, resulting in more efficient delivery of drugs and oxygen to the targeted cancer cells (Fig. 1A) (9). Increased penetration of drugs throughout the tumor would enhance the cytotoxicity of chemotherapy, whereas the ensuing increased level of oxygen would enhance the efficacy of radiation therapy and many chemotherapeutic agents.

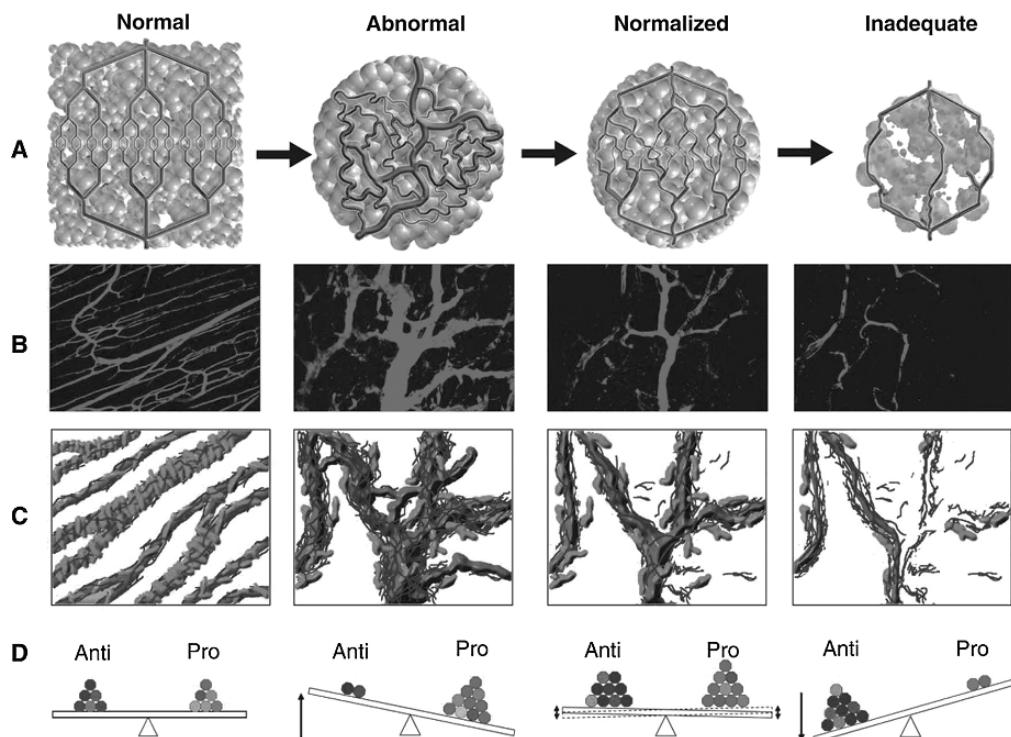


Fig. 1. Proposed role of vessel normalization in the response of tumors to antiangiogenic therapy. (A) Tumor vasculature is structurally and functionally abnormal. It is proposed that antiangiogenic therapies initially improve both the structure and the function of tumor vessels. However, sustained or aggressive antiangiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is both resistant to further treatment and inadequate for delivery of drugs or oxygen (9). (B) Dynamics of vascular normalization induced by vascular endothelial factor receptor 2 (VEGFR2) blockade. Left: two-photon microscopy image showing normal blood vessels in skeletal muscle; subsequent images show human colon carcinoma vasculature in mice at day 0, 3, and 5 after administration of VEGFR2-specific antibody (22). (C) Diagram depicting the concomitant changes in pericyte (red) and basement membrane (blue) coverage during vascular normalization (6, 22). (D) These phenotypic changes in the vasculature may reflect changes in the balance of proangiogenic and antiangiogenic factors in the tissue. Reproduced with permission (8).

Table 1
Morphological and Functional Characteristics of the Vasculature in Normal Tissue, an Untreated Tumor, a Tumor During Early Stages of Treatment with an Antiangiogenic Drug (“Normalized”), and a Tumor Treated with High Doses of an Antiangiogenic Drug over a Long Period (“Regressing”)

| Properties | Vessel type | | |
|---|-------------------------------------|---|------------------------------------|
| | Normal | Tumor (untreated) | Tumor (normalized) (regressing) |
| Global organization | Normal | Abnormal | Normalized |
| Pericyte | Normal | Absent or detached | Closer to normal |
| Basement membrane | Normal | Absent or too thick | Closer to normal |
| Vessel diameter | Normal distribution | Dilated | and some ghost Closer to normal |
| Vascular density | Normal and homogeneous distribution | Abnormal and heterogeneous distribution | Closer to normal |
| Permeability to large molecules | Normal | High | Intermediate |
| MVP and IFP (P)lasma and (I)nterstitial oncotic pressure ^a | MVP > IFP $P > 1$ | MVP ~ IFP $P \sim 1$ | MVP > IFP $P > 1$ |
| pO ₂ | Normal | Hypoxia | Reduced hypoxia |
| Drug penetration | N/A | Heterogeneous | More homogeneous |
| | | | Hypoxia Inadequate |

MVP, microvascular pressure; IFP, interstitial fluid pressure.

^aOsmotic pressure exerted by plasma proteins.
Reproduced with permission (8).

3. RATIONALE FOR NORMALIZING THE TUMOR VASCULATURE

To obtain nutrients for their growth and to metastasize to distant organs, cancer cells co-opt host vessels, sprout new vessels from existing ones (angiogenesis), and/or recruit endothelial cells from the bone marrow (postnatal vasculogenesis) (10, 11). The resulting vasculature is structurally and functionally abnormal (Table 1) (12). These structural abnormalities contribute to spatial and temporal heterogeneity in tumor blood flow. In addition, solid pressure generated by proliferating cancer cells compresses intra-tumoral blood and lymphatic vessels, which further impairs not only the blood but also lymphatic flow (13). Collectively these vascular abnormalities lead to an abnormal tumor microenvironment characterized by interstitial hypertension (elevated hydrostatic pressure outside the blood vessels), hypoxia, and acidosis.

Impaired blood supply and interstitial hypertension interfere with the delivery of therapeutics to solid tumors. Hypoxia renders tumor cells resistant to both radiation and several cytotoxic drugs. And independent of these effects, hypoxia also induces genetic instability and selects for more malignant cells with increased metastatic potential. Hypoxia and low pH also compromise the cytotoxic functions of host immune cells that infiltrate a tumor. Unfortunately, cancer cells are able to survive in this abnormal microenvironment. Interstitial hypertension forces the fluid from the tumor margin to the surrounding tissue (or fluid) contributing to the tumor-associated edema and lymphatic metastasis (14). In essence, the abnormal vasculature of tumors and the resulting abnormal microenvironment together pose a formidable barrier to delivery and efficacy of cancer therapy. This suggests that if we knew how to correct the structure and function of tumor vessels, we would have an opportunity to normalize the tumor microenvironment and ultimately to improve cancer treatment. The fortified tumor vasculature may also inhibit the shedding of cancer cells into the circulation, a prerequisite for metastasis.

4. BLOCKING VEGF SIGNALING NORMALIZES TUMOR VESSELS IN TRANSPLANTED TUMORS

In normal tissues, the collective action of angiogenic stimulators (e.g., VEGF) is counter-balanced by the collective action of angiogenic inhibitors such as thrombospondin-1 (Fig. 1). This balance tips in favor of the stimulators in both pathological and physiological angiogenesis. However, in pathological angiogenesis, the imbalance persists. Therefore, restoring the balance may render the tumor vasculature close to normal. On the other hand, tipping this balance in favor of inhibitors may lead to vascular regression and ultimately to tumor regression.

Of all the known angiogenic molecules, VEGF (also referred to as VEGF-A) appears to be the most critical. VEGF promotes the survival and proliferation of endothelial cells and increases vascular permeability (15). VEGF is over expressed in the majority of solid tumors. Thus, if one were to judiciously down-regulate VEGF signaling in tumors, then the vasculature might revert back to a more “normal” state. Indeed, blockade of VEGF signaling passively prunes the immature and leaky vessels of transplanted tumors in mice and actively remodels the remaining vasculature so that it more closely resembles the normal vasculature (Fig. 1). This “normalized” vasculature is characterized by less leaky, less dilated, and less tortuous vessels with a more normal basement membrane and greater coverage by pericytes (Fig. 1). These morphological

changes are accompanied by functional changes—decreased interstitial fluid pressure, increased tumor oxygenation, and improved penetration of drugs in these tumors (Table 1) (16–25).

5. FIRST CLINICAL EVIDENCE OF NORMALIZATION IN HUMAN TUMORS

In a phase I/II clinical trial in rectal carcinoma patients receiving bevacizumab and chemoradiotherapy, our group recently examined the effect of bevacizumab on human tumors (5,6). Bevacizumab was delivered as a 90-min infusion on day 1 of each cycle. The dose was escalated in successive cohorts of six patients, beginning at 5 mg/kg followed by 10 mg/kg. Infusional 5-FU was administered over 24 h each day at a fixed dose of 225 mg/m² throughout each treatment week of cycles 2–4. External beam irradiation was administered during cycles 2–4 for a total of dose of 50.4 Gy in 28 fractions over 5.5 weeks. The primary clinical objective of this study was to determine the maximum-tolerated dose (MTD) of bevacizumab when delivered concurrently with 5-FU and external beam radiation therapy in patients with locally advanced (stage T3 or T4) rectal cancer prior to surgery. In parallel, a major goal of this study was to clarify through correlative studies the mechanisms by which bevacizumab inhibits angiogenesis and improves the outcome of other therapeutic modalities in the treatment of this malignancy.

We have completed the phase I portion of the study. The first six patients treated with the combination of bevacizumab at the 5 mg/kg dose level with chemotherapy and radiation therapy tolerated this treatment without difficulty. All six patients underwent surgery without complication. In contrast, two of five patients in the second cohort who were given “high-dose” bevacizumab (10 mg/kg) with chemotherapy and radiation therapy experienced grade 3–4 dose-limiting toxicity (DLT) of diarrhea and colitis during the combined treatment. Following recovery from toxicity, these patients were able to resume and complete radiation therapy and 5-FU treatment. Because of these DLTs, accrual at the 10 mg/kg dose was stopped after five patients. All these patients underwent surgery. Of note, one patient on high-dose bevacizumab experienced a pulmonary embolus on day 1 postoperatively and recovered completely with anticoagulation. Another patient in this cohort developed ileostomy obstruction with stent-related ileal perforation 10 days following resection requiring laparotomy and ileostomy revision.

At surgery, patients on the 5 mg/kg bevacizumab showed minimal residual disease, consistent with a significant tumor regression (Mandard Grade 3–4). Of interest, pathologic evaluation of the surgical specimens for staging following completion of all therapy in the patients receiving 10 mg/kg bevacizumab showed two complete pathological responses as compared to no complete pathological response in the 5 mg/kg bevacizumab group.

The study design of this trial permitted a unique opportunity to evaluate the effect of bevacizumab alone (cycle 1) on rectal cancer prior to its concurrent administration (cycles 2–4) with radiation therapy and chemotherapy. Correlative studies were undertaken to clarify the mechanism of action of bevacizumab on rectal cancer. Prior to treatment and 12 days following the first bevacizumab infusion, patients underwent repeat flexible sigmoidoscopy with tumor biopsy, tumor interstitial

pressure measurement, perfusion computed tomography (CT) scan to measure blood flow, positron emission tomography (PET) scan to measure ^{18}F -fluorodeoxyglucose (FDG) uptake, and blood and urine collection for analyses of angiogenesis markers (plasma proteins and viable circulating endothelial cells, CECs). At day 12, no significant clinical response was observed at sigmoidoscopy. However, functional analyses performed at this early time point showed a number of significant antivascular effects induced by bevacizumab. Tumor interstitial pressure measurements showed a significant drop in pressure following bevacizumab administration (Fig. 2A and B). This finding was consistent with preclinical data and supported the vascular normalization hypothesis. At the same time, tumor microvascular density (measured by CD31 immunostaining in serial biopsy tissues) and blood flow (measured by perfusion CT) were also significantly decreased at day 12 after bevacizumab infusion (Fig. 2C). The number of viable CECs (evaluated by flow cytometry) was also significantly reduced by bevacizumab at day 3. In contrast, FDG-uptake in the tumors measured on PET scans

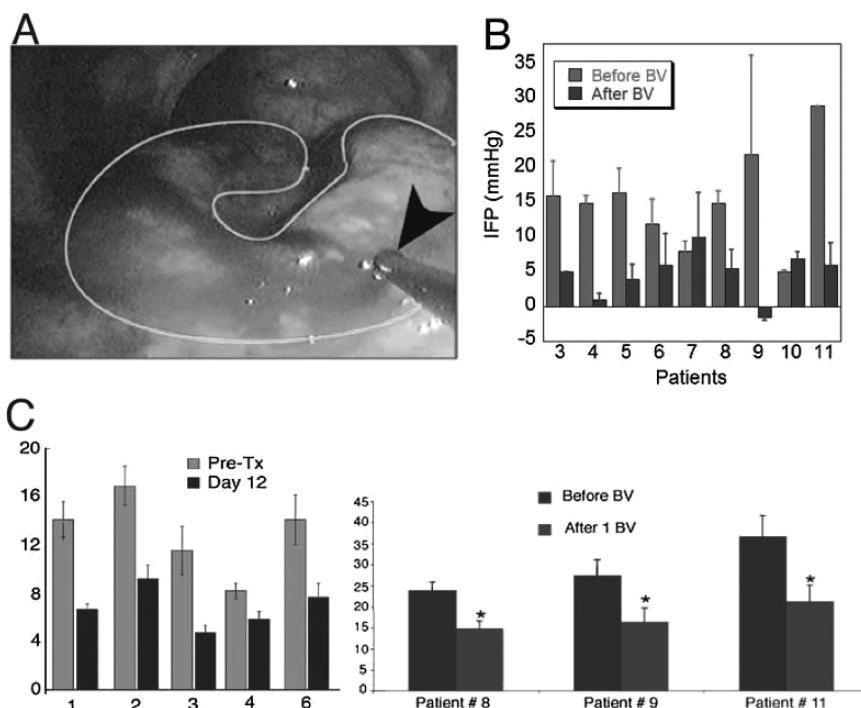


Fig. 2. Tumor response to bevacizumab alone and to combination of bevacizumab with chemoradiotherapy. (A) Endoscopy image showing the interstitial fluid pressure (IFP) measurement in a rectal cancer using the wick-in-needle technique. (B) Changes in IFP 12 days after bevacizumab infusion alone; note the consistent decrease in IFP, particularly in patients with high baseline values. (C) Tumor microvascular density (MVD) measured in serial biopsies before and after bevacizumab infusion; at day 12 bevacizumab alone reduced MVD approximately by half in all analyzable rectal carcinoma biopsies. (D) Tumor ^{18}F -fluorodeoxyglucose (FDG)-uptake before treatment, after bevacizumab alone, and after completion of neoadjuvant treatment in all eleven patients enrolled in the phase I trial. Bevacizumab alone did not significantly reduce the median standard uptake value (SUV) for FDG, but the combined treatment produced significant reductions in SUV prior to surgery. Adapted with permission (5).

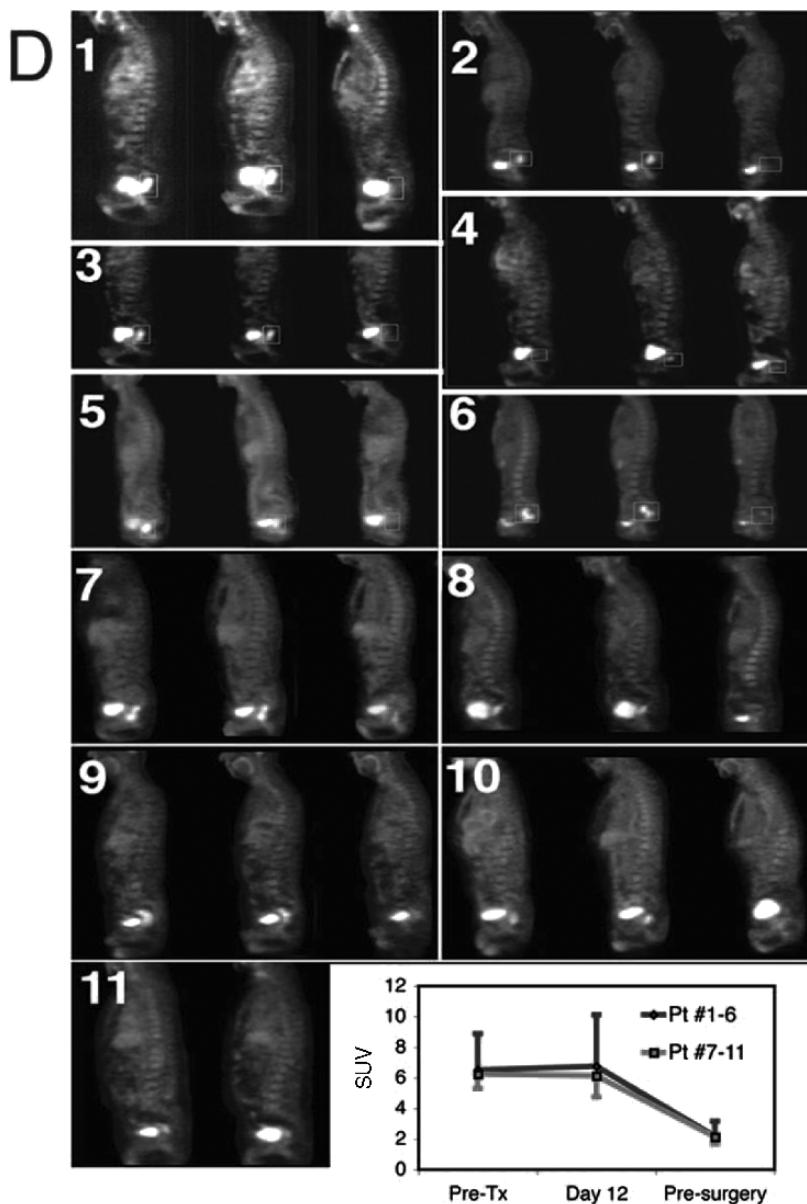


Fig. 2. (Continued).

remained constant (Fig. 2D). Thus, despite decreases in tumor vascular density and blood flow, tumor metabolism as assessed by FDG activity was unchanged, supporting a vascular normalizing effect of bevacizumab at this early time point. Finally, more evidence of the normalization hypothesis was provided by additional immunohistochemical studies in serial tumor biopsies. Whereas apoptosis of cancer cells increased as expected based on the decrease in vessel density, the proliferation rate in cancer cells increased supporting the normalization of microenvironment and potentially explaining the chemosensitization effect of bevacizumab (5, 6).

In summary, the results in patients mirrored those seen in transplanted tumors in mice—2 weeks after a single injection of bevacizumab alone. However, it remained unclear when the vascular normalization had begun and ended during VEGF blockade. Also, the efficacy of bevacizumab with chemoradiation in this neoadjuvant setting is being explored in an ongoing phase II trial at Massachusetts General Hospital in Boston, MA and Duke University Medical Center in Durham, NC.

6. IDENTIFICATION OF THE NORMALIZATION WINDOW IN MICE AND PATIENTS

For the clinical application of anti-VEGF therapy, an optimized scheduling of antiangiogenic therapy with chemotherapy and/or radiation therapy requires knowledge of the time window during which the vessels initially become normalized, as well as an understanding of how long they remain in that state.

Microvascular endothelial cell proliferation is a pathological hallmark of and diagnostic criterion for glioblastoma. VEGF receptor 1 [VEGFR1 or fms-like tyrosine kinase 1 (FLT-1)] and VEGF receptor 2 [kinase insert domain-containing receptor (KDR)] are highly coexpressed on endothelial cells of glioblastoma. In these tumors, VEGF is secreted primarily by the neoplastic cells (26). Angiogenesis in this disease is largely driven by this local overexpression of VEGF (27,28). Furthermore, levels of VEGF protein as well as mRNA correlate with the histological grade and microvascular density of gliomas (29,30). In studies of mice bearing human U87 glioblastoma xenografts and treated with an antibody to VEGF receptor-2, we have previously identified a “normalization time window.” During this period, the addition of radiation therapy yielded a synergistic antitumor effect and the best therapeutic outcome (Fig. 3) (24). The “normalization time window” was shortlived (~6 days) and was characterized by a reduction in tumor hypoxia and vascular permeability. During the normalization window, but not before or after it, VEGFR2 blockade was found to increase pericyte coverage of glioblastoma vessels, reduce vessel diameter, and degrade the pathologically thickened basement membrane in this animal model. Mechanistic studies showed that vascular normalization was causally related to angiopoietin—Tie2 signaling and to activation of matrix metalloproteinases (MMPs) (24).

Building on this preclinical experience, we subsequently demonstrated the existence of a normalization time window in patients with recurrent glioblastoma enrolled in a phase II study of cediranib (AZD2171; AstraZeneca Pharmaceuticals, Cheshire, UK), a pan-VEGF receptor TKI. Using serial MRI sequences—capable of measuring relative vessel size, vascular permeability and tumor-associated edema—we observed 31 patients with recurrent glioblastoma receiving cediranib monotherapy. The serial MRI studies demonstrated reductions in relative vessel size, vascular permeability, and tumor-associated edema as early as day 1 of treatment. These observations are consistent with a rapid vascular normalizing effect of cediranib. In this study, we observed that normalization begins within 1 day of cediranib administration and lasts for a minimum of 28 days (Fig. 4). Furthermore, we found that the tumor vessels become “de-normalized” upon discontinuation of cediranib treatment and “re-normalize” upon drug resumption (Fig. 5) (7). As a consequence of glioblastoma vascular normalization, there was an alleviation of brain edema and a steroid-sparing effect in these patients.

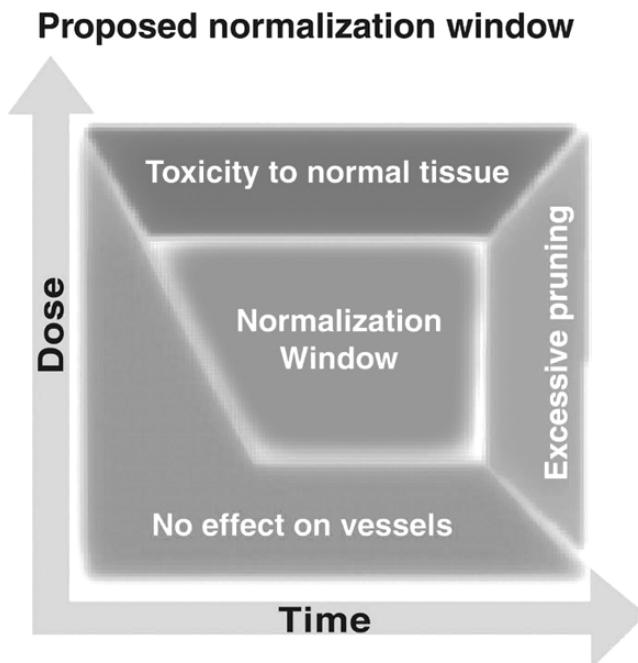


Fig. 3. Proposed effect of drug dose and schedule on tumor vascular normalization. The efficacy of cancer therapies that combine antiangiogenic and cytotoxic agents depends on the dose and delivery schedule of each drug. The vascular normalization model posits that a well-designed strategy should passively prune away immature, dysfunctional vessels and actively fortify those remaining—while incurring minimal damage to normal tissue vasculature. During this vascular normalization time window (red), cancer cells may be more vulnerable to traditional cytotoxic therapies and to novel targeted therapies. Note that the degree of normalization will be spatially and temporally dependent in a tumor. Vascular normalization will occur only in regions of the tumor where the imbalance of pro-angiogenic and antiangiogenic molecules has been corrected. Reproduced with permission (8).

Further studies of cediranib in glioblastoma patients are underway with additional plans to assess the drug in patients with vasogenic brain edema.

This phase II trial included comprehensive correlative biomarker studies at five time points during the first 28-day cycle and one time point after each cycle. Studies conducted in the first 16 consecutive patients showed that the plasma levels of basic fibroblast growth factor (bFGF), SDF1 α , and viable CECs correlated with termination of the normalization time window as measured by increased vessel diameter and treatment failure as measured by volume enlargement of the tumor (7). These observations raise the possibility of utilizing minimally invasive, serial blood biomarkers to monitor the onset and termination of the normalization time window. These observations also provide insight into possible molecular and cellular targets for exploitation to extend the normalization time window in this patient population.

The transient nature of vascular normalization in both preclinical and clinical studies of anti-VEGF therapies in glioblastoma suggests an optimal time or normalization window during which combination therapy (radiation or chemotherapy with anti-VEGF agents) may be critical. Reduction of tumor hypoxia and improved vascular delivery

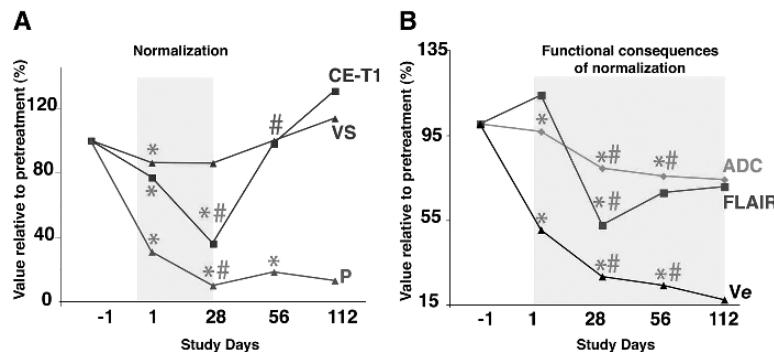


Fig. 4. Changes in MR imaging parameters over time. **(A)** Median values for contrast-enhanced T1-weighted tumor volume (CE-T1), vessel size (VS), and permeability (P) of the tumor over time as measured by an independent expert. Day 1 was set as 100% in all lesions, and changes during cediranib treatment were plotted for all 16 patients. Note the rebound of CE-T1 volume and vessel size after day 28, which indicates a partial closure of the vascular normalization window. **(B)** Median values of T2-weighted abnormality volume measured in fluid-attenuated inversion recovery images (FLAIR), apparent diffusion coefficient (ADC), and extracellular-extravascular volume fraction (v_e) prior to and during treatment showing a sustained decrease of edema while taking cediranib. * $p < 0.05$ for values compared with day 1; # $p < 0.05$ for values compared with day +1. Reproduced with permission (7).

may enhance the cytotoxic effects of chemotherapeutics and ionizing radiation. The addition of anti-VEGF therapy to the 6-week period of standard chemoradiation in patients with newly diagnosed glioblastoma is an attractive option and is currently being explored in ongoing and planned clinical studies at Massachusetts General Hospital.

7. TRASTUZUMAB ACTS AS AN ANTIANGIOGENIC COCKTAIL AND NORMALIZES VESSELS

The constellation of angiogenic molecules expressed in a tumor increases with malignant progression. For example, early stages of breast tumors may require only VEGF for angiogenesis, whereas at later stages, angiogenesis in these tumors may be driven in part by additional factors. Thus, a late-stage breast tumor may escape anti-VEGF treatment by exploiting alternative angiogenic factors to generate/maintain its neovasculature. This may help explain why bevacizumab and chemotherapy did not prolong the survival of breast cancer patients in a recent phase III trial (1). In rectal cancer patients, we discovered that plasma VEGF and placental-derived growth factor (PIGF) levels increase after VEGF blockade with bevacizumab (5, 6) and reproduced these findings in our study of cediranib in recurrent glioblastoma patients (7). Moreover, as noted above, plasma bFGF and SDF1 α levels correlated with relative vessel diameter and disease progression during cediranib treatment in recurrent glioblastoma patients (7). Thus, optimal cancer treatment may require the targeting of multiple angiogenic pathways. In practice, the challenge for the oncologist will be to formulate combinations of antiangiogenic agents specifically tailored to the angiogenic profile of individual tumors.

We have recently shown in preclinical models that anticancer agents such as trastuzumab can “mimic” antiangiogenic combinations (31). Trastuzumab lowers expression

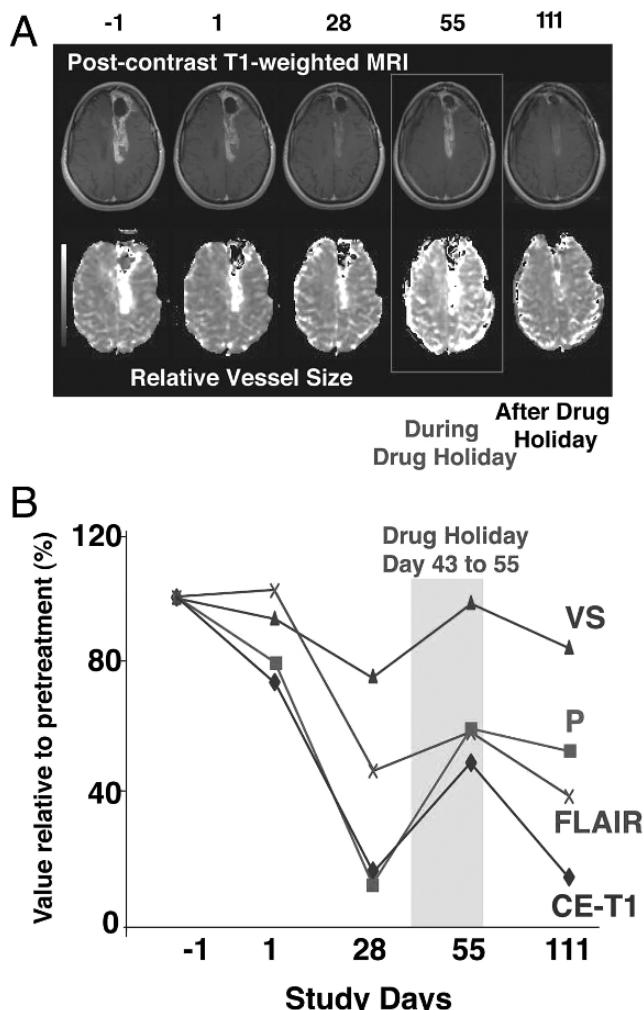


Fig. 5. Reversibility of vascular normalization. (A) Vascular and volume changes as a function of time in a patient who did not take drug from day 43 through 56 and was imaged on day 55 (shown as drug holiday). T1-weighted anatomic images were acquired after intravenous administration of gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA). Note that at day 55 there is a rebound in tumor enhancement, which decreases again after restarting the drug as seen on follow-up imaging on day 110. In this patient, maps of relative vessel size also show fluctuation with the drug holiday and resumption of cediranib treatment. (B) Measurements of MR imaging parameters confirm the reversibility of vascular normalization by drug interruption followed by renormalization after cediranib is resumed. Reproduced with permission (7).

of several pro-angiogenic molecules while increasing expression of the antiangiogenic molecule thrombospondin-1. Interestingly, although trastuzumab lowered the expression of VEGF in tumor cells, tumor stromal cells produced compensatory VEGF; thus, additional anti-VEGF treatment could improve the efficacy of trastuzumab. These findings provide a powerful rationale for the clinical trial in which trastuzumab is combined with bevacizumab for treatment of HER2-positive breast cancer (1).

A further finding from this study was that trastuzumab normalized the vasculature of human HER2-positive breast cancer xenografts. Whereas vessels in the control antibody-treated tumors were dilated and leaky, those in the trastuzumab-treated tumors had diameters and vascular permeability closer to those of normal vessels. Thus, trastuzumab and other drugs that target upstream mutant receptors might act as mimics of antiangiogenic cocktails—that is, these drugs improve their own delivery as well as that of other therapeutics given in combination. This improvement in delivery and alleviation of hypoxia presumably contributes to their efficacy.

8. PERSPECTIVE

At least three major challenges must be met before therapies based on this vascular normalization model can be successfully translated to the clinic. The first challenge is to determine which other direct or indirect antiangiogenic therapies lead to vascular normalization. In principle, any therapy that restores the balance between pro-angiogenic and antiangiogenic molecules should induce vascular normalization. Indeed, withdrawing hormones from a hormone-dependent tumor lowers VEGF levels and leads to vascular normalization (32). Recently, metronomic therapy—a drug delivery method in which low doses of chemotherapeutic agents are given at frequent intervals—has also been shown to increase the expression of thrombospondin-1, which is a potent endogenous angiogenesis inhibitor (33). Conceivably, this therapy might also induce normalization and improve oxygenation and drug penetration into tumors. Whether various synthetic kinase inhibitors, endogenous inhibitors, antivasocrine agents, conventional chemotherapeutic agents, and vascular targeting agents can do the same remains to be seen. Some of these agents may be particularly effective because they target both stromal and cancer cells. To date, most clinical trials are designed primarily to measure changes in the size of the tumor and may therefore not shed light on changes in the vascular biology of tumors. Clinical studies, such as the rectal carcinoma and glioblastoma studies described earlier (5, 7), and other ongoing translational clinical trials should help bridge the gaps in this aspect of our knowledge.

The second challenge is to identify suitable surrogate markers of changes in the structure and function of the tumor vasculature and to develop imaging technology that will help to identify the timing of the normalization window during antiangiogenic therapy. Measurement of blood vessel density requires tissue biopsy and provides little information on vessel function. Although imaging techniques are expensive and far from optimal, they can provide serial measures of vascular permeability, relative vessels size, vascular volume, blood perfusion, and uptake of some drugs and can therefore be used to monitor the window of normalization in patients (7). This is especially important for tumors such as glioblastoma as serial acquisition of tumor specimens is not possible. In glioblastoma patients, bFGF, SDF1 α , and viable CECs are promising biomarkers of vascular normalization and tumor response (7). In rectal cancer patients, the number of viable CECs decreased at day 3 after VEGF blockade with bevacizumab and increased at later time points, but whether their kinetics coincides with the normalization window or predicts tumor response is unclear (34, 35). During the course of therapy, serial blood measurements of molecules involved in vessel maturation have the potential to identify surrogate markers. Positron emission tomography with [F-18] fluoromisonidazole and MRI can provide some indication of tumor oxygenation and

might be useful for tracking the normalization window. Finally, the measurement of the interstitial fluid pressure is minimally invasive, inexpensive, and easy to implement for anatomically accessible tumors.

The third challenge is to fill gaps in our understanding of the molecular and cellular mechanisms of the vascular normalization process so that we may be able to extend this window in patients(36). With rapid advances in genomic and proteomic technology and access to tumor tissues during the course of therapy, we can begin to monitor tumor response to antiangiogenic therapies at the molecular level. Our recent trials have identified PIGF (in rectal cancer), bFGF, and SDF1 α (in glioblastoma) as potential additional targets for extending this window (5–7).

Addressing all of these challenges will not only benefit patients with cancer but possibly patients with other diseases, for example, age-related wet macular degeneration or patients with vasogenic brain edema from any cause. These principles may also be useful for stabilizing plaques, controlling edema, and improving regenerative medicine, where the goal is to create and maintain a functionally normal vasculature.

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16

Metronomic Low-Dose Antiangiogenic Chemotherapy in Mice and Man

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SUMMARY

Metronomic (antiangiogenic) chemotherapy refers to a form of dose dense chemotherapy involving close regular, even daily, administration of conventional chemotherapy drugs at relatively low doses over long periods in the absence of prolonged drug-free periods. Anti-tumor efficacy, which in some cases can be remarkably effective in various experimental mouse models of cancer, even in the absence of toxicity, is thought to be mediated mainly by antiangiogenic effects, either locally, by direct targeting of activated/dividing endothelial cells in the angiogenic tumor neovasculature, or systemically, by effects on circulating (bone marrow derived) endothelial progenitor cells (CEPs). However, additional mechanisms may also be involved, including stimulation of the immune system by targeting regulatory T cells, and possibly also direct effects on tumor cells—which could include the tumor stem cell(-like) subpopulation. Metronomic chemotherapy, because of its relatively nontoxic nature, is ideal for combination therapy using various targeted biologic agents, especially antiangiogenic drugs. Other promising combinatorial strategies include “doublet” metronomic chemotherapy using two different chemotherapy drugs, interspersing low-dose chemotherapy with higher bolus dose (BD) injections of the same drug, or short-course maximum tolerated dose (MTD) chemotherapy followed by long-term metronomic chemotherapy combined with a targeted biologic agent. Such combinations can sometimes have striking preclinical anti-tumor effects, even in models involving large primary tumors or widespread high-volume metastatic disease.

A number of clinical trials and pilot studies testing various combinatorial metronomic chemotherapy regimens have been undertaken which, in aggregate, appear to confirm encouraging clinical activity in certain advanced-stage cancers, with

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only modest or minimal host toxicity being observed. Larger randomized phase III trials are thus warranted, especially considering some of the potential advantages of metronomic chemotherapy. These include increased convenience when using oral chemotherapeutic drugs, reduced costs when off-patent chemotherapeutic drugs are used, and reduced severity of toxic side effects. These features make metronomic chemotherapy-type regimens ideal for adjuvant chemotherapy of early-stage cancers, an example of which is long-term, nontoxic daily oral tegafur-uracil (UFT) (a 5-FU prodrug composed of uracil and tegafur) for treatment of early-stage non-small cell lung cancer (NSCLC).

Key Words: low-dose chemotherapy; tumor angiogenesis; antiangiogenic therapy; clinical trials; endothelial progenitor cells; VEGF; metastasis; breast cancer; ovarian cancer; cyclophosphamide.

1. ORIGIN OF THE CONCEPT OF METRONOMIC ANTIANGIOGENIC CHEMOTHERAPY

Metronomic low-dose chemotherapy refers to a dosing and administration schedule for conventional cytotoxic chemotherapy drugs, which is thought to induce anti-tumor effects indirectly, primarily by antiangiogenic mechanisms rather than by direct targeting of the tumor cell population (1, 2). The term refers to the close, regular administration of a chemotherapy drug in the absence of any prolonged drug-free break periods, generally over long periods of time (even years), using relatively low, nontoxic doses of drug (1, 3, 4). For various reviews and commentaries, see references (4–12). The idea of using chemotherapy drugs as antiangiogenics was initially based on the hypothesis that dividing endothelial cells present in the growing neovasculature of tumors, in theory, should be susceptible to the cytotoxic effects of chemotherapy similar in principle to any other normal rapidly dividing cell population (13). If so, it was hypothesized that it should be possible to induce tumor responses even if the tumor cell population per se is resistant to a given chemotherapy drug, by virtue of targeting the presumably drug-sensitive dividing host endothelial cell population in the tumor's growing neovasculature. In fact, there are numerous studies showing that a wide spectrum of chemotherapy drugs belonging to virtually every class of such drugs can cause antiangiogenic effects in a variety of angiogenesis assays (14). However, Folkman's laboratory reported in 2000 that the potential antiangiogenic effects of chemotherapy as a result of inducing endothelial cell apoptosis in the tumor's vasculature, using cyclophosphamide (CTX), were reversed rapidly if the drug was administered in a conventional, pulsatile fashion using MTDs separated by long 2-week drug-free break periods between successive courses of chemotherapy (1). Such breaks are necessary to allow the host to recover from the harmful side effects of chemotherapy, particularly myelosuppression. Therefore, it was reasoned by Browder et al. (1) that if the drug was administered in a more condensed schedule, e.g., weekly rather than every 2 weeks, this would compromise the repair process involved in replacing damaged or killed vascular endothelial cells. Browder et al. called this schedule of chemotherapy “antiangiogenic chemotherapy,” and its major hallmarks include prolonged treatment, the absence of any excessively long (e.g., 2 or more weeks) drug-free break periods, and hence the need for much lower doses of drug for each unit treatment. In addition, it was reported by Browder et al. that a variety of transplantable mouse tumors that had been previously selected for acquired resistance in

vivo to CTX by using conventional MTD dosing and scheduling of CTX responded to CTX treatment by simply switching to the weekly lower dose metronomic protocol (1). Thus, a state of acquired stable tumor cell drug resistance could be reversed by altering the dosing and administration schedule of the drug. Interestingly, there are a number of precedents for this in the clinic, e.g., ovarian or breast cancer patients who respond to a weekly taxane regimen after seemingly becoming resistant to a taxane regimen administered at the MTD in a once every 3-week schedule (4). Similar findings have been reported for other drugs, e.g., the oral alkylating agent temozolomide (15, 16).

Preclinical results somewhat similar to those of Browder et al. were reported by Klement et al. (3) using vinblastine to treat human neuroblastoma xenografts, where the drug was administered every 3 days at about 1/10 to 1/20 the MTD for mice over a 7-month long period without any longer drug-free break periods. In addition, Klement et al. reported that combining this nontoxic vinblastine protocol with concurrent administration of a targeted antiangiogenic drug, e.g., antibodies to vascular endothelial cell growth factor (VEGF) receptor-2 (VEGFR-2), also administered every 3 days, resulted in remarkably effective tumor responses, which included complete and sustained regressions of large established tumors, without any evidence of either tumor relapse or overt toxicity over the 7-month long period of therapy (3). The rationale for using this particular treatment combination was based on the hypothesis that the presumptive endothelial cell targeting effects of a metronomic chemotherapy regimen might be compromised by locally high levels of endothelial cell survival factors in the tumor vasculature microenvironment, especially VEGF, and thus blocking the pro-survival function of VEGF at the same time as administration of metronomic chemotherapy would, it was hypothesized, significantly improve the effects of the metronomic chemotherapy (3). In addition to antiangiogenic anti-VEGFR-2 antibodies, various metronomic chemotherapy regimens have been combined in preclinical studies with a number of different antiangiogenic drugs including the fumagillin analog TNP-470/AGM-1470 (1), thrombospondin-1 (TSP-1) peptides (17, 18), the multi-receptor tyrosine kinase inhibitor (RTKI) SU11248/sunitinib which targets several receptor tyrosine kinases (RTKs) including VEGFR-2 (19), “PEX,” a fragment of matrix metalloproteinase-2 (20), the matrix metalloproteinase inhibitor (MMPI) BB-94, or the VEGF receptor inhibitor, SU5416 (21). Anti-VEGFR-2 antibodies have also been used by other investigators besides ourselves (22). Table 1 summarizes the various combinations tested and the diverse models used for these studies.

How the combination of metronomic chemotherapy and a targeted antiangiogenic drug causes selective, elevated apoptosis of activated endothelial cells in the tumor-associated neovasculature (1) is a subject of some interest. However, molecular information remains limited. Volpert and colleagues have reported that metronomic chemotherapy, e.g., 1/100 the MTD of doxorubicin, can cause upregulation of CD95 in activated endothelium, whereas CD95L (Fas ligand) can be upregulated by antiangiogenic TSP-1-related proteins (17, 18). Thus, the two types of therapies complement each other, causing the upregulation of an apoptotic death receptor (CD95) and its ligand in endothelial cells, causing inhibition of tumor angiogenesis (17, 18).

The term “metronomic” chemotherapy was first coined by Hanahan et al. (2). It is meant to convey the idea of regular beats of a metronome over long periods of time where each “beat” represents a dose of chemotherapy. Because of the increased frequency of drug administration and the longevity of the treatment, comparatively

Table 1
**Preclinical Studies of Metronomic Chemotherapy in Combination with Biologic
Antiangiogenic Agents**

| <i>Low-dose chemotherapy regimen</i> | <i>Antiangiogenic drug</i> | <i>Tumor model</i> | <i>Reference</i> |
|--|--------------------------------------|---|--------------------------|
| Vinblastine (intraperitoneal) every 3 days, 0.33mg/kg | DC101, anti-VEGFR-2 antibodies | Human neuroblastoma xenografts | Klement et al. (3) |
| Paclitaxel (intraperitoneal) every 3 days, 1 mg/kg | DC101, anti-VEGFR-2 antibodies | Human breast cancer xenografts | Klement et al. (87) |
| Daily oral cyclophosphamide in the drinking water at approximately 20mg/kg/day | DC101, anti-VEGFR-2 antibodies | Human breast cancer xenografts | Man et al. (65) |
| Doxorubicin 1.2mg/kg administered every 3 days | DC101, anti-VEGFR-2 antibodies | Human soft-tissue sarcomas | Zhang et al. (22) |
| Weekly cyclophosphamide at approximately one-third the maximum tolerated dose | TNP-470/AGM-1470 | Transplanted mouse tumors, e.g., Lewis lung carcinoma, EMT-6 breast tumor, L1210 leukemia | Browder et al. (1) |
| Daily oral cyclophosphamide in the drinking water or low-dose intraperitoneal vinblastine every 3 days, approximately 1mg/kg/day | BB-94 MMPI or SU5416 VEGFR inhibitor | Advanced RIP-TAG2 islet cell pancreatic carcinoma | Bergers et al. (21) |
| “Chemo-switch” regimen: short-term MTD cyclophosphamide followed by long-term daily oral cyclophosphamide in the drinking water, at approximately 10mg/kg/day | SU11248/sunitinib multi-RTKI | Advanced RIP-TAG2 islet cell pancreatic carcinoma | Pietras and Hanahan (19) |
| Carboplatin plus etoposide | “PEX” MMPI | Orthotopic glioblastoma | Bello et al. (20) |
| Doxorubicin administered every 5 days 0.2mg/kg | TSP-1 peptide | Human prostate or bladder xenografts | Quesada et al. (18) |

| | | | |
|---|--------------------------------------|--|-----------------------|
| Cyclophosphamide 2–20 mg/kg/day continuously in the drinking water | TSP-1 peptide mimetic, ABT-510 | PC3 human prostate carcinoma xenografts | Yap et al. (17) |
| Weekly one-third MTD i.p. cyclophosphamide | Anti-endoglin antibodies | MCF-1 human breast cancer xenograft | Takahashi et al. (88) |
| Topotecan 5 days per week | Anti-human VEGF antibodies | Orthotopic human Wilms tumor xenograft | Soffer et al. (89) |
| Carboplatin for two cycles days 14 and 21 | Endostatin or TNP-470 | Human germ cell tumors | Abraham et al. (90) |

low doses of chemotherapy drugs are required. In some cases, the cumulative doses of the drug are less than, or equivalent to, the respective chemotherapy drug administered in a conventional MTD fashion. As a result, the toxicities associated with metronomic chemotherapy regimens are often minimal and as a result do not require supportive care drugs, such as hematopoietic growth factors, e.g., G-CSF in clinical studies. This is in marked contrast to “dose dense” and intensive chemotherapy where a chemotherapeutic drug is also administered using a more frequent schedule, by using G-CSF to accelerate recovery from myelosuppression and thus still using fairly high doses such that toxicities remain significant (23). Indeed, it is the use of recombinant G-CSF to accelerate recovery from myelosuppression in the clinic to 2 weeks from 3 weeks, which makes dose dense chemotherapy possible (24).

2. MECHANISTIC BASIS OF ANTIANGIOGENESIS MEDIATED BY METRONOMIC CHEMOTHERAPY

Initially, the presumed sole target for causing the antiangiogenic effects of (metronomic) chemotherapy was hypothesized to be the differentiated dividing vascular endothelial cell in the growing tumor neovasculature (1, 3). Such cells are generally detectable in experimental and human tumors, usually at low percentages (25). However, it is now known that new endothelial cells in growing capillaries can be derived not only from such cells, but also as a result of the mobilization of cells from the bone marrow compartment, which can then enter the peripheral blood circulation and home to sites of angiogenesis where a proportion of them can incorporate into the lumens of such growing vessels and differentiate into endothelial cells (26), i.e., circulating endothelial progenitors (CEPs) (26, 27). Several years ago, it was reported by Bertolini et al. that very shortly after administration of an intensive bolus MTD course of three injections of CTX, the levels of CEPs were substantially reduced, which was followed by an abrupt and marked rebound in such cells, similar in nature to the process of hemopoiesis (28). The rapid mobilization of CEPs following their initial decline could conceivably account for at least part of the repair process to damaged tumor endothelium during the extended drug-free break periods following MTD chemotherapy, if such mobilized cells actually home to sites of tumor angiogenesis/damaged blood vessels. Recently, it was reported by Shaked et al. (29) that such a scenario is exactly the case after administration of so-called “vascular disrupting

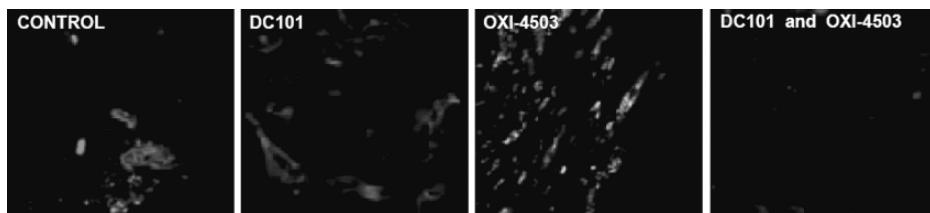


Fig. 1. Acute homing of bone marrow-derived vascular endothelial growth factor (VEGF) receptor-2+ circulating cells (including endothelial progenitor cells) to tumors shortly after tumor-bearing mice are treated with a single injection of a vascular disrupting agents (VDA) (Oxi-4503). Lewis lung carcinoma were grown in syngeneic C57/Bl6 mice that had previously been lethally irradiated and reconstituted with GFP-positive bone marrow cells. Note low levels of GFP-positive cells in tumors from untreated (control) or DC101-treated mice. However, 72 h after Oxi-4503 treatment, a pronounced GFP signal is evident in the tumor, indicating a massive homing of such cells to treated tumors, a process which can be prevented by prior treatment with DC101, the anti-VEGFR-2 antibody. The CEP homing phenomenon was shown to contribute to tumor angiogenesis and tumor growth at the viable tumor rim, which characteristically remains after VDA treatment. Taken from (29) with permission of the publishers. (Please see color insert.)

agents” (VDAs) such as combretastatin, a microtubule inhibitor, or a second-generation derivative of this drug called Oxi-4503. VDAs represent a class of vascular targeting agents which characteristically cause acute drops in tumor blood flow—within minutes to hours—followed by significant, if not massive, central tumor necrosis (30, 31). These effects are due to acute occlusion of established tumor vasculature. However, the striking anti-tumor effects of VDA treatment are compromised by the retention of a viable tumor rim from which rapid tumor re-growth resumes. This is similar in some respects to what can occur after major tumor responses induced by cytotoxic chemotherapy, namely, rapid tumor cell repopulation, which would be accompanied by tumor angiogenesis. We found that VDA treatment can quickly cause a marked increase in the peripheral blood levels of CEPs, which then home to the viable tumor rim (29). These bone marrow-derived cells are then retained at the tumor periphery as shown in Fig. 1, using mice that had been previously lethally irradiated and reconstituted with syngeneic green fluorescent protein (GFP)-positive bone marrow cells. These cells contributed to tumor angiogenesis, and hence tumor growth, but their ability to do so could be almost completely blocked by administration of an antiangiogenic drug (anti-VEGFR-2 antibodies) 24 h prior to VDA treatment (29). If a similar process applies to chemotherapy drugs administered at the MTD (i.e., mobilization of CEPs from the bone marrow and homing to the tumor), this could obviously account for the robust repair that was first noted by Browder et al. (1) to the tumor vasculature after MTD CTX treatment.

3. A PARADOXICAL QUESTION ABOUT METRONOMIC CHEMOTHERAPY

If shortening the breaks compromises endothelial cell repair mechanisms, should this not also apply to other types of normal drug-sensitive cycling cells, thus resulting in an increase in undesirable toxic side effects caused by chemotherapy administered in a metronomic fashion? This question represents an obvious paradox regarding the

mechanistic basis of metronomic chemotherapy. It has been noted by a number of investigators, both clinically (32) and preclinically (33), that metronomic administration of chemotherapy drugs such as CTX, or CTX plus methotrexate, is not associated with common high-grade toxicities such as myelosuppression. So how does one explain maximizing endothelial cell damage by more frequent dosing while not at the same time increasing the severity of other, undesirable side effects such as toxicity? Several possible explanations have been put forward in response to this question. First, for unknown reasons, dividing differentiated endothelial progenitor cells, and possibly bone marrow-derived CEPs, may be exquisitely sensitive to very low doses of chemotherapy, which are otherwise nontoxic or minimally toxic to other types of normal cycling cells that are known to be sensitive to higher doses of chemotherapy. For example, several groups have reported that extremely low concentrations of chemotherapy drugs in vitro can cause anti-endothelial effects, including growth inhibition, inhibition of migration, or apoptosis, at concentrations which do not have such effects on any other type of normal or cancer cell population tested (34–36). So, at extremely low doses of chemotherapy, there might be a marked selective sensitivity of activated dividing vascular endothelial cells. This selectivity might also apply to bone marrow-derived CEPs (37). Indeed, metronomic chemotherapy using CTX administered at an approximate dose of 20mg/kg/day through the drinking water is not associated with any myelosuppression, i.e., neutropenia (33), but was found to cause a marked decline in CEPs (37). A second possibility is that the effects of low-dose metronomic chemotherapy might be mediated by an indirect mechanism that is highly specific for activated vascular endothelial cells and/or CEPs. There is some evidence for this. For example, Bocci et al. (38) first reported that administration of low-dose CTX can result in a systemic induction of the well-known endogenous angiogenesis inhibitor known as thrombospondin-1 (TSP-1). Thus, the effects of metronomic CTX on tumors were largely lost when tumors were grown and treated in TSP-1-deficient mice. Several other groups have now also reported that metronomic chemotherapy can result by unknown mechanisms in an increase in the tissue expression of TSP-1, including in the tumor cells and tumor stroma as well as in increased circulating levels of TSP-1 in peripheral blood (39, 40). A key point in this regard is that TSP-1 would not be expected to affect myeloid bone marrow progenitors, hair follicle cells, or epithelial cells lining the gut, etc. so that cells/tissues that are normally sensitive to the toxic effects of MTD chemotherapy would not be profoundly affected by low-dose metronomic chemotherapy.

4. POTENTIAL ADVANTAGES OF METRONOMIC CHEMOTHERAPY REGIMENS

One significant advantage of most metronomic chemotherapy regimens, as discussed above, would be the absence of high-grade adverse events normally associated with MTD conventional chemotherapy dosing, such as severe nausea, vomiting, neutropenia, thrombocytopenia, mucositis, and hair loss (32, 33, 41). This has been noted preclinically in detailed studies (33, 41) and clinically (32), for example, using daily oral cyclophosphamide. This is not to suggest that toxicities would be absent, but they would be much less severe by comparison, and this obviously represents a significant potential advantage, especially with respect to treatment of elderly patients or children.

A second advantage, at least potentially, is convenience when using orally bioavailable chemotherapy drugs such as CTX (32), etoposide (42), methotrexate (MTX) (32), capecitabine, and UFT (43), among others. Such drugs can be administered at fixed or varying doses on an out-patient at-home basis. This particular advantage, however, must be balanced with the known disadvantages of oral drugs, such as patient compliance, and heterogeneous pharmacokinetics due to variable drug absorption. A third advantage relates to reduced costs (44), when using off-patent inexpensive drugs such as CTX or MTX. This represents a potentially very significant advantage, given the rapidly increasing, if not alarming, costs of most new anti-cancer drugs, the extent of which are placing enormous and growing burdens on health care systems (45). A fourth advantage is the ability to combine targeted biologic agents that are relatively nontoxic in a chronic fashion with metronomic chemotherapy. Such chronic combination treatments would not be possible when using only toxic MTD chemotherapy regimens.

5. OTHER COMBINATIONS THAT CAN BE USED WITH METRONOMIC CHEMOTHERAPY

While most published preclinical studies have shown the benefit of combining a targeted antiangiogenic drug with a particular metronomic chemotherapy regimen, there are other promising drug combinations that have been tested with encouraging results. For example, tumor vaccines can be combined with metronomic chemotherapy to enhance the overall effects of either form of therapy (46). This is particularly interesting since one of the supposed disadvantages of using immunotherapy approaches for cancer treatment is the inability to combine such approaches with potentially immunosuppressive MTD chemotherapy regimens. However, in the case of metronomic chemotherapy (at least with some drugs such as CTX), it has been shown that not only is this way of administering chemotherapy non-immunosuppressive, but it actually can *stimulate* the immune system (47). Indeed, it is known that administration of single low doses of CTX to mice or rats can deplete the host of immunosuppressive regulatory T cells and as a result, amplify the effects of cytotoxic T cells and perhaps other killer cells as well (48). This may be the case in humans as well (49). Thus, low-dose metronomic chemotherapy may be particularly ideal as a combination treatment with tumor vaccines (46). In addition to tumor vaccines, it has also been reported that other agents such as COX-2 inhibitors (50–53), letrozole, an aromatase inhibitor (54), trastuzumab (Herceptin®) (55), and dexamethasone (56) can each be combined with empiric metronomic chemotherapy regimens, often (but not always) involving daily low-dose oral CTX. Some of these studies are preclinical in nature, while others are clinical. It may be that the enhanced anti-tumor effects of combining a drug such as trastuzumab or celecoxib with metronomic chemotherapy are due to the antiangiogenic effects of the aforementioned biologic agents (50, 55). Indeed, the rationale for testing trastuzumab with metronomic CTX was based on previous studies showing that trastuzumab could have antiangiogenic effects that contribute to its overall anti-tumor efficacy (57).

6. DETERMINATION OF THE OPTIMAL BIOLOGIC DOSE (OBD) FOR METRONOMIC CHEMOTHERAPY

The current major disadvantage of metronomic chemotherapy is the empiricism associated with determining what is an optimal biologic low dose (58). In contrast, it is reasonably straightforward to determine the MTD of a given chemotherapy drug.

This handicap is not restricted to metronomic chemotherapy. For example, it is well known that many of the new “biologic” anti-cancer agents do not necessarily have dose-limiting toxicities which are normally used to define an MTD. In addition, even when a particular drug has a definable MTD, the OBD may be less than the MTD (59). However, there has been some progress made recently with respect to defining surrogate pharmacodynamic biomarkers which may be useful for monitoring the biologic activity of antiangiogenic drugs and metronomic chemotherapy, including determining the OBD (27, 37, 60). Shaked et al. (37) reported that the OBD of a number of different metronomic chemotherapy regimens can be determined by using CEPs as such a surrogate biomarker. This is based on prior findings, using mice, that enumeration of peripheral blood CEPs can be used as an *in vivo* assay for angiogenesis and therefore determining the OBD of targeted antiangiogenic agents such as anti-VEGFR-2 antibodies or TSP-1 peptide mimetics (27). The OBD for seven different chemotherapy drugs have been determined for mice using CEPs, including CTX (37), cisplatin (37), vinblastine (37), vinorelbine (37), paclitaxel (37), abraxane, a nanoparticle formulate paclitaxel (61), and UFT, a 5-FU prodrug (62). Whether CEPs can be used successfully in a similar fashion in humans is problematic given the lower numbers of such cells compared to mice (63). Perhaps, a more practical cellular surrogate may be apoptotic circulating endothelial cells (CECs). Recently, Mancuso et al. (64) have shown that apoptotic CECs measured after 2 months of metronomic CTX/MTX chemotherapy treatment in metastatic breast cancer patients has potential as a surrogate marker to monitor biologic activity of metronomic chemotherapy, and quite possibly, to predict future clinical benefit, such as progression-free survival and overall survival. The presumed source of these apoptotic CECs is the tumor vasculature based on preclinical studies. For example, administration of metronomic CTX to normal mice does not result in a detectable increase in the levels of apoptotic CECs, whereas such increases are noted when using biologically active doses of the same drug in tumor-bearing mice (64). Clearly, validation of such markers in humans will be an important step to improving the likelihood of achieving significant clinical benefits using metronomic chemotherapy protocols in patients. In this regard, the benefits that have been noted so far in empirical metronomic chemotherapy trials (summarized below) are particularly encouraging.

7. COMBINING MTD CHEMOTHERAPY WITH METRONOMIC LOW-DOSE CHEMOTHERAPY

In our first preclinical metronomic chemotherapy studies (3), the once every 3-day low-dose vinblastine administration schedule was actually preceded by a 3-week treatment regimen where the drug was given at a cumulative higher dose using a continuous infusion pump, to treat large established neuroblastoma xenografts. The initial upfront higher cumulative dose regimen was used to cause some tumor shrinkage, which would then be followed by the long-term “maintenance” metronomic chemotherapy regimen using the same drug. This highlights the possibility of using conventional chemotherapy dosing in sequence with metronomic chemotherapy. In this regard, another preclinical study by Pietras and Hanahan (19) clearly showed the significant benefits that can be derived by a short upfront course of MTD CTX therapy, immediately followed by long-term daily low-dose metronomic CTX, where the drug was administered daily through the drinking water, as first reported by Man et al. (65).

A variation of the aforementioned two studies was reported by Shaked et al. using three different transplantable tumor models, in which BD intraperitoneal injection of approximately one-third of the MTD of CTX was administered every 3 or 6 weeks, along with daily low-dose oral CTX administered through the drinking water at the OBD (metronomic). This combination of BD plus low-dose metronomic chemotherapy was found to significantly improve the effects of the low-dose metronomic chemotherapy regimen used alone and, in some cases, was associated with surprisingly effective long-term anti-tumor effects (66). Thus, conventional MTD-type chemotherapy and less toxic low-dose metronomic chemotherapy should be considered as a possibly complimentary way of giving the same drug to enhance overall anti-tumor efficacy. Clinically, there are protocols being tested that are somewhat similar in some respects, e.g., daily low-dose oral CTX with weekly vinblastine (67) or weekly platinum and daily oral etoposide (68).

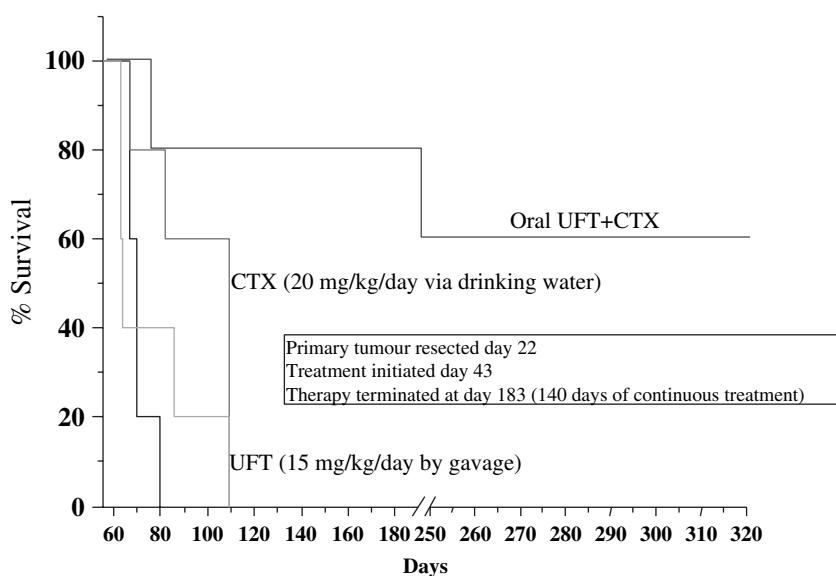


Fig. 2. Effects of combination “doublet” oral low-dose metronomic chemotherapy on survival of mice with advanced, high-volume visceral metastatic breast cancer. A new model of advanced high-volume visceral metastatic breast cancer was developed using the MDA-MB-231 human breast cancer cell line, which involved a combination of orthotopic transplantation, surgical removal of primary tumors, and establishment of cell lines from subsequently forming metastases found in the lungs. One such variant is called LM2-4. This variant was orthotopically transplanted into SCID mice and the tumor removed approximately a month later, and then treatment was initiated approximately 1 month after surgery when extensive macroscopic metastases were established in several different organ sites, such as the lungs, liver, and lymph nodes. Treatment consisted of daily oral low-dose cyclophosphamide (CTX), or daily oral low-dose UFT, administered by gavage at the indicated optimal biologic doses (OBDs), as determined by using circulating endothelial progenitor cell (CEP) as a surrogate biomarker of tumor angiogenesis. Therapy was maintained continuously for 140 days. Taken from (62) with permission of the publishers.

8. COMBINATION DOUBLET METRONOMIC CHEMOTHERAPY USING TWO DIFFERENT CHEMOTHERAPY DRUGS

A question is whether two different chemotherapy drugs, administered as a “doublet” regimen using metronomic dosing and schedules, would improve upon the effects of either drug used alone. It would seem to be a compelling rationale for testing such drug combinations as it is well known that combinations of two chemotherapy drugs administered in an MTD fashion are often more effective than either drug alone, e.g., a platinum drug in combination with a taxane (“PC”) for the treatment of NSCLC, or the combination of 5-FU, leucovorin and irinotecan, or oxaliplatin, for the treatment of colorectal cancer, CTX and adriamycin (AC) for breast cancer, etc. To this end, Munoz et al. (62) investigated the effects of a combination of daily low-dose CTX administered through the drinking water on a non-stop basis combined with UFT administered by gavage, also on a daily non-stop basis, where both drugs were dosed at the OBD, using CEPs as a surrogate biomarker to determine the OBD. This combination chemotherapy treatment was tested in a newly developed model of advanced, high-volume, visceral human metastatic breast cancer in immunodeficient mice. Indeed, evidence was reported showing that the combination of metronomic CTX and UFT could cause remarkable long-term anti-tumor effects, if not cure, and moreover, do so in the absence of any significant discernable toxicity (62), as shown in Fig. 2.

9. ADDITIONAL POSSIBLE MECHANISMS TO ACCOUNT FOR THE ANTI-TUMOR EFFECTS OF METRONOMIC CHEMOTHERAPY

Although the evidence to date suggests that the anti-tumor effects of metronomic chemotherapy have an antiangiogenic basis, there is also some evidence, of other possible mechanisms at play. For example, as already discussed, there are reports showing that low-dose cyclophosphamide chemotherapy can boost the immune system in mice by eliminating regulatory/suppressor T cells (48). Furthermore, Ghiringhelli et al. (49) have recently reported that longer term low-dose CTX therapy in patients can also significantly reduce the levels of T-regulatory cells, and this can have the potential effect of boosting the immune system and other cells such as natural killer cells. Thus, there may be some instances where low-dose metronomic chemotherapy can bring about anti-tumor effects by stimulating the efficacy of various immune surveillance mechanisms. This potentially makes metronomic chemotherapy ideal to combine with immunotherapeutic anti-tumor strategies, including the use of tumor vaccines, for which there is some limited preclinical evidence, as previously discussed (46).

Another obvious possible anti-tumor mechanism for metronomic chemotherapy is direct effects on tumor cells. A basis for this suggestion comes from the fact that most clinical trials showing an ostensible benefit to metronomic chemotherapy utilize chemotherapy drugs that are normally approved for use in treating the particular malignancy being tested, for example, CTX for the treatment of breast and ovarian cancer as well as non-Hodgkin’s lymphoma. In contrast, when metronomic CTX is used for the treatment of a cancer which normally does not respond to CTX, e.g., renal cell carcinoma, the benefits seem negligible by comparison (69). One intriguing possibility with respect to direct effects of metronomic chemotherapy on tumor cells may

be the targeting of the tumor stem cell or stem cell(-like) population present in solid tumors as well as in hematologic malignancies (70–75). Indeed, we recently obtained evidence that metronomic CTX can reduce the fraction of putative tumor stem-like cells in the C6 rat glioma system (76). In contrast, conventional MTD chemotherapy had no such impact on this critically important tumor cell subpopulation (76). Targeting of tumor stem cells by metronomic chemotherapy could conceivably represent a way of obtaining a clinical benefit in the absence of rapidly induced tumor responses.

10. CLINICAL TRIAL RESULTS OF METRONOMIC CHEMOTHERAPY APPEAR TO SUPPORT PRECLINICAL STUDIES

A number of pilot clinical studies as well as phase II clinical trials, including randomized trials, have been initiated to evaluate the metronomic chemotherapy concept. The results, taken together, suggest that metronomic chemotherapy may become a clinically validated concept, though formal confirmation of this awaits initiation and completion of larger randomized phase III clinical trials. Some of the more notable trials include a non-randomized trial of relapsed, refractory non-Hodgkin's lymphoma using daily low-dose CTX in combination with celecoxib (50), a trial of recurrent epithelial ovarian cancer using daily low-dose CTX in combination with biweekly bevacizumab (77, 78), a randomized phase II trial of daily low-dose CTX and low-dose MTX administered 2 days a week in combination with bevacizumab for the treatment of metastatic breast cancer (79), and a non-randomized trial involving different malignancies involving daily low-dose CTX, weekly vinblastine, along with concurrent daily rofecoxib (67). All of these trials have been associated with minimal toxicity along with putative clinical benefit in terms of time-to-progression, response rate, or progression-free survival and sometimes overall survival. The frequent use of CTX, and in some cases, MTX along with CTX, stems from a pivotal non-randomized phase II trial involving 63 women with metastatic breast cancer who were treated with daily low-dose CTX (50 mg orally) and MTX (orally) 2 days a week for a total dose of 10 mg a week by Colleoni et al. (32). A more recent report involving a total of 153 treated patients showed that prolonged clinical benefit 12 months or more, (complete remission, partial remission, or disease stabilization) was achieved in 15.7% of patients (80). Thus, metronomic chemotherapy can induce prolonged clinical benefit in metastatic breast cancer patients (80). Based on a number of preclinical studies, especially Klement et al. (3), it was decided to use this empiric but convenient all-oral metronomic chemotherapy protocol in combination with various targeted biologic agents, as discussed above. In addition, the results of other chemotherapy drugs in clinical trials are being re-examined retrospectively from the point of view of the metronomic chemotherapy concept. For example, over a decade ago, a clinical trial was initiated using the 5-FU oral prodrug, UFT, for the treatment of early-stage, resected NSCLC where the drug was orally administered at low, nontoxic doses every day for 2 years, by tablet with no breaks (43). UFT, or its metabolites, have been shown to have antiangiogenic effects, especially when administered by continuous infusion at lower doses, as opposed to intermittent bolus injections (81). UFT generates three different metabolites—5-FU, gamma butyrolactone (GBL), and gamma hydroxybutyrate (GHB)—all of which have been shown to induce antiangiogenic effects *in vivo* (81).

In addition to the aforementioned clinical trials, results of smaller pilot studies have been reported using low-dose CTX and dexamethasone for advanced prostate cancer (56), rofecoxib and pioglitazone with daily oral low-dose trofosfamide for advanced melanoma and soft-tissue sarcoma as well as malignant vascular tumors (52, 53), among others. Table 2 summarizes many of the clinical trials and pilot studies testing metronomic chemotherapy. In addition to the frequent use of oral CTX, as noted above, another oral alkylating agent—trofosfamide—has been used in a number of clinical studies (51–53, 82–84). This raises the question of whether oral alkylating agents—including temozolomide (15, 16, 85, 86)—are optimal chemotherapeutics for metronomic chemotherapy.

Table 2
Results of Some Clinical Studies Evaluating Metronomic Chemotherapy

| <i>Study or trial</i> | <i>Main details</i> | <i>Results</i> | <i>Reference</i> |
|---|---|--|----------------------|
| Randomized phase II trial of letrozole and low-dose cyclophosphamide in elderly breast cancer patients | 57 patients received letrozole and 57 letrozole plus metronomic cyclophosphamide | Response rate of 87.7% in combination treatment and 71.9% in letrozole arm | Bottini et al. (54) |
| Non-randomized trial in pretreated metastatic breast cancer patients using daily low-dose (50mg) cyclophosphamide and low-dose methotrexate 2 days/week | 64 patients treated (63 evaluable); most (51) patients had progressive disease at entry | Two complete responders (CR), 10 partial responders detected overall clinical benefit, including stable disease of over 6 months was 31.7%. Adverse toxic events mild, e.g., grade 2 or more leucopenia observed in only 13 patients | Colleoni et al. (32) |
| Randomized phase II trial of metronomic cyclophosphamide and methotrexate plus or minus thalidomide in metastatic breast cancer patients | 171 evaluable patients enrolled in trial | Results confirm role for metronomic chemotherapy in breast cancer; very high clinical benefit rates (41.5%) observed with minimal toxicity; addition of thalidomide did not improve results of the metronomic chemotherapy regimen | Orlando et al. (80) |

(Continued)

Table 2
(Continued)

| <i>Study or trial</i> | <i>Main details</i> | <i>Results</i> | <i>Reference</i> |
|---|---|--|-----------------------|
| Pilot study of 3 drugs, pioglitazone, refecoxib, and metronomic trofosfamide, in patients with advanced malignant vascular tumors | 6 patients treated (5 angiosarcomas and 1 hemangioendothelioma) | 2 complete responders, 1 partial responder, and 3 patients achieved disease stabilization | Vogt et al. (52) |
| Pilot study of metronomic temozolomide for recurrent temozolomide-refractory glioblastoma | 12 patients treated with continuous daily dose of oral drug at 40 mg/m ² | Partial responses observed in 2 patients and stable disease in 5; no complete responses; no grade III/IV toxicity observed | Kong et al. (16) |
| Phase II non-randomized clinical trial of pioglitazone, rofecoxib, and metronomic low-dose daily oral trofosfamide for highly advanced malignant melanoma and soft-tissue sarcoma | 40 patients evaluated using daily oral pioglitazone, daily oral rofecoxib, and sequentially added oral trofosfamide, 50mg three times daily | Complete response in 1 melanoma patient and 3 sarcoma patients; stable disease >6 months observed in 11% of melanoma patients and 14% of sarcoma patients; no grade III/IV toxicities observed | Reichle et al. (53) |
| Non-randomized phase II trial in relapsed, or refractory non-Hodgkin's lymphoma with daily oral celecoxib, with no breaks plus cyclophosphamide | 32 of 35 patients evaluable; cyclophosphamide used at 50mg/day and celecoxib at 400m twice/day | Overall response rate 37% including 2 complete responders and 9 partial responders with 22% patients achieving stable disease | Buckstein et al. (50) |
| Phase II trial of oral low-dose cyclophosphamide, weekly vinblastine and rofecoxib in patients with advanced solid tumors | 50 patients treated, 43 of whom received at least one prior chemotherapy regimen; half of the patients also received | 2 complete responders, and 4 partial responders, and 8 patients with stable disease noted giving 30% clinical benefit; minimal toxicity noted | Young et al. (67) |

Table 2
(Continued)

| <i>Study or trial</i> | <i>Main details</i> | <i>Results</i> | <i>Reference</i> |
|--|--|---|---------------------|
| Phase II trial of erbB-2-positive metastatic breast cancer | minocycline as an antiangiogenic agent; 47 patients evaluable 22 patients, all pretreated with trastuzumab and other cytotoxic chemotherapy drugs | Overall clinical benefit (CR+ PR + SD) of 46% in patients overall and 27% in trastuzumab-resistant patients; overall toxicity generally very mild | Orlando et al. (80) |

11. SUMMARY AND CONCLUSIONS

Viewed as a whole, various pilot clinical studies as well as clinical trials suggest that metronomic chemotherapy may be a promising anti-cancer treatment strategy that is associated with considerably reduced toxicity compared to conventional or dose dense chemotherapy-type regimens. Moreover, as stressed throughout this review, it would appear to be an ideal way of administering chemotherapy in combination with many different types of biologic targeted therapies or drugs, including antiangiogenic agents, signal transduction inhibitors such as trastuzumab or sunitinib, aromatase inhibitors such as letrozole, and COX-2 inhibitors such as celecoxib and immunotherapeutic tumor vaccines. This, combined with the various potential advantages of metronomic chemotherapy, clearly warrants a more intensive and expanded effort with respect to larger randomized phase II and phase III clinical trials evaluating metronomic chemotherapy for the treatment of cancer.

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SUMMARY

It is now widely accepted that for any tumor to grow to macroscopic size, a change to a proangiogenic phenotype leading to the formation of new blood vessels is required. This recognition has led to the development and clinical advancement of novel antiangiogenic therapeutics in cancer management. An alternative approach to targeting the neovasculature associated with tumors is not to interfere with new vessel formation but rather to disrupt the function of the tumor vasculature after it has already been formed. Vascular disrupting agents (VDAs) are designed to cause a rapid and selective vascular shutdown in tumors. The resulting ischemia produces rapid and extensive tumor cell kill. Treatment with VDAs has been shown to lead to extensive tumor necrosis in a wide variety of tumor models. VDAs also synergize with conventional anticancer treatments including radiotherapy and chemotherapy, and recent evidence indicates that VDA treatments are complimentary to antiangiogenic therapeutics. Lead VDAs have now entered clinical trials. This chapter focuses on the background and current state of development of VDAs and emphasizes their therapeutic potential when used in combination with conventional anticancer therapies and antiangiogenic agents.

Key Words: Vascular disrupting agents; radiotherapy; chemotherapy; combination therapies; blood-flow inhibition; tumor ischemia; experimental therapeutics.

1. BACKGROUND ON VASCULAR TARGETING THERAPIES

A solid tumor's development, growth, and survival depend on the establishment and expansion of a functional blood vessel network. Consequently, the tumor vasculature has become a major target for the development of new approaches to cancer therapy. Indeed, strategies that emphasize the inhibition of new vessel formation by tumor cell-initiated angiogenic processes, so-called antiangiogenic therapies, have received a great deal of attention. Indeed, this approach has recently resulted in the clinical approval of the first antiangiogenic therapeutics bevacizumab (Avastin), sorafenib (Nexavar), and sunitinib (Sutent). An alternative strategy, based on the recognition of unique

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vascular characteristics and abnormalities in the structure and function of established tumor vessels (1), has led to the development of agents that compromise the function of the abnormal neovasculature already present in the tumor at the time of detection and treatment (2–4). These vascular disrupting agents (VDAs) cause a rapid and catastrophic shutdown in the vascular function of the tumor, which leads to tumor cell death as a result of oxygen and nutrient deprivation and build up of waste products.

Although both strategies target the tumor vasculature, key differences exist between agents that affect angiogenesis and those that lead to selective vascular destruction (5). These differences apply not only in their mode of action but also in their likely therapeutic application. Antiangiogenic therapies interfere with new vessel formation, thereby preventing tumor growth and limiting metastatic potential, and hence such therapies are typically administered chronically over months and years. VDAs compromise established tumor vasculature and have the potential to destroy tumor masses as well as preventing progression. Such agents are designed to be used in an intermittent fashion rather than by means of long-term exposures. Given these differences, it should be clear from a therapeutic perspective that targeting the tumor vasculature with antiangiogenics and VDAs is complimentary and not redundant.

1.1. History of VDAs

Evidence supporting the notion that damaging the existing blood vessel network of tumors could provide therapeutic benefits has existed for some time. Indeed sporadic observations have been made, indicating that solid tumors could be affected or even eradicated when their blood circulation was interrupted (6, 7). Based on such evidence, Denekamp (8) advocated the exploration of therapeutic strategies that could selectively disrupt the existing tumor vasculature. Since tumor blood vessels are constantly growing to meet the needs of the expanding tumor mass, it was postulated that at any given time, some part of the blood vessel network that supplies the tumor will be newly formed and immature (9). Measurements of proliferation indices for endothelial cells have confirmed active angiogenesis in animal and human tumors (10, 11), whereas the vasculature of normal healthy adult tissue typically is extremely stable (11, 12). Such differences between vasculature of normal tissues and that of neoplasia might readily be exploited for therapeutic benefit.

1.2. Classes of VDAs

VDAs are agents that cause direct damage to the already established tumor endothelium (2–4, 13). They are comprised of two main classes—the ligand-based therapies which deliver toxins, procoagulant, or pro-apoptotic effectors to disease-associated vessels and the small molecules, which do not specifically localize to such vessels, but exploit the known differences between them to induce selective vascular dysfunction.

The ligand-based therapies include biological response modifiers or cytokines like tumor necrosis factor (TNF) and interleukins; certain established chemotherapeutic drugs such as vinka alkaloids and arsenic trioxide; and a variety of strategies that use either antibodies, peptides, or growth factors that can selectively bind to tumor vessels (2–4, 13, 14). Gene therapy approaches have been quite appealing for this form of vascular targeting (13). Endothelial cell-specific promoter elements and vectors with restricted cellular tropisms have been examined, and encouraging results

have been reported. Several approaches based on linking antibodies or peptides that recognize tumor-associated vasculature to toxins, procoagulant, and pro-apoptotic effector molecules that can induce endothelial cell damage also have been explored. The utility of such ligand-directed VDA targeting is supported by recent *in situ* studies in preclinical tumor models that demonstrated not only the localization of the therapeutic moiety to tumor vessels but also the induction of thrombi formation and the selective destruction of tumor vasculature (3).

Small-molecule drugs can be subdivided into two classes of agents. The first includes flavone acetic acid (FAA) and its derivative 5,6-dimethylxanthenone-4-acetic acid (DMXAA), which have a complex mechanism of action that is poorly understood, but their main effect on vascular endothelial cells is thought to involve a cascade of direct and indirect effects, the latter involving the induction of cytokines, especially TNF- α , leading to the induction of extensive hemorrhagic necrosis in tumors as a result of vascular collapse (15). A second group includes the tubulin-binding agents combretastatin A-4 disodium phosphate (CA4P), the phosphate prodrug of *N*-acetyl-colchinol (ZD6126), AVE8062, NPI2358, MN-029, and OXi4503 (2,4). These tubulin depolymerizing agents are primarily believed to selectively disrupt the cytoskeleton of proliferating endothelial cells (16), resulting in endothelial cell shape changes and subsequent thrombus formation and vascular collapse. Since they preferentially target dividing endothelial cells, this accounts for their tumor specificity. Both types of small molecule drugs have been shown to have potent antivascular and antitumor efficacy in a wide variety of preclinical models, and the lead agents are undergoing clinical evaluation (4).

2. ANTITUMOR EFFICACY OF VDAS

2.1. VDA *Monotherapy*

The efficacy of VDAs has now been studied in a wide variety of preclinical tumor models, including transplanted and spontaneous rodent tumors, orthotopically transplanted tumors, and human tumor xenografts.

In a promising series of recent studies of ligand-based VDAs, Thorpe and colleagues (17, 18) demonstrated that targeting anionic phospholipids that are differentially expressed on the outer surface on tumor-associated vascular endothelial cells with an unconjugated IgG3 mAb can induce substantial vascular damage and a reduction in both vascular density and tumor plasma volume. Interestingly, the treatment of mice with subtoxic concentrations of docetaxel enhanced the percentage of tumor vessels that express anionic phospholipids on their outer surface in a human breast tumor xenograft, resulting in an enhanced antitumor efficacy that could be achieved with the mAb treatment (19).

Profound disruption of tumor vasculature has also been widely reported following treatment with small-molecule VDAs (20–22). The observed effects include vascular shutdown, reductions in tumor blood flow, and loss of patent blood vessels (Fig. 1). The subsequent impact on tumor tissue has been readily demonstrated by histologic assessments (Fig. 2). Typically, these show extensive, dose-dependent necrosis which can extend to within a few cell layers from the margin of the tumors (14, 21, 23–30). Consequently, a common observation in these studies is that induction of 80–90% tumor necrosis is readily achievable with VDA treatment. The extent of secondary cell

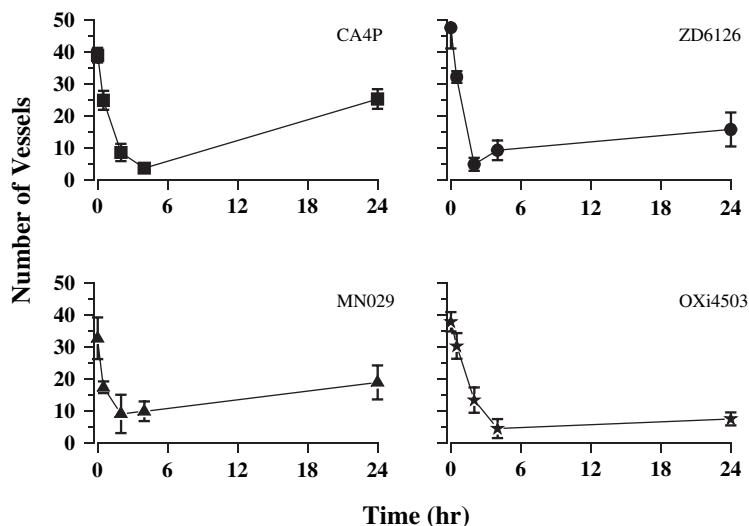


Fig. 1. Effect of vascular disrupting agent (VDA) treatment on patent tumor blood vessels in KHT sarcomas. Tumor-bearing mice were treated with CA4P (100 mg/kg), ZD6126 (100 mg/kg), MN029 (100 mg/kg), or OXi4503 (25 mg/kg), and at various times thereafter, the number of functional blood vessels was determined using fluorescent dye Hoechst 33342. Results are the mean \pm SE of three to five tumors.

death due to ischemia in treated tumors also can be quantified (26, 31) and usually is closely correlated to the extent of tumor necrosis. Importantly, the induction of tumor necrosis has recently been directly linked to reductions in tumor blood flow assessed by non-invasive contrast-enhanced magnetic resonance imaging (32), a technique also utilized in patients to determine the physiologic impact of VDA treatment in cancer patients (4).

Yet, despite the marked induction of tumor necrosis, a hallmark feature of VDA-treated tumors is the survival of a thin layer of neoplastic cells at the tumor periphery (Fig. 2). This is believed to be the consequence of areas at the tumor's edge being nutritionally supported at least in part by vessels in the surrounding normal tissue that are unaffected by VDA treatment (2). This rim can maintain the structure of the tumor, and cells from this area can rapidly re-grow the tumor, leading to the common observation that VDAs induce little or no tumor growth delay (33). A possible exception to this may be the second-generation VDA OXi4503. Recent preclinical work with this agent has shown that it not only leads to more efficient reduction of the viable rim of tumor tissue (Fig. 2) following treatment (30, 32, 34, 35) but also produces measurable tumor growth delays (32, 34, 35). Repeated treatments are particularly effective at retarding tumor growth. Indeed, the effects are even more apparent and achievable at lower drug doses when the agent is administered more than once (Fig. 3). By extending such a repeat treatment regimen over a period of several weeks, even more pronounced antitumor effects can be attained (Fig. 4). Still, because some tumor cells will always be located in areas supported by normal tissue vasculature, monotherapy with VDAs ultimately is unlikely to be curative.

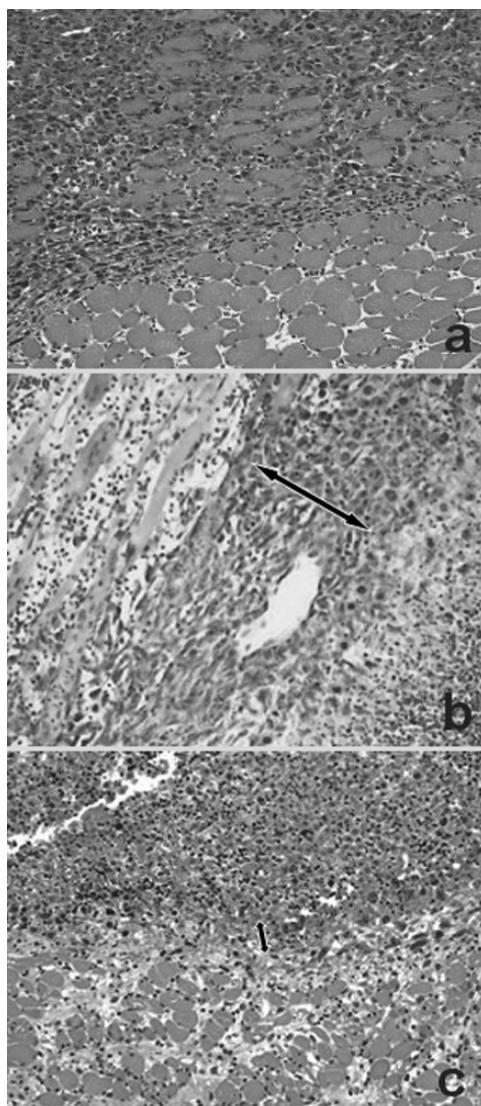


Fig. 2. Histologic evaluations of KHT tumor 24 h after CA4DP (100 mg/kg) or OXi4503 (25 mg/kg) treatment. (a) Untreated KHT sarcoma illustrating infiltrating tumor involving adjacent skeletal muscle. (b) KHT sarcoma after treatment with CA4P showing extensive central tumor necrosis but a persistent multilayered rim of tumor cells (arrow). (c) Tumor necrosis in mice treated with OXi4503 illustrating a reduction in the rim of surviving tumor cells (arrow) and the absence of damage to the adjacent muscle. (Please see color insert.)

2.2. *VDAs as Adjuvants to Conventional Anticancer Therapies*

Neovascularization is intimately involved in tumor survival, progression, and spread. These factors are known to contribute significantly to failures in cancer management, and consequently the tumor vasculature has become a major target for the development of new approaches to cancer treatment. Strategies targeting the tumor blood vessel support network may not only offer unique therapeutic opportunities in their own right

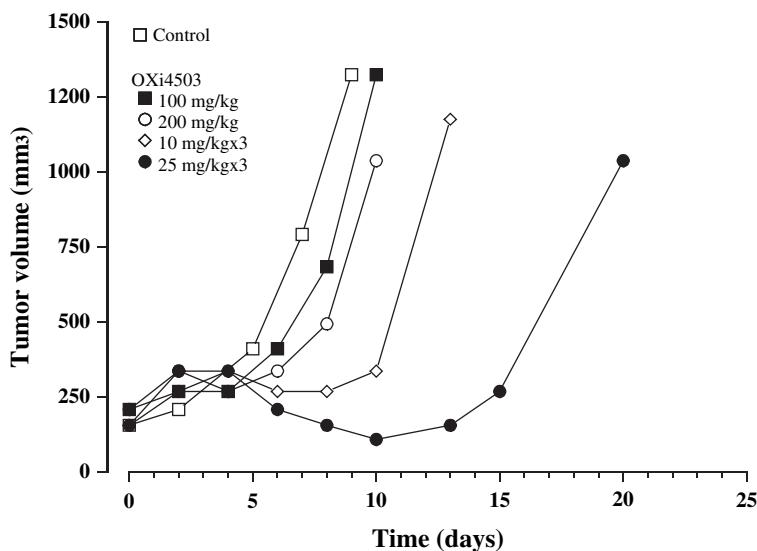


Fig. 3. Growth of median Caki-1 tumors in nude mice. Mice were treated with vehicle control or OXi4503 administered either as a single treatment on day 1 (100 or 200 mg/kg) or as three doses given on days 1, 3, and 5 (10 or 25 mg/kg). Data shown are the median animal of groups of nine mice.

but also novel means of enhancing the efficacies of conventional anticancer treatments such as chemotherapy and radiation therapy.

VDAs offer an attractive potential for combination with conventional cancer therapy due to their effective destruction of interior regions of tumors and excellent activity

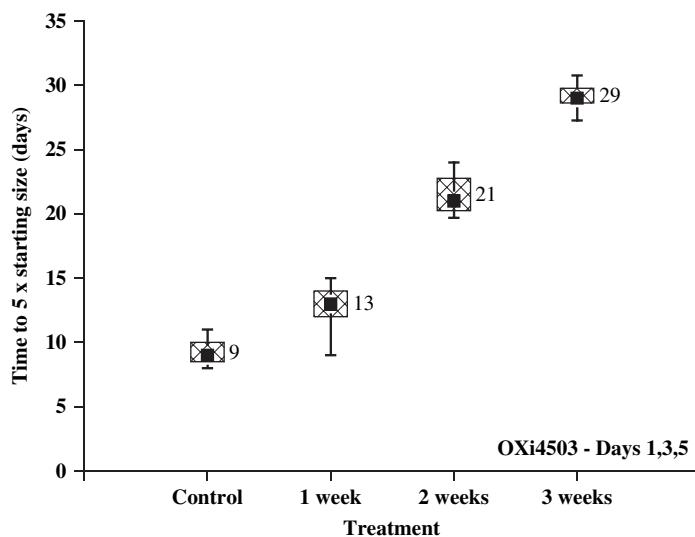


Fig. 4. Re-growth response of Caki-1 tumors in nude mice treated with OXi4503 for a 1-, 2-, or 3-week period. The vascular disrupting agent (VDA) dose was 10 mg/kg. The weekly treatments were on days 1, 3, and 5. Data shown are the median as well as the 25–75% (hatched) and 10–90% (bars) confidence intervals on groups of seven or nine mice.

against large bulky disease (28, 36) typically resistant to conventional anticancer therapies. Conversely, the tumor cells which survive VDA treatment at the border between malignant and normal tissue can repopulate the tumor, thus limiting the use of these VDAs as a single-agent therapy. However, given their location, these cells are likely to be in a state of high proliferation and excellent nutrition. These factors, coupled with their ready accessibility to systemically administered agents, make the surviving tumor cells susceptible to killing by radiation and anticancer drugs. This has led to the concept that VDAs need to be combined with conventional cytotoxic therapies to achieve tumor regression or prolonged disease stabilization (4).

2.2.1. CHEMOTHERAPY

The concept of combining VDAs with conventional anticancer therapies has been extensively tested in preclinical settings using a variety of tumor models (2–4, 13, 18, 37).

Enhanced antitumor effects may be expected on the basis of these therapies having distinctive target cell populations, independent mechanisms of action, and potential interactions. In general, marked enhancements in antitumor activities were observed when VDAs were combined with anticancer drugs. An important consideration in such investigations is the issue of timing and sequencing of the agents. Typically, optimal enhancements were obtained by administrating the VDAs within a few hours after chemotherapy (20, 27, 38–43). The rationale for such sequencing was to minimize possible interference of distribution and uptake of the chemotherapeutic agents by the VDAs. Indeed, some evidence exists that combination therapy may be less effective when VDAs are administrated immediately prior to the administration of the cancer chemotherapy agent (20, 40, 42, 44). One possible explanation for the latter results is that when VDAs are given just before chemotherapy, they may lead to transient reductions in blood flow and hence impaired chemotherapeutic agent delivery. If these blood-flow effects are insufficient to lead to tumor cell death due to ischemia, then a suboptimal antitumor effect could be the consequence. Consequently, investigations combining conventional anticancer drugs and VDAs have either administered the VDA post-chemotherapy or allowed a significant time interval between the two therapies. Under these treatment conditions, efficient enhancements of chemotherapy activity have been widely reported (2, 4, 37). The results indicate that such combinations can markedly enhance the response of tumors to chemotherapy most likely due to the two treatments acting in a complimentary fashion. Specifically, the VDA significantly reduces the central tumor cell burden, while chemotherapy destroys the rim of tumor cells surviving VDA treatment.

Because VDAs and conventional chemotherapy agents have distinctive target populations and mechanisms of action, it should be possible to achieve an increase in treatment efficacy with little or no increase in toxicity. In general, this has been observed in preclinical studies. Combination of VDAs with anticancer drugs have shown improved antitumor effects without concomitant increases in host toxicity or chemotherapy-specific normal tissue side effects (42). Current preclinical data therefore support the notion that combining VDAs with chemotherapy can improve treatment outcomes.

2.2.2. RADIATION THERAPY

In terms of radiation, since the cells surviving VDA treatment receive their nutritional supply from nearby normal tissue vasculature, it is likely that these cells are well oxygenated (26, 31, 44) and as such susceptible to radiation. Thus, a logical rationale for combining a VDA with radiation might be that the two treatments interact in a complimentary fashion at the tumor microregional level, i.e., the former reducing or eliminating the poorly oxygenated and hence radioresistant tumor cell subpopulations, the latter destroying cells not affected by the VDA. Indeed, several studies have now combined radiation with VDAs. Most of these studies have utilized single dose radiation treatments, and in several of these radiation studies the importance of sequence and timing between radiation and the VDA were investigated (26, 44–46). As was the case for chemotherapy, the greatest enhancement of radiation response was observed when VDAs were administered within a few hours after the radiation exposure (Fig. 5). Analysis of dose–response curves following the combination therapy suggested that including the VDA in the treatment led to a significant reduction in the radiation-resistant hypoxic cell subpopulation associated with the tumor. When administered post-radiotherapy, tumor responses have been observed to be significantly enhanced in a range of tumor models as determined by endpoints of growth delay, clonogenic cell survival, and tumor cure (26, 44, 45, 47–49). Increased tumor growth delays and

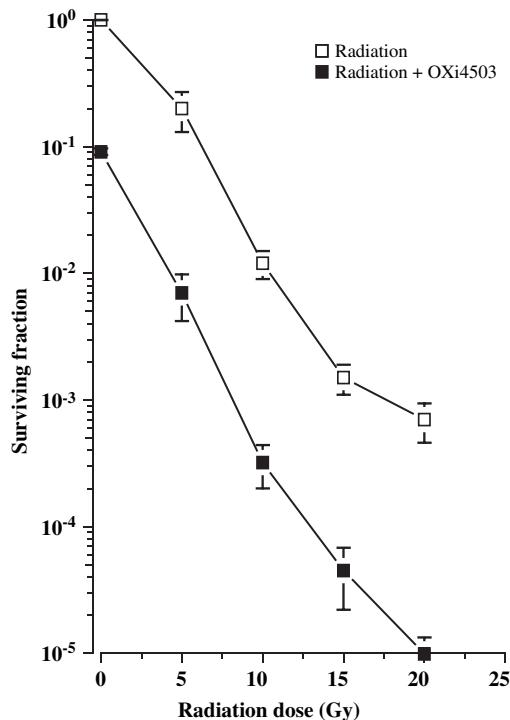


Fig. 5. Survival of clonogens in KHT sarcomas 24 h after treatment with a range of doses of radiation given either alone (open symbols) or in combination with a 2.5 mg/kg dose of OXi4503 (closed symbols). In the combination treatment, OXi4503 was administered 1 h after radiotherapy. Data are the mean \pm SE of three to nine tumors.

tumor cures also have been achieved in the successful application of antibody-targeted radioimmunotherapy and VDA treatments (50).

Though outcomes typically cannot be distinguished from an additive response (the VDA and radiation killing of two different cell populations), there have been some suggestions that some sort of interaction between the two treatments may occur. Precisely how the VDAs and radiation might interact is not clear. Recent studies suggest that tumor vasculature may also be an important target for radiation damage (51), and it is possible that an interaction at the level of the endothelial cells is occurring, perhaps through the VDA, increasing the extent of radiation-induced apoptosis, as has been demonstrated *in vitro* with TNF and radiation (52).

Preclinical observations also may provide important clues for the most appropriate application of VDAs in a clinical setting where fractionated radiation schedules are the norm. To avoid possible complications associated with transient vascular shutdown, the optimal approach would probably involve giving the VDA after the last radiation treatment each week in a conventional fractionated schedule. Using such an approach, several preclinical studies have demonstrated a benefit of combining VDAs and fractionated radiation (20, 26, 53). Giving the VDA more often may still be beneficial (44), but whether this holds true for all VDAs and tumor types is not known.

As is the case for combined modality studies in general, demonstrating improved tumor responses through the combination of VDAs and radiation will only be of benefit if such a treatment does not enhance the response of critical normal tissues. To date, results obtained from preclinical investigations addressing this question have been very encouraging. Indeed, VDAs have shown no influence on the radiation response of early (44–46) or late responding normal tissues (54).

2.2.3. OTHER MODALITIES

Several preclinical investigations have examined the combination of VDAs and heat, and all have reported that such agents enhance the response of tumors to heat (55). This outcome was schedule dependent, with the maximum response generally being observed if the heat was started shortly after VDA administration at times corresponding to the maximal reduction in tumor blood flow (56, 57). The mechanism responsible for this enhancement likely involves both improved tumor heating and reductions in tumor pH (56). Preclinical studies have now also demonstrated that VDAs can significantly improve the antitumor efficacy of combined radiation–heat treatments (55). In these studies, the VDAs were always administered after irradiating and prior to heating, so as to exploit the pathophysiological changes that could enhance both therapies.

3. VDAS IN CLINICAL TRIALS

Several small-molecule VDAs including three combretastatin derivatives (CA4P, OXi4503, and AVE8062), a non-combretastatin-based tubulin depolymerizing agent (MN-029), and the flavonoid DMXAA are now undergoing clinical assessment (Table 1). The evaluation of the clinical status of these agents is outside the scope of the present article, but this topic has been reviewed recently by several authors (4, 58). Suffice to say that proof of concept for the VDAs appears to have been achieved in man. Tumor blood-flow reductions can readily be detected following treatment with these agents in tumors of patients at doses well below the maximum tolerated dose

Table 1
Current Clinical Status of VDAs

| <i>Vascular disrupting agent</i> | <i>Clinical status</i> |
|----------------------------------|--|
| CA4P | Phase II trials in ovarian cancer and NSCLC |
| DMXAA | Phase II trials in ovarian and prostate cancer and NSCLC |
| AVE8062 | Phase IB |
| OXi4503 | Phase I |
| MN-029 | Phase I |

NSCLC, non-small cell lung carcinoma.

(59). On the basis of a large body of preclinical data, lead agents CA4P and DMXAA have now advanced into Phase II studies in combination with conventional treatment modalities (4). Trials in a variety of tumor sites including prostate cancer, ovarian cancer, and non-small cell lung carcinoma (NSCLC) are under active investigation.

4. COMBINING VDAS WITH OTHER VASCULAR DIRECTED THERAPIES

Given their disparate modes of action, the combined application of antiangiogenic therapies and VDAs is likely to lead to complimentary antitumor effects. Since both the initiation of new vessel formation and the integrity of the existing blood vessel network are critical to a tumor's growth and survival, such a double assault on the tumor vasculature would appear to hold considerable promise. Preclinical evidence supports this notion. One experimental example of this strategy is the combination of a selective inhibitor of VEGFR2-associated tyrosine kinase with a microtubulin disrupting VDA (33,60,61). The results showed that such a combination therapy could significantly enhance the tumor response beyond that achieved with either vascular targeting therapy alone. Currently, combinations of other agents seeking to exploit the approach of dual targeting of the tumor vasculature are under active preclinical investigation, and clinical considerations of this concept have begun.

5. CONCLUSIONS

A tumor's critical need for an actively growing vasculature for its progression and spread coupled with the established negative therapeutic consequences associated with its aberrant nature makes targeting tumor vessels an attractive strategy for cancer management. As over 90% of all cancers present as solid tumors, reliant on a functioning vascular network to supply oxygen and nutrients, the therapeutic strategy of interfering with the tumor vasculature holds great promise. Furthermore, targeting a component of a tumor distinct from that targeted by conventional cytotoxic therapies offers the opportunity for significant complementary antitumor activity. Rapid developments in this field in recent years have resulted in the identification of a variety of potential targets and a large number of investigational drugs. Agents such as DMXAA and select tubulin depolymerizing agents, most notably CA4P and OXi4503, are promising lead compounds that have demonstrated potent antivascular

and antitumor efficacy in a wide variety of preclinical tumor models and are currently undergoing clinical trial evaluations. Still, the greatest utility of VDAs will likely lie in combination with conventional anticancer therapies. Such an approach may improve treatment outcomes by capitalizing on principles of enhanced antitumor efficacy, non-overlapping toxicities, and spatial cooperation. Since many of the factors associated with chemotherapy and radiotherapy failures (abnormal tumor microenvironments, tumor progression, and metastatic spread of neoplastic cells) may be affected by VDAs, the combinations of such approaches are likely to improve treatment outcomes.

Finally, it is now apparent from preclinical investigations that the application of angiosuppressive and vascular disrupting strategies can improve treatment outcomes. It therefore is possible to envisage future treatment protocols consisting not only of the current mainstays of cancer management, surgery, radiotherapy, and chemotherapy but also will include a “vascular targeted therapy” consisting of a battery of tumor vessel-directed agents.

ACKNOWLEDGMENTS

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18

Normalization of Tumor Vasculature and Improvement of Radiation Response by Antiangiogenic Agents

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SUMMARY

Recent preclinical studies have suggested that radiotherapy in combination with antiangiogenic/vasculature-targeting agents enhances the therapeutic ratio of ionizing radiation. Because radiotherapy is one of the most widely used treatments for cancer, it is important to understand how best to use these two modalities to aid in the design of rational patient protocols. The mechanisms of interaction between antiangiogenic/vasculature-targeting agents and ionizing radiation are complex and involve interactions between the tumor stroma and vasculature and the tumor cells themselves. These agents can decrease overall tumor resistance to radiation by affecting both tumor cells and tumor vasculature, thereby breaking the codependent cycle of tumor growth and angiogenesis. Because the mechanisms of interaction between ionizing radiation and antiangiogenic/vascular targeting agents are not fully understood, the ideal way to use this potentially powerful combination for tumor cure has yet to be determined. We have described a number of possible mechanisms of interaction between antiangiogenic agents and radiation.

Key Words: Angiogenesis; Antiangiogenic Agents; Cancer; Clinical; Preclinical, Normalization; Tumor; Radiation Response; Radiation Therapy; Tumor Oxygenation; Vasculature.

1. RADIATION THERAPY AND THE IMPORTANCE OF OXYGEN

Elucidation of the mechanisms involved in tumorigenesis reveals that the induction of angiogenesis is a major factor promoting tumor growth and the development of tumor hypoxia. It has also been observed that ionizing radiation can induce angiogenic factors that may contribute to hypoxia or radiation resistance. Because oxygen is a potent radiation sensitizer, tumors that are hypoxic do not receive the full cytotoxic

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Table 1
Studies that Measured Tumor Oxygen Tension and Radiation Response

| <i>Drug</i> | <i>Model</i> | <i>Increased tumor oxygen tension</i> | <i>Delayed tumor growth combined with IR</i> | <i>Author</i> |
|-------------|---|---------------------------------------|--|---------------|
| SU5416 | Transplantable mouse liver tumor model in NMRI mice and FSAII fibrosarcoma in C3H/HeOuJLco mice | Yes | Yes | 22 |
| Thalidomide | FSAII fibrosarcoma in C3H/HeOuJLco mice | Yes | Yes | 21 |
| Anginex | MA148 human ovarian carcinoma athymic mouse SCK murine mammary carcinoma model | No | Yes | 25 |
| DC101 | U87 human glioma xenographs | Yes | Yes | 16 |
| Bevacizumab | Humans Phase I | Yes | Yes | 26 |
| ZD6126 | U87 human glioblastoma xenographs | No | Dependent upon time of application | 14 |
| Anti-VEGF | U87 human glioblastoma xenographs | No | Yes | 17 |

potential of radiation therapy. Recently, it has been observed in some but not all preclinical studies that “normalization” of tumor vascular can occur following treatment with antiangiogenic therapies. Normalization allows for increased tumor perfusion and oxygenation of the tumor. The normalization is of a temporal nature and dependent on drug scheduling. The temporary increase in oxygenation provides a “window of opportunity” where radiation would theoretically be more effective. In this chapter, preclinical and clinical studies that investigated the effect of vascular normalization on radiation response will be explored (Table 1).

2. MECHANISMS OF TUMOR ANGIOGENESIS

The formation of new blood vessels in solid tumors serves to provide blood, oxygen, and nutrients to promote further growth. Angiogenesis is not limited to only tumors but is also seen in other physiological and pathological states (1). Angiogenesis occurs as a result of an imbalance between proangiogenic and antiangiogenic factors (2). There have been many angiogenic factors identified such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor beta-1 (TGF- β 1), and epidermal growth factor (EGF) (1–3). Endogenous antiangiogenic growth factors have also been identified such as endostatin and angiostatin (2). The most widely studied and best characterized angiogenic factor is VEGF. VEGF is the most potent of the growth factors eliciting the most pronounced

affect on neovascularization (3). There have been six members of the VEGF family belonging to the PDGF superfamily identified: VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor. All members of the VEGF family are dimeric glycoproteins. VEGF is expressed in the majority of solid tumors, and survival of newly formed endothelial cells is dependent upon levels of VEGF (1). VEGF is also referred to as vascular permeability factor because of its ability to cause the vasculature to become dilated and leaky. The most striking biological feature of VEGF *in vivo* is its ability to cause rapid vasculature leakage upon injection (4). The proposed sequence of steps of new vessel formation is the onset of hyperpermeability in the vessels resulting in tissue edema. The next step is the formation of “mother vessels” that are characteristically thin walled and deficient in pericytes, which function in stabilizing the endothelial wall of the vessel. These vessels form as a result of basement membrane degradation, with detachment of pericytes from the residual basement membrane, followed by expansion of the remaining endothelium to cover the area where the basement membrane existed (4).

Mediation of VEGF signaling occurs through two transmembrane receptor tyrosine kinases VEGFR-1 and VEGFR-2, which are also observed to be overexpressed in tumors having increased production of VEGF (1, 4). After binding to its receptors, VEGF begins a sequence of signaling events that lead to the activation of several downstream signaling pathways such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) (4). The cascade begins with receptor dimerization upon ligand binding and autophosphorylation of tyrosine residues (3). Although definitive functions of the receptors have not been clearly established, it is accepted that the high-affinity VEGFR-2 is found only in endothelial cells (3) and serves a mediator for the permeability-inducing affects of VEGF followed by proliferation and migration of endothelial cells marking the angiogenic effects of VEGF (1, 4, 5).

Hypoxic conditions serve as a potent activator for the transcription and stabilization of VEGF (4). Hypoxia-inducible factor 1-alpha (HIF-1 α) is a subunit of HIF-1, a heterodimeric transcription factor composed of two subunits: HIF-1 α and HIF-1 β (6, 7). Low oxygen tension directly upregulates transcription of HIF-1 α and transcription of HIF-1 as a hypoxic response is a powerful stimulus for induction of VEGF expression (8). In oxic conditions, HIF-1 α is rapidly degraded (8), whereas in a hypoxic tumor microenvironment, HIF-1 α is stabilized and subsequently dimerizes with HIF-1 β and the complex in turn binds to the VEGF promoter, activating transcription (4). It has also been shown that HIF-1 α expression as a result of hypoxia upregulates the expression of VEGFR-1 (1).

3. RADIATION-INDUCED ANGIOGENESIS

In almost all tumor models, a marked increase in expression of VEGF occurs following radiation. This increase in angiogenic growth factors promotes vessel formation and is protective against the cytotoxic effects of radiation therapy, which in the absence of survival factors leads to the destruction of endothelial cells. Gorski et al. (9) demonstrated radioresistance resulting from an increase in VEGF levels, which was abolished after treatment with anti-VEGF antibodies. Lewis lung carcinoma human xenographs were irradiated, and the subsequent VEGF levels were measured. Northern blot analysis revealed that VEGF transcripts increased threefold by day 2

following radiation and remained elevated for 14 days. It was also observed that VEGF proteins levels increased in a dose-dependent manner (9). An increase in VEGF was also demonstrated by other groups (10–12). This intrinsic upregulation of angiogenic factors is significant in understanding this mechanism of radioresistance to developing therapies to alleviate this obstacle.

Ionizing radiation has been shown to activate other receptor tyrosine kinases such as the ErbB family-like epidermal growth factor receptor (EGFR). EGFR has been observed to become activated upon irradiation in various carcinoma cell types (13). Activation through EGFR can serve to increase signaling of downstream pathways such as MAPK, PI3K, and c-Jun N-terminal protein kinase (JNK). The resulting activation of the RAF/MEK/MAPK and P13K/AKT/FRAP can ultimately serve to activate HIF-1 α . Hypoxia induced by HIF-1 α exacerbates already hypoxic conditions in the tumor microenvironment thereby increasing radioresistance (6).

4. ANTIANGIOGENICS AS A CLASS OF DRUGS

The maintenance and promotion of tumor growth through angiogenesis is a common characteristic in malignant tumors. Therapies aimed at the regression of newly formed tumor vessels is a strategy for improving radiation response (2). There are currently two classes of antivascular drugs used to target the endothelial cells of tumor blood vessels: (i) antiangiogenic drugs that serve to prevent the formation of new blood vessels and (ii) vascular-damaging agents that target existing blood vessels by causing the formation of a thrombus and leading to induced tumor cell death (14). The taxonomy of the agents has been discussed (15).

Numerous antiangiogenics and vascular-targeting agents have been developed and have been used in conjunction with radiation therapy to increase cytotoxic effects. Anti-VEGF and VEGFR monoclonal antibodies and small molecule inhibitors that inhibit VEGFR tyrosine kinase have been studied both preclinically and clinically (2). Inhibition of VEGF and VEGFR2 can increase apoptosis in tumor cells as well as in endothelial cells and significantly decrease tumor growth (2, 16). Neutralizing the action of VEGF and further inhibition of VEGF expression through monoclonal antibodies to VEGF have also shown a marked decrease in tumor growth and an increase in tumor cell apoptosis (17).

5. ENHANCEMENT OF RADIATION THERAPY BY NORMALIZATION OF TUMOR VASCULATURE

Normalization of tumor vasculature is a concept recently developed to explain how antiangiogenic therapies may enhance cytotoxic therapies (18). The seemingly counter-intuitive hypothesis that by destroying emerging tumor vasculature using antiangiogenic therapy will lead to improved oxygen perfusion and ultimately improvement of radiation and chemotherapy is becoming more widely accepted. The notion of “normalization” of the vascular entails a marked decrease in the immature blood vessels that are inefficient in their nutrient/oxygen delivery to the tumor, which contribute significantly to the hypoxia that is characteristic of the tumor microenvironment (18).

In a growing tumor, angiogenic factors are recruited to promote the formation of new blood vessels, which in theory should improve growth by means of increasing

nutrient delivery and oxygen flow to the tumor. The newly formed blood vessels have an abnormal morphology in that they have loosely attaching pericytes, have basement membrane abnormalities, vary in diameter, and are leaky resulting in an increased interstitial fluid pressure (19, 20). The destruction of these unstable vessels through antiangiogenic therapy promotes the further recruitment of pericytes that act to stabilize the remaining vessels in the tumor (18). The resulting normalization provides a potential window for optimal application of radiation therapy, which is crucial to maximizing the effect of radiation or chemotherapy.

Winkler et al. (16) investigated the time course associated with the application of radiation therapy following treatment with the monoclonal antibody to VEGFR2, DC101, in human U87 glioma xenographs. The resulting effect in tumor oxygen perfusion and radiation response was measured. A combination of DC101 or radiation alone or both was applied to tumors in varying time frames. Following DC101 alone, an insignificant delay in tumor growth was observed. Using radiation therapy alone, scheduled as three daily-fractionated doses, a significant delay in tumor growth was observed. However, radiation in combination with DC101 showed an optimal effect when utilized at days 4–6 resulting in the greatest tumor growth delay having a greater than additive effect. After further review, it was observed that the days that showed the greatest delay in tumor growth paralleled a decrease in hypoxia levels in the tumors. On day 2, the oxygen perfusion in the tumor was greatly increased and hypoxia was nearly nonexistent by day 5, increasing again by day 8. These results implicate the mechanism of normalization of the vasculature and were further confirmed by this group (16).

Tong et al. (20) showed that treatment with DC101 in MCaIV murine mammary carcinoma showed a remarkable reduction in vessel tortuosity after 2–3 days, and the vessels took on a more normal morphology. By day 5 of treatment, some regions of the tumor showed complete regression, indicating that normalization occurred before vessel regression. The treatment with DC101 also demonstrated normalization of the wall structure of the tumor vasculature. After treatment, only 8% of the cells showed slight perivascular coverage compared with 25% in the untreated cells (20). This may be model dependent as another laboratory using DC101 did not observe this window of normalization (15).

Combination therapy of ionizing radiation and thalidomide displayed a significant delay in tumor growth. Thalidomide was shown by Ansiaux et al. (21) to inhibit VEGF and bFGF and is widely tested in both preclinical and clinical studies. Statistically different tumor oxygen was seen in the control group and the group treated with thalidomide. At day 2 and 3, a maximum increase in tumor oxygen was observed. The tumors were irradiated on day 2 after the maximum oxygen levels were observed to study the oxygen effect. A significant increase in tumor growth delay was seen when tumors were irradiated 2 days following treatment with thalidomide. No tumor growth delay was observed *in vitro* when tumors were irradiated in the presence of thalidomide, indicating that the oxygen effect was the mechanism for the increased sensitivity to radiation (21).

Mechanisms of tumor oxygenation other than vascular normalization have also been reported after treatment with antiangiogenic drugs. Ansiaux et al. (22) reported significant reoxygenation following treatment with SU5416, an antagonist to the VEGFR inhibiting binding of VEGF, therefore reducing the formation of new vessels. This study showed an increase in tumor oxygen not as a result of remodeling of tumor

vasculature but rather as a result of a decrease in oxygen consumption by the tumor by an inhibition of mitochondrial respiration. Reoxygenation was independent of tumor perfusion, as no significant change was observed between the treated and control groups. There was no observable histological change in the vasculature between the treated group and control group again indicating that a change in vessel architecture did not contribute to the increased oxygen levels observed. The most significant increase in tumor oxygen was observed at day 2 after treatment and continued to decline thereafter. This again supports the idea that there is a window in which the application of radiation therapy will be most efficient (22).

6. ANTIANGIOGENIC COCKTAILS TO PREVENT RESISTANCE

The dogma for improvement in combined therapy using ionizing radiation and antiangiogenics is to destroy tumor endothelial cells (and tumor epithelial cells) while suppressing further regeneration of the tumor vasculature. When combining radiation and antiangiogenics, it may be necessary to use a treatment “cocktail” that targets multiple angiogenic factors. Because tumors tissues may become resistant to antiangiogenics that block certain angiogenic factors, an upregulation of other factors can occur. Herceptin, an antibody to Her2, has action on suite of antiangiogenic factors resulting in vessel normalization. Izumi et al. examined the expression of various angiogenic factors in human breast tumors overexpressing Her2 using a gene array. It was observed that the expression of VEGF, transforming endothelial growth factor alpha, and plasminogen activator inhibitor-1 were decreased, and the expression of the antiangiogenic factor thrombospondin-1 was increased after treatment with herceptin. This sets forth the notion that targeting several angiogenic molecules with a single drug can optimize and possibly customize treatment.

The use of dual inhibitors has proved effective in normalizing tumor vasculature and decreasing cell proliferation. The kinase inhibitor ZD6474 has action on both VEGFR2 and EGFR. In an orthotopic model of gastric cancer, ZD6474 showed a dose-dependent decrease in tumor cell proliferation. Endothelial growth factor-mediated EGFR activation was decreased in tumor cells following treatment with ZD6474 in human umbilical vascular endothelial cells. In TMK-1 human gastric adenocarcinoma cells, a marked decrease was observed in microvessel density. These findings suggest that dual inhibitors targeting both angiogenesis and cell proliferation, two cornerstones of tumor growth, can increase efficacy of antiangiogenic treatment (23).

Through treatment with a dual inhibitor, tumors can be sensitized to other therapies. AEE788 is a specific kinase inhibitor that has shown antitumor activity through inhibition of both EGFR and VEGFR2. Thaker et al. reported the use of AEE788 in human ovarian carcinoma planted into the peritoneal cavity of female nude mice. Administration of the compound showed inhibition of phosphorylation of both EGFR and VEGFR2. Combination treatment of AEE788 and paclitaxel, a first line chemotherapy drug for ovarian carcinoma, showed an increase in apoptosis in tumor cells and tumor-associated endothelial cells. Through the blockade of EGFR and VEGFR2, the tumor-associated endothelial cells showed increased sensitivity to paclitaxel, suggesting that EGFR and VEGFR2 can serve as survival factors. Treating multiple signaling pathways can serve as a means to sensitize and increase efficiency of other treatment modalities (24).

7. VASCULAR-TARGETING AGENTS

Vascular-targeting agents provide a means to examine the role of drug sequencing in relation to radiation- and drug-induced tumor hypoxia. This class of drugs induces vascular collapse of the central tumor leaving an outer ring of oxygenated cells. The viable outer rim of the vasculature not destroyed by antiangiogenics is in theory, still oxygenated and tumor cells may be able to regrow from this rim of cells. However, the majority of the tumor is hypoxic. Wachsberger et al. (14) has measured tumor oxygenation in U87 human glioblastoma human xenographs in response to treatment with the vascular-damaging drug ZD6126 to determine prime intervals of reoxygenation to ultimately develop optimal treatment schedules of combined therapy (14).

Wachsberger shows that if the vascular-targeting agent is supplied before radiation, there is a reduction in radiation response and no significant decrease in tumor doubling time. ZD6126 induced acute hypoxia when given 1 h prior to radiation, and no increase in antitumor effect was detected in comparison to radiation alone. When ZD6126 was given following radiation, either in single or multiple doses, a greater antitumor affect was observed in comparison to radiation alone. This finding is indicative of the importance of dose scheduling to optimize treatment.

8. STUDIES DEMONSTRATING RADIOSENSITIZATION UNDER NORMOXIA OR HYPOXIA

Many studies using antiangiogenic agents demonstrate a radiosensitizing effect without tumor oxygenation. Dings et al. (25) showed that tumor oxygenation which resulted from treatment with other antiangiogenics was not observed after treatment with anginex. An increase in tumor growth delay was observed after treatment with anginex given in conjunction with radiation; however, it was due to direct sensitizing action on endothelial cells. Anginex was administered 2 h prior to irradiation, and a significant tumor growth delay was observed *in vivo* (25). This demonstrated destruction of the endothelial cells supplying the tumor bed was the likely mechanism for radiation enhancement. However, because tumor oxygen in this study was not measured during the time of radiation, the “window of opportunity” by oxygenation cannot be ruled out.

Lee et al. (17) demonstrated that the vascular density is significant in predicting the response to radiation. U87 glioblastoma xenographs were treated with an anti-VEGF monoclonal antibody and then were irradiated in both normoxic and clamped hypoxic conditions. The frequency of low oxygen measurements in the tumors was significantly reduced when treated only with the anti-VEGF antibody. The combined therapy showed a significant increase in tumor growth delay in the presence of both low and normal oxygen levels. When the vasculature was examined following treatment with the anti-VEGF antibody, it was seen that tumor vessel density was reduced by 60% 24 h after the first injection. It can be interpreted that the increased tumor growth delay when combined therapy was given is due to the reduction in tumor vessel density and not an increase in oxygen levels potentiated by the antiangiogenic agent (17).

9. CLINICAL TRIALS

There are few completed clinical trials that have investigated antiangiogenic agents in combination with radiation therapy. At Thomas Jefferson University, angiostatin was evaluated when combined with radiation therapy. Recombinant human angiostatin (rh-angiostatin) is a protein that consists of the first three kringle (amino acids 97–357) of human proplasminogen with a single amino acid substitution (N308–E308) to prevent N-glycosylation. Previous studies (preclinical) that used angiostatin in combination with ionizing radiation indicated that the antitumor activity of human angiostatin is potentiated and results in a significant reduction of tumor volume without increase in toxicity. A single-center, open-label, dose-escalation, phase I clinical study at Thomas Jefferson University evaluated the safety and pharmacodynamics of three dose levels (15, 60, and 240 mg/m²/day) of rh-angiostatin IV protein in combination with external beam radiation therapy (EBRT) for the treatment of patients who had solid cancer tumors. Patients received rh-angiostatin, intravenously (IV), 5 times per week 30 min before EBRT (head and neck, thoracic, or pelvic regions) for a minimum of 25 EBRT fractions. This study had a unique design in that it was performed concurrently with a phase I drug dosage clinical trial at Thomas Jefferson University. As safe dosages were achieved in the drug-alone study, these were used for the radiation study. Twenty-three patients were enrolled and evaluated for safety. Three patients were not evaluable and three who did not complete the minimum EBRT were excluded from response analysis. The 17 remaining patients who had evaluable tumors had advanced head and neck, thoracic, or pelvic cancers. No added toxicity was observed in normal tissue that was contained within the radiation portal. Mild rash was noted in three patients. No clinical thrombotic or bleeding events occurred in any patient. Tumor responses were demonstrated in 90% of patients who entered the trial with measurable disease in the radiation field. The conclusions from this phase I trial are that concomitant administration of daily 10-min infusions of rh-angiostatin and EBRT is safe and does not increase radiation-induced toxicity. Local durable tumor responses [National Cancer Institute (NCI)—Response Evaluation Criteria in Solid Tumors] were observed in this phase I study, although this would be expected with the use of radiation alone.

A translational phase I trial that used antiangiogenic therapy in combination with chemoradiation was reported by Willet and colleagues from Massachusetts General Hospital. This phase 1 clinical trial used bevacizumab (Avastin; Genentech) into preoperative chemotherapy and radiation therapy followed by surgery for patients who had primary and nonmetastatic rectal cancer. A unique aspect of the trial was several translational end points to evaluate the mechanisms of action of bevacizumab. These included (i) tumor physiology [blood perfusion, blood volume, permeability, surface area product, microvascular density (MVD), perivascular coverage, Interstitial Fluid Pressure (IFP), and 18-fluorodeoxy-glucose uptake]; (ii) systemic response (VEGF level in blood, number of circulating endothelial cells, and progenitor cells); and (iii) tumor response. Six patients who had primary and locally advanced adenocarcinoma of the rectum were enrolled in a preoperative treatment protocol of bevacizumab administration alone (5 mg/kg, IV) followed after 2 weeks, the approximate half-life of bevacizumab in circulation, by concurrent administration of bevacizumab with 5-fluorouracil and EBRT to the pelvis and surgery 7 weeks after treatment completion. Twelve days after bevacizumab infusion, flexible sigmoidoscopy revealed that bevacizumab induced tumor regression of more than 30% in one patient with no

change in tumor size in the other five patients. Functional CT scans at this time point indicated significant decreases in tumor blood perfusion (40–44%; $P < 0.05$) and blood volume (16–39% in four of five patients analyzed; $P < 0.05$). This was accompanied by a significant decrease in tumor MVD (29–59% in five patients analyzed; $P < 0.05$). These three sets of data provide direct evidence of the antivascular effects of bevacizumab in human tumors, which is in line with preclinical findings. In addition, 12 days after bevacizumab treatment, IFP was reduced in four of four patients and overall mean IFP decreased significantly from 15.0 mm Hg to 2.0 mm Hg to 4.0 mm Hg to 2.2 mm Hg ($P < 0.01$). This trial stands out for the comprehensive laboratory and clinical correlative findings. The decrease in IFP and increase in the fraction of vessels with pericyte coverage support the normalization hypothesis and provide the first clinical evidence of the mechanism of action of the drug; this supports previous preclinical findings. As a planned continuation of the phase I trial, five additional patients received bevacizumab at 10 mg/kg. Pathologic evaluation of the surgical specimens for staging after the completion of therapy showed two complete pathologic responses. This was not seen in patients who received 5 mg/kg of bevacizumab. However, the complete responses were seen in the two patients who experienced intestinal dose-limiting toxicity (26). The correlative investigations supported the previous findings that bevacizumab has antivascular effects and normalizes the tumor vasculature. These issues and further validation of surrogate markers currently are being explored in the ongoing phase II trial at 5 mg/kg, which was determined to be the maximum-tolerated dose for rectal cancer.

Another phase I trial of bevacizumab + 5-fluorouracil + hydroxyurea + RT every 2 weeks for locally advanced head and neck cancer has been completed at the University of Chicago. The following dose-limiting toxicities were seen at 10 mg/m²: two patients had grade 3 transaminase elevations and one patient experienced grade 4 neutropenia (Ezra Cohen, University of Chicago). The authors concluded in their phase I trial that bevacizumab can be integrated with chemoradiotherapy regimen at a dose of 10 mg/m² every 2 weeks. There were no major additive toxicities observed. In the ongoing phase II study of this regimen, the bevacizumab dose is 10 mg/kg IV q2 weeks (27).

The National Cancer Institute Cooperative Group, The Radiation Therapy Oncology Group (RTOG, <http://www.rtog.org>), has two clinical trials using antiangiogenic agents: RTOG 0615, a phase II study of concurrent chemoradiotherapy using three-dimensional conformal radiotherapy (3D-CRT) or intensity-modulated radiation therapy (IMRT) + bevacizumab (BV) for locally or regionally advanced nasopharyngeal cancer, and RTOG 0417, a phase II study of bevacizumab in combination with definitive radiotherapy and cisplatin chemotherapy in untreated patients with locally advanced cervical carcinoma. Other open trials include the California Cancer Consortium trial, radiation therapy, bevacizumab, paclitaxel, and carboplatin in treating patients with unresectable stage IIIB or stage IV nonsmall cell lung cancer at high risk for hemoptysis caused by bevacizumab. Numerous other trials are planned in combination with radiation therapy but have not been activated.

10. CONCLUSIONS

Various studies have demonstrated that antiangiogenic therapy can induce a normalization of the tumor vasculature. This normalization provides an opportunity to increase the effectiveness of radiation therapy. Studies using vascular-targeting agents also

demonstrate the importance of scheduling to avoid tumor hypoxia. Although normalization is a mechanism of improved radiation response using antiangiogenics, other mechanisms that can improve radiation response also exist. Therefore, investigating the scheduling of antiangiogenics to improve tumor oxygenation as well as mechanisms not involving oxygenation or direct sensitization are warranted.

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Challenges in Translating Antiangiogenic Therapy from the Bench to Bedside

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SUMMARY

Although there are innumerable examples of studies demonstrating the efficacy of antiangiogenic therapy in preclinical models, unfortunately the frequency and magnitude of this effect has been difficult to translate into the clinic. At the current time, with the exception of anti-VEGF therapy for renal cell carcinoma, single agent anti-angiogenic therapy has not lived up to expectations. At this stage it is important to critically evaluate potential explanations for why the success observed in preclinical studies has not translated into patient benefit. This chapter will focus on the biologic and human issues that have led to this apparent discrepancy.

Key Words: Angiogenesis; pharmacokinetics; maximal tolerated dose; integrity; translational research

1. BACKGROUND

The hypothesis that tumor growth can be inhibited by blocking blood vessel growth was first proposed by M. Judah Folkman, MD, more than 30 years ago. Initially, it was believed that blocking the activation of endothelial cells (ECs), blocking the activity of EC growth factors, or increasing the activity of angiogenic inhibitors would lead to tumor dormancy. In theory, tumor dormancy would prolong the lives of patients with malignant disease. The discovery of pro-angiogenic factors led to pharmacologic approaches to block angiogenesis. Initial antiangiogenic approaches fell into two basic categories: (i) agents that blocked the activity of pro-angiogenic molecules (e.g., antibodies to EC growth factors or growth factor receptors) and (ii) agents that directly

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affected EC function or survival (e.g., fumagillin and TNP-470). Early studies in mice demonstrated, for the most part, that “antiangiogenic” therapy led to a decrease in the growth rates of tumors rather than to tumor regression. However, later studies showed that single-agent antiangiogenic therapy could lead to tumor regression. The discovery of endostatin, reported in 1997, raised great expectations for antiangiogenic therapy since the agent led to regression of every tumor type studied in mice. Because many patients and oncologists believed that results in patients would be similar to those in mice, phase I trials supported by the U.S. National Cancer Institute were so popular that lottery systems were implemented for patient enrollment. Unfortunately, no objective responses were observed in three phase I clinical trials conducted at three leading centers in the United States.

Why did the results in the clinical trials bear no resemblance to the results in preclinical studies? This chapter reviews the multiple biologic and logistical reasons why promising antiangiogenic agents have not achieved the same results in the clinic as in murine models.

2. DEFINING EFFICACY IN THE LABORATORY AND THE CLINIC

One reason for the differences in effectiveness of antiangiogenic agents in humans and mice is differences in how effectiveness is defined in clinical and preclinical studies. In preclinical studies of antiangiogenic agents, the agent under investigation is typically administered to tumor-bearing mice, and its effect on tumor growth is compared to that of an innocuous solvent (the control agent). Any delay in tumor growth in mice treated with the investigational agent is considered an indicator of a “successful” experiment, and the agent is deemed effective. However, inhibition of tumor growth is not the same as “response” (a typical secondary endpoint in phase I clinical trials and a primary endpoint in some phase II clinical trials.) A response typically refers to tumor regression, as determined by strict and well-defined criteria (e.g., RECIST and WHO criteria) (1). What appears to be an indicator of effective therapy to a preclinical investigator—slowing of growth—would be considered progressive disease to a clinician (Fig. 1).

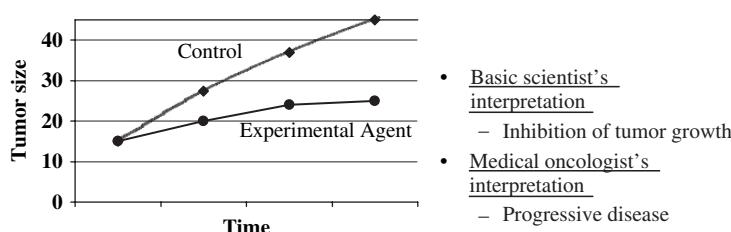


Fig. 1. Differences in interpretation of agents’ efficacy in preclinical studies versus clinical trials. In preclinical studies, slowing of tumor growth with an experimental agent, when compared to a control drug/solvent, is considered a “positive” result, and the drug is deemed active. In contrast, in clinical trials, if a tumor increases in size by 25%, even if the growth rate is slowed with the experimental agent, the patient is considered to have progressive disease, and the agent is deemed ineffective.

3. EFFECT OF TUMOR BURDEN ON EFFICACY OF ANTIANGIOGENIC DRUGS

Another reason for the differences in results of clinical and preclinical studies of antiangiogenic agents is differences in tumor burden in the populations being treated. Most preclinical studies in mice have examined the efficacy of antiangiogenic agents when the mice have a relatively small tumor burden. In fact, tumors are often less than 1–2 mm in diameter, which would be undetectable in humans by current imaging methods. This tumor burden is similar to the tumor burden in the adjuvant therapy setting in humans (note: currently there are ongoing trials with anti-VEGF therapy in the adjuvant setting, but most are done in combination with chemotherapy). However, typical phase I trial participants have advanced-stage bulky disease. To further complicate this issue, nearly all (if not all) patients in phase I trials have progressed on numerous regimens and thus have “refractory” disease. Whether or not chemorefractory disease makes patients more resistant to biologic agents has yet to be determined. Given that antiangiogenic therapy is most effective when the tumor burden is small (2), we are likely expecting far too much from antiangiogenic therapy in phase I clinical trials, whose true goal is to determine the safety profile and the maximal tolerated dose.

4. DIFFERENCES IN MURINE AND HUMAN TUMORS: THE OBVIOUS AND NOT SO OBVIOUS

Intrinsic differences in human and murine tumors likely also contribute to differences in results of clinical and preclinical studies of antiangiogenic agents. Most tumors grown in mice, either syngeneic or transplanted human tumors, grow much faster than tumors in humans. In humans, tumor doubling times are on the order of months (6–12 months for many tumor types, and even longer for some, such as prostate and neuroendocrine tumors). (3) However, the tumor doubling time in mice is typically only about 1–3 weeks. To support the rapidly growing tumor mass, EC division, and migration, must keep pace with tumor growth. It is difficult to compare the proliferation rate of ECs in human tumors to those in mice, but in humans, <10% of tumor vessels will harbor ECs that are undergoing proliferation (4). In contrast, it is estimated that the majority of vessels in tumors growing in mice will harbor ECs that are undergoing proliferation. Murine tumors are thus more likely to “respond” to agents that inhibit EC proliferation and survival than are tumors with less EC proliferation—most human tumors.

The age and maturity of the vasculature in preclinical studies may not be equivalent to that in patients. Tumor xenografts contain newly formed blood vessels, usually only days to weeks old. In contrast, by the time a patient’s tumor becomes clinically apparent (i.e., 1–2 cm), the vasculature has been present for months or even years. Therefore, the vasculature in human tumors is likely more mature and differentiated than that in animal models. One sign of a more mature vasculature bed is the formation of a layer of smooth muscle cells surrounding the endothelium (5). Published data suggest that these pericytes may help to protect ECs from apoptosis (5, 6). Indeed, in preclinical studies in which the expression of VEGF in transgenic mice was under the control of an inducible vector, withdrawal of VEGF led to apoptosis of ECs, many of which were not associated with pericytes (5). Other studies show that pericytes

protect tumor ECs and thus serve as a “barrier” to antiangiogenic therapy. (5, 7). In rapidly growing murine tumors, pericyte coverage of ECs is poor—quantitatively and qualitatively—owing to the rapid proliferation of ECs and lack of time for pericytes to migrate and adhere to ECs. Therefore, murine ECs are more “exposed” and thus more likely to respond to antiangiogenic therapy (8, 9). Furthermore, human tumors are composed of various cell types (fibroblasts and pericytes) and matrices that enhance EC (and tumor cell) survival, whereas human tumor xenografts in mice typically have less matrix and fewer support cells, leaving ECs more “exposed” (Fig. 2).

One certainly could make the argument that transgenic murine models of cancer would improve our ability to screen antiangiogenic drugs since these tumor models grow at a slower pace and are more likely to represent the growth and vessel maturation of human tumors. Logistical issues limit the usefulness of transgenic models, however. For example, transgenic mice, even from the same litter, develop tumors at different points in time that can vary by months. Therefore, a large number of mice are required in order to obtain sufficient numbers of mice to perform studies with therapy when tumor burden is similar, making these studies difficult and costly. This also requires the use of some type of imaging modality. If, the tumor under study is derived from a visceral organ, imaging is necessary to assure that mice with similar tumor burden are appropriately randomized among treatment groups. Furthermore, most transgenic models do *not* mimic the ultimate challenge in the clinic—metastasis. In the clinic, the majority of *primary* tumors can be eradicated by surgery and/or irradiation, but this is not true for metastasis. Unfortunately, transgenic *metastatic* tumor models are rare and even when they exist are subject to variability as described above.

The site of tumor growth is an important determinant of efficacy in preclinical studies (Fig. 3) and may also be important in evaluating the efficacy of antiangiogenic therapy in clinical trials. In humans and mice, tumor growth rates vary according to the site of tumor growth. This has important implications in tumor modeling in mice as in preclinical murine tumor models, tumors are not always grown at orthotopic sites.

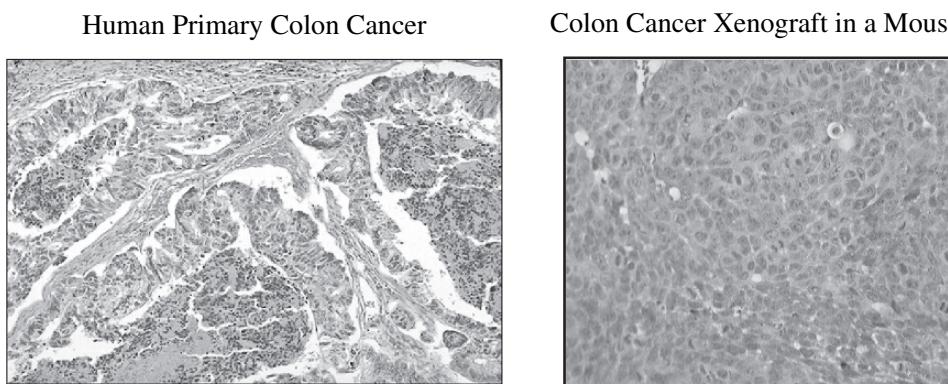


Fig. 2. Differences in cellular and matrix composition in a human tumor and a human cancer cell line xenograft in a mouse. Human tumors are composed of numerous cell types and matrices that enhance tumor cell and endothelial cell (EC) survival. In contrast, xenografts in mice are composed primarily of malignant epithelial cells with few and immature vessels to support tumor growth. With sparse pericyte coverage, ECs in xenografts are more “exposed” and thus sensitive to antiangiogenic agents. (Please see color insert.)

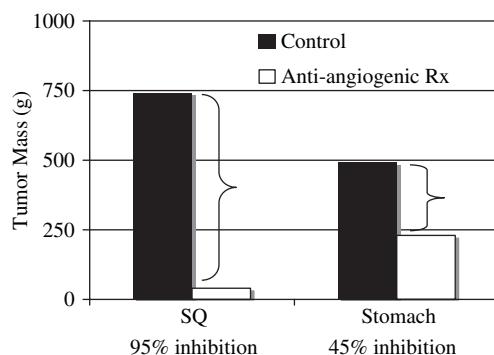


Fig. 3. Effect of site of tumor growth on apparent efficacy of a drug. Tumors growing in the subcutaneous (SQ) tissues are characterized by rapid growth rates and are highly angiogenic. Thus, it is more likely that antiangiogenic therapy will show a desirable effect. In contrast, tumors growing in orthotopic locations (e.g., stomach cancer in the stomach) typically grow at a slower rate than SQ tumors, and these tumors may coopt the pre-existing blood supply of the host organ. Antiangiogenic therapy in the orthotopic site may be less efficacious when compared to the control.

It is important to recognize that ECs from different organs are phenotypically distinct (10, 11) as is EC coverage by pericytes. It therefore follows that antiangiogenic therapy that is effective at one site may be ineffective at another. Therefore, the most relevant models for evaluating antiangiogenic therapy in preclinical studies are orthotopic or transgenic metastatic models where the tumor is growing in the appropriate host microenvironment (12).

5. SPECIES INFLUENCES ON PHARMACOKINETICS

Differences in pharmacokinetic parameters between species may account for some of the discrepancies between results of preclinical studies and of clinical trials. Every substance has a distinct metabolic pathway. For example, monoclonal antibodies are degraded by endogenous protein digestion, while other agents induce the cytochrome-P450 (CYP) enzyme system (13). The VEGF-R tyrosine kinase inhibitor SU5416 is eliminated mainly via the enzyme CYP4A1 in the liver (14). Interestingly, different species have unique patterns of induction of this enzyme, leading to differences in the metabolism of SU5416. One study demonstrated that the intrinsic clearance of SU5416 was almost 20-fold greater in mice than in humans after a single intravenous dose (14). However, clearance of SU5416 increased with repeated dosing in humans, demonstrating auto-induction of drug metabolism, whereas in mice, no such induction was noted. This observation suggests that dosing regimens in clinical trials cannot be directly extrapolated from murine studies in regard to initial dosing and dosing at later times after induction of metabolizing enzymes. This above principle has been observed in clinical trials. Disappointing results from a phase III clinical trial of chemotherapy with or without SU5416 led to the decision that this agent should be eliminated from further clinical development. The poor results with SU5416 were likely due to a poor understanding of pharmacokinetics. Although this is well recognized by those with an interest in pharmacokinetics, this can sometimes be overlooked by enthusiastic investigators.

6. DEFINING THE MAXIMAL TOLERATED DOSE: MICE CANNOT COMPLAIN OF HEADACHES

In preclinical trials, most antiangiogenic agents are well tolerated with few adverse effects, and effectiveness is often dose dependent. However, we must recognize that toxicity in mice can be detected only via objective evidence (e.g., weight loss or failure to groom), whereas toxicity in humans is both objective (e.g., hypertension or proteinuria) and subjective (e.g., headaches and fatigue). Therefore, an antiangiogenic agent that may be effective at high doses in mice and is perceived to be nontoxic may be intolerable in humans at a comparable dose. For example, headache was the dose-limiting toxic effect in a phase II trial of anti-VEGF therapy (bevacizumab) in patients with breast cancer (15). However, since mice cannot complain of headaches, mice may receive a higher relative dose of therapy that is well above the maximal tolerated dose in humans.

7. DATA REPORTING AND ISSUES OF INTEGRITY

It is a simple fact that it is easier to publish positive results than negative results. In fact, nearly all negative preclinical studies never get published (or even submitted), as many journals have little interest in such articles. Furthermore, the suppliers of an agent (i.e., industry) are, for the most part, not interested in publishing negative studies but will publish positive preclinical studies as part of the program that leads to phase I trials. Thus, the “denominator” for preclinical studies is never known when one reads publications touting a new agent in development. For example, if an agent is not effective in one model but the investigator is confident about the agent, the investigator may test the agent in different models. Eventually, the agent may show some activity, and this will be the basis of a presentation or publication (i.e., “if you torture the data long enough, eventually it will give in”). But the number of experiments conducted (i.e., the denominator) before a successful outcome is achieved is rarely reported. In a clinical trial, results on *all* patients must be reported: if only 1 of 10 patients responds to therapy, the response rate is, of course, 10%. However, if a preclinical investigator reports only the positive studies, the efficacy rate is obviously much greater. Credit should be given to those investigators who test and *report* the results of their therapy in multiple models, including ones in which the agent was not effective.

Last, one must address human nature and issues of integrity. There is tremendous pressure on investigators, both trainees and established investigators, to obtain positive data for publication and program advancement. We are all aware that there is great variability in tumor growth in murine models of cancer, and if one “selectively” collects or omits data, one can achieve the highly desired “significant *p* value” that leads to acceptance for publication. Furthermore, immunohistochemical studies can be subjective in nature, and authors can choose to select the photomicrographs that best prove their hypotheses rather than representative photomicrographs that more accurately depict the typical results. And we must face the fact that there is occasionally outright falsification of data. Mentors and senior investigators assign projects to trainees or employees, and it is tempting to them to provide data to support the hypothesis and thus please the mentor or allow completion of a doctoral dissertation or postdoctoral fellowship. Ethics play a role in all aspects of our lives, but in cancer research, one must always remember that there is a patient at the end of all the research. Falsification or

misrepresentation of data can eventually be detrimental to patients, and all investigators MUST conduct themselves with the highest ethical standards.

8. FUTURE DIRECTIONS

We must always interpret findings from preclinical studies with caution due to the discussion above. Activity of an agent in a preclinical study does not guarantee that it will be effective in the clinic. Preclinical investigators should try to study agents in models that more accurately reflect the clinical scenario (e.g., advanced-stage tumors growing in orthotopic locations). Furthermore, negative studies in preclinical experiments must be reported due to the fact that many phase II and phase III clinical trial results suggest that antiangiogenic therapy is best utilized in combination with other therapies (chemotherapy, radiation therapy, and even other targeted therapies), preclinical studies should be designed with this in mind.

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20

Regulation of Angiogenesis in Cancer and Its Therapeutic Implications

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SUMMARY

Starvation of tumors through eliminating their blood supply by blocking angiogenesis is an idea that is elegant in its very simplicity. In this chapter, we describe physiological and pathological angiogenesis and growth factors and microenvironmental influences that govern its initiation, promotion and inhibition. Our current understanding of the gene expression and biology of endothelial cells has led to the development of many therapeutic agents that target molecules in signal transduction pathways critical to angiogenesis in various types of cancer. It is clear that angiogenesis is a complex process which recruits multiple factors that act rapidly to produce a microvasculature in the developing tumor. As such, a multi-pronged attack against the growth factors aimed at early stages of cancer is likely to be most effective.

Key Words: Angiogenesis; VEGF; SAGE; cancer; antiangiogenic.

Angiogenesis as a term to describe the growth of blood vessels was first used in 1787 by the British surgeon, Dr John Hunter. We now define it as the process of formation of new blood vessels from the preexisting vasculature. However, the significance of angiogenesis in cancer development was not recognized until 1971 when work published by Dr Judah Folkman demonstrated that tumor growth is critically dependent on angiogenesis. This finding was initially greeted with much skepticism, but subsequent decades of research have irrefutably confirmed the importance of angiogenesis in supporting cancer growth. It has also led to the realization that inhibition of angiogenesis could provide a powerful new avenue for cancer treatment (1). Due to the identification of numerous angiogenic growth factors and inhibitors in the last 30 years, a deeper understanding of how angiogenesis contributes to cancer development is steadily emerging. Here we discuss the molecular mechanisms underlying the action of angiogenesis-related factors and their potential as targets for therapeutic intervention.

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1. PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS

Angiogenesis is an important physiological phenomenon during development, since embryonic growth requires that sufficient oxygen and nutritional supply be delivered by blood vessels to the dividing and differentiating cells. During embryogenesis, two distinct vascular developmental steps occur in a stepwise order. Vasculogenesis is the first step, characterized by de novo blood vessel formation. During this stage, hemangioblasts, the common cell of origin for endothelial and hematopoietic progenitors, differentiate into a primitive vessel structure with endothelial progenitors surrounding the inner hematopoietic progenitor cells (2). The resulting vascular plexus then undergoes the second step of angiogenesis, mainly through proliferation, migration, maturation, and sprouting of endothelial progenitors. This results in a well-organized and functional vascular network (3). In normal adult tissue, the mature vessels stay quiescent, and no vascular remodeling occurs except during the female reproductive cycle and wound healing, when physiological angiogenesis is required. We now know that the status of angiogenesis is dependent on the relative amount of angiogenic and antiangiogenic factors. In essence, the amount of these two different factors is finely regulated and balanced in most normal tissues. However, this equilibrium is disrupted in pathological states. A shift in this delicate balance can lead to many diseases involving diverse organs. For example, excessive angiogenesis is related to disease states such as psoriasis, inflammatory bowel disease, retinopathy, and arthritis, while deficient angiogenesis plays a role in atherosclerosis, ischemia, and diabetes (4).

In many types of carcinomas, the rapid growth of malignant epithelial cells requires adequate nutritional supply and consequently active angiogenesis. This is achieved by overproduction of angiogenic factors initiated by the cancer cells. Previous studies have provided concrete evidence that angiogenesis is critical for tumor growth and differentiation. For instance, primary human tumors do not grow larger than 1–2 mm in size without new vessel growth (5). The Id1/Id3 loci are implicated in the proper development of blood vessels. Intradermally transplanted lymphoma and breast carcinoma cells do not grow to form tumors in $\text{Id1}^{+/-}\text{Id3}^{-/-}$ mice due to poor angiogenesis and extensive tumor cell necrosis. Consequently, 100% of $\text{Id1}^{+/-}\text{Id3}^{-/-}$ mice injected with the tumor cells survived even after 1 year, while wildtype mice died within 60 days after injection (6). Here, microvessel density (MVD), an indicator of the extent of angiogenesis, was found to be an important prognostic factor. Indeed, high MVD correlated with decreased overall survival rate in clinical breast cancer samples (7).

In addition to its growth-supportive role at the primary tumor site, angiogenesis also provides a physical disseminating route for distant metastasis. After primary tumor cells enter the blood stream through intravasation and colonize at distant organs through extravasation, the few cancer cells that are able to adapt to the new microenvironment at secondary sites will begin to grow and form micrometastases. At this stage, the continuing growth of the small cancer cell cluster elicits a second round of angiogenesis at the secondary sites; the clusters develop into macrometastases and thus complete the cycle of tumor growth and metastasis. Because angiogenesis is critical at almost every step of tumor development, it has become an important candidate pathway for identifying and developing novel targets for cancer treatment.

The vessels formed in physiological angiogenesis are quite different from those arising in pathological states. Normal physiological vessels have well-defined capillary, vein, and arterial structures. The walls of these vessels are covered by pericytes or

vascular smooth muscle cells that mature with normal blood flow. Normal vessels will grow until optimal angiogenesis is reached. In contrast, vessels inside malignant tumors are structurally abnormal. Pericytes or vascular smooth muscle cells are not well associated with endothelial cells (ECs), leading to hemorrhagic leakiness and high interstitial pressure (8) that prevents efficient chemotherapeutic drug delivery into tumor sites. These vessels are irregular in shape and often dilated with relatively slow blood flow in the lumen. The immature and dysfunctional vessels usually keep growing to meet the ever-rising metabolic demand of malignant cells, which are often referred to “wounds that never heal” (9). The molecular mechanisms involved in pathological vessel formation are not completely understood, but in general aberrant vessel development is attributed to excessive production of local angiogenic factors.

2. FACTORS INITIATING ANGIOGENESIS

When solid tumors achieve a size of 4–5 mm, individual tumor cells begin to face an adverse environment. The rapid oxygen consumption and low extracellular pH value are obstacles for further tumor growth. How do the tumor cells react to these unfavorable conditions? One of the known mechanisms is by upregulating a master low oxygen sensor, hypoxia-inducible factor-1 (HIF-1) (10). HIF-1 is a heterodimeric basic helix-loop-helix transcriptional factor composed of HIF-1 α and HIF-1 β subunits. HIF-1 β subunit is constitutively expressed in tissues, while the degradation of the HIF-1 α subunit is regulated by oxygen availability. In normoxia when oxygen is plentiful, HIF-1 α is continually modified by three different HIF prolyl hydroxylase (PHD) enzymes. In the presence of oxygen and other cofactors such as ascorbic acid and iron, PHD enzymes incorporate oxygen atoms from molecular oxygen to hydroxylate a pair of conserved proline residues in the N-terminal transactivation domain (11). The hydroxylation modification allows HIF-1 α to be recognized by von Hippel–Lindau (VHL) tumor-suppressor protein (12). VHL is a subunit of E3 ubiquitin ligase, and the binding to hydroxylated HIF-1 α causes HIF-1 α to be degraded by the proteasome (13). During hypoxic conditions, however, low oxygen prevents the hydroxylation reaction from occurring, and HIF-1 α is stabilized. This allows the binding of HIF-1 α to HIF-1 β to form the active heterodimer and initiate downstream gene transcription. Since HIF-1 is activated in suboptimal conditions, HIF-1 downstream genes usually function to enhance cell survival, including increasing glucose metabolism and angiogenesis. One of the upregulated downstream genes is vascular endothelial growth factor (VEGF), a potent angiogenic factor (14).

3. FACTORS THAT PROMOTE ANGIOGENESIS

VEGF was first named by Ferrara and Henzel for its growth-stimulating effect on ECs (15). Subsequent sequencing of the VEGF cDNA revealed that the encoded protein is identical to a vascular permeability factor (VPF) previously described in the literature (16). The low oxygen-induced HIF pathway is the key regulatory mechanism for inducing VEGF expression. We now know that there are also HIF-independent pathways that upregulate VEGF expression. For instance, hypoxia can increase VEGF expression through the K-ras signal transduction pathway, and HIF is indispensable for this VEGF upregulation (17). Epidermal growth factor receptor (EGFR) inhibitor

can prevent Sp1 from activating VEGF transcription (18). However, the functional relevance of an HIF-independent pathway in tumor angiogenesis is still not clear.

The VEGF family is composed of multiple members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (19). VEGF-C and VEGF-D mainly function in the lymphatic system. By binding to and activating VEGF receptor 3 (VEGFR-3) on lymphatic ECs, they increase cell proliferation and promote lymphangiogenesis (20, 21). In tumor angiogenesis, VEGF-A appears to play a pivotal role. The VEGF-A gene has several isoforms which are produced by alternative splicing (22). These isoforms encode secreted proteins that either directly bind to cognate receptors or are deposited in the extracellular matrix (ECM) to be released upon ECM proteolysis (23). All of these secreted proteins are endothelial mitogenic factors except VEGF₁₆₅b, since amino acid changes in the C-terminal region prevent VEGF₁₆₅b from phosphorylating and activating its receptors (24). Among these isoforms, VEGF₁₆₅ is the most abundant and is the most potent in inducing EC proliferation and vascular permeability (25). After VEGF is released, it interacts with two VEGF receptors on the vascular EC surface, VEGFR-1 and VEGFR-2. Both are receptor tyrosine kinases, but they have different binding affinity for VEGF (26). Although its affinity for VEGF is lower than that of VEGFR-1, VEGFR-2 is found to be the major VEGF signal transducer (27, 28). Upon interaction of VEGF with its receptor, VEGFR dimerizes and autophosphorylates tyrosine residues in the intracellular domain, thus initiating many downstream signal transduction cascades (29, 30).

Different signal transduction pathways have different effects on ECs. For example, activation of the mitogen-activated protein kinase pathway increases EC proliferation (31). PI-3 kinase/AKT pathway activation inhibits pro-apoptotic proteins and confers a survival advantage upon ECs under cell stress conditions (32, 33). VEGF treatment also can induce FAK (focal adhesion kinase) phosphorylation, actin rearrangement, increased EC migration (34), and HSP90 (heat-shock protein 90). Association with VEGFR-2 is essential for the induction of FAK (35). With its central role in tumor angiogenesis, VEGF is found to be correlated with tumor size, stage, and prognosis in different tumors (36, 37). Therefore, VEGF, along with its receptors, forms a group of proteins that are believed to be promising targets for antiangiogenesis therapy. Consequently, numerous drugs aiming to block either the ligands or the receptors of VEGF have been developed.

Another group of potent angiogenesis-promoting factors is the FGF (fibroblast growth factor) family, which now has been expanded to include about 20 members (38, 39). FGF-1 and FGF-2 were first discovered as bovine pituitary-derived factors that strongly increased the proliferation of mouse 3T3 fibroblasts (40). Subsequently, these two factors were found to be implicated in angiogenesis based on a number of findings: (i) These factors promoted EC growth, migration, and invasion. When grown in matrigel, FGF enhanced capillary structure formation of human umbilical vein ECs (HUVEC) (41). (ii) Subcutaneously injected adenovirus-expressing FGF into mice induced higher angiogenesis than the control adenovirus (42). (iii) The expression of FGF-1 and FGF-2 is higher in gliomas than in normal tissue, and FGF-2 expression correlates with increasing grades of malignancy (43, 44). Esophageal cancer patients expressing high levels of FGF-2 have reduced survival rate and are more likely to recur than those with low levels of FGF-2 (45). (iv) In vivo mouse models also support a significant role for FGF-2 in tumor angiogenesis. When

angiogenesis-deficient leukemia cells were subcutaneously injected into mice, these cells hardly grew and stayed dormant. But the tumor cells were reactivated and resumed growth upon treatment with recombinant FGF-2, indicating that FGF-2-induced angiogenesis is critical for tumor growth (46).

Unlike most secreted proteins, FGF-1 and FGF-2 do not possess a signal peptide (47). Therefore, they are not likely to be delivered to the extracellular milieu via conventional vesicular transport. Various alternative release methods have been proposed. For instance, cell death can facilitate FGF release, which may be helpful in wound repair (48). Alternatively, FGF-1 forms a complex with synaptotagmin-1, which is released upon heat-shock stimulation (49). Released FGF binds to heparin, which not only protects FGF from proteolytic degradation but forms an FGF reservoir (50, 51). More importantly, heparin–FGF complex formation is required for FGF receptor binding and activation (52). Four FGF receptors are widely distributed on many cells, and FGF-1 and FGF-2 can bind to all these receptors. Like VEGF receptors, FGF receptors are also receptor tyrosine kinases. Upon heparin–FGF complex binding, the receptors dimerize and different tyrosine residues are phosphorylated (53, 54). Phosphorylation of these tyrosine kinases activates different signal transduction pathways including PLC- γ and Src signaling pathways, initiating diverse effects on various cell types (55, 56).

In summary, more than 20 angiogenic growth factors have now been found, and the list is still expanding. In clinical cancer samples, different combinations of angiogenic growth factors, rather than a single factor, are found to be overexpressed simultaneously (57). This poses a serious challenge for antiangiogenesis therapy, since satisfactory therapeutic effects may only be achieved when multiple angiogenic growth factors are inhibited. Further, each angiogenic growth factor may have a subtly different impact on angiogenesis. Both VEGF and FGF function to increase EC anti-apoptosis, but VEGF is responsible for resistance to apoptosis induced by an extrinsic pathway (activation of transmembrane receptors to initiate cell death), while induction of the intrinsic pathway of apoptosis resulting from cellular stress is repressed by FGF (58).

4. ANGIOGENIC GROWTH INHIBITORS

Apart from the angiogenic growth factors discussed above, many endogenous angiogenesis inhibitors have been identified which balance the angiogenesis-promoting effects of angiogenic growth factors. A number of studies support the finding that the ECM glycoproteins, thrombospondin-1 and thrombospondin-2, are important angiogenesis inhibitors, although some other experiments show conflicting results (59–61). The more convincing studies using mouse models confirm the antiangiogenesis ability of thrombospondin. Thrombospondin-2-knockout mice show twice as many vessels in dermis and adipose tissues as wildtype (62). Subcutaneous implantation of polyvinyl alcohol sponges into thrombospondin-2-knockout mice induces higher vascular network formation growing into them (63). When MMTV-HER2/neu-transgenic mice, which develop spontaneous breast tumors due to specific expression of the oncogene in mammary gland, are crossed with thrombospondin-1-knockout mice, tumor onset is accelerated, with a concomitant increase in the number of enlarged vessels within tumors. Conversely, overexpression of thrombospondin-1 in MMTV-HER2/neu-transgenic mice leads to delayed tumor onset; 20% these mice stayed tumor free after 18 months

(64). However, in another study, the underexpression of thrombospondin correlated with poor prognosis in clinical samples (65, 66). These contradictory results may be due to the complex structural motifs that thrombospondin forms when binding to different receptors, cytokines, and proteases, which may either promote or inhibit angiogenesis, respectively. Currently, there are large ongoing efforts to explore the utility of thrombospondin-1 and thrombospondin-2 as antiangiogenesis therapeutic drugs.

The coexistence of multiple angiogenic and antiangiogenesis factors indicates a very complex mechanism for the regulation of angiogenesis. Although cancer epithelial cells are the main source of these factors, other cell types also contribute to tumor angiogenesis. For example, it is known that the hypoxic tumor microenvironment recruits macrophages, which secrete various cytokines and VEGF, significantly promoting angiogenesis and resulting in a poor clinical prognosis (67, 68). Tumor-secreted stromal cell-derived factor-1 interacts with its receptor CXCR4 on bone marrow-derived endothelial progenitor cells and recruits these cells into tumor sites (69, 70). Another cell type derived from bone marrow that promotes angiogenesis is the hematopoietic stem cell (HSC) (71). These bone marrow-derived cells are an important component of neovessels within tumors. In a heterozygous *Pten*-knockout mouse uterine carcinoma model, these cells contributed to 16% of the neovessels. Their incorporation into neovessels is also of functional significance. For example, when recruitment of HSCs is impaired, tumor growth is reduced and tumor cell death is increased (72). Results reported in a recent study showed an unexpected function of HSCs in tumor metastasis, suggesting that tumor-secreted cytokines attract HSCs to tumor premetastatic sites. The HSCs then create an optimal local microenvironment for tumor growth. Subsequently, tumor cells arrive at these sites and proliferate, forming metastatic lesions (73).

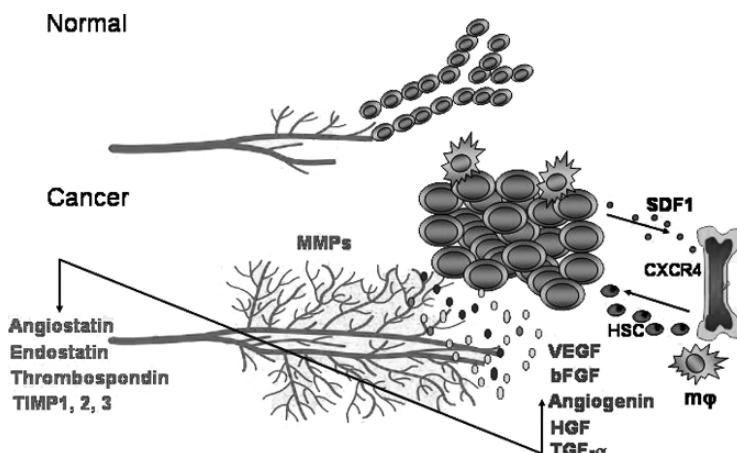


Fig. 1. The imbalance of angiogenesis-related factors produced by a variety of cell types initiates tumor angiogenesis. Illustrated for breast tissue and cancer, the low oxygen content and neoplastic transformation of cells, often by activation of oncogenes, induce cancer cells to increase secretion of angiogenesis-promoting factors and decrease production of angiogenesis inhibitors. The angiogenic factors bind to their cognate receptors on ECs, promote EC proliferation and migration into tumor, induce formation of neovessels within tumor, and support tumor growth. Bone marrow-derived macrophage and HSCs are also recruited into the tumor site, further contributing to angiogenesis by producing more angiogenic factors. They also help maintain vessel wall integrity by incorporating into the vessel wall. (Please see color insert.)

The involvement of multiple angiogenesis-related factors produced by multiple cell types (Fig. 1) may seriously impair the efficacy of antiangiogenesis therapy because it is very difficult to block the activities of many angiogenic factors simultaneously. This implies that, in addition to angiogenic factors, there is a need to search for other targets involved in the initiation of angiogenesis. In recent years, several research groups investigated alterations in blood vessels during tumorigenesis, which occur after the ECs receive signals transmitted by angiogenic factors. Difference in gene-expression profiles in tumor EC as compared with normal ECs could possibly provide potential therapeutic targets. It is hypothesized that targeting gene-expression changes in tumor ECs may improve the specificity and efficacy of antiangiogenesis therapy.

5. APPROACHES TO IDENTIFICATION OF NOVEL THERAPEUTIC TARGETS

There are fundamental biological questions that need to be addressed in order to effectively search for new antiangiogenic targets in tumor ECs. For example, different types of tumors can produce similar angiogenic factors such as VEGF and FGF to initiate angiogenesis. Will these common factors induce the same angiogenic response in all ECs or will each tumor type use different pathways? Even in healthy individuals, vessels in different anatomical sites have different gene-expression profiles. Gene-expression profiles in arterial ECs are not similar to the ones in venous ECs, and large vessels display distinctive gene expression that is not shared by microvessels (74).

In the past, technical difficulties prevented global analysis of gene expression in tumor versus normal ECs. Specifically, the scarcity of ECs and the diffuse distribution of blood vessels within tissues meant that conventional methods for obtaining purified cells, such as by microdissection, were not feasible. This technical problem was finally solved in 2000 when a successful method to purify ECs from colon cancer tissues was developed (75). In this method, magnetic beads coupled to cell type-specific antibodies are used to negatively or positively select specific cell types. This method yields nearly pure populations of ECs.

Serial analysis of gene expression (SAGE) performed on purified tumor and normal ECs revealed two different endothelial gene-expression patterns. One group of genes is expressed at high levels in both normal and tumor ECs, but not in non-ECs are termed “pan-endothelial” markers. The second set of genes is expressed at high levels only in tumor ECs. The strongest 25 markers, whose expression is elevated in tumor ECs compared to normal ECs, consisted mainly of cell-surface proteins, ECM proteins, and uncharacterized transcripts (75). These overexpressed markers in tumor-specific ECs may be promising new targets for antiangiogenesis treatments.

These endothelial gene-expression patterns have other interesting implications as well. For example, some pan-endothelial markers are overexpressed only in primary tumor and normal tissues, but underexpressed in cell lines made from HUVEC and human dermal microvascular ECs (HMVEC). This suggests that *in vitro* cell-culture data may not fully reflect the *in vivo* angiogenesis process and that some informative markers could be lost during cell culture. Some markers are also expressed during the process of wound healing, indicating commonality between physiological and pathological angiogenesis. Moreover, mechanisms of angiogenesis in the primary tumor site and metastasis are similar. Tumor-specific endothelial markers found in primary colon cancer are also shown to have elevated expression in liver metastases (75), thus

raising the possibility that antiangiogenesis therapy can be used to treat both primary and metastatic cancers, potentially benefiting patients at both an early and late stage of disease.

To address the question whether different tumors will induce similar or dissimilar angiogenic responses in EC, SAGE analyses were performed on EC isolated from gliomas, whose growth is highly dependent on angiogenesis, and from breast tumors (76,77). In glioma cells, 14 endothelial markers showed over twofold overexpression in tumors compared to normal ECs, while another set of 14 endothelial markers showed underexpression. As seen in colon cancer tumor endothelial markers, the vast majority of these glioma-endothelial markers are cell-surface or extracellular proteins (76). Other endothelial markers that showed differential expression between tumor and normal tissues are also found in the breast tumor samples. In addition to cell-surface or extracellular proteins, transcription factors and protein tyrosine phosphatases are also found to be elevated in tumor ECs (77). Hairy/enhancer-of-split (HES) related with YRPW motif-like (HEYL) is one such transcription factor and is a member of the HERP family. Like its homologous HES family members, the Notch pathway is believed to be responsible for HEYL induction. HEYL expression in human ECs is associated with faster cell growth and conferred enhanced anti-apoptosis activity, which may play important role in tumor angiogenesis (77). The function of protein tyrosine phosphatase type IVA, member 3 (PRL3), which was elevated in breast tumor vasculature (77), in angiogenesis was tested by overexpression in HMVEC using adenovirus infection. It was found that overexpression of PRL3 increased HMVEC migration. Thus, the breast tumor endothelial markers are also shown to have functional relevance in angiogenesis.

A comparison of the SAGE library data from colon, brain, and breast tumors indicates that tumors do not induce the same angiogenic responses in ECs. For example, only one gene is downregulated by fourfold in both colon cancer and gliomas, and 12 genes show upregulation in both tumors (77). When the SAGE data of breast ECs are included in this comparison, very few genes display differential expression in all three tumor samples (Table 1). Such low similarity in gene expression suggests that different mechanisms are involved in angiogenic responses in different cancer types. As other research groups study endothelial responses of other tumor samples and more gene-expression patterns are discovered, we will have a clearer understanding of angiogenic diversity across the tumor spectrum.

Such innate heterogeneity of tumor angiogenic responses, however, presents a significant obstacle for future development of antiangiogenesis therapies. A therapy targeting one tumor endothelial marker may only be effective for a small proportion of patients who display a specific angiogenesis response to tumor epithelial angiogenic factors. Therefore, different antiangiogenesis therapeutic regimens may be needed in different cancers. It is likely then that a combination of various antiangiogenesis-targeting therapies will be needed to produce the most favorable clinical outcome.

6. ANTIANGIOGENESIS THERAPY

While the gene targets discovered by SAGE and other analyses present a rich resource for future drug development, important advances in antiangiogenesis therapy have already taken place in the past decade. It is clear that angiogenesis is critical at almost every stage of tumor development. Growth and formation of capillary blood

Table 1
Genes Overexpressed in Endothelial Cells (ECs) from Breast Cancer Versus ECs From Normal Breast Tissue Determined by SAGE Analysis and Profile Comparison with Colon and Brain ECs

| <i>Gene description</i> | <i>Breast EC</i> | <i>Colon EC</i> | <i>Brain EC</i> |
|--|---------------------|---------------------|---------------------|
| | <i>Tumor/normal</i> | <i>Tumor/normal</i> | <i>Tumor/normal</i> |
| Matrix metalloproteinase 9 (gelatinase B) | 21 | 3 | 6 |
| Hairy/enhancer-of-split HEYL | 20 | 3 | — |
| <i>Homo sapiens</i> clone FLC1492 PRO3121 mRNA | 15 | 4 | 6 |
| Complement component 4A | 13 | — | — |
| Tumor endothelial marker 7 precursor | 12 | 18 | — |
| Snail homolog 1 (<i>Drosophila</i>) | 10 | — | — |
| Collagen, type IV, α 2 | 9 | 2 | 9 |
| Heat-shock 70kDa protein 1A | 9 | 11 | — |
| Secreted protein, acidic (osteonectin) | 9 | 2 | — |
| Collagen, type XVIII, 1 | 9 | 4 | — |
| Protein tyrosine phosphatase type IVA, member 3 (PRL3) | 8 | — | — |
| Interferon-stimulated protein, 15 kDa | 8 | — | — |
| Tissue inhibitor of metalloproteinase 1 | 7 | 3 | — |
| Secreted protein, acidic, cysteine rich (osteonect) | 7 | 2 | 6 |
| Ras homolog gene family, member C | 7 | — | — |
| Heat-shock 90kDa protein 1 | 7 | 5 | — |
| Calcium channel, voltage dependent, 1H | 7 | — | — |
| Serine/arginine repetitive matrix 2 | 7 | — | — |
| ESTs, highly similar to PLCD_HUMAN 1-acyl-sn-glyc | 7 | — | — |
| Macrophage myristoylated alanine-rich C kinase | 7 | 2 | — |
| Regulator of G-protein signaling 5 | 6 | 2 | — |
| HSPC142 protein | 6 | — | — |
| Tax interaction protein 1 | 6 | — | — |
| DnaJ (Hsp40) homolog, subfamily B, member 1 | 6 | 5 | — |
| ESTs, weakly similar to S55016 protein oaf-fruit | 6 | — | — |
| Nucleophosmin (nucleolar phosphoprotein B23) | 6 | — | — |
| Cadherin 5, type 2, VE-cadherin | 6 | — | — |
| G-protein-12 subunit | 6 | — | — |
| Serine (or cysteine) proteinase inhibitor, clade E | 6 | 2 | 9 |

Genes overexpressed by at least sixfold in ECs isolated from breast carcinoma compared to ECs from normal breast tissue are shown. (—) genes whose expression showed no change in tumor ECs compared to their respective normal ECs (75–77).

vessels within a solid tumor are associated with tumor growth, metastasis, and distant colonization. Also, tumor ECs are usually genetically stable and are thus less likely to spawn drug-resistant variants than malignant epithelial cells. Therefore, antiangiogenesis pathways are a reasonable and effective targets for cancer treatment. We will discuss the ongoing development of some antiangiogenesis drugs mainly aimed at interrupting the VEGF signal transduction pathway (also summarized in Table 2).

6.1. Monoclonal Antibodies

Monoclonal antibodies have gone through the development pipeline the farthest in antiangiogenesis therapy. Bevacizumab (Avastin) is a humanized anti-VEGF monoclonal antibody (mAb) (78). Antibodies to VEGF in combination with chemotherapeutic agents produce synergistic cytotoxicity in a range of cancers. This has now been substantiated in numerous phase I and II trials (79). It appears that bevacizumab can be a component of an effective combination therapy approach to colorectal cancer (CRC) and non-small-cell lung cancer (NSCLC) in particular. Bevacizumab is currently federal drug administration (FDA) approved as a first-line therapy for metastatic CRC in combination with 5-fluorouracil-based chemotherapy. Several phase II clinical trials of bevacizumab as a single agent (80,81) as well as in combination with chemotherapy have shown improvements in long-term survival in metastatic CRC (82) and advanced breast cancer (83,84).

One area of concern is that bevacizumab is a humanized murine antibody rather than a fully human mAb. Advances in molecular immunology have resulted in the engineering of fully human mAbs that are demonstrating fewer adverse events than similar agents that are humanized. To a certain extent, the clinical potential of bevacizumab may depend upon the future development of fully human mAbs directed against VEGF, such as an anti-human VEGF antibody in preclinical development by Merck KGaA. Moreover, apart from any possible relationship to the murine component of bevacizumab, life-threatening hemorrhage in a phase II study of NSCLC patients is a complication that would need to be closely monitored (85). Bevacizumab, in combination with chemotherapy, is likely to be beneficial in the treatment of multiple types of neoplasia.

Other monoclonal antibodies have been produced that neutralize all biologically active forms of VEGF via recognition of the VEGF-binding sites on the VEGF receptors, VEGFR-1 and VEGFR-2. Examples include the fully human anti-VEGFR-2 antibodies IMC-2C6 (86,87) and IMC-1121, the fully human anti-VEGFR-3 mAb (88). All of these antibodies are currently in preclinical development. Such antibodies prevent EC mitogenesis, enhanced vascular permeability, and angiogenesis *in vitro*. Based on *in vitro* and animal studies, human clinical trials were initiated. Researchers are awaiting the outcome of these trials using the fully humanized antibodies because of the promise of equal or better effectiveness as antiangiogenic agents accompanied by fewer side effects.

6.2. Targeting the PKC- β Pathway

Activation of protein kinase C- β (PKC- β) plays a major role in the induction of tumor angiogenesis. Enzastaurin (LY-317615, 317615.2HCl) is an orally active inhibitor of PKC- β and related protein kinases and is being developed as a potential novel treatment

Table 2
Angiogenesis-Targeted Drug Development

| <i>Drug</i> | <i>Other names</i> | <i>Developmental status</i> | <i>Mechanism</i> | <i>Tumor type</i> |
|--------------|---|-----------------------------|--|---|
| Bevacizumab | Avastin | Launched | Monoclonal antibody against VEGF | NSCLC, myelodysplastic syndrome, renal cell carcinoma, melanoma, pancreas tumor, prostate tumor, breast tumor, ovary tumor, colorectal tumor (78–85) |
| Enzastaurin | LY-317615, 317615.2HCl | Phase III clinical | PKC-β inhibitor | Glioma, NSCLC, multiple myeloma, B-cell lymphoma (89) |
| Tacedinaline | CI-994, Goe-5549, PD-123654, acetylindanine | Phase II clinical | Cytostatic histone deacetylation inhibitor | NSCLC, renal cell carcinoma, leukemia, melanoma; pancreas tumor, breast tumor, colon tumor (90–92) |
| ZD-6474 | Zactima, formerly AZD-6474 | Phase III, clinical | Small-molecule inhibitor of VEGF receptor tyrosine kinase that also has activity against the EGF and RET receptor tyrosine kinases | Thyroid tumor, small-cell lung cancer, NSCLC, multiple myeloma, breast tumor, head and neck tumor, solid tumor, central nervous system tumor, brain tumor (95–99) |
| SU11248 | N/A | Phase II clinical | Tyrosine kinase inhibitor that has activity against VEGFR-1 and -2, as well as PDGFR and cKIT | Renal cell carcinoma, lung cancer, metastatic soft tissue sarcoma (101–102) |
| VEGFR-Trap | | Phase I clinical | High-affinity soluble decoy receptor that binds to and inactivates VEGF | Renal cell carcinoma, ovarian cancer, Ewing's sarcoma family of tumors, Hodgkin's lymphoma, colorectal cancer, Wilms tumor (105–109) |
| Ribozymes | Angiozyme™ | Phase II clinical | Ribozyme against the pre-mRNA of VEGFR-1 | Colorectal cancer, glioblastoma, nasopharyngeal carcinoma, and melanoma (110–113) |
| YM-359445 | N/A | Preclinical | VEGFR-2 tyrosine kinase inhibitor | Various established xenografts of tumor cell lines (100) |

^a NSCLC, non-small-cell lung cancer; PKC, protein kinase; VEGF, vascular endothelial growth factor.

for a variety of cancers. Enzastaurin has been shown to induce apoptosis and inhibit the growth of colon cancer cells and glioblastoma cells *in vitro* (89). In March 2006, Lilly began the randomized, open-label, registration STEERING (Study Evaluating Enzastaurin in recurrent Glioblastoma) phase III trial in 397 patients with relapsed glioblastoma multiforme, which is designed to compare oral enzastaurin to CCNU (lomustine). It will be a few years before the outcome of this trial is known, but targeting PKC- β is a reasonable approach directed at debilitating a pathway important in angiogenesis.

6.3. Histone Deacetylase Inhibitors

Tacedinaline (CI-994, Goe-5549, PD-123654, acetyldinaline) is an oral, cytostatic histone deacetylation inhibitor that has antiangiogenic activity and is under development by Pfizer for the potential treatment of solid tumors and leukemia (90). *In vitro* treatment of two NSCLC cell lines, A-549 (adenocarcinoma) and LX-1 (squamous cell carcinoma), with tacedinaline resulted in cell-cycle arrest at the G0/G1 phase (91). In a phase I clinical trial, a total of 30 patients with a variety of cancers were given tacedinaline with or without paclitaxel or carboplatin. Five of these patients who received greater than one cycle of treatment of tacedinaline achieved a partial response (three NSCLC, one CRC, and one unknown primary) and two patients achieved a complete response (esophageal and bladder cancer) (92). Histone deacetylase inhibitors are being tested in a variety of tumor types, both solid and liquid, for their anticancer effects, but their cytostatic effects are probably mediated through changes in expression of a broad variety of molecular targets. Although they may eventually affect angiogenesis, their specificity *in vivo* as antiangiogenesis agents is questionable.

6.4. Small-Molecule Kinase Inhibitors

The promise of targeted therapy was realized with the discovery and successful application of Gleevec, a tyrosine kinase inhibitor, to the treatment of chronic myelocytic leukemia. Using the same principles, a variety of inhibitors targeting the kinase domain of VEGF receptors have been developed. These are in various stages of development, which is summarized below.

ZD-6474 (Zactima; formerly AZD-6474) is a small-molecule inhibitor of VEGF-receptor tyrosine kinase, which also has activity against the EGF and Rearranged in Transfection (RET) receptor tyrosine kinases (93, 94). Clinical trials in NSCLC, small-cell-lung-cancer, myeloma, thyroid cancer, and breast cancer are ongoing (95). The antitumor and antiangiogenesis activity of ZD6474 *in vivo* can be enhanced by its use in combination with a taxane such as paclitaxel. In fact, treatment with the two drugs produced a complete regression of established xenografts of a colon cancer cell line, GEO, in mice (94), a finding that has been confirmed in mouse models of gastric, lung, and central nervous system tumors (96–98). Further evidence for an antiangiogenesis mechanism of its action is that gefitinib, a small-molecule inhibitor of epidermal growth factor receptor tyrosine kinase, is inactive in these models. Phase I clinical trials showed a long half-life for ZD6474 (99). Phase II trials with ZD6474 are currently ongoing in a range of solid tumors.

In mice bearing various established solid tumors, including paclitaxel-resistant tumors, once-daily oral administration of another VEGF receptor tyrosine kinase inhibitor, YM-359445, inhibited tumor growth and reduced its vasculature. In this

study, YM-359445 had greater antitumor activity than other VEGFR-2 tyrosine kinase inhibitors tested, including ZD6474, CP-547632, CGP79787, SU11248, and AZD2171 (100).

SU11248 is an oral tyrosine kinase inhibitor that has activity against VEGFR-1 and VEGFR-2, as well as PDGFR and c-KIT. In an *in vivo* study, athymic nude mice were injected subcutaneously with colon cancer cells (101). Mice were given a single dose of SU11248, and magnetic resonance imaging (MRI) was used to visualize the drug effects. Administration of SU11248 resulted in a 42% decrease in vascular permeability measured in the tumor rim by MRI. Effects verified with histology showed significant reductions in mean tumor vasculature density. A phase II multicenter clinical trial of 63 patients with metastatic renal cancer showed tumor regression in 40% of patients who received SU11248 (102). Phase II clinical trials of SU11248 in combination with carboplatin and paclitaxel in patients with previously untreated NSCLC are underway.

6.5. Decoy Receptors

VEGF trap is a high-affinity soluble decoy receptor that was generated by fusing the extracellular domains of VEGFR-1 and VEGFR-2 to the Fc portion of human IgG1 (103). It binds and inactivates VEGF. A possible advantage of VEGF trap over mAb therapy is that VEGF trap has significantly higher affinity for VEGF. In addition, other members of the VEGF family may also be sequestered by VEGF trap. VEGF trap has been shown effective in blocking angiogenic function in mouse and macaque tumor models (104, 105). Further, other *in vivo* studies using a variety of tumor xenograft models show antitumor efficacy of VEGF trap in reducing metastatic spread of primary tumors (103, 106, 107). In an ovarian cancer mouse model, VEGF trap in combination with paclitaxel reduced tumor burden by 98% compared to control mice (108). A phase I trial is currently underway for patients with Hodgkin's lymphoma. A potential side effect of VEGF trap may be impairment of ovarian function (109).

6.6. Ribozymes

Ribozymes are RNA enzymes that bind and cleave mRNA molecules in a sequence-specific manner. *In vitro* activity of ribozymes against VEGF receptor mRNAs-encoding RNAs has been observed (110). A ribozyme against the pre-mRNA of VEGFR-1 called Angiozyme™ is being developed by Chiron Corp. Ribozyme Pharma. *In vitro*, Angiozyme™ has been shown to reduce glioblastoma cell angiogenesis by reducing the expression of cell-surface and soluble VEGFR-1 (111). A phase I clinical trial, Angiozyme™, was delivered to patients by daily s.c. administration with minimal side effects (112). In a phase II clinical trial, Angiozyme™ administered in combination with irinotecan, 5-fluorouracil, and leucovorin to patients with metastatic CRC showed that Angiozyme™ treatment correlated with improved clinical outcome (99, 113).

7. CONCLUDING REMARKS

In conclusion, although antiangiogenesis therapy holds great promise for novel and effective breast cancer treatment, it also presents great challenges. Anti-VEGF monotherapy often does not show better therapeutic effects than conventional cytotoxic chemotherapeutic drugs. Considering the complex angiogenesis regulatory network

that involves multiple angiogenic factors produced by various cell types, any antiangiogenesis therapy aimed at a single angiogenic factor is not likely to be highly effective. Also, the finding that neovessels that already exist within tumors respond poorly to antiangiogenesis therapy suggests a narrow time window available for effective drug administration. We expect that further research in the antiangiogenesis field will enable us to develop more effective drugs and drug regimens.

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21

Angiogenesis and Angiogenesis Inhibition in Sarcomas

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SUMMARY

Sarcomas are mesoderm-derived malignancies that include tumors arising from the soft tissues, skeleton, and vascular elements. These tumors share a common mesenchymal origin with the vasculature. Many of the signaling pathways involved in angiogenesis also drive sarcoma tumor cell growth. Autocrine and paracrine vascular endothelial growth factor (VEGF)- and platelet-derived growth factor (PDGF)-mediated growth plays a role in the pathogenesis of several sarcoma subtypes. Inhibition of signaling pathways common to sarcoma growth and angiogenesis has been shown to be an effective therapeutic strategy for some patients with sarcoma.

Key Words: Sarcoma; angiogenesis; mesenchymal; VEGF; review.

1. INTRODUCTION

Sarcomas are mesoderm-derived malignant neoplasms. They account for approximately 1% of adult cancers and a somewhat greater proportion of pediatric cancers. This heterogeneous group of tumors originates from a wide range of tissues, including soft tissues, bone, cartilage, and vascular tissues.

Sarcomas provide a unique opportunity to gain insight into the interaction between the process of angiogenesis and neoplasia. As mesenchymal tumors, sarcomas share a common embryologic origin with the vasculature. This common origin raises the possibility of shared signaling pathways that might stimulate both vascular cell and tumor cell growth. Unlike the complex genetic changes often seen in carcinomas, specific mutations and translocations have been implicated in the pathogenesis of many sarcomas. Although still being elucidated, some of these genetic changes appear to either directly or indirectly promote angiogenesis.

While a large amount of cancer angiogenesis research has been conducted in carcinomas, knowledge about angiogenesis in carcinoma may not generalize to sarcoma. To further understand the role of angiogenesis in sarcoma, this chapter will first review the preclinical evidence implicating angiogenesis and shared angiogenesis pathways

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in mesenchymal neoplasia. These preclinical findings have translated into important clinical research on the use of antiangiogenesis therapy in patients with sarcoma. The chapter will conclude with an overview of this research.

2. ANGIOGENESIS AND MESENCHYMAL NEOPLASIA

2.1. Soft Tissue Sarcoma

Soft tissue sarcomas are a heterogeneous group of mesenchymal malignancies arising outside of the bone or cartilage. These heterogeneous tumors have typically been treated with similar approaches, including wide excision with or without chemotherapy and radiotherapy. More recently, biological insights have highlighted important differences within this class of neoplasms, including differences in angiogenesis-related pathways.

2.1.1. RHABDOMYOSARCOMA

Rhabdomyosarcoma is one of the more common soft tissue sarcomas, particularly in children. Several lines of evidence implicate autocrine vascular endothelial growth factor (VEGF) signaling in rhabdomyosarcoma. Rhabdomyosarcoma cell lines express VEGF and VEGF receptors, with VEGFR-1 more commonly observed than the other VEGFRs (1, 2). The PAX3-FKHR fusion oncprotein associated with alveolar rhabdomyosarcoma increases the expression of VEGFR-1 (3). VEGF stimulates rhabdomyosarcoma cell proliferation, and VEGFR-1 inhibition attenuates cell growth *in vitro* (2). Treatment of mice with human rhabdomyosarcoma xenograft tumors with an antibody against human and mouse VEGF completely blocks tumor growth (4). Other mediators of angiogenesis are involved in rhabdomyosarcoma. Rhabdomyosarcoma cell lines express lower levels of thrombospondin than cultured human myoblasts (5). These results demonstrate the importance of angiogenesis in rhabdomyosarcoma.

2.1.2. FIBROSARCOMA

Several aspects of angiogenesis and related signaling have been studied in fibrosarcoma, a soft tissue sarcoma arising from connective tissue. Human fibrosarcoma cells secrete high levels of VEGF-A *in vitro* (6). In a mouse model of fibrosarcoma, cells incapable of producing VEGF produced smaller tumors than cells able to produce VEGF (7). Inhibition of VEGF-A expression with small inhibitory RNA also decreases the growth of human fibrosarcoma mouse xenografts (6). Similar methods using rat fibrosarcoma mouse xenografts demonstrated synergistic tumor growth suppression when VEGF inhibition was combined with thrombospondin-1 expression (8). Several strategies directed at VEGFR inhibition have all slowed the growth of fibrosarcoma xenografts (9, 10).

2.1.3. OTHER SOFT TISSUE SARCOMAS

Synovial sarcoma is another relatively common soft tissue sarcoma. Several features of the angiogenesis system have been evaluated in these tumors. Synovial sarcomas express VEGF, and recent evidence suggests that p53 helps to regulate VEGF production in synovial sarcoma (11). Synovial sarcoma cell lines with mutant *p53* expressed higher levels of VEGF than synovial sarcoma cell lines with wild-type *p53* (11).

Platelet-derived growth factor receptors (PDGFRs) drive the growth of a subset of gastrointestinal stromal tumors (GISTs). These tumors typically arise from the mesenchyme of the stomach or small intestine and are thought to originate from the interstitial cells of Cajal in the myenteric plexus (12). GISTs characteristically harbor activating mutations of *KIT* although approximately 20% of GISTs lack these mutations (12). Of these *KIT* wild-type tumors, approximately 35% harbor activating mutations of *PDGFR-α* (12). GISTs also express VEGF, with an incidence of 26% in one case series (13). Gene expression profiling indicates that GISTs lacking *KIT* mutations have increased *VEGF* expression compared to mutant *KIT* tumors (14).

Preliminary investigations of angiogenesis pathways in a variety of other soft tissue sarcomas have been undertaken. Yoon and colleagues (15) performed gene expression profiling on a panel of 38 soft tissue sarcoma specimens. *Angiopoietin-2* and *bFGF* expression was higher in soft tissue sarcomas than in normal tissues. Other genes that appeared to be up-regulated in soft tissue sarcomas included genes for PDGFR- α and pigment epithelium-derived factor (15).

2.2. Skeletal Sarcomas

2.2.1. OSTEOSARCOMA

Osteosarcoma is the most common primary malignant bone tumor in both adults and children. More than half of osteosarcoma tumors have been shown to express VEGF and VEGFR (16, 17). Several natural inhibitors of angiogenesis have also been evaluated in osteosarcoma. Thrombospondin-1 gene expression was consistently down-regulated in several osteosarcoma cell lines evaluated (18). Administration of endostatin cDNA plasmid-containing liposomes in a rat model of osteosarcoma resulted in diminished tumor growth compared to control-treated animals (Fig. 1) (19). This treatment also significantly reduced the incidence of lung metastases. An antiangiogenic

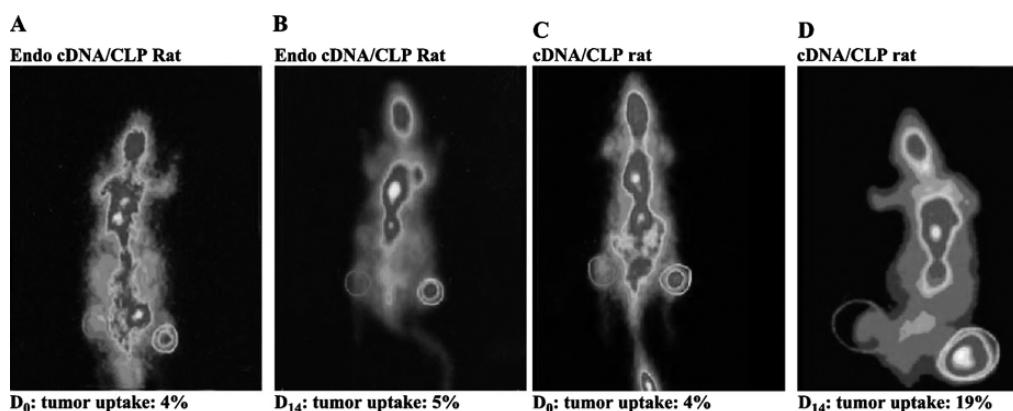


Fig. 1. Treatment with endostatin encoding plasmids suppresses tumor growth in a rat model of osteosarcoma. Panels A and B demonstrate positron emission tomography (PET) images at day 0 and 14 in rats treated with endostatin encoding plasmids. Tumor in the right hind limb remained stable. Panels C and D demonstrate PET images at day 0 and 14 in rats treated with control plasmids. Tumor progression in the right hind limb is evident. Reprinted with permission (19). Copyright 2005, with permission from Elsevier. (Please see color insert.)

fumagillin analog, TNP-470, inhibits the development of pulmonary metastases in rodent osteosarcoma xenografts (20). These preclinical data highlight the importance of angiogenesis in the pathogenesis of osteosarcoma.

2.2.2. EWING SARCOMA

Ewing sarcoma is one of the most common malignant bone tumors, with a peak incidence in the second decade of life. These tumors are highly vascular. Preclinical studies suggest that tumor microvessels in Ewing sarcoma originate from both local endothelial cells and through recruitment of bone marrow-derived endothelial precursor cells (21). The EWS fusion oncoproteins characteristic of this disease may play a role in promoting angiogenesis in Ewing sarcoma. EWS oncoproteins promote VEGF gene expression although this effect does not appear to involve EWS oncoprotein-binding to the VEGF promoter (22). A subset of Ewing sarcoma tumor specimens demonstrate VEGF expression, and Ewing sarcoma cells secrete VEGF, raising the possibility of autocrine or paracrine growth stimulation involving VEGF (23). Blockade of the VEGF pathway in Ewing sarcoma mouse xenograft models inhibits tumor growth (Fig. 2) (24).

2.3. Vascular Sarcomas

2.3.1. ANGIOSARCOMA

Angiosarcomas arise from the endothelium and express endothelial cell markers. Multiple pieces of evidence highlight the importance of VEGF signaling in these tumors. Over-expression of VEGF transforms murine endothelial cells from a hemangioma-forming phenotype to an angiosarcoma-forming phenotype (25). Angiosarcomas express both VEGF and VEGF receptors, suggesting a VEGF-mediated autocrine growth loop in these tumors (26,27).

Other aspects of angiogenesis have also been studied in angiosarcoma. Angiosarcomas highly express angiopoietin-2 and Tie1, whereas angiopoietin-1 and Tie2 are less reliably expressed (28). These tumors also appear to express higher levels of

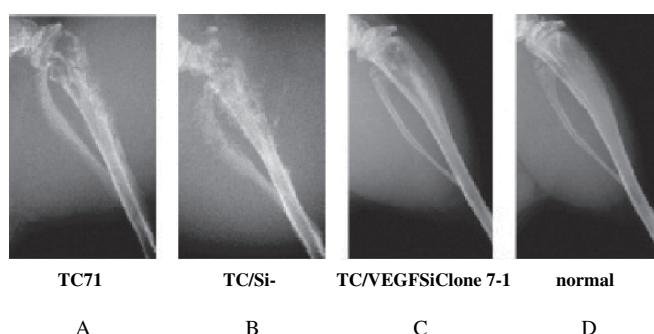


Fig. 2. Small inhibitory RNA against VEGF blocks bone tumor formation in a mouse model of Ewing sarcoma. Mice injected locally with human Ewing sarcoma cells or human Ewing sarcoma cells transfected with control small inhibitory RNA developed bone tumors (panels A and B, respectively). Mice injected with human Ewing sarcoma cells transfected with small inhibitory RNA against vascular endothelial growth factor (VEGF) were less likely to develop bone tumors (panel C). An image from a normal mouse is shown in panel D for comparison. Reprinted with permission (24). Copyright 2005, with permission from American Association for Cancer Research.

bFGF and FGF receptor than normal tissues (29). TNP-470 has been shown to inhibit angiosarcoma growth in mouse models (30).

2.3.2. KAPOSI'S SARCOMA

Kaposi's sarcoma lesions consist of malignant spindle cells interspersed with endothelial cells (31). The spindle cells form vascular channels and are thought to arise from cells in the endothelial cell lineage (31). Infection with human herpes virus 8 (HHV8) plays a major role in the pathogenesis of all subtypes of Kaposi's sarcoma (32). Infection of cultured human endothelial cells with HHV8 results in the formation of immortalized spindle cells (33). These cells retain VEGFR-2 expression well beyond the time at which uninfected endothelial cells begin to lose VEGFR-2 expression. Supernatant from infected cultured endothelial cells stimulates the formation of spindle cells and VEGFR-2 expression from uninfected endothelial cells (33).

Patients with AIDS-associated Kaposi's sarcoma tend to have more rapidly progressive disease than patients with post-transplantation Kaposi's sarcoma (32). This finding suggests that human immunodeficiency virus (HIV) infection may stimulate the growth of these tumors, independent of the degree of immunosuppression. Laboratory studies demonstrate that the HIV Tat protein directly stimulates VEGFR-2, providing one possible explanation for this clinical observation (34).

Other lines of evidence indicate an important role for angiogenesis signaling in Kaposi's sarcoma. Kaposi's sarcoma tumor cells express high levels of VEGF and VEGFR-2 (35, 36). VEGFR-1 appears to be less highly expressed in Kaposi's sarcoma tumor cells (36). Blockade of VEGF production with VEGF antisense oligonucleotides has been shown to inhibit the growth of Kaposi's sarcoma cells *in vitro* and *in vivo* (37). PDGF stimulates the expression of VEGF by Kaposi's sarcoma cells (35). Kaposi's sarcoma cells also express angiopoietin-2, Tie1, and Tie2, whereas angiopoietin-1 and angiopoietin-4 expression is less uniform (28). These findings demonstrate that multiple aspects of the angiogenesis system are involved in the pathogenesis of Kaposi's sarcoma.

2.3.3. OTHER VASCULAR SARCOMAS

Significantly fewer studies have evaluated angiogenesis pathways in other types of vascular sarcomas. Unlike angiosarcoma and Kaposi's sarcoma, VEGF-mediated autocrine growth seems less likely in hemangiopericytomas. Hemangiopericytoma tumor cells express only low levels of VEGF and placental growth factor (38). These cells also express VEGFR-1 and VEGFR-2 at only low levels (38, 39). Instead, PDGF-mediated signaling may be more important in the growth of these tumors. One group reported relatively high levels of PDGF in hemangiopericytoma tumor cells (39). Initial evaluation of the angiopoietin system in these tumors showed little or no Tie expression in tumor cells (38).

3. THERAPEUTIC IMPLICATIONS OF ANGIOGENESIS INHIBITION IN SARCOMA

Despite promising preclinical data supporting a role for angiogenesis inhibition in sarcoma, relatively few clinical trials have evaluated antiangiogenic therapy in sarcoma. Even fewer trials have aimed to determine the therapeutic response of sarcomas to these agents. Instead, much of the data relevant to sarcomas come from general phase I trials in patients with refractory solid tumors. Absence of response in these phase

Table 1
Antiangiogenic and Vascular-Targeting Agents
with Reported Clinical Experience in Patients
with Sarcoma

| |
|---|
| Anti-VEGF agents |
| Bevacizumab |
| VEGF Trap |
| VEGF Antisense |
| Anti-VEGF ribozyme |
| SU5416 |
| SU6668 |
| SU11248 (sunitinib) |
| BAY 43-9006 (sorafenib) |
| AZD2171 |
| PTK787/ZK222584 |
| Tyrosine kinase inhibitors without VEGFR activity |
| BMS 354825 |
| Imatinib |
| Matrix metalloproteinase inhibitors |
| Marimistat |
| COL-3 |
| AG3340 |
| BAY12-9566 |
| Endogenous angiogenesis inhibitors |
| Endostatin |
| ABT510 |
| Vascular targeting agents |
| Comberstatin A4 |
| DMXAA |
| Other agents |
| TNP470 |
| Thalidomide |
| Rapamycin |
| CCI-779 |
| AP23573 |
| Squalamine |
| IM862 |
| MEDI522 |
| MEDI523 |
| Metronomic chemotherapy |

I trials could indicate lack of drug activity or inadequate dose. The Table 1 lists the antiangiogenic agents for which clinical experience in patients with sarcoma has been reported.

3.1. Agents Targeting VEGF Activity

Inhibition of the VEGF pathway has been an active area of clinical cancer research. Multiple strategies have been evaluated, including agents that bind VEGF, agents that decrease VEGF production, and agents that impede VEGF receptor signaling. The first antiangiogenic drug approved for cancer therapy was bevacizumab, a recombinant humanized monoclonal antibody directed against VEGF. Bevacizumab has been utilized extensively in patients with epithelial malignancies, with a relative paucity of data in patients with sarcoma. A phase I study of bevacizumab monotherapy included eight patients with sarcoma (40). A phase Ib trial of bevacizumab in combination with three separate chemotherapy regimens included a total of three patients with sarcoma (41). Two of these patients received doxorubicin combined with bevacizumab, and the third patient received carboplatin and paclitaxel combined with bevacizumab. None of the patients with sarcoma on these two trials responded to treatment (40, 41).

Another study formally evaluated the efficacy of bevacizumab combined with doxorubicin in patients with metastatic soft tissue sarcoma (42). Patients on this phase II trial had not previously received anthracycline-based chemotherapy. The 17 patients on study received bevacizumab and doxorubicin once every 3 weeks. Two patients achieved a partial response, both of whom had uterine leiomyosarcomas. No other patients responded, resulting in a 12% response rate. Eleven patients had disease stabilization for at least 3 months. An increased incidence of cardiac toxicity with this regimen may limit its utility (42). The combination of ifosfamide, carboplatin, and etoposide with bevacizumab is being evaluated for patients with refractory sarcoma, with final results not yet available (43). Bevacizumab will be combined with topotecan and cyclophosphamide in an upcoming Children's Oncology Group phase II trial for patients with Ewing sarcoma.

A bioengineered compound known as VEGF Trap has been tested clinically. VEGF Trap consists of portions of VEGFR-1 and VEGFR-2 fused to the Fc portion of human IgG. This compound avidly binds VEGF121 and VEGF165 in vitro (44). Preliminary reports of a phase I trial with this drug include one patient with uterine leiomyosarcoma who had a minor response to treatment (45). Additional experience with this drug in patients with sarcoma has not yet been reported.

Seven patients with sarcoma and four patients with Kaposi's sarcoma were included in a phase I trial of an intravenous antisense oligonucleotide that has been shown to decrease VEGF protein expression (VEGF-AS) (46). One patient with refractory AIDS-associated Kaposi's sarcoma had a complete remission for 4 months at the lowest dose level on the study. Another patient with refractory chondrosarcoma had stabilization of disease with this agent. A phase II trial of this drug in patients with Kaposi's sarcoma is in progress.

A synthetic ribozyme that specifically degrades VEGFR-1 mRNA has been tested in a phase I clinical trial (47). This drug, angiozyme, was administered subcutaneously daily to a group of patients with refractory solid tumors, including three patients with sarcoma. None of the patients with sarcoma responded to this treatment. One patient with hemangioendothelioma and another patient with endometrial sarcoma had stabilization of disease for at least 6 months (47).

A variety of small molecule VEGF receptor tyrosine kinase inhibitors have been developed. SU5416 primarily inhibits VEGFR-2 (10). This drug has undergone extensive clinical testing, including specific trials in patients with sarcoma. In one

trial that included two patients with sarcoma, one patient with angiosarcoma of the bone attained disease stabilization for approximately 11 months (48). Another general phase I trial included 11 patients with sarcoma (49). One patient on this trial with hemangioendothelioma had disease stabilization for at least 12 weeks.

Two phase II trials of SU5416 in patients with refractory soft tissue sarcoma have been reported with mixed results. In one trial of 26 patients evaluable for response, one patient had a mixed response and five patients had stabilization of disease (50). The median time to progression on this trial was 60 days. A second phase II trial included 10 patients evaluable for response (51). No patients responded, and the median time to progression was 1.8 months, with a 0% progression-free survival rate at 6 months. Pre- and post-treatment biopsies on this study failed to demonstrate a decrease in VEGFR-2 phosphorylation with SU5416 treatment at the recommended phase II dose (51). SU5416 has also been studied in patients with AIDS-associated Kaposi's sarcoma although detailed response data have not yet been published. SU5416 is no longer being developed.

Combined small molecule tyrosine kinase inhibitors of VEGF and PDGF receptors have also been developed and used clinically. SU6668 belongs to this class of compounds with VEGFR-2 and PDGFR- β inhibitory activity. Two phase I trials of oral SU6668 included a combined total of 12 patients with sarcoma (52, 53). Three of these patients experienced prolonged disease stabilization, including patients with GIST, hemangioendothelioma, and leiomyosarcoma. Despite a favorable toxicity profile, this drug has not been developed further due to unfavorable pharmacokinetic characteristics.

SU11248 (sunitinib) inhibits multiple receptor tyrosine kinases, including VEGFR-2, PDGFR- α , PDGFR- β , KIT, and flt3. This oral drug exhibits more favorable pharmacokinetics than SU5416 or SU6668 and is generally well tolerated (54). SU11248 has been most extensively studied in patients with GIST, with much less experience in patients with other types of sarcoma.

SU11248 has been shown to be effective in the treatment of GISTS that are resistant to treatment with imatinib. An initial phase I study of SU11248 in this patient population reported that 61% of tumors responded or stabilized for at least 4 months (55). A follow-up phase II study of SU11248 in imatinib-resistant GIST found that 8% of patients responded and 58% of patients had stabilization of disease for at least 6 months (56). A randomized, placebo-controlled phase III study in patients with imatinib-resistant GIST confirmed the efficacy of SU11248 in this population (57). This trial was stopped at the first planned interim analysis due to a statistically significant improvement in time to progression and overall survival with SU11248. SU11248 appears to benefit approximately half of patients without detectable KIT or PDGFR- α mutations (56). Response of these tumors to SU11248 in the absence of KIT or PDGFR- α mutations suggests a role for VEGFR and PDGFR- β inhibition in inducing the observed treatment effect. Compared to patients who did not benefit from SU11248, biopsy samples from patients with GIST who benefited from SU11248 showed decreased PDGFR- β phosphorylation, but also an increase in both tumor cell and endothelial cell apoptosis (58). The relative contribution of SU11248 on tumor cells as opposed to tumor-associated endothelial cells is not known.

Fewer patients with other types of sarcoma have been treated with SU11248. In one phase I trial, patients with refractory solid tumors were treated with SU11248 once daily for 4 weeks in 6-week cycles (59). A preliminary report of this trial indicated

that the two patients with sarcoma treated on this trial received SU11248 for at least 15 weeks before stopping the drug. Neither patient responded. Another phase I trial with this same dosing regimen included two patients with angiosarcoma, neither of whom responded or benefited from stabilization of tumor growth (54). Further studies evaluating the efficacy of SU11248 are ongoing.

Several other small molecule VEGF receptor tyrosine kinase inhibitors have been tested in clinical trials, though not all have been tested in patients with sarcoma. BAY 43-9006 (sorafenib) has activity against the VEGFR-2 and VEGFR-3, as well as PDGFR- β and Raf kinase (60). Several phase I trials have included patients with advanced solid tumors including sarcomas, but specific outcomes for patients with sarcoma have not yet been detailed. AZD2171 is a small molecule inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR- α , PDGFR- β , and FGFR-1. Preliminary results with this drug have included stabilization of disease in one patient with an ovarian sarcoma (61). PTK787/ZK222584 (vatalanib) has affinity for VEGFR-1, VEGFR-2, VEGFR-3, PDGFR- β , and KIT. A phase I trial of this drug showed a partial response in a patient with refractory GIST who was among the four patients with sarcoma treated (62).

3.2. Endogenous Angiogenesis Inhibitors and Their Analogs

A recombinant human endostatin has been produced for clinical use. In one of the initial phase I studies with this compound, one patient with fibrosarcoma had stable disease in the setting of recently progressive tumor (63). Another phase I study evaluated higher dose levels of daily intravenous endostatin and included five patients with sarcoma (64). In this trial, one patient with synovial sarcoma had a mixed response in which one lesion decreased in size by 60% while another lesion progressed despite treatment. A contemporaneous phase I trial treated four patients with sarcoma with daily intravenous endostatin, none of whom responded (65). To evaluate the effects of continuous exposure to endostatin, patients in another phase I trial received endostatin continuously for 28 days followed by twice daily subcutaneous dosing (66). Of the four patients with sarcoma treated, one patient with hemangiopericytoma attained prolonged disease stabilization with this regimen.

ABT-510 is a peptide that includes the active region of thrombospondin-1 although engineered to have increased antiangiogenic activity. In initial phase I testing of this compound, patients with refractory solid tumors received ABT-510 subcutaneously for 28-day cycles (67). This trial included six patients with sarcoma, of whom one patient with angiosarcoma and one patient with myxoid chondrosarcoma experienced stabilization of disease (67). Another phase I trial reported a partial response in one patient with a soft tissue sarcoma who received ABT-510 (68). A phase I trial combining ABT-510 with 5-fluoruracil and leucovorin included one patient with synovial sarcoma (69). This patient did not respond. Based on these results, a phase II study of ABT-510 in patients with advanced soft tissue sarcoma was conducted (70). Preliminary results of this trial demonstrate a 6-month progression-free survival rate of approximately 35%, which is greater than that observed in historical controls. No patients attained a partial or complete response (70). Further trials will be necessary to confirm the utility of this drug in patients with sarcoma.

3.3. Vascular Targeting Agents

Vascular targeting agents form a class of compounds that decrease tumor blood supply by disrupting existing tumor vascular structures. Combretastatin A4 was the first vascular targeting agent studied clinically. This drug shares homology with colchicine and binds to tubulin. Preclinical studies demonstrated effective reduction in tumor blood flow as well as relatively selective cytotoxicity for stimulated endothelial cells compared to quiescent endothelial cells (71). Several phase I trials of this drug have been performed in patients with refractory solid tumors. Tumor-associated pain has been a common toxicity reported in each of these trials (72–74). In the first trial, patients received combretastatin A4 intravenously once every 21 days (74). Three patients with sarcoma were treated on this regimen, and none of these patients achieved disease response or stabilization. In the second trial, patients received combretastatin A4 intravenously once weekly for 3 out of every 4 weeks (72). None of the seven patients with sarcoma responded. A third trial treated patients with combretastatin A4 intravenously once daily for 5 days every 3 weeks (73). Of the nine patients treated on this schedule, one patient with fibrosarcoma had a partial response lasting eight cycles.

3.4. Other Agents

TNP-470 was one of the first antiangiogenic compounds tested clinically. A phase I trial treated patients with TNP-470 intravenously once weekly (75). This trial included 12 patients with sarcoma, none of whom responded to TNP-470. TNP-470 combined with either paclitaxel or paclitaxel plus carboplatin did not benefit the three patients with sarcoma included in follow-up phase I studies of combined therapy (76,77). TNP-470 has also been evaluated in the treatment of AIDS-associated Kaposi's sarcoma with more promising results. Seven of 39 patients with AIDS-associated Kaposi's sarcoma treated on a phase I trial of TNP-470 monotherapy achieved a partial response at a median of 4 weeks from treatment initiation (78). An additional five patients had stabilization of their disease on treatment.

Thalidomide has been shown to have antiangiogenic properties although its precise mechanism of action remains unclear (79). Several groups have evaluated the antitumor effects of thalidomide, including some trials that included patients with sarcoma. A phase II trial evaluated thalidomide monotherapy in women with uterine sarcoma, including carcinosarcoma and leiomyosarcoma (80). This group of patients did not benefit from thalidomide therapy. Thalidomide monotherapy has had more promising results in the treatment of AIDS-associated Kaposi's sarcoma. One phase II study treated patients with a fixed dose of thalidomide for 8 weeks and reported a 35% partial response rate (81). Another phase II trial allowed thalidomide dose escalation based on individual patient tolerability and treated patients daily until disease progression or other off-therapy indication (82). In this trial, 47% of the evaluable patients had a partial response after a median of 8 weeks of treatment (Fig. 3). An additional two patients had disease stabilization.

Two reports describe thalidomide in combination with other agents. One pediatric phase I trial evaluated thalidomide combined with carboplatin in patients with a range of refractory solid tumors (83). This study included two patients with osteosarcoma, two patients with Ewing sarcoma, and one patient with angiosarcoma. None of these patients responded. In a separate case report, one patient with refractory metastatic osteosarcoma

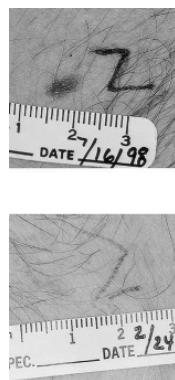


Fig. 3. AIDS-associated Kaposi's sarcoma cutaneous lesion at baseline (top) and after 32 weeks of thalidomide therapy (bottom). Reprinted with permission (82). (Please see color insert.)

received thalidomide and celecoxib as an alternative to conventional chemotherapy (84). This patient had a complete response in his biopsy-proven pulmonary metastases, a remission that has persisted for at least 1 year.

Another emerging class of agents for patients with sarcoma is the family of mTOR (mammalian target of rapamycin) inhibitors. In addition to their known immunosuppressive properties, mTOR inhibitors appear to also have antiangiogenic effects (85). Initial clinical success with mTOR inhibition for sarcoma was reported in patients with transplantation-associated Kaposi's sarcoma, in whom a change in immunosuppression to rapamycin resulted in regression of their lesions (86). A rapamycin analog, CCI-779, has been developed specifically as an anticancer agent. Preliminary results have demonstrated stabilization of disease for some patients with soft tissue sarcoma (87,88). Another mTOR inhibitor, AP23573, showed similar promise in phase I testing (89). This agent has been formally tested in patients with a range of sarcomas, and preliminary results have been favorable (90).

Squalamine is a shark-derived steroid with antiangiogenic properties. Two phase I trials of this drug have been reported, including seven patients with refractory sarcoma. In both trials, patients received continuous infusion of the drug over 5 days. One trial did not provide detailed response data (91). The other trial reported a single patient with synovial sarcoma who had a partial response although this patient received concomitant radiotherapy (92).

A synthetic antiangiogenic dipeptide known as IM862 has had mixed results in patients with AIDS-associated Kaposi's sarcoma. In an initial trial of intranasal IM862, 37% of patients had a complete or partial response (93). The five patients in this trial with complete responses had durable remissions. A follow-up-randomized placebo-controlled trial showed a response rate for intranasal IM862 of 23% compared to a response rate of 21% for placebo (94). No patients on this trial attained a complete response. In addition, the time to disease progression was significantly shorter for patients who received IM862 compared to placebo-treated patients (94). Use of this agent in other types of sarcoma has not been reported.

MEDI-523 (vitaxin) is a humanized monoclonal antibody directed against integrin $\alpha_v\beta_3$. Two trials of MEDI-523 have included patients with sarcoma. One phase I

trial in patients with refractory solid tumors included two patients with sarcoma (95). One patient with non-uterine leiomyosarcoma had a partial response, and this patient received 93 weeks of treatment before his tumor progressed. The other patient with sarcoma did not respond but had stable disease for an unspecified period of time. A follow-up pilot study in patients with leiomyosarcoma found no responses although five patients had brief disease stabilization (96). A derivative of MEDI-523 known as MEDI-522 has increased affinity for $\alpha_v\beta_3$. In a phase I trial of MEDI-522, both patients with sarcoma who were evaluable for response experienced progressive disease (97).

3.5. Metronomic Chemotherapy

The use of conventional cytotoxic chemotherapy at lower doses over an extended period of time has been shown to effectively target tumor angiogenesis (98). Several groups have evaluated this strategy in patients, with some emerging experience in sarcoma. One group has utilized an oral alkylating agent, trofosfamide, given on a metronomic schedule in combination with two oral drugs with antiinflammatory and antiangiogenic properties, rofecoxib and pioglitazone (99,100). In one trial, five patients with angiosarcoma and one patient with hemangioendothelioma received this regimen until disease progression (100). Four patients benefited from this regimen, with two complete responses, one partial response, and one patient with prolonged stable disease. This well-tolerated regimen was then evaluated in patients with progressive soft tissue sarcoma (99). Seven of 21 patients with sarcoma benefited from therapy, including three complete responses in patients with angiosarcoma. One patient with leiomyosarcoma achieved a partial response, whereas one patient each with fibrosarcoma, liposarcoma, and hemangiopericytoma had stable disease for more than 6 months on therapy (99,100).

A pediatric trial has evaluated another combination regimen that utilizes metronomic chemotherapy. In this trial, children with refractory solid tumors received daily thalidomide and celecoxib together with daily oral etoposide alternating every 3 weeks with daily oral cyclophosphamide (101). Four patients with osteosarcoma, two patients with rhabdomyosarcoma, and one patient with Ewing sarcoma received this regimen. None of these patients responded radiographically although one patient with osteosarcoma had stable disease for more than 6 months on treatment. For half of all of the patients on this study, the time to progression with this regimen was greater than with their previous therapy (101). The strategy of metronomic chemotherapy will require further evaluation in patients with sarcoma.

4. CONCLUSION

Sarcomas, like all solid cancers, depend on angiogenesis for tumor growth. In many subtypes of sarcoma, the same growth factors that drive angiogenesis also drive tumor cell growth. Prominent examples of this shared signaling include VEGF signaling in angiosarcoma and Kaposi's sarcoma and PDGF signaling in GIST.

An improved understanding of the importance of angiogenesis-related signaling in sarcomas has allowed for the rational use of antiangiogenic therapies in these patients. This strategy has been used most successfully in patients with GIST, and Kaposi's sarcoma. Additional encouraging results from phase I studies that have included patients with sarcoma should promote formal phase II evaluations of these agents in patients

with specific subtypes of sarcoma. Future trials in patients with sarcoma should be guided by these preliminary results as well as by ongoing biological insights into the growth of these tumors.

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SUMMARY

Advanced colorectal cancer was the first human malignancy in which inhibition of angiogenesis by targeting vascular endothelial growth factor (VEGF) led to a significant survival benefit in a randomized phase III trial. Since then, bevacizumab, a monoclonal antibody against VEGF, has become a standard component of palliative medical therapy in this disease. Various other angiogenesis inhibitors, in particular, inhibitors of VEGF-receptor kinases are currently undergoing testing in clinical trials, either as single agents or in combination with chemotherapy or other targeted agents. In addition, clinical research is currently focusing on the use of anti-VEGF-therapy in the adjuvant and neoadjuvant setting, as well as its usefulness as maintenance therapy to stabilize responses achieved with conventional cytotoxic chemotherapy.

Key Words: colorectal cancer; chemotherapy; bevacizumab; VEGF; EGFR

1. INTRODUCTION

Until recently, systemic treatment of colorectal cancer was synonymous with the use of conventional chemotherapy. Three cytotoxic agents, fluoropyrimidines [5-fluorouracil (5-FU) and oral 5-FU prodrugs], irinotecan, and oxaliplatin, formed the total arsenal of therapeutic options. Although the inclusion of oxaliplatin and irinotecan in standard treatment strategies for advanced colorectal cancer did lead to significant improvements in outcome, a pooled analysis predicted that even if patients received all three active conventional agents in the course of their therapy, median overall survival (OS) in phase III trials was unlikely to exceed 2 years (1). This notion was supported by a recently presented trial that utilized a combination of 5-FU, oxaliplatin, and irinotecan (“FOLFOXIRI”) as first-line therapy for metastatic colorectal cancer demonstrating high antitumor activity of this aggressive regimen [response rate (RR) = 60%] and—as expected—achieving median OS, which fell short of breaking through the 2-year barrier with reported 22.6 months (2).

With the clear demonstration of efficacy of biologic agents in human malignancies, traditional therapeutic concepts and treatment regimens are currently changing. In particular, in colorectal cancer, the survival benefit observed with the addition of the antivascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab to conventional chemotherapy in the first- and second-line setting has rapidly changed

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the standard of care (3, 4). In addition, other targeted agents such as anti-EGF receptor antibodies have shown proof-of-efficacy in advanced colorectal cancer (5, 6). Hence, this disease, which was long regarded as refractory to systemic treatment approaches, is now leading the way in the development of biologic, targeted agents overall, and our understanding of antiangiogenesis as antitumor therapy in particular. To date, clinically relevant antiangiogenic therapy in colorectal cancer is still synonymous with anti-VEGF therapy although it has also become clear that inhibiting VEGF has biologic effects beyond the postulated antiangiogenic mode of action.

2. BEVACIZUMAB

2.1. *First-Line Therapy*

Colorectal cancer was the first tumor in which evidence for the efficacy of an antiangiogenic strategy was obtained on a phase III level. The pivotal phase III trial, initially reported at ASCO 2003 and later published by Hurwitz et al. (3), demonstrated a significant survival benefit when bevacizumab, a monoclonal humanized antibody against VEGF, was added to the historic standard-of-care in chemotherapy, bolus 5-FU/leucovorin (LV) plus irinotecan (IFL).

One generation of trials before a randomized phase II study gave the first indication that bevacizumab is effective in colorectal cancer (7). In this three-arm phase II trial, about 35 patients per arm were randomized to receive either weekly bolus 5-FU/LV (Roswell Park protocol) or the same chemotherapy with either low dose (5 mg/kg) or high dose (10 mg/kg) of bevacizumab every 2 weeks. A safety analysis raised several flags that required further investigation in subsequent trials. As a class-effect of all VEGF-inhibiting agents, a time-dependent development of grade 3 hypertension was observed in about 10–25% of patients. In addition, a higher incidence of minor bleeding (mainly epistaxis), thrombosis, and proteinuria was apparent. In terms of efficacy, the use of bevacizumab was associated with a higher RR, longer progression-free survival (PFS), and OS. Although the phase II design by definition did not allow for direct, *p* value-based comparisons between treatment arms, one of the interesting findings of this trial was that patients in the low-dose bevacizumab arm appeared to benefit more than patients on high-dose bevacizumab in all three efficacy parameters: RR, PFS, and OS.

This observation was instrumental in the design of the pivotal, placebo-controlled phase III first-line trial (3). At that time, the standard first-line chemotherapy had moved to IFL so that this combination regimen was the logical control arm for the study (8). As no experience existed with an IFL-bevacizumab combination, the experimental arm of the trial, a third study arm consisted of the previously tested bolus 5-FU/LV/bevacizumab combination. Based on the experience in the prior randomized phase II study, a dose of 5 mg/kg every 2 weeks was selected for bevacizumab. The trial enrolled a total of 923 patients with previously untreated metastatic colorectal cancer. After an interim analysis, when IFL/bevacizumab was found to be tolerable, the bolus 5-FU/LV/bevacizumab arm was closed with 110 patients enrolled. A key feature of the trial design was that patients who were randomized to the placebo-control arm did not have the chance to ever receive bevacizumab in subsequent lines, whereas patients who had received bevacizumab as part of their first-line therapy had access to bevacizumab after first-line therapy. Thus, the effect of first-line bevacizumab on tumor progression measured by PFS could be maintained over the whole duration of

the trial as no cross-over was allowed. Primary endpoint of the trial was OS. The addition of bevacizumab to IFL dramatically increased OS from 15.6 to 20.3 months ($p = 0.00004$). This effect was paralleled by the same incremental increase in PFS (6.2 vs. 10.6 months, $p < 0.00001$). Interestingly, in view of the impressive magnitude of benefit observed for OS and PFS, the effect of bevacizumab on RR was apparent and statistically significant but rather moderate (35 vs. 45%, $p = 0.0036$). It is intriguing to speculate whether this dissociation of RR and PFS/OS could be an expression of the mechanism of action of bevacizumab as a more cytostatic-antiangiogenic rather than cytotoxic agent.

Most recently, an analysis of the pivotal trial showed that patients who were classified as “non-responders” (i.e., patients who did not have a partial or complete response according to RECIST criteria) benefited from the addition of bevacizumab to IFL in the same magnitude as patients who were classified as “responders” (9). Although it is premature to regard this finding as definitive evidence for the main mechanism of action of bevacizumab as a response-independent inhibition of tumor growth through antiangiogenesis, it clearly demonstrates that traditional response criteria are not able to characterize the full clinical benefit associated with bevacizumab. Based on these considerations, PFS appears to be the best parameter to evaluate the efficacy of bevacizumab and presumably of other antiangiogenic agents, at least in colorectal cancer.

In terms of safety, most of the concerns raised in the initial phase II trial could not be confirmed. Although the addition of bevacizumab to IFL led to an overall increase in grade 3 or 4 adverse events (84.9 vs. 74.0% of patients), these data were not adjusted for the longer duration of therapy on the bevacizumab arm. No increase in venous thrombosis or pulmonary embolism was noted, and the rate of severe bleeding was almost identical. Arterial hypertension of any grade was significantly more frequent in the IFL/bevacizumab arm compared with IFL alone (22.4 vs. 8.3%, $p < 0.01$), with grade 3 hypertension affecting 11.0% of patients. Six patients in the IFL/bevacizumab arm (1.5%) experienced gastrointestinal perforations, an unexpected finding for which a definitive pathomechanism still has to be determined.

It was noted earlier that the pivotal first-line phase III trial was initially designed as a three-arm study with a bolus 5-FU/LV/bevacizumab arm as a safeguard in case unexpected toxicities had been observed in patients receiving IFL/bevacizumab. Before an interim analysis confirmed acceptable safety for IFL/bevacizumab, the 5-FU/LV/bevacizumab arm was discontinued, 313 patients had been concurrently randomized to the three original study arms. Interestingly, within this patient population, all efficacy parameters showed a trend toward superiority for 5-FU/LV/bevacizumab compared with IFL (OS = 18.3 vs. 15.1 months, PFS = 8.8 vs. 6.8 months, RR = 40.0 vs. 37%, median duration of response = 8.5 vs. 7.2 months) (10). Although none of these differences reached statistical significance, conceivably due to the relatively small number of patients in the analysis, it demonstrated the high efficacy of the 5-FU/LV/bevacizumab combination, even when compared with the old standard of care of conventional chemotherapy, IFL. In fact, it is intriguing to speculate that an adequately powered trial would have shown that bevacizumab adds more efficacy to first-line bolus 5-FU/LV than irinotecan.

The activity of this regimen was further highlighted by the results of a randomized phase II trial, which enrolled patients who were not considered optimal candidates

for an irinotecan-based first-line therapy of advanced colorectal cancer (11). A total of 209 patients were randomized to receive either bolus 5-FU/LV (Roswell Park) or bolus 5-FU/LV/bevacizumab. Patients were eligible for this trial if they met at least one of the following criteria: age \geq 65 years, ECOG PS 1 or 2, serum albumin \leq 3.5 g/dL, or prior abdominal/pelvic radiotherapy. Again, the addition of bevacizumab was associated with improvements of all efficacy parameters with significant prolongation of PFS (9.2 vs. 5.5 months, $p = 0.002$) and trends toward superiority for OS (16.6 vs. 12.9 months), RR (26.0 vs. 15.2%), and duration of response (9.2 vs. 6.8 months).

Because three independently conducted studies used the same 5-FU/LV/bevacizumab regimen as first-line therapy of advanced colorectal cancer, a pooled analysis of this treatment arm was conducted, which combined individual patient data from these three trials ($n = 249$) in comparison with the respective combined control group of the trials that received either 5-FU/LV or IFL ($n = 241$) (12). Although the inclusion of patients with IFL as control arm conceivably biased the analysis in favor of the non-bevacizumab arm, a significant survival benefit was found for 5-FU/LV/bevacizumab over the combined control arms (17.9 vs. 14.6 months, $p = 0.008$). Improvements in PFS and RR were also significant (PFS = 8.8 vs. 5.6 months, $p \leq 0.0001$; RR = 34.1 vs. 24.5%, $p = 0.019$). These results confirm that 5-FU/LV/bevacizumab is a valid treatment alternative for patients who are not able to tolerate irinotecan- or oxaliplatin-based chemotherapy.

Based on the pivotal trial published by Hurwitz et al., bevacizumab was approved by the FDA in February 2004 as a component of intravenous 5-FU-based first-line chemotherapy for advanced colorectal cancer. It is of interest that in clinical practice, bevacizumab was predominantly used in combination with 5-FU/LV plus oxaliplatin (FOLFOX), even in the absence of any phase III data for this combination (Table 1 and Fig. 1). This is presumably based on the fact that the oncologic community was convinced of the superiority of FOLFOX over IFL in view of the results of Intergroup

Table 1
Studies on Chemotherapy with or without Bevacizumab in Advanced Colorectal Cancer

| <i>Control arm</i> | <i>N Pts</i> | | <i>RR (%)</i> | | <i>PFS (months)</i> | | <i>OS (mos)</i> | |
|----------------------------|--------------|-------------|---------------|-------------|---------------------|-------------|-------------------|-------------------|
| | <i>-BEV</i> | <i>+BEV</i> | <i>-BEV</i> | <i>+BEV</i> | <i>-BEV</i> | <i>+BEV</i> | <i>-BEV</i> | <i>+BEV</i> |
| First-line therapy | | | | | | | | |
| Kabbinavar | 5-FU/LV | 105 | 104 | 15 | 26 ^a | 5.5 | 9.2 ^a | 12.9 |
| Hurwitz | IFL | 41 | 402 | 35 | 45 ^a | 6.2 | 10.6 ^a | 15.6 |
| Cassidy | FOLFOX4 | 351 | 350 | N/A | N/A | 8.6 | 9.4 ^a | N/A |
| | XELOX | 350 | 350 | N/A | N/A | 7.4 | 9.3 | N/A |
| Fuchs ^b | FOLFIRI | 144 | 57 | 47 | 54 | 7.6 | 9.9 | 23.1 |
| Hochster ^b | mFOLFOX6 | 49 | 71 | 43 | 53 | 8.7 | 9.9 | 19.2 |
| | XELOX | 48 | 72 | 35 | 48 | 5.9 | 10.7 | 17.2 |
| Second-line therapy | | | | | | | | |
| Giantonio | FOLFOX4 | 290 | 289 | 9 | 22 ^a | 4.8 | 7.2 ^a | 10.8 |
| | | | | | | | | 12.9 ^a |

^a Statistically significant.

^b No randomized comparison.

N/A, not available; BEV, bevacizumab.

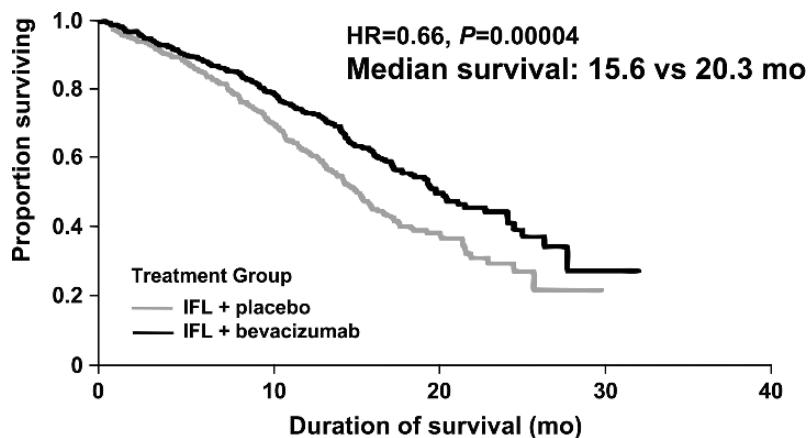


Fig. 1. Phase III trial of bevacizumab in metastatic colorectal cancer: survival. Reproduced with permission from (3).

trial N9741 (13) and thus wanted to combine bevacizumab with what was regarded the most effective chemotherapy backbone. However, definitive proof that bevacizumab can enhance the efficacy of FOLFOX and not only of 5-FU/LV or IFL came not before the results of the second-line phase III trial E3200 became available (see section 2.2) (4). More recently, the results of the sequential TREE trials and the Roche-sponsored phase III trial NO16966 also demonstrated the value of the addition of bevacizumab to oxaliplatin-based first-line therapy in colorectal cancer (14).

TREE-1 investigated the best fluoropyrimidines backbone in combination with oxaliplatin and randomized patients to modified FOLFOX6, bolus 5-FU/LV/oxaliplatin (bFOL), or CAPOX. The FDA approval of bevacizumab warranted a trial amendment so that for the TREE-2 cohort bevacizumab was added to all three arms. It is of note that in view of excess toxicity associated with CAPOX at its original dose of capecitabine 1000 mg/m² BID days 1–14, for TREE-2 the capecitabine dose was reduced to 850 mg/m² BID days 1–14 of the 3-weekly regimen. Reduced-dose CAPOX plus bevacizumab was subsequently found to be tolerable, and no clinically relevant differences in the side-effect profile between the three treatment arms in TREE-2 was noted. In cross-trial comparison (TREE-1 and TREE-2), the addition of bevacizumab appeared to enhance the efficacy of each individual regimen in terms of RR, TTP, and OS. Overall, bFOL was found to be the least effective regimen with CAPOX + BEV and FOLFOX + BEV resulting in similar RRs, time to tumor progression (TTP), and OS. In a pooled analysis of patients enrolled in all arms, median OS of patients in TREE-1 was 18.2 months compared with 24.4 months in TREE-2 after the addition of bevacizumab.

Interestingly, time to treatment failure, a composite endpoint of toxicity and efficacy, was not different between TREE-1 and TREE-2, conceivably due to the dose-limiting toxicity of oxaliplatin, sensory neuropathy, which did not allow patients to remain on continued oxaliplatin-containing therapy for more than 6 months. This finding underscores the clinical need to develop strategies to prevent or ameliorate oxaliplatin-induced neurotoxicity, in particular, in the palliative setting.

This point has gained particular importance in view of the results of the Roche-sponsored phase III trial, NO16966, the first phase III trial investigating the addition of bevacizumab or placebo to an oxaliplatin-based first-line regimen, XELOX (capecitabine/oxaliplatin) or FOLFOX4 (15). This trial included a 2×2 study design component that enrolled 1401 patients. As one of the key results, XELOX was found not to be inferior to FOLFOX4 in terms of the primary endpoint of the trial, PFS. The addition of bevacizumab to both oxaliplatin arms combined did significantly prolong PFS from 8.0 to 9.4 months, but the hazard ratio of 0.83 ($p = 0.0023$) demonstrated a lower benefit than expected from the prior irinotecan- and 5-FU-based trials. In fact, in a subgroup analysis, only patients treated with XELOX enjoyed a significant increase in PFS when bevacizumab was added (7.4 vs. 9.3 months, HR = 0.77, $p = 0.0026$) but not patients on FOLFOX4 (8.6 vs. 9.4 months, HR = 0.89, $p = 0.187$). One explanation for the difference between the bevacizumab effect on XELOX and FOLFOX4 may be found in an apparent imbalance of patients with prior adjuvant chemotherapy between both arms. The overall rather moderate effect of bevacizumab on PFS in both oxaliplatin-containing arms, however, is most likely due to the fact that patients were likely to stop bevacizumab at the same time when oxaliplatin was discontinued because of cumulative neurotoxicity and other side-effects. Thus, the postulated inhibitory effect of bevacizumab on tumor progression could not be fully utilized as the drug was commonly discontinued prior to progression. As a side-note, the addition of bevacizumab did not significantly affect the side-effect profile of XELOX and FOLFOX4, with only a slight increase in grade 3/4 hypertension and no effect on 60-day or overall mortality. The rate of gastrointestinal perforations was 0.6%.

In view of the problems combining oxaliplatin-based regimens, which are invariably associated with issues surrounding cumulative toxicity, with bevacizumab, irinotecan-containing protocols could emerge as more obvious and easier to handle chemotherapy backbones for the addition of bevacizumab. This approach was recently underscored by the BICC-C trial, which was initially designed to compare the efficacy and toxicity of three regimens of irinotecan in combination with different fluoropyrimidine backbones, infusional/bolus 5-FU (FOLFIRI), bolus 5-FU (modified IFL), and capecitabine (CAPIRI) as first-line therapy of advanced colorectal cancer (16). The trial was originally supposed to enroll a total of 1000 patients, but when bevacizumab became approved in the USA, the trial was amended and the statistical assumptions recalculated. Eventually, 430 patients were randomized to the original three arms with PFS as primary endpoint. A second accrual period then saw the addition of bevacizumab to FOLFIRI and mIFL in form of a randomized phase II trial with a total of 117 patients. It is of note that the three-weekly CAPIRI regimen used a relatively high dose of capecitabine ($1000 \text{ mg/m}^2 \text{ BID days } 1-14$) and irinotecan ($250 \text{ mg/m}^2 \text{ BID day } 1$) without upfront dose reductions for elderly patients. BICC-C clearly demonstrated that FOLFIRI is superior to mIFL and also CAPIRI (in the dose and schedule tested) in terms of efficacy and tolerability. In cross-trial (period 1 and 2) comparison, the addition of bevacizumab increased the efficacy of FOLFIRI and mIFL without clinically relevant increase in serious side-effects. The data established FOLFIRI plus bevacizumab as one of the standards of care in front-line therapy of colorectal cancer.

2.2. Second-Line Therapy

The ECOG trial E3200 randomized 822 patients with advanced colorectal cancer that had failed first-line therapy (mainly IFL) into three different arms: FOLFOX4 (control arm), FOLFOX4 plus high-dose bevacizumab (10 mg/kg every 2 weeks), and a high-dose bevacizumab monotherapy arm (4). The rationale for using a higher dose of bevacizumab compared with previous phase II and III trials in colorectal cancer was mainly based on preclinical findings of a dose-dependent effect of bevacizumab. Primary endpoint of this second-line trial was OS with an 88% power to detect a 40% improvement with the addition of bevacizumab to FOLFOX4. An interim analysis had previously shown that the bevacizumab monotherapy arm was inferior to FOLFOX4 so that this arm was closed prematurely. However, at that time, almost all projected patients had already been enrolled so that valid data were available for the bevacizumab monotherapy arm.

At ASCO 2005, the final results of the second-line trial E3200 were presented, which showed that bevacizumab substantially enhanced the activity of FOLFOX. In terms of OS (12.9 vs. 10.8 vs. 10.2 months), PFS (7.2 vs. 4.8 vs. 2.7 months), and RR (21.8 vs. 9.0 vs. 3.0%), FOLFOX plus bevacizumab was clearly superior to FOLFOX and bevacizumab alone.

The toxicity analysis did not reveal any unexpected side-effects. Grade 3/4 hypertension, a characteristic effect of all anti-VEGF agents, affected 6% of patients in the FOLFOX + bevacizumab group, and bowel perforation occurred in 1% of these patients. No increased risk for venous or arterial thrombotic events was noted with the addition of bevacizumab to FOLFOX4. Grade 3/4 sensory neuropathy was significantly more frequent in the FOLFOX/bevacizumab arm (16 vs. 9%, $p = 0.016$), conceivably due to a longer time on therapy with a consequently higher cumulative oxaliplatin dose in patients on bevacizumab. The longer time on therapy might also explain the higher rate of severe nausea (10 vs. 5%) and vomiting (9 vs. 4%) noted in patients on FOLFOX/bevacizumab compared with FOLFOX alone.

E3200 provides the first proof in a phase III setting that bevacizumab adds significant efficacy to an oxaliplatin-based regimen after previous trials had shown significant benefit for patients treated with either 5-FU/LV alone or with an irinotecan combination. This finding is particularly important as even before the results of E3200 were released, FOLFOX plus bevacizumab had already emerged as the most commonly used bevacizumab-containing combination regimen in first-line treatment in the USA. Interestingly, bevacizumab monotherapy was found to have only minimal activity, which supports the notion that this antiVEGF antibody should always be combined with another directly tumor-directed agent, at least in the palliative setting.

2.3. Salvage Therapy

Bevacizumab does not only add efficacy to conventional chemotherapy in advanced colorectal cancer but apparently also to tumor-directed, targeted agents such as cetuximab. This point was illustrated by the results of the so-called BOND-2 trial (17). The trial's logical predecessor, BOND-1, was a European-randomized phase II trial conducted in patients with advanced colorectal cancer who had failed irinotecan-based therapy and in more than 60% also oxaliplatin-based therapy (5). In BOND-1, patients were randomized to receive either cetuximab or cetuximab plus irinotecan as

salvage therapy. The results of BOND-1 firmly established the efficacy of cetuximab in advanced colorectal cancer, confirming the finding of previous sequential phase II trials conducted in the USA and leading to the FDA approval of cetuximab in February 2004. BOND-2 now investigated the effect of the addition on bevacizumab to the BOND-1 design. Again, patients with advanced colorectal cancer who had all failed irinotecan-based therapy, more than 85% of which had also been pretreated with oxaliplatin, were enrolled in a randomized phase II trial comparing cetuximab plus bevacizumab (CB) versus cetuximab/bevacizumab plus irinotecan (CBI) as salvage therapy. Primary objective of the trial was to document the feasibility of the dual-antibody combinations and to assess the RR and TTP in both arms. The implications of the efficacy results recorded in this trial can only be fully understood in comparison with the results of BOND-1. Although cross-trial, historic comparisons are obviously problematic, the addition of bevacizumab appeared to enhance the efficacy of cetuximab and cetuximab/irinotecan in terms of RR but more strikingly in terms of TTP. This effect is even more noteworthy as cetuximab monotherapy in BOND-1 was only associated with a rather disappointing median TTP of 1.5 months. Combining cetuximab with bevacizumab increased median TTP to 5.6 months. A similar effect was seen in the CBI arm (4.1 vs. 7.9 months). It is noteworthy that a lower bevacizumab dose (5 mg/kg every 2 weeks) was used than in E3200, and still, even in this third line setting, bevacizumab's efficacy, in particular with regard to prolongation of PFS, seemed to be maintained. No unexpected side-effects were noted when these two monoclonal antibodies were combined; a welcome finding in view of the ongoing CALGB/SWOG Intergroup 80405 phase III trial that randomizes patients to receive conventional chemotherapy (dealer's choice: FOLFOX or FOLFIRI) combined with either cetuximab or bevacizumab or both antibodies together as first-line therapy for advanced colorectal cancer.

3. RECTAL CANCER

Rectal cancer is clinically a more challenging tumor than colon cancer as its management routinely involves a multimodality approach right from the very beginning in form of neoadjuvant radiochemotherapy, which has recently evolved as standard of care for stage II and III rectal cancer (18). On the contrary, the neoadjuvant treatment concept provides the opportunity for pathologic and molecular examination of tumor tissue before and after a certain treatment modality. This opportunity was used to study the effect of bevacizumab as component of a multimodality therapeutic approach in six patients with adenocarcinoma of the rectum (19). Patients received one dose of bevacizumab (5 mg/kg) followed 2 weeks later by concurrent administration of bevacizumab with 5-FU and external beam radiation. In addition to conventional pathologic analysis of the resected primary tumor, measurements of interstitial fluid pressure (IFP) and imaging studies to document changes in tumor perfusion were performed. The data clearly showed that blood perfusion and IFP decreased rapidly, within 12 days, after administration of single-agent bevacizumab. At time of resection, a marked decrease in microvessel density (MVD) was noted in all specimen, whereas the permeability-surface area product assessed through FDG-PET was virtually unchanged. This findings appear to support preclinical data that anti-VEGF treatment can lead to decreased IFP and in turn to improved quality of blood perfusion and oxygenation,

a precondition for improved efficacy of radiation (20). Future trials in rectal cancer using bevacizumab or other anti-VEGF/antiangiogenic approaches will surely provide further insight into this intriguing, yet hypothetical mechanism of action.

4. ONGOING CLINICAL TRIALS

4.1. *Palliative Therapy*

As bevacizumab has become a standard component of palliative therapy in advanced colorectal cancer, all ongoing phase III first-line trials in the USA, and most trials in Europe, use a bevacizumab-containing regimen as control arm.

The ongoing Intergroup trial CALGB/SWOG 80405 allows the use of FOLFOX or FOLFIRI (investigator's choice) as chemotherapy backbone and randomizes patients to receive either bevacizumab (control arm) or cetuximab or cetuximab/bevacizumab. The trial is powered to detect an increase in median OS from 22 to 27.5 months for which a total number of about 2300 patients are needed.

A company-sponsored trial with a similar design has recently already finished accrual. The so-called PACCE trial (Panitumumab in Advanced Colorectal Cancer Evaluation) randomized patients in two different cohorts (phase III trial with 800 patients on FOLFOX and phase II trial with 200 patients on FOLFIRI) to receive standard chemotherapy (FOLFOX or FOLFIRI) plus bevacizumab or bevacizumab/panitumumab. Primary endpoint of this trial is PFS. Initial efficacy data can be expected for 2007.

A European trial, DREAM (Dual biologics to increase duration of Response with Erlotinib and Avastin Maintenance, planned accrual of 640 patients), will evaluate the role of bevacizumab with or without erlotinib as maintenance treatment in a stop-and-go strategy with oxaliplatin-containing regimens. Patients will receive FOLFOX plus bevacizumab or XELOX plus bevacizumab, followed by maintenance bevacizumab, and then reintroduction of the initial regimen. In addition, in form of a 2×2 randomization, each arm will receive either erlotinib or placebo during maintenance bevacizumab treatment. This trial will assess the effect that VEGF inhibition from bevacizumab and EGFR inhibition from erlotinib will have on PFS, OS, and duration of disease control.

Although bevacizumab has an established role as combination partner for first- or second-line chemotherapy, it is unclear whether patients would benefit from continuation of this drug beyond progression, meaning switching the chemotherapy backbone of therapy but maintaining bevacizumab. Whether the proposed cytostatic effect of bevacizumab results in clinical benefit past progression of metastatic colorectal cancer is going to be evaluated in a phase III US Intergroup trial. In this trial, the Intergroup Bevacizumab Continuation Trial (SWOG/NCCTG 0600), patients who progress on combination therapy with FOLFOX/BEV (or XELOX/BEV) will be randomized to receive (FOLF) IRI plus cetuximab or (FOLF) IRI plus cetuximab and (10 or 5 mg/kg) bevacizumab. The primary endpoint of the trial is OS.

4.2. *Adjuvant Therapy*

Several phase III trials are currently studying bevacizumab as component of adjuvant therapy in early stage colon and rectal cancer. The convincing results of the MOSAIC

and NSABP C-07 trial have established an oxaliplatin-based regimen (FOLFOX or FLOX) as standard adjuvant treatment in colon cancer and de facto also in rectal cancer, all ongoing trials testing bevacizumab in the adjuvant setting in colorectal cancer use FOLFOX as chemotherapy backbone.

NSABP C-08 investigates the activity of bevacizumab added to 6 months of modified FOLFOX6 and continued for another 6 months thereafter in patients with stage II and III colon cancer. The trial completed accrual of 2400 patients in October 2006. Primary endpoint for this, as well as for all other adjuvant trials, is 3-year disease-free survival. The AVANT trial in stage III colon cancer patients, which is mainly conducted outside of the USA, has a similar design but includes a capecitabine/oxaliplatin (XELOX) plus bevacizumab combination as one of the experimental arms to form a three-arm design. Accrual of planned 3400 patients should be completed in early 2007.

The ECOG trial E5202 currently enrolls patients with high-risk stage II colon cancer to 6 months of adjuvant-modified FOLFOX6 with or without the addition of bevacizumab. As in NSABP C-08 and the AVANT trial, the experimental arms continues bevacizumab for another 6 months beyond FOLFOX. The definition of “high-risk stage II” is based on a molecular marker profile (LOH 18q, microsatellite stability—MSS); patients not characterized as high-risk will be observed without adjuvant chemotherapy. It is estimated that about 3600 patients will have to be screened to identify the 1400 patients with molecular high-risk features required for the trial.

In rectal cancer, the ongoing ECOG trial E5204 includes patients with stage II and III disease who have previously received neoadjuvant radiochemotherapy followed by curative surgery. A total of 2100 patients will be randomized to 4 months of modified FOLFOX6 with or without bevacizumab. This is the only ongoing phase III adjuvant trial in colorectal cancer that does not require the continuation of bevacizumab beyond the duration of the chemotherapy component.

5. OTHER ANGIOGENESIS INHIBITORS IN ADVANCED COLORECTAL CANCER

Several other agents targeting the VEGF-signaling pathway have been tested in colorectal cancer. Apart from the decoy receptor VEGF Trap and the anti-VEGF receptor monoclonal antibody IMC-1121b, for which so far very limited experience in colorectal cancer exists, most angiogenesis inhibitors tested in this disease inhibit the VEGF-R tyrosine kinase domain to block VEGF-mediated signaling. These small molecules commonly inhibit kinases beyond VEGF-R2 with varying affinity for VEGF-R1, VEGF-R2, and VEGF-R3 as well as PDGF-R and other kinases. In how far the inhibition of these additional kinases contributes to the observed preclinical and clinical efficacy is not clear.

5.1. Vatalanib (PTK787)

Although bevacizumab's role in colorectal cancer is steadily increasing, a different angiogenesis inhibitor unexpectedly failed to show clear signs of efficacy in advanced colorectal cancer. Vatalanib is an oral tyrosine kinase inhibitor that blocks all known VEGF receptors and also inhibits PDGF-R and c-kit. After encouraging results of phase I/II trials and molecular imaging studies (21–23), vatalanib was evaluated in two placebo-controlled phase III trials in combination with FOLFOX4: as first-line

treatment in 1168 patients with metastatic colorectal cancer (CONFIRM-1) (24) and as second-line therapy in 855 patients with irinotecan-pretreated metastatic colorectal cancer (CONFIRM-2) (25). Data on CONFIRM-1 were presented at ASCO 2005. In this trial, two primary objectives were defined: (i) to achieve a 25% reduction in the risk of progression [hazard ratio (HR) = 0.75] and (ii) to increase 1 year OS from 71 to 76% (HR = 0.80). Although undoubtedly vatalanib-associated toxicities were observed (hypertension, dizziness, and pulmonary embolism), no definitive increase in efficacy could be noted with the addition of vatalanib to FOLFOX4 in terms of independently reviewed PFS (7.7 vs. 7.6 months, HR = 0.88, $p = 0.118$) and RR (42 vs. 46%). Investigator-assessed PFS, however, saw a statistically positive result with a HR of 0.83 ($p = 0.026$). In unplanned, post hoc subgroup analyses, patients with poor prognostic factors (PS1/2 and high LDH) appeared to benefit most from vatalanib. Interestingly, this result was also observed in the second-line CONFIRM-2 trial. Again, investigator-assessed PFS showed an advantage for vatalanib-treated patients (5.6 vs. 4.1 months, HR = 0.83, $p = 0.026$) and a more pronounced effect in patients with high LDH (5.6 vs. 3.8 months, HR = 0.61, $p < 0.001$) (25). A combined analysis of CONFIRM-1 and CONFIRM-2 supported the finding of a differential, beneficial effect of vatalanib in patients with high LDH, which was found in about 30% of all patients with colorectal cancer in both trials (26).

It has to be emphasized that despite the positive results in subgroup analyses, the primary endpoints of the trials (centrally reviewed PFS in CONFIRM-1 and OS in CONFIRM-2) were not met. It is unclear, though, if the once daily dosing schedule was appropriate for an agent with a half-life of 3–6 h and if thus proangiogenic rebound effects could have compromised the overall efficacy. In addition, vatalanib activates its own metabolism over time by inducing the activity of CYP3A4 (27). An analysis of the reasons why this promising agent failed in clinical testing is pertinent in view of the abundance of other small molecules/VEGF-receptor inhibitors that are currently in late-stage development.

5.2. SU5416

In some way, the failure of vatalanib in a phase III trial in advanced colorectal cancer is reminiscent of the fate of a first-generation VEGF-receptor inhibitor, SU5416. SU5416, a competitive inhibitor of the tyrosine kinase domain of VEGF-R2, showed promising results in colorectal cancer and other tumors in phase I and small phase II studies (28, 29). In a phase III setting, however, SU5416 failed to demonstrate efficacy when added to 5-FU/LV in first-line colorectal cancer (30). Subsequently, the development of SU5416 was discontinued in February 2002. Unfortunately, the results of this trial have not yet been published or presented in detail, so that an analysis of reasons for the lack of efficacy of this drug is impossible.

5.3. Sunitinib (SU11248)

Sunitinib is another multitargeted tyrosine-kinase inhibitor that blocks VEGF-mediated signaling. It is currently FDA-approved for the use in renal cell cancer and imatinib-resistant gastrointestinal stromal cell tumors (GIST). A recently presented phase II study in 82 patients with chemotherapy-refractory colorectal cancer demonstrated modest activity of Sunitinib as single agent with TTP of 2.2 months in

bevacizumab-pretreated patients ($n = 42$) and 2.5 months in patients who had not received bevacizumab in prior lines of therapy ($n = 40$) (ASCO 2006, abstract 241) (31). Median OS of patients with prior bevacizumab exposure was rather short with 7.1 months compared with 10.2 months for bevacizumab-naïve patients. In view of the almost identical TTP noted between these groups of patients, the variation in OS could reflect differences in biology of tumors, which had progressed on bevacizumab rather than a differential effect of sunitinib in these patients.

6. CONCLUSION

The treatment of colorectal cancer has entered a new era. Anti-VEGF treatment in the form of the anti-VEGF monoclonal antibody bevacizumab has become an integral part of the standard of care in the management of advanced disease. Studies to evaluate the efficacy of bevacizumab in the adjuvant setting of colon and rectal cancer are ongoing. While to date most trials on antiangiogenesis in colorectal cancer have included bevacizumab, we will soon see an abundance of antiangiogenic agents with proof of efficacy that will be available for testing in phase II and III settings. In conjunction with the development of other biologic agents such as EGF-receptor or cell-cycle inhibitors, the future will conceivably show a further departure from unselective, conventional chemotherapy toward combinations of selective, targeted agents as mainstay of systemic therapy for colorectal cancer. Beyond bevacizumab, several phase II and phase III trials are in various stages of planning and activation, which will test VEGF-receptor inhibitors such as AZD2171, Sunitinib, sorafenib, and AMG706 in first- and second-line setting in advanced colorectal cancer. As colorectal cancer is a very common malignancy in which proof-of-principle for the efficacy of antiangiogenic agents has been established, it appears logical that commercial pharmaceutical interest will continue to drive the development of novel antiangiogenic drugs in this disease. The challenge will be how to best integrate any novel agent into a clinical trial setting and later into clinical practice.

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SUMMARY

Malignant tumors of the central nervous system (CNS) are difficult to cure, despite advances made in the fields of neurosurgery and radiation oncology. These tumors are generally resistant to standard chemotherapeutic agents, and new strategies are needed to overcome these tumors. Tumors of the CNS system demonstrate various features of angiogenesis. Correlation has been made between the degree of increased vascularity and outcome, making these tumors enticing targets for antiangiogenic agents.

Although initial clinical trials of angiogenic inhibitors have been disappointing with regard to improved patient outcome, important advances in utilizing antiangiogenic agents as an additional therapeutic modality for patients with CNS tumors have been identified. Rapid developments in understanding the molecular basis of angiogenesis and brain tumors have been made over the past decade, and application of this knowledge is currently being brought to the clinic. This chapter reviews the current knowledge of angiogenesis as it relates to brain tumors and recent advances that have been made in translating this knowledge into the treatment of patients with CNS tumors.

Key Words: Glioma; glioblastoma; astrocytoma; medulloblastoma; chemotherapy; vasculature.

1. INTRODUCTION

1.1. CNS Tumors

The Central Brain Tumor Registry of the United States (CBTRUS) estimates that over 40,000 new cases of primary malignant and nonmalignant tumors of the CNS will be reported in the United States for the year 2005 (1), and tumors of the CNS remain a leading cause of morbidity and mortality. Gliomas are the most common histologic subtype, and various grades of malignancy exist. High-grade gliomas have a very poor prognosis with an overall 5-year survival for the highest

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grade glioma, glioblastoma, of <5%. Standard treatment for malignant tumors is multimodal, generally involving surgical resection and radiation therapy, sometimes followed by cytotoxic chemotherapy. However, each treatment modality has its limitations. Malignant gliomas are infiltrative and tend to spread along white matter tracts and blood vessels. Locally directed therapies, such as surgical resection and radiation, fail to eradicate all malignant cells, and the tumors tend to recur locally. The addition of systemic chemotherapy has not had a significant impact on overall survival of patients with malignant brain tumors for a variety of reasons, including issues with drug delivery and tumor resistance. New agents with new mechanisms of action are desperately needed.

Malignant gliomas are vascular tumors and high-grade gliomas, particularly glioblastoma, are associated with florid microvascular proliferation. Early studies linked this increased vascularity to glioma growth and spread (2). Subsequent studies correlated microvessel quantification with clinical outcome (3). Angiogenesis in patients with brain tumors, with its subsequent abnormal, compromised vasculature, can also cause peritumoral edema, spontaneous hemorrhage, and hyperemia which can complicate treatment, effect outcome, and impact the quality of life of patients. Angiogenesis is therefore a logical target for brain tumor therapy.

1.2. Blood Vessels in Brain and Brain Tumors

Neural tissue is normally highly vascularized in order to meet the high metabolic demands of the central nervous system (CNS). The vasculature of the CNS is unique in that it is lined by a single layer of highly specialized endothelial cells comprising the blood–brain barrier (BBB), which serves to restrict entry of most hydrophilic and large lipophilic compounds into the brain (4). New vessel formation, or neovascularization, is present during brain development (5) but is repressed in the normal adult brain. Tight regulation of angiogenesis is achieved, as in other areas of the body, by balancing proangiogenic signals with antiangiogenic (inhibitory) mechanisms. For angiogenesis to occur, the balance of proangiogenic and antiangiogenic factors must tip in favor of the proangiogenic factors (termed the “angiogenic switch”) (6). In the brain, this can occur in the presence of ischemia, metabolic abnormalities, and neoplasms (5).

While neovascularization is a feature of many CNS neoplasms, most work on angiogenesis in brain tumors has centered on gliomas because of their higher frequency. However, other tumors, such as primitive neurectodermal tumors/medulloblastomas, which are the most common malignant brain tumors of childhood, have demonstrated marked neovascularization and produce a wide range of angiogenic factors (7). Evidence of increased expression of angiogenic factors has also been demonstrated in pilocytic astrocytomas (8), ependymomas (9), oligodendroglial gliomas (9), and meningiomas (10), among others.

1.3. Angiogenesis in Brain Tumors

The process of new vessel growth in the brain is complex and consists of an interplay amongst a number of growth factors, tumor cells, endothelial cells, and the surrounding basement membranes in which angiogenesis occurs. Several mechanisms of tumor-induced angiogenesis have been elucidated over the past two decades. While the most direct is secretion of cytokines with angiogenic properties by the tumor cells, several

indirect mechanisms also exist, but all depend on the migration and proliferation of endothelial cells and changes in extracellular matrix (ECM) components.

1.3.1. PROANGIOGENIC FACTORS

Several proangiogenic growth factors have been identified in brain tumors. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor due to its effect on vascular permeability (11), has historically been considered the primary angiogenic factor associated with brain tumors. Its expression is stimulated by a variety of mechanisms, including various cytokines, growth factors, and local metabolic derangements, such as hypoglycemia, but hypoxia appears to be its strongest stimulus (12). VEGF mRNA expression is primarily found associated with perinecrotic cells in glioblastoma tissue (13), suggesting that hypoxia is a major stimulus of angiogenesis in these tumors.

VEGF regulates several key processes of the angiogenic cascade, including endothelial cell migration, proliferation, protease expression, and recruitment of endothelial cell precursors (14). Without VEGF, newly formed tumor microvasculature is unstable and the endothelial cells undergo apoptosis (15). VEGF expression correlates with the degree of tumorigenic edema in CNS tumors (16), and, in astrocytomas, VEGF expression correlates with both glioma grade and degree of tumor vascularity(13, 17).

VEGF exerts its effects through three signaling receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4) (18). Activated VEGFR-2 has been shown in several studies to be the major stimulator of angiogenesis (19). The VEGF receptors, VEGFR-1/Flt-1 and VEGFR-2/Flk-1, are not expressed in normal brain endothelium, but, like VEGF, increased levels are found with increasing grades of glioma (20). It is thought that progression of gliomas from low-grade to higher-grade lesions is associated with the induction of VEGF and its receptors (21).

While VEGF is the principal angiogenic factor in gliomas, a number of other growth factors that play key roles in brain tumor angiogenesis have been identified. Fibroblast growth factor (FGF) expression has correlated with tumor progression and prognosis (22). Both acidic (aFGF) and basic (bFGF) FGFs exert their effects via receptor tyrosine kinases and are potent inducers of endothelial cell migration, proliferation, and tube formation *in vitro* and are highly angiogenic *in vivo* (23). FGF also regulates VEGF expression in tumor cells (22). Like VEGF, bFGF levels and expression correlate with microvessel density and tumor grade in gliomas (24). Increased levels of FGF receptors are found in glioma cells and correlate with malignant progression, although whether FGF is acting principally as a tumor autocrine growth factor or an angiogenic factor is not yet clear (24).

Angiopoietins are endothelial growth factors that play a role in the maturation and integrity of new blood vessel formation and have been implicated in glioma angiogenesis. Their receptor, Tie-2, is a tyrosine kinase with immunoglobulin and EGF domains (25). Angiopoietin-1 has been shown to promote blood vessel maturation and counteracts VEGF-induced permeability when chronically administered during vessel formation (26). In endothelial cells, binding of Ang-1 to the Tie-2 receptor leads to activation, while binding of Ang-2 leads to inhibition (27). Ang-1 and Tie-2 are primarily involved in vessel remodeling, maturation, and stabilization (5). Mouse embryos lacking Ang-1 or Tie-2 have endothelial cells that fail to associate with the

ECM and underlying support cells (28), while overexpression results in larger, more numerous, more highly branched vessels (29). In a rat glioma model, overexpression of Ang-1 increased angiogenesis and tumor growth and was associated with an increased number of highly branched vessels (30). Angiopoietin-1, angiopoietin-2, and Tie-2 are upregulated in gliomas (31), and angiopoietin-2 expression correlates with tumor grade (32). Inhibition of Tie-2 signaling interferes with tumor growth and vascularity in animal glioma models, and treatment of mouse xenografts derived from glioma cells with a dominant negative form of Tie-2 resulted in significantly decreased tumor growth (33).

Platelet-derived growth factor (PDGF) is produced by a variety of cells and has a number of mechanisms of action, including angiogenesis (34). PDGF receptors are protein tyrosine kinases, activated by ligand-induced dimerization of receptor subunits. PDGF-A (and to a lesser extent, PDGF-B) is expressed in human gliomas, and expression has correlated with tumor grade (35). PDGFR- β mRNA is not detected in normal brain but is expressed in glioma vasculature, particularly in areas of endothelial proliferation (36). It is hypothesized that astrocytic expression of PDGF and upregulation of the PDGF receptor by surrounding endothelial cells may stimulate VEGF expression and contribute to the regulation of angiogenesis in gliomas (22).

Epidermal growth factor (EGF), transforming growth factor- α (TGF- α), and their receptor, EGFR, have also been implicated in glioma angiogenesis (22). EGF is involved in a number of cell-regulatory pathways, and TGF- α is involved in normal cell growth and development. Both EGF and TGF- α exert their effects via EGFR (ErbB1), a receptor tyrosine kinase. EGFR is not significantly present in normal glial cells, but increased levels have been demonstrated in gliomas (37). TGF- α expression has also been correlated with tumor grade, EGFR, and Ki-67 expression (38). EGF and TGF- α binding to the EGFR stimulates VEGF expression by glioma cells in vitro (39), and therefore this system is likely involved in mediating glioma angiogenesis. TGF- β is also associated with angiogenesis, is markedly elevated in gliomas, and levels correlate with vascularity (40). Studies have demonstrated that TGF- β is synthesized by glioma cells, and it is found within the cyst fluid and cerebrospinal fluid (CSF) of patients with gliomas (41). It plays a role in migration, invasion, and angiogenesis (42). The angiogenic factor, hepatocyte growth factor/scatter factor (HGF/SF), is another angiogenic growth factor whose expression increases with increasing grades of glioma (43). HGF/SF and its tyrosine kinase receptor, c-Met, are both expressed in gliomas, and both are upregulated with progression from lower to higher grade of tumor. VEGF and HGF/SF act synergistically to promote angiogenesis (44).

Stem cell factor (SCF) is a chemokine long known to be important in the proliferation, migration, and maturation of hematopoietic stem cells and melanocytic precursors. SCF is also a powerful chemotactic factor for neural stem cell migration (45). Recent data has shown that SCF is a potent inducer of endothelial proliferation and migration (45). Tumor-associated endothelium expresses high levels of the SCF receptor, c-kit, and gliomas overexpress SCF in a grade-dependent manner. In contrast to VEGF, which is expressed largely within the hypoxic center of malignant gliomas, SCF expression is highest in the glioma cells at the leading front of tumor infiltration, areas within gliomas associated with the most robust angiogenesis. The

negative correlation between the level of SCF expression and the survival of patients with glioblastoma is suggestive of the importance of SCF expression in glioblastoma biology.

1.3.2. TUMOR VESSELS

Fischer et al. have described two vascular phases of growth for brain tumors (46). In this model, the tumor initially co-opts the existing brain vasculature. Glioma cells are found accumulating around the vasculature, disrupting the normal contact between endothelial cells and the basement membrane. This leads to increased expression of angiopoietin-2, which subsequently leads to destabilization of the blood vessel wall and vessel regression. Areas of hypoxia and necrosis develop due to the resulting inadequacy of the blood supply. This hypoxia induces the expression of hypoxia-inducible factor (HIF)-1 α , which increases the production of VEGF (46). This increased VEGF expression ultimately tips the angiogenic balance in favor of angiogenesis, driving new blood vessel formation (47).

It is important to note that blood vessels formed in response to angiogenic signals from brain tumors are not normal blood vessels. Tumor vasculature demonstrates increased leakiness, random branching, irregular vessel diameters, defective vessel walls, and disorganized endothelial cells (48). The BBB is disrupted because of the absence of tight junctions (49). Electron microscopy studies have shown that the vascular structures within malignant gliomas are predominantly composed of immature capillaries; the perivascular spaces are expanded with thickened, irregular, partially interrupted basement membranes (50).

2. TARGETING THE TUMOR VASCULAR SUPPLY

As noted above, these newly formed blood vessels are variable, ranging from delicate capillary networks to areas of glomeruloid microvascular proliferation with endothelial cell hyperplasia. Areas of angiogenesis within a tumor are therefore heterogeneous with regard to function and physiologic features, including blood flow and blood volume. How to best target these variable blood vessels remains to be determined.

2.1. *Endogenous Inhibitors*

An increasing number of endogenous/naturally occurring inhibitors of angiogenesis have been identified (5, 51). These include angiostatin, endostatin, interferon- α (IFN- α), platelet factor-4, and thrombospondin. These endogenous inhibitors have been identified in the normal circulation, but also in the presence of glioma (49), and increasing levels have been demonstrated in higher tumor grades (52). High levels of endogenous inhibitors have been identified in the presence of increased amounts of proangiogenic substances, suggesting their competing influences on the process of angiogenesis (49). The endogenous inhibitors are thought to mediate at least some of their antiangiogenic effects via inhibition or downregulation of VEGF or other proangiogenic molecules (53). Since angiogenesis occurs when the balance of proangiogenic factors outweighs the antiangiogenic factors, it follows that increasing the antiangiogenic factors in relation to the proangiogenic factors would be a strategy to prevent new blood vessel formation, and these naturally occurring inhibitors were the first to be tested clinically.

Angiostatin is an internal fragment of plasminogen generated via a series of proteolytic cleavages (53), and different angiostatin isoforms with differing activities exist (54). Angiostatin exerts its antiangiogenic effect in gliomas via four types of receptors (53), which include $\alpha v\beta 3$. Integrin $\alpha v\beta 3$ mediates cell adhesion to ECM proteins and is upregulated on the surface of proliferating endothelial cells in angiogenic microvessels, including those in glioblastomas (55). Angiostatin is thought to competitively inhibit the interaction of integrin $\alpha v\beta 3$ with its ligands, thereby interfering with cell attachment and adhesion, which are important steps for endothelial cell survival and migration (53).

In immunodeficient mice with xenograft transplants of primary human glioma cells, growth inhibition, reduced vascularity, and increased apoptosis in tumors were observed after systemic administration of angiostatin (56). Both rat and human gliomas were successfully treated in a nude mouse model by systemic administration of angiostatin via gene transfer (57). To overcome issues with long-term systemic delivery of proteins, Tanaka et al. used retroviral and adenoviral vectors to transduce the angiostatin cDNA and again demonstrated angiostatin-related tumor growth inhibition (58). In preclinical studies, the administration of angiostatin in conjunction with radiation also resulted in an antiangiogenic effect, with a synergistic effect noted in a number of solid tumors *in vitro* in mice, including those with D54 gliomas (59). Several approaches are now being utilized to translate the advances in the understanding of angiostatin to human therapy.

Endostatin, a fragment of collagen type XVIII, is an endogenous angiogenesis inhibitor that primarily localizes in the perivascular layer around blood vessels (60). High levels of endostatin are found in human gliomas, and levels of the protein correlate with glioma grade (61). *In vitro* studies demonstrated the importance of continuous elevated circulating levels of endostatin in order to achieve optimal tumor regression in animal tumor models (62). Early preclinical studies were hampered by difficulties with production and reproduction, but many preclinical studies have subsequently demonstrated tumor inhibition by endostatin (63). These studies have included rat glioma (64) and U87 glioblastomas (65). A recent study showed that by administering a direct microinfusion of endostatin for 3 weeks at a dose of 2 mg/kg/day, tumor volume was reduced by 74%, microvessel density was reduced by 33%, and tumor cell apoptosis increased threefold in U87 human brain tumors implanted in the skulls of nude mice, along with a dose-dependent increase in survival (65). In U87 human glioblastomas in nude athymic mice, endostatin, both alone and in combination with the angiogenesis inhibitor, SU5416, was able to significantly delay tumor growth when administered at low doses every 12 h for 14 days (66).

Although initial clinical trials of endostatin and angiostatin in adults with solid tumors were disappointing in terms of significantly improving patient outcome, key lessons, including the method and schedule of administration, and synergistic effects, were gleaned from these trials. Further developments led to advances in the understanding of the relationship of angiogenesis and the molecular biology and the genetics of gliomas, and additional strategies for antiangiogenic therapy were developed.

2.2. *Antiangiogenic Strategies*

Historically, oncologists have treated malignant tumors by delivering cytotoxic therapy aimed at dividing malignant cells in a dose-intensive manner in an effort to achieve rapid cytoreduction of tumor. More recently, “molecularly targeted agents” have focused delivery toward specific disrupted pathways or targets on tumor cells, with

the intention of increasing specificity and reducing toxicity. Antiangiogenic treatment strategies introduce a third focus, primarily that of the endothelial (non-tumor) cell compartment, and numerous experimental models have demonstrated that angiogenesis inhibition using this target can effectively inhibit glioma growth.

Because angiogenesis is a multifaceted process, there are several approaches and potential targets for antiangiogenic therapy that differ from standard cytotoxic therapy. A major obstacle to the delivery of standard antitumor agents to brain tumors is the BBB. One potential benefit of antiangiogenic therapy that is frequently discussed is that since antiangiogenic agents exert their mechanism of action on the endothelial cells of the vasculature, they do not have to cross the BBB. However, this is only true if these agents work on the luminal side of the vasculature. Some angiogenesis inhibitors are considered exclusively antiangiogenic (e.g., bevacizumab), while others have additional antitumor mechanisms in addition to their antiangiogenic activity. As noted above, there are many other targets of antiangiogenic inhibitors, and the ability to cross the BBB may be critical.

Because VEGF is thought to be the primary proangiogenic factor associated with gliomagenesis, many strategies have been developed in an effort to block VEGF, its receptors, or its function. Preclinical models have shown rapid changes to existing microvessels after exposure to VEGF inhibitors, with decreased blood flow and apoptosis occurring within 24 h of exposure (67). VEGF inhibition has been associated with altered structure and function of tumor neovasculature; VEGF inhibition has been shown to prune the smaller, abnormal vessels and transform the larger vessels toward a more normal phenotype (68). Antibodies against VEGF and the VEGF receptor, and VEGF-diphtheria toxin conjugates have all shown inhibitory effects on tumor xenografts in mice (69, 70). Injection of a monoclonal VEGF antibody into nude mice significantly suppressed tumor growth (71). Rats with intracranial GS9L gliomas had increased survival when injected with retroviral cells encoding antisense VEGF RNA (72). Tanaka et al. developed a strategy of targeted antiangiogenesis by constructing retroviral and adenoviral vectors encoding a secreted form of the endogenous inhibitor, platelet factor-4, and transduced RT2 rat glioma cells in vitro (58). They demonstrated that endothelial cell proliferation in vitro was selectively inhibited and that injection of vector-transduced RT2 cells resulted in hypovascular, slow-growing tumors in nude mice. In the intracerebral glioma mouse model, mice treated with the adenoviral vector encoding the angiogenic inhibitor survived significantly longer than control mice receiving the adenoviral vector alone (58).

SU5416 (semaxanib; Pharmacia, San Francisco, CA) is a small-molecule inhibitor that primarily targets VEGFR-2 (Flk-1/KDR) tyrosine kinase (73), VEGFR-1, and VEGFR-3 (74) and secondarily inhibits PDGFR- β and c-kit (74). Systemic administration to mice with subcutaneous xenografts resulted in tumor growth inhibition (75), and systemically administered SU5416 inhibited growth of GS-9L gliomas transplanted in the rat brain, significantly prolonging survival (76). Although initial studies of SU5416 in adults with advanced malignancies showed some activity (73), the randomized phase III study combining SU5416 with cytotoxic chemotherapy in patients with metastatic colorectal carcinoma failed to show a significant survival benefit at interim analysis, resulting in the discontinuation of further development of this agent (77). Results from the phase I/II trials in adults with recurrent gliomas are not yet available.

3. STUDY DESIGN AND ENDPOINT ANALYSIS

3.1. Early Clinical Trial Design

Early phase clinical trials of antiangiogenic agents in patients with brain tumors are complicated by several issues. Conventional endpoints in phase I trials are defined by predetermined toxicity criteria in order to define a maximum tolerated dose (MTD) or, more recently, biologic endpoints in order to define a biologically effective dose. Standard phase I studies are designed to best identify acute, rather than long-term toxicities. Antiangiogenic agents frequently have little acute toxicity and may need to be administered chronically. They may be effective at doses well below the MTD, and therefore dose escalations to the MTD may be unnecessary. However, defining a biologically effective dose is difficult as there is a lack of validated biological surrogate markers.

Efficacy of an agent in patients with brain tumors is historically defined in phase II trials by measuring response of the tumor based on two-dimensional measurements using post-contrast magnetic resonance imaging (MRI) scans. These endpoints are neither ideal nor applicable, when evaluating antiangiogenic agents in patients with CNS tumors. Inhibition of angiogenesis may result in a delayed reduction in tumor size on standard MR imaging after an initial increase in tumor size (known as “tumor flare”). Alternately, the tumor may rapidly appear decreased in size after administration of agents that directly inhibit VEGF (i.e., bevacizumab) since such agents will likely cause decreased gadolinium enhancement and decreased cerebral edema given the vascular permeability effects of VEGF, making it particularly difficult to evaluate the agent’s antitumor effects. Whether these MRI changes will ultimately translate into true long-term tumor control and/or patient benefit remains to be seen. Time-dependent endpoints, such as time to tumor progression or 6-month progression-free survival, have been proposed and may be more appropriate for this group of agents in this patient population.

Quantitative determination of vascularity (i.e., microvessel density) is a histological method that has been proposed to assess response to antiangiogenic agents. However, there are several disadvantages to this method. Obtaining biopsies to evaluate microvascular densities is invasive and dependent on biopsy location as angiogenesis within a tumor is heterogeneous. In addition, this method does not distinguish preexisting from newly formed blood vessels, and tumor microvessel density measurements may not correlate with the degree of endothelial cell proliferation (78).

3.2. Imaging

Newer imaging techniques may be helpful in defining biologic endpoints for antiangiogenic agents. The principles of angiogenesis imaging is that as a tumor grows, new blood vessel formation occurs, which increases capillary density in the tumor environment, which, in turn, leads to higher blood volume and blood flow in the tumor bed. Because tumor blood vessels are abnormal, their characteristics can be imaged, and differences in blood flow, blood volume, and microvascular permeability can be measured. The new vessels and damaged mature vessels are more permeable to contrast agents than normal brain vasculature. MR measurements of microvascularity and hemodynamics are potentially useful in tumor characterization and response to treatment.

The ultimate goal of imaging is to provide an accurate assessment of the effects of antiangiogenic therapy on the tumor microenvironment in real time. The vascularity of brain tumors is commonly quantified using MR perfusion techniques, such as dynamic contrast-enhanced MRI (DCE-MRI) and dynamic susceptibility-contrast MRI (DSC-MRI), which provide quantitative measures of cerebral microvasculature and hemodynamics (79). Relative cerebral blood volume (rCBV), as determined by DSC-MRI, has correlated with tumor grade and microvessel density (80). DCE-MRI is a perfusion technique with the added advantage of estimating permeability. Repetitive image acquisitions made after gadolinium injections over a longer time interval than DSC-MRI allow for any contrast leak out into the extravascular space to reach equilibrium (79). Increased permeability, as measured by the degree of enhancement in the extravascular extracellular space, is increased in high-grade gliomas (81). Both DCE-MRI and DSC-MRI have been used to monitor response to angiogenic therapy (82). There have been contradictory reports regarding the effects of steroids on cerebral perfusion (83). Further studies are necessary to determine the utility of routine assessment of response to antiangiogenic agents using perfusion techniques in the brain tumor patient population.

Many brain tumors have a high rate of glucose utilization that can be imaged by determining the increased uptake of the radioisotope, Fluorine-18 deoxy-glucose (FDG). Because normal brain utilizes glucose, FDG uptake within a brain tumor is compared with the contralateral deep white matter and cortical gray matter. Uptake of FDG by brain tumors has correlated with the grade of tumor and proliferative status, with poorly differentiated, rapidly growing tumors taking up more FDG than low-grade, slowly proliferating tumors. However, the low-grade juvenile pilocytic astrocytoma appears “hot” on FDG positron emission tomography (PET) scans despite absent tumor progression (84). FDG PET imaging has other limitations. Since there is high baseline glucose metabolism in normal cortical gray matter, the tumor to background ratios used to identify hypermetabolism are relatively low. The visual appearance of tumor on PET, and therefore the interpretation of the scan, depends on several factors, including tumor location and size. Newer radioisotopes, such as fluorothymidine, which measures proliferation, are being assessed as indicators of tumor response to treatment.

Given the small diameter of many tumor vessels and the limited resolution of clinically available imaging modalities, it is unlikely that direct visualization of vessels and quantification of changes in blood flow and volume alone will allow meaningful early determination of response to antiangiogenic therapies. Nevertheless, the assessment of an agent’s antiangiogenic activity using a combination of different imaging modalities and biologic markers, rather than a single modality, is most likely to lead to useful surrogate endpoints.

3.3. Biological Markers

The clinical evaluation of antiangiogenic agents has been hampered by the lack of validated surrogate markers to measure their biological effects. These agents are expected to be cytostatic rather than cytotoxic; therefore tumor measurement by conventional radiographic imaging may not be useful as a means to assess drug activity or tumor response. However, measuring circulating angiogenic factors has been inconsistent and difficult to interpret. Markers that are indicative of an antitumor effect and also predictive of patient benefit are necessary. No marker has yet been identified,

although several candidate markers are under investigation. Chan et al. (85) measured urinary VEGF and matrix metalloproteinase (MMP) levels in adult patients undergoing radiation therapy only. Urine samples were collected prior to, during, and 1 month after completion of radiation. An association between levels at presentation and 1-year survival was demonstrated, suggesting that trends of these markers during treatment may be important in monitoring disease. In the phase II trial of thalidomide alone in adults with recurrent high-grade gliomas, changes in serum bFGF correlated with time to progression and overall survival (86). Studies to identify additional reliable surrogate markers are ongoing.

Circulating endothelial cells (CECs) may be useful as a surrogate marker of response to antiangiogenic agents in patients with CNS tumors. CECs are increased in cancer patients (87) and are mobilized in response to VEGF (88). Endothelial progenitor cells (EPC), which originate from the bone marrow, also increase after vascular injury and are thought to have a role in maintenance of vascular integrity and angiogenesis (89). However, their clinical significance is still unknown.

4. CURRENT STRATEGIES

There are three main categories of antiangiogenic agents: true angiogenic inhibitors, vascular targeting agents, and nonspecific agents with antiangiogenic effects. True angiogenic inhibitors target only new blood vessel formation and block vascular sprouting or “neoangiogenesis,” while vascular targeting agents act on preexisting tumor vasculature (49). Vascular targeting agents typically have an acute effect with endothelial cell death within hours, compared to antiangiogenic agents which exert their effects over days to weeks (49). The nonspecific, nonselective antiangiogenic agents may be cytotoxic or antiproliferative agents with antiangiogenic properties whose antiangiogenic effects depend on dosing (49).

True angiogenic inhibitors may target angiogenic factors, their receptors, endothelial cells, and vascular smooth muscle cells. They can prevent tumor cells from producing angiogenic proteins, block receptor interaction with these proteins, or act directly on the microvascular endothelial cells themselves. They may tip the balance in favor of the endogenous inhibitors, or suppress endothelial cell recruitment. Several antiangiogenic inhibitors that have been evaluated in clinical trials or are currently in clinical trials will be discussed.

4.1. Specific Agents

4.1.1. SURAMIN

Early synthetic antiangiogenic agents included suramin, which was originally introduced more than 80 years ago as an anti-trypanozomal agent. Suramin binds to PDGF and inhibits PDGF binding to its receptor (90). It has demonstrated antiangiogenic properties in gliomas (91), including disruption of the PDGF feedback loop, inhibition of VEGF-induced tyrosine phosphorylation of KDR in intact cells, and suppression of bFGF-induced angiogenesis and tube formation (92). Suramin was able to suppress glioma and meningioma growth in vitro (91), and some activity was observed in a phase I trial (93). However, the phase II trial of suramin and radiation therapy in newly diagnosed glioblastoma patients failed to show a significant improvement in overall survival (94).

4.1.2. TNP-470

TNP-470 is one of the earliest antiangiogenic agents identified. It is a potent derivative of fumagillin, an antibiotic secreted from the fungus, *Aspergillus fumigatus* that was found to inhibit endothelial cell proliferation in vitro and in vivo (95). TNP-470 has also been shown to inhibit tumor-induced angiogenesis and tumor growth (95). Preclinical studies have demonstrated growth inhibition by TNP-470 for several types of brain tumors, including gliomas, medulloblastomas, meningiomas, and pituitary adenomas (96, 97). Clinical development of this agent has been hampered by the lack of antitumor responses in early clinical trials, as well as dose-limiting neuropsychiatric toxicities (98).

4.1.3. MARIMASTAT

In order to infiltrate surrounding tissue and induce angiogenesis, brain tumor cells must interact with the ECM. Cell-adhesion molecules within the ECM allow interactions between the cells and the ECM and anchor cells to other cells, blood vessels, and tumor cells. Examples of cell-adhesion molecules include integrins, which are involved early in brain tumor invasion and infiltration and allow tumor cells to adhere to the ECM (99). Once tumor cells adhere, the ECM is degraded by tumor-secreted proteinases, for example, matrix metalloproteinases, creating intercellular spaces into which tumor cells can infiltrate. Marimastat is a low-molecular-weight, broad-spectrum, MMP inhibitor that acts by covalently binding to the active site of activated MMPs (100). Preclinical testing showed that this agent was active against glioma cell lines (101). However, a phase III trial in adults with newly diagnosed glioblastoma treated with or without marimastat after standard radiation did not show any significant benefit for the marimastat cohort using the primary endpoints of progression-free or overall survival (102).

4.1.4. THALIDOMIDE

One of the earliest antiangiogenic agents to be evaluated in clinical trial in patients with CNS tumors was thalidomide, a drug initially developed as a sedative, but subsequently found to have immunomodulatory and antiangiogenic properties. It inhibits growth factor-mediated neovascularization, is a known inhibitor of tumor necrosis factor- α , and has demonstrated inhibition of tumor growth in solid tumor models. Clinical trials of this agent in adults with recurrent high-grade gliomas have been performed (86) with some antitumor activity observed, although the overall response rate was low. Because preclinical investigation has demonstrated synergistic activity when antiangiogenic agents were combined with cytotoxic agents, a study combining thalidomide with carmustine in adults with recurrent high-grade gliomas was performed. In this study, the median progression-free survival was 100 days and the objective radiographic response rate was 24% for patients with glioblastoma, which compared favorably with historical controls (103). In a trial combining thalidomide with radiation therapy followed by thalidomide alone until disease progression in adult patients with newly diagnosed glioblastoma, survival was comparable to historical controls receiving radiation followed by BCNU (102). Further development of thalidomide will likely focus on combinations or more potent analogs, such as CC-5013.

4.1.5. LENALIDOMIDE (CC-5013; CELGENE CORP.)

This is a potent analog of thalidomide that also exerts a broad spectrum of pharmacologic and immunologic effects. It is an immunomodulatory agent that inhibits the release of proinflammatory cytokines, promotes release of anti-inflammatory cytokines, and effects T-cell proliferation. In a phase I trial of lenalidomide in adults with recurrent high-grade gliomas and other refractory CNS tumors, daily doses up to 40 mg/day on a 21-day schedule followed by a 7-day break were well tolerated. There was evidence of some activity with prolonged stable disease observed. Phase I trials are currently being conducted in pediatric patients with recurrent or refractory CNS tumors.

4.1.6. LEFLUNOMIDE (SU101; SUGEN, SAN FRANCISCO, CA)

This was one of the earliest molecularly targeted agents to be clinically tested in patients with malignant gliomas. SU101 and its active metabolite, SU20, inhibit PDGF-mediated signaling events. It demonstrated glioma growth inhibition both *in vitro* in a number of human glioma cell lines and *in vivo* against a variety of xenografts, including gliomas, in athymic mice (104, 105). Activity observed during phase I testing in adults with recurrent malignant gliomas led to single and combination phase II trials (106, 107). In the phase II trial in adult patients with recurrent malignant gliomas, only nine of 15 patients completed cycle 1, one patient had a minor response of 32+ weeks duration, and five had stable disease of 16–41+ weeks duration (106). In the pediatric phase I trial, CNS toxicity was dose limiting (108). No further clinical trials of this agent have occurred due to commercial reasons.

4.1.7. INTERFERONS

The type I IFNs are known to downregulate the expression of proangiogenic molecules, including bFGF, IL-8, MMP-2, and MMP-9 (109). Systemic chronic administration of IFN- α or IFN- β has been shown to produce regression of vascular tumors by downregulation of mRNA expression and protein production of the angiogenic factor, bFGF (110). This effect requires long-term exposure to IFNs, as has been demonstrated clinically (111) and *in vitro* (110). Clinical studies using IFN- α , IFN- β , and IFN- γ have been performed in patients with malignant brain tumors using different dosing schedules and routes of administration. The results have been mixed. Nagai et al. (112) reported a partial response in two of nine patients with glioblastoma treated with rIFN-alpha A and 40% response rate (one complete response, seven partial responses) in 20 patients with glioblastoma treated with human fibroblast IFN- β . In a pediatric study involving children with recurrent malignant tumors treated with recombinant IFN- β , two of nine children with brainstem gliomas had a partial response (>50% reduction in tumor size) and two children had prolonged disease stabilization (one child for 3.5 years) (113). In pediatric patients with brainstem gliomas treated with intravenous recombinant β -IFN during hyperfractionated radiation therapy, 13 of 32 patients required dose modifications due to hepatic or hematologic toxicity (114).

Although IFN- α has been used to treat a variety of neoplasms, its optimal dosing has not yet been established. It has traditionally been administered in high doses, $\geq 3,000,000$ U/m 2 /week. Significant side effects have limited its use. Recent studies have shown that more frequent administration of chemotherapeutic agents using doses well below the MTD may have a more pronounced antiangiogenic effect on tumors

by preventing repair and re-growth of proliferating endothelial cells in the tumor bed (115). Slaton et al. demonstrated that daily administration of IFN- α -2a, at doses below the MTD, produced the most significant inhibition of tumor growth and tumor vascularization and maximal inhibition of angiogenesis-regulating genes (109). This approach using continuous low-dose administration of IFN is currently being tested in clinical trials.

4.1.8. BEVACIZUMAB (AVASTIN®; GENENTECH, INC., SOUTH SAN FRANCISCO, CA)

Bevacizumab is a recombinant humanized monoclonal antibody that binds to and inhibits VEGF, preventing its interaction with the VEGF receptors, Flt-1 and KDR, on the endothelial cell surface. It was the first anti-VEGF agent to be FDA approved for use in cancer patients based on a phase III study that demonstrated an increase in overall survival in previously untreated, metastatic colorectal cancer patients who received bevacizumab in combination with cytotoxic chemotherapy compared to patients receiving cytotoxic chemotherapy alone (74). In a report by Stark-Vance (116), of 21 patients with recurrent glioma treated with irinotecan and bevacizumab, nine patients had an objective response and 11 had stable disease, with one episode of intracranial hemorrhage. In a separate phase II trial of bevacizumab and irinotecan in patients with recurrent malignant gliomas (117), the reported response rate was 63% with 19 partial responses and one complete response. Although some of the radiographic responses may include radiographic permeability effects, median progression-free survival was 24 weeks, and median survival reportedly exceeds 6 months. Because it is unclear whether irinotecan was adding any antitumor activity, a phase II trial of bevacizumab alone is currently underway.

4.1.9. AZD2171

AZD2171 is a highly potent, orally available inhibitor of the VEGF receptor tyrosine kinase (118). It inhibits VEGF-induced signaling in endothelial cells and has demonstrated growth inhibition and antiangiogenic activity in athymic mice with a variety of human tumor xenografts (118). Phase I studies in adults with a variety of solid tumors have shown that it has a half-life of 12.5–35.4 h (119). A phase II trial is currently being performed in adults with recurrent glioblastoma, and in children with recurrent or progressive CNS tumors.

4.1.10. ENZASTAURIN (LY317615; LILLY CORP., INDIANAPOLIS, IN)

The protein kinase C (PKC) family of enzymes are serine/threonine kinases involved in cell signaling. The beta (β) isoform of PKC is involved in the VEGF signaling cascade, and inhibition may lead to inhibition of angiogenesis and tumor growth. As noted earlier, upregulation of the VEGF receptors, Flt-1 and KDR/Flk-1, has been observed in tumor-associated endothelial cells in a variety of tumors, including brain tumors. The signal transduction pathways of these receptors include tyrosine phosphorylation and a number of pathways with downstream activation of PKC and activation of MAP kinase pathway or translocation of PKC into the nucleus (120). Enzastaurin is a potent, selective inhibitor of PKC β , which competitively inhibits its ATP binding site and other intracellular signaling proteins important for tumor

growth (121). Preclinical testing showed that enzastaurin was able to inhibit angiogenesis in a VEGF-impregnated disc in the rat corneal micropocket assay (122), and administration of enzastaurin was associated with decreased microvessel density and VEGF expression in human tumor xenografts (123). Results from phase II testing in adults with recurrent malignant gliomas treated daily at a dose of 500 mg/day and stratified by use of enzyme-inducing antiepileptic agents showed that the agent was well tolerated. Objective responses were observed in 14 of 85 patients, including 10 patients with glioblastoma (124). Preclinical testing has subsequently shown that enzastaurin has direct cytotoxic effects against glioma cell lines in addition to its antiangiogenic effects (121). Additional clinical testing in patients with malignant gliomas is ongoing to test a more frequent dosing schedule. In addition, an international randomized phase III trial of enzastaurin versus CCNU for patients with first recurrence of glioblastoma is ongoing.

4.1.11. GEFITINIB (ZD1839, IRESSA, ASTRAZENECA, WILMINGTON, DE)

EGFR and its ligands, EGF and TGF- α , are important in many cell functions including cell proliferation, mobility, adhesion, invasion, and angiogenesis. EGFR is overexpressed in some malignancies, including gliomas. Treatment of tumor cells in vitro with an anti-EGFR antibody induced cell-cycle arrest (125). Studies have shown that blockade of the EGF-binding site can inhibit tumor cell proliferation in tumor cell culture and in human tumor xenografts (126). Gefitinib is an inhibitor of the EGFR tyrosine kinase and has demonstrated antiangiogenic activity in a number of human tumor xenografts (127). Blockade of the EGF receptor tyrosine kinase in tumor cells by gefitinib results in decreased expression of VEGF, bFGF, and TGF- α by the tumor cells (128). However, when xenograft models of athymic mice expressing EGFRvIII (a mutated variant of EGFR commonly found in gliomas) were treated with gefitinib, there was only a partial decrease in EGFR autophosphorylation and an overall increase in EGFRvIII expression, suggesting that the common EGFR mutation may lead to therapeutic resistance of this agent (129). A single institutional phase II trial and a multi-institutional phase I/II trial of gefitinib in adult patients with malignant gliomas demonstrated no objective tumor responses and only a low response rate, respectively (130,131). Clinical trials using gefitinib in combination with radiation or other agents are ongoing.

4.1.12. ERLOTINIB (OSI-774, TARCEVA; GENENTECH, SOUTH SAN FRANCISCO, CA; OSI PHARMACEUTICALS, MELVILLE, NY; AND F. HOFFMAN-LA-ROCHE, BASEL, SWITZERLAND)

Erlotinib is a potent, oral selective EGFR tyrosine kinase inhibitor. In a recent phase I study of erlotinib alone and combined with temozolomide in adults with malignant glioma, eight of 57 patients evaluable for response had a partial response, six of whom were assigned erlotinib alone and two of whom received erlotinib and temozolomide. In addition, six patients had a progression-free survival of at least 6 months (132). These results suggest possible significant activity of this agent in the glioma population. Interestingly, Mellinghoff et al. performed EGFR molecular analysis on 49 patients with recurrent malignant glioma who had been treated with EGFR kinase inhibitors and found that coexpression of EGFRvIII and PTEN by glioblastoma cells was associated with response (133). If this association holds up, it may be indirect evidence that the

major antitumor mechanism of action of erlotinib is via direct antitumor effects rather than via an antiangiogenic effect.

4.1.13. ZD6474

ZD6474 is a low-molecular-weight tyrosine kinase inhibitor that has been shown to inhibit growth of tumors in *in vitro* models. It differs from other tyrosine kinase inhibitors in that it inhibits two important signaling pathways in glioma, VEGFR and EGFR (134). ZD6474 has significant effects on both endothelial and tumor cells, and when administered to rats with intracerebral gliomas, it significantly reduced tumor volume, increased tumor cell apoptosis, and decreased tumor cell proliferation (135). This agent is currently in phase I and II clinical trials in patients with malignant gliomas.

4.1.14. CILENGITIDE (EMD121974, MERCK KGAA, DARMSTADT, GERMANY)

The angiogenic process is dependent on the ability of proliferating endothelial cells to interact with proteins within the ECM. This interaction is mediated by endothelial receptors and the integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Cilengitide is an $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin inhibitor which demonstrated tumor growth inhibition and an antiangiogenic effect in a chicken chorioallantoic membrane model seeded with human melanoma and lung cancer cell lines, SCID mice, and nude mice inoculated with human tumor cells, including medulloblastoma and glioblastoma (136–138). No dose-limiting toxicity was identified in the phase I trial of Cilengitide in adults with solid tumors when administered at doses up to 1,600 mg/m²/infusion twice weekly (136). In the phase I trial of cilengitide in adults with recurrent malignant gliomas, the drug was well tolerated and some activity was noted, including two complete responses, three partial responses, and four long-term stable disease in the first 51 patients entered on trial (139). Clinical trials in adult and pediatric patients with CNS tumors are ongoing.

4.1.15. PTK787

PTK787 is a potent inhibitor of the VEGFR kinases that also inhibits other kinases, including PDGF- β tyrosine kinase and c-kit, and has demonstrated inhibition of endothelial cell proliferation and migration (140). In an orthotopic rat brain tumor model using C6 rat glioma cells transfected with VEGF, PTK787 significantly inhibited neovascularization and proliferation (141). Phase I/II randomized clinical trials combining this agent with radiation and temozolomide are currently underway in adults with newly diagnosed glioblastoma.

4.1.16. IMATINIB (STI571, GLEEVEC, NOVARTIS, EAST HANOVER, NJ)

Imatinib is an oral agent that inhibits multiple tyrosine kinases, including PDGFR- α and PDGFR- β , c-abl, and c-kit (142). PDGF β receptor mRNA is found within glioma cells and the endothelial cells of hyperplastic capillaries within the tumor bed (143) and PDGFR- α is overexpressed in most glioma cell lines and surgical isolates (144). Kilic et al. were able to demonstrate intracranial inhibition of PDGF-mediated cell growth against intracranial implants of human glioblastoma in nude mice (145). Geng et al. also showed that in irradiated intracranial glioblastoma mouse models, imatinib inhibits phosphorylation of PDGFR and Akt and induces apoptosis, enhancing the effects of

radiation (146). Phase II trials in patients with recurrent glioblastoma showed some activity, with three of 51 patients exhibiting a partial response in one study (147).

4.1.17. COMBINATION THERAPY

Because the process of angiogenesis is complex, targeting multiple pathways simultaneously may increase the effectiveness of antiangiogenic treatment. Studies involving combinations of antiangiogenic agents with radiation therapy have demonstrated synergistic effects in glioma models (148). Combining angiogenic inhibitors that target different pathways and combining antiangiogenic agents with standard cytotoxic agents are strategies currently under clinical investigation.

4.1.18. METRONOMIC THERAPY

Standard cytotoxic therapy kills endothelial cells, but aggressive endothelial proliferation occurs during the recovery phase. Browder et al. demonstrated that administering the same chemotherapy in lower doses over a prolonged period of time allows for more sustained apoptosis of endothelial cells within a tumor vascular bed and had an antiangiogenic effect, with more effective control of tumor growth and decreased likelihood of developing drug resistance (115). Administering chemotherapy on schedules that increase their antiangiogenic potential is referred to as metronomic chemotherapy. A recently reported feasibility trial in which four agents (thalidomide, celecoxib, alternating with etoposide, and cyclophosphamide) were administered on a metronomic schedule to pediatric patients with recurrent or progressive solid tumors for six months demonstrated the tolerability and effectiveness of this approach (149). Of 20 patients enrolled, 40% completed 6 months of therapy and 25% were progression free for >123 weeks (149). In a phase II trial in adults with recurrent gliomas, this regimen was associated with a 12% partial response rate and 59% incidence of stable disease, although the regimen failed to show a significant improvement in overall survival (150).

4.2. Mechanisms of Resistance

The treatment of brain tumors with standard cytotoxic chemotherapy is hampered by tumor heterogeneity, drug resistance, and drug delivery. Antiangiogenic agents face similar obstacles. Both the distribution of areas of angiogenesis and blood flow within the resulting microvasculature of the tumor are heterogeneous, and, as discussed above, morphologically and functionally abnormal. Physiologic differences in the vasculature related to drug delivery and blood flow may lead to important differences in the response to antiangiogenic therapy. Unlike cytotoxic therapy that targets tumor cells, antiangiogenic therapy targets nonmalignant cells (e.g., endothelial cells). Unlike the genetically unstable tumor cells, endothelial cells are thought to be under normal cellular control and thus presumably lack the ability to become drug resistant. The issues of resistance are more likely to be due to the complexity of the angiogenic system and the presence of redundant pathways rather than the ability of a single cell to evade therapy.

Another issue regarding the translation of antiangiogenic inhibitors into therapy for patients with brain tumors relates to the invasive nature of these tumors, particularly the gliomas. Concern has been raised that even if effective inhibition of angiogenesis is attained, invading tumor cells may be able to survive by co-opting existing brain

vasculature (151), resulting in diffuse infiltration (e.g., gliomatosis cerebri) rather than a discrete mass. Other potential mechanisms of resistance include the secretion of alternative angiogenic cytokines or degradative proteases and/or enhanced ability for a given tumor to survive in hypoxic conditions.

5. CONCLUSION

While the past two decades have brought tremendous advances in the field of angiogenesis, the full potential of this strategy as an antitumor therapy remains to be realized, particularly in the field of neuro-oncology. Although preclinical studies have demonstrated tumor growth inhibition, this has not directly translated into a measurable effect in the brain tumor patient population. Whether this is due to deficiencies in study design, a lack of a reliable marker of response, ineffective drugs or ineffectiveness of the overall strategy for these tumor types remains to be determined. What is known is that a number of issues regarding antiangiogenic therapy for brain tumors remain to be resolved. Despite these obstacles, the promise of antiangiogenic therapy remains. The true potential of this therapy will probably be best realized in combination studies in properly designed and properly powered clinical trials.

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24

Angiogenesis Inhibitors for the Treatment of Lung Cancer

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SUMMARY

Non-small cell lung cancer is the leading cause of cancer-related mortality in the United States. In these patients, chemotherapy improves survival compared to supportive care, however, even with modern regimens the median survival is less than one year. As is true for other solid malignancies, vascular density has been demonstrated to be an important prognostic factor in lung cancer, and the extent of tumor vascularization has been linked to expression of proangiogenic molecules and oncogenic mutations. Because an adequate vascular supply is necessary for tumor growth and metastatic spread, much effort has been directed toward the development of therapies targeting the vascular component of lung cancer. In recent studies, the addition of antiangiogenic agents to either chemotherapy or other targeted agents has yielded promising results. This chapter highlights novel antiangiogenic agents and reviews emerging data from lung cancer clinical studies.

Key Words: Lung cancer; angiogenesis; angiogenesis inhibitors

1. ANGIOGENESIS IN LUNG CANCER

Lung cancer is the most frequent cause of cancer death. Despite the development of new chemotherapeutic agents and regimens over 1980s and 1990s, improvements in patient survival have been only incremental (1). Therefore, additional therapeutic approaches are required to improve clinical outcome. Advances in our understanding of cancer biology have led to the development of new therapeutic agents designed to target tumors or the supporting host cells more specifically. Inhibition of tumor angiogenesis is a key therapeutic strategy that holds great promise in the treatment of lung cancer. The extent of tumor vascularization has been found to be an important prognostic factor in many cancer types including lung cancer. Both prospective and retrospective studies have demonstrated that tumor microvessel density (MVD) correlates with disease stage and patient survival (2–6).

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Although vascular density is thought to be relatively homogeneous in normal tissue from healthy individuals, within tumors wide differences occur, and the invading edge of the tumor is more highly vascularized (7). In non-small cell lung cancer (NSCLC), patients with tumors that have a high MVD in the periphery and in the inner tumor region have a worse prognosis than patients with tumors that are poorly vascularized in the inner tumor region or periphery (7).

2. ANGIOGENIC FACTORS IN LUNG CANCER

Vascular endothelial growth factor (VEGF) is one of the most potent mediators of neovascularization and facilitates vascular permeability, endothelial cell proliferation, migration, and survival (8). Expression of VEGF is positively regulated by hypoxia through the stabilization of the transcription factor hypoxia-inducible factor 1 alpha (HIF-1 α) (9, 10). Within tumors, VEGF mRNA is most highly expressed within hypoxic cells proximal to areas of necrosis (11, 12). VEGF is secreted by lung cancer cells, and tumor cell expression of VEGF is associated with an increased vascular density (13–15). Moreover, elevated levels of VEGF correlate with decreased survival time and risk of relapse in retrospective and prospective studies of NSCLC patients (9, 16–20), and high levels of VEGF mRNA are associated with an early post-operative relapse (18).

Basic fibroblast growth factor (bFGF), an endothelial cell mitogen and inducer of angiogenesis, is expressed by 53–74% of NSCLC cells (21, 22). Increased levels of bFGF have been shown to be associated with vascular density and lymph node metastasis (23, 24). Likewise, in a study of 119 NSCLC patients, bFGF expression correlated with poor survival (21). Other molecules thought to contribute to the vascularization of lung tumors include interleukin-8 (IL-8) and platelet-derived endothelial growth factor (PDGF) (Table 1). Increased expression of IL-8 or PDGF has been shown to be associated with angiogenesis and correlate with decreased patient survival (4, 25).

Table 1
Potential Regulators of Angiogenesis in NSCLC

| <i>Proangiogenic molecules</i> | <i>Antiangiogenic molecules</i> | <i>Transcription factors, oncogenes, and other regulators</i> |
|---|---|---|
| Vascular endothelial growth factor (VEGF) | Interferon- α , interferon- β , and interferon- γ | Hypoxia-inducible factor 1- α |
| Basic fibroblast growth factor | Thrombospondin | Nuclear factor- κ B |
| Transforming growth factor- α | Angiopoietin 2 | Epidermal growth factor receptor |
| Platelet-derived growth factor | Tissue inhibitors of MMPs (TIMPs) | Ras |
| Epidermal growth factor | Endostatin | P53 |
| Angiogenin | Angiostatin | |
| Interleukin-6 | Interleukin-12 | |
| Interleukin-8 | | |
| Matrix metalloproteinases (MMPs) | | |

2.1. HIF-1 α

HIF-1 is a transcription factor consisting of two subunits, HIF-1 α and HIF-1 β . Although HIF-1 β is constitutively expressed, expression of HIF-1 α is highly regulated. The stability of HIF-1 α is primarily regulated by hypoxia. When an adequate supply of oxygen is present, prolyl hydroxylases modify proline residues 402 and 564 on HIF-1 α , allowing it to bind the VHL tumor suppressor gene, which targets it for degradation (10). Additionally, HIF-1 α protein stability can be regulated by oxygen-independent mechanisms through activation of the phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) signal transduction pathways (26, 27). Following binding of the HIF-1 α and HIF-1 β subunits, HIF-1 transverses to the nucleus and regulates the expression of many genes involved in angiogenesis, cell survival, invasion, and glucose metabolism (10). Indeed, HIF-1 α is thought to be the key regulator of potent proangiogenic factors including VEGF. HIF-1 α protein levels have been shown to be elevated in many tumor types. In NSCLC tumors, HIF-1 α expression correlates with the expression of proangiogenic molecules including VEGF, PDGF, and bFGF (28). High levels of HIF-1 α or carbonic anhydrase IX (CA IX), a gene regulated by HIF-1 α , are associated with a shorter disease-free survival in lung cancer patients (9). Additional immunohistochemical studies, which utilize CA IX expression as a marker for HIF-1 α activity, link high expression of CA IX to elevated expression of multiple proangiogenic factors and poor clinical outcome in NSCLC.

2.2. Oncogenes as Regulators of Angiogenesis in Lung Cancer

It is widely established that activation of proto-oncogenes can induce tumorigenesis. In tissue culture, expression of activated oncogenes increases cell proliferation and decreases apoptosis (29). Correspondingly, in animal models, tumor cell expression of oncogenes results in enhancement of tumor growth and a shift in the balance between tumor cell proliferation and apoptosis (30, 31). Although oncogenes are believed to promote tumorigenesis by altering the equilibrium between cell proliferation and apoptosis, there is considerable evidence that this alone is not sufficient to produce expansive tumor growth (32). Rather, tumors must also acquire an adequate vascular supply to grow beyond 1–2 mm in diameter. In support of this concept, published reports have demonstrated that transfection of tumor cells with oncogenes results in enhanced production of proangiogenic molecules (33), and the *in vivo* growth of oncogene-driven tumors can be restricted with angiogenesis inhibitors (34). In lung cancer patients, mutations in K-Ras, p53, and epidermal growth factor receptor (EGFR) are among the oncogenes that have been linked to angiogenesis.

2.3. Ras

The Ras gene family includes H-Ras (homologous to the Harvey rat sarcoma virus oncogene), K-Ras (homologous to the Kristen rat sarcoma virus), and N-Ras (first identified in neuroblastoma) (35). These three genes yield highly conserved guanine nucleotide (GDP/GTP)-binding proteins, which localize to the inner face of the plasma membrane. In physiological conditions, activation of cell surface receptors triggers Ras activation and binding to GTP. Wild-type Ras has the intrinsic ability to hydrolyze GTP, and therefore activation is transient and Ras returns to an inactive, GDP-bound

state. Mutant Ras proteins contain an amino acid substitution as a result of a single-point mutation, which results in diminished GTPase activity. Therefore, mutant Ras proteins are constitutively activated (36). Ras is one of the most commonly activated oncogenes, occurring in 17–25% of all human tumors (36). Among NSCLC patients, mutations in Ras are detected in 15–20% of cases, and Ras mutations are detected in 30–50% of NSCLC patients with adenocarcinoma (37). Numerous investigators have sought to determine whether oncogenic Ras or Ras overexpression is a poor prognostic indicator for lung cancer patients, and the results of these studies have been somewhat conflicting. In a recent report, published studies assessing the prognostic value of Ras in lung cancer were identified, and meta-analysis was performed to more thoroughly evaluate the significance of this oncogene in patient survival. This analysis included 28 studies and a total of 3620 NSCLC patients. Results obtained from this analysis indicate that Ras mutations are a poor prognostic factor in NSCLC patients (35).

As mentioned previously, emerging data indicate that oncogenes are capable of activating the angiogenic switch. In tissue-culture studies, transfection of transformed murine endothelial cells with the Ras oncogene results in elevated production of VEGF, and treatment of these cells with the PI3K inhibitor, wortmannin, abrogates VEGF expression, indicating that mutated Ras regulates VEGF expression in a PI3K-dependent manner (38). Moreover, in a retrospective study evaluating 181 human NSCLC tumors, the presence of K-Ras gene mutations was positively associated with high VEGF expression (39).

2.4. p53

The tumor suppressor gene, p53, is a key regulator of cell growth and apoptosis. Activated by DNA-damaging agents or environmental stimuli, p53 induces growth arrest to permit cells to repair damage or promotes apoptosis if the damage is too extreme (40). The effects of p53 on cell cycle and apoptosis are mediated in part through direct transcriptional regulation of proteins including p21, Bcl-1, BAX, and survivin. p53 is frequently inactivated in tumor progression by various mechanisms. Mutations in the p53 gene have a high rate of occurrence in NSCLC and SCLC, with a frequency of 50 and 70%, respectively (41). Thus, mutations in the p53 gene are among the most common known genetic aberrations in lung cancer. Two meta-analyses have investigated the relationship between p53 status and survival of NSCLC patients. In the first study, p53 mutations were a prognostic factor regardless of stage of disease (42). However, in the second study, aberrant p53 was only a significant prognostic factor in lung cancer patients with adenocarcinomas (41, 43).

In addition to its role in the regulation of cell cycle and apoptosis, emerging data indicate that p53 indirectly promotes tumor vascularization by altering the expression of proangiogenic and antiangiogenic molecules. In tissue-culture studies, fibroblasts expressing wild-type p53 secrete high levels of the antiangiogenic glycoprotein, thrombospondin-1 (TSP-1). However, loss of wild-type p53 and expression of the mutant form results in diminished TSP-1 mRNA and protein (44). Furthermore, in fibroblasts transfected with a temperature-sensitive form of p53, which display a mutant phenotype at 37°C and a wild-type phenotype at 32.5°C, VEGF expression is elevated in mutant but not wild-type p53-expressing cells (45). In support of the hypothesis that wild-type p53 regulates angiogenesis in human lung cancer, immunohistochemical

evaluation of 73 NSCLC clinical specimens revealed a strong statistical association between p53 nuclear localization and microvessel count (46). Additionally, in an analysis of 107 NSCLC patients, p53 was determined to be significantly associated with VEGF expression and microvessel count (47). It is likely that loss of wild-type p53 elaborates tumor cell expression of additional proangiogenic factors in NSCLC. Wild-type p53 has been demonstrated to promote Mdm2-mediated ubiquitination and degradation of HIF-1 α (48). The loss of wild-type p53 is associated with elevated levels of HIF-1 α in tissue culture and augments hypoxia-induced VEGF expression (48).

3. EGFR AND LUNG CANCER ANGIOGENESIS

EGFR is a member of the erbB family of receptor tyrosine kinases (RTKs), which also includes HER2/Neu, HER3 (ErbB3), and HER4 (ErbB4) (49). EGFR is composed of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular portion with a catalytic tyrosine kinase domain. Ligands for EGFR include EGF, transforming growth factor-alpha (TGF- α), amphiregulin, betacellulin, and epiregulin (50). Upon ligand binding, EGFR forms homodimers or heterodimers with other erbB family members, which induces conformational changes and results in activation of downstream signal transduction pathways. Signaling molecules activated by EGFR include Src family kinases, signal transducer and activator of transcription (STAT), MAPK, and PI-3K, which ultimately lead to enhanced cell proliferation, migration, and survival. EGFR is overexpressed in many tumor types. In NSCLC, EGFR is expressed in 40–80% of cases (51). The relationship between EGFR overexpression and prognosis in lung cancer remains controversial (52). However, in a recent study, EGFR overexpression was linked to lymph node metastasis and a more aggressive phenotype (53).

The concept that EGFR regulates tumor cell proliferation, survival, and motility led to the development and clinical testing of small-molecule inhibitors such as gefitinib and erlotinib. In the initial clinical testing, these agents yielded a clinical response in 10% of patients with European background and 30% of patients from Japan. These responses were more frequent in female patients than males and in patients with adenocarcinoma than any other histological type (51). It was later identified that in cases where clinical response was observed, tumor cells had somatic EGFR mutations within the tyrosine kinase domain (54, 55). Although these mutations render EGFR constitutively activated, they also enhance sensitivity to EGFR tyrosine kinase inhibitors.

An expanding body of evidence indicates that activation of EGFR leads to enhanced production of proangiogenic molecules. Initial experiments using prostate cancer cell lines demonstrated that stimulation of tumor cells with EGF elevated HIF-1 α expression (56). EGF has been shown to increase VEGF production in some tumor cell lines (57, 58), and conversely, treatment of tumor cells with EGFR inhibitors can decrease VEGF expression in various tumor types (58–61). In NSCLC cell lines, EGF activates HIF-1 α and induces expression of the chemokine receptor CXCR4 in tissue culture (62). Moreover, in an immunohistochemical study of 172 NSCLC patients, expression of EGFR was associated with HIF-1 α positivity (63).

4. PLEURAL EFFUSION

Lung cancer is the foremost cause of malignant pleural effusion (64), and 25% of all lung cancer patients will develop pleural effusion during the course of the disease (65). Malignant pleural effusion leads to significant morbidity from progressive dyspnea, cough, and chest pain. Among lung cancer patients, malignant pleural effusion is associated with end-stage disease and is a poor prognostic indicator (64). The formation of pleural effusion is thought to be due in part to tumor invasion into the pleura and enhanced vascular permeability. Elevated levels of various proangiogenic molecules including angiogenin (66), IL-8 (67), and VEGF (68) have been detected in malignant effusions. In addition to its role in angiogenesis, VEGF is one of the most potent inducers of vascular permeability. Therefore, it is thought to be a key player in the formation of malignant pleural effusion. Several *in vivo* studies support this notion. For example, injection of low VEGF-expressing NSCLC cells into the thoracic cavity failed to produce pleural effusion. Whereas, following sense VEGF gene transfection, these tumor cells induce pleural effusion *in vivo* (69). Furthermore, treatment of nude mice bearing VEGF-positive NSCLC tumors with PTK 787, a tyrosine kinase inhibitor with activity against Flk-1/KDR and Flt-1, reduced tumor vascularization and vascular permeability and inhibited the formation of pleural effusion (70). In similar xenograft studies, treatment of mice bearing NSCLC tumors with ZD6474, a small molecule inhibitor of VEGFR and EGFR tyrosine kinases, inhibited the production of pleural effusion in a dose-dependent manner (71).

5. THERAPEUTIC APPROACHES TO INHIBITING ANGIOGENESIS

Because an adequate vascular supply is critical for tumor expansion and metastasis, many classes of therapeutic agents have been developed to target the multistep process of angiogenesis. VEGF, being one of the most potent inducers of angiogenesis, has been targeted by various strategies including antibodies directed against VEGF (i.e., bevacizumab) and antibodies such as IMC-1121b, which bind the receptor and prevent ligand interactions. Another VEGF-neutralizing agent is VEGF Trap, a soluble decoy receptor composed of the extracellular domain of VEGFR fused to the Fc portion of immunoglobulin (Ig) G1 (8, 72). Multiple oral, small molecule receptor tyrosine kinase inhibitors (RTKIs) have also been developed including ZD6474, sunitinib, and sorafenib (Fig. 1). Because these agents target the ATP-binding site, which is conserved in many RTKs, these drugs typically have activity against multiple receptors (73). ZD6474, for example, inhibits both VEGFR and EGFR activation. Owing to the fact that tumors utilize various ligands and receptors to promote neovascularization, agents targeting multiple RTKs may be most useful therapeutically.

5.1. Monoclonal Antibodies Targeting the VEGF Pathway

Bevacizumab is an intravenously administered, humanized monoclonal antibody that binds VEGF and prevents it from interacting with its receptor (Table 2). In 2004, this agent became the first clinically available “pure” angiogenesis inhibitor in oncology when it received Food and Drug Administration (FDA) approval for first-line use with 5-fluorouracil-based chemotherapy for metastatic colorectal cancer based on improved survival in a randomized trial (74). In October 2005, bevacizumab also

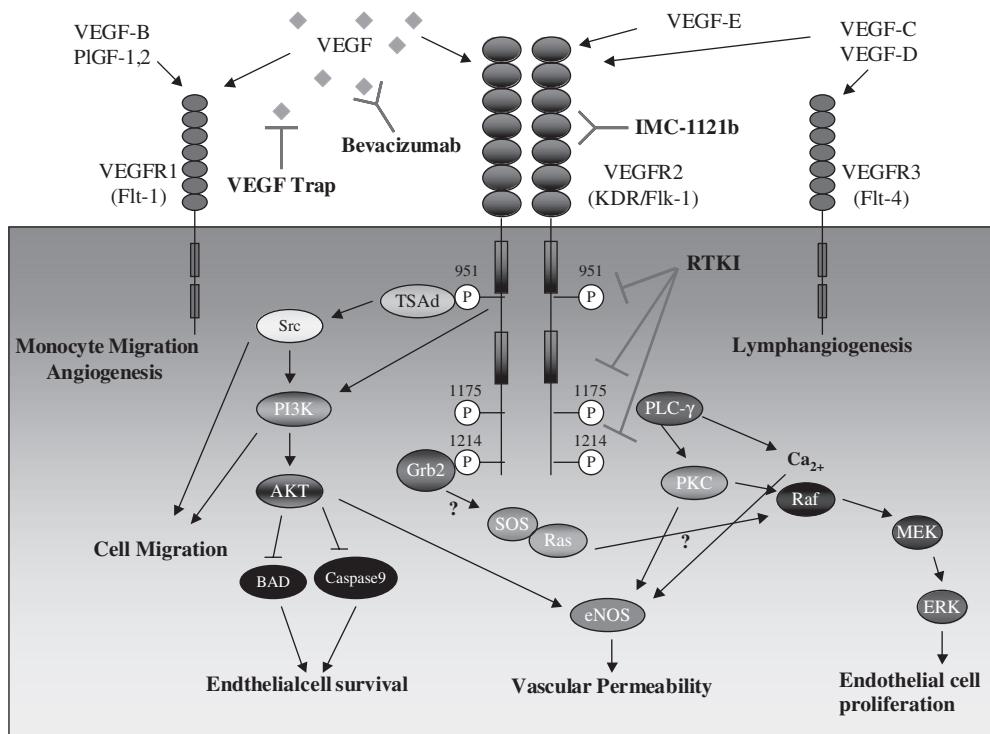


Fig. 1. Vascular endothelial growth factor (VEGF) signal transduction and strategies for pathway inhibition. Upon ligand binding to VEGFR2, signal transduction molecules including Src phospholipase C- γ (PLC- γ), PI3K, Akt, Ras, and mitogen-activated protein kinases (MAPKs) are activated leading to endothelial cell proliferation, migration, survival, and vascular permeability. Tactics to inhibit VEGF signaling include monoclonal antibodies (bevacizumab) or other proteins (VEGF Trap) targeting the VEGF protein, antibodies directed against the receptor (IMC-1121b), and RTKIs. Adapted with permission (8).

received FDA approval for use in non-squamous NSCLC combined with paclitaxel and carboplatin chemotherapy based on the results of the Eastern Cooperative Oncology Group (ECOG) E4599 trial (75).

The activity of bevacizumab in NSCLC was evident in a randomized, phase II trial involving 99 patients with chemonaive stage IIIB (with pleural effusion; “wet”) or IV NSCLC who were treated using first-line therapy with carboplatin (AUC 6) and paclitaxel (200 mg/m²) with or without bevacizumab every 3 weeks (76). Bevacizumab was administered at either 7.5 mg/kg (low dose) or 15 mg/kg (high dose). Treatment with chemotherapy plus high-dose bevacizumab resulted in a higher response rate (RR) than with either low-dose bevacizumab or chemotherapy alone, longer median time to progression, and increased survival. However, severe pulmonary hemorrhage emerged as a serious complication of bevacizumab in this trial. There were six such cases among those who received bevacizumab, four with fatal consequences. Tumor characteristics associated with these hemorrhagic events were central location, proximity to major blood vessels, necrosis and cavitation before or during therapy, and squamous histology. As squamous cell tumors are more often located centrally

Table 2
VEGF Pathway Inhibitors in Clinical Development for NSCLC

| Mechanism | Agent | Target | Phase of development in NSCLC |
|------------------------|-------------|--|--|
| Monoclonal antibody | Bevacizumab | VEGF-A | Phase III-IV. FDA approved for CRC, breast cancer, and NSCLC |
| | IMC-1121B | VEGFR-2 extracellular domain | NA (phase I in solid tumors). |
| Soluble decoy receptor | VEGF Trap | VEGF-A, VEGF-β, and PIGF | Phase II |
| RTKI | ZD6474 | VEGFR-2, EGFR, and RET. | Phase III |
| | Sorafenib | VEGFR-2, VEGFR-3, and PDGFR-β. Also inhibits Raf-1 serine-threonine kinase | Phase III |
| | AZD2171 | VEGFR-1,2,3, PDGFR, and c-kit | Phase II/III |
| | Sunitinib | VEGFR-1, 2, 3, PDGFR, c-kit, and Flt-3 | Phase II |
| | Axitinib | VEGFR-1,2,3, PDGFR, and c-kit | Phase II |
| | AMG 706 | VEGFR-1,2,3, PDGFR, and c-kit | Phase II |

^a Abbreviations: EGFR, Epidermal growth factor receptor; FDA, Food and Drug Administration; NA, not applicable/not studied in NSCLC; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; PIGF, placental growth factor; RTKI, small molecule receptor tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor; and VEGFR, vascular endothelial growth factor receptor.

and have a greater propensity to cavitate than adenocarcinomas, it is unclear whether histology is the primary risk factor for hemoptysis or a surrogate for other risk factors. The pivotal follow-up, phase II/III, randomized E4599 trial excluded patients with squamous histology. E4599 compared standard carboplatin and paclitaxel for six cycles with or without bevacizumab (15 mg/kg) as first-line treatment for wet-stage IIIB/IV non-squamous NSCLC (75). There were 878 patients enrolled. The second interim analysis of this trial found a more than 2 month improvement in median survival with the addition of bevacizumab to chemotherapy, as well as significant improvements in progression-free survival (PFS) and RR (Table 3). The most common grade 3 or higher toxicities associated with bevacizumab were bleeding (4.5 vs. 0.7% in the standard chemotherapy arm) and hypertension (6 vs. 0.7%). There were five deaths due to hemoptysis (1%) and two from gastrointestinal bleeding in the bevacizumab-containing arm. This trial led to the extension of bevacizumab's FDA approval to include use in combination with first-line paclitaxel and carboplatin for advanced non-squamous NSCLC. Interestingly, a recently presented unplanned subset analysis of survival by gender in E4599 found that the survival benefit was confined primarily to male participants although females did benefit in terms of response and PFS (77). The reason for this apparent gender-related difference in benefit is unknown. Bevacizumab is being

Table 3
Randomized Clinical Trials of Angiogenesis Inhibitors for Lung Cancer

| Disease | Treatment | Phase | Endpt | N | ORR (%) | PFS (months) | OS (months) |
|-------------------------|--|--------|-------|-----|---------|------------------|-------------------|
| First-line NSCLC (75) | PC | II/III | OS | 444 | 10 | 4.5 | 10.2 |
| Second-line NSCLC (86) | PCB Doc or Pem [†] + placebo | II | PFS | 434 | 27 | 6.4 | 12.5 ^a |
| | Doc or Pem [†] + BV | | | 41 | 12 | 3 | NR |
| | Erlotinib + BV | | | 40 | 18 | 4.8 | |
| Second-line NSCLC (107) | Doc + placebo | II | PFS | 39 | 18 | 4.4 | |
| | Doc + ZD6474 100 mg | | | 41 | 12 | 2.8 | 13.4 |
| | Doc + ZD6474 300 mg | | | 42 | 26 | 4.3 ^b | 13.1 |
| Second-line NSCLC (83) | Gefitinib | II | PFS | 44 | 18 | 3.9 | 7.9 |
| | ZD6474 300 mg | | | 85 | 1 | 1.9 | NA |
| First-line SCLC (88) | PCDE + placebo | III | OS | 83 | 63 | NR | 8.7 |
| | PCDE + thalidomide | | | 43 | 49 | 82 | 11.7 ^d |

Abbreviations: doc, docetaxel; Endpt, primary endpoint; N, number of patients; NA, not applicable; NR, not reported; ORR, objective response rate; OS, median overall survival; PC, paclitaxel/carboplatin; PCB, paclitaxel/carboplatin/bevacizumab; PCDE, cisplatin, cyclophosphamide, doxorubicin, etoposide; pem, pemetrexed; and PFS, median progression-free survival.

^a*p* = 0.007.

^b*p* = 0.074; trial was designed with pre-specified significance level for PFS as 0.2, so trial met its primary endpoint.

^c*p* = 0.011; trial had crossover design to other treatment, so survival cannot be compared between arms.

^d*p* = 0.02.

^eSquamous histology excluded.

^fChemotherapy was treating physician's choice of pemetrexed or docetaxel.

assessed in combination with other first-line chemotherapy regimens for advanced non-squamous NSCLC, such as gemcitabine and cisplatin.

Numerous clinical trials continue to investigate the use of bevacizumab in other lung cancer settings, including squamous NSCLC and SCLC. A phase II trial is evaluating the safety of bevacizumab-based systemic therapy for advanced squamous NSCLC post-irradiation of the main pulmonary mass. Several clinical trials are considering whether bevacizumab may benefit patients with operable disease in a neoadjuvant or adjuvant setting. ECOG has completed a phase II study (E3501) of first-line cisplatin, etoposide, and bevacizumab in patients with extensive-stage SCLC, and results are awaited. A phase II study with 60 participants with limited-stage SCLC has evaluated chemoradiation with irinotecan and carboplatin, followed by maintenance single-agent bevacizumab (10 mg/kg every 2 weeks for 10 doses) in those with response or stable disease (78). With a median follow-up of 24 months, the median PFS had not been reached, the median survival was 17.5 months, and the 1- and 2-year survival rates

were 70 and 29%, respectively. An ongoing phase II study in the USA is evaluating a paclitaxel–bevacizumab combination in chemosensitive relapsed SCLC.

Antibodies targeting VEGF receptors are in early clinical development. IMC-1121B is a fully human antibody that binds to VEGFR-2 with high affinity, blocking its interaction with VEGF. Among the first 12 patients in a phase I dose-escalation trial in patients with advanced malignancies, there was one unconfirmed partial response (melanoma) and 5 patients with stable disease for greater than 3 months. A fully human monoclonal antibody to VEGFR-3, hF4-3C5, and a bispecific antibody targeting both VEGFR-2 and VEGFR-3 are in preclinical studies.

5.2. *VEGF Trap*

VEGF Trap is a potent angiogenesis inhibitor comprising portions of human VEGFR-1 and VEGFR-2 extracellular domains fused to the Fc portion of human IgG, and it binds VEGF with significantly higher affinity than bevacizumab (Table 2) (79). A number of phase I studies of this agent in patients with advanced solid tumors have shown it to be well tolerated (80). One of these trials reported stable disease of at least 10 weeks duration in 47% of patients with refractory solid tumors. A phase II trial of single-agent VEGF Trap in locally advanced or metastatic platinum- and erlotinib-resistant, non-squamous NSCLC is in progress.

5.3. *VEGF RTKIs*

The most-studied VEGFR TKI in NSCLC to date is ZD6474, an orally administered agent that also inhibits EGFR (Table 2). Its long half-life (>100 h) makes it amenable to once-daily dosing. In phase I studies involving patients with refractory solid tumors, ZD6474 was well tolerated at doses of up to 300 mg/day and demonstrated activity against NSCLC (81, 82). The main reported side effects were facial flushing, facial rash, fatigue, diarrhea, and asymptomatic QTc interval prolongation. A number of phase II studies of ZD6474 alone or in combination with chemotherapy for previously treated NSCLC have subsequently been reported, with promising results and favorable toxicity. These trials included patients with squamous histology.

Single-agent ZD6474 demonstrated significant antitumor activity in a phase II, randomized trial involving 168 patients with locally advanced or metastatic, platinum-refractory NSCLC. Patients received either ZD6474 (300 mg once daily) or gefitinib (250 mg once daily) until disease progression or limiting toxicity, with PFS as the primary endpoint (part A) (83). There was a statistically significant improvement in median PFS with ZD6474 compared with gefitinib (Table 3). Upon progression, patients had the option to crossover to the alternative therapy (part B). In Part B, stable disease for greater than 8 weeks was achieved in 16 of 37 patients (43%) who switched from gefitinib to ZD6474 and in 7 of 29 (24%) who switched from ZD6474 to gefitinib.

In another randomized, phase II trial, ZD6474 was evaluated in combination with docetaxel for the treatment of advanced-stage NSCLC patients previously treated with platinum-containing chemotherapy. One hundred twenty-seven patients were randomized to receive docetaxel (75 mg/m^2) intravenously every 21 days with either placebo, ZD6474 100 mg, or ZD6474 300 mg once daily (84). Toxicities commonly associated with antiepidermal growth factor therapy, such as diarrhea and rash, were

most common with the 300 mg dose of ZD6474. This study met its primary endpoint of prolonged median PFS in the ZD6474 100 mg plus docetaxel arm (Table 3). An international, randomized, phase III trial of docetaxel combined with ZD6474 100 mg or placebo as second-line therapy for locally advanced or metastatic NSCLC is now underway. A randomized, phase II, multicenter study of ZD6474 alone or in combination with standard carboplatin/paclitaxel as first-line treatment for patients with locally advanced, metastatic, or recurrent NSCLC has also been conducted (85), and results are expected in 2007.

ZD6474 is being evaluated in SCLC. The National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG) has completed a phase II randomized trial of maintenance ZD6474 or placebo in patients with either limited- or extensive-stage SCLC who responded to initial chemotherapy or chemoradiation. Results from this trial have not yet been reported.

Sorafenib, another orally bioavailable RTKI, is showing encouraging results in NSCLC (Table 2). It has recently gained FDA approval as monotherapy (400 mg twice daily) for advanced RCC based on significant improvements in PFS in phase II and III trials (86–88). Sorafenib 400 mg twice daily showed evidence of single-agent activity in the second- and third-line settings for advanced NSCLC in a recent single-arm, phase II trial. Although no objective responses by RECIST criteria were reported among 51 evaluable patients, 59% of patients had stable disease and 4 patients had central cavitation of their tumors (89). Thirty-one percent of the participants in this trial had squamous cell carcinoma. There was one case of fatal hemoptysis. This occurred 30 days after stopping sorafenib in a patient with squamous histology and a central cavitary lesion. ECOG is now conducting a phase II randomized discontinuation study of sorafenib in patients with refractory NSCLC (E2501). A randomized, phase III trial is also evaluating sorafenib in combination with 6 cycles of first-line paclitaxel and carboplatin chemotherapy for stage IIIB–IV NSCLC based on evidence of the tolerability and activity of this combination in a phase I trial (90). Patients with squamous histology are eligible provided they have not had significant hemoptysis within the preceding 4 weeks.

AZD2171 is another promising, orally bioavailable RTKI under investigation in lung cancer (Table 2). AZD2171 in combination with paclitaxel and carboplatin chemotherapy has recently been studied in 20 patients with advanced NSCLC in a phase I trial. Toxicities were manageable, including fatigue, anorexia, mucositis, diarrhea, and hypertension (91). Among 15 patients with evaluable disease, there were six partial responses and eight patients with stable disease. NCIC-CTG is now conducting a phase II/III trial of carboplatin and paclitaxel with or without AZD2171 for first-line treatment for stage IIIB–IV NSCLC. Similar to the ZD6474 and sorafenib trials, all NSCLC histologic subtypes are allowed, but patients with a central thoracic lesion with cavitation or clinically relevant hemoptysis within the preceding 4 weeks are ineligible. AZD2171 is also under evaluation as monotherapy for SCLC in a phase II trial.

AMG 706 is yet another multikinase inhibitor with antiangiogenic properties (Table 2). Preliminary data from a phase I trial in patients with advanced NSCLC have shown that AMG 706 can be safely combined with paclitaxel and carboplatin chemotherapy and with panitumumab (a fully human antibody against EGFR), and a phase II extension of this study is planned. A randomized, phase II study of paclitaxel and carboplatin with either bevacizumab or AMG 706 as first-line treatment

for advanced NSCLC is also planned, with objective tumor response as the primary endpoint (79).

Other multitargeted, oral VEGFR TKIs being assessed as monotherapy for previously treated NSCLC are sunitinib and axitinib (AG-013736) (Table 2). Sunitinib (50 mg once daily for 4 out of every 6 weeks) already has FDA approval for use first-line or post-cytokine therapy failure in advanced renal cell carcinoma (RCC) and for use in imatinib-resistant gastrointestinal stromal tumor based on positive findings in phase II and III trials (92–94). Preliminary results from a phase II study of sunitinib (50 mg) monotherapy in 63 patients with previously treated stage IIIB/IV NSCLC reported a partial RR of 9.5% and stable disease in 43% (95). Similar to other TKI trials, squamous histology was allowed, but patients with recent grade 3 hemorrhage or gross hemoptysis were excluded. However, two of the 22 participants with squamous histology died from pulmonary hemorrhage, and another patient with adenocarcinoma had a fatal cerebral hemorrhage, which was subsequently found to be related to a brain metastasis. Nevertheless, further studies with sunitinib continue in NSCLC. A phase II trial of axitinib monotherapy for second-line or later therapy in stage IIIB/IV NSCLC is ongoing, with no available results yet.

5.4. Angiogenesis Inhibitors in Combination with Other Targeted Therapies for NSCLC

The cell signaling pathways involved in the proliferation of cancer cells and tumor angiogenesis are highly complex. If only one point in these cascades is targeted, cancer cells can develop or upregulate alternative survival strategies and therapeutic resistance can emerge. Consequently, combining agents with different targets may obtain greater and more prolonged therapeutic benefit. EGFR signaling plays a role in the regulation of angiogenesis, and the expression of proangiogenic factors, including VEGF and IL-8, is downregulated by EGFR inhibition (61, 96). It has been hypothesized that dual blockade of both the EGFR and VEGF signaling pathways would have additive or synergistic antitumor effects and antiangiogenic effects, and a number of preclinical studies support this concept (97–99). Phase I and II clinical trials of this combination therapeutic strategy are already showing promising results in solid tumors, including NSCLC.

Herbst et al. assessed erlotinib and bevacizumab in a phase I/II study for patients with previously treated non-squamous stage IIIB/IV NSCLC (100). Among 40 participants, there were eight partial responses (20%) and 26 patients with stable disease (65%). The median overall survival and PFS for the patients treated at the phase II doses were 12.6 and 6.2 months, respectively. In a randomized phase II trial, this combination has been compared with chemotherapy alone (docetaxel or pemetrexed) or chemotherapy with bevacizumab (Table 3) (86). The RR was higher in the arm with the combined targeted therapy, and there was a trend to greater PFS and overall survival in both bevacizumab-containing arms compared with the chemotherapy-alone arm, but longer follow-up is awaited. Two multicenter, phase III, randomized, trials in the USA are now considering combination erlotinib and bevacizumab for advanced NSCLC. Several ongoing early-phase studies are also evaluating combinations of VEGFR and EGFR TKIs, such as sorafenib and gefitinib, and sunitinib and erlotinib. Preliminary results on the first 32 patients in a phase I trial of sorafenib and gefitinib found the combination to be well tolerated with one partial response and stable disease in 63% (87).

5.5. Other Angiogenesis Inhibitors

Thalidomide has multiple antitumor effects, including angiogenesis inhibition through unknown mechanisms. It has been assessed in combination with chemotherapy for first-line treatment of extensive-stage SCLC in a randomized, phase III trial (88), with greater toxicities (neuropathy, constipation and requirements for red cell transfusions), higher RR, and greater median survival in the thalidomide-containing arm (Table 3). However, it is difficult to draw any definite conclusions about the efficacy of thalidomide or antiangiogenic agents for SCLC from this trial, because thalidomide has other potential mechanisms of actions (such as its immunomodulatory effects), the trial did not complete accrual, and the number of participating patients was small. Thalidomide has also been evaluated in combination with chemoradiation for locally advanced NSCLC in a completed phase III ECOG trial (E3598), but no results are available yet.

Squalamine is an aminosterol derived from the liver of the dogfish shark and has been shown to have antiangiogenic properties in vitro and in vivo. Its antiangiogenic effects may be due to modulation of the intracellular pH of endothelial cells and inhibition of their proliferation (101). Phase I/II trials of squalamine in combination with paclitaxel and carboplatin chemotherapy for first-line treatment of advanced-stage NSCLC have found the combination to be well tolerated, with RRs of 24–28% and a high rate of disease stabilization (102, 103). However, no phase III trials in NSCLC have been initiated to date.

AE-941 (Neovastat) is a naturally occurring agent derived from shark cartilage, and its proposed mechanisms of antiangiogenesis include inhibition of matrix metalloproteinases, VEGF binding to endothelial cells, and VEGF-dependent tyrosine phosphorylation (104). Based on encouraging phase I/II clinical trial data (105), a phase III, double-blind, placebo-controlled trial of platinum-based chemotherapy and radiotherapy with or without AE-941 in locally advanced NSCLC was conducted. There is preclinical evidence that antiangiogenic agents can synergize with or potentiate the effects of radiotherapy. On interim report of this trial, there was no excess or differential toxicity between the AE-941 and placebo arms (106). The trial has completed accrual, and outcome results are expected in the near future.

6. CONCLUDING REMARKS

Lung cancer remains the leading cause of cancer deaths. In recent years, it had become increasingly apparent that a therapeutic plateau had been achieved with cytotoxic chemotherapy for patients with advanced disease, and new therapeutic approaches would need to be pursued. Recent clinical results suggest that antiangiogenic therapy is one such approach. The addition of bevacizumab to chemotherapy prolonged overall survival and improved RRs in patients with previously untreated NSCLC (75), and promising results have been observed with other antiangiogenic agents, either in combination with chemotherapy or other targeted agents. The improvements have been relatively modest thus far, however, and it appears that tumor progression inevitably occurs. In addition, unexpected toxicities such as pulmonary hemorrhage have been observed with agents in this class. Areas for future investigation include identifying factors other than VEGF that are critical in the angiogenic cascade, elucidating mechanisms of therapeutic resistance, and developing markers for

identifying patients most likely to benefit (or experience toxicity) from antiangiogenic treatment. Progress in these areas will be critical in order for realizing the full potential benefits that antiangiogenic therapy may provide for patients with lung cancer and other solid tumors.

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SUMMARY

The use of antiangiogenic therapy for the treatment of renal cell cancer (RCC) has revolutionized the treatment of a disease, which for the better part of the last generation lacked a highly effective therapy. Prior standard options such as interferon-alpha (IFN- α) or IFN- β were not much better than placebo and were associated with toxicities that worsened the quality of life of many patients. Interleukin-2 (IL-2), while able to induce complete responses in a small percentage of patients, was limited to those subjects who were young enough and healthy enough to tolerate this intensive hospital-based therapy. Appreciating the pathophysiologic role that angiogenesis plays in RCC, numerous investigations have been carried out with a variety of antiangiogenic agents. Superiority of these new drugs to the time-tested standards has been defined, which has resulted in the establishment of effective standard treatments that prolong life for patients with RCC.

Key Words: Renal cell carcinoma; kidney cancer; antiangiogenic; Targeted therapy.

1. INTRODUCTION

Kidney cancer accounts for about 2% of all cancers in the USA each year, and it is estimated that there will be over 35,000 new cases and more than 12,000 deaths because of kidney cancer this year (1). Unlike many cancers that are detectable through screening of serum markers or by simple physical examination, kidney tumors can evolve over long periods of time without causing signs or symptoms and for this reason are typically quite large when initially identified. Surgical resection of isolated primary tumors by either complete or partial nephrectomy is the mainstay of therapy for clinically localized disease. For the majority of patients with early stage disease, this procedure is usually curative though patients with higher Fuhrman grade tumors have a greater likelihood of recurrence. Although numerous trials using radiation therapy and immunotherapy have been conducted, there is currently no effective post-operative

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adjuvant therapy to reduce the risk of relapse after surgery (2–4). Overall, about a third of either present with metastatic disease or develop metastases following treatment for clinically localized disease. Cytotoxic chemotherapy has been mostly ineffective for the treatment of kidney cancer, making it one of the few types of human cancers for which there had been no standard chemotherapy in the twenty-first century (5, 6).

Renal cell carcinoma is composed of three main histological subtypes including the clear cell variant comprising approximately 70–80% of cases, papillary renal cell cancer (~10–20% of cases), and chromophobric renal cell cancer (RCC) (~5–10% of cases) (7). All three subtypes have well-defined, associated chromosomal mutations and familial associations, but it is the clear cell variant for which the use of antiangiogenic therapy has the strongest rationale. Clear cell carcinoma of the kidney (CCRCC) is characterized by the frequent loss of the von Hippel–Lindau (vHL) tumor suppressor gene, resulting in the loss of one of the critical mechanisms for regulating the level of hypoxia-inducible factor 1-alpha (HIF-1 α) and the production of high levels of vascular endothelial growth factor (VEGF) by the tumor cell (8). Although both blood and urinary levels of VEGF have been found to be elevated in RCC, the use of these elevations as prognostic markers is questionable. Elevated levels appear to be associated with more high-grade lesions as well as more advanced staging, both of which are associated with a poorer prognosis (9). Hence, although the use of antiangiogenic therapy, and specifically VEGF-directed therapies, has a sound pathophysiologic rationale in the clear cell variant of RCC, it is different than other molecularly targeted therapies where, as in the case of gastrointestinal stromal tumors, the use of imatinib is based on an addictive mutation of the c-kit protein that drives proliferation of the tumor. In the case of VEGF and clear cell RCC, the mechanism of the antitumor effect is external to the tumor and therefore somewhat less clear.

The use of immunotherapy for the treatment of RCC including high-dose interleukin-2 (HD IL-2), the interferons (IFNs), and combinations of agents in this field helps the minority of patients (10, 11). Only HD intravenous IL-2 has the ability to produce long-lasting responses in patients with metastatic disease though because of its toxicities, this therapy is limited to those younger patients without severe intercurrent medical contraindications. Although selection of patients with a greater likelihood of response to HD IL-2 holds promise that this therapy will continue to be used in selective patients, it is clear that there is a major need for new therapeutic strategies for the treatment of RCC.

2. THE ROLE OF VHL IN KIDNEY CANCER ANGIOGENESIS

The majority of tumors that arise in the kidney are of epithelial origin arising from the renal tubules. Of these, the conventional type of renal cell carcinomas (previously referred to as the clear cell histology) are the most common, accounting for about approximately 70% of all kidney cancers (7). The pathogenesis of RCC was proffered following the discovery of the vHL tumor suppressor gene in the familial cancer syndrome from which the gene was named (12). The vHL syndrome is an autosomal dominant familial cancer syndrome composed of multiple vascular tumors, such as retinal angiomas, cerebellar hemangioblastomas, pheochromocytomas, pancreatic islet cell tumors, and conventional renal cell carcinomas. Based on research conducted by Linehan et al., the vHL tumor suppressor gene was identified and subsequently localized to the long arm of chromosome 3 at 3p25-26 locus. In over half of the family members affected by this mutation, conventional RCC develops as a multi-focal disease due to the inherited germline mutation in

one vHL allele and the acquired loss or silencing of the second vHL allele (12–14). In contrast to the patients with vHL syndrome, most sporadic clear cell (CC)-RCCs have both vHL alleles inactivated, either by mutation (50–60% frequency) or promoter silencing by hypermethylation (an additional 25% frequency) (12, 13, 15, 16).

The vHL gene product (pvHL) is a 213 amino acid protein that typically forms stable complexes containing other proteins such as elongin B and elongin C. The most important function of pvHL appears to be binding to target proteins in the multi-subunit ubiquitin ligase complex (12). The most important pvHL target protein is the alpha subunit of HIF-1 α , and the inherited or acquired loss of functional pvHL leads to the accumulation of high levels of HIF-1 α (14, 17). For this reason, HIF-1 α is often found at high levels in RCCs compared with adjacent normal kidney, where the physiologic post-translational regulation of HIF-1 α maintains levels of this protein that are normally undetectable (18). HIF-1, composed of HIF-1 α and HIF-1 β subunits, controls the expression of hypoxia response genes, such as VEGF, erythropoietin, and GLUT-1, and it is believed that this is the pathophysiologic mechanism for the expression of high levels of VEGF by RCCs (14, 18) (Fig. 1).

3. AGENTS TARGETING THE VEGF PATHWAY

The above noted pathophysiologic rationale has provided significant support for the use of antiangiogenic therapy, specifically drugs targeting VEGF in the treatment of CC-RCC. In contrast, the other types of RCC such as the papillary and chromophobe histologies do not appear to have the same biologic rationale for the use of these agents and likely represent critically different therapeutic targets.

At the present time, numerous antiangiogenic agents have been or are being studied in the treatment of CC-RCC. Antiangiogenic agents being studied in CC-RCC can be classified into those targeting VEGF directly (such as bevacizumab, sunitinib, or sorafenib) and those targeting other aspects of angiogenesis such as the thrombospondin-1 mimetic ABT 510. Thus far, only the former class of agents has demonstrated benefit in randomized trials though the approval of several new agents for this indication has generated a foundation on which subsequent combination trials can be explored.

3.1. Bevacizumab

The first indication of possible activity for bevacizumab in CC-RCC began to emerge from the initial phase I trial of this agent in subjects with refractory cancer (19). Stabilization of previously progressing disease in several patients with metastatic RCC was seen and promoted the rationale for the subsequent trials conducted by Yang et al. In this study, subjects whose tumors were either cytokine refractory or who were IL-2 ineligible were randomized to one of three arms including a placebo arm, low-dose bevacizumab, and HD bevacizumab (20). All patients had measurable disease, and while initially allowed, patients with bone metastases were excluded from the study early on due to the concern that progression in bone metastases may limit the ability for patients to be on a treatment that would likely require a long period of time to work. Overall, 116 patients were randomized to the three arms including low-dose bevacizumab (4.5 mg/kg on day 0 followed by 3 mg/kg on day 7 and every other week thereafter), HD bevacizumab (15 mg/kg on day 0 followed by 10 mg/kg on day 7

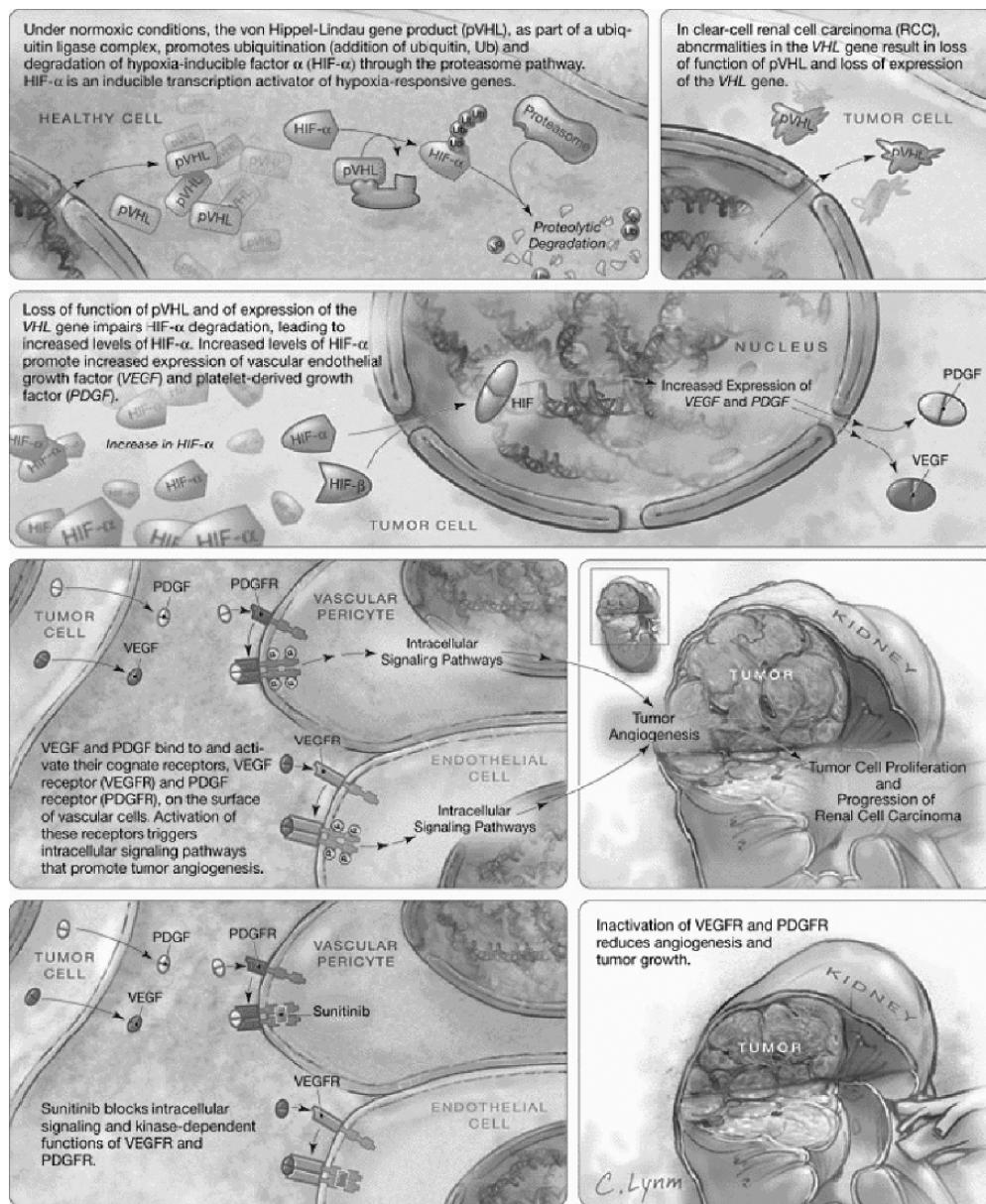


Fig. 1. Role of von Hippel-Lindau (vHL) in pathogenesis of conventional renal cell cancer (RCC). Reproduced with permission (29). (Please see color insert.)

and every other week thereafter), or placebo (administered on the same schedule). The primary endpoint of the trial was time to disease progression, and patients with progressive disease were allowed to cross over to active therapy. For this reason, criteria for progressive disease was conservative and was defined as a 50% increase in the area of any single lesion [as opposed to a more standard response evaluation criteria in solid tumors (RECIST) criteria being applied to more modern studies]. The study was

stopped by the data safety and monitoring board at the second planned interim analysis when the time to progression endpoint passed a pre-specified boundary for efficacy at enrollment of 116 patients. The median time to progression for the HD bevacizumab arm was 147 days compared to only 41 days in the placebo arm ($p < 0.001$). The low-dose bevacizumab arm also has a significantly better median TTP with a value of 97 days ($p = 0.041$) compared with placebo. There were no objective responses seen in either the placebo or the low-dose therapy arm, whereas 4 of the 40 patients on the HD bevacizumab arm experienced a partial response (using standard WHO criteria) lasting 6, 9, 15, and 39+ months. The therapy was well tolerated with expected side effects of hypertension seen in 36% of the patients (20% grade 3 requiring the initiation of antihypertensive therapy)—malaise, mild proteinuria, and epistaxis being seen more frequently in the treated compared with the placebo arm. Importantly, there were no serious tumor-related or unrelated bleeding events seen.

Hainsworth et al. studied the combination of bevacizumab and erlotinib in patients with CC-RCC based on pre-clinical data suggesting synergy for this combined approach to cancer therapy (21). In an initial trial, 63 subjects with metastatic RCC were treated with bevacizumab 10 mg/kg intravenously every 2 weeks and erlotinib 150 mg by mouth (PO) daily (22). Fifteen of 59 patients (25%) assessable for response had an objective partial response, and another 61% had evidence of stable disease after 8 weeks of therapy. The median time to progression was 11 months though 68% of the patients were previously untreated making comparison to the Yang data difficult. As a result of this initial report, a subsequent randomized, placebo-controlled phase II trial of bevacizumab \pm erlotinib was conducted. Presented at the annual meeting of the American Society of Clinical Oncology in 2006, the study enrolled 53 subjects to the bevacizumab arm and 51 to the bevacizumab + erlotinib arm (23). The objective response rate was 13 and 14%, and the progression-free survival (PFS) was 8.5 and 9.9 months for the single-agent and combination arms, respectively. These results were not statistically significantly different, thereby drawing the conclusion that the combination arm is no more active than the single-agent bevacizumab arm.

Two studies assessing the role of bevacizumab in combination with IFN- α in previously untreated patients with metastatic RCC are underway. The first, a European trial termed the AVOREN study, has completed initial analysis and preliminarily reported a statistically significant improvement in PFS for the combination of IFN-alpha + bevacizumab compared with IFN-alpha alone. The second, an American trial being led by the CALGB, has completed accrual though data for the primary endpoint of overall survival is not yet available.

3.2. Sorafenib (Nexavar; BAY43-9006)

Sorafenib is the first multi-kinase inhibitor proven to impact on survival of patients with refractory CC-RCC and the first to be approved by the FDA for this indication. Initially developed as a raf kinase inhibitor for use in melanoma, this agent failed to demonstrate significant single-agent activity in that disease. Further exploration of its kinase inhibitory activity revealed that in addition to the raf kinases, sorafenib also inhibited the VEGF receptor kinases including kinase-insert domain receptor (KDR) (VEGF R2). In an initial phase I trial, approximately half the patients had stable disease, and one objective response was seen in a patient with metastatic RCC suggesting some antitumor activity in this setting (24). As a result, patients with RCC were included

in a subsequent randomized discontinuation study that was performed, which included patients with RCC (25). Overall, 202 patients with RCC were accrued, and all patients received initial therapy with sorafenib 400 mg PO BID. After the 12-week run-in period, 73 patients had at least 25% shrinkage of their tumor and continued on active therapy. In addition, 65 patients had evidence of stable disease and were subsequently randomized to either sorafenib or placebo for the next 12 weeks. At the end of the 24-week period, half of the patients randomized to receive sorafenib remained progression free, whereas only 18% of the placebo-treated patients were free from progression

(Fig. 2). This difference was statistically significant at a *p* value of 0.0077. As a result of the data generated from this trial, a subsequent international randomized, placebo-controlled phase III trial in cytokine-refractory subjects with CC-RCC was conducted. Escudier et al. (26) presented the results of this study at the annual meeting of the American Society of Clinical Oncology in 2006 with 769 patients with CC-RCC randomized to either placebo or sorafenib 400 mg PO BID with a primary endpoint of overall survival and a secondary endpoint of progression-free survival. The partial response rate for sorafenib was 2% with another 78% of patients experiencing stable disease while in the placebo arm, there were no objective responses and only 55% of patients with stable disease. Median PFS for the sorafenib-treated patients was 24 weeks compared with 12 weeks for the placebo patients (*p* < 0.000001) (Fig. 3). In a planned interim analysis, the rate of overall survival was longer with sorafenib than placebo with a hazard ratio of 0.72 based on 220 deaths (95% CI = 0.55–0.95) though the analysis did not meet the pre-specified criteria for statistical significance. In a randomized phase II trial of previously untreated patients with metastatic CC-RCC,

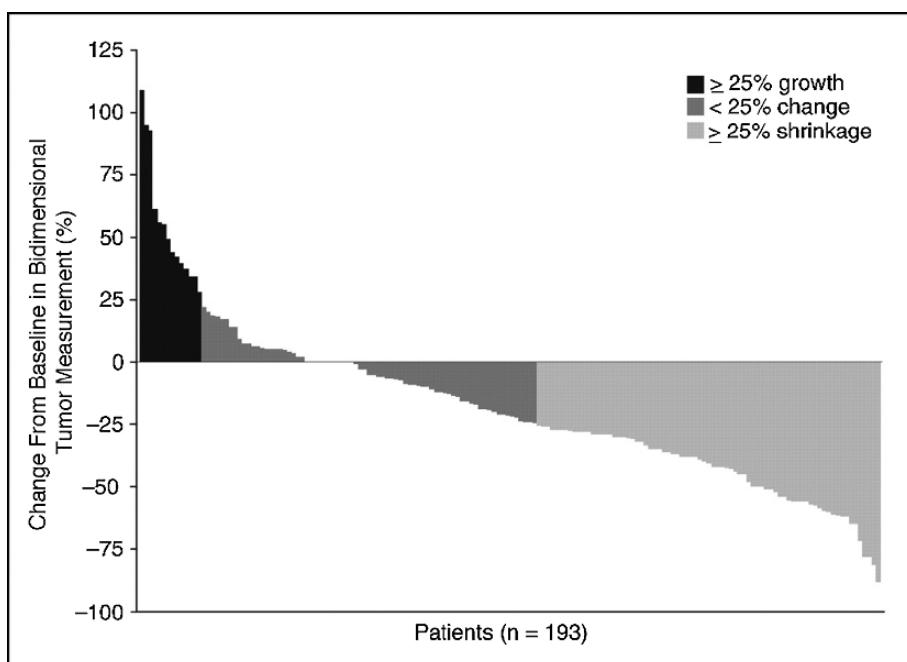
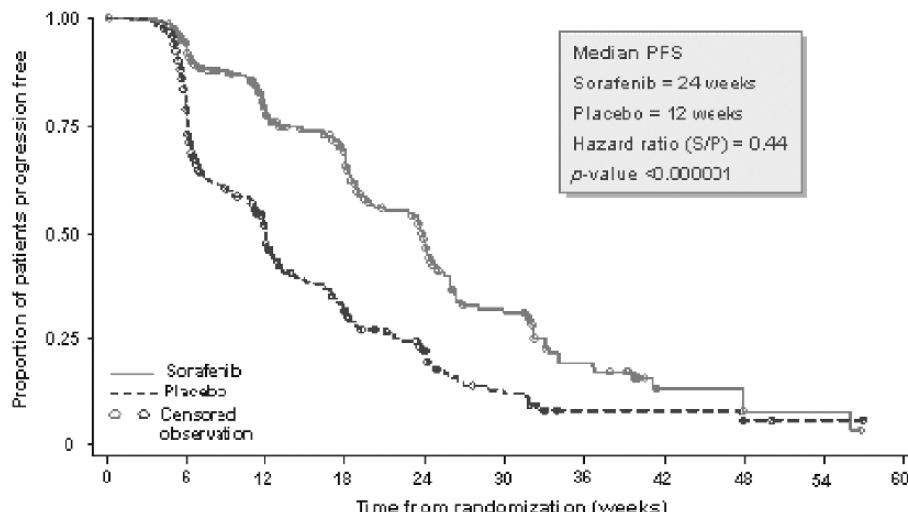


Fig. 2. Changes from baseline in investigator-assessed, bidimensional radiographic measurements at 12 weeks for patients with renal cell carcinoma.

TARGETs***Progression-Free Survival Benefit****

*Independently assessed

Fig. 3. Improvement in progression-free survival for sorafenib compared with placebo in patients with cytokine-refractory renal cell carcinoma. Reproduced with permission (26). (Please see color insert.)

189 patients were randomized to receive either sorafenib 400 mg PO BID or IFN- α 9 MIU s.c. thrice weekly. At the time of its presentation in June 2006, data were not available to assess the role of sorafenib compared with IFN-alpha in this setting, and the data safety and monitoring board recommended an increase in the number of events before an analysis was undertaken (27). On the basis of the dramatic results from the randomized phase III trial, sorafenib was approved by the US FDA and became the first commercially available targeted therapy indicated for patients with metastatic RCC. Trials assessing combinations with sorafenib including combinations with bevacizumab as well as other investigational antiangiogenic agents are ongoing.

3.3. Sunitinib (Sutent; SU 11248)

Sunitinib or SU 11248 is another orally administered agent with broad tyrosine kinase inhibitory activity. Targeting the c-kit, platelet-derived growth factor receptor-beta (PDGFR- β) as well as KDR and the other VEGF tyrosine kinase receptors, it appeared uniquely suited to be applied in patients with metastatic RCC. In an initial single-center trial published by Motzer et al. (28), 63 evaluable cytokine-refractory patients with RCC were treated with sunitinib 50 mg PO daily for 4 out of 6 weeks. Twenty-five (40%) of the 63 patients achieved a partial response with another 17 patients (27%) experiencing stable disease for at least 3 months. The median time to progression was 8.7 months, and toxicities were typical for this class of drugs including fatigue and constitutional symptoms as well as mild rash and leucopenia (Fig. 4). In a subsequent publication, a subsequent phase II multi-center trial was presented with

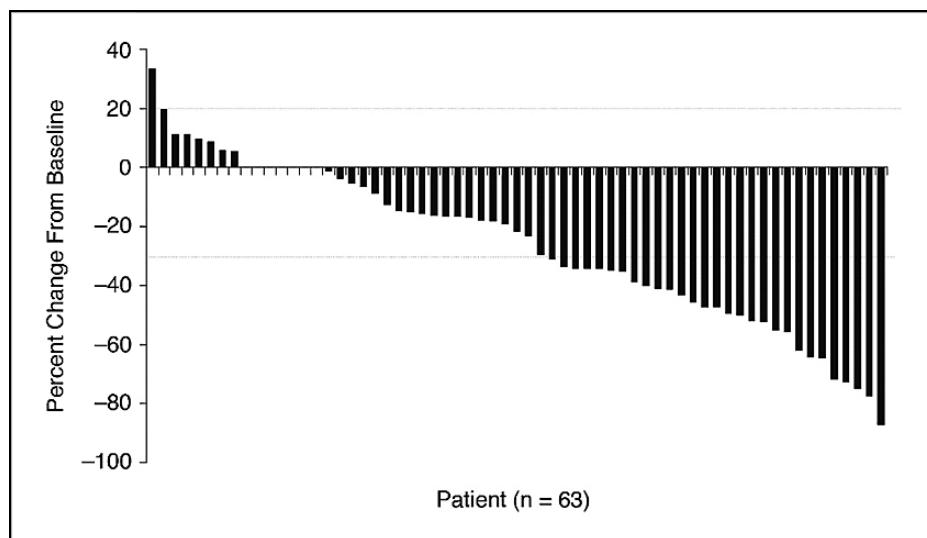


Fig. 4. Maximal percentage of tumor reduction for target lesions by response evaluation criteria in solid tumors (RECIST).

106 patients with metastatic clear cell RCC treated on an identical dose and schedule as the above noted trial (29). One hundred five patients were evaluable for efficacy analysis, and the objective response rate, based on independent radiologic review, was 34% (95% CI = 25–44%). Similar to the initial trial, the median PFS for these patients was 8.3 months. Again, side effects were composed of a now typical listing including fatigue, diarrhea, and a variety of benign laboratory findings. On the basis of the findings of these two phase II trials, a randomized phase III trial comparing sunitinib to IFN- α in previously untreated patients with metastatic clear cell RCC was conducted. Presented at the annual meeting for the American Society of Clinical Oncology in 2006, the trial randomized 750 patients to either standard sunitinib (4 weeks on/2 weeks off) or IFN- α (30). At the time of presentation, there was 1 complete response and 136 partial responses in the sunitinib group (overall RR = 37%) compared with 33 partial responses in the IFN group (ORR = 9%). These numbers dropped slightly to 31 and 6% response rates after independent radiologic review. Median PFS for sunitinib was 11 months compared with 5 months for the IFN arm ($p = 0.000001$) (Fig. 5). Most importantly, although the median overall survival had not been reached for either arm, sunitinib was demonstrating a statistically superior survival compared with IFN in the early analysis (HR = 0.65, $p = 0.0219$). The conclusion of the study was that the superiority seen in this trial established sunitinib as the new reference standard for first-line therapy for CC-RCC, and on the basis of the above data, sunitinib was subsequently approved by the US FDA for the treatment of patients with metastatic renal cell carcinoma. At the present time, additional studies with sunitinib to refine the dose, schedule, and the role for combination therapy are underway.

3.4. Vatalanib (PTK787)

Among the tyrosine kinase inhibitors being studied in CC-RCC, the investigational agent vatalanib was assessed in a phase I/II trial (31). The study assessed doses ranging

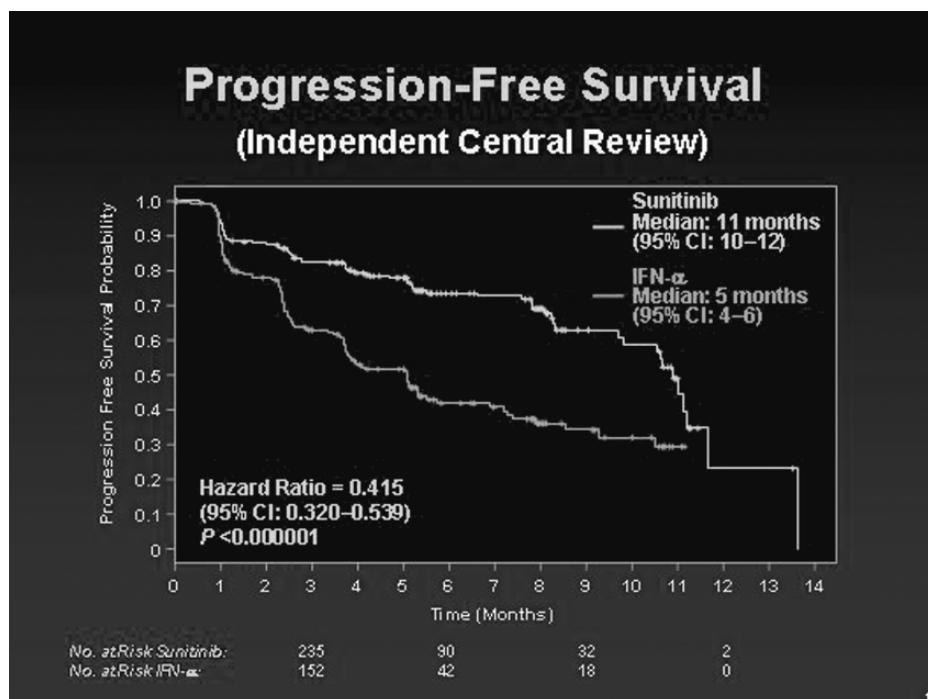


Fig. 5. Improvement in progression-free survival for sunitinib compared with placebo in patients with previously untreated renal cell carcinoma. Reproduced with permission (30). (Please see color insert.)

from 300 to 1500 mg/day administered orally. Patients underwent evaluation for safety, and in addition, diffusion contrast enhanced MRI, and MRI for arterial spin labeling was performed to assess vascular permeability and arterial flow. Forty-nine patients were accrued to the study of whom eighteen were in the dose escalation portion and a total of 42 patients were assessed for tumor response. Dose-limiting toxicity was composed of one patient at the 1000 mg dose level who experienced a grade 3 headaches and one patient at the 1500 mg dose level who had grade 3 hypertension. Other common side effects that occurred at a grade 1 or 2 level included nausea, vomiting, fatigue, headache, and dizziness. Pharmacokinetics appeared to demonstrate a plateau of the area under the curve (AUC) above a dose of 750–1000 mg/day. Only two patients demonstrated a partial response (5%) with 14% experiencing a minor response. Similar to what has been seen with other VEGF inhibitors, stable disease was seen in up to 60% of the patients treated. Median time to progression for this previously treated group of patients was in the range of 5.5 months, which is surprisingly similar to the results with bevacizumab generated by Yang et al. Pharmacodynamic assessment of permeability and flow suggested that PTK787 has the ability to decrease tumor permeability and blood flow at doses above 1000 mg/day. Subsequent trials have suggested that dosing of this agent in a BID schedule may have better tolerability (32). At the present time, no subsequent trials in RCC are underway or planned with this agent.

3.5. SU5416

Less effective signal transduction inhibitors that block KDR have been studied in RCC. Examples include the first generation KDR inhibitor SU5416. This agent specifically inhibited KDR and has been studied in RCC both as a single agent and in conjunction with IFN- α in patients with previously untreated RCC. As a single agent, a study of 29 patients produced one minor response and five with stable disease. The median progression-free survival in these patients was 59 days and tends to be shorter than that seen with more recently studied antiangiogenic agents (33). In the combination trial with IFN- α , 30 patients were accrued with all evaluable for response (34). One patient experienced a minor response, and 15 had stable disease for an overall disease control rate of 53%. Three of these patients received protocol therapy for greater than 8 months. Median survival was 10 months with a median event-free survival of 5 months and a 1-year event free survival (EFS) of 6%, which fell far below the predicted 20% rate for 1-year EFS. As a result of the significant toxicities that was seen, this agent has no significant role in the management of this disease.

Among the TKIs that have been studied in CC-RCC perhaps to one with the most clinically significant data is that of the Pfizer product AG-013736. This orally administered agent was studied in a group of 52 RCC whose tumors had progressed on one prior cytokine-based regimen (35). Dosed at 5 mg PO BID, the drug was well tolerated with toxicities of hypertension, fatigue, and diarrhea seen in 40% or more of the patients though the latter two adverse events were grade 3 or greater in only 8% of the cases. The objective response rate was 46% (95% CI = 32–60) and a stable disease rate of 40% with 38% having some shrinkage as a component of their stable disease. The median time to progression was not reached, and responses were seen in all sites of metastatic disease including bone. The development of AG-013736 is slowed somewhat in RCC by the approval of sunitinib as both agents are being developed by the same company, but consideration for the use of multiple agents offering potentially different spectrums of activity will likely warrant further drug development.

3.6. Other VEGF Inhibitors

A number of other VEGF inhibitors are in earlier phase trials, and activity in RCC is predicted. Examples are the VEGF Trap, a chimeric soluble fusion protein that consists of the active binding site for KDR and flt-1 on a Fc backbone. Phase I trials with this agent have been performed and given the degree of activity noted above, it is anticipated that subsequent studies in RCC may have similar or superior activity to previously explored agents (36). Other agents including antibodies specifically directed against KDR are in phase I trials, and it is expected that as new VEGF inhibitor strategies are developed, they will be focusing on RCC because of the inherent relationship between VEGF and CC-RCC.

4. AGENTS TARGETING OTHER ANGIOGENIC PATHWAYS

The matrix metalloproteinase (MMP) inhibitors were among the first antiangiogenic agents studied in cancer patients. Their ability to inhibit MMP-2 and MMP-9, important in the early stages of angiogenesis, was assessed in a series of large trials in more commonly seen malignancies, with negative outcome. The MMP inhibitor, AE-941 (Neovastat), was initially studied in randomized trial comparing two doses of therapy

(60 vs. 240 ml) in 144 patients with refractory solid tumors of whom 22 had refractory RCC (37). The results of this two-arm study led to a randomized placebo-controlled phase III trial in patients with immunotherapy-refractory RCC (38). In this trial, 302 patients were enrolled and randomized between either AE-941 (at a dose of 120 ml) and placebo orally twice daily. No difference in survival was seen for the overall population indicating that this drug, as a single agent, had no significant activity in patients with refractory RCC.

Additional agents with proposed antiangiogenic that have failed to demonstrate activity in RCC include carboxyamidotriazole (CAI), an agent that inhibits calcium flux and thereby inhibit endothelial cell motility (39, 40), TNP-470 (41), and thalidomide. The latter agent while having some ability to induce stabilization of disease in phase II trials failed to demonstrate an impact on progression-free or overall survival in a randomized phase III trial comparing low-dose IFN- α to low dose IFN- α with thalidomide (42).

One additional agent explored in this disease is the thrombospondin-1 mimetic nonapeptide ABT 510 which was studied in two phase I trials (43, 44). Based on at least one responding patient with CC-RCC, a randomized phase II trial in previously untreated RCC was conducted and reported with minimal antitumor activity though time to progression suggested some activity that may be appropriate to assess in combination with some of the newer targeted therapies approved for this disease (45).

5. CONCLUSIONS

The rationale for the use of antiangiogenic therapy for the management of clear cell RCC is based on the prevalence of the mutations of the vHL gene and the associated up-regulation of VEGF production. The use of VEGF-specific targeted agents such as bevacizumab appears to be associated with an antitumor activity with approximately 10–15% objective response rates. Newer orally administered tyrosine kinase inhibitors such as sunitinib appear to be associated with higher response rates suggesting that an optimized combination of specific targets may produce superior antitumor activity (see Table 1 for comparison of existing response and PFS data). For this reason, combinations with other targeted therapies such as inhibitors of mTOR, other receptor-based pathways, or unique targets make sense in future therapeutic development. Because of the significant activity seen, adjuvant trials in high-risk patients have been initiated and promise to potentially reduce the frequency of relapse, particularly in the patients with highest risk disease. Importantly, it is not clear that these agents, multi-targeted as they are, will

Table 1
Comparison of Outcome Data for Bevacizumab, Sorafenib, and Sunitinib in Patients with Previously Untreated and Cytokine-Refractory Metastatic RCC

| <i>Drug</i> | <i>Previously untreated</i> | | <i>Cytokine refractory</i> | |
|-------------|-----------------------------|----------------------------|----------------------------|----------------------------|
| | <i>ORR (%)</i> | <i>Median PFS (months)</i> | <i>ORR (%)</i> | <i>Median PFS (months)</i> |
| Bevacizumab | 13 | 8.5 | 10 | 5 |
| Sorafenib | 0 | NR | 2 | 6 |
| Sunitinib | 37 | 11 | 34 | 8.3 |

NR, not reported; ORR, objective response rate; and PFS, progression-free survival.

not be efficacious in other histologies of RCC such as the papillary RCC population that has a common mutation of the c-met receptor but typically not vHL or the chromophobie histology, which rarely is associated with vHL abnormalities. Subsequent trials in these specific subgroups would be necessary to definitively answer these questions.

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26

Antiangiogenesis Therapies in Gynecologic Malignancies

Robert A. Burger, MD

SUMMARY

This chapter addresses the epidemiology, pathogenesis and current therapeutic strategy for common gynecologic malignancies; angiogenesis as a mechanism of disease progression; pre-clinical knowledge on angiogenesis-specific therapeutic targets; and the status of clinical trials incorporating antiangiogenic agents.

Key Words: gynecologic; ovarian cancer; endometrial cancer; cervical cancer; biomarkers; clinical trials

1. INTRODUCTION

Malignancies affecting the female genital system are highly diverse with respect to pathogenesis, clinical behavior, and response to therapeutics. Carcinomas of the ovary, uterine corpus, and cervix have by far the greatest impact on public health regard to incidence, morbidity, and mortality. As would be expected, these gynecologic cancers have been the most well studied in terms of angiogenesis as a mechanism of disease progression and as a therapeutic target; hence, they are the focus of this chapter.

2. OVERVIEW OF COMMON GYNECOLOGIC MALIGNANCIES

2.1. Epithelial Ovarian Cancer

As demonstrated in Table 1, ovarian cancers, more than 95% epithelial in origin, represent the most lethal site of gynecologic malignancy in the USA, with an estimated 20,180 new cases and 15,310 deaths per year (1).

The epithelial tumors are thought to arise from inclusion cysts formed by invagination of the surface peritoneal lining during ovulation; less commonly, similar tumors may develop directly from transformed abdominal peritoneum (2). Because of their indistinguishable appearance and biologic behavior, epithelial ovarian and primary peritoneal cancers are generally combined as a single entity. Several histologic types and combinations thereof have been identified, resembling cancers of upper genital tract origin. These include serous, endometrioid, mucinous, and clear cell adenocarcinomas. Following invasion of the external capsule of the ovary, epithelial ovarian cancers tend

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Table 1
US Incidence and Mortality for Female Solid Tumors—2006

| Disease site | New cases | Deaths | Lethality (deaths/new cases) |
|----------------|-----------|--------|------------------------------|
| Pancreas | 16,580 | 16,210 | 0.98 |
| Lung | 81,770 | 72,130 | 0.88 |
| Ovary | 20,180 | 15,310 | 0.76 |
| Uterine cervix | 9,710 | 3,700 | 0.38 |
| Colorectal | 75,810 | 27,300 | 0.36 |
| Breast | 212,920 | 40,970 | 0.19 |
| Uterine corpus | 41,200 | 7,350 | 0.18 |

to exhibit a dual pattern of spread, primarily metastasis along the visceral and parietal peritoneum with subsequent invasion into underlying organs and secondarily through lymphatic metastasis, most commonly to the iliac and aortic-caval lymph nodes.

Most, but certainly not all epithelial ovarian cancers are diagnosed in post-menopausal women. Risk factors include family history of ovarian and/or breast cancer (particularly with pre-menopausal onset and with multi-focal disease) and reproductive factors that appear to relate directly to the total number of lifetime ovulations (3). Despite somewhat effective primary preventive approaches, such as prophylactic surgery in high-risk women and the use of oral contraceptives in the general population, incidence rates have yet to decline convincingly. Furthermore, owing to shortcomings in early detection methods, at the time of initial presentation, the over 80% of patients are found to have metastatic disease, the vast majority beyond the true pelvis (stages III and IV), most with malignant ascites and with the omentum typically representing the most burdensome site of abdominal metastasis. Key clinical and pathologic prognostic factors include stage and extent of residual disease following initial cytoreductive surgery, age, performance status, and tumor grade (4).

After initial abdominal surgery for diagnosis, staging, and cytoreduction (debulking), the standard front-line systemic chemotherapy for women with advanced epithelial ovarian and primary peritoneal cancer consists of a platinum and taxane chemotherapy combination (5), usually carboplatin and paclitaxel (6). With advances in surgical management, peri-operative care, the incorporation of taxanes into standard primary therapy, and the discovery of several other active non-platinum cytotoxic agents, the median survivals for patients with advanced stage III and IV disease appear to have improved somewhat since the mid-1980s, now estimated to be approximately 3 years for patients with greater than 1 cm diameter largest residual tumor implants before primary chemotherapy and 4 years for patients with no greater than 1 cm residual disease (7). Nevertheless, long-term survival rates have still been disappointing. Several alternative approaches have been explored using conventional cytotoxic agents, including the selected use of neo-adjuvant chemotherapy, consolidation (maintenance) therapy, regional (intra-peritoneal) chemotherapy, and the integration of new agents. Still most patients develop recurrent disease, which despite some success with conventional cytotoxic therapy typically leads to refractory abdominal carcinomatosis, intestinal obstruction, and life-threatening malnutrition.

2.2. *Carcinoma of the Uterine Corpus (Endometrial Carcinoma)*

Endometrial carcinoma is the most common gynecologic malignancy, representing the vast majority of the 41,200 new cases of uterine cancer and most of the 3750 uterine cancer deaths estimated in the USA for 2006 (1). The median age at diagnosis is approximately 58 years. Histologically, the majority of endometrial cancers are endometrioid adenocarcinomas; less commonly, other Mullerian histologic types are observed (see Section 2.1.). Two general clinical patterns have been described. The more common type (type I) is comprised almost exclusively of endometrioid tumors engendered in part by endogenous or exogenous estrogen excess. Such tumors appear to arise within foci of atypical endometrial hyperplasia, are usually well or moderately well differentiated, and generally behave in an indolent manner. Risk factors for the development of type I tumors include chronic anovulation, obesity, late menopause, and the use of exogenous estrogens unopposed by progestins. Failure of preventive measures, such as weight control and management of chronic anovulation, is the most likely explanation for the continued rise in incidence for endometrial cancers in the USA. Type II tumors are relatively uncommon, usually are diagnosed in post-menopausal women, tend to be poorly differentiated endometrioid, serous, or clear cell cancers, and behave more aggressively than type I cancers (8). Although risk factors for type II tumors remain relatively elusive, these tumors appear to be more highly associated with African/African-American descent, mutational loss of p53 function, and over-expression of HER-2 than are type I tumors.

Following invasion into the underlying myometrium, carcinomas of the endometrium are associated with a tri-fold pattern of spread: (i) direct extension through the myometrium, caudally into the cervix and cephalically into the adnexal structures, para-cervical connective tissue, and adjacent organs; (ii) lymphatic metastasis, initially to the iliac and aortic-caval nodes; and (iii) intra-peritoneal spread, not unlike that seen with epithelial ovarian cancers. Rarely, distant hematogenous metastases, for example to the liver or lung, are identified at the time of diagnosis. After histologic diagnosis with endometrial biopsy, standard initial evaluation and management includes surgery when feasible, for the purpose of staging and treatment. This usually consists of pelvic peritoneal cytology, standard hysterectomy, bilateral salping-oophorectomy, and iliac and aortic-caval lymph node sampling.

The majority of patients present with symptoms of abnormal vaginal bleeding and are found to have stage I (confined to the uterine corpus) endometrial cancers considered completely treated with surgery alone; these are generally patients with type I tumors. Those patients found to have tumors with direct extension to the cervix (stage II) are generally treated with adjuvant pelvic radiotherapy, whereas those with more distant (stages III and IV) disease usually receive systemic chemotherapy (similar to regimens used to treat epithelial ovarian cancers), with or without tailored radiotherapy. According to 2006 estimates by the ACS, at diagnosis, 72% of invasive cancers in the USA are localized to the uterine corpus, 16% are locally advanced or associated with regional nodal metastases, and 8% are associated with distant metastases. The corresponding 5-year survival rates are 96, 66, and 25%, respectively (1).

2.3. *Carcinoma of the Cervix*

Carcinoma of the uterine cervix is the leading cause of cancer mortality in women worldwide, attributed mostly to the lack of broadly applied cytology screening programs in less industrialized regions. Despite the implementation of effective prevention and early detection methods in the USA, however, the American Cancer Society estimates 3700 cervix cancer deaths for 2006 (Table 1) (1).

Anatomically, the cervix is composed of an external, non-cornified squamous epithelium and an internal, mucinous columnar glandular epithelium. During the reproductive years, owing to metaplastic transformation of columnar glandular epithelium by squamous epithelium, the position of the squamous-columnar transformation zone migrates from a visible ecto-cervical to a concealed endo-cervical location. Unlike epithelial ovarian cancers, for which there is no well-recognized pre-invasive condition or established carcinogenic model, invasive cervical lesions arise within foci of carcinoma *in situ* (predominantly within the squamous-columnar transformation zone) and develop at least in part through oncogenic transformation events associated with persistent infection by high-risk human papillomavirus subtypes (9). Histologically, they may be pure squamous carcinomas (over 75%), pure adenocarcinomas, or mixed (adeno-squamous) tumors. Because of similarities in biologic behavior and response to therapy, these morphologic types have been pooled as a single disease entity, both diagnostically and therapeutically. Following invasion of underlying fibrous stroma, carcinomas of the cervix tend to exhibit a dual pattern of spread, primarily by direct extension to para-cervical connective tissue and adjacent organs and secondarily through lymphatic metastasis, initially to the iliac nodes, then the aortic-caval nodes. Rarely, distant metastases, for example to the lung, can be seen at the time of diagnosis.

In the USA, the median age at diagnosis of invasive cancer is approximately 45 years. Risk factors include multiple sexual partners, history of sexually transmitted infections, socioeconomic or cultural barriers to preventive healthcare, exposure to tobacco-associated products, and compromised cell-mediated immunity. Interestingly and possibly related to cell-mediated immune mechanisms, susceptibility (or protection) has also been linked to specific HLA haplotypes (10). Clinical stage is the most important prognostic factor and appears to be dependent directly on age (hence location of the cervical transformation zone) and access to healthcare (most tumors confined to the cervix, hence stage I, are clinically evident by symptoms of abnormal vaginal bleeding or discharge or by visual and manual pelvic examination). In patients with stage I disease managed initially with surgery, additional factors predictive of recurrence and cancer-related death include size of the primary tumor, depth of invasion into the cervical stroma, the presence or absence of stromal lymphatic space involvement, and the identification of regional nodal metastases (11).

Selection of standard primary treatment (11) is dependent on stage and general health status. Some patients with stage I disease are treated initially with surgery, ranging from standard hysterectomy to extended/radical hysterectomy combined with pelvic lymphadenectomy. Those who undergo surgery and are found to have intermediate- or high-risk factors for recurrence are generally treated with post-operative adjuvant regional therapy (radiation therapy to the pelvis and possibly lower para-aortic lymph nodes) with or without radio-sensitizing platinum-based chemotherapy. Other patients with stage I tumors (typically those with lesions clinically in excess of 4 cm in diameter), as well as all those with locally advanced cancers clinically confined to the

true pelvis (stage II through IVA), tend to receive regional radiotherapy with radiosensitizing platinum-based chemotherapy plus local tumor brachytherapy. Currently, those with distant metastases at the time of diagnosis or who develop recurrent disease outside the central pelvis are treated with palliative measures only. Some of the rare patients who recur in the central pelvis only may be candidates for total or subtotal pelvic exenteration. According to 2006 estimates by the American Cancer Society, at diagnosis, 55% of invasive cancers in the USA are localized to the cervix, 32% are locally advanced or associated with regional nodal metastases, and 8% are associated with distant metastases. The corresponding 5-year survival rates are 92, 55, and 17%, respectively.

3. ANGIOGENESIS AS A MECHANISM OF DISEASE PROGRESSION FOR GYNECOLOGIC MALIGNANCIES

There is abundant evidence that angiogenesis plays a central role in disease progression and prognosis for carcinomas of the ovary, endometrium, and cervix. From a molecular mechanistic standpoint, a great proportion of these tumors display an angiogenic phenotype. Many are associated with suppression of p53 function by mutational events (carcinomas of the ovary and endometrium) and by inactivation by viral oncogenic products (carcinoma of the cervix) (12). This may in part explain relative up-regulation of endogenous angiogenic promoters, such as vascular endothelial growth factor (VEGF) (13), and down-regulation of endogenous angiogenic inhibitors, such as thrombospondin-1 (TSP-1) (14, 15).

3.1. Correlative Studies of Key Biomarkers and Clinical Outcome

The correlation between the expression of key biomarkers of tumor angiogenesis in primary tumor tissue and clinical outcome in gynecologic malignancies is summarized in Table 2. Most notably, micro-vessel density (MVD) in primary epithelial carcinomas of the ovary (16–20), endometrium (21–25), and cervix (26–28) has correlated with extent of disease and has inversely correlated with survival or progression-free survival after initial therapy. Often this relationship to clinical outcome has been found to be independent of important clinical and pathologic prognostic factors (19, 20, 22–28). In addition, VEGF (29–31), TSP-1 (14, 15), angiotensin II type 1 receptor (AT1R) (29), and maspin (32) have demonstrated prognostic value in accordance with their known functional relationships to angiogenesis.

Tumor angiogenesis in gynecologic malignancies has been evaluated *in vivo* using non-invasive imaging modalities, such as contrast-enhanced dynamic magnetic resonance imaging (CD-MRI). CD-MRI examines the transport of contrast agents in tissue vascular and interstitial spaces as a function of time and provides a measure of vascular volume and permeability. Hawighorst et al. reported that pharmacokinetic parameters (amplitude, A; exchange rate constant, k₂₁), calculated from CD-MRI, were directly associated with MVD in cervical cancers treated by radical hysterectomy (33). Interestingly, k₂₁ was shown to be a significant predictor of poor patient survival (34).

Table 2
Association of Angiogenesis Biomarkers and Prognosis in Gynecologic Epithelial Cancers

| Marker | Impact on Outcome | | | | | |
|--------|-------------------|-----|-------------|----------|----------|-----|
| | Ovary | | Endometrium | | Cervix | |
| | OS | PFS | OS | PFS | OS | PFS |
| MVD | ↓(16–18,20) | | ↓(22–25) | ↓(21,22) | ↓(27,28) | |
| VEGF | ↓(29–31) | | ↓ | | | |
| AT1R | ↓(29) | | | | | |
| TSP-1 | ↑(14) | | | | ↑(15) | |

^a AT1R, type 1 angiotensin II receptor; MVD, micro-vessel density; OS, overall survival after primary therapy; PFS, progression-free survival after primary therapy; TSP-1, thrombospondin-1; and VEGF, vascular endothelial growth factor.

4. THERAPEUTIC TARGETS OF ANGIOGENESIS IN GYNECOLOGIC MALIGNANCIES

Data from correlative clinical studies and pre-clinical investigations of the common gynecologic malignancies have provided a strong rationale for antiangiogenic therapeutics in these disease sites. Multiple potential functional targets have been considered, most notably VEGF/VEGF receptors, upstream pathways with pleiotropic effects including up-regulation of VEGF activity, and parallel pathways which appear to work in concert with VEGF.

There is ample pre-clinical evidence in human ovarian cancer xenograft models that direct blockade of VEGF activity alone can result in decreased tumor proliferation and malignant ascites formation, concomitant with vascular remodeling. Notably, this phenomenon has been demonstrated with administration of VEGF-Trap (35), a soluble decoy receptor generated by fusing the constant region of IgG1 with the ligand-binding domains of two principle anti-VEGF receptors, then optimized for VEGF binding affinity and pharmacokinetics.

Upstream targets of VEGF may also be exploited in the treatment of gynecologic malignancies. For example, epidermal growth factor (EGF) (36) and ErbB-2/neu receptor tyrosine kinase inhibition has been shown to down-regulate VEGF expression *in vivo* (36), so it is possible that the use of specific inhibitors may play a complementary role with anti-VEGF agents. Combined anti-VEGF and anti-EGF pathway inhibition is currently being explored in phase II clinical trials (Table 4).

Other examples of angiogenic promoters acting coordinately but upstream of VEGF and having potential therapeutic relevance include angiotensin II, phosphatidylinositol 3-kinase (PI3K), nuclear factor (NF)-κB/relA, and nitric oxide synthase (NOS).

Angiotensin II is a bioactive peptide of the renin-angiotensin system, which may act as a tumor growth promoter through binding to angiotensin II type 1 receptors (AT1R). Suganuma et al. found that angiotensin II significantly enhanced invasive potential and VEGF secretion in AT1R-positive ovarian cancer cells; both effects were completely inhibited by the AT1R blocker candesartan (37). In addition, administration of candesartan in a human ovarian carcinoma xenograft model resulted in inhibition of intraperitoneal tumor growth and tumor angiogenesis.

The PI3K catalytic subunit alpha (PIK3CA) has been found to promote angiogenesis through VEGF in ovarian carcinoma cells *in vivo* (38). Conversely, the PI3K inhibitor LY294002 has been observed to inhibit malignant ascites formation with concomitant blockade of VEGF expression/secretion, endothelial proliferation, and vascular permeability in a mouse model of human ovarian cancer (39).

Inhibition of nuclear factor (NF)-kappaB/relA activity in human ovarian cancer cells has been demonstrated to suppress angiogenesis and tumor growth in an orthotopic nude mouse model; decreased expression of VEGF and IL-8 directly correlated with decreased tumorigenicity, decreased tumor neovascularization, decreased formation of malignant ascites, and prolonged survival (40).

Malone et al. (41) found that tumor angiogenesis and VEGF expression could be inhibited by specific blockade of NOS and stimulated by up-regulation of NOS *in vitro*.

Finally, other processes that appear to act more in parallel with VEGF-related pathways have been explored for their potential therapeutic value.

For example, angiopoietins have been found to play a role in angiogenesis through a cooperative effect with VEGF. Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) have been found to promote tumor angiogenesis in murine human cervical (42) and ovarian carcinoma (43) models, respectively. In latter study, up-regulation of Ang-2 in host tumor endothelial cells was significantly associated with pericyte loss and instability of the tumor vasculature.

The expression of matrix metalloproteinases (MMPs) in ovarian cancer cells has been found to be associated with invasive and metastatic properties. Specifically, Huang et al. (44) investigated angiogenesis and tumor growth of human ovarian cancer cells in MMP-9 wild-type versus MMP-9 knock-out mice. They found that MMP-9 knock-out animals displayed decreased tumor MVD, growth, and macrophage infiltration, which could be partially reversed with adoptive delivery of spleen cells from wild-type mice; these findings suggest that host-derived MMP-9 expression, possibly from tumor-infiltrating macrophages, plays a role in angiogenesis and progressive growth of human ovarian carcinoma.

Some growth factors, such as platelet-derived growth factor (PDGF), have been implicated in tumor angiogenesis at least in part through their impact on pericyte instability (45). The combined administration of paclitaxel with the platelet-derived growth factor receptor (PDGF-R) tyrosine kinase inhibitor ST1571 has been found to inhibit progression of human ovarian carcinoma in the peritoneal cavity of nude mice, in part, by blockade of PDGF; this effect correlated with apoptosis of tumor-associated endothelial cells (46).

There appears to be a key interplay between cell-mediated immunologic functional components and angiogenesis in the tumor microenvironment of gynecologic malignancies. For example, malignant ascites in patients with ovarian carcinomas has been found to be overpopulated by plasmacytoid dendritic cells (PDCs), perhaps due to over-expression of stromal-derived factor (CXCL-12/SDF)-1. In turn, PDCs have been found to induce angiogenesis through the elaboration of pro-angiogenic cytokines, tumor necrosis factor alpha, and interleukin (IL)-8. In contrast, myeloid dendritic cells (MDCs), relatively absent from malignant ascites, are known to suppress angiogenesis through release of IL-12 (47).

5. CLINICAL STUDIES OF ANGIOGENESIS INHIBITORS FOR EPITHELIAL OVARIAN CANCER

5.1. *Strategy for Drug Development*

As one may gather from the earlier description of this disease entity, there are several treatment settings that may be appropriate for the study or practical clinical use of angiogenesis inhibitors as single agents, in combination with cytotoxic drugs or in combination with other biologic therapies. The majority of clinical trials have been single-arm phase II investigations in the second or third-line treatment of patients with relapsed ovarian or primary peritoneal carcinoma who have radiographically or physically measurable disease; some of these trials have included correlative laboratory components. Although cytotoxic drugs such as pegylated liposomal doxorubicin and topotecan are indicated in this treatment setting, the long-term clinical outcomes have been unsatisfying in general, which justifies the study of novel agents. A few consistent observations regarding these patients should be noted before a specific discussion of angiogenesis-targeted therapeutics. First, the odds of response and durability of response to cytotoxic agents correlate with previous disease-free intervals. Second, the primary goal of therapy, unlike absolute cure for patients with newly diagnosed cancers, is the optimization of quality of life over time; this relies on the beneficial effects of treatment outweighing toxicity. Third, ideally therapeutics used to treat patients with disease recurrence include agents with mechanisms of action that do not overlap previous treatments and lack of cross resistance to recently administered drugs (48).

5.2. *Bevacizumab*

The most well studied antiangiogenic agent in patients with epithelial ovarian cancer is bevacizumab, a recombinant humanized monoclonal IgG1 that binds all isoforms of VEGF-A; the estimated half-life when administered systemically is approximately 20 days (49). Two single agent phase II trials have been conducted to date. The first was initiated by the Gynecologic Oncology Group (GOG) in April 2002 and closed to accrual in August 2004 (50). To put things into chronologic perspective, during the last half of the accrual period, bevacizumab received initial US Food and Drug Administration (FDA) approval as part of front-line treatment of metastatic colorectal cancer. As it had become commercially available, clinicians began utilizing the drug off-label in patients with recurrent disease. One year after FDA approval of bevacizumab for colorectal cancer, the first report of single-agent antitumor activity in a patient with a gynecologic malignancy was reported by Monk et al. (51), in this case in a patient with recurrent ovarian cancer who had been treated with ten previous cytotoxic regimens.

Results of the GOG phase II trial were initially reported in May 2005 (50). The primary endpoints included probability of PFS for at least 6 months, clinical response, and toxicity. Eligibility criteria consisted of measurable disease and one to two prior cytotoxic regimens. Patients received bevacizumab at 15 mg/kg IV every 21 days with standard toxicity reporting using common toxicity criteria and disease assessment according to the NCI RECIST system. Based on GOG phase II historical controls, a negative study was defined as both true response rate of less than or equal to 10% and true 6 months PFS rate of less than or equal to 15%. The study population consisted of 62 patients with primary disease characteristics typical of a population of patients with advanced, persistent, or recurrent ovarian and primary peritoneal carcinoma.

Approximately 44% of patients had experienced a progression-free interval of less than 6 months following completion of primary platinum-based therapy. Treatment was generally well tolerated, with only six patients experiencing hypertension requiring treatment (all grade 3), no patients more than grade 1 bleeding, and only two patients with venous thromboembolic events. Importantly, there were no reports of arterial thromboembolic complications, grade 3–4 proteinuria, or gastrointestinal (GI) perforation. With regard to efficacy, 11 (17.7%) clinical responses were observed, and 24 (38.7%) patients were progression free for at least 6 months. Both of these efficacy parameters exceeded pre-defined thresholds to consider bevacizumab active as a single agent. Finally, an exploratory analysis of prognostic factors for PFS was performed using Cox proportional hazards regression, showing no statistically significant association of PFS with performance status, platinum sensitivity, age, or number of prior regimens on the hazard of progression. Correlative imaging and molecular studies examining factors that could potentially predict outcome in patients enrolled in this trial are currently being analyzed.

There has been only one other phase II single-agent bevacizumab trial in patients with this disease, in this case an industry-sponsored third-line therapy trial accruing patients from February to September 2005 (52). This trial was terminated prematurely because of five GI perforations reported out of the first 44 patients enrolled. In October 2005, the FDA released an Action Letter (53) alerting investigators and patients to this risk, even though a black box warning was already present on the package insert. At that time, an overall GI perforation rate of 4.8% had been observed out of 188 patients treated on registered trials. Risk factors specific to this complication remain unclear; and prospective studies of hypothetical factors (e.g., intestinal obstruction, inflammatory bowel disease, peripheral vascular disease, and use of corticosteroids) that may impact risk are of paramount importance. Results of the industry-sponsored trial were presented in May 2006 (52).

Although the two phase II single-agent trials were fundamentally similar in design and treatment regimen, it may be useful to compare outcomes as functions of differences in eligibility. For example, the industry trial enrolled only patients considered either primarily or secondarily platinum resistant and having received two or three previous cytotoxic regimens. These differences in eligibility ultimately translated into a higher level of platinum resistance, a greater number of prior regimens, and a slightly worse performance status profile in the industry trial (Table 3). With regard to toxicity, there appeared to be a greater number of serious adverse events in the industry trial. In terms of efficacy, however, this study was still associated with a response rate of 16% and 6-month PFS rate of 27%. Although it is difficult to compare outcomes across distinct phase II trials, perhaps the slightly lower values for efficacy can be attributed to a higher risk for both disease progression and adverse events requiring discontinuation of study drug.

A number of new strategies of anti-VEGF therapy with bevacizumab are in development in patients with recurrent ovarian cancer, including novel combinations with other biologic therapeutics targeting EGF/EGF-R, PDGF/PDGF-R, metronomic cytotoxic chemotherapy, and immunotherapeutics.

With regard to combined anti-VEGF and anti-EGF therapy, preliminary results of a two-stage phase II trial of bevacizumab (15 mg/kg every 21 days) and erlotinib (150 mg PO daily) were presented in 2006 (54). Eligibility criteria were similar to that for the GOG 170-D single-agent bevacizumab trial. With regard to efficacy, of 13 patients, two

Table 3
Comparison of Two Phase II Trials of Single-Agent Bevacizumab in Patients with Ovarian and Primary Peritoneal Carcinoma

| <i>Trial</i> | <i>GOG 170-D (50)</i> | <i>Industry (52)</i> |
|-------------------------|-----------------------|----------------------|
| Enrollment | 62 | 44 ^a |
| Platinum DFI < 6 months | 36% | 84% |
| Prior regimens (1/2/3) | 34/66/0% | 0/52/48% |
| GOG/ECOG PS (0/1) | 73/27% | 59/41% |
| ≥Grade 3 Toxicity | | |
| GI Perforation | 0 | 5 ^a |
| Arterial TE | 0 | 3 ^b (8%) |
| HTN | 6 (10%) | 6 ^b (14%) |
| CNS | 0 | 1 ^b |
| Proteinuria | 1 | 0 |
| RR | 13 (21%) | 7 (16%) |
| 6-month PFS | 39% | 27% |

^a Trial terminated prematurely.

^b Event fatal in one case.

had clinical responses and seven had stable disease; the median PFS was 4.1 months. Rash, diarrhea, fatigue, and myalgia were the most common adverse effects of treatment. Grade 3/4 toxicities included two patients with grade 3 diarrhea, one patient with hypertension, and two with bowel perforations; the latter two patients had been diagnosed with small bowel obstruction and one had definite disease progression documented within 28 days prior to the event. This trial has proceeded into its second stage of accrual. To determine relative efficacy and toxicity, a randomized phase II trial of bevacizumab–erlotinib versus bevacizumab alone in patients with measurable or evaluable disease and up to three prior treatment regimens is under development within the GOG.

As discussed previously in this book, the rationale for combining cytotoxic drugs with anti-VEGF therapy stemmed initially from additive and in some cases synergistic interaction in pre-clinical models; multiple purported mechanisms exist to explain this interaction. It has also been hypothesized that combining VEGF-targeted agents with frequently administered low dose, so-called metronomic chemotherapy, may have additive or synergistic antiangiogenic or antitumor effects. Preliminary data on 29 patients entered onto a multi-institutional phase II trial combining bevacizumab and low-dose oral cyclophosphamide were presented in 2005 (55). Eligibility criteria were similar to the single-agent bevacizumab GOG 170-D study. Patients were treated with bevacizumab at 10 mg/kg every 14 days with 50 mg of daily oral cyclophosphamide. Patient and disease characteristics were similar to that of the GOG 170-D population. This regimen was associated with a toxicity profile similar to the single-agent bevacizumab trial with the exception of two cases of G4 cerebral ischemia and one G2 GI perforation. With regard to efficacy, 57% of patients were progression-free as of 6 months, and 28% had partial clinical responses. This protocol is now closed and the data are undergoing final analysis, but if consistent with preliminary results, these findings would provide the rationale for a phase II randomized trial of combination versus single-agent therapy.

Additional reports of outcomes for patients with epithelial ovarian and primary peritoneal cancers treated with bevacizumab include three historical case series of patients treated outside clinical trials with single-agent therapy or in combination with cytotoxic drugs, suggesting activity in more heavily pre-treated patients with recurrent disease (56–58) and preliminary data in 2006 demonstrating the feasibility of the combination of traditional carboplatin–paclitaxel chemotherapy combined with bevacizumab in front-line therapy (59).

Based on the above considerations, two phase III trials of bevacizumab in front-line therapy are in progress—GOG 218, activated in September 2005, and ICON7, activated in October 2006. Both trials include six cycles of standard platinum–taxane chemotherapy, but there are important differences between the two trials which should be noted. GOG 218 is a three-arm, placebo-controlled trial, whose primary objective is to determine whether the addition of bevacizumab (15 mg/kg every 21 days) to standard cytotoxic therapy when administered concurrently, or concurrently plus extended for an additional 16 cycles, will produce an improvement in overall survival. It is limited to patients with stage III or IV disease. In contrast, ICON-7 is a two-arm trial without a placebo, with the experimental arm containing bevacizumab (7.5 mg/kg every 21 days) concomitantly with cytotoxic therapy, then extended for 12 cycles, and with the primary endpoint of progression-free survival. The patient population for the ICON trial includes all patients with at least high risk early stage disease. As far as secondary endpoints are concerned, both trials will systematically examine quality of life, whereas translational research will be performed in the context of GOG 218 and a pharmacoeconomic analysis is planned for ICON-7.

Other settings ideal for phase III development of bevacizumab and other angiogenesis-targeted drugs include the treatment of patients with recurrent cancers who have had long initial disease-free intervals (bevacizumab trial under development, GOG 213) and consolidation therapy for patients with advanced disease completing front-line therapy who are considered in complete clinical remission but with a high-relapse risk.

5.3. Clinical Trials of Other Angiogenesis Inhibitors

As shown in Table 4, the study of angiogenesis-targeted therapeutics in patients with epithelial ovarian cancer has been pursued with tremendous enthusiasm. Twenty-three NCI-registered phase II patients are listed, the majority in the second- and third-line treatment setting. Only five of these have closed to accrual, 14 are active, and (at least) four are under development. Eleven of these trials involve agents targeting VEGF or VEGF-R, the vast majority utilizing antibody or decoy soluble receptor specific for the ligand. Five of these 11 trials involve combination therapy, with either cytotoxic or anti-EGF agents. The other 12 trials involve the use of pleiotropic tyrosine kinase inhibitors targeting combinations of VEGF-R1-3, PDGF-R, FGF-R, Raf-kinase, and protein kinase C-β.

6. CLINICAL STUDIES OF ANGIOGENESIS INHIBITORS FOR ENDOMETRIAL AND CERVICAL CARCINOMAS

Clinical trials research in the area of angiogenesis-targeted therapeutics is relatively underdeveloped in these disease sites when compared with epithelial ovarian cancer. There are no current phase III trials involving angiogenesis inhibitors registered to

Table 4
NCI Registered Phase II Trials of Angiogenesis Targeted Therapeutics for Patients with Epithelial Ovarian Cancer

| Protocol | Drug | Class | Target(s) | PI | Status |
|--|---|-------------------------|--|---|---|
| GOG 170-D AVF 2949 | Bevacizumab | MAb ^a | VEGF | Burger (50) Cannistra (52) | Closed |
| NCT00129727 ^b TEACO ^c | Bevacizumab (+CT ^c) Bevacizumab (+oxaliplatin and docetaxel) | MAb MAb | VEGF VEGF VEGF | Penson Not listed Coleman Not listed | Active Active UD ^d Active |
| GOG DTM0507 NCT00327171 | AVE-0005 (VEGF-Trap) | Soluble receptor | VEGF | Friberg Alberts | Active Active |
| NCI NCT00130520 | Bevacizumab (+erlotinib) | MAb | VEGF | Monk | UD |
| GOG DTM0531 CCC PH145 | Bevacizumab (+/- erlotinib) Bevacizumab (+cyclophosphos) | MAb TKI ^e | VEGF VEGF-R | Garcia (55) Not listed | Closed Active |
| NCT00281632 NCT00278343 | GW786034 AZD2171 | TKI | VEGF-R | Hirte Matulonis | Active Active |
| NCT00275028 GOG 170-F | Sorafenib | TKI | Raf-K, VEGF-R, and PDGF-R | Matei | Active |
| NCT00096200 NCT00390611 | Sorafenib (+/- CT) Sorafenib (+CT) | TKI | Raf-K, VEGF-R, and PDGF-R | Von Grunigen | Active |
| GOG DTM0503 NCT00370175 | AMG 706 BIBF 1120 Enzastaurin | TKI | Raf-K, VEGF-R, and PDGF-R VEGF-R, PDGF-R VEGF-R, PDGF-R, and FGF-R | Hainsworth Schilder Ledermann Usha | Active UD Active Active |
| GOG 170-J NCT00039585 | Imatinib | TKI | PKC-β PDGF-R | Schilder Kohn (60) | Closed Closed |
| GOG DTM0618 NCT00388037 | Sunitinib | TKI | VEGF-R and PDGF-R | Chan Biagi | UD Active |

^a Monoclonal antibody.

^b Front line.

^c Carboplatin and paclitaxel.

^d Under development.

^e Tyrosine kinase inhibitor.

Table 5
Phase II Trials of Angiogenesis-Targeted Therapeutics for Patients with Endometrial Cancer

| Protocol | Drug | Class | Target(s) | PI | Status |
|--------------|---------------------|------------------|--------------------------------------|------------|-----------------|
| GOG 229-E | Bevacizumab | MAb ^a | VEGF | Aghajanian | Active |
| GOG 229-F | AVE-0005 | Soluble receptor | VEGF | Coleman | UD ^b |
| GOG DTM 0609 | Sorafenib (+CCI779) | TKI ^c | Raf-K, VEGF-R, Alvarez and PDGF-R | | UD |

^a Monoclonal antibody.

^b Under development.

^c Tyrosine kinase inhibitor.

the NCI database. In fact, as shown in Table 5, all three phase II trials appear to be conducted with the GOG, with only one active (single-agent bevacizumab). The same can be said for cervical carcinoma, for which there is only one known phase II trial (GOG, single-agent bevacizumab). The reason for this has mostly to do with differences in impact on public health in industrialized nations, but there may be other explanations.

As described in the beginning of this chapter, although uterine cancers represent the most common site of gynecologic malignancy, with 41,200 new cases in the USA estimated in 2006, there were only 3750 estimated deaths in the same year (1). According to the same source, 72% of invasive cancers in the USA are estimated to be localized to the uterine corpus, with a corresponding 5-year survival rate of 96%. Furthermore, patients with uterine sarcomas (not discussed in this chapter) are under-represented in total number of cases, but owing to the high risk of mortality associated with these tumors, the survival statistics for patients with endometrial carcinomas are likely more favorable than represented here. Beyond decreased need for systemic therapy, another important factor responsible for relative under-exploration of angiogenesis-targeted agents in patients with endometrial cancers relates to the observation that epithelial ovarian cancers and advanced endometrial cancers appear to be similar with respect to histologic cell types (endometrioid, serous, and clear cell) and biologic behavior; thus the development of systemic therapy for patients with advanced endometrial cancers has tended to shadow the development of systemic therapy for patients with epithelial ovarian cancers.

The situation for carcinomas of the cervix is even more pronounced, with only 3700 cancer deaths estimated for 2006 (1). Of the 9710 annual cases, over half are classified as localized, with over 90% of patients cured using standard modalities. However, given that this disease is a major cause of morbidity and mortality in less industrialized regions, novel approaches to systemic therapy are still needed. Given the potential for angiogenesis inhibitors to restore microcirculation, phase I trials are in progress to explore the interaction between such agents and standard chemo-radiation in the management of patients with advanced disease.

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SUMMARY

The role of angiogenesis in the development and progression of solid tumors has been well established over the 1980s and 1990s. Through more recent investigations, it has become increasingly clear that neovascularization within the bone marrow of patients with hematologic malignancies is of primary importance in the development and progression of these disorders. Evidence of malignant angiogenesis in myeloid malignancies includes increased microvascularity and vascular density within the bone marrow cavity, elevated serum or urine levels of soluble angiogenic peptides, and cellular over-expression of angiogenic molecules and their cognate receptors. Autocrine and paracrine secretion of angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiogenin contributes not only to medullary vasculogenesis but also to the proliferation of myeloid precursors and alteration of their microenvironment. Pro-angiogenic molecules have been linked to prognosis and implicated in the progression of hematologic malignancies. Therapeutic strategies intended to inhibit angiogenesis in myeloid malignancies have reached maturity over the 1990s. Lenalidomide is the first such antiangiogenic agent to receive Food and Drug Administration (FDA) approval for the treatment of myelodysplastic syndrome (MDS) based on hematologic and cytogenetic responses. Other investigational agents in the treatment of myeloid disorders, such as thalidomide, arsenic trioxide (ATO), and bevacizumab, have shown promise as single agents or in combination with chemotherapy in the treatment of leukemia and MDS. Novel small-molecule tyrosine kinase inhibitors (TKIs) with activity against a spectrum of angiogenic receptors have completed early-phase clinical trials showing modest clinical activity. Further investigation of the contribution of angiogenic molecules to the development and progression of myeloid malignancies before this new class of therapeutics can be further integrated into standard practice.

Key Words: Myelodysplastic syndromes; acute myeloid leukemia; chronic myeloid leukemia; myeloproliferative disorders; angiogenesis; neovascularity; VEGF.

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1. INTRODUCTION

Angiogenesis is a complex process of neovascular development that can occur in adult life as either a physiologic or neoplastic phenomenon. Although the role of angiogenesis in the growth of solid tumors has been studied extensively over the 1980s and 1990s, investigation of the importance of this process in hematologic malignancies has been recognized only recently. Evidence to date, however, suggests that angiogenesis and the biologic effects of the corresponding regulatory molecules play a central role in the pathobiology of many hematologic malignancies, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), myelodysplastic syndrome (MDS), myeloproliferative disorders, and multiple myeloma (1–9). This chapter explores the impact of angiogenesis in the development and progression of myeloid malignancies, the prognostic significance of angiogenic biomarkers in MDS and leukemia, and the development of novel antiangiogenic therapeutic agents for these diseases.

2. PATHOBIOLOGY OF ANGIOGENESIS IN MYELOID MALIGNANCIES

Neovascularization in hematologic malignancies, as in solid tumors, is a complex biologic process that is characterized by both *vasculogenesis* and *angiogenesis*. *Vasculogenesis* arises from a mesodermal precursor, or angioblast, that differentiates into endothelial cells within blood islands of the human yolk sac during embryonic development. Concurrent with establishment of such blood islands, primitive hematopoiesis initiates the formation of red blood cells (RBCs) and macrophages from multipotent hematopoietic stem cells, thus highlighting the close integration of the vascular and hematopoietic systems (10). These pluripotent stem cells share a common antigenic phenotype with the expression of the progenitor cell antigen, CD34, and the vascular endothelial growth factor (VEGF) mitogenic receptor, VEGFR-2 (11). Thus, normal embryologic development of blood vessels is dependent on VEGF molecules, their cognate receptors, and other angiogenic regulatory proteins (12). In contrast, *angiogenesis* is characterized by the development of new vessels from preexisting mature vasculature and may occur in adult life as either a physiologic or pathologic process. New vessel formation is differentiated by two distinct phases. The activation phase of angiogenesis leads to basement membrane degradation, proliferation and migration of endothelial cells, and formation of a capillary network. Subsequently, the resolution phase is initiated with stabilization and maturation of the neovasculature, reconstitution of the basement membrane, and resolution of the endothelial cell mitogenic response (13). Vital to the development and expansion of the vascular wall is the presence of Tie-1 and Tie-2 receptors, which are members of the receptor tyrosine kinase family. Angiopoietin-1 and angiopoietin-2 are two ligands that bind to Tie-2 receptors on endothelial cells, activating autophosphorylation of tyrosine residues in the intracellular domain of Tie-2 and in the case of angiopoietin-1, stimulation of growth factor production (14, 15). These interactions are required for differentiation of the surrounding mesenchymal cells into mature smooth muscle cells or pericytes involved in the development of vessel walls, vascular branching, and capillary bed formation. Furthermore, additional factors necessary for the development of a mature vascular network include the extracellular matrix formation with integrin-ligand binding and the development of mature endothelial cell interactions through

adhesion molecules such as vascular endothelial (VE)-cadherin and platelet endothelial adhesion molecule 1 (PECAM-1) (14).

An extensive number of endogenous pro-angiogenic and inhibitory molecules coordinate the intricate balance within these two phases of physiologic and malignant angiogenesis, with VEGF-A being of paramount importance. VEGF-A is a potent angiogenic peptide with multiple biologic effects, including extracellular matrix remodeling, embryonic stem cell development, and paracrine induction of inflammatory cytokines (9, 16). Although first discovered in 1989, it was eventually isolated from a myeloid leukemia cell line, HL-60, and found to be critical to the activation of endothelial cells during vasculogenesis and to capillary formation during angiogenesis (17). Leukemia cells commonly express one or both of the major VEGF receptor tyrosine kinases, the c-fms-like tyrosine kinase (Flt-1 or VEGFR-1), and the kinase domain receptor (KDR or VEGFR-2) and can produce and secrete VEGF (18). Autocrine and paracrine growth stimulation with VEGF results in a mitogenic response within hematologic malignancies and specifically promotes self-renewal of leukemia progenitors (9, 18). Immunohistochemical staining for these angiogenic molecules indicates that the VEGF is produced by the malignant clone and may have prognostic significance. Intense VEGF staining is demonstrable in a diffuse cytoplasmic pattern in myeloid precursors in bone marrow specimens from patients with MDS or AML (9). VEGF protein expression appears restricted to myeloblasts and malignant monocytoid precursors in chronic myelomonocytic leukemia (CMML) while distinctively absent from normal mature myeloid cells or erythroid or lymphoid precursors (9). Moreover, these myelomonocytic precursors also express one or more of the cognate VEGF receptors, implicating autocrine stimulation that may relieve normal dependence upon stromal sources of myeloid growth factors. Indeed, these investigations have shown that receptor-competent myelomonocytic precursors that coexpress VEGF and the VEGF-R1 receptor may dislocate from their normal site of residence along osteoblasts residing in the boney trabeculae to cluster in the central marrow space, recognized morphologically as abnormal localized immature myeloid precursors (ALIPs). The biological consequences of autocrine VEGF expression as yet remains undefined although clonogenic assays indicate that VEGF is mitogenic and enhances colony-forming capacity. The presence of ALIP in MDS portends an aggressive clinical course (19), thus providing a plausible biologic rationale for its prognostic significance in this disorder.

VEGF expression appears to play an important regulatory role in the pathobiology of CML. VEGF over-production is demonstrable both in BCR-ABL-positive leukemia cell lines and in the sera of CML patients in chronic and accelerated disease (4, 7, 20, 21). Increased VEGF expression within the bone marrow of CML patients has been demonstrated in significantly higher levels than control subjects (5, 20). Laboratory studies indicate that the oncogene responsible for malignant transformation in CML, that is, BCR-ABL, is the upstream trigger of malignant angiogenesis. BCR-ABL-positive cell lines, such as K562, secrete VEGF, and BCR-ABL transfection into a naive cell lines induces VEGF expression (21). Furthermore, treatment of BCR-ABL cell lines with imatinib (Gleevec®; Novartis) suppresses VEGF expression in a dose-dependent fashion (20, 21). Imatinib inhibits transcription of BCR-ABL through targeting of the transcription factors Sp1 and Sp3 and prevention of DNA binding (20).

Thus, imatinib may have the additional therapeutic benefit as an antiangiogenic agent through its direct inhibitory effect on the BCR-ABL tyrosine kinase.

Basic fibroblast growth factor (bFGF) is a potent stromal cell mitogen and paracrine stimulant of myelopoiesis (22). High levels of bFGF in the serum or urine are suggestive of increased endothelial activity but may also be associated with infection, inflammation, or tissue breakdown (23). Thus, serum or urine elevations of this soluble peptide may arise from increased angiogenesis or simply an increased systemic tumor burden. Despite this, various studies have identified elevated serum or urine levels of bFGF in leukemia cell lines and patients with MDS, AML, and CML (1, 3, 5, 7). Bone marrow immunostaining for bFGF cellular distribution in myeloproliferative disorders is limited by the strong affinity for platelet and megakaryocyte staining (22). Similarly, human angiogenin, another pro-angiogenic protein that binds to high-affinity receptors on endothelial cells and induces cellular proliferation, migration, and invasion. When compared with control marrows, serum levels of angiogenin are elevated in patients with MDS, AML, and CML and correlate with higher risk disease in MDS (6, 24, 25).

The measurement of microvessel density (MVD) in bone marrow trephine biopsies from patients with myeloid malignancies provides critical insight into the extent of angiogenesis activity and the framework of the neovascular network. MVD measurements have been accomplished by several methods, including hematoxylin and eosin (H & E) staining of endothelial markers as well as immunostains with antibodies recognizing the cell adhesion molecule PECAM or CD31, CD34, UEA-1, von Willebrands factor (vWF), and factor VIII-related antigen (FVIIIrAg) (15). CD31 recognizes platelets, megakaryocytes, some plasma cells, and endothelial cells, whereas CD34 is expressed by both immature myeloid cells and endothelial cells. UEA-1 labels all endothelial cells, megakaryocytes, erythroid cells, stromal cells, and sinusoidal cells. Because of the lack of specificity of these markers, over-counting or under-counting of angiogenic hot spots remains a technical challenge. In addition, myeloid malignancies such as MDS, CML, and AML often harbor increased number of CD34-positive myeloid progenitor cells that may over-estimate MVD when using automated counters. Despite these limitations, measurements of bone marrow MVD in patients with hematologic malignancies have been extensively investigated and yielded important prognostic detail (1, 3, 8, 26–29). When compared with control bone marrow specimens, leukemia and MDS patients display increased number of microvessels (1, 27–30), increased endothelial colonies (26), and increased total vascular area, which directly correlates with myeloblast percentage (28). The complexity of the vascular network has also been implicated in the pathologic features of MDS and CML. Neovascular architecture in MDS exhibits an intricate, disordered network that gives rise to an elevation in intraluminal pressures and impediment to blood flow compared with normal or AML bone marrows, thereby possibly further compounding limited blood cell egress (28). As the pathological progression to acute leukemia occurs, the vessels diameter has been shown to increase, coincident with an expansion in vessel number, resulting in further increase in MVD density (8, 28). Although most studies report greater medullary MVD in AML compared with MDS, others failed to show increased vessel number or total vascular bed area (1). Similar examinations of CML bone marrows have been performed and have demonstrated a tortuous vessel architecture with irregularly shaped vessels (29). Examination of these marrow specimens more than 6 months after receiving allogenic transplant showed normalization of vessel structure and organization. However,

pathologic review of marrows within the first 6 months shows that these changes in vascular architecture after transplantation are delayed, with persistence of vascular dilatation (31).

3. PROGNOSIS

Prognostic variables in myeloid malignancies are critical tools for estimating survival expectation and stratifying patients according to optimal treatment approaches. Multiple angiogenic markers have been found to impact treatment response, disease progression, and survival in myeloid malignancies (Table 1). In MDS, the International Prognostic Scoring System (IPSS) was adopted in 1997 and remains the most widely utilized prognostic tool (32). The IPSS applies three variables, which include the marrow myeloblast percentage, karyotype, and number of peripheral cytopenias, to segregate patients into four distinct prognostic groups. Thus, identification of additional prognostic determinants may further refine differences in clinical outcome and treatment considerations. The prognostic significance of angiogenic markers remains largely conflicting. Measurements of MVD, VEGF, bFGF, and angiogenin have reported correlation with poor prognostic categories, an inferior disease-free survival, or overall survival in many reports in MDS (25, 27, 33–35), whereas others have failed to show consistent prognostic correlations among the myelodysplastic patients (36, 37). Furthermore, there has been no clear association identified between angiogenic markers and cytogenetic pattern, which is a key determinant of prognosis in MDS (26). Discrepancies in outcomes across different reports can largely be attributed to the non-uniform nature of the various methods applied to assess vasculogenesis and the complex biological nature of vessel formation for which additional investigation is needed.

Table 1
Angiogenic Markers of Prognosis in Myeloid Malignancies

| | <i>AML</i> | <i>MDS</i> | <i>CML</i> |
|----------------------|---|---|--------------------------|
| Serum VEGF | + | + | + |
| Serum bFGF | – | + | – |
| MVD | + | + | + |
| VEGFR-1 | + | – | – |
| VEGFR-2 | – | + | + |
| Angiopoietin | + | + | Unknown |
| Endostatin | + | + | Unknown |
| Tie-1 | – | – | + |
| Clinical correlation | Lower remission rate and reduced overall survival | ALIP, higher blast percentage and reduced disease-free and overall survival | Reduced overall survival |
| Reference | 25,27,35,36,82 | 40,82,83 | 4,7,44,45 |

AML, acute myeloid leukemia; bFGF, basic fibroblast growth factor; CML, chronic myeloid leukemia; MDS, ; VEGF, vascular endothelial growth factor.

Variables predictive for response to standard dose induction regimens in AML are limited largely to karyotype analysis, drug resistance antigen profile such as *P*-glycoprotein, selective gene mutations, and presence, or absence of antecedent MDS or chemotherapy-induced leukemia. For those patients able to tolerate intensive chemotherapy or bone marrow transplantation, the choice of post-induction therapy is guided by these various factors. Patients with intermediate risk for relapse do not have an accepted standard optimal treatment strategy, and thus further prognostic discrimination guides those individuals best suited for more aggressive post-induction therapies. Multiple groups have established biomarkers for neovascular angiogenesis as indicators of an unfavorable clinical outcome in AML. For young patients with *de novo* AML, univariate analysis of overall survival in a cohort of 61 patients identified that a day 15 bone marrow exhibiting either aplasia, favorable karyotype analysis, or high levels of angiopoietin 2 are independent predictors of prognosis (38). Significant reductions in MVD were also demonstrated when chemotherapy-sensitive, day 15 bone marrow samples from AML patients as defined by hypoplasia with less than 5% blasts, were compared with chemotherapy-resistant day 15 bone marrows (60 vs. 17%, $p < 0.001$) are demonstrable (39). Additionally, VEGF has been implicated as an independent prognostic factor for overall survival in AML. Using radioimmunoassay, cellular VEGF concentrations prior to induction chemotherapy was predictive of overall survival and disease-free survival (40). VEGF levels did not correlate with blast count or other prognostic features such as age, cytogenetics, or antecedent hematologic disorder. Alternatively, others have reported an association between increased marrow cellularity and higher marrow blast percentage mimicking increased measures of MVD (3, 28). Although elevated VEGFR-1 levels correspond to inferior complete remission (CR) rates in AML, elevated VEGFR-2 levels were associated with response rates in MDS (35). Endogenous inhibitors of angiogenesis, such as endostatin, have similarly been implicated as a biomarker of prognosis. Endostatin is a C-terminal fragment of collagen XVIII studied extensively in solid tumors, and recent investigation has shown it to be of prognostic value in hematologic malignancies. Although prechemotherapy plasma endostatin levels are not significantly elevated in MDS or AML patients compared with controls, patients who achieved CR after induction therapy had lower median endostatin levels compared with resistant patients (41). Furthermore, patients in this cohort who had higher plasma endostatin levels at baseline had a reduced overall survival compared with those patients with lower values.

Assessment of prognosis in CML has evolved considerably following the advent of tyrosine kinase inhibitors (TKIs) that have significantly altered the natural history of the disease. Traditionally, prognostic discrimination in CML was based largely on laboratory and clinical features, such as spleen size, blast percentage, age, basophilia, and platelet count. Scoring systems such as the Sokal (42) or Hasford (43) score were created using these parameters although their utility in directing therapeutic decisions with our current treatment arsenal is uncertain. Reliable measures of angiogenic biomarkers in CML may further delineate patients at risk for disease progression; however, supporting data are limited at this time. Patients with splenomegaly or thrombocytosis had significantly higher levels of serum VEGF (4, 7). Measurements of VEGFR-1 and VEGFR-2 in bone marrow samples in chronic phase revealed that VEGFR-2 expression alone correlated with significantly inferior survival compared with patients without receptor up-regulation (44). Furthermore, increased Tie-1 protein

expression in early-phase CML was reported in older patients or those with thrombocytosis or leukopenia. Measurements of Tie-1 in early phase, but not advanced CML, was independently associated with inferior rates of survival (45). Thus, markers of angiogenesis may eventually prove to be useful to stratify chronic-phase CML patients into categories based on risk of progression with modern kinase inhibition therapy.

4. ANTIANGIOGENIC AGENTS

Treatment of myeloid malignancies such as MDS and leukemia varies widely, depending on the disease type and prognostic features. MDS remains an incurable disease for the majority of patients. The median age at diagnosis is late in the seventh decade, and thus bone marrow transplant, which is the only modality with known curative potential, is not a viable option for most patients. Therapeutic options include growth factors, hypomethylating drugs, and most recently, antiangiogenic agents. The only antiangiogenic agent approved by the Food and Drug Administration (FDA) for treatment of MDS or multiple myeloma is lenalidomide. This agent has potent erythroid-potentiating effects *in vivo* and has produced favorable rates of hematologic and cytogenetic response in phase II trials (46–48). In contrast, there are no antiangiogenic agents approved for the treatment of AML, with strict reliance upon cytotoxic chemotherapy. There is very little data supporting the use of angiogenic-altering therapy in AML although early-phase trials are beginning to emerge. With the advent of BCR-ABL TIKs such as imatinib, the treatment of CML has undergone considerable change in the last several years. Interferon, hydroxyurea, chemotherapy, and allogeneic transplantation are still therapeutic options for chronic myeloproliferative disorders, with bone marrow transplant representing the only curative modality. However, imatinib therapy remains the mainstay of therapy for the majority of patients with CML in chronic phase.

Given the association between angiogenesis and the pathobiology of hematologic malignancies, multiple antiangiogenic agents have been tested in early-phase trials in myeloid malignancies (Table 2). One of the first antiangiogenic agents to show activity in the treatment of MDS or AML is thalidomide (α -N-phthalimidoglutarimide). This immuno-modulatory drug has multiple biological effects in addition to its antiangiogenic properties that may contribute to its activity in these diseases. These include its ability to suppress angiogenic response to VEGF, bFGF, and suppression of TNF- α generation (49–51), enhancement of natural killer cell cytotoxicity (52), and a costimulatory action on T-cell response associated with promotion of interleukin 2 (IL-2) and interferon gamma (IFN- γ) production (53). A phase I/II trial of thalidomide at doses of 100–400 mg daily in patients with MDS reported hematologic improvement in 18% of patients using International Working Group Criteria, with activity largely restricted to the erythroid lineage (54). This modest effect was promising, with hematologic improvement seen across all French-American-British (FAB) subtypes. However, there was a distinct lack of complete hematologic responders, and no cytogenetic remissions were demonstrated. Additionally, only 51 of the 83 patients enrolled in the trial completed the planned 12 weeks of therapy as a result of dose-limiting adverse effects of fatigue, constipation, shortness of breath, and fluid retention.

Thalidomide has also been investigated in AML, with minor responses and similar toxicity profiles. Sixteen patients enrolled in a small, phase II trial with refractory

Table 2
Antiangiogenic Agents in Clinical Trials of Myeloid Malignancies

| Agent | Description | Disease | Route | Reference |
|--|--|---|-------|-------------|
| Lenalidomide (Celgene) | 4-Amino-glutarimide analog of thalidomide ↓bFGF, ↓VEGF, and ↓TNF- α | MDS and MMM | PO | 46–48,65 |
| Thalidomide (Celgene) | Phthalimidoglutarimide ↓bFGF, ↓VEGF, and ↓TNF- α | MDS, AML, and MMM | PO | 54–56,58 |
| Arsenic Trioxide (Cell Therapeutics, Inc) | ↑Apoptosis and ↓VEGF | MDS and AML | PO | 69–71,74–76 |
| Bevacizumab (Genentech) | Humanized MoAb VEGF | AML | IV | 78 |
| PTK 787/ZK 222584 | Small molecule TKI | myeloproliferative disorder (MPD) and AML | PO | 80 |
| (Novartis/Schering AG) | VEGFR-1, VEGFR-2, c-kit, PDGFR, and FMS-like tyrosine kinase (FLT)-3 | | | |
| AG13736 (Pfizer) | Small molecule TKI VEGFR-1, VEGFR-2, c-kit, PDGFR, and FGF | MDS and AML | PO | 81 |

AML, acute myeloid leukemia; bFGF, basic fibroblast growth factor; MDS, myelodysplastic syndrome; MMM, myeloid metaplasia; PDGFR, platelet-derived growth factor receptor tyrosine kinase; TKI, tyrosine kinase inhibitor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

or relapsed AML were treated with thalidomide at daily doses of 200–800 mg for a median of 27 days (55). Although one patient achieved a durable CR for 36 months, only two other patients received benefit with mild transient responses. Toxicities were significant, with most patients unable to tolerate doses higher than 400 mg, thus making this agent unattractive for extensive investigation in acute leukemia.

Myelofibrosis with myeloid metaplasia (MMM) is a hematopoietic stem cell malignancy with few effective therapies outside of stem cell transplantation. Multiple small phase II trials using thalidomide in this disorder have yielded both erythroid and platelet responses and reduction in spleen size. Using a daily dosing schedule of 100–800 mg, improvements in anemia have been reported in 0–60% of patients, platelet responses in 25–100%, and reductions in splenomegaly in 25–60% (56–62). With this standard dose, attrition rates were high, with over 50% of patients discontinuing therapy in 3 months or less because of intolerable side effects or disease progression. The most common dose-limiting symptoms were common to previous studies with thalidomide and include fatigue, constipation, paresthesias, and sedation. However, unique to thalidomide therapy in MMM is the development of a myeloproliferative reaction, characterized by leukocytosis, basophilia, thrombocytosis, and in rare cases splenic

infarct or pericardial effusion associated with extramedullary hematopoiesis (63). In a pooled analysis of five trials, 18% of patients developed a myeloproliferative reaction although clinical sequelae were infrequent (58). Adverse events with thalidomide are dose dependent; however, hematologic responses appear to be unrelated to total dose. A phase II study using low-dose thalidomide with an initial starting dose of 50 mg daily and a median-tolerated dose of 100 mg yielded similar results as the higher dose regimens in terms of efficacy, with only 25% of patients discontinuing treatment at 3 months or less (57). For those patients remaining on therapy longer than 3 months, 40% achieved a clinical or hematologic response.

Because of the beneficial effect seen in multiple myeloma when thalidomide was combined with corticosteroids, low-dose thalidomide has also been investigated in combination with prednisone in MMM. A 3-month oral prednisone taper was combined with 50 mg of thalidomide daily in a phase II trial of 21 patients with MMM (64). Tolerability was excellent, with over 95% of patients receiving more than 3 months of therapy. When compared to thalidomide alone in low or moderate doses, the thalidomide/prednisone combination resulted in favorable overall response rates (62%) with significantly less fatigue, constipation, depression, or cardiovascular symptoms. In addition, 40% of patients achieved transfusion-independence, 75% had an improvement in thrombocytopenia, and 19% had a reduction in spleen size by 50% or more. Venous thrombosis is a significant concern in patients with multiple myeloma who are treated with corticosteroids concomitantly with thalidomide; however, only one patient with MMM developed a clinically significant thrombosis in this study. The rationale for therapeutic benefit in MMM is based on the antiangiogenic and antiinflammatory properties of thalidomide. However, measures of bone marrow MVD or serum or urine levels of angiogenic peptides are only variably affected by thalidomide therapy in MMM and do not consistently correlate with response (58–61). Thus, the precise mechanism by which thalidomide exerts clinical improvement remains elusive.

Lenalidomide (Revlimid®; Celgene) is a 4-amino-glutarimide analog of thalidomide with similar immunomodulatory and angiogenic effects but with superior potency and considerably reduced sedative and neurologic effects. It was the first antiangiogenic agent FDA approved for the treatment of MDS in December 2005 based on a landmark multicenter phase II study. A phase I/II trial involving 43 patients with symptomatic anemia who failed treatment with recombinant erythropoietin investigated three different dosing schedules of oral lenalidomide at 25, 10, and 10 mg daily for 21 of 28 days (46). Hematologic response was analyzed according to modified IWG criteria, and 56% of patients experienced an erythroid response, the majority of which achieved transfusion independence. Unlike recombinant growth factor therapy, cytogenetic responses were common with 50% of patients achieving a complete cytogenetic remission. Interestingly, hematologic response was karyotype dependent with 83% of patients with a chromosome 5q31.1 deletion achieving an erythroid response compared with 56% of patients with a normal karyotype and 12% of patients with other cytogenetic abnormalities. This novel analog of thalidomide was found to have a different toxicity profile, with the major dose-limiting adverse effects relating to moderate to severe neutropenia (65%) and thrombocytopenia (74%) that were dose dependent. Other adverse events such as diarrhea (21%), pruritus (28%), and fatigue (7%) were infrequent and were generally of minor severity. Two multicenter phase II trials evaluated the safety and efficacy of lenalidomide in the treatment of transfusion-dependent lower

risk MDS: the MDS-002 study involved patients without the del5q31, and the MDS-003 study was restricted to patients with the chromosome 5q31 deletion (47,48). In the latter study, 148 patients with transfusion-dependent, low to intermediate-1 risk international prognostic scoring system (IPSS) score were treated with 10 mg either daily or for 21 days every 4 weeks (47). By intention to treat analysis, 67% of patients achieved transfusion independence after 24 weeks of therapy, and 76% of patients achieved a transfusion response characterized by a 50% or greater reduction in transfusion frequency. Responding patients experienced a median 5.4/dL increase in hemoglobin, with a median time to response of 4.6 weeks. Despite the recognized adverse prognosis of additional chromosome abnormalities accompanying deletion 5q, the frequency of transfusion response was independent of karyotype complexity. Median duration of transfusion independence was not reached after 104 weeks of follow-up, indicating a rapid and sustained response to lenalidomide. Erythroid response was closely correlated with cytogenetic response. Seventy-three percent of patients experienced cytogenetic improvement, including complete cytogenetic remissions in 45% of patients, with all cytogenetic responders achieving transfusion independence. Moreover, 36% of patients achieved a complete histological response, all of whom had major cytogenetic responses. The most common adverse events requiring treatment interruption or dose adjustment were neutropenia (55%) and thrombocytopenia (44%), consistent with the agent's suppressive effect on the deletion 5q clone. In comparison, 215 patients without the 5q31 deletion were treated on the same schedule with 27% achieving a major erythroid response and an overall erythroid response rate of 47% (48). Toxicities were similar in both trials; however, there was a higher frequency of grade 3 or higher neutropenia and thrombocytopenia seen in the deletion 5q31 patients (50 vs. 20–25%).

Lenalidomide has also been studied in MMM, with clinical response rates comparable to thalidomide but with a much more favorable toxicity profile. Two simultaneous trials were conducted at MD Anderson and the Mayo Clinic using 10 mg of lenalidomide daily, with dose adjustments based on cytopenias, in a total of 68 patients with MMM (65). The majority of patients had received previous therapy, including 30–32% with previous exposure to thalidomide. Overall response rates of 24–37% were achieved, with major erythroid responses, platelet responses, and spleen reductions seen in both trials. Similar to the MDS experience, responses were achieved in patients both with and without an interstitial deletion of 5q. One patient with chromosome deletion 5q13q33 and heterozygous JAK2 mutation experienced a completed cytogenetic remission accompanied by a significant reduction in JAK2 mutational burden. Nonetheless, JAK2 mutation status was not uniformly affected by lenalidomide therapy nor did its presence correlate with hematologic response. Non-hematologic adverse events were uncommon and included fatigue, pruritus, and rash. However grade 3–4 neutropenia (27–32%) and thrombocytopenia (7–27%) were the most common adverse events and the most frequent reason for dose adjustment. In the Mayo study, bone marrow fibrosis was significantly decreased in lenalidomide responders; however, baseline bone marrow angiogenesis as assessed by CD34+ immunohistochemistry did not correlate with response. Thus, the mechanism of clinical benefit of lenalidomide in MMM remains unclear, but given the favorable toxicity profile and substantial response rates, it remains an attractive agent for further investigational studies.

Another agent with antiangiogenic properties that has shown activity in myeloid disorders is arsenic trioxide (ATO) (Trisenox®; Cell Therapeutics Inc). Its therapeutic

effect can be attributed to depletion of cellular organic thiols, disruption of mitochondrial respiration, and promotion of caspase-dependent apoptosis (66). In MDS, clinical benefit can be attributed to both inhibition of angiogenesis and induction of apoptosis in the latter stages of the disease. ATO has been shown to induce apoptosis in activated endothelial cells within neovasculature and inhibit VEGF in a leukemia cell line (67, 68). As a single agent, ATO was investigated in Europe using an initial loading dose of 0.3 mg/kg/day for 5 days, followed by a maintenance regimen of 0.25 mg/kg ATO twice weekly for 15 weeks (69). Using IWG response criteria, the overall hematologic response rate was 26% in lower risk patients and 17% in higher risk MDS. Although most antiangiogenic agents largely yield erythroid responses, major responses were seen in all hematopoietic lineages, with transfusion independence achieved in 16% of RBC transfusion-dependent patients and 29% of platelet transfusion-dependent patients. Toxicities related to ATO included fatigue, elevations in liver function tests, and prolongation of cardiac QTc interval. A similar trial was conducted in the USA investigating 70 patients with low- to high-risk MDS treated with ATO 0.25 mg/kg/day for 5 days per week for 2 weeks of every 4-week cycle (70). Hematologic improvement rates were comparable to the European study with 34% of lower risk patients and 6% of higher risk patients responding. However, median response duration was superior with this schedule lasting 6.8 months. Responses were again observed in all lineages, and treatment-related toxicities were similar to the previous trial. ATO has also been investigated in combination with low-dose thalidomide (100 mg daily), with a 25% response rate reported in both low- and high-risk disease (71). Overall, multilineage responses with ATO are promising, and treatment-related toxicities are manageable. Biologically, rational-designed drug combinations may yield more favorable response rates and duration of benefit.

The use of ATO in leukemia has been largely limited to the FAB M3 subtype (FAB-M3) or acute promyelocytic leukemia (APL). APL is characterized by a maturation arrest of granulopoiesis at the promyelocyte stage that results from a translocation of the retinoic acid receptor alpha (RAR- α) gene on chromosome 17, t(15;17) to adjoin the promyelocytic leukemia (PML) gene, although additional variants have been described. The resultant fusion protein, PML-RAR- α , blocks retinoic acid-induced myeloid differentiation. The exact mechanism of therapeutic benefit of ATO in treating APL is not clear; however, differentiation of leukemic cells through degradation of the PML-RAR- α fusion protein, caspase-mediated apoptosis with potent inhibition of bcl-2 expression, and glutathione-S-transferase (GSTP1-1) has been implicated in its action (72, 73). ATO alone or in combination with all trans retinoic acid (ATRA) or chemotherapy is effective as both induction and salvage therapy for APL and can result in long-term leukemia-free survival (74–76).

Bevacizumab (Avastin®; Genentech) is a recombinant, humanized, IgG monoclonal antibody that neutralizes VEGF and its binding to cognate receptors. Bevacizumab has shown considerable activity, when combined with cytotoxic chemotherapy, in the treatment of solid tumors, including colon cancer (77). A phase II, randomized study of 48 adult patients with relapsed or refractory AML reported favorable response rates when combined with chemotherapy (78). Patients generally had unfavorable prognostic features, with almost 30% having secondary AML and over 60% with adverse cytogenetic profiles. Treatment consisted of bevacizumab at a dose of 10 mg/kg intravenously on day 8, following induction chemotherapy with cytarabine 2 gm/m²

over 72 h on day 1, and mitoxantrone 40 mg/m² on day 4. Over 90% of patients received either cytarabine or an anthracycline previously. Despite this, 50% of the patients were complete responders, with a median disease-free survival of 7 months. Serum elevation of VEGF was detected in two thirds of the patients prior to treatment, with 93% experiencing a decline in VEGF level and 67% achieving undetectable serum concentrations 2 h after bevacizumab therapy. Similarly, MVD was significantly decreased after antiangiogenic therapy. Given the favorable complete response rates and durability of remission in this population, which is traditionally resistant to chemotherapy, these data suggest a possible beneficial effect of bevacizumab therapy. This combination was relatively well tolerated although concerns of cardiotoxicity with bevacizumab remain. Reductions in left ventricular ejection fraction were detected in 6% of patients, and 4% of patients experienced cerebrovascular bleeding. The contribution of bevacizumab cardiotoxicity in patients who had received anthracyclines in the past or who are receiving anthracycline therapy requires further investigation.

Several small-molecule TIKs have been tested either as monotherapy or in combination with cytotoxic chemotherapy for the treatment of myeloid malignancies and have shown modest antileukemic activity. One such agent is PTK787/ZK 222584 (PTK/ZK) (Novartis), a potent inhibitor of all VEGF RTK, with greater selectivity against VEGFR-2. Other cellular targets include platelet-derived growth factor receptor (PDGFR) tyrosine kinase, c-fms, and c-kit protein tyrosine kinase (79). A phase I study of oral PTK/787 in twice daily dosing was performed in refractory or relapsed AML or advanced MDS patients (80). Following a standard phase I dose escalation with 18 patients receiving PTK/787 monotherapy alone, an additional 45 patients were enrolled into a second arm to receive PTK/ZK monotherapy followed by chemotherapy with daunorubicin (45–60 mg/m²/day for 3 days) with cytarabine (100 mg/m²/day for 7 days) if 28 days of monotherapy alone was ineffective. Dose-limiting toxicities in the monotherapy arm were lethargy and hypertension, with infrequent grade 3 or 4 toxicities and a favorable profile overall. Although no responses were seen in the monotherapy arm, two patients achieved stable disease for up to 14 months. When combined with chemotherapy, 5 of 17 patients achieved a CR, in addition to two CRs with thrombocytopenia and one partial remission (PR). PTK/787 is well tolerated and shows promise as an adjunct to standard chemotherapy in myeloid malignancies.

Modest antileukemic activity was also seen with the antiangiogenesis agent, AG-013736. This agent inhibits phosphorylation of VEGFR-1, VEGFR-2, BEGFR-3, c-kit, and PDGRR-β and exhibited activity in AML cell lines as well as xenograft mice models. Using a daily oral dose of 10 mg for a median of 56 days, AG-013736 was administered to 12 patients with AML and MDS with a median age of 80 (81). Although sustained decreases in soluble VEGFR-2 plasma levels were reported, baseline levels of VEGFR-1 and VEGFR-2 bone marrow expression were minimal and no objective responses were seen. Additionally, grade 3 or 4 toxicities were significant and included hypertension in 43%, mucositis 8%, and venous thrombosis 7%. It is possible that the lack of clinical activity was confounded by the low frequency of bone marrow expression of VEGF receptors in this elderly AML population.

5. CONCLUSION

Angiogenesis is intimately involved in the pathobiology of myeloid malignancies in both preclinical and clinical models. Angiogenic biomarkers such as VEGF, TNF- α , bFGF, and angiogenin and their receptors are elevated in the serum of patients with MDS, AML, and myeloproliferative disorders. The medullary microvascular network in patients with hematologic malignancies is characteristically disordered, with an increased number of vessels and a tortuous appearance. These pathologic vascular changes can be reversed with successful treatment of the underlying malignancy. Angiogenesis biomarkers offer prognostic discrimination with inferior outcomes in patients with MDS, AML, and CML. However, the data is limited and therapeutic decisions cannot be justified on the basis of angiogenic measures alone. Several antiangiogenic agents have shown benefit in the treatment of myeloid malignancies. Lenalidomide is one such agent with proven clinical benefit in the treatment of MDS. Other treatments that show modest activity in the treatment of MDS and leukemias include thalidomide, ATO, and bevacizumab. Small-molecule TIKs of VEGF, c-kit, and PDGF β -R have shown minimal impact as single agents in myeloid malignancies but show promise when combined with cytotoxic chemotherapy. Improvements in therapeutic strategies will only arrive through combinations of chemotherapeutic agents and antiangiogenic agents. Further investigation into the complex pathogenic processes involved in neoplastic angiogenesis is of paramount importance to further our understanding of the pathobiology of myeloid malignancies and develop more effective therapeutic strategies for these frequently incurable diseases.

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SUMMARY

Angiogenesis is a biological process by which new capillaries are formed from preexisting vessels. It occurs in physiological and pathological conditions, such as tumors, where a specific turning point is the transition from the avascular to the vascular phase. Tumor angiogenesis depends mainly on the release by neoplastic cells of growth factors specific for endothelial cells able to stimulate the growth of the host's blood vessels. This article summarizes the knowledge about the role of angiogenesis in the most common forms of pediatric malignant and non-malignant tumors. A major goal is the determination of whether inhibition of angiogenesis is a realistic way of inhibiting tumor cell dissemination and formation of metastasis in pediatric tumors.

Key Words: angiogenesis; antiangiogenesis; pediatric tumors

1. INTRODUCTION

Angiogenesis is the formation of new blood vessels from pre-existing ones and takes place in various physiological and pathological conditions, such as embryonic development, wound healing, the menstrual cycle, chronic inflammation, and tumors (1, 2). It is generally accepted that tumor growth is angiogenesis dependent and that every increment of tumor growth requires an increment of vascular growth (3). Tumor angiogenesis is an uncontrolled and unlimited process essential for tumor growth, invasion, and metastasis, regulated by the interactions of numerous mediators and cytokines with pro-angiogenic and antiangiogenic activity. Tumors lacking angiogenesis remain dormant indefinitely. An expanding endothelial surface also gives tumor cells more opportunities to enter the circulation and metastasize.

New vessels promote growth by conveying oxygen and nutrients and removing catabolites, whereas endothelial cells secrete growth factors for tumor cells and a variety of matrix-degrading proteinases that facilitate invasion. An expanding endothelial

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surface also gives tumor cells more opportunities to enter the circulation and metastasize, whereas their release of antiangiogenic factors explains the control exerted by primary tumors over metastasis. These observations suggest that tumor angiogenesis is linked to a switch in the equilibrium between positive and negative regulators. In normal tissues, vascular quiescence is maintained by the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli. Tumor angiogenesis, on the other hand, is induced by increased secretion of angiogenic factors and/or down-regulation of angiogenesis inhibitors.

Growth of solid and hematological tumors consists of an avascular and a subsequent vascular phase. Assuming that the latter process is dependent on angiogenesis and depends on the release of angiogenic factors, acquisition of angiogenic capability can be seen as an expression of progression from neoplastic transformation to tumor growth and metastasis.

Angiogenic factors can be produced by a number of cells such as embryonic cells, adult resident and inflammatory cells (i.e., fibroblasts, macrophages, T cells, plasma cells, neutrophils, mast cells, and eosinophils), and neoplastic cells. Several angiogenic factors have been identified, including vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), placenta growth factor (PIGF), basic fibroblast growth factor/fibroblast growth factor-2 (bFGF/FGF-2), transforming growth factor beta (TGF- β), hepatocyte growth factor (HGF), tumor necrosis factor alpha (TNF- α), interleukin-8 (IL-8), and angiopoietin-1 and (Ang-1 and Ang-2).

Except for cancers of hematologic origin and of the central nervous system (CNS), pediatric cancers frequently originate from mesenchymal structures, such as bone or muscle. Childhood malignancies tend to have short latency periods and are frequently rapidly growing and aggressively invasive. Unlike adult cancers, most pediatric malignancies have either spread locally or have metastasized at the time of presentation and are not amenable to curative surgical excision.

This chapter summarizes the knowledge about the role of angiogenesis in the most common forms of pediatric malignant and non-malignant tumors.

2. NON-MALIGNANT PEDIATRIC TUMORS

2.1. *Hemangioma and Hemangioblastoma*

Infantile hemangioma is the most common pediatric tumor. Hemangioma is a benign vascular lesion that exhibits rapid growth during the first year of life (proliferating phase) and slow regression during the next 5 years (involutive phase) that is completed by the age of 7–10 years (involved phase) (4).

Proliferating lesions consist of endothelial cells, supporting pericytes, and myeloid cells, but include other cells, such as fibroblasts and mast cells (5, 6). Studies with isolated hemangioma-derived endothelial cells (7) and whole lesions (8) indicate that hemangioma arise from uncontrolled clonal expansion of endothelial cells. Both FGF-2 and VEGF are elevated in proliferating hemangiomas (9, 10).

Hemangioblastomas, either sporadic or associated with von Hippel-Lindau (vHL) syndrome, are highly vascularized and are characterized by an up-regulation of VEGF and VEGFRs expression (11–13).

3. MALIGNANT PEDIATRIC TUMORS

3.1. Acute Leukemia

Acute leukemias originate from immature hematopoietic stem cells (HSCs) that undergo self-renewal, whereas less aggressive forms such as chronic leukemias seem to originate from the more mature, committed HSC.

In a study of 51 children with acute lymphocytic leukemia (ALL), microvessel density in bone marrow was increased six- to sevenfold compared with the control bone marrows of children evaluated for primary tumor (14). A computer-aided three-dimensional reconstruction model of bone marrow vascularity showed a complex, arborizing branching of microvessels in leukemic specimens compared with the single straight unbranched microvessels in controls. Neoplastic cells formed cylindrical cords around new microvessels, as in solid tumors. A non-significant decrease in vessel density after treatment suggests that changes in vascularization occur after the tumor cells are eliminated. Urinary levels of FGF-2 were high in these patients before induction therapy, variable during induction, and normalized when a complete response was achieved (14). Investigation of the association of microvessel density with leukemia phenotype, karyotype, and prognosis showed that, at presentation, the microvessel density was significantly increased in most patients and dropped toward normal in remission (15). There was no significant difference in density at presentation or remission between children in poor prognostic group and those who subsequently relapsed, and there was no association with age, sex, cytogenetic abnormalities, or disease phenotype (15).

Aguayo et al. (16) have provided further evidence of increased bone marrow microvascular density in ALL as well as increased plasma levels of FGF-2, but not VEGF. These investigators also demonstrated that intracellular levels of VEGF in leukemic blasts of patients with acute myelogenous leukemia were higher than in mononuclear cells from controls (17). Increasing VEGF levels were associated with shorter overall and disease-free survival but did not correlate with established prognostic factors, such as blast counts, age, and cytogenetic abnormalities.

Subsets of acute leukemia cells express VEGF and its receptors (VEGFR-2 and VEGFR-3), resulting in autocrine loops that modulate leukemia survival, proliferation, and migration (18, 19). More recently, Fragoso et al. (20) demonstrated that VEGFR-1 modulates acute leukemia distribution within the bone marrow, along VEGF gradients, regulating leukemia survival and exit into peripheral circulation, thus determining the onset of extramedullary disease.

3.2. Lymphomas

The pediatric lymphomas are composed of two prominent forms including Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL). Pediatric HD is composed of histologically distinct subgroups, including nodular sclerosis (70%), mixed cellularity (16%), lymphocytic predominance (7%), and lymphocytic depletion (<2%). The frequency of mixed cellularity and nodular sclerosis subtypes are age- and gender-dependent, with both forms increasing with older age at diagnosis. NHL represents a heterogeneous group of tumors with Burkitt and Burkitt-like tumors predominating in children diagnosed between the ages of 5 and 14 years.

When chorioallantoic membranes of fertilized chicken eggs were engrafted with human lymphoma specimens, they evoked a neovascular response (21). Significantly, higher microvessel counts in high-grade than in low-grade NHL indicate that angiogenesis increases with tumor progression (22, 23). In cutaneous T cells and B lymphoma, microvascular density is higher than in normal skin or a benign cutaneous lymphoproliferative disorder (24–26).

Other studies, however, have shown that high-grade tumors are not more vascularized than either low-grade lymphomas or reactive lymphoid tissues (27). This discrepancy probably results from the use of dissimilar microvessel counting techniques rather than truly different results. Hazar et al. (28) did not find a correlation between microvascular density and histologic subtypes in a series of 71 lymphomas. In a study comprising 36 patients with diffuse large B-cell lymphoma, no differences in microvascular density was found between patients with chemotherapy-resistant lymphomas and those with chemosensitive lymphomas (29). Furthermore, phenotypic differences among blood vessels of reactive lymph nodes, follicular lymphoma, and diffuse large B-cell lymphoma indicate that the clinical significance of lymph node vascularization might vary in different histologic entities (30).

In B-cell lymphoma, expression of VEGF and its receptors is related to tumor grade. The percentage of VEGF-positive cells is higher in the intermediate-high grade than in low-grade lymphomas. In NHLs, an elevated serum FGF-2 level at diagnosis is an independent indicator of poor prognosis (31). Interestingly, the highest prognostic power was obtained when high serum VEGF and FGF-2 levels were combined (32, 33).

Foss et al. (34) investigated the expression of VEGF on lymphoma subtypes. The amount of VEGF transcripts was significantly higher in HD and peripheral T-cell lymphomas than in normal cells, but not in follicle center lymphoma and B-cell CLL. Of particular interest is the discovery of the presence of lymphoma-specific chromosomal translocation in EC in B-cell lymphomas (35). Four different mechanisms were suggested to explain this finding: (i) both lymphoma and endothelial cells have a common malignant precursor; (ii) endothelial cells harboring lymphoma-specific genetic aberrations originate from cells already committed to the lymphoid lineage; (iii) fusion of lymphoma cells and endothelial cells has occurred; and (iv) apoptotic lymphoma cells have been incorporated into endothelial cells.

3.3. Central Nervous System

Central Nervous System (CNS) malignancies are the second most common malignancies in childhood and the most common form of solid tumor.

The expression of certain angiogenic factors and signaling pathways has been documented in pediatric and adult CNS tumors (36). On a molecular basis, the up-regulation of angiogenic genes in pediatric brain tumors further confirms the cytokine expression data (37, 38).

Glioblastoma multiforme (GBM) is one of the most highly vascularized of all human tumors. VEGF is the most important known mediator of angiogenesis in gliomas (39, 40). VEGF expression and microvessel density correlate positively with glioma grade (41), and VEGFR-1 and VEGFR-2 are overexpressed in brain tumor vasculature (39). mRNA of VEGF in GBMs is increased by 50-fold, compared with the relatively low levels of quiescent brain tissue (40). Using a murine glioma model,

overexpression of VEGF was shown to produce formation of hyperplastic microvascular proliferation known as “glomeruloid bodies,” which share structural similarities with the glomeruloid proliferation seen in GBM.

Hypoxia indirectly leads to this marked increase of VEGF in malignant gliomas and in the up-regulation of VEGF in the tumor rim adjacent to necrotic areas (42). Finally, the role of VEGF in the breakdown of the blood-brain barrier in GBM, leading to peritumoral vasogenic edema, is also described.

FGF-2 is also expressed in gliomas, but its expression does not increase with increasing malignancy grade (43). The presence of FGF-2 in cerebrospinal fluids from children with brain tumors has been correlated with tumor microvessel formation (36).

Expression of both Ang-1 and Ang-2 increases with astrocytoma grade, as does expression/activation of Tie-2 in endothelial cells (44–48). Ang-2 is overexpressed in gliomas and in orthotopic glioma animal models and is overexpressed in the tumor rim adjacent to necrotic areas although this may reflect the VEGF-mediated induction of Ang-2 (47,48). On the contrary, Ang-1 is down-regulated by hypoxia in several tumor cell lines, including GBM (47).

The transcription factor, hypoxia-inducible factor-1 (HIF-1), and its target genes play a critical role in glioma-induced angiogenesis. In GBM, HIF-1 α is overexpressed in pseudopalisading cells around necrotic foci, a pattern similar to that of VEGF mRNA (49). Hypoxia, through HIF-1, is one of the most potent stimulators of VEGF expression *in vitro* and *in vivo*.

Matrix metalloproteinases (MMPs) are also expressed in gliomas (50). MMP-9 has been strongly implicated in glioma invasion and angiogenesis. Significantly increased levels of MMP-9 are expressed in human glioma cell lines and human glioma tissue specimens with the degree of expression correlating with tumor grade (51,52).

3.4. Neuroblastoma

Neuroblastoma (NB), most commonly occurring in the adrenal gland, is predominantly a tumor of infancy with 16% of children diagnosed within the first month of life and 41% diagnosed within the first 3 months of life. Little is known about the etiology of NB. Clinical and biological characteristics, such as the very early age at onset, spontaneously regression, amplification of the MYC-N oncogene, hyperdiploidy, and loss of heterozygosity on 1p, generated a great deal of interest in the etiology of NB.

Several recent studies implicate angiogenesis in the regulation of NB growth, and inhibition of angiogenesis is a promising approach in the treatment of NB because of the high degree of vascularity of these tumors. In 1994, Kleinman et al. (53) published a paper in which they showed that the human NB cells induce angiogenesis in nude mouse during tumorigenesis. Meitar et al. (54) evaluated the vascularity of primary untreated NB from 50 patients. They found that the vascularity of NB from patients with widely metastatic disease is significantly higher than in tumors from patients with local or regional disease. Ribatti et al. (55) investigated the angiogenic potential of two human NB cell lines demonstrating their capacity to induce *in vitro* human microvascular endothelial cells to proliferate and *in vivo* angiogenesis in the chick embryo chorioallantoic membrane assay.

Canete et al. (56) in a retrospective study showed that tumor vascularity was not predictive of survival of NB patients and that neither disseminated nor local relapses were influenced by the angiogenic characteristics of the tumors. Eggert et al. (57)

performed a systematic analysis of expression of angiogenic factors in 22 NB cell lines and in 37 tumor samples. They found that high expression levels of seven angiogenic factors correlated strongly with the advanced stage of NB, and this suggests several angiogenic peptides set in concert in the regulation of neovascularization.

Ara et al. (58) found that increased expression of MMP-2, but not of MMP-9, in stromal tissues of NB had significant association with advanced clinical stages. Sakakibara et al. (59) have demonstrated that the higher gelatinases activation ratio resulting from high expression of a novel membrane-type matrix metalloproteinase-1 (MT-MMP-1) on NB specimens is associated significantly with advanced stage and unfavorable outcome. Ribatti et al. (60) showed that the extent of angiogenesis and the expression of the MMP-2 and MMP-9 were up-regulated in advanced stages of NB.

MYC-N may regulate the growth of NB vessels, because its amplification or overexpression is associated with angiogenesis in experimental (61) and clinical settings (54). Amplification of MYC-N is a frequent event in advanced stages of human NB. MYCN amplification correlates with poor prognosis and enhanced vascularization of human NB, suggesting that the MYC-N oncogene could stimulate tumor angiogenesis and thereby allow NB progression (62).

Erdreich-Epstein et al. (63) demonstrated by immunohistochemical analysis that $\alpha_v\beta_3$ integrin was expressed by 61% of microvessels in high-risk NB but only by 18% of microvessels in low-risk tumors.

It has been reported that there is a very low tumor vascularity in Schwannian stroma-rich/stroma-dominant NB tumors and that Schwann cells produce angiogenesis inhibitors, such as tissue inhibitor of metalloproteinase-2 (TIMP-2) and pigment epithelium-derived factor (PEDF), which are capable of inducing endothelial cell apoptosis (64, 65). Chlenski et al. (66) isolated an angiogenic inhibitor in Schwann cell-conditioned medium, identified as Secreted Protein, Acidic and Rice in Cysteine (SPARC), whose expression is inversely correlated with the degree of malignant progression in NB tumors. Furthermore, SPARC inhibited angiogenesis *in vivo* and impaired NB tumor growth.

Leali et al. (67) demonstrated that FGF-2 causes OPN up-regulation in endothelial cells, *in vitro* and *in vivo*, resulting in the recruitment of proangiogenic monocytes. Takahashi et al. (68) demonstrated that OPN-transfected murine NB cells significantly increased neovascularization in mice. Enforced expression of OPN in NB cells significantly stimulated endothelial cell migration and induced angiogenesis in mice, as evaluated by dorsal air sac assay.

3.5. Wilms Tumor

Wilms tumor is the most common primary renal tumor and the second most common solid abdominal tumor of childhood, usually diagnosed between the ages of 2 and 5 years in childhood. Pathological angiogenesis is involved in the progression of Wilms tumor. Increased microvascular density can identify Wilms tumor patients at high risk for relapse, especially those patients with favorable histology tumors (69).

3.6. Bone Tumors

The age-specific incidence of bone tumors is characterized by very low rates before the age of 5 years, followed by increasing rates that peak around 13–15 years of age.

The most frequent tumors are osteosarcoma and Ewing's sarcoma (ES). Osteosarcoma is the most common primary malignant tumor of bone and accounts for approximately 20% of primary bone cancers. Approximately 75% occur in patients younger than 20 years of age. ES accounts for approximately 6–10% of primary malignant bone tumors and follows osteosarcoma as the second most common bone sarcoma in children.

Known risk factors for bone tumors during childhood and adolescence include exposure to radiation/chemotherapy for the treatment of other childhood malignancies and genetic conditions including hereditary retinoblastoma and Li–Fraumeni syndrome (70).

In primary ES, the expression of VEGF positively correlates with microvascular density (71). ES cells express VEGFR-1 and VEGFR-2, suggesting that VEGF may act as paracrine and as autocrine factor in ES. Moreover, platelet derived growth factor-B (PDGF-3), PIGF, and FGF-2 are also expressed by some ES, demonstrating that this tumor can produce several proangiogenic factors; however, their expressions do not correlate with microvascular density.

3.7. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common pediatric soft-tissue sarcoma, with an annual incidence of four to seven cases per million children under 16 years of age (72). Approximately 75% of RMS are embryonal (ERMS), whereas alveolar (ARMS) are less common. ERMS occurs in children under the age of 10 years and typically arises in the nasal cavity, orbit, middle ear, prostate, and paratesticular region. ARMS occurs in early to middle adolescence and commonly arise in the deep musculature of the extremities. A higher frequency of congenital anomalies has been reported among RMS cases, including Li–Frumeni syndrome and neurofibromatosis type I (73).

Gee et al. (74) have shown that multiple RMS cell lines express both VEGF and VEGFRs, suggesting a possible autocrine loop. They showed that RMS cells are responsive to VEGF, and signals are likely transduced through VEGFR-1. Moreover, by blocking VEGFR-1, they were able to reduce RMS cell number, suggesting that they inhibited an autocrine VEGF pathway.

3.8. Retinoblastoma

Retinoblastoma is the most frequent ophthalmic tumor of infancy, with 63% diagnosed within the first 2 years and 95% within the first 5 years. Bilateral retinoblastoma is associated with younger age at diagnosis, occurring in 42% of children less than 1 year old, 21% of those 1 year of age, and only 9% of those 2 or more years of age (75–77). Based on the observed age-specific incidence patterns for children with hereditary and non-hereditary forms of retinoblastoma, the concept of tumor suppressor genes was proposed and validated (78).

The expression of angiogenic cytokines, such as FGF-2 and VEGF, has been found in retinoblastoma (79,80). Rossler et al. (81) quantified vessels in retinoblastoma tissue with different clinical characteristics and histopathological features. They demonstrated that tumors invading the choroid and/or the optic nerve and those with metastases showed higher vessel densities than tumors without invasive growth.

4. CONCLUDING REMARKS AND PERSPECTIVES

Conventional treatments, that is, surgery, chemotherapy, and radiotherapy, have considerably improved the outcome of malignancies in children. Standard chemotherapy regimens for many pediatric solid tumors require courses of intensive agents given in combination every 3–4 weeks to allow recovery of marrow functions prior to each new course. Clinical trials are ongoing in which novel treatment approaches are being evaluated, including immunotherapy, biologically targeted radiotherapy, and the use of agents that induce tumor apoptosis or differentiation. Additional treatment strategies are needed, however. One such strategy involves the use of angiogenesis inhibitors.

Antiangiogenesis as a new treatment option has been tested for pediatric malignancies. It is believed that patients would profit most from a combination therapy consisting of antiangiogenic and chemotherapeutic drugs. Such a combination therapy targets both the endothelial compartment and the tumor cell compartment and seems to be more effective in improving the outcome than either therapy alone.

Children who succumb of pediatric tumors have widespread metastatic disease. NB, for example, is a systemic disease with multiple overt and occult sites of tumor, requiring systemic therapy. Therefore, a mechanism for long-term, systemic delivery of antiangiogenic inhibitors might be required. Long-term, regular low-dose administration of such agents (“metronomic,” that is, very frequent or continuous low-dose chemotherapy) could inhibit endothelial cell proliferation, angiogenesis, and tumor growth (82), and by attacking the vasculature, chemotherapy-induced drug resistance may be circumvented, because endothelial cells are considered to be genetically more stable than tumor cells (83).

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Prognostic and Predictive Significance of Surrogate Biomarkers of Angiogenesis

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SUMMARY

The identification of prognostic factors and the appropriate selection of the patients more likely to benefit of anti-angiogenic therapies is a major area of research. Early experience with other molecular targeted drugs, such as imatinib and/or trastuzumab, has generated the perception that pre-treatment target assessment is a pre-requisite for therapy. However, emerging evidence suggests that presently we have no predictive biomarkers for anti-angiogenic agents. Despite considerable evidence for the association of intratumoral and/or plasma vascular endothelial growth factor (VEGF) levels with tumor progression and/or poor prognosis, pre-treatment VEGF levels are not predictive of response to angiogenesis inhibitors. This may possibly be due to the complexity of the angiogenic pathways and the limitations associated with current methods of VEGF detection and quantification; e.g. low assay sensitivity and lack of standardized methods could prevent detection of very small increases in VEGF, which may be clinically important. In addition to a general lack of agreement as to the relative clinical relevance of circulating versus tumor VEGF levels, the absence of a gold standard VEGF detection assay and the lack of a predefined, clinically relevant cut-off values pose a significant hindrance to the clinical utility of VEGF measurements for therapy selection. Several retrospective studies showed a promising important role of microvessel density and other pro-angiogenic factors (e.g. basic fibroblast growth factor, VEGF, thymidine phosphorylase, etc.) as independent prognostic markers in solid tumors, but these data have to be validated in prospective trials.

Key Words: Prognosis; predictive markers; angiogenesis.

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1. PROGNOSTIC SURROGATE BIOMARKERS

1.1. *Introduction*

A number of studies, most of which are retrospective, correlated potential surrogate markers of angiogenesis with prognostic parameters in different solid tumor types (1). The morphological aspects of angiogenesis, such as microvessel density (MVD), total microvascular areas (TMA) or vascular patterns, as well as the overexpression of angiogenic factors have been correlated with disease outcome (2).

1.2. *Microvessel Density*

Most published prognostic studies are retrospective and are based on the measurement of intratumoral vascularity by counting microvessels identified by panendothelial or other angiogenesis-related markers using immunohistochemistry (IHC) techniques. The basic method for assessment of vascularity was first proposed by Weidner et al. (3). The first step of the method is the use of panendothelial markers, including factor VIII-related antigen (von Willebrand factor, fVIII-RA), monoclonal antibodies to CD31, CD34, or others, to immunostain blood microvessels. The second one is the identification of the single area of highest vascularization (“hot spot”) by scanning of the entire tumor section at low power (400 \times field) and then at higher power (200 \times field) to count each individual microvessel (any stained endothelial cell or separate clusters without vessel lumina is also an evaluable microvessel). To limit the subjectivity of tumor vascularity evaluation, two alternative techniques have been developed: the use of the Chalkley eye piece and multiparametric computerized imaging analysis systems, which evaluate vascular area, microvessel number and perimeter, and intensity of staining (4).

Panendothelial markers do not distinguish blood vessels from lymphatic vessels (as per fVIII-RA) or cross-stain other cells (plasma cells in the case of anti-CD31). The antibody to CD105 (endoglin) or LM609 to the integrin $\alpha_v\beta_3$ more selectively stain proliferating (activated) endothelium. The vascular parameters measured by CD105 appeared to better correlate with overall survival (OS) and disease-free survival (DFS) than panendothelial markers such as CD31 in breast, colon, lung, and prostate carcinomas (2).

1.2.1. BREAST CANCER

Breast cancer (BC) is the widest studied tumor to define the relationship of angiogenesis with clinical outcome. The prognostic value of MVD in BC was first shown independently by two groups in 1992 (5,6). Other authors demonstrated that the degree of vascularization of the primary tumor correlates with the presence of bone marrow micrometastases at diagnosis, and that the degree of vascularity at “hot spots” in axillary lymph nodes is associated with outcome. The Chalkley count seems to be the preferable technique for estimating angiogenesis with regard to the prognostic stratification of BC patients, based on its acceptable reproducibility (7,8).

IHC staining of blood microvessels has been obtained by different markers, mainly with fVIII-RA, CD31, CD34, integrin $\alpha_v\beta_3$, CD105, or type IV collagen. In a series of 197 consecutive patients with invasive BC and long follow-up, the expression of integrin $\alpha_v\beta_3$ has been suggested as the single-most significant prognostic indicator for relapse-free survival (RFS) in both node-negative (N-) and node-positive (N+) BC

patients (9). Overall, CD31 emerged as the marker of choice for prognostic purposes, among studies with multivariate analysis that used the antibodies to CD31, CD34, or fVIII-RA. However, because few comparative studies prospectively tested the different methods, the optimal method to assess tumor vascularity has not been identified yet.

For more than 10 years, MVD has been proposed as surrogate marker of tumoral angiogenesis to identify patients at high risk of recurrence, particularly in N– BC patients (10–12).

Recently, Uzzan et al. (13) systematically reviewed the 87 published studies between 1991 and 2002, linking MVD measured in early BC to RFS and OS. The authors performed four different meta-analyses, the first two including studies involving either N+ or N– patients or both (for OS and for RFS, separately). The two others restricted to studies with a majority ($\geq 75\%$) of or only N– patients. A statistically significant inverse relationship between angiogenesis, assessed by MVD, and survival has been found, confirming that human invasive BC is an angiogenesis-dependent malignancy. High MVD was significantly associated with shorter RFS (RR: 1.54; 25 studies, 6501 patients). Twenty-two studies analyzed separately N– patients ($n = 3580$). This latter meta-analysis included 15 studies for RFS (2727 patients) and 11 for OS (1926 patients). High MVD significantly predicted poor outcome (RR: 1.99 for RFS and RR: 1.54 for OS). However, the authors failed to define the prognostic role of MVD in the subgroup of N– patients not treated with adjuvant chemotherapy. In addition, the prognostic significance resulted from retrospective studies is impaired from relevant selection and information biases, including between-study variations due to different patient selection criteria, techniques to stain and count microvessels, and MVD cut-off selection.

CD105 has been reported to be expressed in activated endothelial cells and, consequently, should better reflect neoangiogenesis in malignant tumors. In a retrospective study involving 905 BC patients, Dales et al. (14) correlated the IHC detection of CD105, CD31, and Tie-2/Tek with patients' long-term outcome (median follow-up 11.7 years). Univariate analysis demonstrated that higher expression of CD31 ($p = 0.032$), CD105 ($p = 0.001$), and Tie-2/Tek ($p = 0.025$) correlated with worse OS. However, only CD105 expression significantly ($p = 0.035$) identifies the subset of N– patients with poorer OS. Moreover, in multivariate analysis, CD105 and Tie-2/Tek, but not CD31, expression proved to be independent and significant prognostic indicators.

1.2.2. NON-SMALL CELL LUNG CANCER

Among the studies on the prognostic value of vascularization in non-small cell lung cancer (NSCLC), two are remarkable. In the first study by Pastorino et al. (15), 515 cases of pathological stage I NSCLC, median follow-up 102 months, several biological markers of angiogenesis were tested. None of the biological markers retained independent prognostic value, but in the subgroup of 137 patients with stage T1N0M0, both MVD (by CD31, Chalkley score) and endothelial growth factor receptor (EGFR) expressions were associated with RFS and OS in multivariate analysis. In the second study, by Fontanini et al. (16) on 407 S1–S3 NSCLC patients, median follow-up 29 months, MVD (CD34) retained a significant and independent prognostic value for OS in multivariate analysis. On the contrary, other studies have not found MVD to be predictive for survival (17).

Recently, in order to verify the impact of MVD as a prognostic marker in lung cancer, Meert et al. (18) performed a meta-analysis of literature. Only 7/14 “fVIII-RA,” 9/10 “CD34,” and 7/8 “CD31” studies (1866, 1440, and 1093 NSCLC patients, respectively) provided sufficient data for meta-analysis on survival and were evaluable for the study. High MVD was a statistically significant poor prognostic factor of OS in NSCLC, independently of the marker used. Eight of the 23 studies did not find MVD to be a significant prognostic factor. Possible explanations for the controversial results include the patient selection criteria, the heterogeneous methodologies used to stain and count microvessels, the identification of “hotspots,” Weidner or Chalkey counting method, and the MVD cut-off selection. In addition, the authors advised that the meta-analysis results are based on the aggregation of data obtained by retrospective trials. They further pointed out that in order to make MVD a reliable prognostic factor, a standardization of angiogenesis quantification would be necessary and the final results need to be confirmed in an adequately designed prospective study, with multivariate analysis inclusive of the classical prognostic factors in NSCLC (18).

Tanaka et al. (19) suggested that CD105 expression, also in limited stage NSCLC, is the best marker of angiogenesis, being a significant predictor of DFS, superior to CD34.

1.2.3. COLORECTAL CANCER

Several retrospective studies have concluded that MVD is inversely related to survival in colorectal cancer (CRC) (20). A systematic review of the literature with a meta-analysis has been performed by Des Guetz et al. (21). Thirty-two independent studies of MVD (3496 patients) reported the correlation with RFS or OS. MVD was assessed by IHC, using antibodies against fVIII-RA (16 studies), CD31 (10 studies), or CD34 (7 studies). Adequate statistics for MVD were available only in 22 of 32 studies, including 9 studies (957 patients) for RFS and 18 for OS (2383 patients). Only 1 study was prospective, and MVD significantly predicted poor RFS ($p = 0.001$) and OS ($p = 0.01$). The meta-analysis demonstrated that MVD is a poor survival predictor in patients with CRC. However, the association of MVD with survival, although statistically significant, was weak, with a global RR of 1.44 for OS and 2.32 for RFS. No study analyzed separately the prognostic role of angiogenic markers between colon and rectum cancers.

A retrospective study on 235 patients with metastatic CRC (mCRC) evaluated MVD quantified by CD34 IHC and Chalkley count. MVD did not result as significant prognostic factor (HR: 0.96) for OS, neither measured as continuous variable, nor if cutoffs were used (22).

Also in CRC, MVD by CD105 was an independent prognostic parameter for survival (23). In a study on 125 CRC patients, median follow-up 70.8 months, the CD105 vessel count was significantly correlated with metastatic disease. The authors calculated that for each one microvessel increase in the vessel count, there was a 1.42-fold increase in the risk of metastases ($p < 0.001$).

1.2.4. PROSTATE CANCER

Most published studies reported positive correlations of vascularity with prognosis in prostate cancer (PC), showing statistically significant correlations between highly vascularized PC, Gleason's score, and the risk of metastases or extraprostatic spread

at diagnosis (24). On the contrary, in a recent study, MVD, stained by fVIII-RA on sections from 104 radical prostatectomy specimens, failed to reveal any prognostic impact (25).

1.3. Angiogenic Factors

Among proangiogenic factors, vascular endothelial growth factor (VEGF) is the most extensively studied for prognostic purposes, either as tissue or as circulating factor. It has been measured most often with IHC, but also with reverse-transcriptase-polymerase chain reaction (RT-PCR) methods or with Northern blot. Basic fibroblast growth factor (bFGF), thymidine phosphorylase (26), hepatocyte growth factor (HGF), Tie2/Tek receptor tyrosine kinase (Tie2/Tek), and thrombospondins (27) have also been measured in a few studies. More recently, also the possible prognostic role of VEGF isoforms and their receptors (VEGFR) have been investigated.

1.3.1. BREAST CANCER

The association between VEGF and clinical outcome in BC has been extensively assessed, but all the published studies are retrospective (28). However, both using univariate and multivariate analyses, VEGF has been recognized to be a significant and independent prognostic indicator for RFS and OS in radically operated patients (29,30).

Linderholm et al. (31), in a study on 362 N+ patients, showed that VEGF also predicted the site of first recurrence; the patients who developed brain, visceral, or soft-tissue metastases had a significantly higher VEGF content in the primary tumor than those with bone recurrences. Similarly, in a smaller study on 125 women with primary BC, multivariate analysis showed that plasma VEGF is an independent predictor of OS (RR: 4.6, $p = 0.02$) and local recurrence (RR: 6.0, $p = 0.04$) (32).

Manders et al. (33) analyzed by enzyme-linked immunosorbent assay (ELISA) tissue VEGF from 574 tumors of N- patients not treated with adjuvant systemic therapy. The median follow-up time was 61 months. VEGF level was positively associated with age and tumor size ($p = 0.042$ and $p = 0.029$, respectively) and inversely correlated with progesterone receptor (PgR) levels ($p = 0.035$). A high VEGF level predicted worse RFS and OS both in the univariate and in the multivariate survival rate analyses ($p = 0.045$ and $p = 0.029$, respectively), in addition to age, tumor size, and PgR.

Three retrospective studies that used IHC methods on a series of 242 N- (34) and 108 N+ BC patients (35) failed to demonstrate the prognostic relevance of VEGF expression. The third retrospective study on 228 stages I-II BC patients also found that the expression of VEGF was not correlated with RFS and OS (36).

Interestingly, Konecny et al. (37) compared the prognostic significance of the six VEGF isoforms measured by ELISA in 611 primary tumor tissues of patients with a median follow-up of 50 months. When the analyses were performed separately for N- and N+ patients, VEGF(121–206) and VEGF(165–206) were of prognostic significance for survival only in N+ patients (multivariate analysis: VEGF(121–206), $p = 0.0103$; VEGF(165–206), $p = 0.0150$). The authors also analyzed the potential prognostic combined effects of HER-2/neu and VEGF measurement. The combined analysis of HER-2/neu and VEGF isoforms VEGF(121–206)/VEGF(165–206) resulted in additional prognostic information for survival. Furthermore, a biological concentration-effect relationship between VEGF expression and survival has been found (VEGF(121–206), $p = 0.0280$; VEGF(165–206), $p = 0.0097$).

Nakamura et al. (38) explored the role of VEGF-C and VEGF-D, which are selective growth factors of lymphangiogenesis, by IHC in 123 and 105 BC patients, respectively. Both factors significantly correlated with lymph node metastasis and poorer DFS and OS by univariate analysis. In multivariate analysis, only VEGF-D emerged as an independent indicator for DFS ($p = 0.045$).

Also the prognostic value of VEGFR-1/2, and Tie2/Tek has been investigated by IHC assays in a series of about 900 patients. After a median follow-up of 11.3 years, in multivariate analysis, VEGFR-1, but not VEGFR-2, or Tie2/Tek immunoexpression was identified as independent prognostic indicator, allowing the identification of patients with poor outcome, particularly among N– disease (39). Accordingly, Bando et al. measured the protein levels of free and total VEGF, VEGFR-2, and sVEGFR-1 from 202 primary BC tissues. A significant inverse correlation between free or total VEGF and estrogen receptor (ER) status was found. Multivariate analysis confirmed the independent prognostic values of total VEGF and the ratio of sVEGFR-1 to total VEGF. In subgroup analysis, total VEGF was a significant prognostic indicator in ER+ tumors, but not in ER– tumors, whereas sVEGFR-1 was significant only in ER– tumors (40).

The relationship between bFGF and VEGF expression with other BC prognostic factors, metastatic site, and survival after adjuvant therapy has been examined by Linderholm et al. (41) by ELISA in cytosol specimens obtained from 1307 patients with T1-3 primary BC (789 N–, 518 N+); the ER– patients were excluded from the analysis. The median follow-up was 70 months. bFGF overexpression was more frequently found in tumors with low VEGF expression ($p = 0.095$). Increased bFGF was associated with smaller tumors ($p < 0.001$), absence of axillary metastasis ($p = 0.003$), low S-phase fraction ($p < 0.001$), and longer RFS ($p = 0.0038$) and OS ($p = 0.0316$). VEGF was a prognostic factor of worse RFS ($p < 0.0001$) and OS ($p < 0.0001$) in uni- and multivariate analyses, whereas bFGF expression was not. Increased VEGF content was correlated with shorter survival after adjuvant endocrine therapy (RFS, $p = 0.0004$; OS, $p = 0.0009$). High bFGF expression was related to good prognostic features and longer survival times but did not add prognostic information in multivariate analysis.

1.3.2. NON-SMALL CELL LUNG CANCER

Although a few studies did not show any prognostic value for VEGF expression, probably due to small number of patients involved, in the majority of studies overexpression of VEGF particularly in early-stage NSCLC, demonstrated a significant prognostic role (42). The only published meta-analyses (43) on the prognostic significance of VEGF expression in lung cancer included both SCLC and NSCLC (1549 NSCLC patients). The analyses revealed that VEGF expression is a statistically significant unfavorable prognostic factor in NSCLC (HR: 1.48).

Nakashima et al. (44) explored the expression of different classes of VEGF mRNAs, assessed by RT-PCR. VEGF-C was found to be a significant prognostic factor in patients with squamous cell carcinoma (OR: 3.9), while VEGF-A was a significant prognosticator for those with adenocarcinoma (OR: 3.8). Seto et al. (45) examined the prognostic value of both VEGF and its receptor (flt-1 and KDR) expression in 60 patients with surgical stage I NSCLC. Specimens were IHC stained. Median follow-up was =5 years. Multivariate analysis identified the expression of both flt-1/KDR and VEGF/KDR as independent prognostic factors.

Results from the studies that explored the clinical significance of circulating VEGF are conflicting. Bremnes et al. (46) reported that among 16 published studies on the prognostic impact of elevated levels of VEGF in blood, 10 analyzed the prognostic impact of circulating VEGF on survival. Only four of these revealed a statistically significant inverse correlation between circulating levels and survival. However, the limited number of patients or the lack of adjustments of circulating VEGF for platelet counts represents relevant biases. Among the six studies evaluating the prognostic impact on intermediate markers such as clinical stage, vessel invasion, and lymph node metastasis, only the earliest published study was negative (34). Notably, in latest studies VEGF-C blood assessment has been included in addition to VEGF-A, and a higher specificity for VEGF-C than VEGF-A to predict lymph node metastasis has been suggested (47).

bFGF is frequently overexpressed in NSCLC, but its prognostic role remains controversial. Also for bFGF receptor-1 has been suggested a potential, but positive prognostic role (48). The clinical significance of circulating bFGF seems to be more promising in lung cancer than in other malignancies. In a study on 58 NSCLC patients, univariate analysis found tumor burden, platelet counts, sVEGF, and serum bFGF to be prognostic indicators for survival, but only serum bFGF remained significant in multivariate analysis (49).

1.3.3. COLORECTAL CANCER

Des Guetz et al. (21) performed a systematic review of literature. In the 18 selected studies (2050 patients), tissue VEGF expression was mostly assessed by IHC method. Statistics were performed for VEGF in 17 studies, including 9 studies for RFS (1064 patients) and 10 for OS (1301 patients). The authors highlighted that VEGF expression significantly predicted poor RFS (RR: 2.84) and OS (RR: 1.65). However, they emphasized the need of future prospective studies after a better standardization of assay methods.

In a recent study (14), 312 tissue samples were collected from patients affected by mCRC. Both epithelial and stromal VEGF expression, assessed by *in situ* hybridization and IHC on tissue microarrays, were not significant prognostic factor for OS and PFS.

Regarding circulating VEGF, it is well known that the major amount of sVEGF derives from *in vitro* degranulation of granulocytes and platelets. Therefore, plasma may be preferred for VEGF measurements. The Danish RANX05 Colorectal Cancer Study Group (50) evaluated the prognostic value of matched preoperative serum and plasma VEGF concentrations in 524 CRC patients. This study suggests that preoperative serum VEGF is a better predictor of OS than preoperative plasma VEGF. In addition, tumor drainage VEGF level seems to provide better prognostic information (disease recurrence) than its assessment in peripheral venous blood by multivariate analysis (51).

The preoperative plasma VEGF was measured by ELISA method in combination with serum carcinoembryonic antigen (CEA) in 279 patients with primary CRC. Preoperative VEGF levels were positively correlated with tumor stage ($p < 0.01$), but not with nodal status, tumor site, or grade. The combined assessment of VEGF and CEA was superior to each individual marker. In N– tumors, the patients with elevated VEGF had worse DFS ($p = 0.037$) (52).

Also postoperative circulating VEGF has been evaluated as a prognostic marker. In 318 consecutive patients who had undergone radical resection of primary CRC, VEGF concentrations in plasma and serum obtained 6 months after surgery was analyzed and the results compared with the prognostic value of postoperative CEA. In multivariate analyses, the combination of high CEA and high VEGF was significantly associated with poor survival compared to high CEA and low sVEGF (HR: 3.0; $p = 0.02$). High VEGF in the subset with elevated CEA was an even better predictor of OS than only CEA (53). In addition, it has been found that plasma VEGF levels, as well as bFGF and HGF levels, which remain permanently high 3 months after radical resection of CRC liver metastases, accurately identified the patients at increased risk of recurrence (54).

1.3.4. PROSTATE CANCER

Plasma VEGF level at diagnosis is an independent prognostic marker for survival in patients with hormone refractory prostate cancer (HRPC). In CALGB 9480, an inter-group study of suramin in patients with HRPC, pre-treatment plasma and urine median levels were analyzed by ELISA. As a continuous variable, VEGF levels inversely correlated with OS ($p = 0.002$). In multivariate analysis including serum prostate-specific antigen (PSA), alkaline phosphatase, measurable disease, VEGF levels maintained significance at various cut points tested (55). Also pre-treatment urine VEGF levels were predictive of survival. In multivariate analysis, patients whose baseline urine VEGF median level was >28 pg/ml had a significantly shorter survival (HR: 1.72, $p = 0.02$). In addition, when VEGF levels were investigated after radical prostatectomy, no statistically significant association with risk groups or known tumor-associated prognostic factors has been demonstrated (56).

1.4. Limits of Angiogenesis Prognostic Surrogate Markers

MVD demonstrated to be a weak prognostic tool in all the published meta-analyses, but several discrepancies emerged among the published studies, related to the criteria of selection of patients, the small number of cases, heterogeneity of therapies, inadequate follow-up, the choice of antibody used to stain endothelium, the experience of the observer, and the appropriateness of statistical analysis. A consensus among experts has been proposed (57, 58). It is suggested that determination of angiogenesis is presently investigational, to be used for determining biological characteristics rather than for routine clinical application.

In addition, other biological issues appear relevant. First, MVD does not necessarily correlate with proliferation indices of tumor cells or intratumoral endothelial cells. Hlatky et al. (59) stated that MVD is not a measure of the angiogenic dependence of a tumor but rather reflects the metabolic burden of the supported tumor cells. Accordingly, Hayes et al. (60), in their review of prognostic factors in BC, did not recommend the use of MVD as a basis for making clinical decisions.

Advanced solid tumors are characterized by heterogeneous vascularity. Panendothelial markers (CD34, CD31, and fVIII-RA) react better with larger vessels than microvessels, while CD105, a proliferation-associated and hypoxia-inducible protein, has been demonstrated to be preferentially expressed in small activated endothelial cells. Tie-2/Tek and VEGF/VEGFR also identify stromal vessels of tumor neoangiogenesis. As a consequence, more specific markers of angiogenesis should be more rationally used to estimate the prognostic relevance of quantitative expression of neoangiogenesis.

Last, other angiogenesis parameters including qualitative aspects, such as microvascular shape and complexity, seems to be important for accurate prognosis, in particular in squamous head and neck carcinomas, glioblastomas, and ocular melanomas, in addition to quantitative measures such as MVD (2).

Regarding a potential prognostic value of tumor and/or circulating VEGF levels, there are several unresolved issues to be taken into account (61). The first limit relay on the wide variation between published reports including method of sample collection, processing, data interpretation, and controversy whether plasma, serum, or whole blood provide the optimal prognostic information (62). Unfortunately, an international standard agreement on the preferable method to assess angiogenic factors in the blood is lacking. Some investigators use assays detecting the total amount of circulating VEGF, whereas others measure only free VEGF, or use antibodies specific for single VEGF isoforms (63). To date, it is impossible to establish whether the levels of free VEGF truly reflect tumor VEGF production or relate to degradation rates and/or altered binding to carrier proteins. Furthermore, it has been demonstrated that VEGF can be variably released from platelets and leukocytes during sampling and handling (62). In addition, a soluble form of VEGFR-1 (sFlt-1) interacts with VEGF functioning as a “decoy” receptor (64), hampering plasma VEGF evaluation.

2. SURROGATE PREDICTIVE MARKERS

As targeted therapies for cancer become increasingly integrated into standard practice, appropriate selection of patients most likely to benefit from these therapies is receiving critical scrutiny. Early experience with therapies directed at targets that are definitively overactive (e.g., the bcr-abl and c-kit tyrosine kinase targeted by imatinib) or overexpressed (e.g., the human epidermal growth factor receptor 2 [HER2] targeted by trastuzumab) has generated the perception that pre-treatment target assessment is necessary for a rationally based therapy. Despite evidence for the association of intra-tumoral and/or plasma VEGF levels with tumor progression and/or poor prognosis, pre-treatment VEGF levels do not appear to be predictive of response to antiangiogenic therapy. This may possibly be a function of the complexity of the angiogenic pathways and the limitations associated with current methods of VEGF detection and quantification; for example, low assay sensitivity and lack of standardized methods could prevent detection of very small increases in VEGF, which may be clinically important in patients with tumors that are highly dependent on this growth factor. In addition to a general lack of agreement as to the relative clinical relevance of circulating versus tumor tissue VEGF levels, the absence of a “gold standard” VEGF detection test and the lack of a predefined, clinically relevant cut-off pose a significant hindrance to the clinical utility of VEGF measurements for therapy selection.

There are also several important aspects that must be taken into account, including:

1. Why the use of surrogate predictive biomarkers of efficacy is needed in the clinical setting?
2. When do we use these markers during treatment? At diagnosis and baseline or later after therapy considering that antiangiogenic agents are preferentially cytostatic compounds?
3. Which is the best predictive factor?

4. How do we determine these factors and interpret the data? In the primary tumor or in metastatic sites? Which methods of detection and quantification must be used (e.g., ELISA, PCR, microarrays, and proteomics)?

All these questions require specific answers that could guide the oncologist in the clinical practice.

2.1. Why Predictive Biomarkers for Antiangiogenic Therapy Are Needed?

Considering the mechanism of action of angiogenic inhibitors (AIs), one of the most important problems is the choice of the optimal biological dose (OBD) as the maximum tolerate dose (MTD) and the dose-limiting toxicity (DLT) are probably not the optimal parameters for the development of these agents. In fact, at a particular dose level, there is not a linear relationship between dose and efficacy, whilst the toxicity may increase with the dose (65).

Indeed, tumor stabilization rather than objective response is probably a more appropriate endpoint as well as the time to progression (TTP) (65–68).

Early evaluation of responses and clinical benefit by surrogate indicators is fundamental to better decide on the future therapeutic program, in order to minimize drug toxicity and to optimize the costs of therapy.

There is evidence suggesting that higher doses of AIs might lead to excessive vascular pruning with reduced penetration of cytotoxic drugs into solid tumors. Jain et al. (67) suggest the role of AIs for “normalization” of tumor vasculature by decreasing the interstitial pressure and thus favoring the penetration of the drug into the tumor extracellular space.

The titration of drug dosage to achieve equivalent tumor exposure could increase the efficacy of the antiangiogenic treatments (67, 68).

In mCRC, the survival benefit of the addition of bevacizumab to chemotherapy is irrespective of objective response status. Considering all the patients, bevacizumab was associated with a decreased HR and a longer PFS. The analysis of two subgroups, responders and non-responders, found a similar incremental survival benefit, statistically significant, suggesting that a standard endpoint, such as RR, is not an appropriate measure of the efficacy of antiangiogenic compounds (66). Consequently, strategies that discontinue AIs in patients without an objective response could compromise the clinical benefit.

Another important aspect is the choice of the antiangiogenic drug or schedule of treatment in first-line setting. The efficacy of first-line antiangiogenic therapy is likely to depend on several factors, including: tumor stage, the degree of vasculature, and genotype of neoplastic cells (65–68). But with other molecular targeting agents, such as trastuzumab, the efficacy is correlated to the expression of its target. Analysis of retrospective published studies did not show a correlation of VEGF, the target of bevacizumab, and the efficacy of treatment (69, 70) (Table 1).

2.2. Microvessel Density

MVD depends on the balance of pro- and antiangiogenic factors (65–68). In the literature, there are conflicting results probably associated with methodologic discrepancies.

Willet et al. (71,72) showed a decrease of MVD in locally advanced rectal cancer following the first infusion of bevacizumab, both at low and at high doses.

Table 1
Relationship Between Vascular Endothelial Growth Factor (VEGF) Levels and Response Rate in Randomized Trials

| Tumor | VEGF Evaluation | Results | References |
|-------|--|---|------------|
| RCC | Plasma VEGF protein measured in 113 patients | No significant associations with either response or ttp | (68) |
| MBC | Tumor VEGF mRNA in FFPE tissue by ISH | No significant associations with response | (95) |
| CRC | Tumor VEGF mRNA in FFPE tissue by ISH | No significant associations with OS | (70) |
| CRC | Plasma VEGF protein by ELISA | Significant associations with OS | (22) |

^a Results of the principle published studies evaluating plasma and/or tumor VEGF as potential predictive marker of efficacy for bevacizumab treatment

CRC, Colorectal cancer; IS4, *in situ* hybridization, MBC, Metastatic breast cancer; OS, overall survival; RCC, renal cell carcinoma;

However, in a retrospective review of treated mCRC patients, MVD did not predict the survival benefit associated with the addition of bevacizumab to chemotherapy (22) and, in inflammatory and locally advanced BC, no changes in MVD were reported in the experimental group (73).

2.3. Circulating and/or Tumor VEGF and (Soluble) s-VEGFR2

Until now, several studies evaluated the role of these factors as potential surrogate biomarkers of efficacy of AIs.

In a study conducted in normal and in tumor-bearing mice with escalating dose of DC 101, a monoclonal antibody against VEGFR2, Bocci et al. showed a significant increase of mouse plasma VEGF levels after 24 h of treatment in all the treated mice when compared with the control groups which had undetectable levels reaching a plateau around the experimentally determined optimal therapeutic dose of 800 to 1200 µg/mouse. Indeed, escalating doses of DC 101 showed a marked dose-dependent antitumor activity, with a maximum effect between these two doses (74).

In 63 patients with metastatic RCC treated with sunitinib, an oral active VEGFR TKI, there were significant increases of plasma VEGF-A and placental growth factor (PIGF) but decreases of sVEGFR2 after drug exposure during each cycle of treatment. After the 2 weeks off, the levels of all the three biomarkers returned to near baseline levels. The differences between days 1 and 28 in the levels for the biomarkers were highly significant in all cycles through cycle 8 ($p \leq 0.002$). No correlation was reported between clinical response and plasma changes of these factors (75).

VEGF levels are known to increase in response to hypoxia and pharmacologic angiogenesis inhibition. There are several hypotheses that could explain this phenomenon, including the dislodgement of VEGF bound to the external domain of VEGFR-2, the rapid release of stored VEGF from known sources (e.g., platelets, α2-macroglobulin, and thrombospondin-1), the compensatory increase of VEGF in various tissues secondary to induced state of local hypoxia, the block of the VEGF-A clearance by the kidney due to the inhibition of VEGFR-2, and finally, the lack of VEGF clearing by VEGFR-2 after anti-VEGFR-2 therapy (75).

The mechanism related to the consistent decrease in plasma sVEGFR-2 levels observed in SU11248 studies is not entirely understood, as biochemical characterization of the naturally occurring sVEGFR-2 protein has only recently begun. Probably, these data could reflect a feedback regulatory loop (75).

Consistent with these data, in 28 patients treated with sunitinib, the plasma VEGF concentrations increased slightly during the first month of treatment, whereas sVEGFR2 plasma levels decreased (76).

Jubb et al. conducted a retrospective analysis in 813 patients with untreated mCRC randomly assigned to receive IFL regimen plus bevacizumab or placebo. Of 312 tissue samples collected (285 primaries and 27 metastases), outcome data were available for 278 patients (155 bevacizumab and 125 placebo). Epithelial and stromal VEGF expressions were assed by *in situ* hybridization (ISH) and IHC on tissue microarrays and whole sections. Stromal thrombospondin-2 (THBS-2) expression was examined by ISH on tissue microarrays. MVD was quantified by Chalkley count and assessed in "hot spots" by IHC as a continuous variable, or using different cut-offs to define high versus low MVD. OS was associated with these variables in retrospective subset analyses. In all the subgroups, estimated HRs for risk of death were less than 1 for bevacizumab-treated patients regardless of the level of VEGF or THBS-2 expression or MVD. Patients with high THBS-2 score (>2) showed a statistically nonsignificant improvement in survival following bevacizumab treatment compared to patients with a low score. Similar results were found in analyses of PFS and objective RR. Regarding a potential prognostic role for OS, PFS, and RR, the results were all not significant. These data, in contrast with the results of other studies, are probably correlated to clinicopathologic differences among the cohorts of patients evaluated, methodologic diversity, such as the type or the site of specimens evaluated, and probably, the small number of patients examined (22).

Based on past evidence (71), Willet et al. explored the effects of higher bevacizumab doses (10 mg/kg every 2 weeks) in two consecutive cohorts of 3 patients with locally advanced rectal carcinoma after concurrent administration of 5-FU and pelvic radiotherapy. All the 6 patients underwent surgery, and 2 complete pathologic responses were shown as compared with no complete responses in the lower bevacizumab dose group. The responses were also evaluated by computed tomography (CT) and positron emission tomography (PET) scan. Twelve days after the higher bevacizumab dose, MVD, blood flow, and IFP were reduced, but fluorodeoxyglucose uptake measured on PET scan did not change. These results, although were statistically significant only in a combined analysis, were consistent with those ones found with the lower doses (72).

In contrast with these results, a recent phase II study by Kindler et al. in 52 patients with advanced pancreatic cancer treated with bevacizumab and gemcitabine showed that pre-treatment plasma VEGF levels did not correlate with outcome. Although patients who obtained PR or SD had slightly higher baseline VEGF levels than those with PD, the difference was not statistically significant ($p = 0.37$). There was no significant difference in OS ($p = 0.2$) or PFS ($p = 0.37$) between patients whose VEGF levels were above or below the median (77).

A number of different techniques have been used to evaluate VEGF expression in human cancers, each with their associated advantages and drawbacks (Table 2), and to date there is no "gold standard" test (78).

Table 2
Summary of Common Vascular Endothelial Growth Factor (VEGF) Detection Methods

| <i>Method and description</i> | <i>Comments</i> |
|--|---|
| Immunohistochemistry (IHC) detects VEGF protein expression in whole tissue sections (usually formalin-fixed, paraffin-embedded tissue) | Easily to perform with low cost Widely applicable method No standardized methodology or scoring procedure Results variable and subjective |
| Enzyme-linked immunosorbent assay (ELISA) and chemiluminescence immunoassay (ICMA) detect VEGF protein expression in tissue homogenate (fresh-frozen tissue), serum, or plasma | Can be automated for high throughput Cannot distinguish between tumor and non-tumor sources of VEGF Circulating VEGF may be bound to serum proteins and unavailable to ELISA antibodies Serum measurements may be confounded by release of VEGF from platelets |
| Western blotting detects VEGF protein expression in tissue homogenate (fresh-frozen tissue) | Cannot distinguish between tumor and non-tumor sources of VEGF More complex than IHC |
| <i>In situ</i> hybridization (ISH) detects VEGF mRNA in whole tissue sections (ideally, fresh-frozen tissue) | Can distinguish between tumor and non-tumor VEGF expression May not directly relate to VEGF protein expression |
| Northern blotting detects VEGF mRNA from tissue homogenates (fresh-frozen tissue) | Less simple to perform than IHC Cannot distinguish between tumor and non-tumor VEGF expression May not directly relate to VEGF protein expression |
| Reverse-transcription polymerase chain reaction (RT-PCR) detects VEGF mRNA in tissue homogenates (usually fresh-frozen) | Less simple to perform than IHC Quantitative method that can be automated for high throughput Cannot distinguish between tumor and non-tumor sources of VEGF Sensitive to contamination May not directly relate to VEGF protein expression |
| RNase protection assay detects VEGF mRNA in cellular extracts (tissue or circulating) | Cannot distinguish between tumor and non-tumor VEGF expression May not directly relate to VEGF protein expression Relatively complex to perform |

Accurate and meaningful quantification of VEGF can be confounded by a number of factors; for example, increased VEGF mRNA expression is found in tumor cells adjacent to necrotic foci (65). In addition, VEGF mRNA expression correlates with vascular density in certain (e.g., carcinomas of the cervix or breast) but not all cancers (70). IHC studies have shown that in addition to VEGF staining on tumor cells,

antibodies to VEGF also often stain tumor-associated blood vessels (79,80), indicating that the vessels may provide a “sink” for binding and retaining tumor-derived VEGF (since endothelial cells do not produce VEGF) (78). Results from protein detection techniques involving tissue homogenates reflect a combination of tumor cell VEGF and associated blood vessel VEGF and thus may not accurately reflect the degree of active tumor VEGF expression at a given time. Finally, different primary tumors and metastases from the same patient may differ in their level of VEGF expression (79,80), further complicating or confounding the interpretation of data.

2.4. Determination of Circulating Endothelial Cells (CECs) and Endothelial Progenitors Cells (EPCs)

VEGF stimulates bone marrow-derived EPC to mobilize from the bone marrow compartment to enter the peripheral blood circulation, where they are referred to as circulating EPC (CEPs). CEPs move to sites of ongoing angiogenesis, incorporate into growing blood vessels, and differentiate to endothelial cells (81,82). Thus, although their role in tumor vascularization remains to be determined, it is thought that CEPs contribute to angiogenesis and therefore have the potential to become surrogate markers. However, questions remain regarding the optimal approach and the reproducibility of methods (e.g., flow cytometry) used for measuring CECs/EPC, and whether sufficient EPC are mobilized to be detected by routine testing in clinical practice.

At least two distinct populations of CECs have been identified: bone marrow-derived CEPs, which may contribute to pathologic neovascularization, and mature CECs, which are thought to be derived from mature vasculature (81–83).

Preclinical and clinical studies support a role for CEPs in angiogenesis as a measure of antiangiogenic therapy. The effect of AIs on CECs/EPC was recently evaluated in mice bearing Lewis lung carcinoma (83). In control mice, exogenous administration of VEGF increased the levels of both CEC and ECPs. Co-administration of ZD6747 inhibited this increase in CEC and CEP number but had no significant effect in the absence of exogenous VEGF. In contrast, in mice bearing Lewis lung carcinoma, ZD6474 had differential effects, causing a dose-dependent increase in mature CECs but not EPC, accompanied by a decrease in tumor MVD and tumor volume after 3 days of treatment. The apoptotic fraction of the mobilized CECs was not significantly increased by treatment. In the same study, ZD6126, a vascular-targeting agent, was evaluated. After treatment with this agent, a fivefold induction of mature CECs was observed ($p = 0.04$). These CECs were predominantly (95%) mature CECs, although a small increase in EPC was observed (83).

Moreover, treatment with a targeted VEGFR-2 antibody (DC101) caused a dose-dependent reduction in viable EPC that correlated with antitumor activity in tumor-bearing mice (84).

There is evidence suggesting that measurement of CECs could be used to determine the response to antiangiogenic therapy. A phase II study with the thrombospondin-1 mimetic peptide, ABT-510, in patients with soft-tissue sarcomas showed that patients with high baseline CEC levels exhibited reduced TTP (85). Similarly, changes in the levels of viable CECs from baseline to week 3 inversely correlated with PFS ($p = 0.015$) in patients with metastatic BC treated with letrozole plus bevacizumab (86).

In locally advanced rectal carcinoma, one bevacizumab infusion at high doses reduced the percentage of viable CECs at day 3 in 3 of 5 patients who had high CEC counts at baseline. The decrease in blood concentration of viable CECs in patients occurred at day 12, despite the significant increase in the levels of plasma VEGF and PIGF (72).

A recent study investigated the correlation between CEC kinetics and clinical outcome in patients with advanced BC receiving metronomic chemotherapy (73). CECs decreased in patients with no clinical benefit (defined as a clinical response or a stable disease) as compared with those who had a clinical benefit ($p = 0.015$). This difference was due to an increased fraction of apoptotic CECs. After a median follow-up of 17.4 months, univariate and multivariate analyses indicated that CEC values greater than $11/\mu\text{L}$ after 2 months of therapy were associated with longer PFS ($p = 0.001$) and improved OS ($p = 0.005$). In the same study, there was neither clinical benefit nor effect on CEC or CEP count and viability in patients receiving thalidomide combined with chemotherapy (data not shown) (87).

So, it would seem that a decrease in viable CECs, or an increase in non-viable CECs (resulting in an overall increase in total CECs compared to the baseline), might be surrogate biomarkers of efficacy of antiangiogenic therapy. One proposed mechanism for the observed changes is that antiangiogenic treatments damage and/or kill endothelial cells, either in circulation or in tumor-associated blood vessels, with their subsequent release into the circulation (81, 87).

A phase I study evaluated the kinetics of CECs in 32 patients with advanced solid tumors treated with ZD6126. CECs numbers increased after a median of 4 h after infusion, either after the first (week 1) or after the second (week 2) dose. The ZD6126 dose had no apparent correlation with the magnitude of CEC increase, and CEC increase did not correlate with the peak plasma concentrations or drug exposure (88).

The study by Rugo et al. (89) found that the clinical benefit of the combination of bevacizumab and erlotinib in metastatic BC was associated with post-treatment increase in non-viable CECs at 3 weeks after treatment versus the baseline.

In another study, in 16 patients treated for imatinib-resistant gastrointestinal stromal tumor (GISTs)) with sunitinib, there was a statistically significant increase of mature CECs after 6–20 days of therapy in responders versus non-responders (90).

However, there are a number of unresolved questions: (a) the sensitivity and reproducibility of the methods employed; (b) the challenge if tumors may mobilize sufficient CEPs to be detected in clinical practice as surrogate predictive markers; (c) the best antigen panel for characterization of these cells, and, finally, (d) the role of viable and non-viable CEPs (91).

2.5. Vascular Imaging

Imaging modalities are non-invasive techniques that assess a larger volume of the tumor than histology, reducing the potential of bias. Although tumor size is not always relevant for the efficacy of AIs, combinations of AIs and cytotoxic drugs make it advantageous to simultaneously assess both tumor vascularity (a marker of antiangiogenesis efficacy) and size (a marker of cytotoxic efficacy). Very few techniques have been evaluated in conjunction with antiangiogenic therapies in the clinic. There is good evidence to support a potential role for 3 techniques: dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), PET scan, and dynamic CT scan (92, 93).

2.5.1. DCE-MRI

DCE-MRI is a technique that yields parameters related both to tissue perfusion by T2-methods and to permeability by T1-methods.

Morgan et al. identified a significant inverse correlation between the reduced percentage of baseline bidirectional transfer constant (K_i), a measure of tumor permeability and vascularity, and increase in PTK 787/ZD plasma levels. Twenty-six patients with MCRC were treated with PTK 787/ZD, an oral available AI, at doses from 50 to 2000 mg once daily. The percentage of baseline K_i at day 2 and at the end of each 28-day cycle was compared to pharmacokinetic and clinical endpoints. A substantial reduction in contrast enhancement was evident for all doses on day 2 and at the end of cycle 1 (EC1). At the higher doses, the reduction was greater with a mean reduction of K_i of 43%. A significant inverse relationship was found between increasing PTK dose, area under the curve (AUC), and reducing K_i on both day 2 and EC1. Responsive patients had a significantly greater reduction of enhancement on day 2 and EC1. The authors identified a dose in which the lower limit of exposure was associated with at least 40% reduction in contrast enhancement (60% baseline K_i) and with non-progressive disease (94).

Another phase I study evaluated the role of DCE-MRI as a pharmacodynamic measure of response after therapy with AG-013736, an oral AI, in 31 patients with advanced solid tumors. Twenty-six patients were evaluable, but only 17 had data interpretable from baseline and day 2 scans. AG-013736 caused significant decreases in DCE-MRI vascular parameters by day 2 of treatment, and this decrease seemed dose dependent. However, there was no association between vascular changes and clinical response (97).

In the study by Wedam et al., 21 patients with inflammatory and locally advanced BC were treated with bevacizumab for cycle 1 at 15 mg/kg on day 1, followed by 6 cycles of bevacizumab with doxorubicin and docetaxel every 3 weeks. Tumor biopsies and DCE-MRI were obtained at baseline, and after cycles 1, 4, and 7 of therapy. The decrease of K_{trans} , representative of vascular permeability and flow measured from the two compartment model, was observed after the first infusion of bevacizumab. However, no significant difference was found between clinical responders and non-responders (73).

A critical issue is to establish the reproducibility of the measurement. For example, if an agent causes a 20% decline in a vascular parameter measured by MRI but the day-to-day variation in that parameter is 25%, then it would not be possible to understand whether that drug is active or not. Indeed, studies in the upper abdomen and thorax can be compromised by respiratory motion artefacts.

Finally, the results should be validated in larger prospective studies (92, 93).

2.5.2. PET SCAN

PET is a sensitive and quantitative technique used to monitor the pharmacokinetics and pharmacodynamics of radiolabeled drugs with positron-emitting radioisotopes. It has been used to assess tumor blood flow with oxygen-labeled water and tumor metabolism with fluorolabeled fluorodeoxyglucose as biologic endpoints of response to antiangiogenic agents. The oxygen-labeled water is freely diffusible, has a very short half-life (2 minutes), and has favorable dosimetric properties. However, there are some potential limitations. First, in small tumors, partial volume effects may be significant

if the tumor size is less than twice the resolution of the scanner. Second, there is the phenomenon called “spill over” or “spill in” of counts from surrounding structures with high blood flow, such as the heart and the aorta, or within areas of relatively high flow, such as the liver, thereby limiting the use of PET scan in lung, liver, and mediastinum. In addition, tumors may not have a uniform exchange of water between blood and tissue. Necrotic areas may have a poor exchange between blood and tissue and a lower volume of distribution of tracer, and the heterogeneity of delivery of drugs to solid tumors may lead to variability in the results obtained from PET scan (92, 93).

2.5.3. DYNAMIC OR FUNCTIONAL CT SCAN

Using dynamic or functional CT scan, it is possible to determine absolute values of tissue perfusion, relative blood volume, capillary permeability, and leakage. All these parameters provide physiological correlates for microscopic changes that occur with tumor angiogenesis. Tumor microvessels are too small to image directly, but their increased density translates *in vivo* to increased tumor perfusion and blood volume. Dynamic CT is simple, widely available, and reproducible and has been validated against oxygen-labeled water PET scan. A major problem is that this technique uses ionizing radiation, and there is limit to the number of studies that can be performed in a patient. Finally, it may be possible to label monoclonal antibodies to VEGF. This technique is currently under evaluation at several institutions (92, 93).

3. CONCLUSIONS

VEGF is a rational target for anticancer therapy, and clinicians are now faced with the challenge of how best to integrate anti-VEGF agents into clinical practice. The lack of a “gold standard” VEGF detection test is a significant hindrance to the clinical utility of VEGF measurements for selection of patients. In addition, there is no consensus regarding the most relevant form (e.g., tumor or circulating) of VEGF to be measured. Given the complexity of the VEGF signaling network, it is important to consider VEGF expression in the context of other determinants of molecular activity, such as specific isoforms, receptors and co-receptors, downstream components, and cross-talks with other molecular pathways. Recent data suggest that VEGF bioavailability, not total expression, determines the response to VEGF inhibition (94).

In recent studies, VEGF polymorphisms have been correlated with VEGF protein expression in cancer cells and tumor angiogenic activity (95, 96). The improvement of angiomics and proteomic technologies may render possible to define a comprehensive genetic or protein biomarker expression pattern, using panels of genes or proteins that will allow the physician to define a specific, individualized, profile to select the most appropriate antiangiogenic therapy for each single patient.

Despite the rational basis for antiangiogenic therapy, the identification and validation of prognostic and predictive markers still remains a challenge. Preliminary data suggest promising results with sVEGFR-2 and CEC/EPC evaluation in the circulation and new imaging strategies, including DCE-MRI, PET, and dynamic CT scan, but the data were obtained only in small phase I/II studies, so they need to be confirmed in larger prospective trials.

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Endpoints for the Determination of Efficacy of Antiangiogenic Agents in Clinical Trials

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SUMMARY

Agents targeting angiogenesis have proven efficacy and an expanding role in cancer therapy. Preclinical development has been rapid, in part because assays exist to determine biological activity. In early clinical trials, the modest single agent efficacy in phase I trials requires surrogate or tumor assays to determine proof of mechanism and early proof of efficacy. Many theoretically attractive assays have been disappointing in clinical practice. New assays have been introduced or are about to be introduced into clinical development. Experience with existing agents provides benchmarks to evaluate these proposed assays.

Key Words: angiogenesis; clinical trials; cancer; biomarkers.

1. INTRODUCTION

The first trials of therapeutic agents targeting the proliferation of the endothelial vascular network in tumors faced a potential dilemma in determining an appropriate endpoint for evaluating efficacy. Preclinical experiments provided conflicting expectations whether these agents would be cytostatic or cytotoxic, as different agents gave different outcomes and sometimes even the same agent could produce disparate results, depending on the exact experimental conditions. A second class of agents directed at tumor vasculature, the vascular-targeted agents (VTA), produced a cytotoxic effect within the tumor vascular endothelial cells, with endothelial cell death, tumor hemorrhage, and tumor necrosis. However, there was a potential for a transient increase in tumor volume or bidimensional area, the traditional endpoint for the determination of efficacy in phase II—or even phase I—trials. The preclinical investigator, with efficacy models that produced results in days to weeks, received definitive endpoints in a reasonable time. In a clinical trial, even transient progression in tumor size is frequently an indication to remove a patient from a study and consider the investigational agent of no benefit. The long-term effects might never be seen, simply because

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of standard trial design. The development of interim surrogate endpoints to evaluate any possible efficacy of a potentially cytostatic or slow-acting agent became a new challenge for clinical investigators developing antiangiogenic agents. These methods would be under study as much as the agents themselves. The surrogate endpoints clinical investigators and drug developers have employed include clinical, radiological, and pathological studies. The discussion of these surrogates in 2006 is much more erudite because of a decade of trial, error, observation, and results, and because there are now several new clinically approved agents that were specifically developed to target tumor angiogenesis. Proper attribution to the trial and error of a decade of clinical drug development of these and other agents must be considered part of any discussion.

2. CLINICAL SURROGATES

When the first specifically targeted angiogenesis agents (as opposed to clinically approved agents where angiogenesis was not the original target but a secondary mechanism, such as interferon, celecoxib, and thalidomide) went into clinical trials, no toxicity phenotype was known. The remarkable safety in animals was a class effect, certainly of the purely angiogenesis-targeted agents, as opposed to the VTA. Concerns for bleeding, impaired wound healing, and ischemia to normal tissues predominated (1,2). In fact, all of these have been observed, albeit only in a relatively small number of patients for bevacizumab, sorafenib, and sunitinib (1, 3, 4). These events do not occur clinically in a gradual manner, in that mild or moderate events that would not be of a dose-limiting severity occur regularly in phase I trials and could serve as useful markers of a biological activity. These events are almost always clinically significant, often severe, life threatening, or fatal. Unless an individual patient demonstrated a significant benefit from the study agent, it would be unethical to re-administer the agent to the patient. Rare events are often not appreciated in phase I studies, where most patients receive a suboptimal dose of the agent (for either clinical benefit or toxicity), relatively few patients are treated, and selection/eligibility criteria exclude patients with demonstrable risk factors. The uncertainty about attribution to the study agent in the clinical setting of an advanced cancer that may be progressing is often difficult unless the toxicity is completely unrelated to any effect the cancer itself may have on a patient, such as a stroke, myocardial infarction, or abdominal perforation at site of a previous surgical anastomosis, diverticulus, or ulcer (5). These events have been seen with bevacizumab, sorafenib, and sunitinib, but at incidences less than 3% in the large phase II and III trials, where rapid accrual and specific tumor factors, such as the absence of prior therapy predominate over physiological eligibility factors (1, 3, 4). Bleeding was seen in the phase I and II trials, particularly with bevacizumab, but investigators attributed these to specific tumor-host factors such as pulmonary location or specific tumor type (squamous cell histology, central location, and presence of necrosis with bevacizumab in phase II lung cancer trials).

3. HYPERTENSION

In retrospect, it is now clear that agents that target the vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor 2 (VEGFR-2) axis produce hypertension as a class effect. This includes bevacizumab, a monoclonal antibody that removes circulating VEGF, and both of the clinically approved small

molecule VEGFR-2 receptor tyrosine kinase inhibitors (RTKIs) sorafenib and sunitinib. The overall incidence at the clinically approved dose of each agent is approximately 60% (1, 3–5). The heterogeneity of patients, such as the presence or absence of preexisting hypertension or concomitant use of antihypertension drugs, may influence the incidence. Unlike myocardial infarction, stroke or rupture of abdominal viscera, hypertension is a continuous variable and may be a very useful surrogate for activity. Hypertension may not only determine the final dose recommendation but also be a surrogate that a particular agent at a specific dose demonstrates inhibition of the VEGF/VEGFR-2 axis. This is important not only when an agent specifically targets this axis, but also when an agent has an extended spectrum of activity with VEGF/VEGFR-2 as a component of multiple tyrosine kinase inhibition. It may be possible then to determine whether an inhibitor has a balanced spectrum of activity, inhibiting VEGFR-2 at a dose that also inhibits other tyrosine kinases. VEGF-Trap, AG 13736, and AZD2171 are examples of VEGF/VEGFR-2-targeted agents (although each has an extended spectrum, either exclusively directed at angiogenesis or other targets).

It appears that hypertension combined with evidence of target interaction at other sites might help in the development of new, multi-targeted agent. Recently, an extended spectrum selective kinase inhibitor, XL880, began clinical trials. XL880 is a small molecule RTKI that targets VEGFR-2, the platelet-derived growth factor B receptor (PDGFR B), TIE-2, and the c-MET receptor. Immunohistochemistry studies on tumor samples showed significant inhibition of phosphorylation (>75%) in MET and its closely related receptor RON, at doses well below where hypertension was observed and in fact below the maximum-tolerated dose (6). Thus, if an agent is supposed to target VEGF/VEGFR-2, hypertension serves as a surrogate for target interaction, even if it is not a dose-limiting toxicity. If such an agent does not produce hypertension at tolerable doses, it is unlikely that the agent will be useful for inhibiting VEGF/VEGFR-2 in the clinic.

The mechanism of hypertension that results from inhibiting the normal physiologic action of VEGF is unknown (2, 7). Preeclampsia, a condition that occurs in pregnancy, offers clinical and biological insight into the mechanism of hypertension associated with disruption of VEGF/VEGFR pathway signaling. Preeclampsia is a condition that occurs in 5% of pregnancies (8–10). It is diagnosed by the occurrence of hypertension (systolic blood pressure > 140 mm Hg and/or diastolic pressure > 90 mm Hg), proteinuria [>0.3 g/24 h or clinical dipstick 1⁺ positive (30 mg/dl)], decreased glomerular filtration due to vasospasm and microthrombi in the afferent arterioles, elevated liver transaminases, and evidence of brain dysfunction. This may be demonstrated as headache, blurred vision, scotoma, and/or confusion. [The full syndrome overlaps or is the same as the posterior reversible encephalopathy syndrome (PRES) seen in hypertensive encephalopathy (11).] Histopathologic changes in the brain include edema, ischemia/hemorrhagic changes, vasculopathy, and fibrinoid necrosis. Preeclampsia/eclampsia is due to increased binding of VEGF to soluble placental VEGF receptor 1 (soluble Flt-1 receptor) produced by the ischemic placenta. Consequently, the mother experiences the result of depriving normal tissues and organs of the normal physiologic amount of VEGF signaling, with serious adverse effects as noted above.

The actual cause(s) of hypertension are still under investigation. Postulated mechanisms include rarefaction, the decrease in microvascular density that results from the reduced number of capillaries as a result of antiangiogenic activity (12). There may also be direct vasomotor effects to angiogenic growth factors and receptor activation. VEGF causes endothelial cell-dependent vasodilation and dose-dependent hypotension in animals (13, 14). Activation of *AKT/PKB* by VEGF stimulates the phosphorylation of endothelial cell nitric oxide synthase (eNOS), with increased calcium-independent activity leading to increased nitric oxide production (12, 13). Nitric oxide, the endothelial-derived relaxing factor (EDRF), is a potent vasodilator. VEGF increases the messenger RNA, protein, and activity of eNOS in endothelial cells. VEGFR-2 may be activated by shear stress in absence of ligand to increase eNOS. Abrogation of nitric oxide production in endothelial cells abolishes the effect of VEGF on proliferation, permeability, and vasodilatation (12, 15–19).

The direct hemodynamic effects of angiogenic growth factors have been described. VEGF administered intracoronary or intravenously causes hypotension, as does fibroblast growth factor (FGF). Hepatocyte growth factor, the ligand for c-MET, causes a decrease in systemic vascular resistance when given intravenously. The potential clinical significance of decreased VEGF signaling resulting in hypertension include tissue ischemia or hypoxia, endothelial cell dysfunction, platelet activation, and thrombosis, with contributions to peripheral edema, PRES, arterial plaque angiogenesis, nephropathy/albuminuria, and a generalized inflammatory or pro-thrombotic state (12).

Clinical studies of blood markers such as renin, angiotensin, catecholamines, endothelin I, or VEGF itself and the like in patients treated with sorafenib did not indicate any significant correlations with any value and hypertension (20). Experimental work with AZD 2171 in rats suggested that at high doses, the calcium channel blocker nifedipine was the most effective treatment (21). No systematic therapeutic stratagem has been devised for clinical use.

4. PROTEINURIA

Proteinuria, specifically albuminuria, occurs with VEGF/VEGFR-2-targeted agents. The incidence is 40–60% with bevacizumab if all degrees of severity are calculated. VEGF is essential to glomerular podocyte integrity and function. The podocytes, through their slit diaphragm complexes, regulate protein filtration and damage to the podocytes or any of the slit diaphragm proteins produces protein loss in the urine. Inactivation or mutation in the genes producing nephrin—Neph 1 and 2—CD2AP, and podocin causes human and experimental glomerular disease with proteinuria (22).

During development, decreased or absent VEGF production from the podocytes causes the endothelial cells lining the glomerulus to hypertrophy, round up, detach from the glomerular basement membrane, and disrupt the slit diaphragms. VEGR-directed antibodies significantly reduce the levels of nephrin in mice and are associated with proteinuria, consistent with the crucial role nephrin expression and function has in proteinuria (7, 23).

As VEGF signaling is essential to endothelial cell and podocyte slit diaphragm structural integrity, resolution of proteinuria will likely not be immediate upon removal of drug. Proteinuria will likely persist until the ultra structure of the glomerular filtration apparatus is restored. Moreover, as clinicians now have multiple choices of

VEGF/VEGFR-2-targeted agents, such as renal cell cancer, rapid succession of agents may have a compound or cumulative effect on proteinuria, resulting in more severe loss with subsequent agents (9, 10, 23).

Proteinuria and urine protein to urine creatinine ratios (U_p/U_c) may be a sensitive indicator of drug effect, as it is in assessing renal damage in diabetes mellitus. Although proteinuria is not necessarily dose-limiting toxicity in itself, proteinuria is less amenable to intervention than hypertension, and nephrotic range proteinuria in conjunction with the nephrotic syndrome (albuminuria > 3 gm/day, serum albumin < 3.0 gm/dl, and peripheral edema) will be dose limiting and clinically intolerable. Patients on phase I and II clinical trials who have received prior nephrotoxic drugs (i.e., platinating agents, ifosfamide, gemcitabine, etc.) and prior VEGF/VEGFR-2-targeted agents and/or are hypoalbuminemic as a result of anorexia may be more sensitive than otherwise healthy patients. As a result, dose-limiting toxicity may occur more easily in these early studies than it might otherwise, and specific eligibility criteria might be utilized to avoid a premature cessation of dose escalation.

5. TISSUE BIOPSIES

The presence of an increased number of small blood vessels within a tumor (microvessel density, MVD) is indisputable as an adverse prognostic factor in a number of cancer types, including breast, colon, and sarcoma (24). As the objective of targeting the VEGF/VEGFR-2 axis and the angiogenesis of cancer is to reduce the viability of the tumor neovascular network, it has been proposed that the ideal surrogate assay for antiangiogenic activity is reduced MVD within the tumor itself. Although an attractive idea, there are conceptual and logistical problems with tumor sampling for MVD or other histopathologic markers of antiangiogenic activity. The different possible methodologies for measuring MVD could produce discrepancies in results, as there is no standard assay method in use. The choice of antibody for staining, such as CD34, CD31/PECAM, or factor VIII/von Willibrands factor, for example, used to detect endothelial cells can potentially account for this. The selection of where in the tumor to look, such as the center or periphery, may influence the score of the MVD. The reproducible finding of increased MVD at the most vascular portion of the tumor as a poor prognostic fact, regardless of the above, suggests that too much attention to differences may be unwarranted, and valid comparisons can be made between and among various studies.

As pointed out by Hlatky et al. (24), MVD differs widely from tumor type to tumor type. MVD depends on the metabolic requirement of a particular tumor, specifically on oxygen delivery and nutrient supply. MVD is a measure of the capillary density, and the distance of tumor cells from the capillary depends on the oxygen consumption and nutrient requirement of the tumor cells. This differs between tumor types and at different stages of a tumor's growth. All tumor blood vessels are not alike. There are many cul-de-sacs and areas of pooled, oxygen-depleted blood, with little active flow and consequently, little or no oxygen and nutrient exchange. Certain tumors, perhaps especially so with melanoma, may employ vascular mimicry, whereby the cells lining the blood capillaries may not be endothelial cells at all but tumor cells adapted to a new role. Thus, some tumors may have more blood vessels than needed to adequately supply the cancer cells, and the loss of some vessels will be of no consequence for

proliferation. Other tumor masses may have an abundance of poorly functioning vessels whose loss will be inconsequential and may even involute without any intervention. If these factors remain constant, a tumor may decrease in size but have its MVD remain unchanged, as the ratio of tumor cells supplied by each endothelial cell will remain constant even as a tumor progressively shrinks in size. These theoretical concerns may affect the significance of the findings and lead to misleading conclusions about the effect of any treatment.

Changes in MVD would be expected to result from inhibition of VEGFR-2 or other endothelial cell targets, with presumably endothelial cell apoptosis preceding a decrease in MVD. However, there are many technical steps in immunohistochemistry or *in situ* hybridization that are very demanding and can make results difficult to interpret or reproduce, even within one laboratory as well as between studies. The timing of tissue sampling is important. If the agent has a short plasma half-life or more important a short duration of target inhibition in tissue (often not readily known), a tissue sample may be obtained too late to show an inhibitory effect on receptor phosphorylation, for instance. By the same token, a sample that shows good target interaction may be too early to demonstrate endothelial cell apoptosis and certainly too early to see changes in MVD. Investigators might come away thinking that the agent does not even inhibit the target at maximal clinical dose or assuming that inhibition does not produce either endothelial cell death or a decrease in tumor vascularity. Even a well-designed immunohistochemistry (IHC) or *in situ* hybridization (ISH) protocol, with reliable antibodies and excellent tissue handling and preparation, may give uninformative or misleading results just because of timing. The spatial heterogeneity within a tumor results in well-vascularized areas, particularly at the edges, and poorly vascularized areas, often necrotic areas. Biopsies may be obtained from different areas within a tumor at different time points and produce contradictory or misleading results having nothing to do with therapeutic or biologic effect of an agent under study. These issues are very important, and the difficulty of obtaining paired pretreatment and post-treatment in clinical studies, at the appropriate time and location, handling, processing, and preparing these samples, should never be underestimated!

The requirement of a tumor biopsy before and after treatment significantly limits the pool of potential patients. This necessarily slows the rate of accrual and prolongs a study of a new agent. Biopsies also add to the risk, duration, and expense of a trial, often significantly, on all accounts. It would be useful to see what significance the use of biopsies has had on the ultimate development of antiangiogenic drugs or drug combinations.

Conventional chemotherapy in breast cancer has minimal effect on MVD even when the treatment produces significant clinical responses (25). Breast cancer is a useful model, as pretreatment biopsies from locally advanced cancers where preoperative chemotherapy is the treatment standard that can be compared with the findings at definitive surgery. In the study reported by Bottini et al. (26), endothelial cells were stained with CD34, and vascularity defined as number of vessels counted in the area of highest vascular density. They found that anthracycline-based chemotherapy was able to slightly but significantly decrease MVD, but the decrease did not correlate with the degree of response, including complete responses. Regardless of the therapy and the therapeutic outcome, there remained a strong correlation between pretreatment and post-treatment MVD, suggesting that conventional cancer therapy directed at cancer

cells as opposed to endothelial cells does not significantly affect the degree of baseline angiogenesis despite even a significant reduction in tumor volume. Thus, the MVD remains the same, regardless of whether the tumor increases or decreases in size.

The addition of the humanized monoclonal antibody bevacizumab to irinotecan/5-fluorouracil/leucovorin (IFL) chemotherapy was the first demonstration that VEGF/VEGFR-targeted therapy had meaningful clinical benefit, as the combination treatment group had a significantly longer time to tumor progression and overall survival compared with IFL placebo-treated patients (5). In a retrospective study of archived primary tumor tissue, MVD and *in situ* hybridization of VEGF expression in tumor epithelial and endothelial cells were not significant predictive factors for treatment response, time to progression, or overall survival (26). It may be that the angiogenic content of metastatic tumors is different from the primary tumor and that archived material is of little or no value in predicting outcome in metastatic disease. Nevertheless, the results of this study are an important cautionary point in the use of MVD or VEGF expression as decision criteria in selecting antiangiogenic therapy in metastatic colorectal cancer (and possibly other tumor sites).

Many agents that target endothelial cell angiogenesis have been tested. Many of these agents are ineffective and have had their clinical development stopped after phase I or II trials. Many other agents remain under active investigation and few or no reports are yet in print or subject to peer review. Nevertheless, several agents have had experience with measurement of MVD or receptor phosphorylation published. SU5416 is a specific and very potent TKI of VEGFR-2 ($IC_{50} = 0.160 \mu\text{mol}$), and SU6668 is a RTKI of VEGFR-2, basic fibroblast growth factor (bFGF), and platelet-derived growth factor(s). Considerable effort went in to determining whether each had antiangiogenic in patients at the clinical doses used (27). Clinical development of both these agents has been stopped. SU5416 had minimal clinical activity and undesirable pharmacologic properties. SU6668 had a broader spectrum of angiogenesis that was intended to overcome the limited activity of SU5416. SU6668 had unacceptable toxicity at doses below those demonstrating clinical activity in humans. Sunitinib (SU011248) demonstrated remarkable clinical activity at tolerable doses, without the need to demonstrate target activity in surrogate or tumor tissue, and has been approved for the treatment of renal cell cancer and gastrointestinal stromal tumors (3).

Tumor biopsies from several clinical studies of SU5416 and SU6668 were analyzed for the effect on receptor phosphorylation of VEGFR-2 by IHC and ISH by Davis and colleagues (28) after careful testing first in xenografts to establish optimal conditions. Considerable heterogeneity was observed for receptor phosphorylation and no correlation with clinical response was observed. They proposed that the duration of receptor inhibition, not merely the degree to which occurs, might have a more marked effect. As with all clinical investigations, the heterogeneity between and among tumors may be a more significant factor than can be accounted for by even the latest technology and techniques. Neither agent has sufficient clinical activity to preclude the possibility that the lack of significant correlations is not simply because of ineffective agents. There are no published results of these types of correlative studies with either sunitinib or sorafenib. Sunitinib had a 25% objective response in the phase I trial by response evaluation criteria in solid tumors (RECIST), a standard far more meaningful and interpretable than surrogate tissue or tumor biopsy correlates of angiogenic efficacy, which are often substitutes for the lack of demonstrable clinical efficacy.

The repeat biopsy of tumor masses that persist despite treatment might actually be expected to yield no significant changes; otherwise, the tumor should be smaller. The previously cited study of XL-880 was a series of biopsies of superficial skin metastasis in a patient with malignant melanoma responding to therapy (6). That significant target inhibition was seen, with a resulting increase in tumor cell apoptosis in clinically shrinking lesion should not be surprising, as this is the perfect scenario to demonstrate drug activity but one that is extremely rare in early clinical trials. This also emphasizes how difficult tissue biopsy is in drug development, and why it is essential to develop some other marker that is simpler to obtain, more reproducible, and less expensive.

6. BLOOD MARKERS

Markers of angiogenesis and angiogenic inhibition in circulating blood would be an easily obtained, easily repeated means to test the efficacy of agents targeted at tumor angiogenesis. Such assays could be done on every patient, with multiple time points repeated as often as necessary to obtain an adequate profile of drug activity. The same considerations apply to blood tests as to tissue samples: the assay must be sensitive, specific, accurate, and precise and the measurement must be of something that is involved in the pathogenesis of tumor angiogenesis. Two principal assays type have come into wide usage: angiogenic proteins, notably VEGF itself and circulating endothelial cells.

The measurement of circulating VEGF levels is complicated. Jelkman (29) has reviewed some of the important considerations in VEGF measurement in clinical samples. The sensitivity, accuracy, and precision have improved, and the availability of several reliable commercial antibodies has made comparisons between studies easier. However, besides the technical aspects of the test, the analyte itself is important. Different assays may measure different isoforms of VEGF, and it is critical to determine whether VEGF is free or bound to soluble receptors, such as VEGFR-1/flt1. Serum contains more VEGF because of the release of VEGF from the platelet-release reaction upon blood clotting. Platelet counts are often increased in cancer patients, particularly with advanced disease.

Levels of VEGF itself have a genetic component. Genetic polymorphisms affect the expression and release of VEGF (30–32). Persons with a single-nucleotide polymorphism (SNP) at promoter site VEGF⁺⁴⁰⁵ have different levels of VEGF expression. VEGF G⁺⁴⁰⁵G has higher levels of VEGF than VEGF G⁺⁴⁰⁵C, which in turn are higher than VEGF C⁺⁴⁰⁵C. The incidence of preeclampsia increases in nulliparous women with CC > GC > GG SNPs at position 405⁺. A second SNP at VEGF-2578 also regulates VEGF expression with CC > CA > AA production and same relative risk of preeclampsia (30). High VEGF expressers have a greater risk of IgA nephropathy (31) as well. These factors have not been explored in cancer and may complicate studies in this area, introducing unaccounted variability into interpretation of results.

Plasma and urinary levels of VEGF decrease after treatment with a number of VEGFR-2-directed RTKIs, including SU5416, SU6668, valatinib, and sunitinib (3, 27, 28, 33). In the phase I trial of sunitinib, the only approved agent in this group, dose-dependent increases in VEGF and decreases in soluble VEGFR-2 were observed, suggesting that these assays may be clinically useful in establishing a threshold of target

inhibition. The newly hypoxic tumor likely increased VEGF expression in compensation, similar to the findings in mice through inhibition of VEGF signaling by the murine monoclonal antibody DC101 (34).

Other studies have looked for changes in other angiogenic factors such as bFGF, ICAM, and soluble VCAM (25). The reports on these are few, and no significant effects were observed, but these were in clinical trials of unapproved agents (35). The results with sorafenib or sunitinib, which are not yet in print, may be more helpful as these agents have useful clinical activity.

7. CIRCULATING ENDOTHELIAL CELLS

Circulating endothelial cells (CEC) contribute to vasculogenesis in physiologic and pathologic states. Vasculogenesis is differentiation of circulating angioblasts to form a basic vascular network. The physiologic roles of vasculogenesis in the post-natal state include maintenance of the corpus luteum and proliferative uterus in menses, pregnancy, in the response to ischemia in the diabetic retina and myocardium, corneal neovascularization, and normal wound healing. CEC may be multipotential (heme-) angioblasts with progenitor capability, senescent cells shed physiologically from the vascular network, a variable proportion of which are apoptotic, and endothelial cells shed from the disorganized “chaotic” vascular bed of tumors (36).

Assays for CEC have included cell culture and flow cytometry. Flow cytometry is less expensive, rapid, and far less technically dependent than culture methods and has largely supplanted it. Which surface markers signify a specific subset of CEC is not fully established, but there is emerging consensus that CD133 is present on the surface of true multipotential progenitor cells (a very rare population of the total CEC) and CD146 is present on the much more numerous differentiated CEC population. The basic surface marker CEC profile is CD31+ CD45-. Studies in colorectal cancer patients with bevacizumab demonstrated a decrease in CD133 bright cells with only a week of therapy (37, 38). More extensive studies have shown that CD146+ cells are not only more abundant and more readily detectable in plasma but that CD146+ cells were present in vascular endothelial cells, pericytes, and surrounding small round stromal cells. Thus, the CD146+ CEC population has a direct role in cancer angiogenesis and is amenable to repeat sampling of patient blood and effective VEGF-targeted antiangiogenic therapy that reduces the size of tumors also reduces plasma levels of CECs.

CECs offer another non-invasive assay for angiogenesis. Although requiring dedicated investigators, flow cytometry is a widely used, accurate, precise, and reproducible technique that can be repeated frequently to every patient at minimal risk and discomfort. CEC analysis requires additional study, particularly with agents of differing mechanism of action but appears to be very promising as a surrogate for drug action.

8. RADIOLOGIC IMAGING

The efficacy of cytotoxic chemotherapy has been traditionally assessed by measurement of the cross-sectional diameter of tumors on repeated computed tomography (CT). Formal guidelines for tumor measurement and the assignation of different degrees of response have been laid out in the RECIST (39). Angiogenesis inhibitors, however, are primarily cytostatic in their effects and do not induce rapid tumor shrinkage as seen on CT. Therefore, conventional imaging may be insensitive to the

therapeutic effects of this newer class of agents. Clinical pathologic studies have demonstrated a correlation between the levels of plasma or urine angiogenic factors like VEGF and prognosis in several tumor types, including breast, colorectal, non-small cell lung cancer, and melanoma although they have not been validated as surrogate markers of response (40–42). Assessment of MVD on tissue biopsy, while more reliable than circulating biomarkers in predicting disease response, is subject to sampling variability and is invasive (24).

In addition to the different mechanism of action from cytotoxic chemotherapy, the antiangiogenic agents tend to have fewer traditional side effects (such as GI toxicity or bone marrow suppression). Although of obvious benefit to the prospective patient, this lack of predictable side effects has brought into question the standard dose-escalation paradigm used in early phase drug development and highlighted the need for an alternative methodology for dose selection with antiangiogenic agents. Phase I studies of this class of drugs need to focus more on establishing the optimal biological dose (OBD) rather than the maximal-tolerated dose (MTD), and phase II studies require a means of assessing response to therapy other than conventional CT.

Radiographic imaging of tumor blood flow, tumor vascular volume, and/or vascular permeability offers a mechanism-based approach to monitoring the biologic effect of these agents. We review the use of dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), dynamic (or functional) CT, ultrasound (US) and positron emission tomography (PET), and their advantages and disadvantages in assessing changes in tumor microvasculature.

The goals of molecular imaging of angiogenesis and angioactive agents are threefold: (i) to better understand underlying tumor angiogenesis and biology, (ii) to obtain pharmacodynamic data about the activity of cytostatic antiangiogenesis drugs, and (iii) to confirm that such pharmacodynamic surrogates predict for meaningful clinical endpoints like overall survival.

9. DCE-MRI

DCE-MRI has emerged as the predominant modality for determining vascular changes for several reasons. First, MRI in general is excellent for soft-tissue imaging and is already commonly used to define tumor staging and response to therapy. Second, the standard contrast agent, gadolinium diethyltriamine pentaacetic acid (Gd-DTPA), is readily available and relatively non-toxic. Third, there is no ionizing radiation, so multiple studies can be performed to monitor response to therapy (43). Finally, there is increasing evidence that DCE-MRI results correlate with certain immunohistochemical features of tumor angiogenesis, such as tumor grade and VEGF expression in breast cancer and tumor MVD in cervical cancer (44–46). In one study, a higher degree of vessel permeability, a hallmark of the “leaky” vessels induced by tumor angiogenesis, predicted for worse survival (45).

DCE-MRI essentially assesses changes in the tumor microvasculature by measuring changes in vessel permeability and blood volume. T2-weighted images provide information about tumor perfusion (blood flow and blood volume) as the contrast agent is viewed passing through the tumor vasculature a few seconds after contrast injection. T1-weighted images provide information about tumor permeability by measuring the rate and volume of contrast accumulation into the tumor extravascular-extracellular space

(EES) on delayed images. Blood volume and flow to the tumor is dependent upon arterial input, and this requires that the MRI take at least one slice through the aorta or another large artery in the region of interest to calculate an arterial input function (AIF) (43).

Changes in tumor microvasculature are measured in terms of changes in blood flow and volume and vessel permeability. The following hemodynamic parameters have been recommended as standards for describing the kinetics of a given tracer in DCE-MRI: K^{trans} , v_e , and k_{ep} . K^{trans} is the volume transfer constant, which refers to the rate at which the contrast agent moves from the plasma into the EES; v_e is the volume of the EES; and k_{ep} is the rate of reflux of contrast agent from the EES back into the plasma (47).

In one of the first studies of antiangiogenic agents, Eder et al. (35) administered recombinant human endostatin once daily as IV infusion to fifteen patients with refractory solid tumors. Comparative DCE-MRI studies were used before treatment and after each of three monthly cycles of therapy to assess quantitative effects on tumor blood flow and volume in ten of the fifteen patients. No consistent effect was seen in either the volume transfer constant, K^{trans} , or the volume of the EES, v_e . The investigators also noted that image acquisition of lung and liver metastases was difficult to reproduce on serial examinations because of motion artifact. The inability to capture an effect on DCE-MRI, however, is difficult to interpret as there was only one objective response among the fifteen patients, and the duration of time that serum levels of endostatin exceeded those required to exert an *in vitro* antiangiogenic effect was brief.

For the first time, there is now persuasive evidence that DCE-MRI can be used to establish a dose–response relationship and, possibly, to predict for a clinical response to antiangiogenic therapy. In a phase I study, Morgan et al. gave twenty-six patients with metastatic colorectal cancer an oral VEGF receptor inhibitor, PTK787/ZK 222584, in a standard dose-escalation schema and performed DCE-MRI at baseline, day 2, and at the end of each 28-day cycle (48). They were able to demonstrate a significant negative correlation between increasing doses of PTK/ZK and decreases in vessel permeability. Furthermore, those patients who had a reduction in tumor vessel permeability on DCE-MRI were statistically more likely to have stable disease rather than progression.

Many issues surrounding DCE-MRI remain to be resolved prior to its widespread incorporation into early clinical trials. Standardization of image acquisition and methods and software for analysis are required for any multicenter trials and to allow for comparison of results between trials. The majority of DCE-MRI data in the current literature were derived from institution-specific computer programs for data analysis. Intrapatient reproducibility from scan-to-scan is also an issue given the natural fluctuation in dynamic parameters like blood flow and vessel permeability. Some experts have recommended obtaining two baseline studies to ensure that percent changes observed after antiangiogenic therapy are not simply within normal variation (49). However, Morgan et al. (50) have recently published data using a single slice DCE-MRI protocol that was highly reproducible, with a coefficient of variation in K^{trans} of only 16–19% between studies. Finally, the most important task is to validate DCE-MRI (or any other biomarker image) as a surrogate for real clinical endpoints like time to progression or overall survival. The Morgan study is the first step in this direction, and we await other trials to confirm this finding.

10. PET

PET scanning has become widespread within oncology for diagnosis, staging, re-staging, and assessing response to therapy, and its clinical applications continue to evolve (51). The most commonly used tracer, ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG), permits visualization of highly metabolic tissues like that seen in many neoplasms. The technology has been adapted to measuring tumor perfusion by substituting ¹⁸F-FDG with ¹⁵O-water, which is freely diffusible, has a short half-life (2 minutes), and has favorable dosimetric features (52).

There are several limitations to PET scanning, however. The exposure to ionizing radiation makes it difficult to design trials that require serial examinations. Tumors near areas of naturally occurring high blood flow, like the heart or aorta, may be obscured from “spill-in” or “spill-over” of the tracer. This may make it difficult to use PET scanning to assess antiangiogenic effects on lesions located in the lungs or mediastinum. Also, tumors are heterogeneous in terms of their distribution and functionality of blood vessels, and there may be non-uniform diffusion of tracer, particularly in necrotic areas within the tumor. Finally, PET tends to underestimate blood flow in areas with high flow rates (53).

One example of the utility of PET scanning was demonstrated in a phase I study of endostatin in patients with advanced solid tumors in which changes in blood flow (using ¹⁵O-water) and tumor metabolism (using ¹⁸F-FDG) were measured at different doses (53). The study demonstrated a decrease in tumor blood flow in patients receiving between 180 and 300 mg/m²/day of endostatin. These radiographic findings seemed to correlate with an increase in both tumor cell and endothelial cell apoptosis found on repeat tumor biopsies. As in the aforementioned Eder et al. study, however, there was no significant anticancer effect seen in any of the treated patients.

11. DYNAMIC OR FUNCTIONAL CT

Dynamic or functional CT scan, such as DCE-MRI, can be used to obtain quantitative information regarding blood volume, blood flow, and vessel permeability by timing the injection of a contrast agent with conventional CT image acquisition (54). As tumors signal through angiogenic factors, the vessels they create are too small for conventional imaging. However, they increase perfusion and blood volume by an *in vivo* increase in vessel surface area that can be assessed by dynamic CT (49). As in PET scanning, the exposure to ionizing radiation limits the number of studies that can be performed serially on a single patient and represents the biggest impediment to incorporation into early phase trials. However, it is simple to use, widely available and often incorporated into dual PET-CT scanners.

In an elegant phase I study of neoadjuvant therapy in rectal cancer patients, Willett et al. (38) investigated the effects of VEGF blockade with bevacizumab with both imaging and invasive biopsies. Six patients were given bevacizumab 5 mg/kg as a single dose 2 weeks prior to combined chemoradiation and weekly bevacizumab. Dynamic CT and PET scans were obtained prior to the initial dose of bevacizumab and then 12 days later. Furthermore, flexible sigmoidoscopies with tissue biopsies and measurement of interstitial fluid pressure (IFP) were performed both before and 12 days after the bevacizumab. The investigators were able to correlate the effects of antiangiogenic therapy on tumor biology (blood perfusion, blood volume, vessel permeability, IFP, MVD, and FDG uptake)

to tumor response. Twelve days after receiving bevacizumab, only one of six patients had a gross tumor response when re-assessed on flexible sigmoidoscopy. Decreases in blood perfusion by 40–44% ($p < 0.05$) and blood volume by 16–39% ($p < 0.05$) on dynamic CT scan and MVD and IFP on tissue biopsy after a single dose of bevacizumab supported the principle that antiangiogenic therapy mediates both a decrease in overall microvasculature and a “normalization” of aberrant tumor vessels (35). All six patients had an excellent response to therapy, but the relative contribution of VEGF inhibition in comparison to the standard chemoradiation program could not be derived from this study.

12. US

Standard Doppler US can resolve structures down to the millimeter level and has therefore been unable to image changes that occur at the level of the tumor microvasculature (49). US technologies continue to evolve, and recent techniques using high frequency (20–100 MHz range) have been reported to detect flow in vessels down to 15–20 μm in a murine ear model (55). Nonetheless, the poor tissue penetration and the heavy operator reliance of US have so far limited the role of this imaging modality in studying the effects of antiangiogenic agents in solid tumors.

13. CONCLUSIONS

Tumor angiogenesis is now a validated clinical target for cancer therapy, as three agents developed specifically for this purpose have demonstrated sufficient clinical efficacy to be approved for clinical use. All three happen to target the VEGF/VEGFR-2 axis, but other agents with different mechanisms of action or expanded target spectrum built on the existing platform of VEGF axis inhibition are under active investigation. Although sunitinib retains a clinical profile similar to classic DNA-targeted agents with measurable radiological shrinkage of tumors, sorafenib and bevacizumab do not. Alternative means of determining target activity, without relying on all-to-infrequent tumor regression in phase I trials, will be important to the development of these agents. At present, the only validated surrogates are in the VEGF axis, so some of the observations may not be generalized to other angiogenic targets (56).

The basic clinical findings in patients on study provide valuable information. For agents that potently target VEGF/VEGFR-2, hypertension is a continuous marker of increased VEGF inhibition. Although individual patients will vary in their susceptibility to hypertension, the appearance of hypertension in population of patients receiving a VEGF axis-targeted agents signifies target interaction. Proteinuria will follow, but likely in a delayed manner. VEGF-targeted RTKI will cause a rise in plasma and urine VEGF levels.

Radiologic imaging is also an assessment that can be repeated frequently and provides dual information on blood flow and tumor response. DCE-MRI appears most advanced and while expensive in general, it may be very cost effective in decisions about whether an antiangiogenesis agent is effective and appropriate to study further.

The appeal of looking directly at tissue for efficacy is alluring and often overpowering to investigators. The data available suggest that either the methods used are insufficiently sensitive or that issues around timing and tumor heterogeneity are so formidable that this is not a practical tool in drug development at this time. Much more translational work needs to be done to validate IHC and ISH in patient material before the risk and expense justifies biopsies as a matter of routine practice. Circulating endothelial cell assays are

very promising and could be of general use. Too little has been done yet to be certain, but early clinical work in VEGF- and non-VEGF-targeted agents looks promising.

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The Role of Imaging in the Clinical Development of Antiangiogenic Agents

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SUMMARY

Angiogenesis, the process of new blood vessel development from the pre-existing vasculature, is vital for tumor growth and subsequent metastasis and has become an important target for novel anticancer therapeutics. One major difficulty in the development of these antiangiogenic agents is the lack of a robust biomarker of target inhibition that will enable establishment of an optimal biological dose (OBD). Recently, several non-invasive imaging modalities have been adopted to try and address this problem, and these will be discussed in this chapter. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) has been most extensively investigated, and published guidelines are available detailing best practice. Emerging data indicate that changes in DCE-MRI measurements of tumor perfusion on treatment with antiangiogenic agents may correlate with clinical outcome. However, reproducibility, in particular between centers makes intertrial comparisons difficult and the problem of tumor heterogeneity, needs to be fully addressed.

Key Words: Angiogenesis; clinical trials; imaging; antiangiogenics; DCE-MRI; PET; functional CT; ultrasound.

1. INTRODUCTION

Angiogenesis, the process of new blood vessel development from the pre-existing vasculature, is vital for tumor growth and subsequent metastasis. In the absence of a vascular network, neoplastic deposits remain dependent on diffusion for nutrient delivery, oxygenation, and the removal of waste metabolites. Their growth is therefore restricted to 1–2 mm³ and they remain clinically quiescent (1). The clinical importance of tumor angiogenesis is reinforced by the demonstration of a correlation between microvascular density and both the rate of metastasis and patient survival for most types of solid malignancies (2–7). These findings indicate the broad applicability of

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antiangiogenic strategies to the management of human malignancy and are reflected in the large number of targeted antiangiogenic agents under clinical development.

Although the classical phase I trial design paradigm for cytotoxic agents is based on recommending the highest administered dose associated with acceptable toxicity, such an approach is not appropriate for antiangiogenic agents. This is because the relationship between toxicity, which may be unpredictable, and efficacy is far from clear, meaning that the optimal biological dose (OBD) may be significantly lower than the maximum-tolerated dose (MTD) (8). Despite this problem, the majority of phase I trials conducted with targeted agents have until recently been reliant on toxicity criteria for recommending doses for future development (9), because of the lack of robust validated biomarkers of target inhibition. Further clinical development of these agents may also be challenging because of their predominantly cytostatic mode of action indicating that conventional response criteria measured on cross-sectional imaging are unlikely to be the most sensitive means of assessing whether antiangiogenic agents have clinically relevant anticancer activity (10). Despite these problems, however, antiangiogenic agents, in particular bevacizumab, a monoclonal antibody to vascular endothelial growth factor (VEGF), have been shown to improve survival when combined with conventional chemotherapy in colorectal, breast, and lung cancers (11–13).

Much recent research has therefore focused on the development of biomarkers that can be utilized to determine whether antiangiogenic agents are having their predicted effect on the tumor vasculature (8). Although determining changes directly in intratumoral microvessels by serial biopsies is a key approach to addressing this question, it is invasive and restricts significantly the patient population suitable for entry into these clinical trials. It also suffers from problems associated with sampling and tumor heterogeneity. The use of imaging methodologies to measure angiogenesis *in vivo* has therefore received much attention and has been utilized in several early-phase clinical trials. In this chapter, we will discuss the imaging modalities that have been used recently to aid the clinical development of antiangiogenic agents, their relative advantages and drawbacks, and try to define the role that angiogenesis imaging may play in future.

2. ROLE OF A BIOMARKER FOR ANGIOGENESIS INHIBITION

A useful biomarker of angiogenesis inhibition should allow us to address several key factors in antiangiogenic drug development. These include

1. Proof of principle. Does the agent inhibit angiogenesis? Is there clear evidence of decreased intratumoral blood flow/vascular permeability?
2. Dose selection. Is there a relationship between the dose/exposure to the agent given and the size of the effect on tumor perfusion? What is the dose of the drug required to achieve the optimal degree of angiogenesis inhibition?
3. Predictive factor. Is the magnitude of effect on tumor perfusion related to the clinical outcome? Can the biomarker be used quickly to determine which patients are likely to respond to the drug and which not?

Clearly, the identification of such a biomarker would allow the rapid screening of novel antiangiogenic agents, allowing the development of those that are ineffective to be rapidly halted and those that are promising to be utilized at their OBD in the right patients. However, such a biomarker would need extensive validation and to be highly

Table 1
Angiogenesis Imaging Modalities

| <i>Imaging modality</i> | <i>Benefits</i> | <i>Drawbacks</i> | <i>Clinical validation</i> | <i>Reproducibility</i> |
|-------------------------|---|--|--|--|
| DCE-MRI | Published best practice guidelines | Lack of uniform software/analysis algorithms Motion artefact | Correlation with microvessel density, tumor hypoxia, and VEGF expression | Difficult to correlate between centers |
| Functional CT | Commercially available analysis software | Radiation exposure | Correlation with microvessel density and PET | |
| | Linear relationship signal-contrast concentration | Contrast allergy | | |
| PET | Sensitivity | Short tracer half-life Limited availability Radiation exposure | | Most reproducible technique |
| Ultrasound | Real-time imaging Portability | Poor depth of penetration Poor sensitivity of Doppler imaging Operator dependent | None in humans | Operator dependent |

CT, computed tomography; DCE-MRI, dynamic contrast-enhanced magnetic resonance imaging; PET, positron emission tomography; VEGF, vascular endothelial growth factor.

reproducible across clinical centers. It is important that these factors are considered when discussing the four main angiogenesis imaging methodologies used in clinical trials to date (Table 1).

3. DYNAMIC CONTRAST-ENHANCED MAGNETIC RESONANCE IMAGING

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) has become the most extensively utilized angiogenesis imaging modality. It is non-invasive, avoids ionizing radiation, and can be performed on scanners used for routine clinical applications. It works by tracking the pharmacokinetics of a low-molecular weight paramagnetic contrast medium, generally gadopentetate dimeglumine (Gd-DTPA), which alters

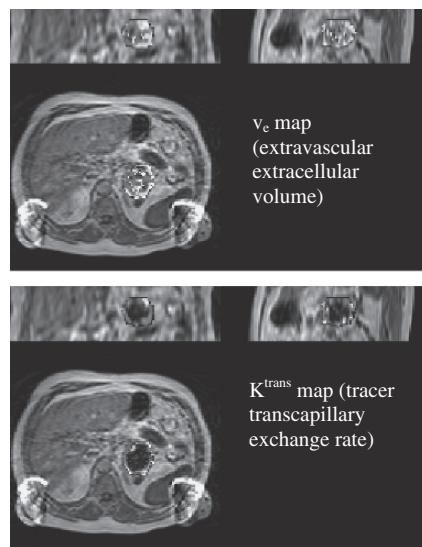


Fig. 1. T1-weighted DCE-MRI images of a pancreatic neuroendocrine tumor. Representative coronal (top left), sagittal (top right), and axial (bottom) images showing maps of v_e and K^{trans} of the tumor region of interest. Courtesy of Dr. Geoff Parker, Imaging Science and Biomedical Engineering Group, University of Manchester, UK.

the magnetic state of the hydrogen atoms in water. After administration of Gd-DTPA as a rapid intravenous bolus injection, first-pass T2*-weighted imaging that detects high-contrast concentrations that cause dephasing (also known as T2* relaxation) provides information on blood flow and blood volume within the region of interest (ROI) that is being imaged (14, 15). Technical limitations, however, restrict the utility of T2-weighted DCE-MRI imaging in oncology primarily to brain tumors (16). Subsequent T1-weighted imaging is sensitive to the low concentrations of contrast that leak out of the microvasculature into the extravascular extracellular space (EES) (Fig. 1), which causes signal enhancement. The change of T1-weighted signal intensity with time is acquired using serial imaging and provides information on tissue perfusion, endothelial permeability, and EES volume—this technique is particularly sensitive to intratumoral vasculature due to its dependence on high concentrations of VEGF, which increase vascular permeability (14).

The calculation of parameters that describe the microvasculature from DCE-MRI data is complex. Many techniques have been described, but these fall into two main groups: semi-quantitative parameters that describe the shape of the signal intensity–time curve and parameters derived from pharmacokinetic modeling. Although semi-quantitative parameters are relatively easy to calculate, they are dependent on intratumoral physiology and contrast agent kinetics in a complex and incompletely defined manner. They are also influenced by scanner settings, contrast injection technique, and position of the ROI. As such, direct comparisons between patients and imaging centers are difficult (14, 17). Pharmacokinetic parameters, however, are theoretically independent of the scanning acquisition protocol and are therefore more likely to depend solely on tissue characteristics. This supports their use in multicenter studies and to allow cross-study comparison (16).

Table 2
Summary of Key Recommendations for DCE-MRI Analysis of Antiangiogenic Agents

| |
|---|
| Assessment should be based on T1-weighted studies of low molecular weight Gd-chelates |
| K^{trans} or IAUGC should be primary endpoint |
| Vascularized tumor volume should be calculated |
| 3D-measurements of ROI are preferred |
| Tumor arterial input function should be calculated individually |
| Baseline reproducibility should be assessed in each case |

DCE-MRI, dynamic contrast-enhanced magnetic resonance imaging; IAUGC, initial area under the gadolinium concentration time curve.

Source: Reproduced with permission (14).

Current guidelines (Table 2) indicate that K^{trans} (transfer constant) and initial area under the gadolinium concentration time curve (IAUGC) should be the primary endpoints reported for studies employing DCE-MRI as a pharmacodynamic measure of angiogenesis inhibition as these are the most reproducible between centers. K^{trans} describes the passage of contrast across the endothelium into the EES and as such is dependent on both the perfusion of the ROI and both the permeability and surface area of the endothelium (Fig. 1). It therefore provides an indication of a composite of these factors. Tumor-vascularized volume can then be easily calculated by summing the voxels above a predetermined threshold of K^{trans} or IAUGC within the ROI.

3.1. The Reproducibility of DCE-MRI

One key factor in interpreting DCE-MRI data is ensuring reproducibility. Consensus recommendations indicate that two baseline scans are performed to establish intratumoral reproducibility prior to drug administration. Studies have indicated that changes in vascular parameters of 20–45% can be detected with confidence in normal tissues/tumors (18, 19). Although reproducibility appears better for gliomas as these are confined by the skull, in one study of hepatic metastases conducted over an 8-h period, the coefficient of variation in K^{trans} was 11% and the percentage change required to prove drug activity was 15% (20). However, the imaging of tumor deposits within the thorax and liver remains difficult due to the significant movement artefacts created by respiration and the heart beat.

Another factor impinging on reproducibility, particularly in longitudinal studies, is the selection of the ROI for analysis. Ideally, this should encompass the entire tumor deposit volume, as single-slice 2D measurements are more prone to bias due to incomplete sampling and positioning errors. The data should be analyzed on a voxel-by-voxel basis although average measurements may be taken if there is significant motion artefact (14). Although summary statistics as described above are the recommended way of presenting DCE-MRI data, these may hide important findings related to intratumoral heterogeneity that reflects variations in microvessel density, expression of growth factors, hypoxia, and necrosis (10, 17). Newer techniques are under development to address this. Histogram analysis of individual voxel data has allowed the demonstration of subtle therapeutic effects in clinical studies (21). Fractal analysis of DCE-MRI data and principal component analysis to identify macroscopic patterns of heterogeneity (vascular domains) within the defined ROI are also under exploration (22, 23).

Although validation of DCE-MRI data against patient outcome data is still in its early stages (see Section 3.2.), a number of studies have addressed its correlation with other markers of angiogenesis including microvessel density (summarized in refs (10, 17)). Although these studies are somewhat confounded by the difficulty of comparing histological evaluations at the micrometer level with radiological data, the resolution of which is measured in centimeters, significant correlations have been noted in most studies.

3.2. The Clinical Application of DCE-MRI in Studies of Antiangiogenic Agents

Although DCE-MRI has been utilized as a correlative pharmacodynamic endpoint in the evaluation of several antiangiogenic agents in the last 5 years (Fig. 2), there is currently insufficient technical standardization to allow direct comparisons between these studies. Important findings, however, have emerged from these early-phase clinical trials. In the first reported study to use this method (24), the humanized monoclonal anti-VEGF antibody HuMV833 was noted to reduce first pass K^{trans} at 48 h at all dose levels tested with a median fall of 44% (range 5–91%). Sustained reduction in K^{trans} was noted at 35 days for all but the lowest dose level (0.3 mg/kg) tested. No dose response relationship was found suggesting a threshold effect. Notably, variation of up to one order of magnitude was noted in baseline K^{trans} , between patients reflecting variations in functional anatomy and pathophysiology within human tumors.

The investigation of AZD2171, an oral VEGF receptor tyrosine kinase inhibitor (25), in a phase I/II study again performed in a patient population with multiple cancer types also demonstrated a threshold effect on iAUC₆₀ with reductions seen only in doses above 10 mg/day. However, further analysis suggested that the extent of reduction was

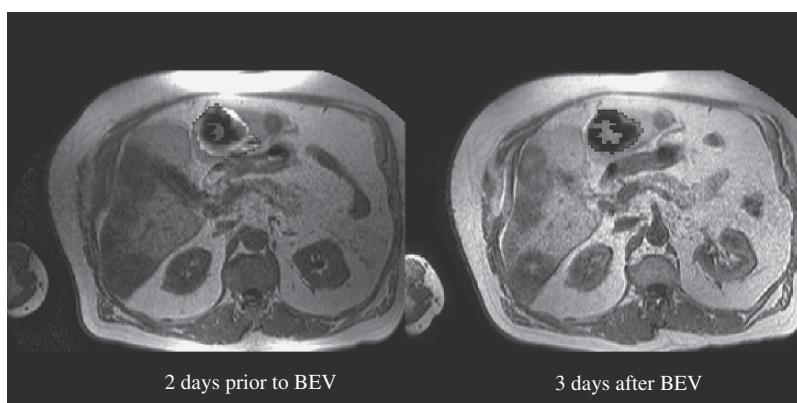


Fig. 2. K^{trans} parameter maps of a patient with hepatic metastases from a colorectal primary tumor. Images taken 2 days before and 3 days after administration of 10 mg/kg bevacizumab (BEV). A marked reduction in K^{trans} after treatment can be seen in the region of interest. Courtesy of Dr. Geoff Parker, Imaging Science and Biomedical Engineering Group, University of Manchester, UK.

correlated with total drug exposure as defined by the steady state AUC for AZD2171 clearance measured at day 28.

It is possible that the marked heterogeneity in tumoral vascular parameters seen in studies recruiting patients with advanced cancer from multiple tumor types such as those described above could be a potentially significant impediment to the utility of DCE-MRI in early clinical studies. This may be overcome by selecting cohorts of patients with a defined disease subgroup. Such an approach has yielded intriguing findings with PTK787/ZK222584, an oral inhibitor of VEGFR1, 2 and 3, c-kit, and PDGFR. An analysis of DCE-MRI data obtained from 22 patients with advanced colorectal carcinoma and hepatic metastases who were treated daily on a continuous basis over seven dose levels on two concurrent phase I clinical trials has been reported (26). Bidirectional K^{trans} (K_i) was measured on single slice images at baseline and days 2 and 28 after starting treatment. Significant inverse correlations were detected between percentage decrease in K_i and increase in oral dose and plasma AUC for PTK787/ZK222584 ($p = 0.0001$). Importantly, patients with stable disease at day 28 ($n = 12$) had a significantly greater reduction in K_i at both days 2 and 28 than those patients whose disease progressed ($n = 9$) (K_i at day 28 as percentage of baseline = 48 vs. 99%). These decreases in K_i were maintained at day 56 in patients whose disease remained stable. Subsequent mathematical modeling suggested that DCE-MRI may have potential for detecting OBD—a 40% reduction from baseline K_i was associated with clinical benefit. The oral dose of PTK787/ZK222584 required to achieve this was 1000 mg/day. These findings have subsequently been confirmed in a study using twice-daily dosing in patients with multiple tumor types (although 24 of 43 patients had colorectal carcinoma with liver metastases) (27).

A study utilizing sorafenib, an oral targeted inhibitor of Raf kinase, VEGFR-2, and PDGFR- β in patients with metastatic renal cell carcinoma (28) has also produced interesting results, with changes in DCE-MRI parameters correlating with patient outcome. Imaging was performed at baseline and a median of 6.1 weeks after commencing treatment in 15 patients. K^{trans} reduced by a median of 60% and notably the size of change in K^{trans} correlated with progression-free survival (PFS) ($p = 0.01$). Intriguingly, baseline K^{trans} was also associated with PFS indicating that it may be a predictive factor for responsiveness to sorafenib. Although these results are intriguing, and are the first to address validation of this marker against clinical outcome, the patient numbers investigated in these studies were small.

The validity of DCE-MRI has also been assessed in a pharmacodynamic study of bevacizumab in 21 patients with locally advanced breast cancer (29). Bevacizumab 15 mg/kg was administered as a single agent for one dose prior to combination with chemotherapy. This initial dose was associated with a median decrease of 34% in K^{trans} and 15% in k_{ep} . Simultaneous tumor core biopsies were taken to assess phosphorylated VEGFR-2 levels. Although decreased receptor activation was noted in tumor cells, no meaningful data were obtained for endothelial cell VEGFR-2 activation so preventing firm conclusions being drawn as to whether the changes in K^{trans} were caused by inhibition of proangiogenic pathways.

DCE-MRI has also shown promise in determining the mechanism of toxicity associated with novel antiangiogenic agents. In a phase II study (30), we administered CDP860 humanized PEGylated di-FAb' directed against PDGFR- β to patients

with advanced ovarian or colorectal cancer. Three of eight patients rapidly developed clinically significant ascites and seven of eight had signs of fluid retention. DCE-MRI demonstrated rapid increases in tumor-vascularized volume consistent with the recruitment of previously poorly perfused vessels elucidating a putative mechanism for these toxicities.

4. POSITRON EMISSION TOMOGRAPHY AND ANGIOGENESIS IMAGING

Positron emission tomography (PET) imaging is a sensitive and quantitative technique that allows the detection of very low amounts of positron-emitting tracer molecules. Although the use of ¹⁸F-labeled fluorodeoxyglucose PET is well established as a physiological imaging modality to stage cancer and assess response to anticancer treatment (31), there is much less research using other PET tracers to directly image angiogenesis. Although inhalational ¹¹CO and ¹⁵C have been used as markers of blood volume through assessment of carboxyhemoglobin levels, these and the use of ^{H₂15}O as a marker of blood flow are hampered by the short half-life of the tracer molecules (2 min for ¹⁵O and 20 mi for ¹¹C). This limits these techniques to centers with on-site cyclotron facilities. Although ^{H₂15}O is freely diffusible, a clear advantage for imaging angiogenesis, its use has other limitations. For instance, the imaging of small tumors is complicated by partial volume effects due to scanner resolution and counts from tissues with high blood flow, such as the heart, aorta, and liver, can mask tumors close to these areas, the so-called spill-over phenomenon (32). The use of direct arterial sampling is also required to obtain the most value from this technique although modeling has been used in some studies (17,33).

Because of these problems, the use of ^{H₂15}O PET to assess antiangiogenic agents has been limited. In one phase I study of endostatin administered as a short daily infusion (33), a modest 20% reduction in index tumor blood flow was detected after 28 days treatment at dose levels above 120 mg/m²/day. Studies using the vascular-disrupting agent combretastatin A4 have also indicated the potential utility of this technique (34), and it is possible that with the recent development of combined PET-computed tomography (CT) scanners that allow the co-registration of functional and anatomic data, its use to assess antiangiogenic agents will increase.

5. FUNCTIONAL CT AND ANGIOGENESIS

Functional CT imaging can be readily incorporated into standard CT scanning protocols and provides excellent anatomical resolution. It also has the advantage of providing absolute values for tissue perfusion, capillary permeability, and relative blood volume (10,17) although sensitivity is relatively low compared with DCE-MRI. Functional CT imaging using multislice and helical scanners has also been validated against intratumoral microvessel density and ^{H₂15}O PET imaging (17,35–37). However, the use of functional CT for the multiple imaging assessments required in early-phase clinical trials evaluating antiangiogenic agents is limited by repeated significant exposures to both ionizing radiation and potentially toxic intravenous contrast agents.

Despite this, functional CT has been incorporated as a pharmacodynamic endpoint in several studies. In an important study exploring the antiangiogenic effects of

bevacizumab in rectal carcinoma (38), six patients who received 5 mg/kg bevacizumab underwent functional CT assessment and sigmoidoscopic tumor biopsies at baseline and 12 days after bevacizumab administration. A 40–44% reduction in tumor perfusion was noted on imaging at day 12, and this was associated with reductions in tumor microvessel density, intratumoral interstitial fluid pressure, and tumor ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) uptake. Such findings are consistent with a hypothesis of bevacizumab-induced vessel normalization and can be considered as preliminary validation for the use of functional CT. An association between decreased tumor perfusion on functional CT and response by cross-sectional imaging/clinical benefit has also been demonstrated in a phase II study of AG-013736, an oral VEGFR-1, VEGFR-2, and PDGFR inhibitor (39) in metastatic renal cell carcinoma. Dose-dependent changes in functional CT imaging parameters have been detected in a phase I study of MEDI-522, a monoclonal antibody against $\alpha_v\beta_3$ integrin (40). Increases in mean transit time of contrast through the designated ROI were noted at 8 weeks in a dose-dependent manner although no significant changes were seen in mean blood flow, blood volume, or permeability surface product. It was postulated by the authors that this finding reflects a biological effect on the tumor microvasculature.

6. ULTRASOUND IMAGING

Ultrasound has many potential attractions for imaging angiogenesis. It is a relatively low-cost imaging modality, is portable, allows imaging in real-time, and can be repeated without exposing the subject to significant risks. However, its use in the evaluation of antiangiogenic agents has lagged behind DCE-MRI for several reasons. Notably, until recently, the resolution of standard Doppler imaging was restricted to blood vessels above a millimeter in diameter (41). However, the development of novel gas-encapsulated microbubble contrast agents has improved sensitivity to allow vessels as small as 70 μ m in diameter to be detected. These bubbles act as reflectors of the ultrasound pressure wave at low energy levels but are destroyed at high energy intensities resulting in an intense echo signal known as flash ultrasound scintillation that allows the effects of contrast reperfusion to be assessed (41). These agents allow assessment of tumor blood flow and volume but are dependent on the skill of the operator. The utility of ultrasound for imaging angiogenesis is also limited by depth of tissue penetration.

Initial attempts using color Doppler ultrasound have failed to detect significant changes in tumoral blood flow after patient exposure to PTK787/ZK222584 (42) or endostatin (43). In the case of PTK787/ZK222584, DCE-MRI detected changes in K^{trans} in the same patient group. However, recent preliminary results have been published using microbubble contrast-enhanced Doppler to assess blood flow within hepatocellular carcinoma nodules in patients treated with thalidomide (44). Significant changes in both blood volume and flow were detected indicating that this technique may hold promise and should be investigated further.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

The use of angiogenesis-imaging modalities to assess pharmacodynamic endpoints has been used in many early-phase clinical trials of antiangiogenic agents as discussed above. Most experience has been gained with DCE-MRI, and it is clear that reductions

in K^{trans} and IAUC have been documented in several studies (24, 26, 28, 29). Indeed preliminary validation against clinical outcomes have been noted in two small trials (26, 28) although the sobering results from the CONFIRM trials place in doubt the clinical utility of PTK787/ZK222584 (45, 46).

However, despite consensus guidelines (14), intertrial comparisons remain difficult due to problems with reproducibility between centers. This is compounded by heterogeneity of the intratumoral vasculature that may mask significant antiangiogenic effects in phase I trials, in particular in those recruiting patients with multiple tumor types. It is clear that more sensitive and reproducible analysis techniques that take heterogeneity into account are required and these are under development. One other approach to this problem would be to use each patient as their own comparator and employ intrapatient dose escalation, thus removing interpatient heterogeneity. It is also important to note that the recommended method for presenting DCE-MRI data, K^{trans} , is an artificially derived value that is dependent on both blood flow and vascular permeability and so essentially reflects a composite of these two factors. It may be possible in future to analyze blood volume/flow separately using high-molecular weight paramagnetic contrast agents that are under development (17). These agents will not extravasate and therefore have potentially superior biophysical performance characteristics.

Improvements in technology are also required that will allow imaging of smaller target lesions and lesions in organs subject to motion artefacts so as to increase the utility of angiogenesis imaging. Currently there is significant attrition in evaluable patients with all imaging modalities. For instance, in one study employing DCE-MRI (47), only 65% of patients had assessable lesions and McNeel et al (40) could only analyze 50% of their patients using functional CT.

It is clear, however, that as we gain more experience using the imaging modalities discussed in this chapter, it will enable us to accelerate and target the clinical development of antiangiogenic agents and potentially select patients who are more likely to benefit from these targeted therapies.

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