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The Biology of Vascular Endothelial Growth Factor

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I. Introduction

THE development of a vascular supply is a fundamental requirement for organ development and differentiation during embryogenesis (1, 2) as well as for wound healing and reproductive functions in the adult (3, 4). Angiogenesis is also implicated in the pathogenesis of a variety of disorders: proliferative retinopathies, age-related macular degeneration (AMD), tumors, rheumatoid arthritis, psoriasis, etc. (3, 4). In the case of proliferative retinopathies and AMD, the new blood vessels are directly responsible for many of the destructive events characteristic of these conditions. Leakage and bleeding, followed by organization of the clot and fibrosis, may ultimately lead to retinal detachment or irreversible damage to the macula (5). Conversely, tumor-associated neovascularization, by establishing continuity with the systemic circulation, allows the tumor cells to express their critical growth advantage and also facilitates metastatic spreading (3, 4). Accordingly, a correlation has been observed between density of microvessels in primary breast carcinoma sections, nodal metastases, and survival (6–8). Similarly, a correlation has been reported between vascularity and invasive behavior in a variety of other tumors (9–12). These findings led several investigators to conclude that the number of vessels in tumor sections is an independent predictor of outcome in cancer patients (9–12).

The search for potential regulators of angiogenesis has yielded numerous candidates: acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), transforming growth factor-α (TGF-α), TGF-β, hepatocyte growth factor, tumor necrosis factor-α (TNF-α), angiogenin, interleukin-8 (IL-8), etc. (13, 14). Although these molecules are able to promote angiogenesis, at least in certain model systems, it has been difficult to correlate such activity with the physiological or pathological regulation of blood vessel growth.

Work done by several laboratories over the last few years has elucidated the pivotal role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (15). In particular, the recent finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (16, 17). Furthermore, VEGF-induced angiogenesis has been shown to result in a therapeutic effect in animal models of coronary (18–20) or limb (21–23) ischemia and, most recently, in a human patient affected by critical leg ischemia (24).

II. Biological Activities of VEGF

VEGF is a potent mitogen (ED_{50} 2–10 pM) for micro- and macrovascular endothelial cells derived from arteries, veins, and lymphatics, but it is devoid of consistent and appreciable mitogenic activity for other cell types (25–31). The denomination of VEGF was proposed to emphasize such narrow target cell specificity (25, 26). VEGF promotes angiogenesis in tridimensional *in vitro* models, inducing confluent microvascular endothelial cells to invade collagen gels and form capillary-like structures (32). These studies provided evidence for a potent synergism between VEGF and bFGF in the induction of this effect (32). Also, VEGF induced sprouting

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from rat aortic rings embedded in a collagen gel (33). This model emphasizes the specificity of VEGF, as the proliferation induced by this growth factor consisted almost exclusively of vascular endothelial cells. In contrast, insulin-like growth factor-I (IGF-I) or platelet-derived growth factor (PDGF) induced endothelial cell sprouting accompanied by extensive fibroblastic proliferation (33). VEGF also elicits a strong angiogenic response in a variety of *in vivo* models including the chick chorioallantoic membrane (26, 29), the rabbit cornea (34), the primate iris (35), the rabbit bone (27), etc.

VEGF induces expression of the serine proteases urokinase-type and tissue-type plasminogen activators (PA) and also PA inhibitor 1 (PAI-1) in cultured bovine microvascular endothelial cells (36). Moreover, VEGF increases expression of the metalloproteinase interstitial collagenase in human umbilical vein endothelial cells but not in dermal fibroblasts (37). The coinduction of PA and collagenase by VEGF is consistent with a prodegradative environment that facilitates migration and sprouting of endothelial cells. Pepper and Montesano (38) proposed that PAI-1 provides a negative regulatory step that serves to balance the proteolytic process. Other studies have shown that VEGF promotes expression of urokinase receptor (uPAR) in vascular endothelial cells (39). Considering that the PA-plasmin system and in particular the interaction of uPA with uPAR is an important element in the chain of cellular processes that mediate cellular invasion and tissue remodeling (40), these findings are consistent with the proangiogenic activities of VEGF.

VEGF is known also as vascular permeability factor (VPF) based on its ability to induce vascular leakage in the guinea pig skin (41, 42). Dvorak and colleagues (43, 44) proposed that an increase in microvascular permeability is a crucial step in angiogenesis associated with tumors and wounds. According to this hypothesis, a major function of VPF/VEGF in the angiogenic process is the induction of plasma protein leakage. This effect would result in the formation of an extravascular fibrin gel, a substrate for endothelial and tumor cell growth. Recent studies have also suggested that VEGF may be a factor that induces fenestrations in endothelial cells (45). Topical administration of VEGF acutely resulted in the development of fenestrations in the endothelium of small venules and capillaries, even in regions where endothelial cells are not normally fenestrated, and was associated with increased vascular permeability (45). Interestingly, Dellian *et al.* (46) have described the quantification and long-term physiological characterization of microvessels induced by gels containing either VEGF or bFGF in transparent chambers in the dorsal skin or in the cranium of mice. These studies indicate that VEGF- or bFGF-induced vessels have similar diameter, permeability to albumin, and red cell velocities. However, permeability and red cell velocities were higher in the cranium than in the dorsal skin. These findings led to the conclusion that the steady-state physiological properties of blood vessels, including permeability, are primarily determined by the local microenvironment, rather than the initial angiogenic stimulus (46).

An additional effect of VEGF on the vascular endothelium is the stimulation of hexose transport (47). Exposure of bovine aortic endothelial cells to VEGF or TNF-α resulted in a significant increase in the rate of hexose transport. The combination of factors had an additive effect. This action may have relevance for increased energy demands during endothelial cell proliferation or inflammation.

Recently, Melder *et al.* (48) have shown that VEGF promotes expression of VCAM-1 and ICAM-1 in endothelial cells. This induction may result in the adhesion of activated natural killer (NK) cells to endothelial cells, mediated by specific interaction of endothelial VCAM-1 and ICAM-1 with CD18 and VLA-4 on the surface of NK cells (48). It has been suggested that these effects may provide an explanation for the previously observed preferential adhesion of IL-2-activated NK cells to the tumor vasculature (49).

VEGF has been reported to have regulatory effects on certain blood cells. Clauss *et al.* (50) reported that VEGF may promote monocyte chemotaxis. More recently, Broxmeyer *et al.* (51) have shown that VEGF induces colony formation by mature subsets of granulocyte-macrophage progenitor cells that had been stimulated with a colony stimulating factor. These findings may be explained by the common origin of endothelial cells and hematopoietic cells and the presence of VEGF receptors in progenitor cells as early as hemangioblasts in blood islands in the yolk sac (see *Section VIII*). Furthermore, Gabrilovich *et al.* (52) have reported that VEGF may have an inhibitory effect on the maturation of host professional antigen-presenting cells such as dendritic cells. VEGF was found to inhibit immature dendritic cells, without having a significant effect on the function of mature cells. These findings led to the provocative hypothesis that VEGF may facilitate tumor growth also by allowing the tumor to avoid the induction of an immune response (52).

VEGF induces vasodilatation *in vitro* in a dose-dependent fashion (53) and produces transient tachycardia, hypotension, and a decrease in cardiac output when injected intravenously in conscious, instrumented rats (54). Such effects appear to be caused by a decrease in venous return, mediated primarily by endothelial cell-derived nitric oxide, as assessed by the requirement for an intact endothelium and the prevention of the effects by *N*-methyl-arginine (53, 54). Accordingly, VEGF has no direct effect on contractility or rate in isolated rat heart *in vitro* (54). These hemodynamic effects, however, are not unique to VEGF: other angiogenic factors such as aFGF and bFGF may also induce nitric oxide-mediated vasodilatation and hypotension (55).

III. Organization of the VEGF Gene

The human VEGF gene is organized in eight exons, separated by seven introns, and its coding region spans approximately 14 kb (56, 57). The human VEGF gene has been assigned to chromosome 6p21.3 (58). cDNA sequence analysis of a variety of human VEGF clones had indicated that VEGF may exist as one of four different molecular species, having, respectively, 121, 165, 189, and 206 amino acids ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) (26, 28, 56, 57). It is now well established that alternative exon splicing of a single VEGF gene is the basis for this molecular heterogeneity. $VEGF_{165}$ lacks the residues encoded by exon 6, while $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Compared with

$VEGF_{165}$, $VEGF_{121}$ lacks 44 amino acids; $VEGF_{189}$ has an insertion of 24 amino acids highly enriched in basic residues, and $VEGF_{206}$ has an additional insertion of 17 amino acids. Interestingly, there is no intron between the coding sequence of the 24-amino acid insertion in $VEGF_{189}$ and the additional 17-amino acid insertion found in $VEGF_{206}$. The 5′-end of the 51-bp insertion of $VEGF_{206}$ begins with GT, the consensus sequence for the 5′-splice donor necessary for mRNA processing. Therefore, the definition of the 5′-splice donor site for removal of a 1-kb intron sequence is variable (57). Analysis of the VEGF gene promoter region reveals a single major transcription start that lies near a cluster of potential Sp1 factor-binding sites. Also, several potential binding sites for the transcription factors AP-1 and AP-2 are present in the promoter region (56). $VEGF_{165}$ is the predominant molecular species produced by a variety of normal and transformed cells. Transcripts encoding $VEGF_{121}$ and $VEGF_{189}$ are detected in the majority of cells and tissues expressing the VEGF gene (56, 57). In contrast, $VEGF_{206}$ is a very rare form, so far identified only in a human fetal liver cDNA library (57). The organization of the murine VEGF gene has been also described (59). Similarly to the human gene, the coding region of the murine VEGF gene encompasses approximately 14 kb and is comprised of eight exons interrupted by seven introns. Analysis of exons suggests the generation of three isoforms, $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$. Therefore, murine VEGFs are shorter than human VEGF by one amino acid. A fourth isoform comparable to $VEGF_{206}$ is not predicted, since an in-frame stop codon is present in the region corresponding to the human $VEGF_{206}$ open reading frame. Analysis of the 3′-untranslated region of the rat VEGF mRNA has revealed the presence of four potential polyadenylation sites (60). A frequently used site is about 1.9 kb further downstream from the previously reported translation termination codon (30). The sequence within this 3′-untranslated region reveals a number of motifs that are known to be involved in the regulation of mRNA stability (60). (See also *Section V. A.*)

IV. Properties of the VEGF Isoforms

Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45,000 daltons (61). These properties correspond to those of $VEGF_{165}$, the major isoform. $VEGF_{121}$ is a weakly acidic polypeptide that fails to bind to heparin (62). $VEGF_{189}$ and $VEGF_{206}$ are more basic and bind to heparin with greater affinity than $VEGF_{165}$ (62). Previous studies demonstrated that such differences in the isoelectric point and in affinity for heparin may profoundly affect the bioavailability of VEGF (62, 63). $VEGF_{121}$ is a freely soluble protein; $VEGF_{165}$ is also secreted although a significant fraction remains bound to the cell surface and the extracellular matrix. In contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the extracellular matrix (63). However, these isoforms may be released in a soluble form by heparin or heparinase, suggesting that their binding site is represented by proteoglycans containing heparin-like moieties. Interestingly, the long forms may be released by plasmin (62, 63) after cleavage at the COOH terminus. This action generates a bioactive proteolytic fragment having a molecular mass of ~34,000 daltons (62, 63). Plasminogen activation and generation of plasmin have been shown to play an important role in the angiogenesis cascade. Thus, proteolysis of VEGF is likely to occur also *in vivo*. Generation of bioactive VEGF by proteolytic cleavage may be especially important in the microenvironment of a tumor where increased expression of proteases, including PA, is well documented (64, 65). Keyt *et al.* (66) have shown that the bioactive product of plasmin action is comprised of the first 110 NH_2-terminal amino acids of VEGF. These findings suggest that the VEGF proteins may become available to endothelial cells by at least two different mechanisms: as freely diffusible proteins ($VEGF_{121}$, $VEGF_{165}$) or after protease activation and cleavage of the longer isoforms. However, loss of heparin binding, whether it is due to alternative splicing of RNA or plasmin cleavage, results in a substantial loss of mitogenic activity for vascular endothelial cells: compared with $VEGF_{165}$, $VEGF_{121}$ or $VEGF_{110}$ demonstrate 50-fold reduced potency when tested in endothelial cell growth assay; the $VEGF_{165/110}$ heterodimer resulting from limited proteolysis of $VEGF_{165}$ demonstrated a 5–10-fold loss in potency when compared with wild type $VEGF_{165}$ (66). It has been suggested that the stability of VEGF-heparan sulfate-receptor complexes contributes to effective signal transduction and stimulation of endothelial cell proliferation (66). Thus, VEGF has the potential to express structural and functional heterogeneity to yield a graded and controlled biological response. Figure 1 illustrates some of the actions of the VEGF isoforms on the vascular endothelium and possible regulatory mechanisms.

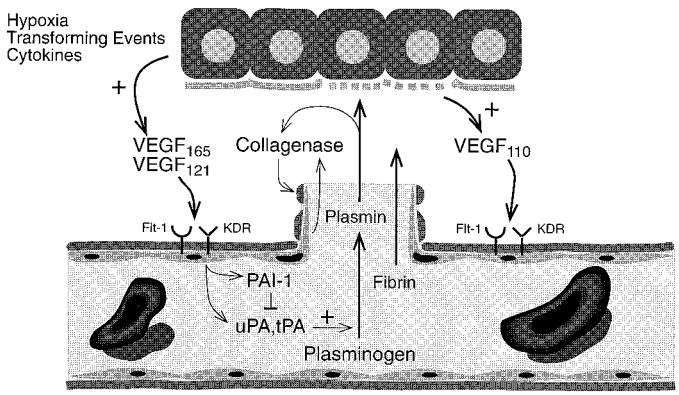

FIG. 1. Schematic representation of the actions of VEGF isoforms on the vascular endothelium. Several stimuli may result in the release of the diffusible alternatively spliced VEGF isoforms ($VEGF_{165}$, $VEGF_{121}$) from a variety of cell types. These proteins may induce a complex series of effects on the vascular endothelium, including cell sprouting, induction of interstitial collagenase, plasminogen activators (PA), and plasminogen activator inhibitor I-1 (PAI-1), as well as extravasation of plasma proteins. Plasminogen activation results in generation of plasmin, which may cleave extracellular matrix-bound VEGF ($VEGF_{189}$ or $VEGF_{206}$) to release a diffusible proteolytic fragment ($VEGF_{110}$). Plasmin may also activate procollagenase. Activation of PAI-1 may constitute a negative regulatory step, by inhibiting the action of PA.

V. Regulation of VEGF Gene Expression

A. Hypoxia

Several mechanisms have been shown to participate in the regulation of VEGF gene expression. Among these, oxygen tension plays a major role, both *in vitro* and *in vivo*. VEGF mRNA expression is rapidly and reversibly induced by exposure to low pO_2 in a variety of normal and transformed cultured cell types (67–70). In glioblastoma multiforme and other tumors with significant component of necrosis, the VEGF mRNA is highly expressed in ischemic tumor cells that are juxtaposed to areas of necrosis (68), suggesting that local hypoxia is a major inducer of VEGF gene expression in the microenvironment of a tumor. Also, ischemia caused by occlusion of the left anterior descending coronary artery results in a dramatic increase in VEGF RNA levels in the pig myocardium, suggesting the possibility that VEGF may mediate the spontaneous revascularization that follows myocardial ischemia (71). Furthermore, Stone *et al.* (72) proposed that hypoxic up-regulation of VEGF mRNA in neuroglial cells, secondary to the onset of neuronal activity, plays an important physiological role in the development of the retinal vasculature.

Similarities exist between the mechanisms leading to hypoxic regulation of VEGF and erythropoietin (Epo) (73). Hypoxia inducibility is conferred on both genes by homologous sequences. By deletion and mutation analysis, a 28-base sequence has been identified in the 5′-promoter of the rat and human VEGF gene that mediated hypoxia-induced transcription in transient assays (60, 74). Such sequence reveals a high degree of homology and similar protein- binding characteristics as the hypoxia-inducible factor 1 (HIF-1) binding site within the Epo gene, which behaves like a classic transcriptional enhancer (75). HIF-1 has been purified and cloned as a mediator of transcriptional responses to hypoxia and is a basic, heterodimeric, helix-loop-helix protein (76, 77). Forsythe *et al.* (78) presented more direct evidence that HIF-1 is indeed implicated in the activation of the VEGF gene transcription during hypoxia. When reporter constructs containing the VEGF sequences that mediate hypoxia inducibility were cotransfected with expression vectors encoding HIF-1 subunits, reporter gene transcription was much greater than that observed in cells transfected with the reporter alone, both in hypoxic and normoxic conditions (78).

It has been shown that accumulation of adenosine, which occurs under hypoxic conditions, is involved in the induction of the VEGF gene during hypoxia (79). According to these studies, adenosine, by activating adenosine A_2 receptors, results in elevated cAMP concentrations that in turn increase VEGF mRNA levels, possibly through a protein kinase A-mediated pathway (79). Activation of c-*Src* also has been shown to participate in the hypoxic up-regulation of the VEGF gene (80). Hypoxia increases the kinase activity of $pp60^{c\text{-}src}$ and its phosphorylation on tyrosine 416. Expression of a negative dominant mutant of c-*Src* significantly reduced the hypoxic induction of VEGF (80).

It is noteworthy that several studies have shown that transcriptional activation is not the only mechanism leading to VEGF up-regulation in response to hypoxia. Increased mRNA stability has been identified as an important post-transcriptional component (81–83). Sequences that mediate increased stability were identified in the 3′-untranslated region of the VEGF mRNA (see also *Section III*). Also, a hypoxia-induced protein that bound to such sequences was identified (81).

B. Cytokines

Several cytokines or growth factors up-regulate VEGF mRNA expression and/or induce release of VEGF protein. Exposure of quiescent human keratinocytes to serum, epidermal growth factor (EGF), TGF-β, or keratinocyte growth factor results in a marked induction of VEGF mRNA expression (84). Also, primary, nontransformed, keratinocytes show VEGF up-regulation in response to TGF-α (85, 86). EGF also stimulates VEGF release by cultured glioblastoma cells (87). In addition, treatment of quiescent cultures of several epithelial and fibroblastic cell lines with TGF-β resulted in induction of VEGF mRNA and release of VEGF protein in the medium (88). Based on these findings, it has been proposed that VEGF may function as a paracrine mediator for indirect-acting angiogenic agents such as TGF-β (88). Furthermore, IL-1β induces VEGF expression in aortic smooth muscle cells (89). Both IL-1α and PGE_2 have been shown to induce expression of VEGF in cultured synovial fibroblasts, suggesting the participation of such inductive mechanisms in inflammatory angiogenesis (90). IL-6 has been also shown to significantly induce VEGF expression in several cell lines (91). Not only promoter elements, but also motifs in the 5′-untranslated region of the VEGF mRNA were found to be involved in such up-regulation (91). IGF-I, a mitogen implicated in the growth of several malignancies, has also been shown to induce VEGF mRNA and protein in cultured colorectal carcinoma cells (92). The induction was mediated by a combined increase in transcriptional rate of the VEGF gene and in the stability of the mRNA. Thus, IGF-I, in addition to its direct mitogenic effects on malignant cells, may facilitate tumor growth via an increase in the vascular supply, mediated by VEGF.

C. Differentiation and transformation

Cell differentiation has been shown to play an important role in the regulation of VEGF gene expression (93). The VEGF mRNA is up-regulated during the conversion of 3T3 preadipocytes into adipocytes or during the myogenic differentiation of C2C12 cells. Conversely, VEGF gene expression is repressed during the differentiation of the pheochromocytoma cell line PC12 into nonmalignant, neuron-like cells. These studies also indicate that induction of VEGF mRNA expression in preadipocytes requires pathways mediated by both protein kinase C and protein kinase A activation (93). Consistent with the presence of AP-1 and AP-2 sites in the VEGF gene promoter, phorbol esters and forskolin, a potent activator of adenylate cyclase, induce VEGF mRNA expression (94). Accordingly, luteotrophic hormone, a known activator of adenylate cyclase, has been shown to induce expression of VEGF mRNA in cultured bovine ovarian granulosa cells (94).

Specific transforming events also result in induction of VEGF gene expression. A mutated form of the murine p53 tumor suppressor gene ($Ala^{135} > Val$) has been shown to induce VEGF mRNA expression and potentiate phorbol ester-stimulated VEGF mRNA expression in NIH 3T3 cells in transient transfection assays (95). Likewise, oncogenic mutations or amplification of *ras* lead to VEGF up-regulation (96, 97). This effect is blocked by treatment with inhibitors of *ras* farnesyl transferase. Interestingly, expression of oncogenic *ras*, either constitutive or transient, potentiated the induction of VEGF by hypoxia (98). Also, overexpression of v-*raf* (97) or v-*Src* (99) lead to VEGF up-regulation. Moreover, the von Hippel-Lindau (VHL) tumor suppressor gene has been recently implicated in the regulation of VEGF gene expression (100). Human renal cell carcinoma cells either lacking endogenous wild type VHL gene or expressing an inactive mutant demonstrated altered regulation of VEGF gene expression, which was corrected by introduction of wild type VHL gene. Essentially all of the endothelial cells mitogenic activity released by tumor cells expressing mutant VHL gene was neutralized by anti-VEGF antibodies (100). These findings suggest that VEGF is a key mediator of the abnormal vascular proliferations and solid tumors characteristic of VHL syndrome (101). Most recently, Iliopulos *et al.* (102) have shown that a function of the VHL protein is to provide a negative regulation of a series of hypoxia-inducible genes, including the VEGF, platelet-derived growth factor B chain, and the glucose transporter GLUT1 genes. In the presence of a mutant VHL, mRNAs for such genes were produced both under normoxic and hypoxic conditions. Reintroduction of wild type VHL cDNA resulted in inhibition of mRNA production under normoxic conditions and restored the characteristic hypoxia inducibility of those genes (102).

Taken together, these findings indicate that several, unrelated, alterations in cellular regulatory pathways result in VEGF up-regulation. Therefore, this event may be a final common pathway necessary for uncontrolled proliferation *in vivo*.

VI. The VEGF Receptors

A. Characterization and distribution of VEGF-binding sites

Two classes of high-affinity VEGF-binding sites were initially described in the surface of bovine endothelial cells, with dissociation constant (K_d) values of 10 pM and 100 pM, respectively, and molecular mass in the range of 180–220 kDa (103, 104). Lower affinity binding sites on mononuclear phagocytes were subsequently described (105). It has been suggested that such binding sites are involved in mediating chemotactic effects for monocytes by VEGF (50). Recently, it has been suggested that low-affinity, low molecular mass (120–130 kDa), receptors exist on endothelial and tumor cells (106, 107). Such receptors cross-link $VEGF_{165}$ but not $VEGF_{121}$. Thus, certain tumor and endothelial cells express lower affinity sites that bind selectively exon 7-encoded sequences. The molecular nature and biological significance of these receptors remain to be elucidated.

Ligand autoradiography studies on fetal and adult rat tissue sections demonstrated that high-affinity VEGF-binding sites are localized to the vascular endothelium of large or small vessels *in situ* (108, 109). These findings represented direct evidence for the hypothesis that the vascular endothelium is the major target of VEGF action. Interestingly, VEGF binding was apparent not only on proliferating but also on quiescent endothelial cells (108, 109). Also, the earliest developmental identification of high-affinity VEGF binding was in the hemangioblasts in the blood islands in the yolk sac, suggesting that expression of VEGF receptors is one of the earliest events in endothelial cell differentiation (109).

B. The Flt-1 and Flk-1/KDR tyrosine kinases

1. Binding characteristics. Two VEGF receptor tyrosine kinases (RTKs) have been identified (110–116). The Flt-1 (*fms*-like-tyrosine kinase) and KDR (kinase domain region) receptors bind VEGF with high affinity. Flk-1 (fetal liver kinase-1), the murine homolog of KDR, shares 85% sequence identity with human KDR (114). Both Flt-1 and KDR/Flk-1 have seven immunoglobulin (Ig)-like domains in the extracellular domain (ECD), a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain (110–116). Figure 2 shows the alignment of the amino acid sequences of the ECD of Flt-1 and KDR. Flt-1 has the highest affinity for $rhVEGF_{165}$, with a K_d of approximately 10–20 pM (110). KDR has a somewhat lower affinity for VEGF: the K_d has been estimated to be approximately 75–125 pM (111).

A cDNA coding an alternatively spliced soluble form of Flt-1 (sFlt-1), lacking the seventh Ig-like domain, transmembrane sequence, and the cytoplasmic domain, has been identified in human umbilical vein endothelial cells (117, 118). This sFlt-1 receptor binds VEGF with high affinity (K_d 10–20 pM) and is able to inhibit VEGF-induced mitogenesis, suggesting that it may be a physiological negative regulator of VEGF action (117, 118).

An additional member of the family of RTKs with seven Ig-like domains in the ECD is Flt-4 (119–122) which, however, is not a receptor for VEGF but rather binds a newly identified ligand called VEGF-C or VEGF-related peptide (VRP) (see *Section VII*).

2. Signal transduction. Our understanding of the signal transduction properties of the VEGF receptors is still incomplete. VEGF has been shown to induce the phosphorylation of at least 11 proteins in bovine aortic endothelial cells (113). PLC-γ and two proteins that associate with PLC-γ were phosphorylated in response to VEGF (123). Furthermore, immunoblot analysis for mediators of signal transduction that contain SH2 domains demonstrated that VEGF induces phosphorylation of phosphatidylinositol 3-kinase, *ras* GTPase activating protein, and several others. These findings suggest that VEGF promotes the formation of multimeric aggregates of VEGF receptors with proteins that contain SH2 domains. These studies, however, did not identify which VEGF receptor(s) are involved in these events.

Several studies have indicated that Flt-1 and KDR have different signal transduction properties (124, 125). Porcine aortic endothelial cells lacking endogenous VEGF receptors display chemotaxis and mitogenesis in response to VEGF

FIG. 2. Alignment of the extracellular domains of human Flt-1 and KDR. The seven immunoglobulin (Ig)-like domains are shown as individual *boxed areas*.

when transfected with a plasmid coding for KDR (124). In contrast, transfected cells expressing Flt-1 lack such responses (124). Flk-1/KDR undergoes strong ligand-dependent tyrosine phosphorylation in intact cells, while Flt-1 reveals a weak or undetectable response (110, 124, 125). Also, VEGF stimulation results in weak tyrosine phosphorylation that does not generate any mitogenic signal in transfected NIH 3T3 cells expressing Flt-1 (125). These findings agree with other studies showing that placenta growth factor (PlGF), which binds with high affinity to Flt-1 but not to Flk-1/KDR, lacks direct mitogenic or permeability-enhancing properties or the ability to effectively stimulate tyrosine phosphorylation in endothelial cells (126) (see *Section VII*). Therefore, interaction with Flk-1/KDR is a critical requirement to induce the full spectrum of VEGF biological responses. In further support of this conclusion, VEGF mutants that bind selectively to Flk-1/KDR are fully active endothelial cell mitogens (see *Section VI.B.5*) (127). Furthermore, Kendall *et al.* (118) suggested that sFlt-1 may form heterodimeric complexes with KDR, which could potentially exert a dominant-negative effect on KDR signal transduction. These findings contributed to cast doubt on the role of Flt-1 as a truly signaling receptor. However, more recent evidence indicates that Flt-1 indeed signals, although our understanding of these processes is clearly fragmentary. Cunningham *et al.* (128), using the yeast two-hybrid system, have demonstrated an interaction between Flt-1 and the p85 subunit of phosphatidylinositol 3-kinase. Mutagenesis analysis revealed that change of a tyrosine residue at position 1213 to phenylalanine completely abolished such interaction. These data suggest that p85 couples Flt-1 to intracellular signal transduction systems and implicate elevated levels of PtdIns(3,4,5)P3 levels in this process (128). Also, members of the *Src* family, such as *Fyn* and *Yes*, show an increased level of phosphorylation after VEGF stimulation in transfected cells expressing Flt-1 but not KDR (124). Furthermore, Barleon *et al.* (129) have shown that a specific biological response, the migration of monocytes in response to VEGF (or PlGF), is mediated by Flt-1. However, the most compelling evidence so far for an important biological role played by the Flt-1 receptor has been provided by gene knockout studies (see *Section VIII. B*).

3. Regulation. The expression of Flt-1 and Flk-1/KDR genes is largely restricted to the vascular endothelium (see *Section VIII.A*). The promoter region of Flt-1 has been cloned and characterized and a 1-kb fragment of the 5′-flanking region essential for endothelial-specific expression was identified (130). Likewise, a 4-kb 5′-flanking sequence has been identified in the promoter of KDR that confers endothelial cell-specific activation (131).

Similarly to VEGF, hypoxia has been proposed to play an important role in the regulation of VEGF receptor gene expression. Exposure of rats to acute or chronic hypoxia led to pronounced up-regulation of both Flt-1 and Flk-1/KDR genes in the lung vasculature (132). Also, Flk-1/KDR and Flt-1 mRNAs were substantially up-regulated throughout the heart after myocardial infarction in the rat (133). However, *in vitro* studies have yielded unexpected results. Even though Thieme *et al.* (134) have shown that hypoxia increases VEGF receptor number by 50% in cultured bovine retinal capillary endothelial cells, the expression of KDR is not induced but paradoxically shows an initial down-regulation (135). Brogi *et al.* (136) have proposed that the hypoxic up-regulation of KDR observed *in vivo* is not direct but requires the release of an unidentified paracrine mediator from ischemic tissues. Also, recent studies have shown that both TNF-α (137) and TGF-β (138) are able to inhibit the expression of the KDR gene in cultured endothelial cells.

4. Structural requirements for ligand binding in Flt-1 and KDR. As noted above, the VEGF receptors have seven Ig-like do-

mains in the ECD. Until now, the significance and function of these domains for ligand binding and receptor activation were unknown. Recently, the domains in the ECD of Flt-1 and KDR responsible for specific ligand recognition were identified by constructing and analyzing a variety of receptor variants (139). These included individual Ig-like domain (140) deletions, as well as chimeras in which domains of either KDR or Flt-4 were exchanged for the homologous sequences from Flt-1. Deletion of the second Ig-like domain of human Flt-1 completely abolishes VEGF binding (Fig. 3). Introduction of the second domain of KDR into an Flt-1 mutant lacking the homologous domain restored VEGF binding. However, PlGF was unable to displace VEGF bound to such mutant, a pattern characteristic of the KDR but not the Flt-1 receptor (Fig. 3). Also, "swap" experiments in which the second Ig-like domain of Flt-1 replaced the corresponding domain in Flt-4 demonstrated that such a chimeric receptor had the ability to bind VEGF with affinity nearly identical to that of wild type Flt-1. Furthermore, transfected cells expressing this chimeric Flt-4 receptor exhibited increased DNA synthesis in response to both VEGF and PlGF (139). Thus, VEGF binding to domain 2 of Flt-1 is able to initiate a signal transduction cascade, even in the context of the ECD of a foreign receptor. Further studies are required to elucidate the significance of the remaining Ig-like domains in receptor dimerization (141) and in coupling binding with signal transduction.

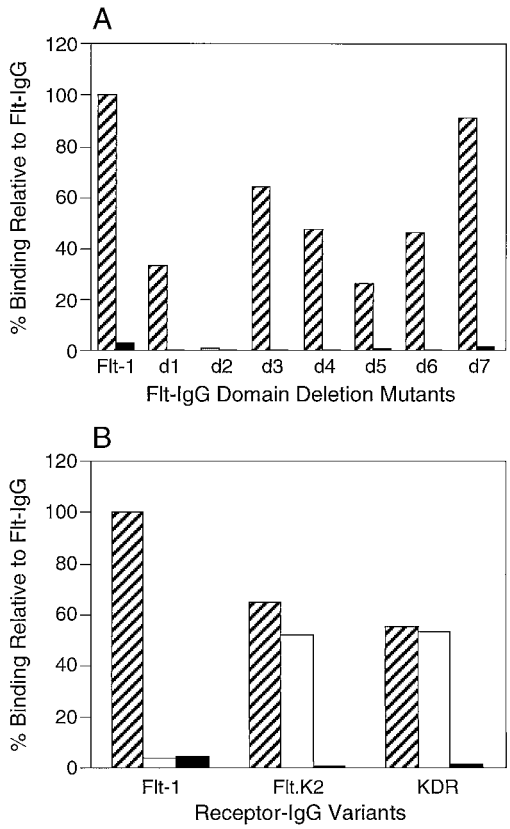

FIG. 3. The second Ig-like domain contains the major determinants for binding and ligand specificity in the VEGF receptors. In panel A, Flt-1-IgG individual domain deletion variants (5 ng per reaction) were tested for their ability to bind [^{125}I]VEGF$_{165}$ in the absence (*striped bars*) or presence of 50 ng cold VEGF$_{165}$ (*solid bars*). Deletion of the second Ig-like domain completely abolishes the binding of VEGF. The second Ig-like domain of KDR was cloned into the Flt-1 domain 2 deletion construct to produce "swap" mutants (panel B). Replacement of the second domain of Flt-1 with the homologous domain of KDR reestablished VEGF-binding. However, PlGF$_{152}$ (*open bars*) could not displace VEGF$_{165}$ bound to Flt.K2, a pattern characteristic of the KDR but not the Flt-1 receptor.

5. VEGF determinants for binding Flt-1 and KDR. Site-directed mutagenesis has been used to localize the determinants on VEGF that mediate binding to the KDR and Flt-1 receptors. Alanine-scanning analysis was performed to identify a positively charged surface in VEGF that mediates receptor binding (127, 142). A model based on the crystal structure of PDGF-BB was used (143). Arg^{82}, Lys^{84}, and His^{86}, located in a hairpin loop, were found to be critical for binding KDR, while negatively charged residues, Asp^{63}, Glu^{64}, and Glu^{67}, were primarily responsible for Flt-1 binding. The single mutations R82A, K84A, and H86A were found to display modestly decreased KDR binding. The triple mutants involving alanine replacement or neo-glycosylation sites, R82A, K84A, H86A VEGF and R82N, I83L, K84S VEGF, exhibited minimal binding to KDR receptor. The half-maximally effective concentrations (EC$_{50}$) to stimulate bovine capillary endothelial cell growth for most of the VEGF mutants were similar to those observed for wild type VEGF. The most significant effect on endothelial cell proliferation was observed with mutations in the 82–86 region. The EC$_{50}$ of R82A, K84A, H86A VEGF increased 20-fold such that mitogenic potency of this mutant was decreased to 5% of wild type VEGF. In contrast, the mutants that failed to bind Flt-1 were fully active endothelial cell mitogens (127, 142).

VII. VEGF-Related Molecules

Over the last few years, three VEGF-related genes have been identified from mammalian sources. The encoded factors are known as PlGF, VEGF-B, and VEGF-C/VRP. In addition, two sequences in the genome of the parapoxvirus *orf* virus show homology to VEGF. Figure 4 shows the alignment of the amino acid sequences of these molecules with the sequence of VEGF$_{165}$. Although the biological role of these factors is still largely unclear, their structural homology to VEGF suggests that they may play a role in the regulation of blood vessel growth. The first VEGF-related factor identified is PlGF. This molecule shares a 53% identity with the PDGF-like region of VEGF. The encoded protein was expected to have 149 amino acids, including the signal peptide (144). Subsequently, a longer form characterized by a 21-amino acid insertion was identified (145). Similar to the 24-amino acid insertion in the longer forms of VEGF, this insertion is highly enriched in basic residues. These two isoforms, which arise from alternative splicing of mRNA, are known as PlGF-1 and PlGF-2 or PlGF$_{131}$ and PlGF$_{152}$, respectively. Similar to VEGF, these molecules are dimeric glycoproteins. Park *et al.* (126) have shown that PlGF binds with high affinity (K_d ~250 pM) Flt-1 but not KDR. Purified PlGF demonstrated minimal activity in vascular endothelial cell growth and vascular permeability assays, suggesting that binding to KDR is a requirement for both activities. However, PlGF was able to

FIG. 4. Amino acid sequence of $VEGF_{165}$ and VEGF-related molecules: VEGF-B, VEGF-C, $PlGF_{152}$, and a VEGF-like sequence identified in the genome of the parapoxvirus orf virus. The conserved cysteine residues are *boxed*.

potentiate the bioactivity of low, marginally efficacious, concentrations of VEGF, both on endothelial cell growth and on vascular permeability (126). The molecular basis of this effect remains to be fully elucidated. Interestingly, naturally occurring heterodimers between VEGF and PlGF have been identified in the conditioned medium of a rat glioma cell line (146). In agreement with previous studies, the PlGF homodimer demonstrated minimal mitogenic activity on endothelial cells. However, the VEGF:PlGF heterodimer was active, although its potency was approximately 7-fold lower than the VEGF homodimer. It has been suggested that the formation of heterodimers with PlGF constitutes a mechanism of negative regulation of VEGF bioactivity, by shifting the balance toward less potent molecules (147).

As previously noted, similarly to the VEGF receptors, Flt-4 is a RTK with seven Ig-like domains in the ECD (119–122). Interestingly, the expression of Flt-4 mRNA, which is initially localized to angioblasts and venules in the early embryo, becomes restricted to lymphatic endothelium at later stages of development (148). This expression pattern suggested that Flt-4 may play a role in the regulation of lymphangiogenesis (122, 148). A ligand selective for Flt-4 has been recently identified by two groups and has been named VEGF-C (149) or VRP (150). VEGF-C/VRP is a secreted protein with 399 amino acid residues and has a 32% identity to VEGF. Its COOH-terminal half contains a 180-amino acid region that is not found in VEGF. This region contains cysteine-rich motifs similar to a protein component of silk produced by the larval salivary gland of the midge *C. tentanus* (149, 150). VEGF-C/VRP has been reported to stimulate the growth of human lung endothelial cells, albeit at 100-fold less potency than $VEGF_{165}$ (150). It is still unclear whether VEGF-C/VRP is capable of high-affinity interaction with KDR (149, 150).

A newly identified member of the VEGF gene family is VEGF-B (151, 152). This molecule consists of 188 amino acids, including the signal peptide. VEGF-B has been reported to stimulate the growth of human and bovine vascular endothelial cells (151). Interestingly, VEGF-B is distributed primarily in the skeletal muscle and myocardium and is coexpressed with VEGF (151). Similar to the long forms of VEGF, VEGF-B is expressed as a membrane-bound protein that can be released in a soluble form after addition of heparin. VEGF-B and VEGF are also able to form heterodimers, when coexpressed (151). These findings led to the hypothesis that VEGF-B may participate in the regulation of angiogenesis, particularly in muscle (151). Figure 5 schematizes the interaction of VEGF and VEGF-related factors with their tyrosine kinase receptors.

Intriguingly, two sequences having a significant homology to VEGF have been identified in the genome of two different strains of *orf* virus, a parapoxvirus that affects goats, sheeps, and occasionally humans (153). This suggests that the viral VEGF-like gene has been acquired from a mammalian host and is undergoing genetic drift. Interestingly, the lesions of goats and humans after *orf* virus infection are characterized by extensive microvascular proliferation in the skin, raising the possibility that the product of the viral VEGF-like gene is responsible for such lesions.

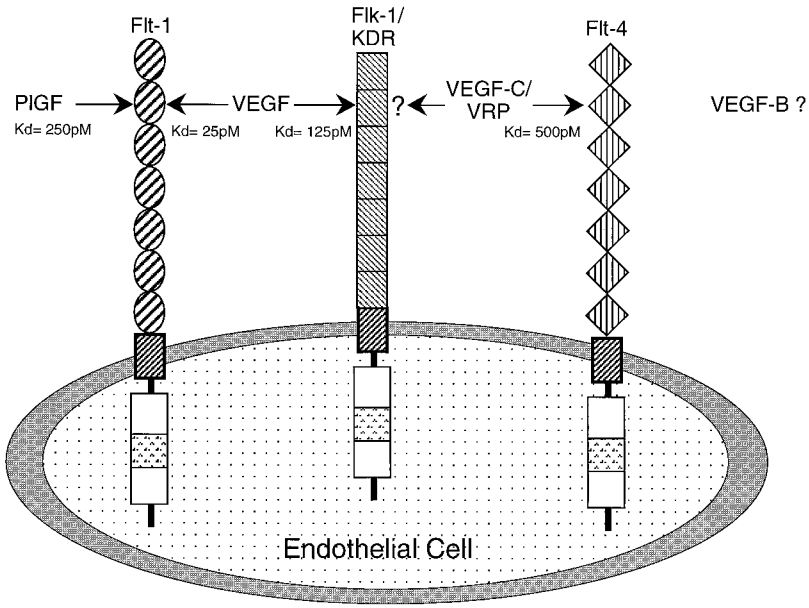

FIG. 5. The diagram illustrates the interaction of VEGF and VEGF-related molecules with the three known members of the family of RTKs with seven Ig-like domains in the ECD. VEGF interacts with Flt-1 and KDR; PlGF binds only Flt-1 and VEGF-C/VRP binds with high affinity to Flt-4. It is unknown at the present time whether VEGF-B binds to any of these receptors.

VIII. Role of VEGF and Its Receptors in Physiological Angiogenesis

A. Distribution of VEGF, Flk-1/KDR, and Flt-1 mRNA

The proliferation of blood vessels is crucial for a wide variety of physiological processes such as embryonic development, normal growth and differentiation, wound healing, and reproductive functions. Previous studies have indicated that the VEGF mRNA is temporally and spatially related to the proliferation of blood vessels in the rat, mouse, and primate ovary and in the rat uterus, suggesting that VEGF is a mediator of the cyclical growth of blood vessels that occurs in the female reproductive tract (154–157). In fact, *in situ* hybridization studies in the rat ovary provided the first evidence that VEGF may be a regulator of physiological angiogenesis (154).

During embryonic development, VEGF expression is first detected within the first few days after implantation in the giant cells of the trophoblast (109, 158), suggesting a role for this factor in the induction of vascular growth in the decidua, placenta, and vascular membranes. At later developmental stages in the mouse or rat embryos, the VEGF mRNA is expressed in several organs, including heart, vertebral column, kidney, and along the surface of the spinal cord and brain (109, 158). In the developing mouse brain, the highest levels of mRNA expression are associated with the choroid plexus and the ventricular epithelium (158). In the human fetus (16–22 weeks), VEGF mRNA expression is detectable in virtually all tissues and is most abundant in lung, kidney, and spleen (159). VEGF protein, as assessed by immunocytochemistry, is expressed in epithelial cells and myocytes, but not vascular endothelial cells (159).

In situ hybridization studies have shown that the Flk-1 mRNA is expressed in the yolk sac and intraembryonic mesoderm and later on in angioblasts, endocardium, and small and large vessel endothelium (115, 116). There is evidence that the Flk-1 mRNA is down-regulated in adult endothelial cells as compared with fetal endothelial cells (115, 116). These findings strongly suggested a role for Flk-1 in the regulation of vasculogenesis and angiogenesis. Other studies have demonstrated that expression of Flk-1 mRNA is first detected in the proximal-lateral embryonic mesoderm, which gives rise to the heart (160). Flk-1 is then detectable in endocardial cells of heart primordia and subsequently in the major embryonic and extraembryonic vessels (160). These studies have indicated that Flk-1 may be the earliest marker of endothelial cell precursors (160). The Flt-1 mRNA is selectively expressed in vascular endothelial cells, both in fetal and adult mouse tissues (161). Similar to the high-affinity VEGF binding (108, 109), the Flt-1 mRNA is expressed in both proliferating and quiescent endothelial cells (161), suggesting a role for Flt-1 in the maintenance of endothelial cells.

Interestingly, VEGF expression is also detectable around microvessels in areas where endothelial cells are normally quiescent, such as kidney glomerulus, pituitary, heart, lung, and brain (61, 162, 163). These findings raised the possibility that VEGF may be required not only to induce active vascular proliferation but, at least in some circumstances, also for the maintenance of the differentiated state of blood vessels (61). In agreement with this hypothesis, Alon *et al.* (164) have shown that VEGF acts as a survival factor, at least for the developing retinal vessels. They propose that hyperoxia-induced vascular regression in the retina of neonatal animals is a consequence of inhibition of VEGF production by glial cells. Accordingly, intraocular administration of VEGF to newborn rats at the onset of hyperoxia was able to prevent cell apoptosis and regression of the retinal vasculature (164).

It has been suggested that VEGF is also involved in a major pathophysiological process such as wound healing (84–86). Keratinocytes in a healing wound express VEGF mRNA.

Interestingly, a decreased expression of VEGF mRNA has been observed in the skin of genetically diabetic db/db mice (84), suggesting that an altered regulation of VEGF gene expression contributes to defective angiogenesis and impaired wound healing characteristic of this disorder.

B. Analysis of Flk-1/KDR, Flt-1, and VEGF gene knockouts

Recent studies have demonstrated that both Flt-1 and Flk-1/KDR are essential for normal development of embryonic vasculature. However, their respective roles in endothelial cell proliferation and differentiation appear to be distinct (165, 166). Mouse embryos homozygous for a targeted mutation in the Flt-1 locus died *in utero* between day 8.5 and 9.5 (165). Endothelial cells developed in both embryonic and extraembryonic sites but failed to organize in normal vascular channels. Mice in which the Flk-1 gene had been inactivated lacked vasculogenesis and also failed to develop blood islands (166). Hematopoietic precursors were severely disrupted and organized blood vessels failed to develop throughout the embryo or the yolk sac, resulting in death *in utero* between day 8.5 and 9.5 (166).

However, these findings do not necessarily imply VEGF as being equally essential, since other ligands might potentially activate the Flt-1 and Flk-1/KDR receptors and thus substitute VEGF action. Very recent studies (16, 17) have generated direct evidence for the role played by VEGF in embryonic vasculogenesis and angiogenesis. Unexpectedly, inactivation of the VEGF gene in mice resulted in embryonic lethality in heterozygous embryos, between day 11 and 12. The VEGF+/− embryos were growth retarded and also exhibited a number of developmental anomalies (167). The forebrain region appeared significantly underdeveloped. In the heart region, the outflow region was grossly malformed; the dorsal aortas were rudimentary, and the thickness of the ventricular wall was markedly decreased. The yolk sac revealed a substantially reduced number of nucleated red blood cells within the blood islands. Also, the vitelline veins failed to fuse with the vascular plexus of the yolk sac. Significant defects in the vasculature of other tissues and organs, including placenta and nervous system, were evidenced. For example, in the nervous system of heterozygous embryos at day 10.5, vascular elements could be demonstrated in the mesenchyme but not in the neuroepithelium (17) (Fig. 6). This failure of blood vessel ingrowth was accompanied by apoptosis and disorganization of neuroepithelial cells (Fig. 6). The VEGF+/− embryos survive approximately 2 days longer than the Flt-1 or Flk-1/KDR null embryos, presumably reflecting a partial activation of these tyrosine kinases by VEGF. *In situ* hybridization confirmed expression of

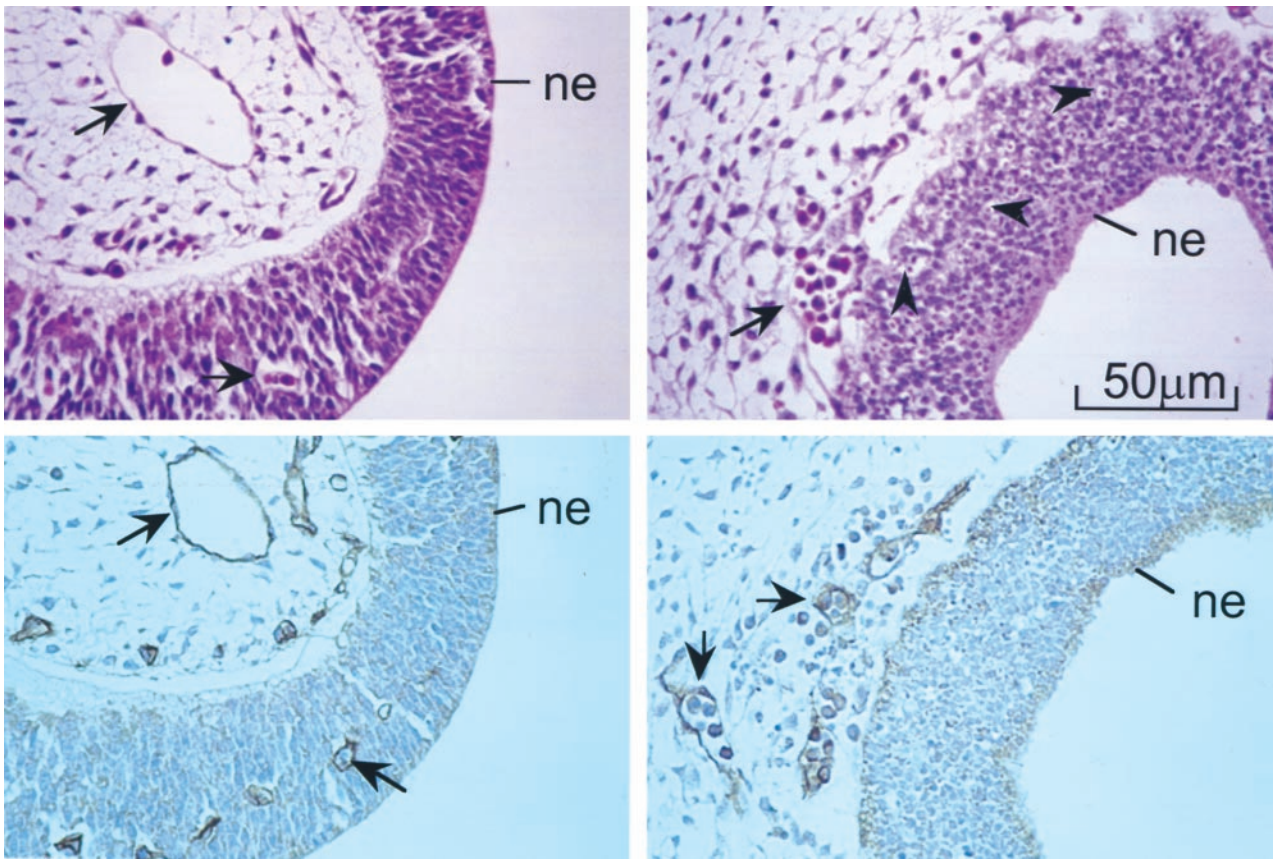

FIG. 6. Hematoxylin and eosin staining (*upper panels*) and CD34 immunostaining (*lower panels*) on sections of neuroepithelium (ne) from wild type (*left*) and VEGF+/− (*right*) E 10.5 mouse embryos. *Arrows* indicate blood vessels. Blood vessel lumina can be identified in the mesenchyme adjacent to the ne in both groups. However, they are absent within the ne of the heterozygous embryos. Note also the presence of apoptotic cells in the ne of the heterozygous embryos. This contrasts with the well differentiated and vascularized ne in the wild type. [Reproduced with permission from N. Ferrara *et al.*: *Nature* 380:439–442, 1996 (17). ©1996 Macmillan Magazines Limited.]

VEGF mRNA in heterozygous embryos. Thus, the VEGF+/− phenotype is due to gene dosage and not to maternal imprinting.

Although several heterozygous phenotypes have been described (168), this may be the first report that the loss of a single allele of a gene that is not maternally imprinted can be lethal. Therefore, VEGF and its receptors are essential for blood island formation and angiogenesis such that even reduced concentrations of VEGF are inadequate to support a normal pattern of development. These findings also indicate that, in the VEGF+/− mutant, the mechanisms that normally up-regulate VEGF gene expression, such as hypoxia, are unable to provide an effective compensatory response. It is tempting to speculate that, as VEGF concentrations and angiogenic gradients fall below a threshold during critical periods, this can cause irreversible disruption of normal organogenesis.

IX. Role of VEGF in Pathological Angiogenesis

A. Tumor angiogenesis

1. Expression of VEGF in human tumors. In situ hybridization studies have demonstrated that the VEGF mRNA is markedly up-regulated in the vast majority of human tumors so far examined. These include: lung (169), thyroid (170), breast (171, 172), gastrointestinal tract (173, 174), kidney and bladder (175), ovary (176), and uterine cervix (177) carcinomas, angiosarcoma (178), germ cell tumors (179), and several intracranial tumors including glioblastoma multiforme (68, 180, 181) and sporadic, as well as VHL syndrome-associated, capillary hemangioblastoma (182, 183) (Table 1). Only sections of lobular carcinoma of the breast and papillary carcinoma of the bladder failed to show significant VEGF mRNA expression (184). As already indicated in *Section V.B*, the expression of VEGF in glioblastoma multiforme and other tumors with significant necrosis is highest in hypoxic tumor cells adjacent to necrotic areas (68, 180, 181). A correlation has been noted between VEGF mRNA expression and vascularity of the tumor (169, 174, 177, 182, 183). In the tumors where VEGF and PlGF were coexpressed, only VEGF expression correlated with the degree of malignancy and vascularity (170, 179). In virtually all specimens examined, the VEGF mRNA was expressed in tumor cells but not in endothelial cells. In contrast, the mRNAs for Flt-1 and KDR were up-regulated in the endothelial cells associated with the tumor (173, 180, 185). These findings are consistent with the hypothesis that VEGF is primarily a paracrine mediator (186). An interesting exception may be angiosarcoma, where VEGF and Flt-1 mRNA were found to be coexpressed in angiosarcoma cells, raising the possibility that in this malignancy VEGF may play a role as an autocrine factor (178). Angiosarcoma cells, however, arise from the endothelium. Recently, Freeman *et al.* (187) have suggested that lymphocytes infiltrating the tumor may constitute an additional source of VEGF, which contributes to tumor angiogenesis. Immunohistochemical studies have localized the VEGF protein not only to the tumor cells but also to the vasculature (173, 180, 185). This finding indicates that tumor-secreted VEGF accumulates in the target cells. Ultrastructural studies have localized VEGF bound to tumor endothelial cells to the abluminal plasma membrane and to the recently described vesiculovacular organelles, cytoplasmic structures that are thought to be involved in macromolecular transport across the tumor endothelium (188).

TABLE 1. Human tumors overexpressing the VEGF mRNA *in situ*

Tumor type	Reference
Intracranial tumors	
Glioblastoma multiforme	68, 180–182
Meningioma	182
Capillary hemangioblastoma	182, 183
Thyroid carcinomas	170
Lung carcinomas	169
Breast carcinomas	171, 172
Gastrointestinal tract tumors	
Esophageal carcinomas	173
Gastric carcinomas	173, 191
Small bowel carcinomas	173
Colorectal carcinomas	173, 194
Hepatocellular carcinoma	174
Urinary tract tumors	
Kidney carcinomas	175
Bladder carcinomas	175
Female reproductive tract tumors	
Ovarian carcinomas	176
Uterine cervix carcinomas	177
Germ cell tumors	179
Angiosarcoma	178

Elevations in VEGF levels have been detected in the serum of some cancer patients (189). Also, a correlation has been observed between VEGF expression and microvessel density in primary breast cancer sections (190). A postoperative survey indicated that the relapse-free survival rate of patients with VEGF-rich tumors was significantly worse than that of VEGF-poor tumors, suggesting that expression of VEGF is associated with stimulation of angiogenesis and with early relapse in primary breast cancer (190). A similar correlation has been described in gastric carcinoma patients (191). VEGF positivity in tumor sections was correlated with vessel involvement, lymph node metastasis, and liver metastasis. Furthermore, patients with VEGF-positive tumors had a worse prognosis than those with VEGF-negative tumors (191).

2. Inhibition of VEGF action in vivo. The availability of specific monoclonal antibodies capable of inhibiting VEGF-induced angiogenesis *in vivo* and *in vitro* (192) made it possible to generate direct evidence for a role of VEGF in tumorigenesis. In a study published by Kim *et al.* in 1993 (193) , such antibodies were found to exert a potent inhibitory effect on the growth of three human tumor cell lines injected subcutaneously in nude mice, the SK-LMS-1 leiomyosarcoma, the G55 glioblastoma multiforme, and the A673 rhabdomyosarcoma. The growth inhibition ranged between 70% and more than 95%. Figure 7 illustrates the effects of the anti-VEGF-neutralizing antibody on the *in vivo* growth of such cell lines. These findings provided the first direct demonstration that inhibition of the action of an endogenous endothelial cell mitogen may result in suppression of tumor growth *in vivo*. Subsequently, other tumor cell lines were found to be inhibited *in vivo* by this treatment (194–198) (Table 2).

In agreement with the hypothesis that inhibition of neo-

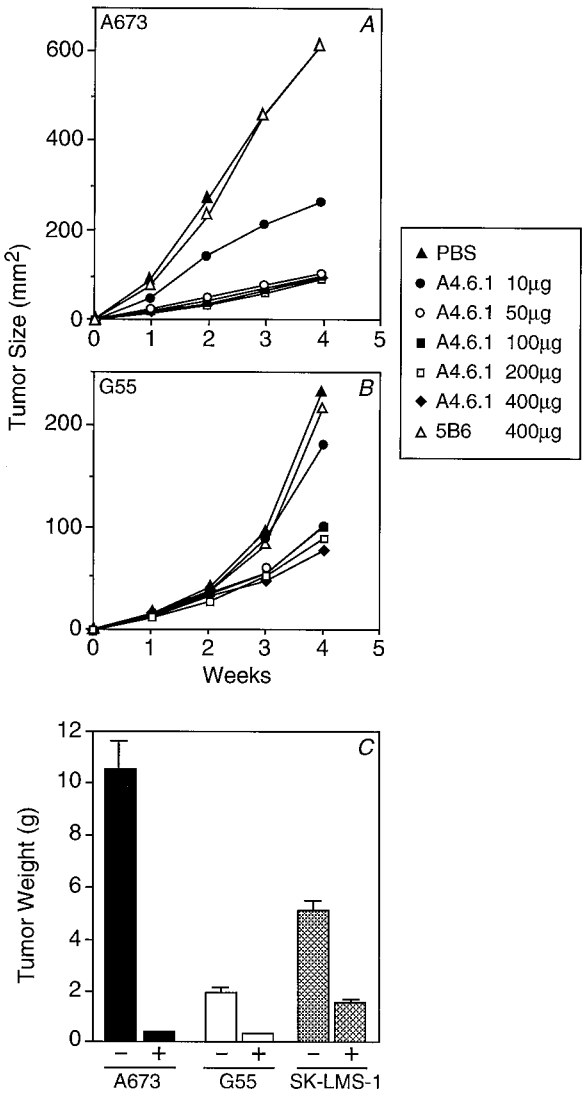

FIG. 7. Effects of anti-VEGF monoclonal antibody on tumor size (A, B) and weight (C). A673, G55, and SK-LMS-1 cells were injected subcutaneously in nude mice. Animals were then treated with anti-VEGF neutralizing antibody (A.4.6.1) or a control antibody (5B6) twice weekly intraperitoneally, at the indicated doses. In A and B, tumor size was measured weekly. Panel C illustrates the weight of the tumors at the end of the experiment. Data shown reflect the response to 100 μg (5 mg/kg) of antibody twice weekly. Plus and minus signs denote the presence or absence of antibody treatment. A673 and G55 tumors were collected 4 weeks after tumor cell injection. SK-LMS-1 tumors were harvested after 10 weeks. [Reproduced with permission from J. Kim *et al.*: *Nature* 362:841–844, 1993 (193). © 1993 Macmillan Magazines Limited.]

vascularization is the mechanism of tumor suppression, the density of blood vessels was significantly lower in sections of tumors from antibody-treated animals as compared with controls (193, 194). Furthermore, neither the antibodies nor VEGF had any effect on the *in vitro* growth of the tumor cells (193, 194). Intravital videomicroscopy techniques have allowed a more direct verification of the hypothesis that anti-VEGF antibodies indeed block tumor angiogenesis (195). Tumor spheroids of A673 cells were implanted in dorsal skinfold chambers inserted in nude mice. Noninvasive imaging of the vasculature revealed a nearly complete suppression of tumor angiogenesis in anti-VEGF treated animals as compared with controls, at all time points examined. These findings were corroborated by histological analysis that showed a dramatic difference in the density of CD34-positive vascular elements between the two groups (195). Thus, inhibition of VEGF-induced angiogenesis caused a dramatic change in growth characteristics of this cell line, from a rapidly growing malignancy to a dormant tumor seedling. Very similar findings were subsequently obtained with other tumor cell lines (P. Borgström *et al.*, submitted).

TABLE 2. Human tumor cell lines inhibited *in vivo* by anti-VEGF neutralizing antibodies

Cell line	Tumor type	Reference
A673	Rhabdomyosarcoma	193, 195
G55	Glioblastoma multiforme	193
SK-LMS-1	Leiomyosarcoma	193
HM 7	Colon carcinoma	194
LS LiM6	Colon carcinoma	194
A-431	Epidermoid carcinoma	196
HT-1080	Fibrosarcoma	197
U87	Glioblastoma multiforme	198
LS174T	Colon carcinoma	198
P-MEL	Melanoma	198

The following data represent additional human tumor cell lines inhibited *in vivo* by anti-VEGF neutralizing antibodies: MCF-7 cells from breast carcinoma (P. Borgström *et al.*, submitted); D-145 cells from prostatic carcinoma (P. Borgström *et al.*, submitted); SK-OV-3 cells from ovarian carcinoma (S. Mesiano, unpublished observations); SK-AS cells from neuroblastoma (N. Ferrara, unpublished observations); and Calu-6 cells from non-small cell lung carcinoma (F. Kabbinavar, unpublished observations).

Warren *et al.* (194) have demonstrated that VEGF is a mediator of the *in vivo* growth of human colon carcinoma HM7 cells in an orthotopic nude mouse model of liver metastasis. Similar to human tumors, in this murine model the expression of Flk-1 mRNA was markedly up-regulated in the vasculature associated with liver metastases. Treatment with anti-VEGF monoclonal antibodies resulted in a dramatic decrease in the number and size of metastases. Most of the tumors in the treated group were less than 1 mm in diameter and all were less than 3 mm. Also, neither blood vessels nor Flk-1 mRNA expression could be demonstrated in such metastases. Also, administration of anti-VEGF-neutralizing antibodies inhibited primary tumor growth and metastasis of A431 human epidermoid carcinoma cells in *scid* mice (196) or HT-1080 fibrosarcoma cells implanted in BALB/c nude mice (197).

Recently, Borgström *et al.* (submitted) have shown that a combination treatment that includes anti-VEGF monoclonal antibody and doxorubicin results in a significant enhancement of the efficacy of either agent alone and led in some cases to complete regression of tumors derived from MCF-7 breast carcinoma cells in nude mice. Combination treatments that include anti-VEGF monoclonal antibody and cisplatin have resulted in similar enhancement of the efficacy of each agent (our unpublished observations).

Intravital fluorescence microscopy and video imaging analysis have been also applied to address the important

issue of the effects of VEGF on permeability and other properties of tumor vessels (198). Three different human tumor cell lines (U87, P-MEL, and LS174T) were implanted in two locations in immunodeficient mice, the cranium and the dorsal skinfold. Treatment with an anti-VEGF monoclonal antibody (192) was initiated when the tumor xenografts were already established and vascularized and resulted in time-dependent reductions in vascular permeability (198). These effects were accompanied by striking changes in the morphology of vessels, with dramatic reduction in diameter and tortuosity (198). This reduction in diameter is expected to block the passage of blood elements and eventually stop the flow in the tumor vascular network. Accordingly, a regression of blood vessels was observed after repeated administrations of anti-VEGF antibody. These findings led to the intriguing conclusion that tumor vessels require constant stimulation with VEGF to maintain not only their proliferative properties but also some key morphological features (198).

An additional verification of the hypothesis that VEGF action is required for tumor angiogenesis has been provided by the finding that retrovirus-mediated expression of a dominant negative Flk-1 mutant, which inhibits signal transduction through wild type Flk-1/KDR receptor, suppresses the growth of glioblastoma multiforme as well as other tumor cell lines *in vivo* (199, 200).

Further evidence that VEGF action is necessary for effective tumor angiogenesis has been recently obtained in an *in vivo* model of embryonic stem (ES) cell tumorigenesis (17). ES cells are able to form highly vascularized teratocarcinomas when injected in nude or syngeneic mice (201). VEGF null ES cells were dramatically impaired in their ability to form tumors in nude mice. The number of vessels in the VEGF$-/-$ group was substantially reduced and showed a much less complex branching pattern than that observed in controls. Thus, even in a pluripotent system such as the ES cells, which is expected to have the potential to activate redundant pathways, VEGF is required for *in vivo* growth.

B. Intraocular neovascular syndromes

Diabetes mellitus, occlusion of central retinal vein, or prematurity with subsequent exposure to oxygen can all be associated with intraocular neovascularization (202, 203). The new blood vessels may lead to vitreous hemorrhage, retinal detachment, neovascular glaucoma, and eventual blindness (5). Diabetic retinopathy is the leading cause of blindness in the working population (203). All of these conditions are known to be associated with retinal ischemia (202). In 1948, Michaelson proposed that a key event in the pathogenesis of these conditions is the release by the ischemic retina into the vitreous of a diffusible angiogenic factor(s) ("factor X") responsible for retinal and iris neovascularization (204). Factors such as FGF and IGF-I do not show a consistent elevation as would be expected if they played a major pathogenic role (205, 206). VEGF, by virtue of its diffusible nature and hypoxia inducibility, was an attractive candidate as a mediator of intraocular neovascularization. Accordingly, elevations of VEGF levels in the aqueous and vitreous of eyes with proliferative retinopathy have been described (207–209). In a large series where ocular fluids from 165 patients were examined, a strong correlation was found between levels of immunoreactive VEGF in the aqueous and vitreous humors and active proliferative retinopathy (207). VEGF levels were undetectable or very low (<0.5 ng/ml) in the eyes of patients affected by nonneovascular disorders or diabetes without proliferative retinopathy. In contrast, VEGF levels were in the range of 3–10 ng/ml in the presence of active proliferative retinopathy associated with diabetes, occlusion of central retinal vein, or prematurity. Remarkably, the VEGF levels were again very low in the eyes of patients with quiescent proliferative retinopathy, a phase of vascular regression that follows the period of active vascular proliferation in diabetic and other retinopathies (207). Thus, although the involvement of other factors cannot be ruled out, VEGF is the molecule that correlates best with ocular angiogenesis (210). In agreement with these findings, *in situ* hybridization studies have demonstrated up-regulation of VEGF mRNA in the retina of patients with proliferative retinopathies secondary to diabetes, central retinal vein occlusion, retinal detachment, or intraocular tumors (211). Interestingly, VEGF mRNA expression was localized to the specific retinal layer(s) expected to be ischemic in each of these conditions (211).

More direct evidence for a role of VEGF as a mediator of intraocular neovascularization has been generated in a primate model of iris neovascularization and in a murine model of retinopathy of prematurity (212, 213). In the former, intraocular administration of anti-VEGF antibodies dramatically inhibits the neovascularization that follows occlusion of central retinal veins (214). Likewise, soluble Flt-1 or Flk-1 fused to an IgG suppresses retinal angiogenesis in the mouse model (215).

Neovascularization is a major cause of visual loss also in AMD, the overall leading cause of blindness (216). Most AMD patients have atrophy of the retinal pigment epithelial cells and characteristic formations called "drusen." A significant percentage of AMD patients (~20%) manifest the neovascular (exudative) form of the disease. In this condition, the new vessels stem from the extraretinal choriocapillary. It is well established that the appearance of such choroid neovascularization coincides with a dramatic worsening in prognosis (216). Leakage and bleeding from these vessels may lead to damage to the macula and ultimately to loss of central vision. Because of the proximity of the lesions to the macula, laser photocoagulation or surgical therapy are of very limited value. Very recent studies have documented the immunohistochemical localization of VEGF in surgically resected choroidal neovascular membranes from AMD patients (217, 218). Transdifferentiated retinal pigment epithelial cells in the highly vascularized regions of the membranes were the major sources of VEGF. Also, Kvanta (219) has shown that choroid fibroblasts are able to release VEGF and also that various cytokines are able to induce VEGF gene expression in these cells. These findings suggest a role for VEGF in the progression of AMD-related choroidal neovascularization, raising the possibility that a pharmacological treatment with monoclonal antibodies or other VEGF inhibitors may constitute a therapy for this condition.

C. Other pathological conditions

Two independent studies have suggested that VEGF is involved in the pathogenesis of rheumatoid arthritis (RA), an important disease in which angiogenesis plays a significant role (220, 221). The RA synovium is characterized by the formation of pannus, an extensively vascularized tissue that invades and destroys the articular cartilage (222). By its vascularity and rapid proliferation rate, the RA synovium has been likened to a tumor (223). Levels of immunoreactive VEGF were high in the synovial fluid of RA patients whereas they were very low or undetectable in the synovial fluid of patients affected by other forms of arthritis or by degenerative joint disease. Furthermore, anti-VEGF antibodies significantly reduced the endothelial cell chemotactic activity of the RA synovial fluid (220).

It has been shown that VEGF expression is increased in psoriatic skin (85). Increased vascularity and permeability are characteristic of psoriasis. Also, VEGF mRNA expression has been examined in three bullous disorders with subepidermal blister formation: bullous pemphigoid, erythema multiforme, and dermatitis herpetiformis (224). In all of these conditions, VEGF mRNA was markedly up-regulated not only in the epidermis over blisters, but also at a distance from blisters, in areas adjacent to dermal inflammatory infiltrates.

Angiogenesis is also important in the pathogenesis of endometriosis, a condition characterized by ectopic endometrium implants in the peritoneal cavity (225). Recently, elevation of VEGF in the peritoneal fluid of patients with endometriosis has been reported (226, 227). Immunohistochemistry indicated that activated peritoneal fluid macrophages as well as tissue macrophages within the ectopic endometrium are the main source of VEGF in this condition. Interestingly, VEGF secretion by macrophages was enhanced by ovarian steroids (226, 227). VEGF up-regulation has been also implicated in the hypervascularity of the ovarian stroma that characterizes Stein-Leventhal syndrome (228).

Moreover, Sato *et al.* (229) proposed that VEGF is implicated in the pathogenesis of Graves' disease. TSH, insulin phorbol ester, $(Bu)_2$cAMP, and Graves' IgG were found to stimulate VEGF mRNA expression in cultured human thyroid follicles (229). These findings suggest that VEGF, secreted by thyroid follicles via the protein kinase A and C pathways, may be responsible for the characteristic hypervascularity (230) of Graves' syndrome.

Furthermore, it has been suggested that VEGF up-regulation plays a pathogenic role in the capillary hyperpermeability that characterizes ovarian hyperstimulation syndrome (231) as well as in the dysfunctional endothelium of preeclampsia (232).

X. Therapeutic Applications of VEGF-Induced Angiogenesis

The availability of agents able to promote the growth of new collateral vessels would be potentially of major therapeutic value for disorders characterized by inadequate tissue perfusion and might constitute an alternative to surgical reconstruction procedures. For example, chronic limb ischemia, most frequently caused by obstructive atherosclerosis affecting the superficial femoral artery, is associated with a high rate of morbidity and mortality, and treatment is currently limited to surgical revascularization or endovascular interventional therapy (233, 234). No pharmacological therapy has been shown to be effective for this condition. It has been shown that intraarterial or intramuscular administration of $rhVEGF_{165}$ may significantly augment perfusion and development of collateral vessels in a rabbit model where chronic hindlimb ischemia was created by surgical removal of the femoral artery (21, 22). These studies provided angiographic evidence of neovascularization in the ischemic limbs. Figure 8 illustrates the development of collateral vessels in this ischemic hindlimb model at various time points after a single intraarterial administration of VEGF (1 mg). Arterial gene transfer with cDNA encoding VEGF also led to revascularization in the same rabbit model to an extent comparable to that achieved with the recombinant protein (23, 235). In addition, the hypothesis that the angiogenesis initiated by the administration of VEGF improved muscle function in ischemic limbs was tested by Walder *et al.* (236). A single intraarterial injection of $rhVEGF_{165}$ augmented muscle function in this rabbit model of peripheral limb ischemia. Also, exercise-induced hyperemia was significantly increased in ischemic limbs treated with $rhVEGF_{165}$ (224). Such improvement in perfusion was, however, not seen in other nonischemic tissues including the contralateral limb. Similarly, Bauters *et al.* (237) have shown that both maximal flow velocity and maximal blood flow are significantly increased in ischemic limbs after VEGF administration. Other studies have shown that VEGF administration also leads to a recovery of normal endothelial reactivity in dysfunctional endothelium. After obstruction of a large artery and development of collateral vessels, the increase in blood flow that normally follows acetylcholine infusion is severely blunted; serotonin paradoxically leads to a decrease in blood flow (238). Thirty days after a single intraarterial bolus of $VEGF_{165}$, restoration of normal increase in blood flow in ischemic rabbit hindlimb after acetylcholine or serotonin infusion was demonstrated (239).

Banai *et al.* (18) have shown that VEGF administration results in increased coronary blood flow in a dog model of coronary insufficiency. After occlusion of the left circumflex coronary artery, daily intraluminal injections of rhVEGF distal to the occlusion resulted in a significant enhancement in collateral blood flow over a 4-week period. In addition, Harada *et al.* (19) have demonstrated that extraluminal administration of as little as 2 μg of rhVEGF by an osmotic pump results in a significant increase in coronary blood flow in a pig model of chronic myocardial ischemia created by ameroid occlusion of the left proximal circumflex artery. Also, magnetic resonance imaging was employed to provide a noninvasive assessment of the benefits secondary to VEGF administration in the porcine model (20). Image series converted to a space-time map demonstrated reduction in the size of the ischemic zone and decreased delay in contrast arrival after VEGF treatment (20). These findings demonstrated improvement in cardiac global and regional function and reduced infarct size, resulting from enhanced collateral blood supply (20).

A further potential therapeutic application of VEGF is the

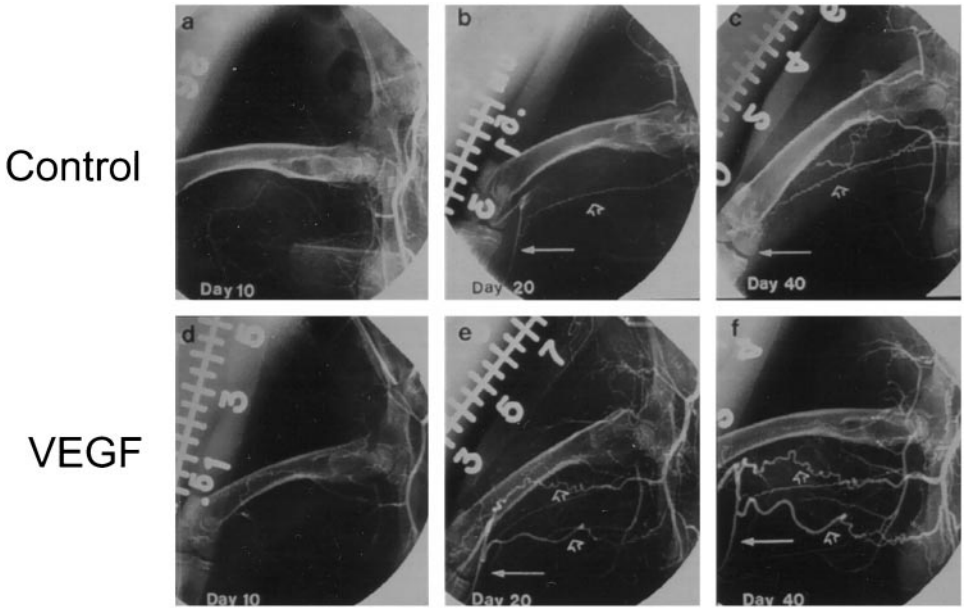

FIG. 8. Selective internal iliac angiography of control and VEGF-treated rabbit at (a) day 10 (baseline), (b) day 20, and (c) day 40. VEGF was administered as single intraarterial bolus in the iliac artery of rabbits in which the ipsilateral femoral artery had been removed to induce sustained, severe ischemia. Note the modest collateral vessel development in the control, which contrasts with the marked improvement observed after VEGF treatment. [Reproduced with permission from S. Takeshita *et al.*: *J Clin Invest* 93:662–670, 1994 (21) by copyright permission of The American Society for Clinical Investigation.]

prevention of restenosis after percutaneous transluminal angioplasty. Between 15% and 75% of patients undergoing percutaneous transluminal angioplasty for occlusive coronary or peripheral arterial disease develop restenosis within 6 months (234). It has been proposed that damage to the endothelium is a crucial event triggering fibrocellular intimal proliferation (240). Therefore, the induction of rapid reendothelialization may be an effective strategy to prevent the cascade of events leading to neointima formation and ultimately to restenosis in patients. Recent evidence shows that VEGF accelerates reendothelialization and also attenuates intimal hyperplasia in balloon-injured rat carotid artery or rabbit aorta (241, 242).

Very recently, the hypothesis that VEGF may result in therapeutically significant angiogenesis in humans has been tested by Isner *et al.* (243) in a gene therapy trial in patients with severe limb ischemia. A case report of an interim analysis of this trial has been published (24). Arterial gene transfer of 2000 μg of naked plasmid DNA encoding $VEGF_{165}$, applied to the hydrogel polymer coating of an angioplasty balloon, resulted in angiographic and histological evidence of angiogenesis in the knee, midtibial, and ankle levels 4 weeks after the transfer. Such effects persisted at a 12-week view.

XI. Perspectives

The findings that heterozygous mutations inactivating the VEGF gene and homozygous mutations inactivating the Flt-1 or Flk-1/KDR genes result in profound deficits in vasculogenesis and blood island formation, leading to early intrauterine death, emphasize the pivotal role played by the VEGF/VEGF-receptor system in the development of the vascular system. Future studies, using inducible gene knockout technology (244), should help determine when the embryo is most vulnerable to VEGF deficiency. Such studies may also be of major value in establishing the role of VEGF and its receptors in the maintenance and homeostasis of endothelial cells in the adult animal. It will be of considerable interest also to determine the role of the other members of the VEGF family, both in the context of developmental angiogenesis and in the physiology of the adult animal.

The elucidation of the signal transduction properties of the Flt-1 and Flk-1/KDR receptors may provide the key to dissection of the pathways leading to such fundamental biological events as endothelial cell differentiation, morphogenesis, and angiogenesis. Furthermore, a more complete understanding of the signaling events involving other endothelial cell-specific tyrosine kinases (122, 245, 246) as well as cell adhesion molecules (48, 247) and their interrelation with the VEGF/VEGF receptor system should provide a more integrated view of the biology of vascular cells, both in normal and abnormal circumstances. In this context, recent studies have shown that VEGF-mediated angiogenesis requires a specific vascular integrin pathway, mediated by $\alpha v\beta 5$ (247).

An attractive possibility is that recombinant VEGF or gene therapy with VEGF gene may be used to promote endothelial cell growth and collateral vessel formation. This would represent a novel therapeutic modality for conditions that frequently are refractory to conservative measures and unresponsive to pharmacological therapy. Surprisingly, gene therapy with naked plasmid DNA has resulted in a demonstrable therapeutic effect, both in experimental animals and in humans (23, 24, 235). This raises the possibility that more efficient expression systems such as adenoviral vectors (248)

may achieve even greater pharmacological effect. However, the finding that the VEGF protein is able to promote therapeutic angiogenesis even at minute concentrations (19, 20) suggests that gene therapy may not offer advantages over the recombinant protein.

The high expression of VEGF mRNA in human tumors, the presence of the VEGF protein in ocular fluids of individuals with proliferative retinopathies, and the localization of VEGF in AMD lesions strongly support the hypothesis that VEGF is a key mediator of angiogenesis associated with various disorders. Therefore, anti-VEGF antibodies or other inhibitors of VEGF action such as small molecules inhibiting signal transduction of Flk-1/KDR (249), soluble receptors (118, 126, 215), or antisense oligonucleotides (250) may be of therapeutic value for a variety of malignancies as well as for other disorders, used alone or in combination with other agents. The recent elucidation of the minimal structural elements required for VEGF binding in Flt-1 may lead to the generation of smaller soluble receptors, with improved bioavailability and *in vivo* efficacy (139). Although the safety of such treatment has not been yet established, it is tempting to speculate that an anti-VEGF therapy may have low toxicity, possibly limited to inhibition of wound healing and ovarian and endometrial function, since endothelial cells are essentially quiescent in most adult tissues. Very recently, a humanized version of a high-affinity anti-VEGF monoclonal antibody, which retains the same affinity and efficacy as the original murine antibody, has been generated and may be used in future clinical trials (L. G. Presta *et al.*, submitted).

In conclusion, in spite of the plurality of factors potentially involved in angiogenesis, one specific factor, VEGF, appears to play an irreplaceable role in a variety of physiological and pathological circumstances.

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