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**SURVEY**

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## **Transforming Growth Factor-beta: Vasculogenesis, Angiogenesis, and Vessel Wall Integrity**

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Genetic studies have recently revealed a role for transforming growth factor-beta-1 (TGF- $\beta$ 1) and its receptors (TGF- $\beta$ Rs I and II as well as endoglin) in embryonic vascular assembly and in the establishment and maintenance of vessel wall integrity. The purpose of this review is threefold: first, to reassess previous studies on TGF- $\beta$  and endothelium in the light of these recent findings; second, to describe some of the well-established as well as controversial issues concerning TGF- $\beta$  and its regulatory role in angiogenesis; and third, to explore the notion of 'context' with respect to TGF- $\beta$  and endothelial cell function. Although the focus of this review will be on the endothelium, other vascular wall cells are also likely to be important in the pathogenesis of the vascular lesions revealed by genetic studies. © 1997 Elsevier Science Ltd. All rights reserved

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**Key words:** Vascular biology · Endothelium · Cytokine · Development

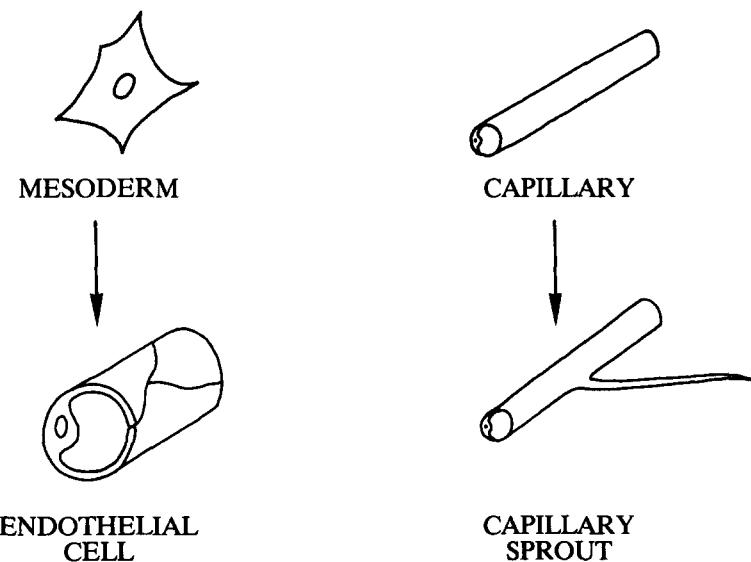
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The establishment and maintenance of a vascular supply are absolute requirements for the growth of normal and neoplastic tissues, and as might be predicted, the cardiovascular system is the first organ system to develop and to become functional during embryogenesis. Both during development and in postnatal life, all new blood vessels begin as simple endothelial-lined capillaries. Some remain as capillaries and develop an intimate association with other vessel wall cells including pericytes, while others develop into larger vessels and acquire a variable number of concentrically-disposed smooth muscle cell layers. Two processes are responsible for the formation of new blood vessels, both of which result in the formation of endothelial-lined tubes (Figure 1). The first is vasculogenesis, which is the primary *in situ* differentiation of endothelial cell precursors from mesoderm, and their subsequent organization into a primary capillary plexus [1]. The second is angiogenesis, which is the formation of new vessels by a process of sprouting from preexisting vessels [2]. It is currently assumed that vasculogenesis is limited to early embryogenesis, while angiogenesis occurs both during development and in post-natal life. However, the possibility that endothelial 'stem cells' persist into

adult life where they may contribute to the formation of new blood vessels through the formation of circulating endothelial precursors, remains to be explored.

With respect to vasculogenesis, observations made in the first half of this century suggested that endothelial cells arise either from angioblasts (which differentiate exclusively into endothelial cells) or from haemangioblasts (which have the dual capacity to differentiate into either endothelial or haematopoietic cells) [3, 4]. The existence of the angioblast is now well established; definitive proof for the existence of the haemangioblast is, however, still lacking. It is important to note that the definition of vasculogenesis includes both the primary differentiation of mesodermal precursors into endothelial cells, as well as the organization of these endothelial cells into capillary-like tubes. At the present time, primary differentiation from mesoderm appears to be a process that is limited exclusively to vasculogenesis, while the formation of capillary-like tubes is implicit in current definitions of both vasculogenesis and angiogenesis.

A contemporary view of early development suggests that the formation of the embryonic vasculature occurs in two separate stages, and by two different processes. The first stage commences in the extraembryonic membranes shortly after the establishment of the mesodermal layer, by the formation of clusters of haemangioblasts, which subsequently give rise to endothelial-lined cavities containing centrally located blood islands. This is fol-



**Figure 1.** Vasculogenesis and angiogenesis. Capillary blood vessels are formed by two processes. Vasculogenesis is the *in situ* differentiation of mesodermal precursors into endothelial cells and their subsequent organization into capillary-like tubes. This results in the formation of a primary capillary plexus. Angiogenesis is the formation of new capillaries by a process of sprouting from pre-existing capillaries or post-capillary venules. From Pepper *et al.* *Curr Top Microbiol Immunol* 1996, **213/II**, 31–67. Copyright Springer Verlag.

lowed by a vasculogenic process in the embryo itself, which results from the *in situ* differentiation of angioblasts and possibly haemangioblasts from mesodermal precursors which subsequently organize into a primary capillary plexus. Extra- and intraembryonic vessels subsequently anastomose, and, following the onset of cardiac action, constitute the earliest circulatory system. The second stage occurs during organogenesis, and results not only from *in situ* differentiation of angioblasts from mesoderm (vasculogenesis), but also from migration of preformed angioblasts as well as sprouting of endothelial cells from the established primary embryonic vasculature (angiogenesis). Therefore, although of necessity vasculogenesis must initially precede angiogenesis, the two processes appear to continue in parallel during both extra- and intraembryonic blood vessel formation, and subsequently during organogenesis [1, 4, 5]. As will be described below, the role of transforming growth factor-beta (TGF- $\beta$ ) in capillary morphogenesis and the maintenance of vessel wall integrity both during development and in post-natal life, has only recently become apparent from genetic studies in man and mouse. Prior to this, however, most work on TGF- $\beta$  was conducted in the context of angiogenesis. An overview of the biological significance of angiogenesis will therefore be presented.

In addition to its role during development, angiogenesis is required for the maintenance of functional and structural integrity of the organism post-natal life [2, 6]. Thus it occurs during wound healing, in inflammation, in situations of ischaemia, and in female reproductive organs (ovary—ovulation and corpus luteum formation; placenta and mammary gland during pregnancy). Angi-

ogenesis in these situations is tightly regulated, and is limited by the metabolic demands of the tissues concerned. Angiogenesis also occurs in pathological situations, such as proliferative retinopathy, rheumatoid arthritis and juvenile haemangioma [2, 6]. However, much of our interest in angiogenesis comes from the notion that, for tumours to grow beyond a critical size, they must recruit endothelial cells from the surrounding stroma to form their own endogenous microcirculation [2, 6]. Thus, during tumour progression, two phases can be recognized: a prevascular phase and a vascular phase. The transition from the prevascular to the vascular phase is referred to as the 'angiogenic switch' [7]. The prevascular phase is characterized by an initial increase in tumour growth followed by a plateau in which the rate of tumour cell proliferation is balanced by an equivalent rate of cell death (apoptosis). This phase may persist for many years, and can be recognized clinically as carcinoma *in situ*, which is characterized by few or no metastases. During the vascular phase, which is characterized by exponential growth, tissue invasion and the haematogenous spread of tumour cells, the rapid increase in tumour growth is largely due to a decrease in the rate of tumour cell apoptosis [8, 9]. An inverse relationship between tumour dormancy/tumour cell apoptosis and tumour angiogenesis thus exists. In a sense, tumour angiogenesis might almost be considered as 'appropriate', in that newly formed vessels serve to meet the metabolic demands of the rapidly growing tumour. Although this may be beneficial to the tumour itself, it is clearly detrimental to the organism, since it is permissive for continued tumour growth and also allows for the

dissemination of tumour cells and the formation of metastasis.

It is usually stated that, with the exception of angiogenesis, that occurs in response to tissue injury or in female reproductive organs, endothelial cell turnover in the healthy adult organism is very low [10–12]. The maintenance of endothelial quiescence is thought to be due to the presence of endogenous negative regulators, since positive regulators are frequently detected in adult tissues in which there is apparently no angiogenesis. The converse is also true, namely that positive and negative regulators often co-exist in tissues in which endothelial cell turnover is increased. This has led to the notion that endothelial activation status is determined by a balance between positive and negative regulators: in activated (angiogenic) endothelium, positive regulators predominate, whereas endothelial quiescence is achieved and maintained by the dominance of negative regulators [2, 13]. With respect to activated endothelium, an important distinction needs to be made between physiological and pathological settings: although many of the same positive and negative regulators are operative in both, endothelial cell proliferation in the former is tightly controlled, whereas in the latter, uncontrolled angiogenesis implies the continuous dominance of positive regulators, which results in unchecked endothelial cell growth. It should also be appreciated that the ultimate target for both positive and negative regulators is the endothelial cell. This has led to the notion that angiogenesis regulators may either act directly on endothelial cells, or may act indirectly by inducing the production of direct-acting regulators by inflammatory and other non-endothelial cells.

Used initially in the context of tumour progression to

describe the passage from the prevascular to the vascular phase (see above), the notion of the 'switch' also can be used to include developmental, physiological as well as pathological angiogenesis. Although it still remains to be definitively demonstrated *in vivo*, the current working hypothesis is that the 'switch' involves either the induction of a positive regulator and/or the loss of a negative regulator. Among the factors that affect endothelial cell activation status, either positively or negatively, are cytokines and chemokines (chemotactic cytokines) produced by normal and tumour cells. Cytokines are polypeptide regulatory factors involved in the control of cellular proliferation and differentiation. Released by living cells or from extracellular matrix, cytokines act at picomolar to nanomolar concentrations to affect cellular function [14]. Based on the observation that a given tissue can profoundly influence the way in which its cellular components respond to a given cytokine, it has been suggested that cytokines should be seen as "specialized symbols in a language of intercellular communication, whose meaning is controlled by context" [14] (Figure 2). Context is determined by (at least) three parameters: first, by the presence and concentration of other cytokines in the pericellular environment of the responding cell; second by interactions between cells, cytokines and the extracellular matrix; and third by the geometric configuration of the cells (and thus their cytoskeleton). With respect to angiogenesis, the molecular mechanisms underlying the notions of both the angiogenic 'switch' as well as 'context', are likely to be central to our understanding of this process. With respect to vasculogenesis, although the notions of the 'switch' and 'context' are not at present widely used, both are also likely to be important.

The multiple cell functions that occur during angi-

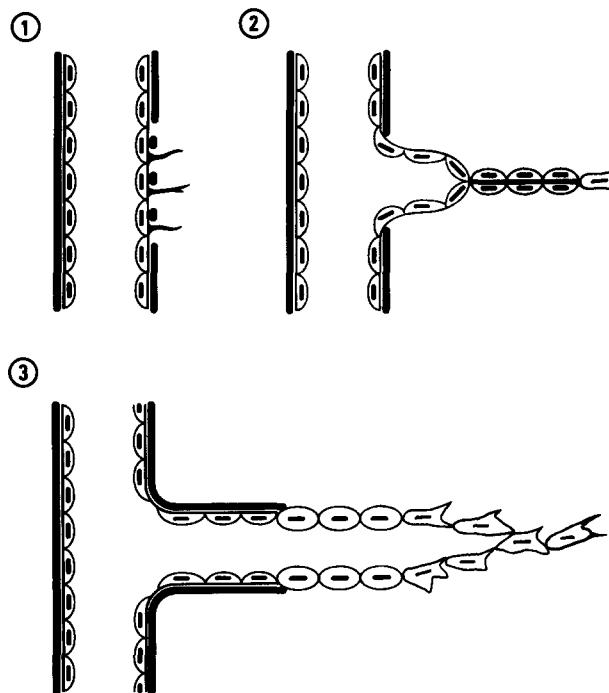
## CONTEXTUAL ACTIVITY OF ANGIOGENESIS REGULATING CYTOKINES

**Cytokines:** "Specialized symbols in a language of intercellular communication, whose meaning is controlled by context"

(Nathan and Sporn, 1991)

- |                 |   |
|-----------------|---|
| <b>Context:</b> | <ul style="list-style-type: none"> <li>• Cytokines</li> <li>• Extracellular matrix</li> <li>• Geometry</li> </ul> |
|-----------------|---|

Figure 2



**Figure 3.** Schematic representation of the angiogenic process. (1) Endothelial cell activation following exposure to an angiogenic stimulus is followed by basement membrane degradation and the extension of thin cytoplasmic processes in the direction of the stimulus. (2) This is followed by cell migration into the surrounding matrix and the formation of a capillary sprout. Proliferation occurs in cells just behind the migrating front, and lumen formation begins in the proximal region of the sprout. (3) Sprout maturation occurs by reconstitution of the basement membrane. A functional capillary loop is formed following fusion of the tip of the sprout with a contiguous sprout. (From Ref. 193; with kind permission from S. Karger AG, Basel, Switzerland.)

ogenesis belong either to a phase of activation or to a phase of resolution (Figure 3 and Table 1). The activation phase encompasses initiation and progression, and includes: (a) increased vascular permeability and extravascular fibrin deposition; (b) basement membrane degradation; (c) cell migration and extracellular matrix invasion; (d) endothelial cell proliferation; and (e) capillary lumen formation. The phase of resolution encompasses termination and vessel maturation, and includes: (a) inhibition of endothelial cell proliferation; (b) cessation of cell migration; (c) basement membrane reconstitution; and (d) junctional complex maturation. As indicated above, the definition of vasculogenesis includes both primary endothelial cell differentiation, as well as the organization of these endothelial cells into capillary-like tubes. With respect to the phases of activation and resolution, many components are equally as applicable to vasculogenesis as they are to angiogenesis.

While a great deal is known about those factors that induce the activation phase, very little is known about the factors involved in the phase of resolution, in which the dominant activity of negative regulators is called into play. Furthermore, it is at present, unclear as to whether the resolution phase is an active phase, or whether it is

**Table 1. Phases of angiogenesis**

Activation: initiation and progression

- Increased vascular permeability and extravascular fibrin deposition
- Basement membrane degradation
- Cell migration/matrix invasion
- Cell division
- Lumen formation

Resolution: termination and maturation

- Cessation of migration
- Inhibition of cell division
- Basement membrane reconstitution
- Junctional complex maturation

Endothelial cell functions that result in the formation of new capillary blood vessels during angiogenesis can be separated into two phases: a phase of activation, which encompasses initiation and progression, and a phase of resolution including termination and vessel maturation. Although most of these functions are equally applicable to vasculogenesis, they have been less well studied in this context. (Modified from Ref. 25; with kind permission from Springer, Berlin.)

the consequence of exhaustion of positive regulators that predominated during the phase of activation. If the latter hypothesis is correct, this would assume that endothelial cells have the inherent capacity to synthesize their own basement membrane and to organize into capillary-like tubes, and that this is mediated in part by the autocrine activity of endogenous regulators.

The most extensively studied cytokines involved in the positive regulation of new blood vessel formation are vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (aFGF, bFGF, which will be collectively referred to as FGF) [15–17]. However, although a role for VEGF in developmental and tumour angiogenesis has been well defined [18–24], much controversy still exists as to whether or not the FGFs are relevant to the endogenous control of neovascularization *in vivo*. The finding that, *in vitro*, VEGF and FGF positively regulate many endothelial cell functions, including proliferation, migration and extracellular proteolytic activity [25] as well as the capacity of stimulated endothelial cells to invade three-dimensional extracellular matrices within which they form capillary-like tubes [26, 27], has led to the notion that these factors are direct-acting positive regulators. It is crucial to bear in mind, however, that, although a large number of factors have been shown to be active in the experimental setting [25, 28, 29], it does not necessarily follow that these factors are relevant to the endogenous regulation of new blood vessel formation, i.e. that they are relevant to the control of vasculogenesis or angiogenesis in the intact organism. In the case of molecules that are active during the phase of activation, only one, namely VEGF, meets most of the criteria required for the definition of a vasculogenic or angiogenic factor [25, 30].

In addition to VEGF and the FGFs, TGF- $\beta$ , and in particular TGF- $\beta$ 1, have featured prominently amongst cytokines studied for their capacity to regulate new blood

vessel formation, both *in vivo* and *in vitro*. TGF- $\beta$ 1 is a member of a large superfamily of cytokines including activins, inhibins, bone morphogenetic proteins and others. Three TGF- $\beta$ s (1, 2 and 3) have been described in mammals. TGF- $\beta$ s are secreted from cells or purified from platelets as a high molecular weight latent complex in which the C-terminal mature homodimer is non-covalently associated with a dimer of its N-terminal pro-region (also known as latency-associated peptide or LAP). Cleavage of the dimerized TGF- $\beta$  precursor to form the TGF- $\beta$ /LAP complex occurs in the secretory pathway and is mediated by a furin peptidase. LAP in turn may be disulphide-bonded to structurally and genetically unrelated TGF- $\beta$  binding proteins. Following secretion, the latent TGF- $\beta$ /LAP complex, which is unable to bind TGF- $\beta$  receptors, is activated in the extracellular milieu. Although the latent complex can be activated *in vitro* by plasmin, cathepsin D and low pH, the physiological mechanisms that activate TGF- $\beta$  *in vivo* are unknown. LAP, when independently expressed, associates non-covalently with mature TGF- $\beta$ , thereby inactivating its biological activity [31–33].

TGF- $\beta$ s achieve their biological effects through binding to cell surface receptors (TGF- $\beta$ Rs) designated types I, II and III. TGF- $\beta$  binds directly to TGF- $\beta$ R II, which exists on the cell surface as a homo-oligomer. Binding is followed by recruitment of TGF- $\beta$ R I and the formation of a stable ternary complex. The cytoplasmic domain of TGF- $\beta$ R II is autophosphorylated and constitutively active. Following recruitment, TGF- $\beta$ R I, which is not phosphorylated in the absence of TGF- $\beta$ , is phosphorylated on serine/threonine by TGF- $\beta$ R II. This is followed by TGF- $\beta$ R I-mediated activation of intracellular signal transduction. Therefore, the kinase activities of TGF- $\beta$ Rs I and II are both required for transducing TGF- $\beta$ 's signals. Furthermore, the components of the heterotrimeric complex are interdependent, as TGF- $\beta$ R I requires TGF- $\beta$ R II to bind TGF- $\beta$ , while TGF- $\beta$ R II requires TGF- $\beta$ R I to signal. TGF- $\beta$ R III is betaglycan, a transmembrane proteoglycan with a short cytoplasmic domain, containing both heparan sulfate and chondroitin sulphate glycosaminoglycans. TGF- $\beta$ R III does not appear to be required for signal transduction, but may serve to present or deliver TGF- $\beta$  to the signalling receptors. Endothelial cells also express endoglin, a protein with structural homology in the TGF- $\beta$ R III. TGF- $\beta$  also binds to the decorin core protein that neutralizes its activity, as well as to thrombospondin, a large multifunctional glycoprotein that mediates the adhesion of both endothelial and non-endothelial cells to the extracellular matrix [34–37].

Although there has been much controversy concerning the interpretation of studies with TGF- $\beta$  on experimental angiogenesis both *in vivo* and *in vitro*, genetic studies in man and mouse have recently revealed a role for this cytokine in embryonic vascular assembly and in the maintenance of vessel wall integrity. The purpose of this review is threefold: first, to reassess previous studies on TGF- $\beta$  and endothelium in the light of these recent findings;

second, to describe some of the well-established and controversial issues concerning TGF- $\beta$  and its regulatory role in angiogenesis, in an attempt to place it in an appropriate context *in vivo*; and third, since it is highly likely that endothelial cells are rarely (if ever) exposed to a single cytokine during physiological and pathological processes *in vivo*, some of the interactions that have been described between TGF- $\beta$  and other cytokines will be described to illustrate the notion of 'context', a notion to which TGF- $\beta$  appears to be particularly well suited. The focus of this review will be on the endothelium; earlier studies on the role of TGF- $\beta$  in the regulation of endothelial cell function and angiogenesis have been previously reviewed [38–41]. It should be pointed out, however, that other vascular wall cells, either as producers of, or as responders to, TGF- $\beta$  may be important in the pathogenesis of the vascular lesions that have been revealed by genetic studies.

## STUDIES *IN VIVO*

### Targeted disruption of the *TGF-β1* gene

TGF- $\beta$ 1 is expressed in many tissues during embryogenesis (including endothelial and haematopoietic precursors) [42], and targeted disruption of the TGF- $\beta$ 1 gene results in mortality at two distinct times: either during midgestation (group A) or at 3 weeks post-partum (group B) [43–45]. With few exceptions (see below), embryos and offspring in virtually all cases reported thus far have been conceived in heterozygous mothers (TGF- $\beta$ 1+/-). With respect to group A, mortality occurred in approximately 50% of homozygotes (TGF- $\beta$ 1-/-) and 25% of heterozygotes (TGF- $\beta$ 1+/-) after 10.5 days of gestation, and this resulted from defects in the extraembryonic tissues, namely the yolk sac vasculature and the haematopoietic system. TGF- $\beta$ 1-/- embryos *per se*, unlike their yolk sacs, had no specific abnormalities, although generalized developmental retardation, ischaemia and necrosis did occur, which may have been secondary to the extraembryonic lesions. In particular, intraembryonic endothelial cells, expressing high levels of VEGF receptor-2 (VEGFR-2—also known as KDR or Flk-1), appeared to have developed normally. With respect to the yolk sac, initial differentiation of mesodermal precursors into endothelial cells appeared to have occurred, although there was a reduction in the number of VEGFR-2-expressing cells. Differentiation into capillary-like tubes was also defective, resulting in vessels with increased wall fragility: contacts between endothelial cells had either not formed or had been disrupted, resulting in leakage of blood cells into the yolk sac cavity. As indicated above, the definition of vasculogenesis includes both the primary differentiation of mesodermal precursors into endothelial cells as well as the organization of these endothelial cells into capillary-like tubes. At present, it is not possible to say whether the reduction in the number of VEGFR-2-expressing endothelial cells in the yolk sac was due to reduced angioblast differentiation or to inefficient net-

work formation by newly differentiated endothelial cells. (With respect to the latter, TGF- $\beta$ 1 has been shown to potentiate VEGF and bFGF-dependent capillary sprout formation *in vitro*—see below.) However, what is clear is that TGF- $\beta$ 1 deficiency markedly affects the establishment and maintenance of vessel wall integrity.

With respect to group B, these mice completed embryogenesis successfully in heterozygous mothers, and showed no overt developmental abnormalities. However, at 3–4 weeks of age they developed a rapidly progressive and fatal wasting syndrome associated with massive and generalized inflammatory cell infiltration and multiple organ failure [43, 44]. Although these mice lack a functional copy of the TGF- $\beta$ 1 gene, maternal TGF- $\beta$ 1 derived either from transplacental transfer or from milk during lactation might have rescued the homozygous null embryos from developmental abnormalities. This might also explain why the inflammatory dysfunction was delayed until weaning [46]. An alternative explanation for the different stages at which the two lethal phenotypes occurred [namely mid-gestation (group A) and at 3 weeks of age (group B)] may be redundancy, in that other foetal or maternal isoforms of TGF- $\beta$  might have substituted for the lack of TGF- $\beta$ 1. Finally, polymorphisms in genetic modifiers that exist in the different genetic backgrounds might dictate sensitivity to reduced TGF- $\beta$ 1 levels in these two groups of mice. With respect to the limited number of embryos that developed in homozygous null mothers (some of which had to be treated with high doses of dexamethasone to prevent the fatal wasting syndrome), both foetuses and neonates appeared to have acquired severe cardiac abnormalities. However, it has been suggested that the cardiac lesions may have been secondary to the extraembryonic defects [45, 46].

Taken together, the findings in TGF- $\beta$ 1 null mice imply that this cytokine is an important positive regulator of extraembryonic endothelial cell differentiation, the establishment of extraembryonic vessel wall integrity, and yolk sac haematopoiesis. They also suggest that intraembryonic and extraembryonic vasculogenesis and haematopoiesis are regulated differently.

### **Expression of TGF- $\beta$ 1 during angiogenesis**

At least two major paradigms can be invoked to account for the transition of endothelial cells from a quiescent to an activated or angiogenic state. These include the ‘angiogenic switch’ and the notion of ‘context’. With respect to the former, there is, at present, no good direct evidence *in vivo* demonstrating that TGF- $\beta$  is responsible for the transition from the prevascular to the vascular state during tumour progression, or in any other setting in which angiogenesis is induced. The same can be said regarding the notion of context. None the less, models employing exogenous TGF- $\beta$ 1 together with *in vitro* systems that recapitulate various stages of the process (described below), clearly point to a role of this cytokine in the regulation in angiogenesis in the exper-

imental setting. Furthermore, descriptive studies have clearly demonstrated the presence of TGF- $\beta$ 1 in tissues in which there is active neovascularization, thereby providing correlative evidence in favour of a role for this cytokine.

With respect to carcinogenesis, TGF- $\beta$ s are expressed in a large number of tumour types as well as by a variety of cultured tumour cells. This might appear paradoxical in light of TGF- $\beta$ 's well established antiproliferative effects. However, it has recently become apparent that many tumours as well as tumour cell lines bear mutations that inactivate the TGF- $\beta$  receptor complex. This has led to the suggestion that TGF- $\beta$  resistance is a primary progression event that is selected for during carcinogenesis, and that the TGF- $\beta$  receptor complex should be added to the family of tumour suppressor genes [47]. In light of these findings, the seemingly paradoxical observation that many tumours express TGF- $\beta$ s, can be reconciled by assuming that once a tumour cell has escaped from TGF- $\beta$ 's negative regulatory effects, TGF- $\beta$  can promote tumour growth indirectly by stimulating angiogenesis and desmoplasia and by suppressing immune surveillance. However, despite this emerging paradigm, there is no consistent pattern of expression that suggests that TGF- $\beta$ s are involved in tumour angiogenesis. For example, elevated plasma TGF- $\beta$ 1 levels have been reported in patients with invasive prostate cancer [48] and hepatocellular carcinoma [49], and in the latter study, this correlated positively with tumour vascularity but not with tumour size or underlying liver disease. However, in malignant brain tumours, including gliomas, which are highly vascular and in which the role of VEGF in the induction of angiogenesis has been clearly defined [50], although one study found that the level of expression of TGF- $\beta$ 1 was higher in metastatic than in non-metastatic tumours [51], in a second study, mRNA levels for VEGF but not for TGF- $\beta$ 1, TGF- $\alpha$  or bFGF, were found to correlate significantly with vascularity [52].

A role for TGF- $\beta$  has also been proposed during angiogenesis in situations of tissue hypoperfusion. For example, occlusion of cerebral vessels leads to ischaemia and infarction (stroke), and this is accompanied by neovascularization which is particularly marked in the border zone of the infarct (the ischaemic penumbra). TGF- $\beta$ 1 mRNA and active protein were found to be significantly higher in the ischaemic penumbra than in the infarct zone, which in turn displayed greater levels of cytokine than in the normal contralateral hemisphere [53]. Organ transplantation constitutes a second example of tissue hypoperfusion in which the expression of angiogenic factors is upregulated in hypoxic devascularized tissues. For example, it has been demonstrated that endogenous VEGF is increased *in vitro* as a function of time in devascularized rat islets of Langerhans [54]. When the ovaries of juvenile rats are autotransplanted to an ectopic site, they become profusely revascularized within 48 h and recover the ability to control gonadotropin secretion via negative feedback within 1 week. Vascular ingrowth in this model was shown to be accompanied by

a 40- to 60-fold increase in expression of VEGF and TGF- $\beta$ 1 [55]. Although a less marked and slightly delayed increase in TGF- $\beta$ 3 was also observed, there was no increase in expression of other cytokines, including bFGF, transforming growth factor-alpha (TGF- $\alpha$ ) or TGF- $\beta$ 2. In accord with the long-standing proposal that angiogenesis is driven by tissue hypoxia in both prenatal and postnatal life, it has now been clearly demonstrated that hypoxia is a major positive regulator of VEGF synthesis [56–58]. With regard to TGF- $\beta$ , mRNA and protein for TGF- $\beta$ 1, but not for TGF- $\beta$ 2, have been shown to be increased in human dermal fibroblasts in response to hypoxia (2% O<sub>2</sub> for 48–72 h), and this effect was mediated by an increase in transcription rather than in mRNA stability [59]. Active TGF- $\beta$  was likewise increased in the conditioned medium of hypoxic bovine smooth muscle cells (1 and 5% O<sub>2</sub> for 12–24 h) [60], and hypoxia (1% O<sub>2</sub> for 16 h) increased TGF- $\beta$ 1 mRNA in human hepatoma and trophoblastic cell lines [61].

With respect to wound healing, in which angiogenesis is a prominent component, all three isoforms of TGF- $\beta$  have been reported to be induced in full-thickness cutaneous wounds in mice [62]. During the early phases of wound healing, TGF- $\beta$ 1 was the predominant isoform, while in the later period, during which maximal granulation tissue formation occurs (day 7), the greatest induction was seen with TGF- $\beta$ 3, although absolute levels of TGF- $\beta$ s-1 and -3 were similar.

### **Regulation of angiogenesis in experimental models**

The two most widely used *in vivo* angiogenesis assays are the chick embryo chorioallantoic membrane (CAM) [63, 64] and the rabbit corneal micropocket [65]. These assays have been used for many years to describe the morphologically identifiable events that occur during angiogenesis, and have also been important in the identification of positive and negative regulators. Direct subcutaneous injection or infusion of substances of interest has also been used to assess their pro- or anti-angiogenic effects. More recently described quantitative *in vivo* assays involve subcutaneous implantation of various three-dimensional substrates to which angiogenesis-regulating factors can be added. These include polyester sponges [66], expanded polyfluorotetraethylene (ePTFE) tubes filled with collagen [67], polyvinyl-alcohol foam discs covered on both sides by millipore filters (the disc angiogenesis system) [68], and Matrigel, a basement membrane-rich extracellular matrix [69].

TGF- $\beta$  has been described as being either angiogenic or anti-angiogenic *in vivo*, depending on the nature of the assay. Thus TGF- $\beta$  is a potent inducer of angiogenesis when administered subcutaneously to new born [70] or adult [71] mice or adult rats [72], when applied to the chick embryo CAM [73], when tested in the rabbit cornea [74, 75], when added to subcutaneously implanted collagen-filled ePTFE tubes in rats [67], when applied to full thickness wounds in a rabbit ear dermal ulcer model

[76] or when tested in the disc angiogenesis system [77]. Spontaneous angiogenesis in the latter system was inhibited by local administration of anti-TGF- $\beta$ 1 neutralizing antibodies [77]. Angiogenesis, in most of these situations, was associated with a marked inflammatory cell infiltrate. When skeletal myocytes stably transfected with TGF- $\beta$ 1 were injected into the myocardium of the left ventricle of syngeneic mouse hosts, this also resulted in a measurable angiogenic response [78]. Similarly, stable transfection with TGF- $\beta$ 1 was shown to confer a growth advantage on Chinese hamster ovary (CHO) cells *in vivo* but not *in vitro*, and this was associated with the development of a metastatic phenotype [79, 80]. Tumour growth in this system was accompanied by an increase in capillary density, and local administration of neutralizing antibodies to TGF- $\beta$ 1 reduced both capillary density and tumour growth. In contrast to the above findings, it has been observed that TGF- $\beta$ 1 on its own has no effect when added to subcutaneously implanted polyester sponges (T-P Fan, personal communication) or to subcutaneously injected Matrigel [69], and that it inhibits aFGF/heparin-induced angiogenesis when the two cytokines are co-added in the latter system [69]. Similarly, TGF- $\beta$ 1 inhibited the growth of vascular tumours induced by Polyoma virus middle T antigen-transformed endothelial cells in syngeneic mice [81]. In keeping with these discrepancies, it is interesting to note that overexpression of TGF- $\beta$ 1 in a tissue-specific manner does not necessarily induce angiogenesis *in vivo*. Thus, overexpression of TGF- $\beta$  in the vessel wall following direct arterial gene transfer [82], or in the mammary gland [83–85], the liver [86, 87], the central nervous system [88], the epidermis [89–91], respiratory epithelial cells [92] or insulin-producing  $\beta$ -cells of the endocrine pancreas [93, 94] in transgenic mice, resulted in dramatic alterations in tissue function and organization (including increased extracellular matrix deposition) without inducing a marked inflammatory cell infiltrate and angiogenic response. It has also been reported that double transgenic mice expressing TGF- $\beta$ 1 under the control of the mouse mammary tumour virus (MMTV) promoter display a marked reduction in mammary tumour formation induced by a MMTV-TGF- $\alpha$  transgene [85], an effect that may have been mediated by inhibition of mammary epithelial cell growth. However, in this setting, angiogenesis does not appear to have been stimulated as a consequence of TGF- $\beta$ 1 transgene expression.

One explanation for these apparently discrepant observations is that in many of the settings in which TGF- $\beta$ 1 is angiogenic, this is preceded either by the influx of inflammatory cells or by chemotaxis and activation of connective tissue or epithelial cells [73, 75, 77, 95, 96]; the angiogenesis that follows is then mediated by positive regulators such as VEGF (see below) produced by TGF- $\beta$ 1-recruited inflammatory or connective tissue cells [74, 97–100]. Indeed, it is possible that the inflammatory and tissue repair processes that are initiated in the process of applying exogenous TGF- $\beta$ 1 (subcutaneous injection, rabbit cornea and chick embryo CAM assays, the disc

angiogenesis system) or following the injection of TGF- $\beta$ 1-transfected tumour cells or myoblasts into recipient mice, are exacerbated by the presence of this cytokine. In contrast, in most transgenic animals in which TGF- $\beta$ 1 is overexpressed endogenously in a tissue-specific manner, there is little or no inflammation or tissue repair, and consequently no angiogenesis.

In agreement with the notion of indirect angiogenesis, TGF- $\beta$ 1 has been shown to increase VEGF production *in vitro* by cells from a variety of origins, including human histiocytic lymphoma [101], vascular smooth muscle [102], human lung adenocarcinoma [103] and human glioma [104], as well as choroidal [105] and mouse embryo-derived fibroblasts [103]. TGF- $\beta$ 1 also increases its own production as well as the production of bFGF, platelet-derived growth factor (PDGF), tumour necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1 (alpha and beta) and transforming growth factor-alpha in human peripheral blood monocytes [97, 99, 100, 106], and induces bFGF in mouse embryo fibroblasts [107].

## Summary

The phenotype of vessel wall fragility which occurs in a subset of TGF- $\beta$ 1 null mice clearly points to the importance of this cytokine in the establishment and maintenance of vessel wall integrity. A number of studies have demonstrated that exogenous application of TGF- $\beta$ 1 induces angiogenesis in the experimental setting. However, the lack of extensive angiogenesis in the face of other major tissue alterations in transgenic mice which overexpress TGF- $\beta$ 1, suggests that when angiogenesis does occur *in vivo*, this is dependent on local inflammation, which may either be initiated or exacerbated by TGF- $\beta$ .

## STUDIES *IN VITRO*

As indicated above, *in vivo* assays of angiogenesis are frequently accompanied by inflammation; under these conditions, the angiogenic response appears to be elicited indirectly, at least in part through recruited inflammatory cells. Although this may be relevant to some settings in which angiogenesis occurs *in vivo*, it does not allow one to study the consequences of direct interaction of angiogenesis regulators with endothelial cells, which, it should be recalled, are the ultimate target cells in this process. To circumvent these drawbacks, *in vitro* assays, using populations of cultured endothelial cells, have been developed for several of the cellular components of the angiogenic process, and, based on the geometry of the assay, these can be classified as either two-dimensional or three-dimensional. Conventional two-dimensional assays include measurement of cell proliferation and migration, as well as the study of gene expression in cytokine-stimulated cells. Three-dimensional assays have, as their end point, the formation of capillary-like cords or tubes by endothelial cells cultured either on the surface of (planar models) or within simplified extracellular matrices [108–

118]. The relevance of planar models to the processes of angiogenesis, in which endothelial cell invasion and capillary morphogenesis are the hallmarks, has been questioned [119].

## Two-dimensional assays

Two-dimensional *in vitro* assays have revealed that TGF- $\beta$  has a pronounced inhibitory effect on endothelial cell proliferation and migration. With respect to cellular proliferation and DNA synthesis, most studies have demonstrated that TGF- $\beta$ 1 is inhibitory over a wide range of concentrations (10 pg/ml–10 ng/ml) when tested on endothelial cells from a large number of sources including bovine aorta and pulmonary artery, adrenal cortex, retinal, brain and renal glomerular capillaries (BAEC, BPAEC, BCE/BMEC, BREC, BBEC, BGCEC), foetal bovine heart (FBHEC), porcine aorta (PAEC), rat epididymal fat pad, brain and heart (RFPEC, RBEC, RHEC), human umbilical and saphenous vein (HUVEC, HSVEC) as well as mouse endothelial cells transformed by the middle T oncogene of polyoma virus isolated from brain, heart, skin and thymus (bEND.3, B9V, HSV, sEND.1, tEnd.1) [60, 81, 114, 120–153]. The inhibitory effect of TGF- $\beta$ 1 could be overcome by increasing the concentration of exogenously added bFGF to BAECs, BRECs and BBECs [120, 121, 127, 130], by the addition of aFGF to BAECs and EGF to RHECs [124], and by adding HGF to BCE cells [152]. A possible mechanism includes the downregulation of TGF- $\beta$  receptor type II (with an increase in affinity) by bFGF [130]. These findings highlight the importance of cytokine interactions in regulating endothelial cell growth. Although the mechanisms of inhibition are not known, it has been reported that TGF- $\beta$ 1 decreases the number of high affinity EGF receptors on RHECs without altering their affinity, and that TGF- $\beta$ 1 inhibits the induction by EGF in these cells of the early response gene *c-myc* but not *c-fos* [124]. It has also been reported that TGF- $\beta$ 1 decreases the binding of aFGF to the surface of FBHECs by 45% [154], and that replenishment of cellular glutathione with thiol-amino acids counteracts the growth inhibitory effect of TGF- $\beta$ 1 on BPAECs [139]. Since the addition of exogenous fibronectin mimicks TGF- $\beta$ 1's inhibitory effect, it has also been suggested that the inhibition of proliferation by TGF- $\beta$ 1 is due, in part, to the concomitant induction of fibronectin synthesis [114]. Finally, the structural requirements for the inhibitory effect of TGF- $\beta$ 1 on endothelial cell proliferation have been localized to a region between amino acids 40–82 [144].

In contrast to the above-mentioned studies, it has been reported that TGF- $\beta$ 1 has no effect on the proliferation of a mouse endothelial cell line transformed by the entire polyomavirus genome (Py-4-1 [148]), that TGF- $\beta$ 1 has a biphasic effect on the growth of foetal bovine heart endothelial cells (FBHECs) (stimulation at 50 pg/ml and inhibition at concentrations greater than 100 pg/ml), and that TGF- $\beta$ 1 slightly increases the proliferation of

HUVECs [154]. In addition, it has been reported that anchorage-independent but not anchorage-dependent growth of RHEC can be reversibly inhibited by TGF- $\beta$ 1 [134]. Finally, using a planar model of angiogenesis in post-confluent BAEC cultures, it has been demonstrated that TGF- $\beta$ 1 (0.5 pg/ml–5.0 ng/ml) selectively stimulates proliferation of cells that are undergoing cord formation [147]. These authors suggest that the specific ‘angiogenic’ phenotype of the cord-forming cells (including the composition of their extracellular matrix) distinguishes the way in which they respond to TGF- $\beta$ 1. It should be noted, however, that, with the exception of studies just cited, the vast majority of reports indicate that TGF- $\beta$ 1 inhibits proliferation of endothelial cells from a large number of different tissues in a wide variety of species. Exceptions to this general rule may none the less, provide useful models for studying the mechanisms of TGF- $\beta$ 1-mediated endothelial cell growth control.

With respect to endothelial cell migration, numerous studies have demonstrated that TGF- $\beta$ 1 is inhibitory from 10 pg/ml to 10 ng/ml. Assays used include mechanical wounding of a confluent monolayer [122, 123, 136, 140, 142, 146, 155–157], the Boyden chamber assay [62, 123], and a Teflon or stainless steel ‘fence’ assay [125, 132, 133, 144, 150, 158]. As for proliferation, structural requirements for the inhibitory effect of TGF- $\beta$ 1 on endothelial cell migration have been localized to a region between amino acids 40 and 82 [144]. As a general rule, an excellent correlation exists between endothelial cell migration in two-dimensional assays and the invasive behaviour of these cells in three-dimensional collagen gels [146, 159]. These findings suggest that, with regard to two-dimensional assays, migration may be closer to the behaviour of cells in three dimensions than is proliferation.

TGF- $\beta$  regulates the expression of a number of gene products in endothelial cells (summarized in Table 2). Thus TGF- $\beta$ 1 promotes the synthesis and deposition of matrix components such as fibronectin (including alternative splice variants), collagens I, IV and V, thrombospondin and SPARC [114, 123, 125, 128, 130, 131, 135, 147, 148, 151, 160, 161], although the magnitude of induction in most cases is considerably lower than that reported for other cell types [147]. With respect to integrins, TGF- $\beta$ 1 (6 ng/ml) upregulates  $\alpha_2$ ,  $\alpha_5$  and  $\beta_1$  integrin subunit expression in subconfluent cultures of neonatal (human) dermal microvascular endothelial cells (HDMECs) [162]. TGF- $\beta$ 1 (0.5 ng/ml) upregulates  $\alpha_5$ ,  $\beta_1$  and  $\beta_3$  expression in subconfluent/migrating BAECs [161]. TGF- $\beta$ 1 (0.5–10 ng/ml) also upregulates  $\alpha_5$  and  $\alpha_v$  expression in confluent monolayers of BMECs (MS Pepper and G Collo, unpublished data). In contrast, TGF- $\beta$ 1 (1 ng/ml) downregulates  $\beta_3$  expression in monolayers of HDMECs [163]. TGF- $\beta$ 1 (0.5–50 ng/ml) also downregulates  $\alpha_3$ ,  $\alpha_5$ ,  $\alpha_6$  and  $\beta_1$  expression in the same cells [164]. With respect to proteases and protease inhibitors, TGF- $\beta$ 1 either decreases [165, 166] or increases [151, 166] uPA expression depending on the cell type (decrease in BAECs and CPAECs; increase in BMECs and RFPECs).

**Table 2. Regulation of endothelial cell gene expression by TGF- $\beta$ 1 *in vitro*<sup>a</sup>**

Up-regulation	Matrix components: fibronectin, collagens I, IV and V, thrombospondin, SPARC Integrin subunits: $\alpha_2$ , $\alpha_5$ , $\alpha_v$ , $\beta_1$ , $\beta_3$ Proteases and inhibitors: uPA, PAI-1 Others: endothelin, ecNOS, PDGF-A and -B chains, $\alpha$ -SM actin
Down-regulation	Integrin subunits: $\alpha_3$ , $\alpha_5$ , $\alpha_6$ , $\beta_1$ , $\beta_3$ Proteases and inhibitors: uPA, TIMP-1 Others: E-selectin, thrombomodulin, Gro- $\alpha$ , MCP-1, von Willebrand factor, VEGFR-2

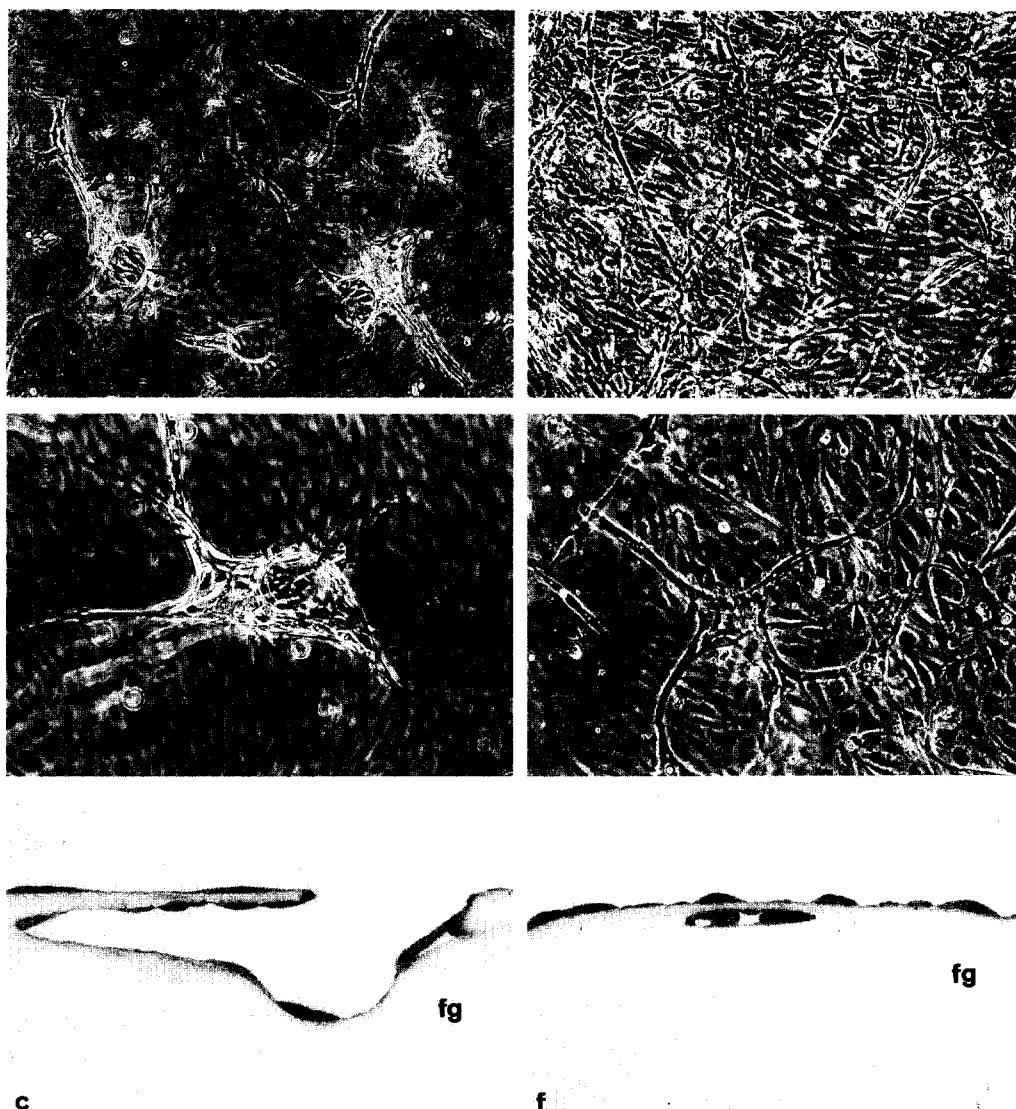
<sup>a</sup> See text for references.

As with many of the direct effects of TGF- $\beta$ 1 on endothelial cells, modulation of gene expression is often biphasic. For example, integrin subunits  $\alpha_5$ ,  $\beta_1$  and  $\beta_3$ , as well as uPA, are either upregulated or downregulated, depending on the endothelial cell type and the culture conditions used. The most striking and consistent effects *in vitro* are upregulation of fibronectin and PAI-1 synthesis. However, to date, none of these *in vitro* effects has been confirmed *in vivo*.

In contrast, TGF- $\beta$ 1 consistently increases PAI-1 expression in virtually all endothelial cell lines tested [130, 138, 140, 147, 150, 151, 157, 165–172], and downregulates TIMP-1 expression in BMECs (MS Pepper, unpublished data). Other gene products that are regulated by TGF- $\beta$ 1 include an increase in alpha smooth muscle actin ( $\alpha$ -SM actin) in RPFCs [173] and in BAECs [174], an increase in endothelin production by BAE cells [175], increased endothelial cell nitric oxide synthase (ecNOS) expression and NO production in BAECs [176] and induction of the A and B chains of PDGF in human kidney cortex microvascular endothelial cells [177–179]. In contrast, TGF- $\beta$ 1 downregulates E-selectin [180] and thrombomodulin [181] expression by HUVE cells, as well as the chemokines Gro- $\alpha$  (growth-related oncogene- $\alpha$  or KC) and MCP-1 (monocyte chemoattractant protein-1 or JE) in RHECs [124]. TGF- $\beta$ 1 also decreases von Willebrand factor expression in BAECs [174]. Finally, it has recently been demonstrated that TGF- $\beta$ 1 downregulates VEGFR-2 in BAE and BME cells [182], thereby implicating TGF- $\beta$ 1 as a major regulator of VEGF-induced angiogenesis.

Other effects of TGF- $\beta$ 1 *in vitro* include induction of a ‘prooxidant’ effect in BPAECs [183], and a sevenfold decrease in the surface density of endothelial fenestrae in BMECs [184]. TGF- $\beta$ 1 also induces cytotoxicity in endothelial cells whether cultured in two- or three-dimensions (Figure 4: compare panels (a) and (d)) [138, 185]. This reflects the capacity of TGF- $\beta$ 1 to induce apoptosis in endothelial cells, and to concomitantly decrease expression of bcl-2 [149, 186, 187].

The effects of TGF- $\beta$ 1 on endothelial cell functions are highly dependent on culture conditions and on the time of exposure to the cytokine. Thus, endothelial cell DNA synthesis and proliferation are inhibited by TGF- $\beta$ 1 in



**Figure 4.** TGF- $\beta$ 1 modulates lumen size during *in vitro* angiogenesis. bFGF (30 ng/ml) was added without (a, b, c) or with 500 pg/ml TGF- $\beta$ 1 (d, e, f) to confluent monolayers of microvascular endothelial cells grown on fibrin gels in the presence of Trasylol. The resulting capillary-like tubular structures were viewed by phase-contrast microscopy (a, b, d and e) and semi-thin sections (c and f). bFGF induced endothelial cells to invade from a circular opening in the surface monolayer (arrow-heads in a and b), to form well-organized cell cords with a clearly visible refringent lumen (arrows in a and b), which tapered down progressively in the distal part of the cords. Semi-thin sectioning revealed that the proximal part of the cords was often cavernous (c). When 500 pg/ml TGF- $\beta$ 1 was co-added with bFGF, the total additive length of the invading cell cords was increased (compare a and d, and see Figure 4 for quantitation), and clearly distinguishable lumina were present beneath the surface monolayer (white refringent line indicated by the arrows in e). Semi-thin sectioning revealed that the lumen was reduced to a more physiological size when compared to cultures treated with bFGF alone (compare c and f). Note also the greater number of detached cells in (d) (bFGF alone) as opposed to (a) (bFGF + TGF- $\beta$ 1). Magnification in (a, d) = 50 $\times$ , in (b, e) = 100 $\times$ , and in (c, f) = 200 $\times$ . (From Ref. 185; with kind permission from Academic Press, Orlando, FL.)

sparse or sub-confluent cultures (for references, see above), but are stimulated by TGF- $\beta$ 1 in post-confluent cord forming BAECs in a planar model of angiogenesis [147]. When cultured in three dimensions, endothelial cells cease to proliferate; under these conditions, TGF- $\beta$ 1 induces a small reduction in cell number and DNA content [114, 138, 151]. Endothelial cells cultured in three dimensions show a marked reduction in expression of TGF- $\beta$  receptors II and III [151]. TGF- $\beta$ 1 significantly increases protein synthesis in two-dimensional cultures, but has little effect on total protein synthesis when cells

are cultured in three dimensions [114, 138]. However, specific proteins including fibronectin, uPA and PAI-1, which are induced in three dimensions are increased, to a greater degree, under these conditions than when cells are grown in two dimensions [151]. In contrast, the induction of  $\alpha$ -SM actin by TGF- $\beta$ 1 in RFPECs was seen in two-, but not in three-dimensional cultures [173]. Finally, endothelial cell density in two-dimensional cultures and the time of exposure to TGF- $\beta$  have been shown to be important parameters in determining net proteolytic activity: BAECs plated at high density display a net

decrease in PA activity irrespective of the time of exposure to TGF- $\beta$ 1; in contrast, sparsely plated cells exposed to TGF- $\beta$ 1 for a short period of time (4 h) display a net increase in PA activity, whereas sparse cultures exposed to TGF- $\beta$ 1 for a longer time period (24 h) display a net decrease in PA activity [171].

### Three-dimensional assays

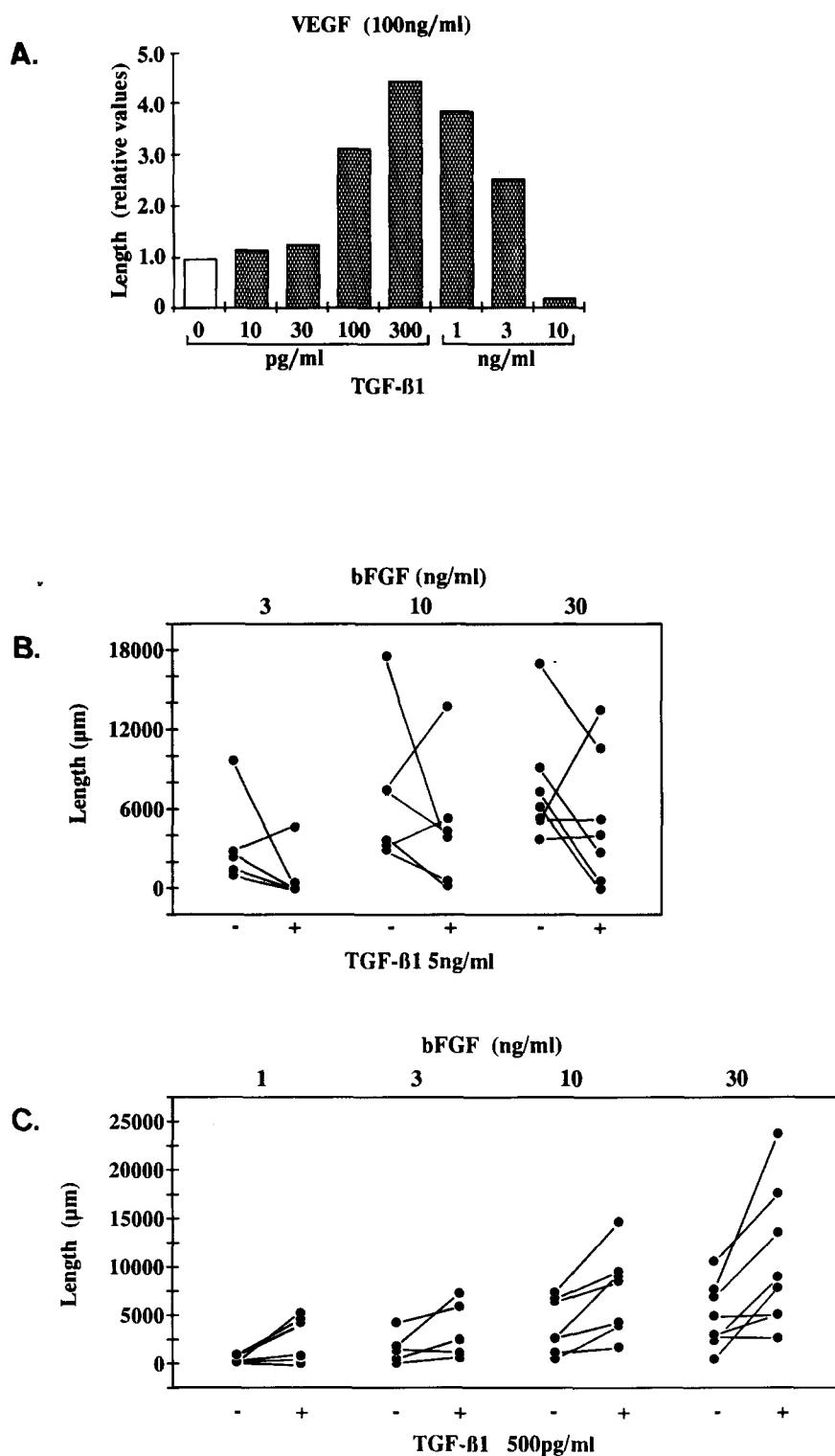
Results from three-dimensional *in vitro* assays demonstrate that the response to TGF- $\beta$ 1 varies depending upon the assay used. Thus, TGF- $\beta$ 1 inhibits endothelial cell invasion of three-dimensional collagen or fibrin gels, as well as the explanted amnion [115, 123, 166, 172, 188, 189]. When seeded on the surface of Matrigel, endothelial cells organize into a network of cell cords, which subsequently regress; in this planar model of angiogenesis, TGF- $\beta$ 1 was shown to accelerate cord regression [190]. These results demonstrate the TGF- $\beta$ 1 is a direct-acting inhibitor of extracellular matrix invasion and tube formation, suggesting that it is a negative regulator of the phase of activation. However, it has also been reported that when endothelial cells are cultured within three-dimensional collagen gels, TGF- $\beta$ 1 promotes rapid gel contraction and organization of the cells into tube-like structures [114, 131]. This model closely mimicks the phase of resolution. Taken together, findings in three-dimensional systems suggest that when interacting directly with endothelial cells, TGF- $\beta$ 1 might have different functions on vessel formation at different stages of the angiogenic process. Thus it may prevent endothelial cell activation, in part, by down-regulating tyrosine kinase receptor expression in endothelial cells (e.g. VEGFR-2) [182] and thereby inhibit proliferation and invasion and thus sprout formation. On the other hand, once a new vessel has formed TGF- $\beta$ 1 may promote the phase of resolution by inducing endothelial cell quiescence (by inhibiting proliferation and migration) and vessel maturation (through the deposition and organization of a functional basement membrane and the promotion of intercellular junctional complex maturation) (Table 2).

### Biphasic effect of TGF- $\beta$ 1

An additional possibility is that TGF- $\beta$ -mediated angiogenesis is contextual, i.e. that this requires the presence of other positive regulators, whose activity is potentiated in the presence of TGF- $\beta$ . Furthermore, since TGF- $\beta$  has been described as a bifunctional regulator in a variety of biological processes, it is likely that the direct effect of this cytokine on endothelial cell function is concentration-dependent [14]. To address these issues, an *in vitro* model of angiogenesis, which assays both for endothelial cell invasion and capillary morphogenesis in three-dimensional collagen or fibrin gels [112, 191], was used to assess the effect of a wide range of concentrations of TGF- $\beta$ 1 on the response of BMECs to VEGF or bFGF. Unlike VEGF and bFGF, which, *in vitro*, induce a predictable

angiogenic response [26, 27], when tested on its own over a wide range of concentrations, TGF- $\beta$ 1 had no effect. However, VEGF- or bFGF-induced invasion of collagen or fibrin gels was significantly increased when TGF- $\beta$ 1 was co-added to the system at 200–500 pg/ml, and decreased when TGF- $\beta$ 1 was added at 5–10 ng/ml (Figures 4 and 5) [185]. Similar findings have been reported with BAECs grown on the surface of three-dimensional collagen gels [146], and a biphasic effect has been noted when RFPECs are grown in suspension in collagen gels: TGF- $\beta$ s-1 and -3 at 500 pg/ml promoted angiogenesis in a manner similar to that seen with bFGF, whereas TGF- $\beta$ 1 at 5 ng/ml was slightly inhibitory [132, 133]. This biphasic effect is in accord with the observation that endothelial cell wound-induced migration [122, 123] and invasion of three-dimensional collagen gels [123] or the explanted amnion [115] are inhibited at relatively high concentrations (1–10 ng/ml) of TGF- $\beta$ 1, whereas 500 pg/ml TGF- $\beta$ 1 potentiated two-dimensional wound-induced migration [146; MS Pepper, unpublished observation]. With respect to endothelial cell proliferation, although one study has demonstrated a similar biphasic effect of TGF- $\beta$ 1 in subconfluent cultures [154], most studies have demonstrated that this cytokine is inhibitory over a wide range of concentrations (see above). Finally, not only was invasion affected by TGF- $\beta$ 1 in a concentration-dependent manner, but lumen size in the resulting structures was progressively reduced with increasing concentrations of cytokine. Thus, in the absence of TGF- $\beta$ 1, bFGF-induced cell cords within fibrin gels contain widely patent lumina. Co-addition of TGF- $\beta$ 1 at 500 pg/ml markedly reduced lumen diameter, perhaps to a more physiological size (Figure 4) [185]. Addition of TGF- $\beta$ 1 at a 10-fold higher concentration, namely 5 ng/ml, completely inhibited lumen formation in the invading cell cords [166]. The presence of ectatic or cavernous lumina in the absence of TGF- $\beta$ 1, which are reduced in size in the presence of this cytokine *in vitro*, is strikingly reminiscent of the vascular phenotype seen in TGF- $\beta$ 1-deficient mice (see above).

The mechanisms responsible for the *in vitro* biphasic effect are not known. One hypothesis is based on alterations in the net balance of extracellular proteolysis [192, 193]. Thus at the dose of TGF- $\beta$ 1 that potentiates bFGF- or VEGF-induced invasion, an optimal balance between proteases and protease inhibitors may be achieved, which allows for focal pericellular extracellular matrix degradation, while at the same time protecting the matrix against inappropriate degradation. This hypothesis may also apply to the regulation of lumen formation, in as much as the linear dose-dependent increase in PAI-1 can be correlated with a progressive reduction in lumen size. A second explanation might be related to alterations in integrin expression and ligand binding affinity [194, 195]. Since TGF- $\beta$  alters endothelial cell integrin expression (see above), it is likely that maximal invasion occurs in the presence of an optimal degree of cellular adhesion, and that submaximal invasion occurs when adhesion is either greater or less than that achieved with the



**Figure 5.** Biphasic effect of TGF- $\beta$ 1 on VEGF- or bFGF-induced *in vitro* angiogenesis. (A) Confluent monolayers of bovine microvascular endothelial cells were treated for 4 days with VEGF (100 ng/ml) and/or TGF- $\beta$ 1, and the total additive length of all invading cell cords was measured. (B and C) Co-treatment of confluent monolayers with bFGF and TGF- $\beta$ 1 at the indicated concentrations for 7 days. bFGF-treated cultures with (+) or without (-) co-added TGF- $\beta$ 1 from the same experiment are joined by a single line. bFGF- or VEGF-induced invasion was potentiated by TGF- $\beta$ 1 at 200–500 pg/ml and inhibited by TGF- $\beta$ 1 at 5–10 ng/ml. (From Ref. 185; with kind permission from Academic Press, Orlando, FL.)

potentiating dose of TGF- $\beta$ 1. The notion that 500 pg/ml TGF- $\beta$ 1 stimulates adhesion to an extent that is optimal for migration, is consistent with the observation that 500 pg/ml TGF- $\beta$ 1 potentiates, whereas 5 ng/ml inhibits bFGF-induced wound-induced two-dimensional

migration [146, MS Pepper, unpublished data]. Differential regulation of integrin expression may also contribute to the alterations in lumen size that occur with different concentrations of TGF- $\beta$ 1 in fibrin gels. Finally, the complete absence of lumen formation at 5 ng/ml

implies that at this concentration TGF- $\beta$ 1 inhibits the cellular machinery responsible for maintaining endothelial cell polarity, which, in the case of angiogenesis, is likely to be a major factor in driving histotypic morphogenesis, namely the formation of tube-like structures.

### Maintenance of endothelial quiescence and tissue avascularity

*In vitro* co-culture experiments have demonstrated that non-endothelial vascular wall cells induce endothelial cell quiescence through the activation of latent TGF- $\beta$ 1 [196, 197]. In these studies, only pericytes and smooth muscle cells, but not fibroblasts or epithelial cells, inhibited endothelial cell growth and migration; this effect was dependent on close physical proximity between the co-cultured cells. Inhibition was mediated by the rapid and transient activation of latent TGF- $\beta$  which was produced by both co-cultured cell types; activation of TGF- $\beta$  was dependent on the protease plasmin, which in turn was generated by activation of plasminogen by uPA [126, 156, 157, 198]. Furthermore, in heterotypic co-cultures as well as homotypic endothelial cell cultures, the synthesis of PAI-1 in response to TGF- $\beta$  suppressed further activation of latent TGF- $\beta$  by inhibiting uPA and thereby plasminogen activation. These findings suggest that the process of TGF- $\beta$  activation is self-limiting [140, 157].

The above-mentioned *in vitro* observations have led to the proposal that pericytes and smooth muscle cells, possibly through the activation of latent TGF- $\beta$ , are involved in the induction and maintenance of endothelial quiescence *in vivo*. The observations that, during angiogenesis, pericytes are absent from newly forming capillaries, and that 'pericyte dropout' precedes endothelial cell proliferation in diabetic proliferative retinopathy, have been used in support of this idea [196]. However, in an extensive and critical review of the existing literature, others have seriously questioned these concepts [199]. It has been suggested that "disappearance of intramural pericytes and proliferation of endothelial cells may be explained without postulating that pericytes inhibit endothelial proliferation", and that "although frequently claimed, there is hardly any evidence for a selective death of capillary pericytes during the development of diabetic retinopathy". The precise role of pericytes and smooth muscle cells in the maintenance of endothelial cell quiescence *in vivo*, and the possibility that this may be mediated by TGF- $\beta$ , are therefore issues that remain to be resolved.

TGF- $\beta$  has also been identified as a potential negative regulator of endothelial cell growth in certain avascular tissues. Thus, in three-dimensional assays in which endothelial cells were co-cultured with chondrocytes, endothelial cell invasion and tube formation were shown to be inhibited, and this effect was mediated by a chondrocyte-derived TGF- $\beta$  [172, 188]. In addition, the endothelial cell growth inhibitory activity of aqueous and vitreous humours as well as the inner part of the aortic media was recognized by antibodies to TGF- $\beta$ 1 [137].

### Summary

It is likely that different *in vitro* models recapitulate different phases of the angiogenic process. Results from these systems imply that when interacting directly with endothelial cells, TGF- $\beta$  has different functions on vessel formation at different stages of the process. Thus, it regulates the phase of activation by potentiating or inhibiting the activity of positive regulators like bFGF and VEGF in a concentration-dependent manner. On the other hand, once a new vessel has formed, TGF- $\beta$ 1 promotes the phase of resolution by maintaining endothelial cell quiescence and inducing vessel maturation. However, very few of the effects of TGF- $\beta$ 1 on endothelial cells *in vitro* have been confirmed *in vivo*. These include inhibition of proliferation and migration, the maintenance of endothelial quiescence, alterations in gene expression affecting matrix synthesis and extracellular proteolysis, and the induction of apoptosis (summarized in Table 3). None the less, genetic studies in man and mouse have revealed increase vessel wall fragility in the absence of TGF- $\beta$ 1 and TGF- $\beta$ Rs (see below). Future studies *in vivo* are therefore likely to confirm that TGF- $\beta$  does indeed affect endothelial cell function by promoting basement membrane deposition and capillary sprout maturation, as well as by promoting interactions with other vascular wall cells including recruitment and differentiation of smooth muscle cells.

### TGF- $\beta$ RECEPTORS

#### Genetic studies

The phenotype of TGF- $\beta$ R II-deficient mice has recently been reported [200]. These mice are highly reminiscent of TGF- $\beta$ 1 null mice described above. Thus, homozygous deficiency was lethal at about 10.5 days gestation, and this resulted from defects in yolk sac haematopoiesis and vasculogenesis. As with TGF- $\beta$ 1-deficient mice, TGF- $\beta$ R II null mice were capable of forming blood vessels, but these were dilated and incompletely attached to the adjacent mesothelial and endodermal cell layers. Generalized embryonic growth retardation also occurred, although this was thought to result from the extraembryonic defects. It is striking that the expression patterns of TGF- $\beta$ 1 and TGF- $\beta$ R II are highly coincidental during embryogenesis [201].

The phenotype of vessel wall fragility in homozygous TGF- $\beta$ 1- and TGF- $\beta$ R II-deficient embryos is strikingly reminiscent of the vascular lesions that occur in patients with hereditary haemorrhagic telangiectasia (HHT). HHT is an autosomal dominant single gene disorder characterized by multisystemic vascular dysplasia and recurrent haemorrhage. The earliest detectable change in the telangiectatic lesions is dilatation of post-capillary venules in the upper dermis; the endothelial cells themselves, including intercellular junctions, appear to be normal [202]. The genes for HHT have recently been identified, and their identification has led to the definition of two

**Table 3. Regulation of endothelial cell function by TGF- $\beta$ 1<sup>a</sup>**

<b>Effects <i>in vivo</i></b>
Direct effect on endothelial cells:
• nothing definitive demonstrated to date
• may promote stabilization of the vessel wall
Indirect effect:
• nothing definitive demonstrated to date
• may stimulate angiogenesis through recruitment and activation of inflammatory, epithelial and connective tissue cells
<b>Effects <i>in vitro</i> (direct effects): biphasic</b>
(a) Lower concentrations stimulate migration and invasion and possibly proliferation through the potentiation of VEGF and bFGF activity
(b) Promotion of quiescence and vessel maturation:
• inhibition of proliferation
• inhibition of migration and invasion
• reduction in extracellular proteolysis
• increase in matrix synthesis and basement membrane deposition
• promotion of junctional maturation
• downregulation of VEGFR-2 expression
• induction of apoptosis

<sup>a</sup> See text for references.

*In vivo*, TGF- $\beta$ 1 may promote stabilization of the vessel wall through its direct interaction with endothelial cells as well as by promoting differentiation and recruitment of other vessel wall cells. *In vivo*, TGF- $\beta$ 1 appears to stimulate angiogenesis indirectly by inducing chemotaxis of inflammatory, epithelial and connective tissue cells, which are subsequently activated to produce direct-acting positive regulators such as VEGF. The consequences of the direct interaction of TGF- $\beta$ 1 with endothelial cells *in vivo* are unknown. *In vitro*, most reports indicate that TGF- $\beta$ 1 inhibits proliferation (although a stimulatory effect has been observed under certain circumstances), and the effects on migration and invasion are biphasic. *In vitro*, TGF- $\beta$ 1 reduces extracellular proteolytic activity and promotes matrix synthesis, the net result of which is matrix accumulation. TGF- $\beta$ 1 also induces vessel maturation *in vitro* by promoting the deposition and organization of a basement membrane and the maturation of intercellular junctional complexes. Finally, TGF- $\beta$ 1 decreases the expression of VEGFR-2 and induces endothelial cell apoptosis *in vitro*. (Modified from Ref. 25; with kind permission from Springer, Berlin).

HHT subtypes. The gene for HHT type I is endoglin [203], which displays regions of structural homology to betaglycan, the type III TGF- $\beta$ R [204, 205]. The gene for HHT type 2 is ALK-1 [206], a TGF- $\beta$ -binding type-I receptor that is expressed at high levels in endothelial cells *in vitro* [207].

### Studies *in vivo*

The expression of specific TGF- $\beta$ R subtypes has been studied in settings in which angiogenesis is prominent. These include embryonic development [201, 208–212] and the ovary during the oestrous cycle [213]. However, with the exception of the endocardium [211] and possibly small blood vessels in the developing lung [212], expression of TGF- $\beta$ R subtypes I, II and III has generally not been observed in either quiescent or activated endothelial cells. These descriptive findings are in accord with functional

studies in mice in which steady-state levels of PAI-1 mRNA were found to be increased in all tissues within 3 h of treatment with TGF- $\beta$ 1 [214]. Morphological analysis revealed that under non-stimulated conditions, smooth muscle, but not endothelial cells, expressed PAI-1 mRNA. Following exposure to TGF- $\beta$ 1, PAI-1 mRNA was increased in smooth muscle cells, but not in the endothelium of the same vessels [215]. The finding that PAI-1 is synthesized by virtually all endothelial cell lines *in vitro* and that this can be increased by TGF- $\beta$ 1 (see above), suggests that the TGF- $\beta$  signalling receptors are themselves upregulated *in vitro* as a consequence of culture (see below). These findings also suggest that *in vivo*, the lack of induction of PAI-1 in endothelium is a reflection of the absence of TGF- $\beta$  signalling receptors in these cells, and that the binding sites on endothelial cells of the microvasculature *in vivo* that are accessible to circulating [<sup>125</sup>I]-labelled TGF- $\beta$ 1 [216], are unrelated to TGF- $\beta$ Rs I and II. Despite these observations, it is none the less possible that with studies designed to specifically address the issue of TGF- $\beta$ Rs in endothelial cells, previously undetectable but biologically relevant low levels of expression will be revealed.

In contrast to TGF- $\beta$ R subtypes I, II and III, endothelial cells do express endoglin (CD 105) *in vivo*. Endoglin is a homodimeric transmembrane glycoprotein, with subunits of 95 kDa, that shares regions of sequence identity with TGF- $\beta$ R III (betaglycan), particularly in the transmembrane and intracellular domains [204, 205, 217, 218]. Although constitutively expressed by endothelium in a wide variety of tissues *in vivo*, endoglin immunoreactivity appears to be increased in endothelial cells in a variety of tissues in which angiogenesis is prominent [reviewed in 219].

### Studies *in vitro*

In contrast to the above-mentioned studies *in vivo*, type-I and type-II TGF- $\beta$ Rs have been well characterized in microvascular and large vessel-derived endothelial cells *in vitro* [123, 128, 129, 132, 141, 154, 207, 220]. Thus, expression of a truncated form of TGF- $\beta$ R I in endothelial cells inhibited TGF- $\beta$ 1's stimulatory effect on fibronectin synthesis in a dominant-negative manner [151]. It has also been shown that TGF- $\beta$ R II is down-regulated by bFGF, which concomitantly decreases the growth inhibitory response to TGF- $\beta$ 1 [129], and that the inhibitory effect of TGF- $\beta$ 1 on proliferation is suppressed in a dominant-negative manner in endothelial cells expressing a truncated form of TGF- $\beta$ R II [149, 151]. Similarly, loss of the growth inhibitory effect of TGF- $\beta$ 1 has been correlated with a glycosylation defect in TGF- $\beta$ R II in mutagenized endothelial cells [130], and expression of a truncated form of the type II receptor prevents TGF- $\beta$ 1-induced angiogenesis in a planar *in vitro* model [149]. It has also been reported that TGF- $\beta$ R II is down-regulated when endothelial cells are grown in suspension in a three-dimensional *in vitro* model of angiogenesis that mimicks

the phase of resolution [151]. Finally, TGF- $\beta$ 1 has been shown to induce apoptosis in endothelial cells in a dose-dependent manner, with a concomitant decrease in expression of bcl-2; apoptosis was inhibited in cells expressing a truncated TGF- $\beta$ R II, which acted in a dominant-negative fashion [149, 187]. Taken together, these findings clearly point to the importance of TGF- $\beta$ Rs I and II signalling in mediating the effects of TGF- $\beta$ 1 in endothelial cells.

Although a number of reports have suggested that endothelial cells do not express TGF- $\beta$ R III, it has now been established that this receptor is expressed at high levels *in vitro* by microvascular but not by large vessel-derived endothelial cells [132, 220]. Since TGF- $\beta$ 2 has a lower affinity for TGF- $\beta$ R II than TGF- $\beta$ s-1 and -3, but binds with high affinity to TGF- $\beta$ R III, this may explain, in part, why large vessel-derived endothelial cells respond equally well to TGF- $\beta$ s-1 and -3 (which have similar activity profiles) and poorly, if at all, to TGF- $\beta$ 2. This may also explain why isoform discrimination is less marked in endothelial cells from the microvasculature [132, 133, 220–222]. In support of this hypothesis, it has recently been demonstrated that large vessel endothelial cells become responsive to TGF- $\beta$ 2 (inhibition of migration and proliferation as well as induction of PAI-1) when stably transfected with TGF- $\beta$ R III [150]. Furthermore, when microvascular endothelial cells are transfected with a TGF- $\beta$ R III construct in the antisense orientation, they display a selective reduction in the inhibitory effect of TGF- $\beta$ 2 on proliferation, with no change in the inhibitory effect of TGF- $\beta$ 1 [151]. It has been suggested that isoform discrimination may also be due to the differential sequestration of the TGF- $\beta$  isoforms by  $\alpha_2$ -macroglobulin [223]. Like TGF- $\beta$ R II, TGF- $\beta$ R III is down-regulated in a three-dimensional *in vitro* model of angiogenesis that mimicks the phase of resolution [151]. Cultured endothelial cells also express endoglin, which binds TGF- $\beta$ s 1 and 3 with high affinity, but fails to bind TGF- $\beta$ 2 [205]. The observation that only a small percentage of endoglin molecules on cultured endothelial cells are capable of binding TGF- $\beta$  avidly [205], suggests that a rate-limiting event (possibly acquisition of an active conformation) is required for TGF- $\beta$  binding.

The observation that the type-II and III receptor subtypes are downregulated in three-dimensional cultures *in vitro* suggests that TGF- $\beta$ Rs may be downregulated during the process of vessel maturation that occurs in the phase of resolution. These findings may also explain the inability thus far to detect specific receptor subtypes in endothelial cells *in vivo*, and the apparent lack of induction of PAI-1 in these cells following intravenous injection of TGF- $\beta$  (see above). Conversely, expression of TGF- $\beta$ Rs in two-dimensional cultures *in vitro* is consistent with the notion that, when placed in culture, endothelial cells are phenotypically closer to activated/angiogenic endothelium than to the resting endothelium from which they were derived. It has been suggested that TGF- $\beta$ R I mediates the matrix synthesis response

of endothelial cells to TGF- $\beta$ 1, and that TGF- $\beta$ R II mediates TGF- $\beta$ 1's antiproliferative effect [151], which is consistent with the hypothesis that receptor I and II may signal via distinct pathways [224]. However, it has been pointed out that the threshold of TGF- $\beta$ R I-mediated signalling may be significantly different for different downstream pathways; a much stronger signal may be required for initiating the signal cascade required for inhibiting growth than for altering gene expression [37]. Whether the differential responses to TGF- $\beta$ 1 are mediated by different receptors, or whether TGF- $\beta$ 1 signalling is threshold-dependent, remains to be clarified.

## Summary

Genetic studies have clearly revealed a role for TGF- $\beta$ Rs I and II as well as endoglin in the establishment and maintenance of vascular integrity in man and mouse, and *in vitro* findings point to the importance of TGF- $\beta$ Rs I, II and III in TGF- $\beta$ -mediated signalling in endothelial cells. However, the consistent inability to detect TGF- $\beta$ Rs in endothelial cells *in vivo* may point to other vascular wall cells as the mediators of the phenotypes of vessel wall fragility. Finally, the inability to detect specific receptor subtypes in endothelial cells *in vivo*, and the observation that the type-II and III receptors are downregulated in three-dimensional cultures *in vitro*, suggest that expression of TGF- $\beta$  receptors in two-dimensional cultures *in vitro* is a consequence of endothelial cell activation.

## CONCLUSIONS

From genetic studies, it appears that TGF- $\beta$  and its receptors are important positive regulators of endothelial cell differentiation, vascular network formation and the establishment and maintenance of vessel wall integrity. Vessels that form in the absence of the ligand or its receptors are ectatic both *in vivo* and *in vitro* (Figure 4). This is likely to be due to incomplete vessel wall maturation, which, following an increase in haemodynamic pressure during development, results in vessel wall rupture and haemorrhage. It is noteworthy that during development in the mouse, this occurs in extraembryonic but not in intraembryonic tissues, and that in man, the phenotype of vessel wall fragility generally becomes apparent during the second or third decades, although lesions can appear at any time from infancy to old age.

The pathogenesis of the vascular lesions seen in TGF- $\beta$ 1- and TGF- $\beta$ R II-deficient mice as well as in individuals with HHT is not known. None the less, since TGF- $\beta$  induces the synthesis and assembly of the endothelial cell extracellular matrix (see above), one of the consequences of defective TGF- $\beta$ R signalling may be the formation of structurally incompetent basement membranes. It is striking that with respect to the vasculature, the phenotype of mice lacking either fibronectin or the  $\alpha_5$  integrin subunit [226, 227] closely mimicks the phenotype of TGF-

$\beta$  and TGF- $\beta$ R-deficient mice, particularly since TGF- $\beta$ 1 has been shown to increase expression of fibronectin and its specific integrin  $\alpha_5\beta_1$  (see above). It is also noteworthy that vascular lesions in HHT are well localized, and that vascular integrity is maintained outside of the lesions; this suggests that some local event, possibly trauma, initiates vascular repair, which, in the case of HHT patients, is defective. An additional and intriguing possibility comes from the observation that a similar phenotype of vessel dilatation and increased vessel wall fragility occurs in mice deficient in PDGF-B or in PDGF receptor-beta (PDGFR- $\beta$ ) [228] as well as in mouse embryos exposed to anti-PDGF-A neutralizing antibodies *in utero* [229]. Recall that TGF- $\beta$ 1 induces PDGF-A and -B chain synthesis in endothelial cells [177–179]. In addition, TGF- $\beta$ 1 increases PDGFR- $\beta$  expression in fibroblasts and smooth muscle cells [230, 231]. Since it is possible that endothelial-cell derived PDGF may mediate the differentiation of vascular wall cells (pericytes and smooth muscle cells) from the surrounding mesenchyme and promote their recruitment to newly formed capillaries, the absence of TGF- $\beta$  signalling in endothelial cells may result in defective assembly of the cellular components of the vessel wall, which, under normal circumstances, would be expected to contribute to vascular stability. It should also be recalled that TGF- $\beta$ s themselves affect many smooth muscle cell functions, including migration and proliferation, and that some of these effects may be in turn mediated through the autocrine regulation of PDGFs and their receptors [40, 41, 232, 233]. Finally, ectatic vessels, which form in the absence of TGF- $\beta$  *in vitro* do so in the absence of circulating blood. This suggests that, although intravascular haodynamic pressure is likely to be responsible for vessel rupture and haemorrhage, the formation of dilated vessels can occur in its absence.

With respect to angiogenesis, it is still unclear as to whether TGF- $\beta$  is involved in the endogenous regulation of this process. None the less, an indirect inflammatory cell-mediated mode of activity may be applicable to angiogenesis associated with acute and chronic inflammation, wound healing and tumour growth. With respect to its direct effect on endothelial cells, *in vitro* studies suggest that the response depends on whether TGF- $\beta$ 1 is present during the activation or resolution phases of angiogenesis, as well as on the local concentration of active cytokine.

Pharmacological stimulation and inhibition of angiogenesis are currently envisaged as an important adjunct to conventional therapeutic strategies: stimulation of angiogenesis for the treatment of ischaemia and wound healing, and inhibition of angiogenesis for the treatment of juvenile haemangiomas and rheumatoid arthritis, as well as for inhibiting tumour growth and the spread of metastases [2, 6]. Where does TGF- $\beta$  fit in into this paradigm? With respect to ischaemia, although stimulation of angiogenesis has proven to be of enormous therapeutic benefit in a variety of animal models [6], the potential use of TGF- $\beta$ s in this setting has not been reported. With

respect to wound healing, TGF- $\beta$  has been shown to accelerate this process by directly stimulating fibroblasts to synthesize and deposit a collagen-rich matrix (with a minimal increase in glycosaminoglycans) with a consequent increase in tensile strength; although this process is thought to bypass the acute inflammatory phase of tissue repair, TGF- $\beta$ 1-accelerated wound healing is invariably associated with an inflammatory cell infiltrate [95, 234–239]. TGF- $\beta$ 1 has been reported to specifically stimulate angiogenesis during wound healing, with a resulting increase in vessel number, size and branching; this response was however less marked than in response to bFGF, TNF- $\alpha$  or PDGF-BB, but was greater than the response elicited by EGF or IGF-1 [76, 240, 241]. Angiogenesis which occurs during wound healing has been interpreted as being ‘supportive neovascularization’ in contrast to bFGF-induced angiogenesis, which occurs independently of matrix accumulation and granulation tissue formation [76].

With regard to inhibition of angiogenesis, it has been shown that CHO cells stably transfected with TGF- $\beta$ 1 induce tumours in nude mice and that tumour growth is accompanied by an increase in capillary density [79, 80]; local administration of anti-TGF- $\beta$ 1 neutralizing antibodies to tumour-bearing nude mice was reported to reduce both capillary density and tumour growth. With the exception of these studies, the potential role of interfering with TGF- $\beta$  signalling as a means of inhibiting tumour angiogenesis, has not been reported. None the less, it is conceivable that TGF- $\beta$  and TGF- $\beta$  receptor antagonists might well inhibit tumour growth, both through the inhibition of angiogenesis, as well as by reducing the suppressive effect of TGF- $\beta$  on immune surveillance. The precise role of TGF- $\beta$  in the regulation of angiogenesis in other pathological situations such as rheumatoid arthritis and juvenile haemangioma, as well as the effect of anti-TGF- $\beta$ -based therapeutic strategies in these settings, remains to be explored.

In conclusion, the role of TGF- $\beta$  and its receptors in the maintenance of vascular integrity has been clearly established from genetic studies in man and mouse. Future studies are, however, likely to reveal that the phenotype of TGF- $\beta$  and TGF- $\beta$ R null mice is dependent on genetic background, which in turn will affect levels of modifier proteins and thus the penetrance and severity of the phenotype. This, in itself, may turn out to be an excellent example of ‘context’. The lack of identity of the cells that express TGF- $\beta$  as well as the nature of the target cells that express TGF- $\beta$ Rs, together with the paucity of knowledge concerning the mechanisms by which vascular integrity is maintained, promises to stimulate a large amount of exciting research in this area in the near future. With respect to angiogenesis, the notion of the ‘angiogenic switch’ is, at present, not as clearly applicable to TGF- $\beta$  as it is to other cytokines such as VEGF; correlative *in vivo* and *in vitro* data none the less suggest that, in addition to its indirect angiogenic effect, TGF- $\beta$ 1 can either promote or inhibit angiogenesis when interacting directly with endothelial cells, depending on whether it is

present during the activation or resolution phases of this process. In this respect, the response of endothelial cells to TGF- $\beta$ 1 during angiogenesis further highlights the notion of 'context', a notion to which TGF- $\beta$  appears to be particularly well suited.

*Note added in proof:* A third phenotype resulting in foetal loss prior to organogenesis, has recently been described in TGF-B1-deficient mice [242]. Of great significance was the observation that the three categories of lethality (pre-organogenesis loss, mid-gestation yolk sac failure and postnatal death) which occur in the absence of TGF-B1, are determined by genetic background. A major co-dominant modifier gene, which is responsible in part for the distribution of the three lethal phenotypes, was mapped to proximal mouse chromosome 5.

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