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## Angiogenesis-Regulating Cytokines

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### INTRODUCTION

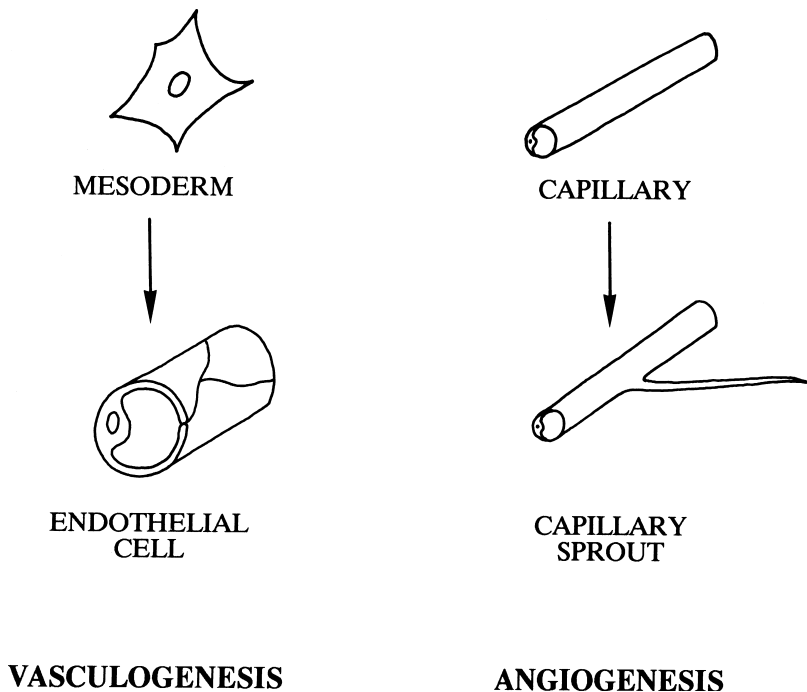
The establishment and maintenance of a vascular supply is an absolute requirement for the growth of normal and neoplastic tissues, and as might be predicted, the cardiovascular system is the first organ system to develop and to become functional during embryogenesis. Both during development and in postnatal life, all new blood vessels begin as simple endothelial-lined tubes. Some become capillaries after developing an intimate association with pericytes, whereas others develop into vessels of larger diameter (arteries and veins) and acquire a variable number of concentrically disposed smooth-muscle cell layers. Traditionally, the formation of new blood vessels has been ascribed to two inter-related but separable processes, vasculogenesis, and angiogenesis (Fig. 1).

### *Definitions*

Vasculogenesis is a series of differentiation and morphogenetic events which result in the formation of a primary capillary plexus, and is comprised of at least three stages: 1) the *in situ* differentiation of mesodermal cells into angioblasts or hemangioblasts; 2) the differentiation of angioblasts and hemangioblasts into endothelial cells (and hematopoietic cells in the case of the hemangioblast); and 3) the organization of newly formed endothelial cells into a primary capillary plexus. The existence of the angioblast, which differentiates exclusively into endothelial cells, has been well-established. However, definitive proof for the existence of the hemangioblast, which is purported to have the dual capacity to differentiate into either endothelial or hematopoietic cells, is still lacking (reviewed in refs. 1–4).

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**Fig. 1.** Vasculogenesis and angiogenesis. Blood vessels are formed by two processes: vasculogenesis, the *in situ* differentiation of mesodermal precursors into endothelial cells, which subsequently organize into tube-like capillaries to form a primary capillary plexus, and angiogenesis, in which new capillaries are formed by a process of sprouting from pre-existing capillaries or post capillary venules. Adapted from ref. 21, with copyright permission from Springer-Verlag GmbH & Co. KG.

The term angiogenesis, derived from the Greek words “angeion” and “genesis,” meaning vessel and production respectively, was coined by Hertig in 1935 (5) to describe the formation of new blood vessels in the placenta. As will become apparent however, angiogenesis is not limited to this setting, and a more contemporary definition would be “the formation of new capillary blood vessels by a process of sprouting from pre-existing vessels in a variety of developmental, physiological, and pathological settings.” A similar although far less well-studied process also occurs in the lymphatic system, and is sometimes referred to as lymphangiogenesis.

Evidence has recently been provided for the existence, in the peripheral circulation, of an endothelial-cell precursor that contributes to the formation of new blood vessels in postnatal life (6). These findings are likely to have a major impact on the current definition of angiogenesis. Recall that although the formation of capillary-like tubes is implicit in the definitions of both vasculogenesis and angiogenesis, primary differentiation of mesoderm into angioblasts is a process that is limited exclusively to vasculogenesis. Because mesoderm does not persist into postnatal life, the definition of vasculogenesis is unlikely to be affected by this new finding. However, the existence of a circulating endothelial precursor does mean that the source of new endothelium during angiogenesis can no longer be ascribed exclusively to sprouting from pre-existing vessels. Thus the definition of angiogenesis will have to be extended to include the incorporation of

endothelial progenitors and/or their progeny into newly forming vessels. Important questions concerning the origin of circulating precursors (possibly from the bone marrow) as well as their precise relationship to angioblasts (and hemangioblasts) remain to be answered.

As indicated earlier, the immature endothelial-lined tubes that arise during vasculogenesis and angiogenesis subsequently differentiate into capillaries (after association with pericytes) or into larger vessels such as arteries and veins (after forming a media composed essentially of smooth-muscle cells). Furthermore, capillaries in many organs undergo further differentiation and develop organ-specific functions. Examples include formation of the blood-brain barrier (BBB) in the central nervous system (CNS), formation of fenestrated endothelium in endocrine and other organs, formation of sinusoids in the liver and spleen, and formation of high endothelial venules in lymph nodes. These processes, which occur in primitive vessels resulting either from vasculogenesis or angiogenesis, should be referred to as secondary differentiation, in order to distinguish them clearly from primary angioblast and endothelial cell differentiation.

The multiple cell functions that occur during angiogenesis belong either to a phase of activation or to a phase of resolution. The activation phase encompasses initiation and progression, and includes: 1) increased vascular permeability and extravascular fibrin deposition; 2) basement-membrane degradation; 3) cell migration and extracellular matrix invasion; 4) endothelial-cell proliferation; and 5) capillary lumen formation. The phase of resolution encompasses termination and vessel maturation, and includes: 1) inhibition of endothelial cell proliferation; 2) cessation of cell migration; 3) basement-membrane reconstitution; and 4) junctional complex maturation. As indicated earlier, 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. Although 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 the consequence of exhaustion of positive regulators that predominated during the phase of activation. If the latter hypothesis is correct, this assumes 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.

### ***Angiogenesis in Pre- and Postnatal Life***

Although, by definition, vasculogenesis must precede angiogenesis, the two processes continue in parallel during early development. However, unlike vasculogenesis, which appears to be restricted to early development, angiogenesis is also required for the maintenance of functional and structural integrity of the organism in postnatal life. Thus it occurs during wound healing, in inflammation, in situations of ischemia, and in female reproductive organs (in the ovary during ovulation and corpus luteum formation; in the placenta and mammary gland during pregnancy). Angiogenesis 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 (RA) and juvenile hemangioma (reviewed in refs. 7–9).


Much of our interest in angiogenesis comes from the notion that for tumors to grow beyond a critical size, they must recruit endothelial cells from the surrounding stroma to form their own endogenous microcirculation (reviewed in ref. 10). Thus during tumor 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.” The prevascular phase is characterized by an initial increase in tumor growth followed by a plateau in which the rate of tumor-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 hematogenous spread of tumor cells, the rapid increase in tumor growth is largely because of a decrease in the rate of tumor-cell apoptosis (11,12). An inverse relationship thus exists between tumor dormancy/tumor-cell apoptosis and tumor angiogenesis. In a sense, tumor angiogenesis might almost be considered as “appropriate,” in that newly formed vessels serve to meet the metabolic demands of the rapidly growing tumor. Although this may be beneficial to the tumor itself, it is clearly detrimental to the organism, because it is permissive for tumor growth, for the dissemination of tumor cells, and for the formation of metastasis.

### ***Regulation of Angiogenesis: Balance and Context***

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. The maintenance of endothelial quiescence is thought to be caused by the presence of endogenous negative regulators, because 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 of the “angiogenic switch,” in which 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 (Fig. 2) (reviewed in ref. 13). Used initially in the context of tumor progression to describe the passage from the prevascular to the vascular phase, the notion of the “switch” can also be applied in the context of developmental, physiological, and 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. 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.

Among the factors that affect endothelial-cell activation status, either positively or negatively, are cytokines and chemokines (chemotactic cytokines) produced by normal and tumor 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. 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

## POTENTIAL ENDOGENOUS REGULATORS OF ANGIOGENESIS

POSITIVE	NEGATIVE
	
<b>Adipocyte lipids</b>	<b>Angiostatin</b>
<b>Angiogenin</b>	<b>C-X-C chemokines:</b>
<b>Angiopoietins</b>	- Platelet factor 4
<b>EGF/TGF-<math>\alpha</math></b>	- IP-10, gro- $\beta$
<b>FGFs</b>	<b>Endostatin</b>
<b>G-CSF</b>	<b>Hyaluronan</b>
<b>HGF</b>	<b>IL-12</b>
<b>Hyaluronan oligosaccharides</b>	<b>Interferons</b>
<b>Hypoxia</b>	<b>MMP &amp; PA inhibitors</b>
<b>IL-8</b>	<b>16Kd prolactin fragment</b>
<b>PDGF-BB</b>	<b>Proliferin-related protein</b>
<b>PIGF</b>	<b>Retinoids</b>
<b>Proliferin</b>	<b>Ribonuclease inhibitor</b>
<b>Prostaglandins</b>	<b>Steroids/metabolites:</b>
<b>TGF-<math>\beta</math></b>	- glucocorticoids
<b>Thymidine phosphorylase/PD-ECGF</b>	- 2-methoxyestradiol
<b>Tissue factor</b>	<b>TGF-<math>\beta</math></b>
<b>TNF-<math>\alpha</math></b>	<b>Thrombospondin</b>
<b>VEGFs/VPF</b>	<b>TNF-<math>\alpha</math></b>

**Fig. 2.** Potential endogenous positive and negative regulators of angiogenesis. A large number of factors, listed here alphabetically, have been shown to regulate angiogenesis in the experimental setting. For many of these factors, definitive studies are still required to demonstrate their role in the endogenous regulation of angiogenesis. It is nonetheless generally assumed that the switch to the angiogenic state may involve either the loss of a negative regulator or the induction of a positive regulator, or both, although definitive proof for this notion is also still awaited. Adapted from ref. 8, with copyright permission from Arnold Publishers.

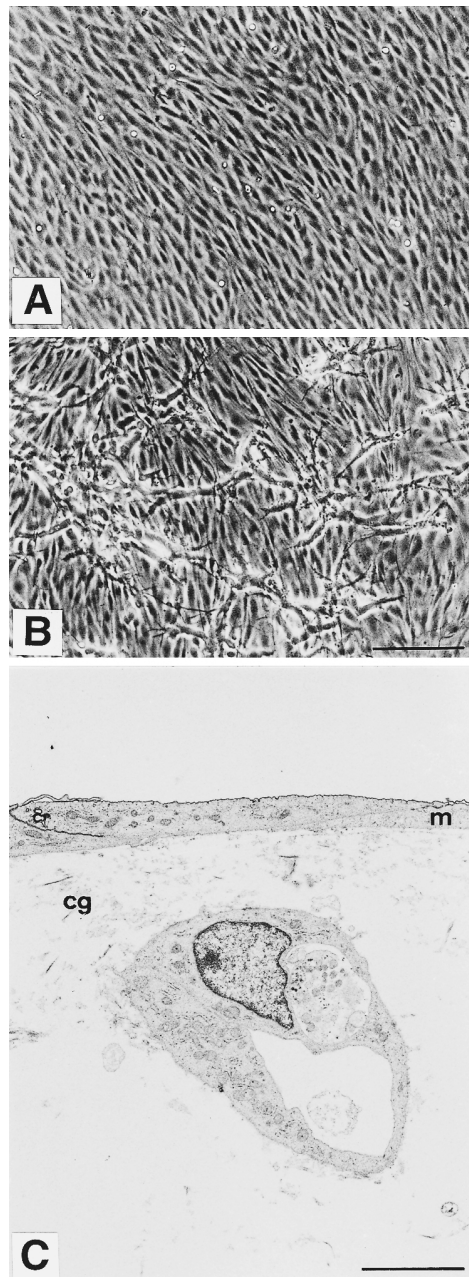
should be seen as “specialized symbols in a language of intercellular communication, whose meaning is controlled by context” (14). Context is determined by (at least) three parameters: first, by the presence and concentration of other cytokines in the pericellular environment of the responding cell; 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 the regulation 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.

## IN VIVO AND IN VITRO MODELS FOR THE STUDY OF ANGIOGENESIS

The two most widely used *in vivo* angiogenesis assays are the chick-embryo chorioallantoic membrane (CAM) and the rabbit corneal micropocket. Direct subcutaneous injection or infusion of substances of interest has also been used to assess their pro- or anti-angiogenic effects. Quantitative *in vivo* assays involve subcutaneous implantation of various three-dimensional substrates to which putative angiogenesis-regulating factors can be added. These include polyester sponges, expanded polyfluorotetraethylene (ePTFE) tubes filled with collagen, polyvinyl-alcohol foam discs covered on both sides by millipore filters (the disc angiogenesis system), and Matrigel, a basement membrane-rich extracellular matrix. These assays are essential to establish whether a given molecule stimulates blood-vessel formation in the intact organism; however, their interpretation is frequently complicated by the fact that the experimental conditions may inadvertently favor inflammation, and that under these conditions the angiogenic response is elicited indirectly, at least in part, through the activation of inflammatory or other nonendothelial 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 the direct interaction of angiogenesis regulators with endothelial cells. To circumvent this drawback, *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 endothelial cell proliferation, migration, and production of proteolytic enzymes such as matrix metalloproteinases (MMPs) and plasminogen activators (PAs). 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. These assays include: 1) long-term culture of endothelial cells in dishes coated with a thin layer of matrix proteins; 2) short-term culture of endothelial cells on a thick gel of basement membrane-like matrix; 3) suspension of endothelial cells within three-dimensional gels composed of collagen or fibrin; 4) radial growth of branching tubules from rings of rat aorta or from fragments of either rat adipose-tissue microvessels or human placental blood vessels embedded in collagen or fibrin gels; 5) radial growth of tubular sprouts from endothelial cells grown on microcarrier beads embedded in a fibrin gel (reviewed in ref. 15).

In our own studies, we have employed an *in vitro* model of angiogenesis that assays both for the invasive capacity of stimulated endothelial cells as well as their capacity for histotypic morphogenesis, i.e., the formation of capillary-like tubes. The model consists of cultivating endothelial cells on the surface of three-dimensional collagen (16) or fibrin (17) gels; under these conditions, the cells form a monolayer on the surface of the gel and do not invade the underlying matrix (Fig. 3A). When the monolayer is treated with an angiogenic factor such as basic fibroblast growth factor (bFGF) (18) or vascular endothelial growth factor (VEGF) (19), the cells are induced to invade the underlying gel, and by adjusting the plane of focus beneath the surface monolayer, branching and anastomosing cell cords can be seen within the gel (Fig. 3B). In cross-section, the presence of tube-like structures resembling capillaries can be observed beneath the surface monolayer (Fig. 3C). Invasion can be quantitated by measuring the total additive length of all cells that have penetrated into the underlying gel to form cell cords (19). Unlike planar models of *in vitro* angiogenesis, the model we have developed has the advantage of accurately recapitulating the invasive nature of the angiogenic process, and by virtue of its three-dimensional nature, is also permissive for histotypic morphogenesis, i.e., for the formation of patent capillary-like tubes whose abluminal surfaces are in direct contact with the extracellular matrix.



**Fig. 3.** Collagen gel invasion model for the study of angiogenesis in vitro. **(A)** When viewed from above by phase-contrast microscopy, endothelial cells grown on the surface of a three-dimensional collagen gel form a confluent monolayer without invading the underlying matrix. **(B)** Addition of angiogenic cytokines such as bFGF or VEGF induces the cells to invade the underlying gel and to form a network of branching cords, which can be viewed by focussing beneath the surface monolayer. **(C)** When the invading cell cords are viewed in cross-section by electron microscopy, their tubular nature, morphologically similar to capillaries seen in vivo, can be appreciated (“cg” = collagen gel, and “m” = surface monolayer.) Bar in **(A,B)** = 150  $\mu\text{m}$  and in **(C)** = 10  $\mu\text{m}$ . **(A,B)** adapted from ref. 18; **(C)** adapted from ref. 21, with copyright permission from Springer-Verlag GmbH & Co. KG.

## ANGIOGENESIS-REGULATING CYTOKINES

The ultimate target for both positive and negative regulators of angiogenesis is the endothelial cell. This has led to the notion that angiogenesis regulators may either act directly on endothelial cells, or indirectly by inducing the production of direct-acting regulators by inflammatory and other nonendothelial cells. The most extensively studied cytokines involved in the positive regulation of angiogenesis are VEGF and acidic and basic FGFs (aFGF, bFGF). However, although a regulatory role for VEGF in developmental, physiological, and pathological angiogenesis has been well defined, much controversy still exists as to whether the FGFs are relevant to the endogenous control of angiogenesis *in vivo*. The finding that *in vitro*, VEGF and FGF positively regulate many endothelial-cell functions including proliferation, migration, and extracellular proteolytic activity, has led to the notion that these factors are direct-acting positive regulators. In contrast, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) inhibit endothelial-cell growth *in vitro*, and have therefore been considered as direct-acting negative regulators. However, both TGF- $\beta$ 1 and TNF- $\alpha$  are angiogenic *in vivo*, and it has been demonstrated that these cytokines induce angiogenesis indirectly by stimulating the production of direct-acting positive regulators from stromal and chemoattracted inflammatory cells. In this context then, TGF- $\beta$ 1 and TNF- $\alpha$  are considered to be indirect positive regulators. In view of TGF- $\beta$ 's capacity to directly inhibit endothelial-cell proliferation and migration, to reduce extracellular proteolysis, and to promote matrix deposition *in vitro*, as well as to promote the organization of single endothelial cells embedded in three-dimensional collagen gels into tube-like structures, TGF- $\beta$  has also been proposed to be a potential mediator of the phase of resolution (reviewed in refs. 20–22).

Other cytokines which have been reported to regulate angiogenesis *in vivo* include hepatocyte growth factor (HGF), epidermal growth factor/transforming growth factor- $\alpha$  (EGF/TGF- $\alpha$ ), platelet-derived growth factor-BB (PDGF-BB), interleukins (IL-1, IL-6, and IL-12), interferons, granulocyte colony stimulating factor (G-CSF), placental growth factor (PIGF), proliferin and proliferin-related protein. Chemokines that regulate angiogenesis *in vivo* have to date only been identified in the -C-X-C- family, and include IL-8, platelet factor 4, and gro- $\beta$ . Angiogenesis can also be regulated by a variety of noncytokine factors including enzymes (angiogenin, platelet-derived endothelial-cell growth factor/thymidine phosphorylase [PD-ECGF/TP], inhibitors of matrix-degrading proteolytic enzymes (tissue inhibitors of metalloproteinases [TIMPs] and plasminogen-activator inhibitors [PAIs]), extracellular matrix components/coagulation factors or fragments thereof (thrombospondin, angiostatin, hyaluronan and its oligosaccharides, endostatin), soluble cytokine receptors, prostaglandins, adipocyte lipids, and copper ions (Fig. 2) (reviewed in refs. 7,20,21,23).

It is crucial to bear in mind that although a large number of factors have been shown to be active in the experimental setting, 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 (21,24).

### ***Vascular Endothelial Growth Factor***

Vascular endothelial growth factor (VEGF) is a highly conserved multifunctional glycoprotein that exerts several possibly independent functions on vascular endothelium.



VEGF was initially described as a tumor-secreted protein that increases the permeability of microvessels, hence its alternate (and possibly more appropriate) name, vascular permeability factor. This extremely potent function of VPF/VEGF is 50,000 times greater than that of histamine. In vivo, VEGF is also a potent positive regulator of angiogenesis, and in vitro, VEGF induces endothelial-cell migration and proliferation and alters endothelial cell gene expression (including the production of matrix-degrading proteolytic enzymes). Although the mitogenic properties of VEGF appear to be endothelial cell-specific, in vitro this effect is relatively weak when compared to other positive regulating cytokines such as bFGF. VEGF contains a signal peptide and is therefore secreted from producer cells. At least three VEGF isoforms, which vary in their relative proportions in different tissues, are generated through alternative splicing of a single mRNA that arises from a gene containing 8 coding exons. The most abundant and most extensively studied 165 amino acid isoform (VEGF<sub>165</sub>; 164 amino acids in rodents), arising from exons 1–5, 7, and 8, has been detected in both soluble and cell/matrix-bound forms. (This isoform will be referred to simply as VEGF throughout this chapter.) A 121 amino acid form (VEGF<sub>121</sub>; 120 amino acids in rodents), arising from exons 1–5 and 8, has been detected only in soluble form, whereas a 189 amino acid isoform (VEGF<sub>189</sub>; 188 amino acids in rodents), arising from exons 1–8 appears to be localized exclusively to the cell surface and extracellular matrix. A polymerase chain reaction (PCR) product inferring the presence of a fourth isoform, VEGF<sub>206</sub>, has also been described in humans, although its biological significance remains to be determined. Although VEGF was initially purified on the basis of its affinity for heparin, this is substantially lower than that of other heparin-binding growth factors such as bFGF. VEGF<sub>121</sub> does not bind to heparin. VEGF isoforms exist as disulfide-bonded homodimers, and have a significant degree of similarity to placenta growth factor (PlGF, approx 50% identity) and platelet-derived growth factor (PDGF, approx 20% identity). The PlGF gene contains 7 coding exons from which two alternatively spliced forms can be generated. PlGF can form biologically active heterodimers with VEGF. VEGF expression is regulated by hypoxia, glucose deprivation, prostaglandins and estrogens, as well as by a number of cytokines (reviewed in refs. 25–28).

The importance of VEGF in experimental primary and metastatic tumor growth in vivo has been clearly demonstrated using a variety of approaches including anti-VEGF antibodies, soluble VEGF receptors (VEGFRs), antisense VEGF, a VEGF-toxin conjugate, as well as a dominant negative approach using a truncated form of VEGFR-2. The inhibitory effect of soluble VEGFR chimeric proteins and antisense VEGF oligonucleotides in a murine model of proliferative retinopathy has also been described (reviewed in ref. 9). However, the most dramatic demonstration of the requirement for VEGF in the development of the vascular tree comes from genetic studies involving targeted gene disruption in mice. In a manner that is unprecedented for a gene that does not undergo imprinting, heterozygosity for VEGF inactivation was embryonic lethal (29,30). The observation that the phenotype of VEGF<sup>–/–</sup> mice was more severe than that of VEGF<sup>+/–</sup> mice, demonstrates the existence of a dose-dependent requirement for VEGF during embryogenesis, and implies that minimal amounts of VEGF are required in a tightly regulated manner for normal vascular development. Essentially, although endothelial-cell development was delayed in VEGF-deficient mice, resulting in the formation of abnormal vascular structures and massive tissue necrosis, it was not entirely aborted. This is in contrast to VEGF receptor-2 (Flk-1)-deficient mice, in which endothelial-cell development was completely absent (*see below*). These findings point to the existence

of VEGFR-2 ligands other than VEGF. Because of embryonic lethality, the homozygous phenotype was inaccessible by standard (germ-line) breeding; this required the use of embryonic stem (ES) cell aggregates combined with tetraploid mouse embryos. Under these conditions, the resulting fetuses contain a mutant ES-derived embryonic compartment (in which VEGF is inactivated) and wild-type, tetraploid-derived extraembryonic membranes. This approach allows for rapid examination of mutant phenotypes derived from genetically altered ES cells without the need for germ-line transmission (31). With the exception of conditional VEGF knockouts, which are likely to add significantly to our understanding of the role of this cytokine, the ES-tetraploid system is currently the only method available to assess the effect of homozygous deficiency of a gene that is embryonic lethal in the heterozygous state. Finally, when a nude mouse model was used to determine the role of VEGF in ES-cell tumorigenesis (i.e., formation of teratomas), VEGF<sup>-/-</sup> ES cell-induced tumor and associated blood-vessel growth were strikingly reduced when compared to wild-type ES cells (30).

Two proteins with structural homology to VEGF have recently been described. The first has been called VEGF-B (32) or, alternatively, VEGF-related factor (VRF) (33,34). VEGF-B transcripts are alternatively spliced, and the overall genomic organization of VEGF-B is conserved between other members of the VEGF gene family (VEGF, PlGF, PDGF). VEGF-B is primarily cell-associated, and is capable of forming heterodimers with VEGF. VEGF-B increases [<sup>3</sup>H]thymidine incorporation in human and bovine endothelial cells. Whether VEGF-B/VRF binds to the same receptors as other members of the VEGF family (*see below*) remains to be elucidated. VEGF-B is co-expressed with VEGF in some tissues, and is particularly abundant in embryonic and adult striated (cardiac and skeletal) muscle; however, unlike VEGF, elevated levels of VEGF-B mRNA were not found in glioblastomas, breast, or renal carcinomas (32,33,35).

The second protein with structural homology to VEGF has been called VEGF-C (36), or alternatively, VEGF-related protein (VRP) (37). VEGF-C was isolated during a search for a ligand for VEGFR-3 (Flt-4). VEGF-C displays a high degree of similarity with VEGF, including conservation of the eight cysteine residues involved in intra- and inter-molecular disulfide bonding. It appears that the VEGF-C mRNA is first translated into a precursor from which the mature ligand is derived by cell-associated proteolytic processing. The cysteine-rich C-terminal half, which increases the length of the VEGF-C polypeptide relative to other ligands of this family, shows a pattern of spacing of cysteine residues reminiscent of the Balbiani ring 3 protein repeat. Like VEGF and VEGF-B, VEGF-C/VRP transcripts are alternatively spliced to give a number of major isoforms. VEGF-C binds to the extracellular domain of VEGFR-3 and induces VEGFR-3 tyrosine phosphorylation. In addition to VEGFR-3, VEGF-C appears to bind to and induce phosphorylation of VEGFR-2 (Flk-1/KDR). VEGF-C/VRP transcripts are detectable in many adult and fetal human tissues and in a number of cell lines. Patterns of VEGF-C expression during development suggest that this cytokine plays an important role in lymphangiogenesis (38).

Alterations in endothelial-cell function induced by members of the VEGF family are mediated via transmembrane tyrosine kinase receptors that at present include VEGFR-1 (Flt-1), VEGFR-2, and VEGFR-3. Ligands for VEGFR-1 include VEGF and PlGF; ligands for VEGFR-2 include VEGF and VEGF-C; whereas the only ligand reported so far for VEGFR-3 is VEGF-C (27,39; for references on VEGF-B/VRF and VEGF-C/VRP, *see above*). Whether VEGF-B binds to the same receptors as other members of the

VEGF family remains to be described. VEGFRs are expressed in many adult tissues, despite the apparent lack of constitutive angiogenesis. VEGFRs are however clearly upregulated in endothelial cells during development and in certain angiogenesis-associated/dependent pathological situations including tumor growth (reviewed in refs. 25,28). The phenotypes of both VEGFR-1- and VEGFR-2-deficient mice have been described. VEGFR-1-deficient mice die in utero at mid-somite stages, and although homozygous deficient mice are capable of forming endothelial cells in both intra- and extra-embryonic regions, assembly of these cells into vessels is perturbed, resulting in the formation of abnormal vascular channels. The authors conclude that VEGFR-1 signaling pathways may regulate normal endothelial cell-cell or cell-matrix interactions during vascular development (40). VEGFR-2-deficient mice also die in utero between 8.5 and 9.5 d postcoitum, although in contrast to VEGFR-1, this appears to be owing to abortive development of endothelial-cell precursors. Yolk-sac blood islands and organized embryonic blood vessels were not detectable at any stage of development. The development of hematopoietic precursors was also severely reduced (41). By using a dominant-negative approach, the requirement for VEGFR-2 has also been clearly demonstrated in tumor angiogenesis (42,43). Gene targeting and dominant negative approaches have therefore clearly defined an essential role for VEGFRs in developmental and tumor angiogenesis.

Because VEGFR-expressing endothelial cells are located adjacent to regions of tumor ischemia and necrosis, it is possible that the increase in VEGFR expression is mediated by hypoxia (44; reviewed in ref. 45). In this context, it has been demonstrated in some *in vitro* studies that hypoxia increases high-affinity VEGF binding to endothelial cells and induces an increase in VEGFR-2 number (without alterations in receptor affinity), and that this is associated with increased VEGF-induced mitogenicity and VEGFR-2 tyrosine phosphorylation in endothelial cells (46,47). It has also been reported that hypoxia increases expression of VEGFR-1 and -2 mRNA in endothelial cells both *in vivo* and *ex vivo* in rat lungs (48). Of particular interest is the observation that conditioned medium from hypoxic skeletal myoblasts or smooth muscle cells contains a factor that markedly increases VEGFR-2 number (without alterations in receptor affinity) in endothelial cells *in vitro* (49). On the basis of neutralizing antibody studies, it was concluded that this factor is neither VEGF, bFGF, TNF- $\alpha$ , or TGF- $\beta$ 1. Taken together, these findings suggest that hypoxia can increase the effect of VEGF via the paracrine induction of VEGFRs in metabolically deprived tissues. With the exception of hypoxia, other factors that increase VEGFR expression have not been published. However, we have observed that high and low glucose concentrations increase VEGFR-2 mRNA levels in bovine microvascular but not large vessel-derived endothelial cells *in vitro* (S.J. Mandriota and M.S. Pepper, unpublished observations). We have also studied the effect of bFGF on VEGFR-2 expression in bovine endothelial cells, and have found that although bFGF increases levels of VEGFR-2 mRNA and total protein, cell surface protein is diminished. This may be owing to the fact that bFGF concomitantly increases expression of VEGF and VEGF-C, which upon secretion may interact with and promote internalization of VEGFR-2 (S. J. Mandriota and M. S. Pepper, unpublished observations). Downregulation of VEGFR expression in endothelial cells *in vitro* has been seen with nitric oxide (NO) or an NO-related metabolite in rat lungs *ex vivo* (48), with TGF- $\beta$  (50), and with TNF- $\alpha$  (51).

### ***Basic Fibroblast Growth Factor***

Basic fibroblast growth factor (bFGF), also known as FGF-2 (or heparin-binding growth factor-2), is a member of the FGF superfamily that comprises more than 14 distinct gene products. bFGF is a cationic polypeptide (pI 9.6) with potent angiogenesis-inducing properties *in vivo* (reviewed in refs. 52–56).

Application of neutralizing antibodies to bFGF to the chick embryo CAM has been reported to inhibit vascularization (57). Although these findings suggest that endogenous bFGF is rate-limiting in CAM vascularization, they do not allow one to determine whether the target for bFGF in this process is the endothelium. In contrast to the clear inhibitory effect on tumor growth observed in studies with neutralizing antibodies to VEGF, use of neutralizing antibodies to bFGF has been reported to inhibit tumor growth in some studies (58–60) but not in others (61,62). One study reported a significantly lower degree of neovascularization in animals that received anti-bFGF antibody (60), suggesting that in this tumor model, angiogenesis is bFGF-dependent. It is difficult however to conclude whether the reduction in tumor growth in the other studies was mediated through inhibition of angiogenesis, because bFGF mitogenicity is not endothelial cell-specific. With respect to genetic studies in transgenic mice, unlike FGFs -3, -4, and -5 (reviewed in refs. 54,63), the effects of a homozygous null mutation in bFGF have not been reported. The phenotype of transgenic mice in which full length human bFGF was overexpressed in all tissues and through all stages of development under the control of a phosphoglycerate kinase promoter has recently been described (64). These mice develop severe abnormalities in the skeletal system with no obvious features related to aberrant vascular morphogenesis or growth. In particular, there were no signs of hemangioma or microangiopathy, and overexpression of bFGF did not increase the overall susceptibility to tumor formation.

Basic FGF-induced endothelial responses are mediated via transmembrane tyrosine kinase receptors (FGFRs). To date, high-affinity FGFRs 1–4 have been described, and the existence of a large number of FGFR variants (generated by alternative mRNA splicing and differential polyadenylation) further increases FGFR diversity. This diversity results in a complex pattern of overlapping binding specificities for the various FGFs. It has been demonstrated that bFGF binds with high affinity to all four FGF receptors (or alternatively spliced variants) (reviewed in refs. 56,65–67). The importance of FGFR-1 has recently been demonstrated by targeted gene disruption in mice. Post-implantation growth and mesodermal patterning were affected, resulting in recessive embryonic lethality during gastrulation (68,69). Although mesoderm was formed, specification of cell fate and regional patterning were severely disrupted. With respect to the cardiovascular system, in a few embryos that had progressed to the appropriate stage, the heart and blood islands were present, and VEGFR-2-positive cells (endothelial precursors) were found in appropriate locations in the lateral plate and yolk-sac mesoderm. This suggests that FGFR-1 is not required for differentiation of endothelial-cell precursors. In humans, genetic alterations in the FGFRs 1–3 have recently been linked to craniofacial and limb developmental defects and achondroplasia (reviewed in refs. 54,63).

The controversy that has arisen over the role of bFGF as an endogenous regulator of angiogenesis stems from the following observations. First, as indicated earlier, there is a lack of consensus regarding inhibitory studies, and bFGF gain of function and FGFR gene-inactivation approaches have failed to clearly delineate a requirement for bFGF in endothelial function during development and in postnatal life. Second, the stimulatory effects of bFGF on proliferation and migration are not restricted to endothelial cells. Third, in contrast to VEGF, bFGF lacks a signal peptide and therefore fails to enter the classical secretory pathway. Its mode of cellular export is at present unknown. bFGF is synthesized as both an 18 kDa and higher molecular weight (22–25 kDa) isoforms, resulting from the use of alternate start codons, in which translation is initiated at CUG rather than AUG. The 18 kDa form is stored in the cytoplasm of its producer cells,

whereas the higher molecular weight forms contain an amino-terminal nuclear localization/retention sequence that appears to mediate intranuclear accumulation. Although bFGF does not enter the classical secretory pathway, the 18 kDa form can be detected outside the cell. 18 kDa bFGF export may involve cell injury/death and possibly an active and regulatable nonclassical secretory mechanism. As a consequence of its extracellular localization, it appears that access to the transmembrane FGFRs is limited only to 18 kDa bFGF and not to its high molecular-weight isoforms (reviewed in refs. 52,70,71).

Although endothelial cells express FGFRs 1, 2 and 4 *in vitro*, which may be a consequence of culture conditions and/or serial passaging, the fourth and perhaps most significant issue concerning the endogenous angiogenic activity of bFGF is whether or not endothelial cells of the microvasculature express FGFRs *in vivo*. This is important because new capillary blood vessels arise from pre-existing capillaries or postcapillary venules. A limited number of studies have reported the presence of immunoreactive FGFR-1 in endothelial cells of the microvasculature (predominantly postcapillary venules) of a wide range of normal and neoplastic adult tissues, as well as in newly formed vessels of atherosclerotic plaques and underlying adventitial vessels. However, FGFRs have been undetectable in microvascular endothelial cells in virtually all settings in which there is active angiogenesis, and in which expression of high-affinity VEGFRs has been clearly demonstrated (reviewed in refs. 21,44,56).

bFGF immunoreactivity has been demonstrated in the vascular intima (endothelial cells and underlying basement membrane) in a wide variety of settings *in vivo*. These include embryonic and normal adult tissues, chronic inflammatory tissues, juvenile hemangiomas, the endothelium of newly formed vessels in atherosclerotic plaques in human vessels, angiogenesis associated with thyroid hyperplasia, and a variety of tumors including glioblastomas. However, the presence of bFGF immunoreactivity gives no information as to the origin of the molecule nor the molecular nature of the structures to which it is bound. Extracellular matrix-bound bFGF is extremely stable, and because bFGF immunoreactivity but not mRNA is detectable in quiescent endothelial cells *in vivo*, it is possible that endothelium-associated bFGF may have a nonendothelial origin (circulation or medial smooth muscle cells), or that it may have been deposited in the matrix during development and postnatal growth. In contrast, bFGF mRNA has been detected by *in situ* hybridization in endothelial cells of brain tumors including glioblastomas, and bFGF mRNA is induced during endothelial regeneration in the rat aorta, which demonstrates that activated endothelium has the capacity to synthesize bFGF. bFGF-like activity, protein, and mRNA are present in cultured endothelial cells, all of which can be increased by serial passaging, and bFGF expression by cultured endothelial cells is density-dependent: levels are greater in sparse (low-density) cultures, in which cells migrate and proliferate, than in confluent (high-density) cultures in which cell migration and proliferation are virtually absent. The aforementioned observations suggest that cultured endothelial cells are phenotypically closer to activated/angiogenic endothelium than to the resting endothelium from which they were derived (reviewed in ref. 21).

If one accepts that the FGFs are indeed endogenous regulators of angiogenesis despite the fact that microvascular endothelial cells cannot be convincingly shown to express FGFRs *in vivo*, a number of alternative hypotheses can be envisaged. The first two follow on from the observation that bFGF immunoreactivity can be detected in endothelium *in vivo*. First, bFGF-dependent, FGFR-independent signaling may occur in endothelial cells through other cell surface molecules such as heparan sulfate proteoglycans (HSPGs)

(72). Second, the observation that bFGF lacks a signal peptide and that its high molecular-weight forms have nuclear localization/retention signals, raises the possibility of an autocrine/intracrine role for bFGF in vivo. A third scenario might be that bFGF is an indirect angiogenic factor that acts by stimulating the production of direct acting cytokines by adjacent nonendothelial cells. However, it is important to note that none of these alternate hypotheses that have been proposed to explain bFGF-dependent, FGFR-independent endothelial-cell activation have yet been clearly substantiated in vivo.

As a general observation, it appears that providing justification for the role of bFGF in the regulation of endogenous angiogenesis requires extensive and elaborate argument when compared to VEGF, although it is possible that the role of bFGF may be more subtle than that of VEGF.

### ***Transforming Growth Factor- $\beta$***

TGF- $\beta$  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 noncovalently 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 disulfide 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 noncovalently with mature TGF- $\beta$ , thereby inactivating its biological activity (reviewed in refs. 73–75).

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- $\beta$ , whereas 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 sulfate 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 signaling receptors. Endothelial cells also express endoglin, a protein with structural homology to the TGF- $\beta$ R III. TGF- $\beta$  also binds to the decorin core protein, which neutralizes its activity, as well as to thrombospondin, a large multifunctional glycoprotein that mediates the adhesion of both endothelial and nonendothelial cells to the extracellular matrix (reviewed in refs. 76–79).

TGF- $\beta$ 1 has featured prominently among cytokines studied for their capacity to regulate new blood vessel formation. However, it is still unclear as to precisely how TGF- $\beta$

is involved in the endogenous regulation of this process. Thus, a number of *in vivo* 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 that overexpress TGF- $\beta$ 1 in a tissue-specific manner, suggests that when angiogenesis does occur *in vivo*, this is dependent on local inflammation, which may either be initiated or exacerbated by TGF- $\beta$ . If one accepts that TGF- $\beta$  is important for angiogenesis outside of the experimental setting, then an indirect inflammatory cell-mediated mode of activity may be applicable to physiological or pathological angiogenesis associated with acute and chronic inflammation, wound healing, and tumor growth. With respect to *in vitro* studies, it is likely that different models recapitulate different phases of the angiogenic process, and therefore when interacting directly with endothelial cells, TGF- $\beta$  has different functions on vessel formation at different stages of the process. Thus TGF- $\beta$  regulates the phase of activation by potentiating or inhibiting the activity of positive regulators like bFGF and VEGF in a concentration-dependent manner (*see below*). 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. Thus, 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. 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. With respect to TGF- $\beta$ Rs, *in vitro* findings point to the importance of TGF- $\beta$ Rs I, II and III in TGF- $\beta$ -mediated signaling in endothelial cells. However, the consistent inability to detect TGF- $\beta$ Rs in endothelial cells *in vivo* and the observation that the type II and III receptors are downregulated in three-dimensional cultures *in vitro*, suggests that expression of TGF- $\beta$  receptors in two-dimensional cultures *in vitro* is consequence of endothelial cell activation (reviewed in refs. 22,80–83).

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 humans and mouse have recently revealed a role for this cytokine in embryonic vascular assembly and in the maintenance of vessel wall integrity. TGF- $\beta$ 1 is expressed in many tissues during embryogenesis (including endothelial and hematopoietic precursors) (84), and targeted disruption of the TGF- $\beta$ 1 gene results in mortality at three distinct times: prior to organogenesis, during midgestation, or at 3 wk postpartum (85–88). With respect to the group that dies during mid-gestation, this is owing to defects in the extraembryonic tissues, namely the yolk-sac vasculature and the hematopoietic system. TGF- $\beta$ 1 $^{-/-}$  embryos *per se*, unlike their yolk sacs, had no specific abnormalities, although generalized developmental retardation, ischemia, and necrosis did occur, which may have been secondary to the extraembryonic lesions. In particular, intraembryonic endothelial cells expressing high levels of VEGFR-2 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 earlier, 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. It is not possible at present to say whether the reduction in the number of VEGFR-2-expressing endothelial cells in the yolk sac was owing to reduced angioblast differentiation, or to inefficient network 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 is an important positive regulator of extraembryonic endothelial-cell differentiation, the establishment of vessel-wall integrity, and yolk-sac hematopoiesis. The earlier findings also suggest that intraembryonic and extraembryonic vasculogenesis and hematopoiesis are regulated differently.

Of great significance is a recent report that has revealed that the three categories of lethality that occur in the absence of TGF- $\beta$ 1, namely early pre-organogenesis loss, mid-gestation yolk-sac failure, and postnatal death, are determined by genetic background. A major co-dominant modifier gene, which is responsible in part for the distribution of the three lethal phenotypes, has been mapped to proximal mouse chromosome 5 (88).

The phenotype of TGF- $\beta$ R II deficient mice has also recently been reported (89). These mice are highly reminiscent of TGF- $\beta$ 1 null mice described earlier. Thus, homozygous deficiency was lethal at about 10.5 d gestation, and this resulted from defects in yolk-sac hematopoiesis 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 (90).

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 hemorrhagic telangiectasia (HHT). HHT is an autosomal, dominant, single-gene disorder characterized by multisystemic vascular dysplasia and recurrent hemorrhage. The earliest detectable change in the telangiectatic lesions is dilatation of postcapillary venules in the upper dermis; the endothelial cells themselves, including intercellular junctions, appear to be normal (reviewed in ref. 91). The genes for HHT have recently been identified, and their identification has led to the definition of two HHT subtypes. The gene for HHT type I is endoglin (92), which displays regions of structural homology to betaglycan, the type III TGF- $\beta$ R. The gene for HHT type 2 is ALK-1 (93), a TGF- $\beta$ -binding type I receptor that is expressed at high levels in endothelial cells *in vitro* (94).

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. Nonetheless, because TGF- $\beta$  induces the synthesis and assembly of the endothelial-cell extracellular matrix (*see above*), one of the consequences of defective TGF- $\beta$ R signaling 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 (95,96) closely mimicks the phenotype of TGF- $\beta$ - and TGF- $\beta$ R-deficient mice, particularly because TGF- $\beta$ 1 has been shown to increase expression of fibronectin and its specific integrin,  $\alpha_5\beta_1$  (reviewed in ref. 22). 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 (97), in PDGF receptor- $\beta$  (PDGFR- $\beta$ ) (98) as well as in mouse embryos exposed to anti-PDGF-A neutralizing antibodies *in utero* (99). It has been demonstrated that TGF- $\beta$ 1 induces PDGF-A and -B chain synthesis in endothelial cells (100–102). In addition, TGF- $\beta$ 1 increases PDGFR- $\beta$  expression in fibroblasts and smooth-muscle cells (103,104). Because 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- $\beta$  signaling in endothelial cells may result in defective assembly of other 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 in turn be mediated through the autocrine regulation of PDGFs and their receptors (reviewed in refs. 82,83,105,106). Vessels that form in the absence of the ligand or its receptors are ectatic both *in vivo* and *in vitro*. This is likely to be owing to incomplete vessel-wall maturation, which following an increase in hemodynamic pressure during development results in vessel-wall rupture and hemorrhage. It is noteworthy that during development in the mouse this occurs in extraembryonic but not in intraembryonic tissues, and that in humans, 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. However, ectatic vessels which form in the absence of exogenous TGF- $\beta$  *in vitro* (*see below*) do so in the absence of circulating blood; this suggests that although intravascular hemodynamic pressure is likely to be responsible for vessel rupture and hemorrhage, the formation of dilated vessels can occur in its absence.

In summary, the role of TGF- $\beta$  and its receptors in the maintenance of vascular integrity has been clearly established from genetic studies in humans and mouse. They appear to be required for capillary sprout maturation, as well as for promoting interactions with other vascular-wall cells including recruitment and differentiation of smooth-muscle cells. The observation that the phenotype of TGF- $\beta$  null mice depends on genetic background points to the importance of modifier proteins in determining the penetrance and severity of the phenotype. This in itself may turn out to be an excellent example of “context.” 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 nonetheless 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.

## CONTEXTUAL ACTIVITY OF ANGIOGENESIS-REGULATING CYTOKINES

Despite the unambiguous demonstration of VEGF’s relevance in the endogenous regulation of angiogenesis, a number of important questions remain. The first arises from the observation that both VEGF and its receptors are expressed at high levels in a variety

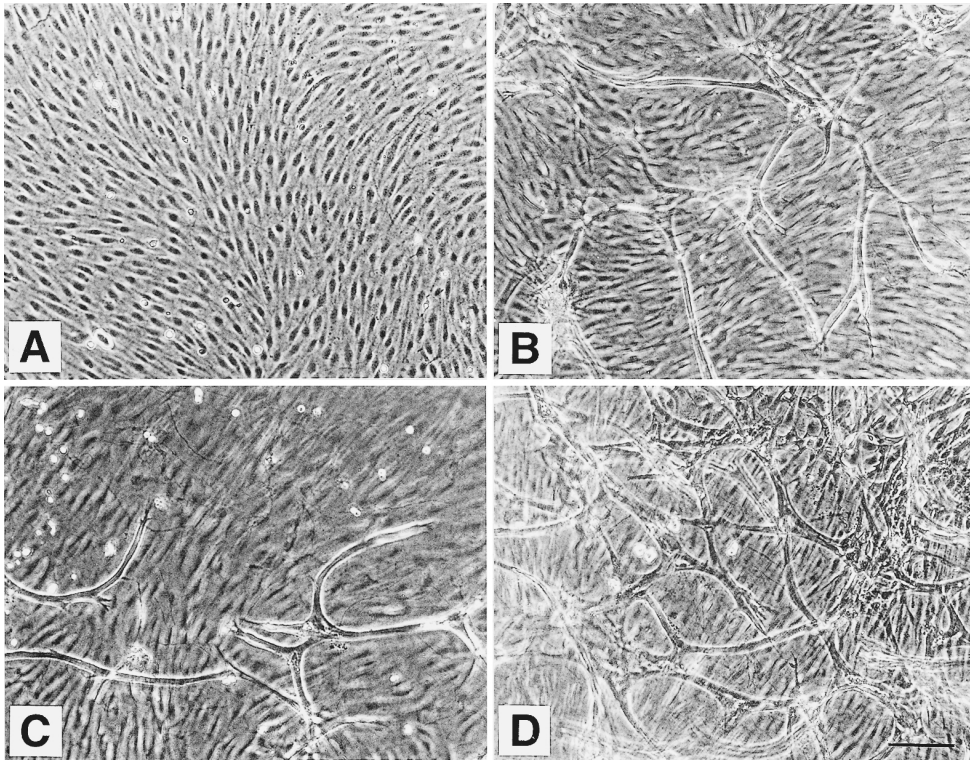
of adult tissues in which there is apparently no angiogenesis. This might mean that VEGF has additional functions not directly related to angiogenesis: 1) VEGF is clearly involved in the regulation of vascular permeability (25); 2) VEGF may be important for endothelial survival (trophic effect); 3) VEGF may be required for the maintenance of organ-specific endothelial characteristics such as fenestrae (107,108). Despite the dogma that endothelial cell turnover in the adult organism is very low, the presence of VEGF may indicate the requirement for a constant low-grade angiogenesis that is necessary to regenerate capillaries in organs with high rates of blood flow (lung and kidney). The absence of extensive angiogenesis in the face of high levels of VEGF (and its receptors) might also indicate the dominance of negative regulators, which serve to prevent uncontrolled angiogenesis in these organs.

The second question arises from the observation that levels of VEGF and its receptors appear to be unchanged in certain organs and tumors in which there is extensive angiogenesis. These include, although are unlikely to be limited to, the mammary gland during pregnancy (108a) and some experimental tumors, in which the levels of VEGF are similar in the prevascular and vascular phases of tumor progression (109). One explanation might be that in these tissues, angiogenesis is in fact VEGF-dependent, but that the angiogenic switch involves the loss of a negative regulator. Alternatively, it may imply that some other positive regulator might be acting in these settings. A third hypothesis, namely that of context, implies that VEGF-induced angiogenesis depends on interactions with other regulatory molecules. Based on results from our own laboratory, we will describe three examples of context.

### ***Synergism Between VEGF and bFGF***

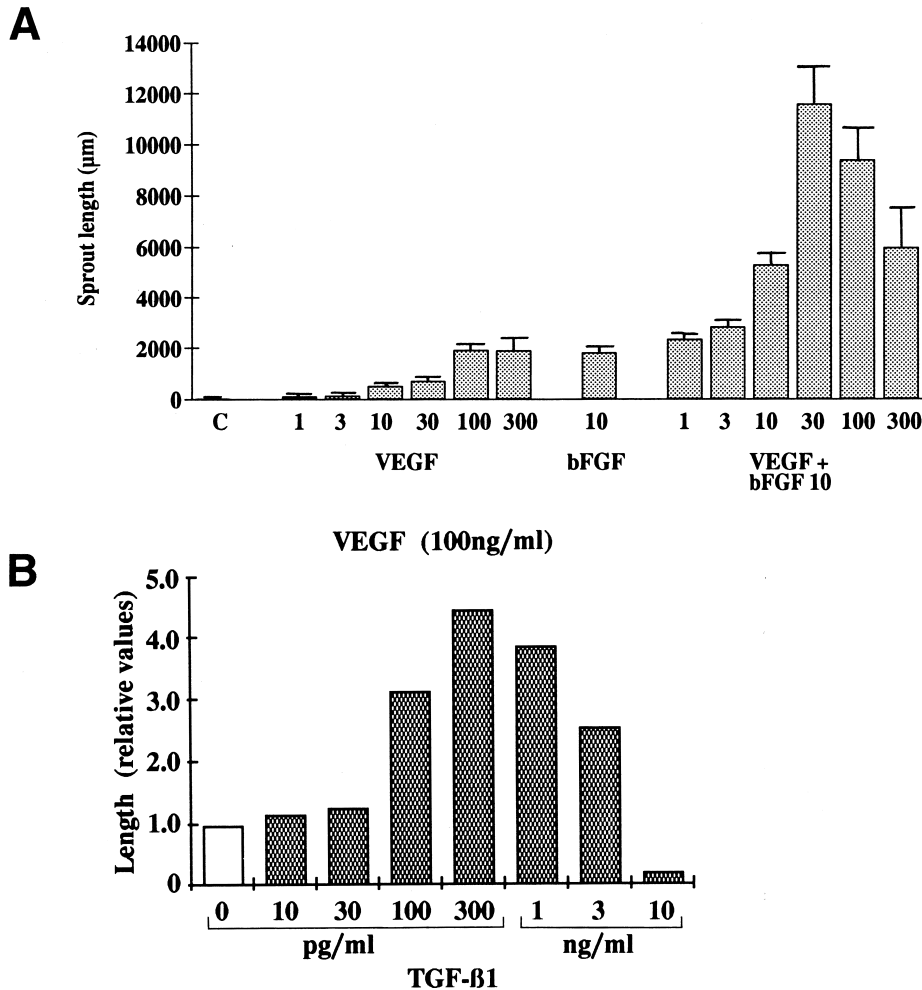
Because it is likely that endothelial cells are rarely if ever exposed to a single cytokine, and based on the observation that VEGF and bFGF are often co-expressed in settings in which angiogenesis occurs in vivo, we assessed the effect of simultaneous addition of VEGF and bFGF on the in vitro angiogenic response. We found that when added separately at equimolar concentrations, bFGF was about twice as potent as VEGF. However, when added simultaneously, VEGF and bFGF induced a synergistic invasive response (Figs. 4 and 5A), which occurred with greater rapidity than the response to either cytokine alone (19). The synergistic interaction between VEGF and bFGF was subsequently confirmed in an independent study using a related three-dimensional in vitro assay (110), and synergism has been observed in vivo in a rabbit model of hind-limb ischemia (111) as well as in the rat sponge implant model (112). These results demonstrate that, when acting in concert, VEGF and bFGF have a potent synergistic effect on the induction of angiogenesis both in vivo and in vitro.

What are the mechanisms responsible for this synergistic effect? Although co-addition of VEGF and bFGF in conventional two-dimensional in vitro assays of endothelial-cell proliferation, migration, and PA-mediated extracellular proteolysis has failed to reveal synergism (113–115), synergism is detected at the level of endothelial proliferation when cells are grown in a three-dimensional collagen matrix (110,110a). An additional approach has been to determine whether bFGF and VEGF modulate expression of FGFRs and VEGFRs in monolayer culture. None of the endothelial-cell lines we have used express VEGFR-1 (50). However, although neither cytokine, either alone or in combination, is capable of significantly modulating expression of FGFR-1, bFGF increases expression of VEGFR-2 at the level of mRNA and total protein. bFGF also induces the



**Fig. 4.** Synergistic effect of co-added bFGF and VEGF on angiogenesis in vitro. (A) Endothelial cells are grown on the surface of a three-dimensional collagen gel as described in Fig. 3. Under these conditions, the cells do not invade the underlying matrix. When treated with bFGF (10 ng/mL) (B) or VEGF (30 ng/mL) (C) for 4 d, the cells invade the underlying matrix and form branching cell cords within the gel. When co-added (D), bFGF and VEGF induce an invasive response that is greater than additive. Bar = 100  $\mu$ m. Adapted from ref. 15, with copyright permission from Marcel Dekker, Inc.

ligands for this receptor, namely VEGF and VEGF-C, which may explain why in contrast to total protein, bFGF decreases cell surface VEGFR-2, possibly by promoting internalization of the VEGF/VEGFR-2 complex (S.J. Mandriota and M.S. Pepper, unpublished observation). The observation that bFGF increases both VEGF and VEGFR-2, raises the possibility that bFGF-induced in vitro angiogenesis is mediated by an autocrine VEGF/VEGFR-2 loop. Indeed, we have observed that bFGF-induced in vitro angiogenesis can be inhibited by at least 50% by co-administration of VEGF antagonists (J.-C. Tille et al., manuscript submitted). At least in vitro therefore, VEGF-induced invasion might require the presence of a second cytokine such as bFGF, which increases VEGFR-2-mediated signal transduction above a critical threshold required for mitosis, migration, and increased proteolytic activity, all of which are necessary for the formation of new capillary sprouts. It is important to bear in mind that if we wish to extrapolate the results from our in vitro studies to the in vivo setting, we must assume that in vivo, endothelial cells are able to respond to exogenous bFGF through its interaction with the cell surface (e.g., FGFRs or HSPGs). Findings obtained thus far in vivo do not allow us to comment on this possibility. Nor do they allow us to rule out the possibility that in vivo, bFGF is acting



**Fig. 5.** Interactions between angiogenesis-regulating cytokines. Randomly selected fields of bovine microvascular endothelial cell monolayers treated with VEGF, bFGF, and/or TGF- $\beta$ 1 for 4 d were photographed at a single level beneath the surface monolayer. Endothelial-cell invasion was quantitated by measuring the total additive length of all cell cords, and values are expressed as mean  $\pm$  SEM from at least three experiments per condition. **(A)** Synergistic effect of bFGF and VEGF on in vitro angiogenesis. VEGF-induced invasion is dose-dependent. Co-addition of bFGF at a single concentration induces an invasive response that was greater than additive. **(B)** Biphasic effect of TGF- $\beta$ 1 on VEGF-induced in vitro angiogenesis. VEGF (100 ng/mL)-induced invasion is potentiated by TGF- $\beta$ 1 at 100 pg/L–3 ng/mL and inhibited by TGF- $\beta$ 1 at 10 ng/mL. (A) is adapted from ref. 19 and (B) is adapted from ref. 117, with copyright permission from Academic Press.

indirectly by inducing the production of a positive regulator by nonendothelial cells, which in turn contributes to the synergistic effect. Recent observations using inhibitory anti-bFGF antibodies indicate that VEGF-induced in vitro angiogenesis as well as the VEGF-induced increase in urokinase-type PA and tissue-type PA activity are dependent on endogenous bFGF (115a). These findings raise the possibility that VEGF-induced endothelial responses are dependent on endogenous bFGF. Taken together, these findings demonstrate that VEGF- and bFGF-mediated in vitro angiogenic responses are

themselves a form of synergism, in that they are dependent on the presence of both cytokines concomitantly.

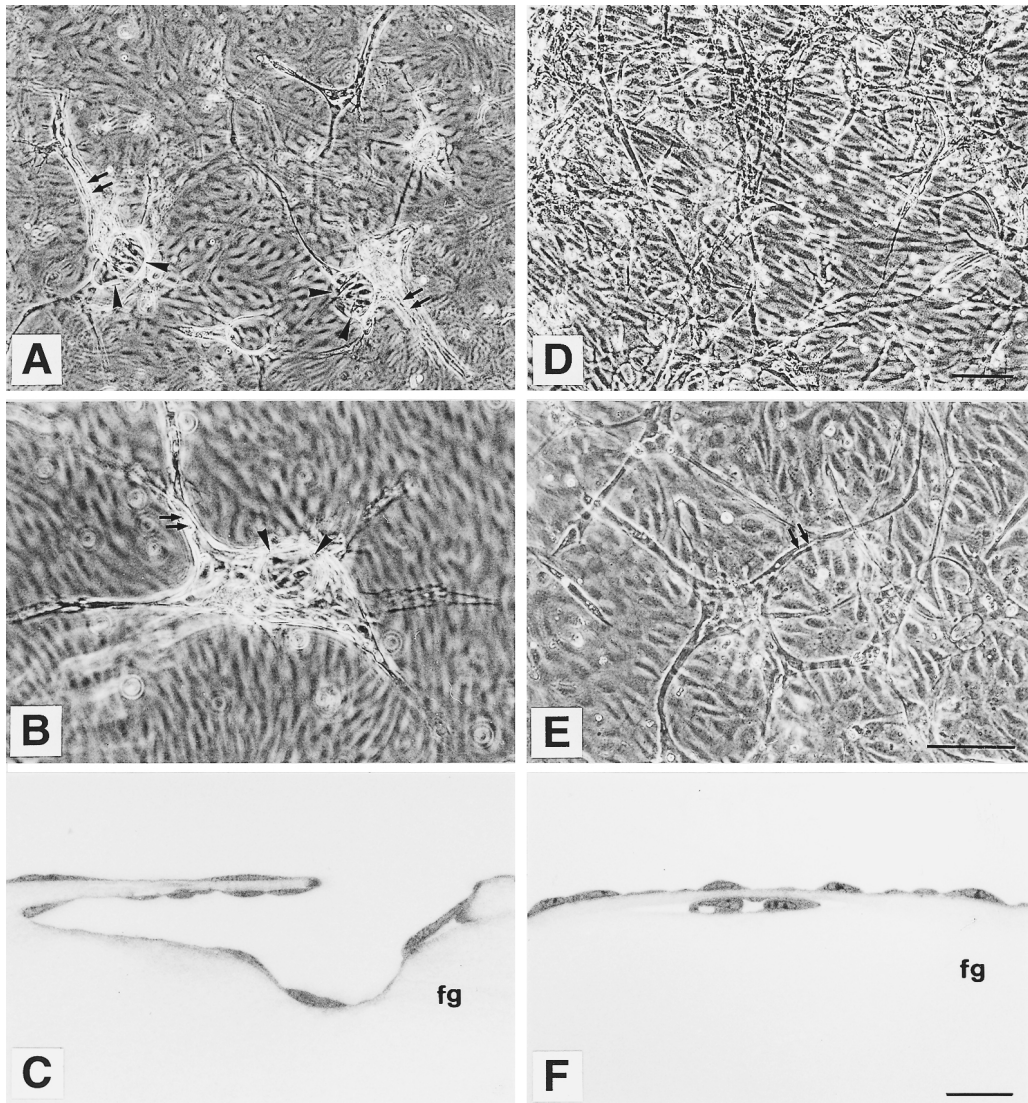
What is the significance of these findings? First, the levels of VEGF and its receptors appear to be unchanged in certain organs and tumors in which there is extensive angiogenesis (*see above*). Although, as mentioned earlier, some controversy still exists concerning its role as an endogenous regulator of angiogenesis, it has recently been demonstrated that bFGF is expressed in the mammary gland during early pregnancy, i.e., during the phase of active angiogenesis, and declines during late pregnancy and lactation (116), at which time angiogenesis is markedly reduced. In addition, induction of the vascular phase in certain experimental tumors in which VEGF levels are unchanged appears to coincide with the export of bFGF from tumor cells (117). These findings suggest that VEGF-dependent angiogenesis in certain settings may require the presence of a second positive regulator. Although this hypothesis is based on findings obtained from our *in vitro* invasion assay, in which bFGF is clearly angiogenic, endogenous regulators of synergism might include cytokines other than bFGF as well as hypoxia. Second, our observations might provide a partial explanation for the observation that VEGF and its receptors are expressed, sometimes at relatively high levels, in adult tissues in which angiogenesis is apparently not occurring. The lack of angiogenesis in these tissues might indicate the absence of a synergistic co-factor required for VEGF-dependent angiogenesis (and/or the presence of a dominant, negative regulator). Third, modulation of new capillary blood-vessel formation may serve as an alternative/adjunct to current therapeutic modalities in several angiogenesis-associated diseases. At first sight, the redundancy of angiogenesis-regulating cytokines might suggest that therapeutic strategies based on neutralization of single angiogenic factors might be unrealistic. If however the synergism which we have observed *in vitro* is relevant to the endogenous regulation of angiogenesis *in vivo*, angiogenesis would be more prominent in tumors or other pathologic settings in which more than one angiogenic factor is produced. This may justify anti-angiogenesis strategies based on the neutralization of a single angiogenic factor, because this would reduce the synergistic effect. On the other hand, recent work has demonstrated that administration of angiogenic factors can enhance the growth of collateral vessels in animal models of coronary, peripheral and cerebral arterial occlusion (reviewed in ref. 9). In this context, we predicted that the therapeutic effect of co-addition of two cytokines whose interaction is synergistic would be greater than that derived from addition of one of these cytokines alone (19). Support for this hypothesis has been provided by an *in vivo* study in which co-administered VEGF and bFGF synergized in the induction of collateral blood-vessel formation in a rabbit model of hind-limb ischemia (111). In summary, our findings on the synergism between VEGF and bFGF may have relevance both to understanding the mechanisms of angiogenesis as well as to positive and negative therapeutic modulation of this process. Furthermore, our observations highlight the importance of a three-dimensional environment for the study of angiogenesis *in vitro*: had we relied exclusively on traditional two-dimensional assays of proliferation, migration, or proteolysis, synergism between VEGF and bFGF would not have been detected.

### ***Biphasic Effect of TGF $\beta$ -1***

TGF $\beta$ -1 is an angiogenesis-modulating cytokine that has been described as being pro- or anti-angiogenic depending on the nature of the assay (*see previous Subheading*). These findings have led to the proposal that this cytokine has different functions on vessel

formation at different stages of the angiogenic process. Thus when acting directly on endothelial cells, TGF- $\beta$ -1 may inhibit invasion and vessel formation, and once sprout formation has occurred, it may be necessary for the inhibition of further endothelial-cell replication and migration, and may induce vessel organization and functional maturation. An additional possibility is that TGF- $\beta$ -mediated angiogenesis is contextual, in that this requires the presence of other positive regulators, whose activity is potentiated in the presence of TGF- $\beta$ . Furthermore, because 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, the effect of a wide range of concentrations of TGF- $\beta$ 1 on the response of microvascular endothelial cells to VEGF or bFGF was assessed in our in vitro model of angiogenesis, which assays both for endothelial cell invasion and capillary morphogenesis. Unlike VEGF and bFGF, 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 markedly increased when TGF- $\beta$ 1 was co-added at 200–500 pg/mL, and decreased when TGF- $\beta$ 1 was added at 5–10 ng/mL (Fig. 5B and 6) (118). Similar findings have been reported with large-vessel (aortic) endothelial cells (119), and a biphasic effect has been noted when endothelial cells 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 (120,121). This biphasic effect is in accord with the observations that endothelial-cell wound-induced migration (122,123) and invasion of three-dimensional collagen gels (123) or the explanted amnion (124) 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 (118; M.S. Pepper, unpublished observation). With respect to proliferation, although one study has demonstrated a similar biphasic effect of TGF- $\beta$ 1 in subconfluent endothelial-cell cultures (125), the vast majority of studies have demonstrated that this cytokine is inhibitory over a wide range of concentrations (reviewed in ref. 22). Finally, not only was invasion affected by TGF- $\beta$ 1 in a concentration-dependent manner, but lumen formation in the resulting tube-like 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 reduced lumen diameter to a more physiological size (Fig. 6) (118). Addition of TGF- $\beta$ 1 at a 10-fold higher concentration, namely 5 ng/mL, completely inhibited lumen formation in the invading cell cords (126). It is interesting to note that 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, are 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 (127,128). Thus at the dose of TGF- $\beta$ 1 that potentiates bFGF- or VEGF-induced invasion, an optimal balance between proteases and protease inhibitors might be achieved, which allows for focal pericellular 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. The observation that PAI-1 competes with the urokinase-type PA receptor and integrin  $\alpha_v\beta_3$  for binding to vitronectin (129,130), also raises the possibility that higher concentrations of TGF- $\beta$ 1-induced PAI-1 might inhibit migration by interfering with cell



**Fig. 6.** 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* Fig. 5B 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**). Bars in (**A**), (**D**) = 100  $\mu$ m, in (**B**), (**E**) = 50  $\mu$ m, and in (**C**), (**F**) = 20  $\mu$ m. Adapted from ref. 117, with copyright permission from Academic Press.

adhesion. A second explanation may come from the observation that TGF- $\beta$ 1 decreases VEGFR-2 expression at concentrations that inhibit in vitro angiogenesis (50,118). A third possibility might be related to alterations in integrin expression and ligand-binding affinity (131,132). Because TGF- $\beta$  alters endothelial-cell integrin expression (reviewed in ref. 22; 133a), 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 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 (119; M.S. Pepper, unpublished data). Differential regulation of integrin expression may also contribute to the alterations in lumen size that are seen with different concentrations of TGF- $\beta$ 1 in fibrin gels. Finally, the complete absence of lumen formation at 5 ng/mL may imply 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.

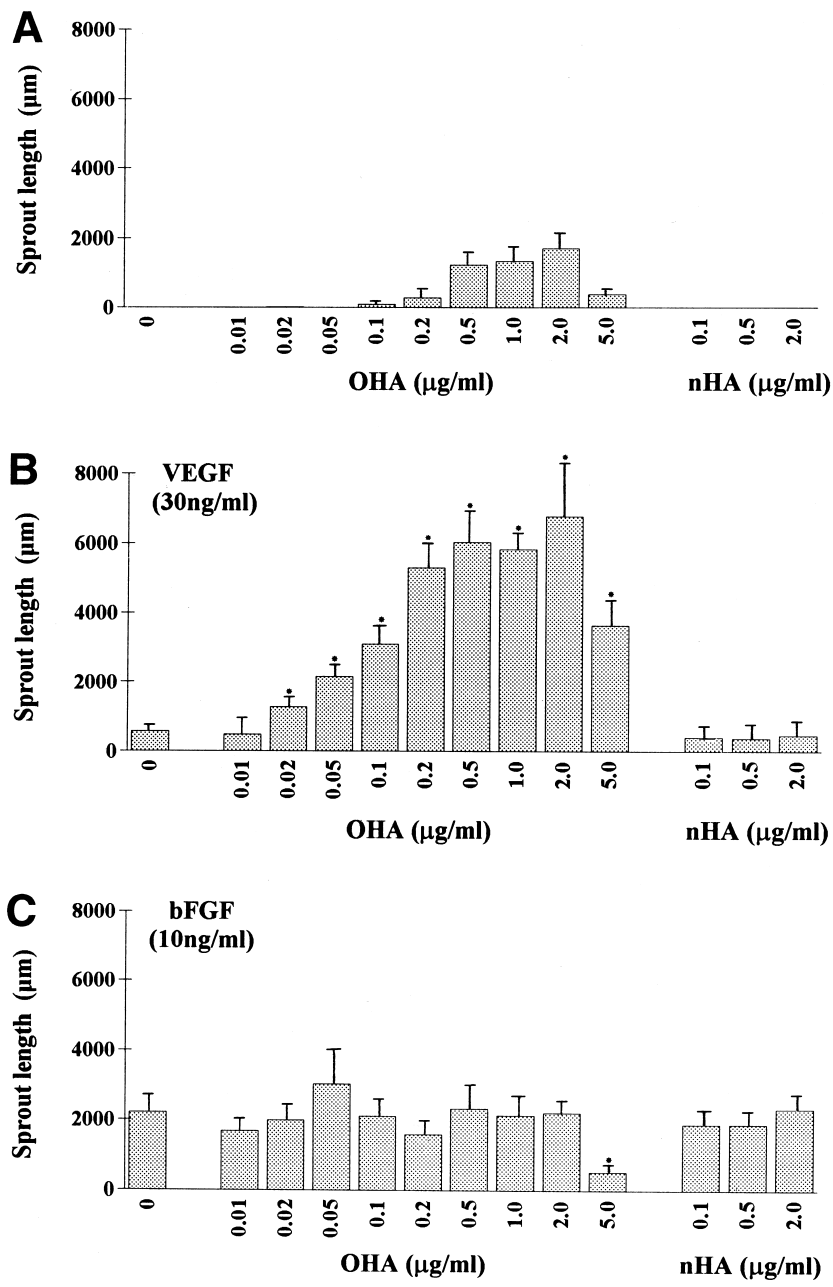
### ***Synergism Between Hyaluronan Oligosaccharides and VEGF***

In addition to diffusible cytokines, extracellular matrix components including collagens, fibronectin, laminin, and other glycoproteins have been shown to be important in angiogenesis. Hyaluronan (hyaluronic acid, HA), a glycosaminoglycan composed of repeating disaccharide units of D-glucuronate and N-acetylglucosamine, is one of the most abundant extracellular matrix constituents. Although initially considered primarily as a structural moiety, HA has now emerged as an important signaling molecule. Thus, HA is involved in a number of developmental processes, and has been shown to promote cell proliferation, differentiation, and motility. The diverse biological activities of HA are believed to be mediated, at least in part, through interaction with specific cell-surface receptors such as CD44, which results in activation of intracellular signaling events. HA obtained from different tissue sources exhibits considerable variation in size, and its biological activity has been shown to be critically dependent on molecular mass in a number of experimental systems, including angiogenesis. Thus, native high molecular-weight HA is anti-angiogenic, whereas HA degradation products of specific size (3–10 disaccharide units) stimulate endothelial-cell proliferation and migration, and induce angiogenesis in the chick chorioallantoic membrane (CAM) assay, in rat skin, and in a cryoinjured skin-graft model (reviewed in ref. 15).

We have used our collagen gel assay to determine whether HA and/or its degradation products influence endothelial-cell invasion. We found that like bFGF and VEGF (*see above*), oligosaccharides of HA (OHA) induce endothelial cells to invade the underlying gel within which they form capillary-like tubes, with an optimal effect at approx 0.5–2.0  $\mu$ g/mL OHA. Co-addition of OHA (0.5–2.0  $\mu$ g/mL) and VEGF (30 ng/mL), but not of OHA and bFGF (10 ng/mL), induced a striking in vitro angiogenic response that was greater than the sum of the effects elicited by either agent separately. In contrast to OHA, native high molecular-weight HA (nHA) was consistently inactive, either when added alone or when added in combination with VEGF or bFGF (Fig. 7) (133).

The mechanisms by which OHA stimulate endothelial-cell invasion of collagen gels are not known. However, in bovine endothelial cells, OHA have recently been found to induce phosphorylation and activation of MAP kinase (M. A. Slevin et al., manuscript





**Fig. 7.** Synergistic effect of OHA and VEGF on angiogenesis in vitro. Confluent monolayers of endothelial cells on collagen gels were treated with OHA or nHA at the indicated concentrations (A), cotreated with OHA or nHA and VEGF (B), or cotreated with OHA or nHA and bFGF (C). Invasion was quantified after 4 d and results are expressed as mean total additive length (in m)  $\pm$  s.e.m. of all sprouts that had penetrated beneath the surface monolayer in three randomly selected photographic fields from each of at least 3 separate experiments per experimental condition. \* $p < 0.001$ . OHA stimulate angiogenesis in vitro in a dose-dependent manner (A) and synergize with VEGF (B) but not with bFGF (C). nHA has no significant effect on invasion, either when added alone (A) or in combination with VEGF or bFGF (B,C). Adapted from ref. 132, with copyright permission from Williams and Wilkins.

submitted), as well as upregulation of early response genes such as *c-fos*, *c-jun*, and *jun-B* (134), which are known to control the expression of a number of other genes including those of matrix-degrading proteases. Because HA receptors, including a CD44-like transmembrane protein, have been identified in bovine endothelial cells (135–138), it is conceivable that OHA promotes endothelial-cell invasion and tube formation by activating intracellular signaling pathways that ultimately result in modulation of pericellular proteolysis. The molecular mechanisms responsible for the specific synergistic interaction between OHA and VEGF in the induction of angiogenesis *in vitro* are also unknown. OHA and VEGF might activate independent but converging intracellular signaling pathways, resulting in a synergistic effect, or OHA might upregulate expression of high-affinity VEGF receptors such as Flk-1. Alternatively, as has been shown for heparin-like glycosaminoglycans, OHA may complex with VEGF molecules, thereby increasing ligand half-life or facilitating multivalent VEGF binding and receptor oligomerization.

Although exogenously-added OHA promotes angiogenesis in *in vitro* and *in vivo* assays, it has not yet been clearly established whether endogenous OHA can act as a physiological regulator of angiogenesis. Several observations nonetheless suggest the potential involvement of OHA in angiogenesis associated with reparative and pathological processes (reviewed in refs. 15, 139). In a number of clinical settings, including wound healing, rheumatoid arthritis (RA), vasoproliferative retinopathy, and cancer, angiogenesis occurs in close proximity to HA-rich tissues or fluids. HA catabolism has been shown to be very rapid: in skin for instance, up to 25% of injected HA is degraded locally in 24 h. Although most vertebrate hyaluronidases so far characterized are lysosomal, HA-degrading activities with near neutral pH optima have recently been shown to be expressed by tumor cells and to induce angiogenesis *in vivo* (140, 141). It is therefore conceivable that breakdown of high molecular-weight HA occurs in the extracellular space during pathological processes. This would result in the production of HA oligosaccharides, which in addition to being angiogenic on their own, could synergize with VEGF, which has been shown to be expressed in all the clinical settings mentioned earlier. Based on our *in vitro* studies, we propose that the potential therapeutic effect of co-administration of VEGF and OHA deserves to be investigated in situations that would benefit from stimulation of angiogenesis, particularly in animal models of coronary or peripheral arterial insufficiency.

## FUTURE PERSPECTIVES

Although an enormous amount of progress has been made in identifying cytokines that regulate blood-vessel formation either positively or negatively, many important fundamental questions remain. A number of issues which merit further investigation are discussed below.

First, although it is currently assumed that vasculogenesis is limited to early development, the observation that circulating endothelial-cell precursors contribute to new blood-vessel formation in postnatal life is likely to significantly enhance our understanding of angiogenesis. Two important questions raised by this seminal observation include the origin of circulating precursors as well as their precise relationship to angioblasts (and hemangioblasts). From a therapeutic point of view, the existence of circulating precursors may have important implications both for stimulation and inhibition of angiogenesis.

Second, both vasculogenesis and angiogenesis result in the formation of simple endothelial-lined capillary-like tubes, and a significant body of information is now available concerning the mechanisms of these processes. However, the mechanisms of vessel-wall maturation, which include differentiation of contractile cells (pericytes and smooth-muscle cells) from

adjacent mesenchyme as well as their organization into a functional vessel, remain for the present poorly understood. Nonetheless, in addition to TGF- $\beta$  and PDGF as well as their respective cell-surface receptors (*see above*), evidence is at present emerging that implicates a third cytokine family, namely the angiopoietins, in the process of vessel-wall maturation. Angiopoietin-1 has recently been identified as the ligand for the TIE-2 tyrosine kinase receptor (*142*), and gene-deletion studies have revealed an important role for this ligand-receptor pair in vessel-wall maturation (*143–145*). It appears that angiopoietin-1 is expressed by stromal cells and TIE-2 by endothelial cells, and that in the absence of either the ligand or its receptor, smooth-muscle cell differentiation and recruitment are significantly reduced, resulting in the formation of dilated endothelial-lined vascular channels that fail to mature into arteries and veins. It will now be important to know which genes (including possibly TGF- $\beta$  and PDGF) are regulated by angiopoietin-1 in TIE-2 expressing endothelial cells.

Third, we are clearly entering an era in which a genetic approach to understanding the pathogenesis of vascular disorders (reviewed in ref. *146*) will require identification of mutations in endothelial-cell receptor tyrosine kinases (VEGFRs-1, -2 and -3; TIE-1 and TIE-2) and other molecules involved in new blood-vessel formation. Mutations in these receptors would be expected to be important in the pathogenesis of vascular malformations, and may play a role in the development of hemangiomas as well as in chronic vasoproliferative disorders (cancer, arthritis, retinopathy), which are likely to be multigenic in origin. It will also be important to determine whether increased susceptibility/predisposition to some of these chronic disorders is linked to a genetically-based pro-angiogenic state that may result from increased activity of positive regulators (angiogenic factors and receptors) or decreased activity of inhibitors. With respect to vascular malformations, an activating mutation in Tie-2 has recently been shown to segregate with an autosomal dominant form of venous malformation in two independent families (*147*), and candidate loci for the Klippel-Trenauny-Weber syndrome (*148*) and cerebral cavernous malformations (*149–151*) have been identified.

Finally, novel pharmacological and gene-therapy approaches need to be developed for the stimulation and inhibition of angiogenesis. In addition, extensive clinical evaluation of current therapeutic strategies will almost certainly require testing in multicenter trials. It will also be important to develop animal models that are relevant to angiogenesis-associated diseases, and that could be exploited in the search for novel therapeutic strategies. For example, the establishment of transgenic mice by site-directed overexpression of positive regulators (e.g., VEGF in the skin as a model for hemangiomas) could be combined with assessment of the therapeutic potential of novel angiogenesis inhibitors.

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