Mechanisms of Normal and Tumor-Derived Angiogenesis

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Preface

Often those diseases most evasive to therapeutic intervention usurp the human body’s own cellular machinery or deregulate normal physiologic processes for propagation. Tumor-induced angiogenesis is a pathological condition that results from aberrant deployment of normal angiogenesis, an essential process in which the vascular tree is remodeled by the growth of new capillaries from pre-existing vessels. Normal angiogenesis ensures that developing or healing tissues receive an adequate supply of nutrients. Within the confines of a tumor, the availability of nutrients is limited by competition amongst actively proliferating cells, and diffusion of metabolites is impeded by high interstitial pressure (1). As a result, tumor cells induce the formation of a new blood supply from the pre-existing vasculature, and it affords tumor cells the ability to survive and propagate in a hostile environment. Because both normal and tumor-induced neovascularization fulfill the essential role of satisfying the metabolic demands of a tissue, the mechanisms by which cancer cells stimulate pathological neovascularization mimic those utilized by normal cells to foster physiological angiogenesis.

The following review investigates mechanisms of tumor-induced angiogenesis. The strategies used by cancer cells to develop their own blood supply are discussed in relation to those employed by normal cells during physiological angiogenesis. With an understanding of blood vessel growth in both normal and abnormal settings we are better suited to design effective therapeutics for cancer.
Normal Angiogenesis

The adult vasculature is derived from a network of blood vessels that is initially created in the embryo by vasculogenesis, a process whereby vessels are formed de novo from endothelial cell precursors termed angioblasts (2). During vasculogenesis, angioblasts proliferate and coalesce into a primitive network of vessels known as the primary capillary plexus. The endothelial cell lattice created by vasculogenesis then serves as a scaffold for angiogenesis.

After the primary capillary plexus is formed, it is remodeled by the sprouting and branching of new vessels from pre-existing ones in the process of angiogenesis. Most normal angiogenesis occurs in the embryo where it establishes the primary vascular tree as well as an adequate vasculature for growing and developing organs (3). Angiogenesis occurs in the adult during the ovarian cycle and in physiological repair processes such as wound healing (4). However, very little turnover of endothelial cells occurs in the adult vasculature (5).

Maturation and remodeling of newly-formed microvessels is accomplished by the coordination of several diverse processes in the microvasculature (6) that are summarized in figure 1. In order for new blood vessel sprouts to form, mural cells (pericytes) must first be removed from the branching vessel. Endothelial cell basement membrane and extracellular matrix is then degraded and remodeled by specific proteases such as matrix metalloproteinases (7), and new matrix synthesized by stromal cells is then laid down. This new matrix, coupled with soluble growth factors, fosters the migration and proliferation of endothelial cells. After sufficient endothelial cell division has occurred,
endothelial cells arrest in a monolayer and form a tube-like structure. Mural cells (pericytes in the microvasculature, smooth muscle cells in larger vessels) are recruited to the ablumenal surface of the endothelium, and vessels uncovered by pericytes regress. Blood flow is then established in the new vessel.

Under normal circumstances, angiogenesis is a highly ordered process under tight regulation because it requires inducing quiescent endothelial cells in a monolayer to divide and spread the vascular network only to the extent demanded by the demands of growing tissues. Many positively and negatively acting factors influence angiogenesis; among these are soluble polypeptides, cell-cell and cell-matrix interactions, and hemodynamic effects. The soluble growth factors, membrane-bound molecules, and mechanical forces that mediate these signals are summarized in Table 1 and discussed below in terms of their contribution to the mechanism of normal angiogenesis.

**Soluble Factors**

**Vascular Endothelial Growth Factor (VEGF)**

Perhaps the most well characterized angiogenic factor is vascular endothelial growth factor (VEGF). Alternative splicing of a single gene generates six isoforms of VEGF composed of 121, 145, 165, 183, 189, and 206 amino acids, though VEGF\(_{165}\) is the most commonly expressed isoform (8,9). Interestingly, although all isoforms demonstrate identical biological activities, VEGF\(_{121,165}\) are secreted into the extracellular environment while VEGF\(_{189,206}\) and to some extent VEGF\(_{189}\) remain cell- or matrix-associated via their affinity for heparan sulfates (10). VEGF is a highly conserved disulfide-bonded dimeric
glycoprotein of molecular weight 34-45 kDa that loses biological activity in the presence of reducing agents (11).

A wide variety of human and animal tissues express low levels of VEGF, but high levels are produced where angiogenesis is required such as fetal tissue, the placenta, and the corpus luteum and in a vast majority of human tumors (8). Many mesenchymal and stromal cells produce VEGF (12). VEGF binds to at least three known tyrosine kinase receptors: flt-1 (VEGFR1) (13), kdr/flk-1 (VEGFR2) (14), and flt-4 (VEGFR3) (15). Functional VEGF receptors were originally characterized as endothelial-cell specific (16), but they have recently been found on other normal cell types including vascular smooth muscle cells (17) and monocytes/macrophages (18). Therefore, VEGF mediates several actions derived from varied sources and may utilize endothelial as well as other cell types as effectors.

VEGF receptors belong to the “7-Ig” or flt gene family characterized by seven extracellular immunoglobulin-like domains, one membrane spanning segment, and a conserved intracellular tyrosine kinase domain (19,20). VEGFR-1 has the highest affinity for VEGF ($K_d = 10-30$ pM) (13) and it is expressed in the endothelium of adult and embryonic mice as well as in healing skin wounds (21) VEGFR-1 is also expressed on vascular smooth muscle cells (17) and monocytes (18). Interestingly, no direct migratory, proliferative, or cytoskeletal effects appear to be mediated by VEGFR-1 (22). Nonetheless, VEGFR-2, a tyrosine kinase with lower affinity ($K_d \sim 75-760$) for VEGF than VEGFR-1, mediates endothelial cell mitogenesis, chemotaxis, and shape changes (22). VEGFR-2 is expressed on endothelial and hematopoietic precursors (23) as well as proliferating endothelial cells in the embryo (24), but in quiescent endothelium of the
adult vasculature, VEGFR-2 RNA is dramatically reduced (24). A third receptor tyrosine kinase, VEGFR-3, is mainly expressed adult lymphatic endothelium and may be involved in lymphangiogenesis (25). VEGFR-3 does not bind VEGF but rather complexes VEGF-related proteins VEGF-C and VEGF-D (see below).

A VEGF receptor distinct from flt family members is neuropilin. Neuropilin-1 is a neuronal receptor for members of the collapsing/semaphoring family (26, 27). However, neuropilin-1 is also expressed on normal endothelial cells (28). Neuropilin-1 binds VEGF_{165}, but not VEGF_{121}, fosters its binding to VEGFR-2, and enhances its chemotactic effects (28). Neuropilin-1 may be involved in angiogenesis because in transgenic mice, neuropilin-1 overexpressing mice have a high density of dilated blood vessels and die at embryonic day 17.5 (29). Furthermore, neuropilin-1-deficient mice exhibit disrupted blood vessels and insufficient development of vascular networks (30).

VEGF exerts several effects on vascular endothelial cells. It was initially isolated from tumor cell conditioned medium as a protein that increased the permeability of small blood vessels to circulating metabolites (31). VEGF may increase endothelial cell permeability by enhancing the activity of vesicular-vacuolar organelles, clustered vesicles in endothelial cells lining small vessels that facilitate transport of metabolites between lumenal and ablumenal plasma membranes (32). Alternatively, VEGF may enhance permeability by loosening adherens junctions between endothelial cells in a monolayer via rearrangement of cadherin/catenin complexes (33-34). Increased vascular permeability may allow for the extravasation of plasma proteins and formation of extracellular matrix favorable to endothelial and stromal cell migration (35). In addition, VEGF stimulates endothelial cell the production of plasminogen activators (u-PA and t-
PA) (36), plasminogen activator inhibitor-1 (PAI-1) (36), and interstitial collagenase (37). Therefore, VEGF induces a balanced system of proteolysis that can remodel extracellular matrix components necessary for angiogenesis.

Many laboratories have described VEGF’s ability to stimulate endothelial cell proliferation in vitro (38-40). This effect is specific to vascular endothelial cells because VEGF does not induce proliferation of other cell types such as smooth muscle cells, corneal endothelial cells, lens epithelial cells, fibroblasts, and adrenal cortex cells (39-40). VEGF also enhances endothelial cell migration in vitro (41), an initial step in the branching of endothelium from the pre-existing vasculature. Interestingly, VEGF inhibits endothelial cell apoptosis (42) and thus acts as a survival factor. Thus, VEGF demonstrates many effects on endothelial cells.

Many experiments also implicate VEGF in angiogenesis in vivo. In the cornea and healing bone grafts, VEGF induces growth of capillary sprouts from pre-existing blood vessels (38). Mice deficient in the gene for VEGF and the VEGF receptor Flk-1 are virtually devoid of vascular structures and are thus defective in the very early events in blood vessel formation that characterize vasculogenesis (43-44). However, Flt-1 (VEGF receptor-1)- null mice form vascular structures but are impaired in the assembly of vessels (45). Therefore, Flt-1 appears to have a role in vascular remodeling in angiogenesis rather than creation of blood vessels de novo in vasculogenesis. Interestingly, these results suggest that Flk-1 and Flt-1 effect different signaling cascades upon VEGF binding. The molecular nature of these differences, however, remains unclear.
VEGF production is regulated by local oxygen concentration (46). Hypoxia stimulates VEGF production through the binding of hypoxia inducible factor (HIF) to cis-elements in the VEGF promoter, and HIF increases VEGF gene transcription and mRNA stability (47). Therefore, not only does VEGF stimulate the entire angiogenic process through its various effects on endothelial cells, but it also acts as a physiological sensor that stimulates angiogenesis upon demand of low oxygen concentration. Although this mechanism ensures that developing tissues and hypoxic environments become vascularized and oxygenated, as described below, it also underlies pathologies associated with angiogenesis.

Interestingly, VEGF is one member of a family of growth factors that share significant amino acid homology. Platelet-derived growth factor (PDGF) shares between 18 and 24% total amino acid homology (48, 49) and eight conserved cysteine residues (48) with VEGF. Though these conserved cysteine residues suggest a common mode of intra- and interchain disulfide bond formation (50), PDGF and VEGF bind distinct receptors. Other VEGF-related molecules bind VEGF receptors. For example, placenta growth factor (PlGF), which shares 53% amino acid identity with the PDGF-like region in VEGF (51), binds VEGFR-1 (52) and neuropilin-1 (53). Under normal conditions, PlGF is preferentially expressed in placenta (54). Interestingly, embryonic angiogenesis is unaffected in PlGF-deficient mice (55). However, PlGF may synergize with VEGF by forming PlGF/VEGF heterodimers (56,57) or by potentiating VEGF signaling (52).

VEGF-B is a growth factor that shares ~43% amino acid sequence identity with VEGF164 and 30% identity with PlGF (58). It is expressed predominantly in embryonic and adult muscle tissues and to a lesser extent in many other tissues such as brain, lung,
and kidney (58). VEGF-B binds and activates VEGFR-1 as well as neuropilin-1 (59). Because mice deficient in VEGF-B are overtly normal and exhibit only minor cardiac defects (60,61), VEGF-B does not appear necessary for angiogenesis. Nonetheless, VEGF-B is mitogenic for endothelial cells (58) and, similar to PlGF, VEGF-B may cooperate with VEGF through its ability to from heterodimers with VEGF (58,62).

Other VEGF-related molecules share less homology with VEGF. VEGF-C and VEGF-D form a subfamily of their own based on their structural similarity (9). VEGF-C (63) and VEGF-D (64), respectively, share 32% and 31% identity with VEGF_{121} and VEGF_{165}. Interestingly, both bind and activate VEGFR-2 and VEGFR-3 and are mitogenic for endothelial cells \textit{in vitro} (63,64). This mitogenic activity, however, is significantly less potent (5-100 fold) than that induced by VEGF (63,64) Both VEGF-C (65,66) and VEGF-D (67) stimulate angiogenesis \textit{in vitro} and \textit{in vivo}. Nonetheless, localization of VEGF-C and its preferred receptor VEGFR-3 suggest that VEGF-C may play a paracrine role in angiogenesis of lymphatic vessels during development (68) and maintenance of differentiated lymphatic endothelium in the adult (25). VEGF-D is induced by c-fos (69) and its high expression in embryonic lung suggests a role in lung development (70). Physiological roles of VEGF-C and VEGF-D are, however, still largely undefined.

Finally, VEGF-E refers to a group of VEGF-related proteins encoded by the orf virus, a parapoxvirus that infects sheep, goats, and occasionally humans, that share between 16 and 27% amino acid identity to mammalian VEGF (71). Interestingly, these viral proteins have retained VEGF function because they signal through VEGFR-2 and stimulate angiogenesis \textit{in vitro} and \textit{in vivo} (72). Furthermore, orf virus lesions exhibit dermal vascular endothelial proliferation and dilation (71). VEGF-E may be a product of genetic
drift from a VEGF gene acquired by the orf virus from a mammalian host (71). Use of VEGF and a VEGF-related protein’s actions for propagation and growth in organisms as distantly related as mammals and viruses underlies its functional importance and versatility in fostering processes inherent for survival.

**Angiopoietins and Tie Receptors**

The angiopoietins belong to a family of secreted proteins, four of which have been identified to date, that bind Tie family receptors. Angiopoietins and Tie receptors have been reported to play a major role in angiogenesis. Tie receptors were discovered before angiopoietins in attempts to characterize novel tyrosine kinases in endothelium and heart tissue (73-74).

**Tie Receptors**

Expression patterns of the two Tie receptors identified so far, Tie1 and Tie2 (Tek), mimic those of VEGF receptors and appear to be specific for vascular endothelium (75), though cells in the hematopoietic cell lineage such as the tumor cell line K562 (76) also express Tie receptors. Tie1 mRNA is robustly expressed in embryonic angioblasts (endothelial cell precursors), vascular endothelium, and endocardium, while in adult tissues Tie1 mRNA is expressed weakly in endocardium but strongly in lung capillaries (77). Tie2 mRNA shows a similar embryonic localization but is detected earlier (day 7.5) than Tie1 (day 8.5), and it is expressed weakly in adult endocardium and vasculature endothelium (74). Therefore, expression patterns of both Tie receptors suggest a role in developmental angiogenesis.
Genetic studies also implicate the Tie receptors in angiogenesis. Tie1-deficient mice develop extensive edema and hemorrhage and die either perinatally (78) or at embryonic day 14.5 (79). Although blood vessels are established in these mice, vascular integrity is severely compromised suggesting that Tie1 is not necessary for endothelial cell differentiation in vasculogenesis but rather for integrity and survival of endothelial cells during angiogenesis (79). Tie2-deficient mice die embryonically and exhibit a reduction in the number of endothelial cells in blood vessels compared to wild-type littermates, underdeveloped hearts, vasodilation, and abnormal vascular network formation including lack of sprouting and branching vessels (78,80). Therefore, whereas these studies suggest that both Tie1 and Tie2 are important for vascular integrity, they imply that Tie2 is crucial for sprouting and branching of vessels characteristic of angiogenesis.

**Angiopoietins**

The angiopoietins are ~70 kDa secreted ligands for Tie2. A ligand for Tie1 has not yet been identified. mRNA for angiopoietin-1 (Ang1), the most extensively characterized member of this family, is found at embryonic day 9-11 in heart myocardium surrounding the endocardium and later in mesenchyme surrounding blood vessels (81). Although human neuroepithelioma and mouse myoblast cell lines are sources of Ang1, *in situ* localization studies suggest that mesenchymal cells closely associated with endothelium produce Ang1 (6). Interestingly, Ang1 does not induce endothelial cell proliferation or tube formation *in vitro* (81), but it does stimulate sprout formation from confluent endothelial cells cultured on microcarrier beads and embedded in three dimensional fibrin gels (82). Accordingly, mice deficient in Ang1 exhibit many defects similar to those seen
in Tie2-deficient mice: a grossly normal primary vasculature develops, but the mice die at embryonic day 12.5 because of incomplete vascular remodeling (83). The most severe defects are in the heart, where endocardial and trabecular development is notably impaired. Furthermore, branching of the vascular network and organization into large and small vessels is defective, blood vessels are dilated, and periendothelial cells are absent from underdeveloped tissue folds that are thought to be involved in normal vessel branching. When overexpressed in transgenic mice, Ang1 induces blood vessels that are more numerous, more highly branched, and larger in diameter than those in wild-type mice (84). In addition, overexpression of Ang1 causes blood vessels to be resistant to leakage induced by inflammatory agents or coexpressed VEGF (85). Hence, the vessel branching and remodeling stimulated by Ang1 signaling appears to be mechanistically related to Ang1’s ability to increase the girth and stability of endothelium in newly formed angiogenic sprouts.

Another angiopoietin family member, angiopoietin-2 (Ang2), was first characterized as a structural homolog of Ang1 that bound Tie2 and antagonized Ang1 (86). However, even though Ang2 blocks Ang1-mediated Tie2 autophosphorylation in endothelial cells, which express endogenous Tie2, Ang2 stimulates Tie2 autophosphorylation in NIH 3T3 cells ectopically expressing Tie2 (86). Thus, Ang2 may be an Ang1 antagonist only in the context of the vasculature. Ang2 mRNA is expressed embryonically in the dorsal aorta and in punctate regions of the vasculature, and in adult tissues it is expressed in the placenta, ovary, and uterus, primary sites for angiogenesis in the adult (86). Furthermore, overexpression of Ang2 in vascular structures results in embryonic lethality with similar, yet more severe, defects (such as endothelial discontinuities) as those observed in mice
lacking Ang1 or Tie2 (86). Therefore, Ang2 antagonizes Ang1 in the vasculature \textit{in vivo} and may act as a check on Ang1/Tie2 mediated angiogenesis to prevent excessive branching and sprouting of blood vessels by promoting destabilization of blood vessels. In addition, vessel destabilization induced by Ang2 may allow angiogenic sprouts to be plastic and sensitive to remodeling factors. The angiogenic mechanism established by stimulation from VEGF and Ang1 and inhibition from Ang2 thus plays a major role in the regulation of normal blood vessel remodeling.

\textit{Fibroblast Growth Factor (FGF)}

Basic (pI = 9.6) and acidic (pI = 5) fibroblast growth factors are ubiquitously-expressed 18-25 kDa polypeptides that are members of a large family of structurally related growth regulators (87) and have been thought to play a role in normal angiogenesis. In fact, acidic FGF was the first growth factor to be associated with angiogenesis. Similar to VEGF, both bFGF and aFGF induce processes in endothelial cells \textit{in vitro} that are critical to angiogenesis. FGFs stimulate endothelial cell proliferation (88) and migration (89) as well as endothelial cell production of plasminogen activator and collagenase (90). In addition, bFGF causes endothelial cells to form tube-like structures in three-dimensional collagen matrices (91). Thus, FGFs appear to induce many processes involved in angiogenesis. Nevertheless, unlike VEGF which is mitogenic primarily for endothelial cells, FGF stimulates proliferation of most, if not all, cells derived from embryonic mesoderm and neuroectoderm, including pericytes, fibroblasts, myoblasts, chondrocytes, and osteoblasts (87).
Perhaps the most convincing evidence for a role of FGFs in angiogenesis is the fact that, like VEGF, FGFs induce sprouting of pre-existing blood vessels towards an implanted bolus \textit{in vivo} in the cornea and chick chorioallantoic membrane (92-93). Nevertheless, it appears that FGF’s do not play a major role in angiogenesis \textit{in vivo} because vascular development is normal in mice deficient in both aFGF and bFGF (94). A clue to the way in which FGF's are delivered and signal to cells \textit{in vivo} comes from the observations that aFGF and bFGF lack a signal sequence and are therefore not secreted proteins. Most FGF remains cytoplasmic or is bound to the extracellular matrix (95-96) because of an intrinsic affinity for heparin. Thus, FGF may be released upon cell disruption by an injury and might have a role in local reparative angiogenesis following tissue injury where it is deposited in the extracellular matrix. Indeed, mice deficient in FGFs display mild defects in wound healing (94). Therefore, bFGF does not appear to play a general role in all angiogenic responses but rather may be necessary for blood vessel remodeling associated with tissue repair.

\textit{Platelet Derived Growth Factor (PDGF)}

As its name suggests, platelet derived growth factor (PDGF) was originally purified from platelets; however, it has since been found in many other cell types including fibroblasts, keratinocytes, myoblasts, astrocytes, epithelial cells, and macrophages (for review see reference 97). PDGFs exist as 45 kDa homodimers (PDGF-AA or –BB) or heterodimers (PDGF-AB) composed of PDGF chains A and B. Most cells express both PDGF A and B, though a few only express only one isoform. PDGF receptors are also dimeric in nature; they are made up of complexes between \(\alpha\) and \(\beta\) subtypes (97). \(\alpha\)
receptor can bind both PDGF A and PDGF B chains while β receptor can only bind PDGF B. Therefore, of the three types of receptors (αα, αβ, and ββ), only 1 (αα) can bind all three PDGF isoforms. PDGF receptor expression follows a similar pattern as PDGF; however, a majority of cell types express only one isoform (α or β).

The effects of PDGF on vascular cells in vitro and in vivo suggest a role for this growth factor in angiogenesis. Capillary endothelial cells express PDGF receptor β and are stimulated by PDGF-BB not only to increase DNA synthesis (98-99) but also to form angiogenic chords and sprouts in vitro (99-100). Although endothelial cells produce PDGF-BB, an autocrine feedback loop for PDGF in endothelial cells is unlikely because little convincing evidence exists regarding coexpression of PDGF-BB and PDGF receptor-ββ in endothelial cells (12,99). PDGF also stimulates the proliferation of cultured smooth muscle cells and pericytes (101), both of which have been shown to express PDGF-β receptor (97). In addition, PDGF may contribute indirectly to cardiac angiogenesis as PDGF-AB induces von Willebrand factor as well as VEGF and VEGF-R2 in cardiac microvascular endothelial cells in vitro (102).

PDGF has also been shown to be important for angiogenesis in vivo. Although mice deficient in PDGF-B or PDGF receptor-β develop blood vessels that appear normal by gross inspection, they die perinatally from hemorrhage and edema and lack mesangial cells, the counterparts of pericytes in the kidney (103-104). Closer inspection indicates that a lack of pericytes (PDGF β receptor-positive mural cells) in the microvasculature of these mutant mice is responsible for capillary dilation and leakiness (105). Interestingly, PDGF β receptor-positive mural cells are found around arteries in these mutant mice. Further study indicated that pericytes are initially recruited to microvessels independent
of PDGF, but proliferation and migration of pericytes along angiogenic sprouts is mediated by PDGF (106). However, because pericytes were localized indirectly by PDGF receptor (105) as well as desmin and smooth muscle actin (106) staining in these studies, accurate delineation of microvascular pericytes in control and mutant mice remains questionable. Nevertheless, electron microscopic analysis of PDGF-B-deficient mouse brain capillaries (105) clearly shows the absence of pericytes and dilated vessel lumen. Therefore, PDGF may play a role in recruitment of pericytes to preformed capillaries or in inducing the proliferation of pericytes previously recruited by a PDGF-independent mechanism, and it thus helps to maintain capillary wall stability.

*Transforming Growth Factor-β (TGF-β)*

The transforming growth factor-βs represent a family of highly conserved 25 kDa disulfide-linked homodimeric cytokines typified by TGF-β1 (107). Before secretion from the cell, cleavage by a furin peptidase generates a C-terminal 112 amino acid peptide that noncovalently associates with the N-terminal pro region (called latency-associated peptide or LAP) and dimerizes to form mature TGF-β (108). Secreted TGF-β cannot bind TGF-β receptors and is biologically inactive; the latent complex is activated by proteases such as plasmin and cathepsin D, low pH, chaotropic agents such as urea, and heat (109-110). Exposure to low pH or protease cleavage most likely activates latent TGF-β *in vivo*. TGF-β is expressed by a wide variety of normal and transformed cells while TGF-β receptors are broadly expressed in virtually all mammalian and avian cells (107,111). Similar to bFGF, TGF-β is found in extracellular matrix of many tissues (112). In the microvasculature, both endothelial cells and pericytes produce TGF-β (113-114) and
possess TGF-β receptors. Therefore, TGF-β exerts its effects in many cell types including those comprising the vasculature.

TGF-β was originally characterized by its ability to support anchorage-independent growth of fibroblasts (115) and has since been associated with a variety of functions in several different cell types. It can stimulate or inhibit cell proliferation, control cell adhesion by regulating production of extracellular matrix, protease inhibitors, and integrins, and induce cellular differentiation (107). Much evidence points to an important role for TGF-β in the vasculature.

Several in vitro studies have demonstrated the importance of TGF-β in vascular cells. TGF-β significantly inhibits the proliferation and migration of endothelial cells (116). However, one study claims that it stimulates growth at low doses and inhibits at high doses (117). TGF-β also regulates endothelial cell migration and formation of tube-like structures in a collagen gel, features characteristic of in vitro angiogenesis. Interestingly, similar to its effects on endothelial cell proliferation, TGF-β may either stimulate (118) or inhibit (119-120) in vitro endothelial tube formation. At a lower doses (≤ 0.5 ng/ml), TGF-β1 stimulates tube formation (118), but at higher doses (1-5 ng/ml) it inhibits this angiogenic activity (118-119). This effect of TGF-β is also isoform-specific; unlike TGF-β1, TGF-β2 has no effect on in vitro vessel formation at low concentrations, but at higher doses it stimulates endothelial tube formation (118).

TGF-β’s effects on endothelial tube formation may be mediated by its effects on proteolytic activity. TGF-β can produce a net antiproteolytic activity in these cultures by modulating uPA (urokinase-like plasminogen activator), and PAI (plasminogen activator inhibitor) levels (111). Furthermore, TGF-β can inhibit the production of proteases, such
as transin, and stimulate the production of protease inhibitors, such as tissue inhibitor of metalloproteinase (TIMP) (116). These effects prevent matrix remodeling and inhibit angiogenesis.

Other studies show that TGF-β promotes angiogenesis by another mechanism. When endothelial cells are co-cultured with either pericytes or vascular smooth muscle cells, latent TGF-β is cleaved, most likely by plasmin, to generate active TGF-β (113-114) that affects both endothelial cells and pericytes. Active TGF-β mediates the inhibition in endothelial cell growth observed upon endothelial cell:mural cell contact (120). In addition, active TGF-β binds to pericytes and induces expression of vascular smooth muscle actin (VSMA) and myogenic determination (122). Taken together, these studies suggest that TGF-β may function to establish the structural integrity of newly formed capillary sprouts during angiogenesis. By inhibiting endothelial cell proliferation and promoting mural cell differentiation, it helps to form and strengthen the vessel wall, and its matrix-modulating effects stimulate tube assembly.

TGF-β has also been shown to be involved in angiogenesis in vivo, though results vary depending on the experimental conditions. TGF-β will stimulate robust angiogenesis if administered subcutaneously into mice (123), applied to the chick embryo CAM (124), or implanted into the rabbit cornea (125) and rabbit ear dermal ulcers (126). However, in most cases, new blood vessels were accompanied by inflammation. Because TGF-β is chemotactic for a wide range of cells, including monocytes (127) and fibroblasts (128), angiogenesis in these studies is likely indirectly mediated by TGF-β’s effect on recruiting these cells and directly mediated by angiogenic factors produced by them. Moreover, when overexpressed in the vessel wall and a variety of other tissues, TGF-β does not
induce angiogenesis or an inflammatory response (116). Thus, TGF-β is not angiogenic in vivo in the absence of inflammatory mediators. Another possibility, however, is that TGF-β may not have been activated from its latent form in the instances angiogenesis was not observed in vivo. Activation of TGF-β requires certain conditions that have been mimicked in vitro, including direct contact between two specific cell types (113-114), but may not be present those systems studied in vivo.

Genetic studies also suggest a role for TGF-β in angiogenesis. In embryos of mice lacking TGF-β1, differentiation of mesodermal precursors into endothelial cells appears normal, but embryonic lethality results because of defects in the yolk sac vasculature and hematopoietic system (129). In these embryos, blood vessel walls are frail because of disrupted endothelial cell contacts. Similarly, TGF-β receptor I-deficient mice show a similar phenotype: blood vessels are formed but are dilated and exhibit disrupted cell contacts (130). Thus TGF-β does appear to play a role in establishing vessel wall integrity.

Taken together, the in vitro and in vivo studies demonstrate important roles for TGF-β in angiogenesis. Through modulation of the synthesis of extracellular matrix components, proteases, and protease inhibitors, TGF-β establishes a scaffold favorable to formation of vessel tubes. This cytokine also acts to establish and strengthen the vessel wall through regulation of endothelial cell quiescence, stability of cell-cell contacts, and differentiation of mural cells. Furthermore, TGF-β indirectly stimulates angiogenesis by the recruitment of inflammatory mediators that secrete angiogenic factors. Therefore, TGF-β stimulates vascular remodeling through its pleiotropic effects on different cell types.
**Other soluble factors**

Many other soluble factors have been proposed to function in angiogenesis, but their effects on the vasculature are not as widespread as the above growth factors. For example, other growth factors such as tumor necrosis factor-alpha (TNF-α), epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), and the colony stimulating factors (CSFs) exhibit angiogenic properties. TNF-α is secreted mainly by activated macrophages and some tumor cells, and although it is primarily involved in inflammation and immunity (131), it shares many properties with TGF-β. Both stimulate angiogenesis in vivo (in the CAM and cornea for TNF-α) (132), promote endothelial cell tube formation in vitro (133), and inhibit endothelial cell growth (4,132). Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α), which both bind the EGF receptor (134), are 5-6 kDa proteins that are mitogenic for endothelial cells in vitro and induce angiogenesis in vivo in the hamster cheek pouch (135). In addition, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF), proteins required for growth and differentiation of hematopoietic precursors (136), induce migration and proliferation of endothelial cells to a limited extent (137).

In addition to well-characterized growth factors, several other soluble substances have been shown to affect angiogenesis. Angiogenin is a 14.1 kDa polypeptide isolated from a human adenocarcinoma cell line that induces angiogenesis in the CAM and rabbit cornea but is not mitogenic or chemotactic for endothelial cells in vitro (138). Angiogenin also binds to extracellular matrix components and can support the adhesion and spreading of endothelial cells in vitro (139). However, angiogenin is synthesized minimally in the
developing fetus, when angiogenesis occurs the most, and maximally in the adult (140), when angiogenesis rarely occurs. Therefore, its timing of synthesis is inconsistent with a major role in blood vessel growth.

Angiotropin is a 4.5 kDa polyribonucleopeptide that was purified from the conditioned medium of activated peripheral monocytes. It induces random capillary endothelial cell migration and tube formation but is not mitogenic for endothelial cells (141). Furthermore, angiotropin stimulates angiogenesis in the CAM, cornea, and ear lobe that is accompanied by epidermal and stromal cell proliferation (142).

2 proteins involved in the coagulation cascade, tissue factor and factor V, have been linked to angiogenesis because mice deficient in either factor die in utero as a result of abnormal development of the yolk sac vasculature (143-144). These proteins, as well as certain non-peptide low molecular weight molecules such as prostaglandins (145), nicotinamide (146), and monobutyrin (147), appear to contribute to angiogenesis, but their roles are controversial and their mechanisms of action unknown.

Still other natural factors have been demonstrated to inhibit angiogenesis. For example, gamma interferon inhibits capillary formation and endothelial cell proliferation in vitro (148-151). In addition, cortisone (152), thrombospondin (153), platelet factor IV (154), protamine (155), and the more recently discovered angiostatin (156) and endostatin (157) inhibit angiogenesis in vivo in the CAM or corneal pocket assays. Although thrombospondin (153) inhibits endothelial cell migration and platelet factor IV (154) inhibits the proliferation of endothelial cells in vitro, the mechanisms whereby most of these substances inhibit angiogenesis in vivo are not clear. Whether these substances
inhibit physiological angiogenesis is unclear, but they are potent inhibitors of tumor angiogenesis (see below).

The soluble factors mentioned above are, in many cases, derived from several different sources, but in all cases their effects are felt directly or indirectly at the level of the endothelial and mural cells. That such a large number of discovered (and probably yet undiscovered) soluble factors contribute to angiogenesis attests to its complex nature and highlights its multiple modes of positive and negative regulation \textit{in vivo}.

\textit{Membrane-bound factors}

In addition to factors that are secreted from cells and act at a distance from their sites of synthesis, several membrane-bound proteins play prominent roles in angiogenesis. These molecules require close cell:cell or cell:matrix contact in order for their effects to be felt. Integrins, cadherins, and ephrins are endothelial membrane proteins that mediate many functions involved in blood vessel assembly. In particular, integrin $\alpha_v\beta_3$, VE cadherin, and ephrin-2B have been reported to play important roles in normal angiogenesis.

$\alpha_v\beta_3$ \textit{integrin}

Integrins are heterodimeric complexes composed of $\alpha$ and $\beta$ subunits that are receptors for extracellular matrix proteins and membrane-bound polypeptides on other cells. Over 16$\alpha$ and 8$\beta$ subunits can combine to form a diverse array of over 20 different integrins (158). Extracellular matrix substrates for integrins include polysaccharide
glycosaminoglycans as well as fibrous proteins such as fibronectin, vitronectin, collagen, laminin, and elastin (159). Some integrins bind short peptide sequences, such as the RGD (Arg-Gly-Asp) sequence found in fibronectin and vitronectin, but others recognize three-dimensional conformations (160).

Because angiogenesis involves invasion of the extracellular matrix and migration of endothelial cells through it, the cell-matrix interactions mediated by integrins seem likely to play important roles in vascular remodeling. Indeed, the integrin $\alpha_v\beta_3$, which binds von Willebrand factor, vitronectin, fibronectin, and fibrin (161), is highly expressed in vitro on endothelial cells exposed to growth factors such as bFGF (162) and VEGF (163). Integrin $\alpha_v\beta_3$ also mediates in vitro endothelial cell attachment, spreading, and migration (164), and it is transiently localized to endothelial cells at the tips of capillary sprouts during wound repair (165). $\alpha_v\beta_3$ is thus important for endothelial cell functions in neovascularization. In addition, $\alpha_v\beta_3$ is abundantly expressed on angiogenic blood vessels in granulation tissue but not on vessels from normal skin. Newly formed blood vessels induced by bFGF in the CAM assay also show robust $\alpha_v\beta_3$ expression that is not seen in untreated CAMs (166).

A requisite role for $\alpha_v\beta_3$ integrin in angiogenesis is suggested by the observation that neutralizing antibodies to $\alpha_v\beta_3$ inhibit bFGF-induced vessel sprouting in the CAM while the antibody has no effect on pre-existing vessels (166). In addition, $\alpha_v\beta_3$ is highly expressed on angioblasts before and during vasculogenesis in the quail embryo, and injection of an antibody to $\alpha_v\beta_3$ results in abnormal patterning of blood vessels characterized by discontinuous lumens and incomplete vascular networks (167). Therefore, $\alpha_v\beta_3$ appears to be crucial for aspects of vasculogenesis as well as angiogenesis.
in the developing embryo. Therefore, $\alpha_\beta_3$ mediates endothelial cell functions in vitro and plays an important role in angiogenesis in vivo.

The role of $\alpha_\beta_3$ in blood vessel growth has been examined in embryonic vascular development. $\alpha_\gamma$ integrin knockout mice exhibit vessel abnormalities and hemorrhaging in brain and intestinal vasculatures (168), and mice deficient in $\beta_3$ integrins exhibit extensive bleeding but demonstrate grossly normal vasculatures (169). Surprisingly, in both knockout experiments, extensive vasculogenesis and angiogenesis proceeded normally. Nevertheless, these studies are difficult to interpret because the functional redundancy of many integrins (158) raises the possibility of compensatory mechanisms in the absence of $\alpha_\gamma$ and $\beta_3$ integrins.

The role of $\alpha_\beta_3$ in mediating angiogenesis is not limited to binding of extracellular matrix components. $\alpha_\gamma\beta_3$ also binds matrix metalloproteinase-2 and localizes the active form of the enzyme at the tips of angiogenic blood vessels (170). Therefore, $\alpha_\beta_3$ may regulate localized degradation of the extracellular matrix and then mediate endothelial cell migration by adhering to the modulated matrix. $\alpha_\gamma\beta_3$ ligation also induces MAP kinase activation (171) and suppresses apoptosis (172) in endothelial cells. Thus, $\alpha_\gamma\beta_3$ integrin may mediate endothelial cell survival by activating intracellular pathways that promote proliferation and activation. By several mechanisms, then, $\alpha_\gamma\beta_3$ mediates angiogenesis.

Other integrins have been implicated in regulating angiogenesis. For example, in both the CAM and rabbit corneal pocket assays, anti-$\alpha_\beta_3$ inhibits bFGF-induced angiogenesis while anti-$\alpha_\gamma\beta_3$ suppresses VEGF-stimulated angiogenesis (173). In addition, the collagen receptor integrins $\alpha_\gamma\beta_1$ and $\alpha_\gamma\beta_1$ are induced by VEGF, and antibodies to them drastically
inhibit VEGF-driven angiogenesis (174). Antibodies to $\alpha_5\beta_1$ inhibit angiogenesis induced by several growth factors but not that induced by VEGF (175). These studies suggest that different growth factors may induce the expression of similar, yet distinct, integrins that mediate the growth of new blood vessels. These different integrins may regulate adhesion of endothelial cells to distinct substrates and facilitate migration through several extracellular matrices. Furthermore, alpha 5 integrins, in general, appear to be involved in angiogenesis because though $\alpha_5$-null mouse embryos develop a vascular system, their blood vessels are dilated and leaky (176). Given the diverse functions of integrins in angiogenesis, including adhesion to extracellular matrix, localization of proteases to capillary sprouts, and enhancement of endothelial cell survival, endothelial cell expression of a variety of integrins may stimulate distinct intracellular pathways that all contribute to the progression of angiogenesis.

**VE-Cadherin**

Cadherins comprise a large family of Ca$^{2+}$-binding transmembrane molecules that promote homotypic cell:cell interactions (177). These proteins serve diverse purposes in many cells. The intracellular domain of cadherins mediate a linkage to the cytoskeleton by binding to $\beta$-catenin and plakoglobin, two proteins that are anchored to cortical actin by $\alpha$-catenin (177). Cadherins also mediate intracellular signaling by controlling cytoplasmic levels of $b$-catenins and plakoglobin which, when released from cadherins, can translocate to the nucleus and regulate gene transcription (178).

Endothelial cells possess two cadherins: VE-cadherin, which is localized to adherens junctions exclusively in endothelial cells (179), and N-cadherin, which is not found at
Many studies highlight the importance of VE-cadherin in neovascularization. First, VEGF-mediated enhancement of endothelial permeability is accompanied by tyrosine phosphorylation and dissociation of VE-cadherins (33-34). These results suggest that VE-cadherin may regulate the passage of molecules across the endothelium. Also, VE-cadherin mediates contact inhibition of endothelial cell growth (181). This suppression of proliferation ensures that endothelial cells maintain a patent, stable monolayer in the vessel wall. In addition, erythroid bodies derived from embryonic stem cells that harbor a targeted null mutation in VE-cadherin remain dispersed and do not develop into the organized vessels characteristic of wild-type erythroid bodies (182). Furthermore, mice deficient in VE-cadherin exhibit extreme vascular abnormalities (183). Although angioblasts differentiate into endothelial cells and a primary capillary plexus is formed in these mice, later stages of vascular development are impaired. Endothelial cells become progressively disconnected, branching and sprouting into a network of larger and smaller blood vessels is severely diminished, and vessels eventually regress and disintegrate (183). Presumably, cadherins not only establish endothelial cell junctional stability in the vessel wall but also enhance endothelial cell survival by promoting transmission of VEGF's anti-apoptotic signal to the nucleus (183). Therefore, although VE-cadherin does not function in vasculogenesis, it is crucial for remodeling and maturation of vessels in angiogenesis.

**Eph-B4/Ephrin-B2**

A unique class of receptor/ligand pair, eph receptors and ephrin ligands, plays a prominent role in blood vessel development. Eph receptors belong to the largest known
family of receptor tyrosine kinases consisting of at least 14 membrane-bound proteins, and eight transmembrane ligands (ephrins) for them have been identified (184). Interestingly, not only does an ephrin expressed on the surface of one cell bind and activate its cognate eph receptor on another cell, but through a reciprocal signaling mechanism the ephrin is also activated upon receptor engagement (185). These molecules have been well characterized in the nervous system where they appear to assist axon guidance through repulsive signals and establish borders between neuronal compartments (186). They are also found at compartment boundaries in several other embryonic tissues, including early somites and limb precursors (187). The requirement of cell-cell contact for their engagement and activation suggested that they were involved generally in the formation of spatial boundaries that establish the developing body plan during embryogenesis (187).

One member of the ephrin family, ephrin-B2, is expressed on arterial endothelial cells of the developing embryo, and its receptor eph-B4 is exclusively localized to venous endothelial cells; ephrin-B2 colocalizes with eph-4B at arterial/venous interfaces after vasculogenesis has established the primary capillary plexus but before angiogenesis remolds it (188). Indeed, the importance of ephrin-B2 and its interaction with eph-4B during angiogenesis is highlighted by mice with a null mutation in ephrin-B2 that exhibit normal vasculogenesis but demonstrate defects in angiogenesis of the head and yolk sac vasculatures and in myocardial trabeculation (188). Interestingly, ephrin-2B is also expressed in a variety of nonvascular tissues, including caudal somites (189). However, eph-4B is exclusively localized on vascular endothelial and endocardial cells, and mice with a targeted mutation in eph-4B exhibit similar phenotypes as seen in the ephrin-2B-
null mice (190). These results suggest that establishment of contact and signaling between arterial and venous compartments mediated by ephrin-B2 and eph-4B is necessary for remodeling of the established primary capillary plexus.

Other ephrin/eph family members appear to play a role in angiogenesis as well. Ephrin-A1 is required for angiogenesis stimulated by TNF-a, but not bFGF, in the rat cornea (191). Interestingly, a soluble Ig chimera of ephrin-A1 is chemotactic for endothelial cells in vitro (191). Furthermore, transfection of human umbilical cord endothelial cells (HUVECs) with a dominant negative form of eph-2A, the receptor for ephrin-2A, results in an impaired ability to form capillary tubes in vitro (192). Thus, ephrin family members other than ephrin-2B are important for endothelial cell events involved in angiogenesis.

Interestingly, ephrins (particularly ephrin-2A) exhibit growth factor specificity in angiogenesis (191) in a similar manner as is reported for integrins (173,175). These results reinforce the concept that diverse members of transmembrane signaling molecule families are induced by distinct stimuli during angiogenesis and that each has an important role in mediating the varied angiogenic processes in vivo. Thus, mechanisms of angiogenesis cannot be modeled merely as summations of growth factor signals through singular pathways. Rather, angiogenesis results from a complex coordination of positive and negative regulators on many different cellular systems.

**Biomechanical Forces**

In addition to the soluble and membrane-bound molecules described above, mechanical forces acting on vascular endothelium also contribute to the pruning and
remodeling processes characteristic of normal angiogenesis. The mechanical forces mediated by blood flow have profound effects on vessel growth. Vessels that are not perfused with blood eventually regress (2). This phenomenon is most apparent in the regulated cycles of angiogenesis occurring in the female reproductive system where periodic growth and regression of blood vessels cyclically remodel the ovarian, uterine, and placental tissues (193). For example, some of the highest rates of blood flow on a weight basis are observed in these tissues and are associated with extensive proliferation of vascular endothelial cells (193). On the contrary, it has been suggested that a reduction in ovarian blood flow leads to luteal regression (194), a process associated with extensive capillary bed degeneration. Furthermore, in skeletal muscle, increased blood flow induced by electrical or chemical stimulation results in capillary angiogenesis and arterial growth (195), and decreased blood flow causes a reduction in size and number of arterioles (196).

Careful in vitro and in vivo characterization of blood flow’s effects on capillary cells revealed a mechanism by which it affects vessel growth. In vitro, fluid shear stress induces a dramatic increase in endothelial cell stress fiber expression (if flow is laminar) (197), promotes endothelial cells to divide (if flow is turbulent) (198), and stimulates the transcription of genes for PDGF and TGF-β (199) which promote angiogenesis as described above. In vivo, increased shear stress in rabbit ear vessels associated with enhanced blood flow correlates with an increase in microvascular area (200). Therefore, shear stress induced by blood flow modulates blood vessel morphogenesis. Laminar flow stabilizes and protects the vessel wall by increasing stress fiber expression in endothelial cells, and turbulent flow leads to further blood vessel growth. This is an efficient
mechanism of remodeling the primary vascular plexus because vasculogenesis results in
the overproduction of blood vessels. Unperfused capillaries regress, probably by
endothelial cell apoptosis (201), while those in which blood flow is established persist
and become a stable part of the vasculature.

Thus, microvascular blood vessels are remodeled in angiogenesis through several
diverse mechanisms. Growth factors secreted from distant cells, transmembrane proteins
binding to extracellular matrix components or receptors on other cells, and hemodynamic
forces all act in concert to regulate normal angiogenesis. In a physiological setting, these
factors exert both positive and negative influences on blood vessel growth to ensure that
angiogenesis is confined to metabolic demands of growing and healing tissues. However,
certain pathological conditions usurp these mechanisms to enhance the spread of disease.
One of the most characterized of these is tumor angiogenesis and is discussed below in
terms of its relation to normal angiogenesis.

**Tumor-Induced Angiogenesis**

Tumors are populations of host-derived cells that have lost the ability to regulate
growth and therefore proliferate aberrantly. Though several features distinguish them
from their non-transformed counterparts, many aspects of tumor cells are similar to those
of normal ones. One major similarity is the requirement for an adequate supply of oxygen
and nutrients and an effective means to remove wastes in order for metabolic processes to
occur and survival to be maintained. Proximity to a vascular supply fulfills these requirements for mammalian cells. Normal cells and tissues rely on physiological vasculogenesis and angiogenesis (described in detail above) to provide them with a vasculature that fulfills their metabolic demands. Tumor cells, on the other hand, can induce their own blood supply from the pre-existing vasculature in a process that mimics normal angiogenesis.

**The Tumor Vasculature**

Tumors can establish their own blood supply by several means. Figure 2 is a schematic of tumor-induced neovascularization. In a process very similar to normal angiogenesis, a tumor may elicit the formation of blood vessels from pre-existing capillaries. In addition, tumor cells are able to grow around an existing vessel and hence, at least initially, do not need to induce angiogenesis for adequate vascularization (202). Furthermore, circulating endothelial precursors (CEPs), angioblast-like cells derived from bone marrow but reported to be present in the adult circulation, have recently been suggested to contribute to tumor-derived blood vessels (203). Though tumor-induced vessels form a conduit for the delivery of metabolites, ultrastructurally they are abnormal. Many lack functional pericytes (204), they are dilated and convoluted, and they are exceptionally permeant due to the presence of fenestrae and transcellular holes and lack of a complete basement membrane (205) (see figure 2). Furthermore, tumor vessel walls may be made up of both endothelial cells and tumor cells (206). These structural abnormalities in tumor vessels reflect the pathological nature of their induction, yet their ability to support cell growth
also underlies the use of physiological mechanisms of angiogenesis that tumors commandeer for their propagation.

**Factors Involved in Tumor Angiogenesis**

The induction of new blood vessel growth by a tumor is mediated through the action of many molecules, some of which are involved in normal angiogenesis. Those substances that are well characterized in tumor neovascularization are summarized in Table II and described below.

**Vascular Endothelial Cell Growth Factor (VEGF)**

As in normal angiogenesis, tumor angiogenesis appears to rely heavily on VEGF. Many tumor cell lines secrete VEGF *in vitro* (207), and by *in situ* hybridization VEGF mRNA is highly upregulated in most human cancers including lung, breast, gastrointestinal tract, kidney, bladder, ovary, and endometrial carcinomas, intracranial tumors, glioblastomas, and capillary hemangioblastomas (19). Both VEGF and its receptor (flk-1) are highly expressed in metastatic human colon carcinomas and their associated endothelial cells, respectively, and production of these two proteins correlates directly with the degree of tumor vascularization (208). Furthermore, increased VEGF expression is closely associated with increased intratumoral microvessel density (MVD) and poor prognosis in breast cancer patients (209). In addition to producing VEGF themselves, tumors may induce the production of VEGF in their surrounding stromal tissue (210). In this study, GFP driven by the VEGF promoter was robustly expressed for weeks in fibroblasts surrounding both implanted and spontaneous tumors. Therefore, high
levels of VEGF production in a wide variety of tumor and tumor-associated cells and robust expression of its receptor in tumor-associated blood vessels suggest that VEGF plays an important role in tumor angiogenesis.

A causative role for VEGF in tumor angiogenesis is suggested by inhibition studies. Intraperitoneal administration of anti-VEGF antibody in nude mice harboring tumors derived from injected sarcoma and glioblastoma cells significantly decreases tumor vessel density and suppresses tumor cell growth (211). By intravital examination of blood vessels stimulated by tumor spheroids administered to mice, anti-VEGF was shown to almost completely inhibit tumor neovascularization (212). These observations indicate that a general inhibition of VEGF activity \textit{in vivo} results in reduced tumor angiogenesis and tumor growth. A more direct role of tumor cell-derived VEGF in stimulating angiogenesis \textit{in vivo} was suggested by the dramatically impaired ability of embryonic stem (ES) cells with a targeted inactivation of the VEGF gene to form teratocarcinomas in nude mice compared to control ES cells (44). Interestingly, blood vessels induced by the VEGF\textsuperscript{−} ES cells were lower in number and less branched than those induced by control ES cells. Inhibition of VEGF receptor signaling also suppresses tumor growth \textit{in vivo}. Retrovirus-mediated expression of a dominant negative VEGF receptor (Flk-1) dramatically inhibits the growth of a variety of tumors, including mammary, ovarian, and lung carcinomas (213) as well as C6 glioblastomas (214), in nude mice. Histological examination of these inhibited neoplasms demonstrates that the growth of blood vessels in the tumors is also severely reduced. These studies have far-reaching clinical implications because aside for the reduction in tumor vascularization and growth, host animals were largely unaffected by inhibition in VEGF or its receptor.
The mechanism of VEGF-mediated tumor angiogenesis most likely relies on regulation by oxygen tension. That solid tumors contain a central region of necrotic tissue resulting from poor delivery of oxygen has been known for some time (215). In fact, hypoxia associated with poorly vascularized areas of tumors selects for cells that are resistant to damaging effects of hypoxia or that can induce oxygenation. In the former case, hypoxia is able to select for apoptosis-defective cells lacking p53 and may be partially responsible for the observation that p53 is one of the most commonly mutated genes in human cancer (216). The latter case is indicative of the hypoxic tumor environment to stimulate VEGF production and angiogenesis by cancer cells. *In situ* hybridization has identified VEGF mRNA in hypoxic regions of glioblastoma immediately adjacent to necrotic areas, and capillary bundles are found next to the VEGF-producing cells (46). These observations suggest that induction of VEGF mRNA in tumor cells by exposure to hypoxia *in vitro* (47) is also relevant *in vivo*.

Taken together, these results strongly implicate VEGF as having a prominent role in inducing tumor angiogenesis. Mechanistically, the mediation of blood vessel growth by VEGF in tumors is similar to that of physiological angiogenesis, i.e. low oxygen tension induces neovascularization to satisfy metabolic demands (217). Furthermore, perhaps the most clinically relevant aspect of a tumor is its ability to metastasize to distant sites. In addition to stimulating blood vessel growth, VEGF increases vascular permeability (31-33). In such a manner VEGF can induce the formation of leaky blood vessels with fragmented membranes that can easily be penetrated by neoplastic cells (218) to disseminate a primary tumor. Thus, VEGF may have multiple roles in tumor
angiogenesis. Nevertheless, although VEGF is important for these processes, other polypeptides complement VEGF’s actions in inducing blood vessel growth in tumors.

**Fibroblast Growth Factor (FGF)**

The first tumor-derived factor that stimulated endothelial cell proliferation and induced neovascularization *in vivo* was isolated by Folkman *et al.* in the early 1970’s (219). However, purification of this factor was difficult because of a lack of suitable bioassays, and the angiogenic activity was simply known as “tumor angiogenesis factor” (219). The advent of heparin affinity chromatography facilitated the identification of FGF as the first known tumor-derived angiogenic factor (220-222).

A direct role for FGF in tumor angiogenesis is suggested in an inhibition study using a soluble form of the bFGF receptor. Administration of a soluble form of the FGF receptor to mice injected with pancreatic β-cell tumors induced by SV40 T antigen expression driven by the rat insulin promoter dramatically suppresses tumor growth and decreases tumor vessel density (223). In addition, if biopsied pancreatic β-cell tumors are incubated with human umbilical cord endothelial cells (HUVECs) in a three-dimensional collagen matrix, the HUVECs proliferate and migrate towards the tumor. However, HUVEC’s induced to secrete soluble FGF receptor demonstrate a very limited angiogenic response (223). Therefore, these results suggest that FGF production by neoplastic cells induces angiogenesis that stimulates tumor survival and growth *in vivo*.

Due to the observation that soluble FGF receptor impedes tumor growth at a later timepoint after tumor induction than does soluble VEGF receptor, it was speculated that while VEGF acts to initiate tumor angiogenesis, FGF is important for its maintenance
Indeed, bFGF has been reported to cooperate with VEGF in stimulating angiogenesis. VEGF and bFGF synergized in vitro to increase the rate of proliferation and formation of cord-like structures by bovine capillary endothelial cells in a collagen gel (224) and in vivo to induce collateral vessel development following hindlimb ischemia in rabbits (225). FGF may also help to augment the production of VEGF. Exogenous expression of FGF4, a fibroblast growth factor family member that is secreted from cells, in normal mouse mammary cells renders them tumorigenic in nude mice and angiogenic for HUVECs cultured in a collagen gel (226). The angiogenic effect is mediated by stimulation of VEGF mRNA and protein production by FGF4 expression (226). In addition, bFGF induces an increase of VEGF mRNA in vascular smooth muscle cells (227) and an increase in VEGF receptor in microvascular endothelial cells (228). It is very likely, then, that FGF can stimulate tumor angiogenesis in vivo via several mechanisms including activation of and synergism with VEGF.

Heparanase can also be considered as a separate inducer of tumor angiogenesis, but its mechanism of action appears to be mediated by bFGF. Heparanase promotes angiogenesis directly by stimulating invasion of endothelial cells and vascular sprouting as well as indirectly by releasing heparan sulfate-bound bFGF from its sites of deposition in the extracellular matrix (229). Heparanase mRNA and protein are enriched in metastatic cell lines as well as specimens of human melanomas and carcinomas vs. normal tissues, and transfection of nonmetastatic T lymphoma and melanoma cell lines with the heparanase gene renders them highly metastatic in vivo (229). Furthermore, in vivo angiogenic activity of heparanase is evidenced by the significant increase in neovascularization induced by T lymphoma cells in the Matrigel plug assay when these
cells are transfected with heparanase (229). Thus, heparanase may be necessary to evoke the blood vessel growth by tumor cells.

FGF may therefore both directly and indirectly stimulate tumor angiogenesis. Inhibition studies indicate that FGF is in part necessary, but not sufficient, to induce blood vessel growth by tumors. FGF and VEGF are two of the many factors that cooperatively mediate neovascularization in the tumor microenvironment.

**Angiopoietin-2 (Ang2)**

Recent evidence strongly implicates angiopoietin 2 (Ang2) in tumor angiogenesis. As mentioned above, the angiopoietins are play prominent roles in normal angiogenesis. Ang1 signaling through the Tie2 receptor remolds newly formed capillary tubes and stabilizes them through interactions between endothelial cells and surrounding support cells (83-85). Ang2 is an antagonist of Ang1 and destabilizes blood vessels (86). In the absence of VEGF production, Ang2 mediates blood vessel regression; however, in the presence of VEGF, Ang2-induced destabilization of vessels renders them plastic and more responsive to VEGF-mediated growth (86).

Based on Ang2 and VEGF functions in normal angiogenesis, an interesting model has been proposed for angiogenesis induced by several tumors. Contrary to initial reports that most tumors, especially metastases, originate in an environment devoid of blood vessels, many tumors, start growing around existing vessels and initially do not need to induce angiogenesis to survive (75). As the tumor grows larger, however, mural cells progressively disengage from the endothelium of these co-opted vessels, and the blood vessels regress (230) by endothelial cell apoptosis. Interestingly, Ang2 is induced in the
endothelium of these vessels even before they regress (202). Furthermore, robust expression of VEGF in the growing tumor cells then results in angiogenesis, and the newly formed vessels also express high levels of Ang2 mRNA (202). Therefore, Ang2 plays a dual role in tumor angiogenesis. In the early stages of tumor cell growth around an existing blood vessel, tumor cells do not produce VEGF. Instead, they induce Ang2 expression in the blood vessel which results in vessel destabilization and regression. As the tumor grows and its metabolic demands become greater, VEGF production by the tumor induces neovascularization, and Ang2 induction by tumor cells in endothelial cells of newly formed vessels facilitates this process by rendering endothelium unstable and plastic. Indeed, by in situ hybridization, Ang-2 mRNA is expressed in endothelial cells of tumor vessels, but not in normal blood vessels, and it is one of the earliest markers of tumor-induced neovascularization (231). Blockage of Ang1’s stabilizing effect on newly formed blood vessels by Ang2 is probably a major contribution to leakiness and fragility of tumor vessels (205). Therefore, Ang2 contributes significantly to tumor angiogenesis. Similar to FGF, it cooperates with VEGF to induce blood vessel growth.

**Interleukin-8 (IL-8) and Matrix Metalloproteinase-2 (MMP-2)**

A growth factor that is not well characterized in normal angiogenesis but has attracted attention in tumor neovascularization is interleukin-8 (IL-8). An angiogenic role for IL-8 in angiogenesis was first suggested by the observation that macrophages produce IL-8 and mediate angiogenesis in chronic inflammatory diseases such as psoriasis and rheumatoid arthritis (232-233). Subsequently, it was shown that not only is IL-8
mitogenic and chemotactic for HUVEC’s in vitro, but it also stimulates angiogenesis in the rat cornea (233).

A role for IL-8 in tumor angiogenesis is suggested by the findings that IL-8 mRNA is upregulated in neoplastic tissues, such as non-small-cell lung cancer (NSCLC) (234) and melanoma (235), vs. normal ones in vivo and its expression correlates with the extent of neovascularization. In addition, overexpression of IL-8 in nonmetastatic, IL-8-negative melanoma cells not only increases their ability to invade Matrigel-coated filters but also renders them highly tumorigenic and metastatic in nude mice (236). Also, stable transfection of gastric carcinoma cells that produce low amounts of endogenous IL-8 with the IL-8 gene allows them to produce rapidly growing, highly vascular neoplasms that are not seen with control-transfected cells (237). Furthermore, conditioned medium from the IL-8-transfected cells stimulates HUVEC proliferation (237). Although these results suggest a role for IL-8 in the induction of endothelial cell proliferation in the tumor vasculature, another mechanism may mediate IL-8’s role in tumor angiogenesis.

An important observation made in the studies using IL-8-transfected melanoma cells was that the cells exhibit an increase in matrix metalloproteinase-2 (MMP-2) mRNA and activity and an increase in MMP-2 promoter-driven reporter gene activity (235). Therefore, angiogenesis induced by IL-8 may have been mediated in part by its ability to stimulate production of MMP-2 which degrades basement membranes and remolds the extracellular matrix for cell invasion and migration. One of the first steps in angiogenesis, normal or pathogenic, is degradation of the extracellular matrix (6). Indeed, MMP-2 has been shown to directly modulate melanoma cell adhesion and spreading on extracellular matrix (238), and an inhibitor of MMP-2 significantly inhibits growth and
neovascularization of tumors implanted into CAM’s (239). Thus, MMP-2 plays an important role in tumor angiogenesis.

Interestingly, although MMP-2 expression is increased in cells transfected with IL-8, VEGF and bFGF mRNA levels are unchanged (236-237). Therefore, the IL-8-mediated stimulation of tumorigenicity and blood vessel growth are independent of upregulated VEGF and bFGF activity in the tumor cells. These results suggest that IL-8 induced MMP-2 production is a major mechanism by which tumor cells induce angiogenesis. The other factors outlined above that mediate normal angiogenesis, such as PDGF, TGF-b, and angiogenin, likely participate in tumor neovascularization, but their roles in this pathological condition are not well characterized, particularly in the clinic, and thus they may play only minor roles. Experimental evidence provided above indicates that VEGF plays a dominant role in tumor neovascularization. Nevertheless, bFGF, angiopoietins, IL-8, and, most likely, other less-characterized inducers all cooperate with VEGF to mediate blood vessel formation by tumors. The identification of these inducers has stimulated extensive interest in discovering angiogenesis inhibitors that can be useful as therapeutics for cancer.

**Inhibitors of Tumor Angiogenesis**

In addition to the numerous factors that stimulate angiogenesis, both physiologically and pathologically, many substances including those mentioned above can inhibit blood vessel growth. Over forty endogenous angiogenesis inhibitors have been characterized, and these can be divided into four major groups: interferons, proteolytic fragments, interleukins, and tissue inhibitors of metalloproteinases (TIMPs) (240). Many of these
agents are in clinical trials as cancer therapeutics because inhibition of angiogenesis usually results in suppression of tumor growth. Representative members of each of the four classes of inhibitors are highlighted below.

**Interferons**

Interferons (INF-α, β, and γ) are members of a family of secreted glycoproteins that were initially characterized for their antiviral effect (241). Nonetheless, one of the first pieces of evidence that endogenous angiogenesis inhibitors exist was demonstrated by the ability of IFN-α to inhibit endothelial cell chemotaxis *in vitro* (242). Tumor cell extracts induce motility of endothelial cells across gold-plated cover slips, and IFN-α suppressed this activity in a dose-dependent manner (242). More recently, interferons have been shown to inhibit angiogenesis *in vivo*: IFN-α suppresses the vascularization of the chick embryo area vasculosa (243). It is possible that the ability of IFN-α and IFN-β to downregulate bFGF mRNA and protein levels in bladder, renal, colon, breast, and prostate carcinoma cells (244) as well as its inhibitory effect on endothelial cell migration (242) that underlie this *in vivo* suppression.

**Interleukins**

Interleukins are proteins secreted from leukocytes that mediate a wide spectrum of activities ranging from lymphocyte activation and proliferation (245) to stimulation of IgE release from B cells (246). A subset of these lymphokines has been found the affect blood vessel growth. Interestingly, interleukins having a glu-leu-arg (ELR) motif at the
N-terminus, such as IL-8, enhance angiogenesis, and those that lack this sequence, such as IL-4, inhibit it (247).

IL-4 is well characterized to inhibit tumor growth (248). Although it may directly inhibit proliferation of some tumor cells (249) or induce a host immune reaction against the tumor (250), some tumors are inhibited by other means (251). Inhibition of neoplastic angiogenesis is another likely mechanism by which IL-4 inhibits tumor growth. IL-4 inhibits in vivo neovascularization induced by bFGF in the rat cornea and blocks the migration of microvascular endothelial cells towards bFGF in vitro (252). Therefore, inhibition of neovascularization is an important mechanism mediated by IL-4 in suppressing tumor growth.

**Tissue Inhibitors of Metalloproteinases (TIMPs)**

An important theme that has emerged in the angiogenesis field is that not only are directly effects on endothelial cell growth and migration important in regulating blood vessel growth, but a wide variety of additional interactions are crucial. In particular, the extracellular matrix is an essential component of the angiogenic response (253). Remodeled extracellular matrix components comprise a scaffold upon which endothelial cells can adhere, migrate, and form tubes, and deposition of these components forms the basal lamina that ensheaths endothelium and mural cells. As mentioned above, many proteases, including those of the metalloproteinase family, are important in effecting this remodeling necessary for progression of angiogenesis. Accordingly, the tissue inhibitors of metalloproteinases (TIMPs) have been found to inhibit angiogenesis. For example, transfection of the highly metastatic B6F10 murine melanoma cell line with TIMP-2
cDNA inhibited its invasive potential in vitro as well as its growth, associated neovascularization, and metastatic potential in vivo (254). Furthermore, conditioned medium from these cells exhibited a reduced ability to induce endothelial cell migration and invasion through Matrigel, and the transfected tumor cells were also suppressed in vitro invasive potential (254). In addition, in vitro migration of endothelial cells through gelatin is significantly inhibited by overexpressed TIMP-1 (255). The multiple effects of TIMPs on both endothelial and tumor cell migration render MMPs attractive targets for tumor therapy.

**Proteolytic fragments**

Numerous potent anti-angiogenesis agents are proteolytic fragments of larger naturally occurring proteins. Interestingly, most of these cleavage products are derived from extracellular matrix components, such as collagen or fibronectin, or from enzymes such as plasminogen and MMP-2 that remodel extracellular matrix. Perhaps the most characterized inhibitors in this class are angiostatin and endostatin.

Interestingly, angiostatin was discovered as a factor somehow produced or generated by a primary tumor that circulates and inhibits the growth of remote metastases (256). Angiostatin is a 38 kDa internal fragment of plasminogen that potently inhibits capillary endothelial cell growth in vitro (146,256). In addition, intraperitoneal administration of angiostatin potently inhibits the neovascularization and metastasis formation in mice observed after a primary tumor has been removed (256). Furthermore, by engineering various cell lines, including those derived from melanoma (257) and glioma (258), to express angiostatin, tumors induced by them in mice are significantly inhibited in growth
and neovascularization. Although the mechanism by which angiostatin is produced or generated from plasminogen \textit{in vivo}, human prostate carcinoma cells have been reported to express a serine protease that generates biologically active angiostatin from purified human plasminogen or plasmin (259). However, the identity of this protease is unknown.

Endostatin is a 20 kDa fragment of type XVIII that was identified as a factor produced by hemangioendothelioma cells that specifically inhibits endothelial cell proliferation (157). Similar to angiostatin, endostatin dramatically inhibits angiogenesis \textit{in vivo} in the CAM assay, potently inhibits the growth of metastases of a primary Lewis Lung tumor, and induces almost complete regression of a wide variety of primary tumors (157). Interestingly, repeated administration (2-6 treatment cycles) of endostatin to mice bearing tumors derived from Lewis Lung carcinoma, T241 fibrosarcoma, or B16F10 melanoma induces no drug resistance in the host and results in tumor dormancy which requires no further treatment (260). These results indicate that endostatin can irreversibly halt tumor progression most likely through its anti-angiogenic effects.

\textit{Other anti-angiogenic molecules}

Several other factors mediate inhibition of angiogenesis, but their effects have not been extensively characterized perhaps because they are not as potent or are not amenable to therapeutic delivery as the agents mentioned above. One of these molecules is thrombospondin-1 (TSP-1). The anti-angiogenic activity of TSP-1 was discovered during a screen for genes that promote tumorigenesis (tumor promoters) or inhibit it (tumor suppressors). One screen for such regulatory genes revealed that a nontumorigenic hamster cell line became tumorigenic upon mutational inactivation of a tumor suppressor
gene (153). Interestingly, the nontumorigenic cell line secreted a potent inhibitor of endothelial cell chemotaxis in vitro and corneal neovascularization in vivo, and the tumorigenic cell lines secreted much less of this activity (153). The inhibitory activity was shown to be due to TSP-1, and purified TSP-1 was shown to be a potent inhibitor of in vitro as well as in vivo neovascularization (261). Subsequent work indicates that the tumor suppressor gene p53 regulates TSP-1 expression in certain cell lines (262). Furthermore, thrombospondin-2 (TSP-2), which shares high structural similarity with TSP-1 but has a distinct expression pattern (263), is a potent inhibitor of angiogenesis in vitro and in vivo (264) and inhibits tumor growth and neovascularization (265).

The experiments with TSP-1 were among the first that demonstrated the significance of an angiogenesis inhibitor in suppressing tumor growth. The list of angiogenesis inhibitors is expanding even today, and their clinical use in cancer therapy (266) is becoming evident. As more angiogenesis inhibitors are discovered it will be easier to delineate a common mechanism underlying their actions and perhaps eventually design a highly effective cancer therapeutic.

**Summary and Concluding Remarks**

Angiogenesis is a complex process that relies on the coordination of many different activities in several cell types. Endothelial cells, pericytes, fibroblasts, and immune mediators express many different cytokines and growth factors that react with other cells or extracellular matrix components to effect endothelial cell migration, proliferation, tube formation, and vessel stabilization. Under physiological conditions, angiogenesis is a highly ordered process required for the normal remodeling of the primary vascular plexus
formed during vasculogenesis. Not only are chemical mediators such as cytokines and membrane proteins essential for remodeling the vascular tree, but biomechanical forces such as blood flow and shear stress are also critical angiogenic factors. The reliance of a newly-formed vessel on a flow of blood for survival ensures that only those vessels supporting a physiological function become a part of the vascular network. Because any nonfunctional component is eliminated from the system early in development, such a mechanism is an extremely efficient use of resources, space, and energy.

In pathological states such as cancer, mechanisms of normal angiogenesis are used to promote disease. The fact that tumors use the same angiogenic molecules to commandeer a blood supply as those utilized by the host to develop a functional blood vessel network is advantageous for the tumor in several ways. First, angiogenesis is a highly effective means of establishing a system to deliver nutrients and remove wastes from a source of metabolic demand. Normal mammalian tissues thrive because blood vessel growth meets their metabolic needs (217), and tumors rely on a similar highly effective mechanism to promote survival. Also, because the tumor vasculature relies on a system created by the host, it evades host defenses. Immunologic attack on this system is prevented by recognition of it as self. Furthermore, its pathological nature also makes it abnormally plastic and renders normally quiescent endothelial cells proliferative. In the adult, angiogenesis does not occur in most tissues (2,4), but tumors establish conditions which foster continued blood vessel growth for their propagation.

The discovery of endogenous angiogenesis inhibitors not only reveals another aspect of exquisite neovascular regulation but also reinforces the notion that tumors are indeed dependent on angiogenesis (92). Angiogenesis is a dynamic process driven by many
positive factors but also curtailed by several negatively-acting molecules, and at any time it can be seen as an equilibrium between these stimulators and inhibitors. Tumors rely on the creation of angiogenic stimulators for their survival (92). Angiogenesis inhibitors push the equilibrium in favor of vessel quiescence and thus deprive tumors of a means to grow. Therefore, anti-angiogenic therapy of cancer (218) theoretically represents a highly effective strategy for destroying tumors. Multiple agents that target individual factors involved in blood vessel growth as well as endogenous angiogenesis inhibitors demonstrate promise in eradicating established tumors, and several of these are in the process of being tested in the clinic (240). Endogenous angiogenic inhibitors probably represent the most effective approach to tumor therapy to date because not only have they shown efficacy in destroying established tumors (240), but at least one of these agents, endostatin, does not promote resistance to therapy upon repeated administration, a common drawback to conventional chemotherapy (260). Anti-angiogenic agents, if administered before a tumor develops or becomes dependent on a vascular supply, would therefore theoretically act similar to a vaccine in preventing tumor development, not just tumor growth. Important to note, however, is that anti-angiogenic therapy represents a treatment, not a cure, for cancer. Only by targeting agents and mechanisms that cause normal cells to become tumorigenic can a cure for cancer be realized. Angiogenesis inhibitors, nonetheless, take great strides toward that goal because they block a fundamental requirement of tumor growth.
ACKNOWLEDGEMENTS

We are grateful to Pat D'Amore for insight comments. Studies from the Herman lab described in this review were supported in part by NIH GM 55110 and NIH EY 09033 (IMH).
Figure 1. Mechanisms of Physiological Angiogenesis

Normal angiogenesis depends on the coordination of several independent processes. Removal of pericytes from the endothelium and destabilization of the vessel by Ang2 shift endothelial cells from a stable, growth-arrested state to a plastic, proliferative phenotype. VEGF-induced hyperpermeability allows for local extravasation of proteases and matrix components from the bloodstream. Endothelial cells proliferate and migrate through the remodeled matrix, then they form tubes through which blood can flow. Mesenchymal cells proliferate, migrate along the new vessel, and differentiate into mature pericytes. Establishment of endothelial cell quiescence, strengthening of cell:cell contacts, and elaboration of new matrix stabilize the new vessel.
Figure 2. Mechanisms of Tumor Angiogenesis

A. Schematic of tumor blood vessel. Notice the thin walls, tortuous shape, absence of pericytes, and variations in diameter. Numerous gaps or fenestrae are found between endothelial cells. The vessel wall is mosaic and can consist of both tumor cells as well as endothelial cells. B. Model of tumor-induced neovascularization. In i, an initially avascular tumor grows until inner regions become hypoxic and upregulate production of angiogenic factors such as VEGF, FGF, and IL-8. In ii, a tumor grows on an existing blood vessel. Soon the tumor induces Ang2 expression in the pre-existing vessel, and it regresses due to endothelial cell apoptosis. The tumor is now avascular and by upregulating angiogenic factors as in i, it induces the production of a new blood supply.

= normal tumor cell  = necrotic tumor cell
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### TABLE 1. Factors that regulate normal angiogenesis

<table>
<thead>
<tr>
<th>Factor</th>
<th>Biological Actions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Soluble mediators</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>1. Increases endothelial cell permeability</td>
<td>31-34</td>
</tr>
<tr>
<td></td>
<td>2. Stimulates endothelial cell uPA/PAI-1 production</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>3. Stimulates endothelial cell proliferation</td>
<td>38-40</td>
</tr>
<tr>
<td></td>
<td>4. Inhibits endothelial cell apoptosis</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>5. Enhances endothelial cell migration</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>6. Stimulates in vivo angiogenesis</td>
<td>38</td>
</tr>
<tr>
<td>Angiopoietin-1 (Ang1)</td>
<td>1. Stimulates in vitro endothelial cell sprout formation</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>2. Increases girth and stability of endothelium</td>
<td>83,85</td>
</tr>
<tr>
<td>Angiopoietin-2 (Ang2)</td>
<td>1. Antagonizes Ang1 signaling/destabilizes endothelium</td>
<td>86</td>
</tr>
<tr>
<td>aFGF, bFGF</td>
<td>1. Stimulates endothelial cell proliferation</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>2. Enhances endothelial cell migration</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>3. Stimulates endothelial cell PA/collagenase production</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>4. Stimulates endothelial cell tube formation</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>5. Stimulates in vivo angiogenesis</td>
<td>93,221</td>
</tr>
<tr>
<td>PDGF</td>
<td>1. Stimulates DNA synthesis in endothelial cells</td>
<td>98,99</td>
</tr>
<tr>
<td></td>
<td>2. Stimulates endothelial cells to form chords in vitro</td>
<td>99,100</td>
</tr>
<tr>
<td></td>
<td>3. Stimulates proliferation of smooth muscle cells and pericytes</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>4. Induces vWF, VEGF, and VEGF receptor-2 expression in cardiac endothelial cells</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>5. Increases capillary wall stability</td>
<td>103-106</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1. Supports anchorage-independent growth of fibroblasts</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>2. Inhibits proliferation and migration of endothelial cells</td>
<td>116</td>
</tr>
</tbody>
</table>
3. Stimulates/inhibits formation of endothelial cell tubes 
   \textit{in vitro}  
   119
4. Produces net antiproteolytic activity via modulation of 
   uPA/PAI-1 expression levels  
   121
5. Inhibits production of other proteases/stimulates 
   production of protease inhibitors  
   120
6. Stimulates VSMA production by pericytes  
   122
7. Chemotactic for monocytes and fibroblasts  
   127,128
8. Stimulates \textit{in vivo} angiogenesis in presence of 
   inflammatory response  
   116,123-126
9. Increases vessel wall stability  
   129,130

| TNF-\(\alpha\) | 1. Stimulates angiogenesis \textit{in vivo}  
                  132 |
|---------------|---------------------------------|
|               | 2. Stimulates formation of endothelial cell tubes \textit{in vitro}  
                  133 |
|               | 3. Inhibits endothelial cell proliferation  
                  132 |

| EGF, TGF-\(\alpha\) | 1. Stimulate endothelial cell proliferation  
                           135 |
|                     | 2. Stimulate angiogenesis \textit{in vivo}  
                           135 |

| G-CSF, GM-CSF | 1. Stimulate endothelial cell proliferation and migration  
                           137 |

| Angiogenin | 1. Stimulates angiogenesis \textit{in vivo}  
                     138 |
|           | 2. Supports endothelial cell binding and spreading  
                     139 |

| Angiotropin | 1. Stimulates random capillary endothelial cell migration  
                          141 |
|            | 2. Stimulate endothelial cell tube formation  
                          141 |
|            | 3. Stimulates \textit{in vivo} angiogenesis  
                          142 |

| Tissue Factor | 1. Contributes to development of yolk sac vasculature  
                          143 |

| Factor V | 1. Contributes to development of yolk sac vasculature  
                           144 |

| Prostaglandin | 1. Stimulates \textit{in vivo} angiogenesis  
                           145 |

| Nicotinamide | 1. Stimulates \textit{in vivo} angiogenesis  
                           146 |
Monobutyrin

1. Stimulates *in vivo* angiogenesis 147
2. Stimulates endothelial cell migration *in vitro* 147

II. Membrane-bound proteins

αvβ3 Integrin

1. Highly expressed on activated endothelial cells 162,163
2. Mediates endothelial cell attachment, spreading, and migration 164
3. Present on angiogenic capillary sprouts 165-166
4. Required for bFGF-stimulated angiogenesis *in vivo* 166-167,169
5. Localizes MMP-2 to capillary sprouts 170
6. Suppresses endothelial cell apoptosis 172

αvβ5 Integrin

1. Required for VEGF-stimulated angiogenesis *in vivo* 173

α5β1 Integrin

1. Required for non-VEGF growth factor-stimulated angiogenesis *in vivo* 175

VE Cadherin

1. May mediate permeability of endothelium 33-34
2. Required for *in vivo* angiogenesis 182-183
3. Prevents endothelial cell apoptosis 183

Eph 4B/Ephrin-B2

1. Colocalize at venous/arterial interfaces of developing embryo 188
2. Required for angiogenesis of head and yolk sac and for myocardial trabeculation 188

Ephrin A1

1. Required for *in vivo* angiogenesis induced by TNF-α 191
2. Chemotactic for endothelial cells *in vitro* 191

Eph-2A

1. Required for endothelial cell tube formation *in vitro* 192

III. Biomechanical Forces

Blood flow/shear stress

1. Increases endothelial stress fiber formation
1. Promotes endothelial cell division (if turbulent)  
2. Stimulates transcription of bFGF and TGF-β genes

**TABLE 2. Factors that regulate tumor angiogenesis**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Role in tumor neovascularization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Soluble mediators</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>1. Secreted by many tumor cells <em>in vitro</em></td>
<td>207</td>
</tr>
<tr>
<td></td>
<td>2. Highly upregulated in most human cancers</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>3. Expression correlates with intratumoral microvessel density and poor prognosis in cancer patients</td>
<td>208,209</td>
</tr>
<tr>
<td></td>
<td>4. Inhibition decreases tumor vessel density and tumor growth</td>
<td>44, 211-214</td>
</tr>
<tr>
<td>FGF</td>
<td>1. Inhibiton suppresses generation of tumor vessels (<em>in vitro</em> and <em>in vivo</em>) and tumor growth <em>in vivo</em></td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>2. Important for maintenance, vs. induction, of tumor angiogenesis</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>3. Synergizes with VEGF to promote angiogenesis <em>in vitro</em> and <em>in vivo</em></td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>4. Induces VEGF expression in tumor cells and VEGF receptor expression in endothelial cells</td>
<td>226-228</td>
</tr>
<tr>
<td>Heparanase</td>
<td>1. Stimulates invasion and vascular sprouting of endothelial cells</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>2. Releases bFGF from extracellular matrix</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>3. mRNA and protein are enriched in metastatic tumor cell lines and human tumors vs. normal tissues</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>4. Overexpression renders nonmetastatic cell lines metastatic <em>in vivo</em> and increases tumor neovascularization</td>
<td>228</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>1. Induced in endothelial cells of pre-existing vessels co-opted by a tumor, leading to vessel regression</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>2. Induced in endothelial cells of newly-formed vessels of tumor, leading to vessel plasticity and VEGF-mediated growth</td>
<td>197</td>
</tr>
</tbody>
</table>
IL-8
1. Mitogenic and chemotactic for HUVECs in vitro 233
2. Stimulates angiogenesis in vivo 233
3. mRNA is upregulated in neoplastic tissues vs. normal ones in vivo; expression correlates with extent of neovascularization 234,236
4. Overexpression increases invasiveness, tumorigenicity, neovascularization, and metastatic potential of tumor cells 236,237
5. Mediates stimulation of MMP-2 gene transcription 235

MMP-2
1. Directly modulates melanoma cell adhesion and spreading on extracellular matrix 238
2. Mediates tumor growth and neovascularization in CAM 239