Regulation of Angiogenesis via Vascular Endothelial Growth Factor Receptors

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Introduction

Endothelial cell signal transduction mechanisms involved in angiogenesis have come into focus in cancer research when it was realized that solid tumors are dependent on neovascularization (1). Unlike normal human endothelial cells, which are quiescent except in the reproductive organs of fertile women, endothelial cells in tumors express several target features associated with their angiogenic activation. On the other hand, unlike the tumor cells, endothelial cells are readily accessible from the blood circulation, and they are not likely to develop resistant variants to cytostatic therapy (see Ref. 2). Vascular targeting may involve either the destruction of existing vessels by exploitation of differences between normal and tumor vessels (3–6) or inhibition of the tumor angiogenesis process per se (7–9). In both scenarios, knowledge of the endothelial cell-specific growth factor receptors and their signal transduction and effector mechanisms is essential and will undoubtedly provide additional points of attack to human cancers. Here we discuss recent results on one important family of endothelial growth factor receptors implicated in angiogenesis. Several excellent reviews have appeared on the same topic and related topics (10–14).

The VEGF1 family currently includes five members in addition to the prototype VEGF, namely, PIGF, VEGF-B, VEGF-C, VEGF-D, and Orf virus VEGFs (also called VEGF-E; Refs. 15–18 and Refs. below). The VEGFs mediate angiogenic signals to the vascular endothelium via high-affinity RTKs. To date, three receptors for the VEGFs have been identified. All three are relatively specific for endothelial cells and demonstrate structural and functional similarities to the PDGF receptor family (see Refs. 14 and 19). These receptors are currently designated VEGFR-1, VEGFR-2, and VEGFR-3 and were originally named flt (fms-like tyrosine kinase), KDR (kinase insert domain-containing receptor)/flk-1 (fetal liver kinase-1), and FLT4, respectively (11, 12, 20–23). All have seven immunoglobulin domains in their extracellular part and an intracellular tyrosine kinase signaling domain split by a kinase insert (Fig. 1). In adults, VEGFR-1 and VEGFR-2 are expressed mainly in the blood vascular endothelium, whereas VEGFR-3 is restricted largely to the lymphatic endothelium.

The VEGF molecule is an antiparallel disulfide-linked homodimer with several different isoforms generated by alternative splicing, consisting of polypeptides of 121, 145, 165, 189, or 206 amino acid residues (15, 24–26). VEGF binds both VEGFR-1 and VEGFR-2 and is apparently capable of inducing heterodimers between the two (27–30). VEGF uses symmetrical binding sites at each pole of the dimer for receptor binding (31, 32). It has been shown that the second immunoglobulin homology domain of VEGFR-1 is critical for ligand binding, but the first three immunoglobulin domains are required to reconstitute full affinity (31, 33–35). Of the other VEGF family members, PIGF and VEGF-B bind only VEGFR-1, the Orf virus VEGFs bind only VEGFR-2, and VEGF-C and VEGF-D interact with both VEGFR-2 and VEGFR-3 (16–18, 36–42). Recently, NRP-1, a cell surface glycoprotein that acts as a receptor for collapsins/semaphorins and controls axon guidance during embryonic development, has been identified as an isoform-specific receptor for VEGF165, PIGF-2, VEGF-B, and Orf virus VEGFs (39, 43–45). Heterodimers of PIGF and VEGF are produced by certain cell lines, and they exert mitogenic activity toward endothelial cells (46, 47). VEGF-B/VEGF heterodimers have also been obtained in expression vector cotransfection experiments (48). In addition to the high-affinity receptors, certain splice isoforms of the VEGFs also bind heparan sulfate proteoglycans on the cell surface and in the pericellular matrix via a distinct heparin binding domain (16, 49–51).

VEGFR-1 is a Mr 180,000 transmembrane glycoprotein, but its mRNA can also be spliced to produce a shorter soluble protein consisting of only the first six extracellular immunoglobulin homology domains (20, 27, 52). Such a RNA splice variant, originally detected in a HUVEC cDNA library, encodes 31 unique amino acid residues before a stop codon (52). VEGFR-2 is a Mr 230,000 protein, and no splice variants have been reported for this receptor. In human VEGFR-3, alternative 3’ polyadenylation signals result in a 4.5-kb transcript and a more prevalent 5.8-kb transcript (53). The latter transcript encodes 65 additional amino acid residues and is the major form detected in tissues. After biosynthesis, the glycosylated Mr 195,000 VEGFR-3 is proteolytically cleaved in the fifth immunoglobulin homology domain, but the resulting Mr 120,000 and Mr 75,000 chains remain linked by a disulfide bond (41, 54).

Because hypoxia is a major inducer of VEGF expression in tumors, and the VEGFRs are enhanced in tumor endothelia (see Fig. 2), hypoxic regulation of the VEGFR genes has been studied by several groups. In vivo, VEGFR-1 and VEGFR-2 appear to be up-regulated by hypoxia (55–57). In vitro, VEGFR-1 expression was increased by hypoxia, whereas VEGFR-2 was either down-regulated or not affected (58–60). On the other hand, culture medium from hypoxic cells up-regulated VEGFR-2 protein (59). The latter effect was probably mediated by VEGF in the culture medium because VEGF treatment has been shown to up-regulate VEGFR-2 expression in endothelial cells and in cerebral slice cultures (61, 62). The promoter for VEGFR-1 contains sequences matching the HIF-1α consensus binding site, whereas the closely related HIF-2α may stimulate VEGFR-2 promoter activity (60, 63, 64).

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PIGF, placenta growth factor; RTK, receptor tyrosine kinase; PDGF, platelet-derived growth factor; NRP-1, neuropilin-1; HUVEC, human umbilical vein endothelial cell; HIF, hypoxia-inducible factor; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PLC, phospholipase C; P3-K, phosphatidylinositol 3’-kinase; STAT, signal transducer and activator of transcription; KS, Kaposi’s sarcoma; VE-cadherin, vascular endothelial cadherin; ES, embryonic stem; VHL, von Hippel-Lindau; HHV-8, human herpes virus-8/KS herpes virus; Ang, angiotensin.
VEGFR Signaling

Like other RTKs, the VEGFRs are thought to dimerize and undergo trans-autophosphorylation on ligand binding. Both VEGFR-2 and VEGFR-3 are tyrosine phosphorylated when stimulated with their respective ligands (40, 65), but VEGFR-1 autophosphorylation is less obvious and has been studied mostly in receptor-overexpressing transfected cells (37, 65–67). Phosphorylated tyrosine residues may serve to control the kinase activity of the receptor and to create docking sites for cytoplasmic signaling molecules, which provide substrates for the kinase. These molecules, either adapters or enzymes themselves, link VEGFRs to the signaling pathways that are discussed below.

VEGFR-1 and VEGFR-2. Activation of the MAPK pathway in response to VEGF has been observed in many types of endothelial cells (66–74). Interestingly, MAPK activation was delayed in VEGFR-2-transfected fibroblasts, and the mitogenic response was weaker than in endothelial cells, suggesting the involvement of cell type-specific signaling mechanism(s) (71). In postcapillary venular endothelium, VEGF-mediated induction of MAPK was blocked by inhibitors of nitric oxide synthase, suggesting that nitric oxide contributes to MAPK activation (75). The role of Ras in the VEGFR-MAPK pathway remains to be elucidated. In primary endothelial cells, Ras was not activated in response to VEGF, and MAPK activation was mediated mainly via a PKC-dependent pathway (74). PKC has also been implicated in VEGF-induced MAPK activation in VEGFR-2-transfected fibroblasts (71). Seetharam et al. (66) have observed activation of the Ras guanine nucleotide exchange factor Sos in VEGF-stimulated endothelial cells, but phosphorylation of the adapter protein Shc was barely detectable. On the other hand, Shc phosphorylation in response to VEGF stimulation has been detected in porcine aortic endothelial cells overexpressing VEGFR-2 (69), and Sck, a Shc homologue, has been shown to interact with the VEGFR-2 cytoplasmic domain in the yeast two-hybrid system (76). Several investigators have reported phosphorylation of the Ras GTPase-activating protein in endothelial cells after VEGF stimulation (65, 66, 77). Ras GTPase-activating protein activation has also been observed in VEGFR-1-transfected fibroblasts and porcine aortic endothelial cells,
but these cells showed no clear MAPK activation or proliferation in response to VEGF (65, 66). Interestingly, activation of MAPK in bovine brain capillary endothelial cells by VEGF was selectively inhibited by the $M_{16}$,000 NH$_2$-terminal fragment of prolactin, an antiangiogenic factor that also inhibited proliferation of these cells (68, 78). In addition to MAPK, VEGF has been shown to activate the p38 stress kinase pathway in HUVECs, and this response has been linked to F-actin reorganization and cell migration (72).

The PLC-$\gamma$-PKC pathway has also been implicated in the mitogenic action of VEGF. VEGFR-1 interacts with the PLC-$\gamma$ SH2 domain in the yeast two-hybrid assay (79), and several groups have reported that VEGF induces the phosphorylation and activation of PLC-$\gamma$ (66–68, 70, 71, 73, 77, 80), leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerols and inositol 1,4,5-trisphosphate. Inositol 1,4,5-trisphosphate is likely to be responsible for the increase in intracellular Ca$^{2+}$ after VEGF stimulation (27), whereas diacylglycerol, in turn, activates certain PKC isoforms expressed in the target cells. VEGF selectively activated the Ca$^{2+}$-sensitive PKC isoforms $\alpha$ and $\beta$2 in bovine aortic endothelial cells, and the mitogenic effect of VEGF in these cells was inhibited by a PKC-$\beta$-selective inhibitor (80).

Xia et al. (80) reported activation of PI3-K in response to VEGF and demonstrated that PI3-K activity is not required for mitogenesis or activation of PKC (74, 80). However, PI3-K was shown to activate Akt, a serine kinase involved in antiapoptotic signaling, and to subsequently deliver a survival signal in HUVECs treated with a VEGFR-2-selective VEGF mutant (81). VEGFR-2 and the PI3-K/Akt pathway may therefore play a role in VEGF-mediated survival of immature vessels (82). Akt has also been reported to directly activate endothelial nitric oxide synthase, suggesting that Akt may regulate the increased production of nitric oxide in response to VEGF stimulation (83, 84).

STATs are latent cytoplasmic transcription factors. STAT activation by the VEGFRs has been studied in transient transfection assays (85). All three receptors were shown to be strong activators of STAT3 and STAT5, whereas STAT1 was not activated by the VEGFRs. However, the role of this pathway in endothelial cell biology is unknown.

**VEGFR-3.** Whereas both VEGFR-3 isoforms bind to and phosphorylate adapter protein Shc, phosphorylation is stronger in cells expressing the long isoform (54, 86, 87). The Shc PTB domain is required for ligand-induced Shc tyrosine phosphorylation by VEGFR-3 (88), and mutations in Shc phosphorylation sites increased VEGFR-3 transforming activity in the soft agar assay, indicating that Shc has a negative role in VEGFR-3 signaling.

Both VEGFR-3 isoforms bind Grb2 via its SH2 domain in an inducible manner (54, 87). Stimulation of VEGFR-3 also activates MAPK, at least in transfected cells (89). Similarly, both isoforms bind to a glutathione S-transferase-SH2 (PLC-$\gamma$) fusion protein on stimulation (54, 86). In contrast, no direct interaction has been observed with PI3-K (86). In a human erythroblleukemia cell line and in KS cells, VEGF-C-induced tyrosine phosphorylation of the cytoskeletal protein paxillin by related adhesion focal tyrosine kinase, a recently identified member of the focal adhesion kinase family (90, 91).

Although very little is known about the specific signal transduction of VEGFR-3 in the lymphatic endothelium, mutations in VEGFR-3 have recently been linked with hereditary lymphedema, an autosomal dominant disorder of the lymphatic system that leads to disabling swelling of the extremities and, in rare cases, to lymphangiosarcomas (see Fig. 1; Ref. 92). This indicates that disturbed VEGFR-3 signaling may play a key role in the development of this disease.

**VEGFRs Associate with Cell Adhesion Receptors.** According to new data, endothelial cell proliferation and survival in response to VEGF may require the association of VEGFR-2 with cell surface adhesive proteins. Activated VEGFR-2 was found in a complex with integrin $\alpha_\beta_3$, an adhesion molecule specifically expressed on angiogenic endothelium (93). $\alpha_\beta_3$ binds to pericellular matrix proteins containing an arginine-glycine-aspartic acid (RGD) peptide motif, such as vitronectin and fibronectin. $\alpha_\beta_3$ has been shown to be involved in the regulation of the cell cycle and the survival of endothelial cells (94–96). Various antagonists of this integrin, such as antibodies and cyclic binding peptides, as well as cytokines that interfere with $\alpha_\beta_3$ activity, are capable of inhibiting tumor angiogenesis (97). VEGFR-2 tyrosine phosphorylation and VEGF-induced mitogenicity were enhanced when endothelial cells were plated on the $\alpha_\beta_3$ ligand vitronectin, demonstrating that this integrin contributes to VEGFR-2 signaling (93).

VE-cadherin, an endothelium-specific cell-cell adhesion protein, has also been implicated in molecular interactions with the VEGFRs. Deficiency or truncation of VE-cadherin was lethal in mouse embryos due to increased endothelial cell apoptosis (98). In normal endothelial cells, VEGFR-2 colocalized with VE-cadherin, and VEGF stimulation resulted in the formation of a complex between VE-cadherin, $\beta$-catenin, PI3-K, and VEGFR-2 and subsequent Akt activation. On the other hand, disruption of VE-cadherin function prevented the endothelial cells from responding to survival signals induced by VEGF. Interestingly, VEGFR-3, but not VEGFR-1, also communoprecipitated with VE-cadherin.

**Vascular Permeability May Be Transduced via an Unknown VEGFR.** Tumor vessels are leaky in general, and VEGF is one of the most potent inducers of vascular permeability (99). VEGF-C also has vascular permeability activity, but the response is not transduced via VEGFR-3 (89). On the other hand, PlGF, which only binds to VEGFR-1 (36, 37), is incapable of inducing permeability. These data imply that either VEGFR-2 or some other as yet unidentified receptor mediates the permeability response. When the third variable domain in the VEGF polypeptide was substituted with the analogous region of PlGF, creating a chimeric molecule with significantly reduced binding to VEGFR-2, the mutant still induced vascular permeability with an activity similar to that of wild-type VEGF (100). Therefore, it may be that vascular permeability is not mediated by any of the known VEGFRs. Interestingly, the c-Src protein seems to be a necessary mediator of the permeability response because adenoviral vectors expressing VEGF do not induce vascular permeability in mice deficient in c-Src (101).

**Importance of the Cellular Background in VEGF Signaling Studies.** Because most VEGFR signal transduction studies have been carried out using endothelial cells that express more than one type of VEGFR, it has not been possible to attribute the results to a particular receptor. Attempts to study signal transduction by individual receptors using transfected cells have been compromised by the lack of a proper cellular background, which may be an important factor in VEGF signaling, depending on the substrate under study. Use of receptor-specific ligands or mutants of the VEGF family may offer an alternative approach to these questions (89, 102). Another variable in the signal transduction studies is introduced by the heterogeneity of endothelial cells and their tendency to lose differentiated properties in culture. Keeping these aspects in mind, the signaling mechanisms discovered thus far are of interest and may offer possible targets for therapeutic intervention (103). For example, global assessment of endothelial cell gene expression by RNA microarray analysis may help to better define the activated state of the endothelial cells on angiogenesis and drug treatment. However, for candidate drugs, additional validation has to be provided by experiments in transgenic and gene targeted mice.
The VEGFRs in Vascular Development

The various VEGFRs are first expressed in the mouse embryonic mesoderm between days 7.5–9.5 of development and distinct expression patterns are also found in 17 week human fetuses (104–106). Disruption of any of the three VEGFR genes leads to embryonic lethality. In embryos with targeted null mutations of VEGFR-2, differentiation of both endothelial and hematopoietic cells is blocked, and no blood vessels are formed (107). However, at least in vitro, endothelial/hematopoietic precursor cells can be derived from VEGFR-2-deficient ES cells, demonstrating that VEGFR-2 is not required for the formation of the common hematopoietic/endothelial progenitor cell, the so-called hemangioblast (108, 109). Rather, VEGFR-2 signaling seems to be necessary for endothelial commitment because endothelial development is terminated at an early stage in VEGFR-2-negative ES cell cultures (109). Hematopoietic requirement for VEGFR-2, on the other hand, is conditional and depends on the cell culture conditions (108, 109). Although VEGFR-2 is not required for hemangioblast formation, it appears to be essential for the subsequent VEGF-directed hemangioblast migration to appropriate environments in the developing embryo (108–110). In the absence of VEGFR-2 signaling, such cells may rapidly disappear, explaining the lack of endothelial and hematopoietic precursors in VEGFR-2 null embryos.

In human postnatal hematopoietic tissues, VEGFR-2 apparently persists as a specific marker of hematopoietic stem cells, differentiating them from the lineage-committed hematopoietic precursor cells (111). Endothelial progenitors or angioblasts have also been isolated from peripheral blood and claimed to be incorporated into sites of active angiogenesis (112, 113). Apparently, tissue ischemia or systemic VEGF treatment can mobilize such bone marrow-derived mononuclear progenitor cells (114, 115).

Mouse embryos homozygous for a targeted mutation of the VEGFR-1 locus develop an excess of endothelial cells in both embryonic and extraembryonic locations, but the endothelial cells fail to organize into normal vascular channels (116). The increase in endothelial cell numbers is due to an alteration in cell fate determination among mesenchymal cells, leading to increased hemangioblast commitment (117). However, when only the tyrosine kinase domain of VEGFR-1 was deleted, leaving the ligand binding extracellular part and the transmembrane domain intact, the gene-targeted mice developed normal vessels and survived (118). One possible explanation for these and other findings is that during embryogenesis, VEGFR-1 acts as a VEGF sink, regulating the amount of free VEGF available for vascular development. In the absence of VEGFR-1, there would be an excess of free VEGF available to its major signal transducing receptor, VEGFR-2. Therefore, coordinated expression of both VEGFR-1 and VEGFR-2 would be essential for controlled early vascular development.

Disruption of VEGFR-3 led to a defective remodeling of the primary vascular plexus and cardiovascular failure after embryonic day 9.5, but differentiation of endothelial cells, formation of primitive vascular networks, and vascular sprouting occurred normally (119). This phenotype suggests that VEGFR-3 is required for maturation of the primary vascular plexus into a hierarchy of large and small vessels. Therefore, VEGFR-3 seems to have a general blood vascular function during early development and only later becomes restricted mostly to the lymphatic vascular system. Conditional gene targeting is needed to better define the lymphatic functions of VEGFR-3.

Other VEGF-binding proteins, such as NRP-1, also seem to be involved in the regulation of angiogenesis during development. In normal mouse embryos, NRP-1 is expressed in a temporally restricted manner in endothelial cells of capillaries and blood vessels and in limb buds, and expressed more widely in the mesenchyme (43, 120). Targeted disruption of the mouse NRP-1 gene caused severe abnormalities in the peripheral nervous system (121). Interestingly, VEGF has recently been reported to act also as a neurotrophic survival factor and as a mitogenic agent for Schwann cells (122). Mice overexpressing NRP-1 formed excess capillaries and abnormal blood vessels and died in utero (120). It is possible that aberrant angiogenesis in NRP-1 transgenic embryos resulted from inappropriate VEGF activity.

VEGF and VEGFRs in Tumors

Expression of VEGF and its receptors correlates with the degree of vascularization of many experimental and clinical tumors as detected by in situ hybridization and immunohistochemistry (123–132), and both have been used as prognostic indicators of an increased metastatic risk (see Ref. 133). Although the detailed molecular mechanism of the “angiogenic switch” by which quiescent endothelium becomes activated is unknown, VEGF seems to be the main inducer of tumor angiogenesis. Tumor hypoxia and oncogenes up-regulate VEGF levels in the neoplastic cells, and hypoxia, in combination with the locally increased VEGF concentrations, up-regulates VEGFR-1 and VEGFR-2 on tumor endothelial cells (62, 127, 131). VEGF-negative ES cells or Ras-transformed fibroblasts grow poorly as tumors in syngeneic mice (134, 135). Interestingly, ES cells lacking HIF-1α (deficient in hypoxic induction of VEGF) are tumorigenic. The tumors formed by HIF-1α-deficient ES cells are poorly vascularized, but they are apparently more resistant to tumor cell apoptosis under hypoxic conditions (136). A recent report has suggested that in some cases, tumor vessels can also be formed without endothelial cells (137).

Autocrine VEGF and VEGFR-1 expression has been shown to occur in angiosarcomas (138). Of the nonendothelial tumors studied, only a few melanomas and leukemia cell lines aberrantly expressed VEGFRs, whereas several tumor cell lines expressed NRP-1 (22, 139–141). In tumors associated with the VHL disease, VEGF and VEGFRs are constitutively up-regulated in the absence of hypoxia (see Ref. 142). Whereas the normal VHL protein functions as a component of a ubiquitin-protein ligase complex that targets selected proteins such as HIF-1α for ubiquitin-mediated proteolysis (143), the cells containing a defective VHL protein fail to degrade HIF-1α, leading to constitutive up-regulation of many hypoxia-regulated genes, including VEGF (144).

VEGF-3 is up-regulated in tumor angiogenesis in general, for example, in breast carcinomas (145, 146). In addition, VEGF-3 has been shown to be increased in the endothelium of lymphatic vessels in metastatic lymph nodes and in lymphangiomas, vascular skin tumors, and KS spindle cells (105, 147, 148).

VEGF and VEGFRs in the Development of AIDS-linked KS.

KS, which is the major neoplastic manifestation of HIV-induced AIDS, is an angiogenic tumor composed of endothelial and spindle cells (for a review, see Ref. 149). AIDS-linked KS is always accompanied by infection with HHV-8. In KS lesions, HHV-8 is present in the neoplastic cells, and hypoxia, in combination with the local increase in VEGF concentrations, up-regulates VEGF-R1 and VEGF-R2 on tumor endothelial cells (62, 127, 131). VEGF-negative ES cells or Ras-transformed fibroblasts grow poorly as tumors in syngeneic mice (134, 135). Interestingly, ES cells lacking HIF-1α (deficient in hypoxic induction of VEGF) are tumorigenic. The tumors formed by HIF-1α-deficient ES cells are poorly vascularized, but they are apparently more resistant to tumor cell apoptosis under hypoxic conditions (136). A recent report has suggested that in some cases, tumor vessels can also be formed without endothelial cells (137).

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Michael Klagsbrun, personal communication.
proliferation of KS cells in vitro (158). It thus seems probable that an autocrine VEGF/VEGFR activation loop plays a part in the development of AIDS-linked KS.

Role of VEGF-induced Vessel Permeability in Tumor Angiogenesis. In contrast to the well-organized normal blood vessels, tumor vessels are characteristically poorly functioning, leaky endothelial channels with incomplete arteriovenous and perivascular differentiation (159). Tumor blood flow is temporally and spatially heterogeneous, and the interstitial pressure in solid tumors is generally much higher than that in normal tissues. Lack of functional lymphatic vessels is another factor leading to interstitial hypertension (159). The microvascular hyperpermeability in tumors causes plasma proteins to leak into the extravascular space, leading to clotting of extravasated fibrinogen and introduction of a provisional plasma-derived matrix. Matrix formation precedes and accompanies the onset of endothelial cell migration and vessel sprouting (160). VEGF derived from hypoxic areas of tumors is likely to mediate increased permeability. VEGF therefore contributes to tumor angiogenesis by both direct and indirect mechanisms: on one hand, VEGF stimulates endothelial cell proliferation and migration; on the other, it renders vessels hyperpermeable, leading to formation of a matrix that supports blood vessel growth. Interestingly, new studies have shown that both VEGF-induced permeability and pathological angiogenesis are attenuated in PlGF-deficient mice.5

Angiogenesis Inhibitors and VEGFR Function

Studies in animal models have illustrated the dependence of tumor vascularization and progression on VEGF signaling. A heterodimeric VEGF variant in which the receptor binding sites at one pole of the ligand were mutated while the other pole was left intact was shown to act as an inhibitor of VEGF function.6 A four-fold excess of this mutant VEGF, which bound to its receptors but failed to mediate receptor dimerization and subsequent signaling, was sufficient for almost complete inhibition of vascular permeability and tissue factor expression induced by wild-type VEGF. A dominant negative mutant of VEGFR-2 has been expressed from a retroviral construct and was found to prevent glioblastoma vascularization and growth in nude mice (161). The study was later extended to other types of tumors, and in most cases, tumor growth was inhibited (162). Antibodies directed against either VEGFR-2 or the VEGFR-2/VEGF complex have also been effective in inhibiting VEGF-mediated signaling and endothelial cell proliferation in vitro (163–165). Because the dominant negative VEGFR-2 inhibits angiogenesis in vivo, compounds that block the tyrosine kinase activity of VEGFR-2 should also prevent neovascularization. Indeed, specific VEGF-2 tyrosine kinase inhibitors have been shown to abolish VEGF-induced mitogenesis of human endothelial cells in vitro and to have an antitumor effect in mice (103). Several such compounds (see Ref. 166) are currently under evaluation in clinical trials for the treatment of human cancers.

Because soluble extracellular domains of the VEGFRs compete with endothelial cell surface receptors for VEGF binding and inhibit VEGF-induced mitogenicity (52, 167, 168), recombinant VEGFRs retaining high ligand binding affinity also provide the potential for inhibition of tumor angiogenesis. Tumor growth in mice has been shown to be suppressed by the soluble VEGFR-1 and VEGFR-2 receptor “bodies” (168–170). Adult mice seem to tolerate such therapy well, whereas newborn mice that receive the inhibitor during their first two postnatal weeks fail to thrive and die (171). In young animals, systemic administration of soluble VEGFR-1 leads to inhibition of growth plate differentiation and growth of long bones, apparently because VEGF-mediated blood vessel invasion is essential for coupling the resorption of cartilage with bone formation (172). Interestingly, VEGF also supports osteoclast recruitment and bone resorption, and this effect has been shown to be mediated via VEGFR-1 expressed in osteoclasts (173). Given the role of VEGF in bone development, anti-VEGF therapy could be useful in treating bone-associated pathologies, for example, bone metastases and osteosarcomas.

Cross-Talk between Different Receptor Families Required for Angiogenesis

Recent reports on the association of activated VEGFR-2 with other cell surface receptors raise questions on the possible roles these coreceptors play during angiogenesis in vivo. Both NRP-1 and integrin αβ3 have been shown to increase the mitogenic effects of VEGF (43, 93). Because NRP-1 binds only to certain VEGFs (39, 43–45), it could function to specifically potentiate the effects of these family members. In addition, NRP-1 may be able to induce biological responses in cells that express NRP-1 but not VEGFRs. VEGFR-2 association with VE-cadherin and αβ3 may serve to create active signaling complexes by clustering proteins involved in VEGF-mediated survival signaling (81, 98). αβ3 is required for the full activation of VEGFR-2, suggesting that the complex might regulate tyrosine phosphatase activity or ensure correct juxtaposition of the receptor and putative cytoskeletal substrates.

In addition to the VEGF/VEGFR system, Tie and Eph families of endothelial tyrosine kinases have important functions in the formation and maintenance of the vascular system (174–180). Tie-1 and Tie-2 are restricted to the vascular endothelium and certain hematopoietic progenitor cells. Four known ligands, the Angs, bind to Tie-2, whereas there are currently no ligands for Tie-1 (Fig. 3; Refs. 181–185). Interestingly, recent findings demonstrate that overexpression of

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5 Peter Carmeliet and M. Graziella Persico, personal communication.
6 Robert de Waal, personal communication.
Ang-1 leads to the formation of vessels resistant to vascular leakage induced by VEGF (186). Plasma leakage contributes not only to tumor angiogenesis but also to a variety of other disease processes (160), and Ang-1 could perhaps be used therapeutically to reverse such adverse effects of VEGF. The Tie-2 pathway has been reported to be essential for tumor growth and angiogenesis (168, 187, 188). In the study by Siemeister et al. (188), the VEGFR pathway was not sufficient to compensate for the lack of Tie-2 signaling, suggesting the existence of two independent mechanisms, both of which are required for tumor angiogenesis (188). Interestingly, the antagonistic Tie-2 ligand Ang-2 has been shown to be up-regulated in the endothelial cells of coopted host vessels of certain tumors, and its increased expression preceded and coincided with apoptotic regression of these vessels (189). Hypoxia, fibroblast growth factor, and VEGF have been shown to increase Ang-2 mRNA in endothelial cells, suggesting important pathways of signal trans-regulation (see Fig. 3; Refs. 190, 191). The intense autocrine expression of Ang-2 by endothelial cells may represent a host defense mechanism against the growing tumor because Ang-2 expression in the absence of VEGF has been implicated in destabilizing the interactions of endothelial cells, pericytes, and the surrounding extracellular matrix, which are necessary for endothelial cell survival (182).

In contrast to the VEGFs and the Angs, the ligands of the large Eph RTK family, ephrins, do not function as soluble molecules but are attached to membrane via either a transmembrane domain or a glycosylid anchor (180). In gene-targeted mice, ephrin-B2 was shown to specifically mark arterial endothelial cells, whereas its EphB receptors specifically and reciprocally marked the venous endothelium (178, 179). Ephrins have also been linked to integrin function because a recent study showed that ephrin-B1 can promote the attachment of endothelial cells to extracellular matrix components by activating integrins, including αβ1 (192). Because inhibition of integrin function has been shown to disrupt many different aspects of angiogenesis, it is intriguing that αβ1 has been shown to be associated with both VEGFRs and the Ephps.

Of other receptor-ligand families, PDGF-B and PDGF receptor β have been implicated in the establishment of endothelial cell-pericyte interactions (193, 194). Transforming growth factor β is also an important regulator of vascular structures (195). Endothelial cell-specific members of the TGF-β receptor family include endoglin and ALK-1. Lack of endoglin led to poor vascular smooth muscle development, arrested endothelial remodeling, and lethality in mouse embryos, demonstrating that endoglin is essential for developmental angiogenesis (196). On the other hand, elevated endoglin expression has been shown to disrupt many different aspects of angiogenesis, two independent mechanisms, both of which are required for tumor angiogenesis but also to a variety of other disease processes (160), and Ang-1 could perhaps be used therapeutically to reverse such adverse effects of VEGF. The Tie-2 pathway has been reported to be essential for tumor growth and angiogenesis (168, 187, 188). In the study by Siemeister et al. (188), the VEGFR pathway was not sufficient to compensate for the lack of Tie-2 signaling, suggesting the existence of two independent mechanisms, both of which are required for tumor angiogenesis (188). Interestingly, the antagonistic Tie-2 ligand Ang-2 has been shown to be up-regulated in the endothelial cells of coopted host vessels of certain tumors, and its increased expression preceded and coincided with apoptotic regression of these vessels (189). Hypoxia, fibroblast growth factor, and VEGF have been shown to increase Ang-2 mRNA in endothelial cells, suggesting important pathways of signal trans-regulation (see Fig. 3; Refs. 190, 191). The intense autocrine expression of Ang-2 by endothelial cells may represent a host defense mechanism against the growing tumor because Ang-2 expression in the absence of VEGF has been implicated in destabilizing the interactions of endothelial cells, pericytes, and the surrounding extracellular matrix, which are necessary for endothelial cell survival (182).

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Of other receptor-ligand families, PDGF-B and PDGF receptor β have been implicated in the establishment of endothelial cell-pericyte interactions (193, 194). Transforming growth factor β is also an important regulator of vascular structures (195). Endothelial cell-specific members of the TGF-β receptor family include endoglin and ALK-1. Lack of endoglin led to poor vascular smooth muscle development, arrested endothelial remodeling, and lethality in mouse embryos, demonstrating that endoglin is essential for developmental angiogenesis (196). On the other hand, elevated endoglin expression has been correlated with the proliferation of tumor endothelial cells (197), and expression of ALK-1 coincides with sites of vasculogenesis and angiogenesis in early mouse development (198). Mutations in endoglin or ALK-1 are the cause of hereditary hemorrhagic telangiectasia (199, 200). Endothelial cell-specific members of the transmembrane tyrosyl phosphatase and Notch receptor families have also been described (201, 202). The functions of some or many of these may at least partially fail in the poorly organized tumor vessels, which in general do not contain pericytes or well-formed basement membranes. A better understanding of the endothelial signal transduction pathways should lead to the development of additional molecular tools for the prevention of tumor angiogenesis.

References


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REGULATION OF ANGIOGENESIS VIA VEGFRs


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