

## Endothelial Receptor Tyrosine Kinases Involved in Angiogenesis

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**D**EVELOPMENTAL growth, the remodeling and regeneration of adult tissues as well as solid tumor growth, can only occur accompanied by blood vessel formation. Angioblasts and hemopoietic precursor cells differentiate from the mesoderm and form the blood islands of the yolk sac and the primary vascular system of the embryo (vasculogenesis). The formation of the rest of the vascular tree is thought to occur as a result of vascular sprouting from pre-existing vessels, a process called angiogenesis (9).

Endothelial cells can give rise to several types of functionally and morphologically distinct vessels. Upon angiogenic stimuli, endothelial cells can re-enter the cell cycle, degrade the underlying basement membrane and migrate, forming capillary sprouts that project into the perivascular stroma, and again withdraw from the cell cycle and subsequently differentiate to form new vessels that are functionally adapted to their tissue environment. Thus, angiogenesis, concurrent with tissue development and regeneration depends on the tightly controlled processes of endothelial cell proliferation, migration, differentiation, and survival. On the other hand, dysfunction of the endothelial cell regulatory system is a key feature of many diseases. Most importantly, tumor growth and metastasis have been shown to be angiogenesis dependent (9).

Key signals regulating cell growth and differentiation are mediated by polypeptide growth factors and their transmembrane receptors, many of which are tyrosine kinases. Several families of receptor tyrosine kinases have been characterized (46). The main currently known growth factors and receptors transducing angiogenic stimuli are schematically shown in Fig. 1. Some of them, such as the receptors for fibroblast growth factors (FGFR),<sup>1</sup> platelet-derived growth factor-BB (PDGFR $\beta$ ), transforming growth factor- $\alpha$  (epidermal growth factor receptor, EGFR), and hepatocyte growth factor (Met oncoprotein) are widely expressed in many tissues and cell types, whereas others are strictly endothelial cell specific (46). FGFs (for a review see reference 1) have been shown to be mitogenic and chemotactic for cultured endothelial cells. FGFs also stimulate the production of proteases such as col-

lagenases and plasminogen activators and induce tube formation by endothelial cells. Cultured endothelial cells express FGF receptor-1 but no significant levels of other high-affinity FGF receptors. Curiously, in situ hybridization analyses show very little or no FGF receptor expression in endothelia of mouse embryos or human melanomas (16, 48). Thus, at least part of the angiogenic effects of FGFs in vivo may be mediated indirectly.

### *Vascular Endothelial Growth Factor Is a Major Endothelial Cell-specific Angiogenesis and Permeability Factor*

Striking new evidence shows that there are endothelial cell specific growth factors and receptors that may be primarily responsible for the stimulation of endothelial cell growth, differentiation, and differentiated functions. The best studied of these is vascular endothelial growth factor (VEGF), a member of the PDGF family. VEGF is a dimeric glycoprotein of 23-kD subunits, mitogenic for endothelial cells and able to induce vessel permeability (alternative name vascular permeability factor; see references 7, 28). Other effects of VEGF include the mobilization of intracellular Ca<sup>2+</sup>, the induction of plasminogen activator, urokinase receptor (22), and plasminogen activator inhibitor-1 synthesis, stimulation of hexose transport in endothelial cells, and promotion of monocyte migration in vitro. Four VEGF isoforms encoded by distinct mRNA splicing variants appear to be equally capable of stimulating mitogenesis of endothelial cells, but have different affinities for cell surface heparan sulfate proteoglycans (HSPGs) (see reference 28). Soluble non-heparin-binding and heparin-binding forms have been also described for the related placenta growth factor (PlGF) (23, 33).

The pattern of VEGF expression suggests its involvement in the development and maintenance of the normal vascular system and in tumor angiogenesis (2, 27, 44). During murine development, the entire 7.5-d postcoitum endoderm expresses VEGF and the ventricular neuroectoderm produces VEGF at the capillary ingrowth stage (3). In addition, epithelial cells next to fenestrated endothelia in adult mice show persistent VEGF expression, suggesting a role in the maintenance of this specific endothelial phenotype and function (3).

### *Characterization of VEGF Receptors*

Two high affinity receptors for VEGF have been characterized, VEGFR-1/Flt-1 (*fms*-like tyrosine kinase-1) (4, 43)

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1. *Abbreviations used in this paper:* EGFR, epidermal growth factor receptor; FGFR-1, 2, and 4, fibroblast growth factors 1, 2, and 4; HSPG, heparan sulfate proteoglycans; PDGFR $\beta$ , platelet-derived growth factor-BB; PlGF, placenta growth factor, VEGF, vascular endothelial growth factor.

and VEGFR-2/Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1) (25, 27, 29, 45). The expression of VEGFRs occurs mainly in vascular endothelial cells although some may also be present on certain hematopoietic cells, such as monocytes, and in melanoma cell lines. Only endothelial cells have been reported to proliferate in response to VEGF and endothelial cells from different sources show different responses.

VEGFR-1 and VEGFR-2 bind VEGF with high affinity ( $K_d$  1–20 and 75–770 pM, respectively) (4, 27, 45, 47). VEGFR-2-mediated signals cause striking changes in the morphology, actin reorganization and membrane ruffling of porcine aortic endothelial cells overexpressing this receptor (47). In these cells, VEGFR-2 also mediated ligand-induced chemotaxis and mitogenicity, whereas VEGFR-1-transfected cells lacked mitogenic responses to VEGF. In contrast, VEGF had a strong growth stimulatory effect on rat sinusoidal endothelial cells expressing endogenous VEGFRs (47). There is also evidence that the two forms of PlGF bind to VEGFR-1 ( $K_d$  about 200 pM) but not to VEGFR-2. Although PlGF is not a major mitogen for most endothelial cells, it potentiates the mitogenic activity of low concentrations of VEGF, whereas at higher VEGF concentrations PlGF had no effect (33). Heterodimerisation between VEGF and PlGF polypeptides has not yet been reported.

In situ hybridization studies, mouse VEGFR-2 mRNA expression was found in yolk sac and intraembryonic mesoderm (E7.5), from which the endothelium is derived, and later in presumptive angioblasts, endocardium and large and small vessel endothelium (E12.5) (39). Abundant VEGFR-2 mRNA in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic and early postnatal brain and decreased expression in adult brain suggested that VEGFR-2 is a major regulator of vasculogenesis and angiogenesis (27). VEGFR-1 expression was similarly associated with early vascular development in mouse embryos and with neovascularisation in healing skin wounds (36). However, high levels of VEGFR-1 expression were also detected in adult organs, suggesting that VEGFR-1 has a function in quiescent endothelium of mature vessels not related to cell growth. The avian homologue of VEGFR-2 was observed in the mesoderm from the onset of gastrulation, whereas the VEGFR-1 homologue was first found in cells coexpressing endothelial markers (8). In human fetal tissues VEGFR-1 and VEGFR-2 showed overlapping, but slightly different expression patterns (15). In vitro quail epiblast cultures FGF, which is required for vasculogenic differentiation of these cells, upregulated VEGFR-2 expression (8).

Targeted homozygous null mutations of VEGFRs indicate that VEGFR-2 is required for the formation of blood islands and later blood vessels and for hematopoiesis whereas VEGFR-1 plays an essential role in endothelial organization during vascular development. Interestingly, the block in hematopoiesis occurred at an early stage of differentiation in VEGFR-2  $-/-$  embryos, whereas differentiation of endothelial cells proceeded as shown by the expression of early vasculogenesis markers, e.g. VEGFR-1, Flt4 and Tek. Tie, which marks relatively later stages of endothelial cell development was not expressed, indicating the absence of more mature endothelial cells (personal communications from Drs. G. H. Fong, F. Shalaby, and J. Rossant). These data suggest that VEGF and its receptors act in a paracrine and co-

operative manner to regulate the differentiation of endothelial cells and neovascularization of tissues.

### *Flt4, a VEGFR Related Receptor of the Lymphatic Endothelium*

Flt4 is a receptor tyrosine kinase closely related in structure to the products of the VEGFR-1 and VEGFR-2 genes (10, 32). However, the mature form of Flt4 differs from the VEGFRs in that it is proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides (30, 31). VEGF did not show specific binding to Flt4 or induce its autophosphorylation (31) and our recent results show that Flt4 transmits signals for a novel growth factor (Joukov, V., and K. Alitalo, unpublished data).

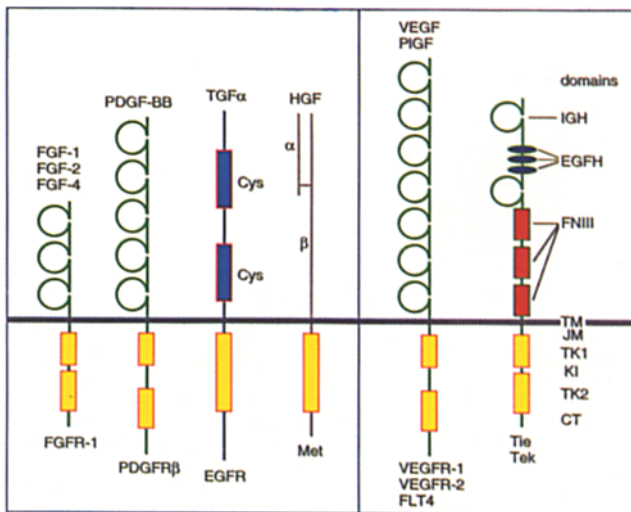
*FLT4* gene expression appears to be more restricted than the expression of VEGFR-1 or VEGFR-2 (14, 15). The expression of Flt4 first becomes detectable by in situ hybridization in angioblasts of head mesenchyme and veins of the embryo. During later stages of development, Flt4 mRNA becomes restricted to developing lymphatic vessels. Only lymphatic endothelia and some high endothelial venules express Flt4 mRNA in adult human tissues. The results support the theory of the venous origin of lymphatic vessels (14).

### *Receptor Tyrosine Kinases Tie and Tek*

Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) and Tek (tunica interna endothelial cell kinase) comprise another family of endothelial cell specific receptor tyrosine kinases (5, 13, 24, 34, 40, 41, 50). Tie cDNA was originally cloned from human megakaryoblastic leukemia cell lines. In vivo, its expression is restricted to endothelial cells and certain hematopoietic cells, including hematopoietic stem cells (13, 20, 21, 34, our unpublished data).

During embryonic development the Tie and Tek receptors are uniformly expressed in endothelial cells of blood vessels. Tek mRNA signals appear between days 7.5 and 8.0. In addition to the mesoderm cell layer of the amnion, Tek marks vascular endothelial cells and also their precursors, von Willebrandt factor-negative angioblasts (5). Tie is first expressed on embryonic day 8.5 in angioblasts of the head mesenchyme, in the splanchnopleure, dorsal aorta, and vitelline veins. The migrating endothelial cells in the developing heart are also positive for Tie, as is the allantoic membrane (21). In adult mice the expression of Tie mRNA persists in vessels of the lung whereas in the brain, heart, and liver it appears to decrease (21). However, newly formed capillaries in hormone-induced, maturing ovarian follicles and in the granulation tissue of skin wounds show somewhat enhanced Tie expression in the adult mouse (20).

The Tie and Tek receptors are required for endothelial cell function as disruption of the corresponding gene loci by targeted mutagenesis was fatal. Tek-deficient mouse embryos died very early, soon after the gene should have been expressed. The number of endothelial cells was dramatically decreased, the heart was underdeveloped and severe hemorrhaging was detected (6). Tie-deficient embryos survived after the time point where Tie expression normally begins, but died of hemorrhage later (Sato, T., M. Puri, and J. Partanen, personal communication). Further studies should clarify the specific functions of Tie and Tek, identify their ligand(s) and characterize the signal(s) mediated by these receptors.



**Figure 1.** Major endothelial cell receptor tyrosine kinases and growth factors involved in vasculogenesis and angiogenesis. The structures of the receptors are shown schematically, and their major protein domains have been indicated (IGH, immunoglobulin homology domain; EGFH, epidermal growth factor homology domain; FNIII, fibronectin type III homology domain; TM, transmembrane domain; JM, juxtamembrane domain; TK1 and TK2, tyrosine kinase catalytic domains; KI, kinase insert; CT, carboxyl terminal tail; Cys, cysteine-rich domains). Only ligands and receptors with proven angiogenic activity have been included.  $\alpha$  and  $\beta$  mark the proteolytically cleaved, disulfide-linked subunits of the Met receptor. A detail not shown in the figure is that the Flt4 receptor is also proteolytically processed, unlike the VEGF receptors. Other names and abbreviations appear in the text.

### Endothelial Growth Factors and Receptors in Tumor Angiogenesis

Angiogenesis has been proposed to be a rate limiting factor for tumor growth, playing an important role in metastasis (see reference 9). In several tumors, increased vascularization has been directly correlated with a poor prognosis. Consistent with this, highly malignant gliomas showed higher VEGF expression than tumors of lower malignancy (12). Besides blood flow into the tumor, entry of tumor cells into the blood stream and subsequent metastasis may be facilitated by enhanced vascular permeability and fibrin deposition induced by VEGF.

VEGF expression in cultured cells has been shown to be elevated by hypoxia (44), apparently due to hypoxia-responsive elements regulated by a putative heme iron-containing sensor system similar to the one regulating erythropoietin expression (11). This provides a plausible mechanism for induction of tumor angiogenesis in the hypoxic areas of solid tumors. In support of this hypothesis, elevated VEGF mRNA levels were detected in non-mitotic palisading cells of glioblastoma surrounding necrotic areas (38, 44). Growth factors produced by tumor cells as well as mutations in the tumor suppressor genes may also induce VEGF secretion (18, 35). Growth of several solid tumors in nude mice was inhibited in vivo by treatment with monoclonal antibodies specific for VEGF (19).

Significantly elevated levels of the Tie, VEGFR-1 and

VEGFR-2 mRNAs, but no Flt4 mRNA, have been observed in vascular endothelial cells of malignant glioblastoma. In comparison, there was little or no expression in endothelia of normal brain vasculature (12, 37). Enhanced expression of the Tie receptor mRNA was also observed in the vasculature of metastatic melanomas (16).

Inhibition of tumor angiogenesis may become effective therapy in several types of tumors. Of key importance for this kind of therapy is a clear understanding of the processes and regulatory mechanisms involved in blood vessel formation. For example, it may become possible to inhibit the induction of VEGF expression by hypoxia, once the specific signal transduction mechanisms have been elucidated. Several kinds of potential angiogenesis inhibitors have been reported (9). Extracellular domains of certain growth factor receptors have been detected in conditioned media of cultured cells and in human blood, and they form the basis for competitive inhibition of ligand binding and activation of cell surface signal transducing receptors. Alternative splicing of VEGFR-1 transcripts generates a truncated soluble high-affinity receptor, which can inhibit VEGF-induced mitogenesis of cultured endothelial cells (17). So-called dominant negative forms of growth factor receptors, engineered by in vitro mutagenesis (46) provide a potential tool for anti-angiogenesis gene therapy. A dominant negative Tek receptor in transgenic mice produced similar phenotypic effects as the Tek gene knockout, e.g., dramatic hemorrhaging and decreased overall number of endothelial cells (6). Moreover, glioblastoma growth in nude mice was inhibited by retrovirus-mediated expression of a dominant negative VEGFR-2 (26). Inhibitors of low-affinity binding to HSPGs or specific tyrosine kinase inhibitors may also have therapeutic potential. Finally, the promoter elements controlling endothelial-specific expression may be useful in targeting human gene therapy against angiogenesis associated with tumors or other disease processes involving the vascular system in, e.g., blood cell trafficking, hemostasis, wound healing, and atherosclerosis.

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