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PERSPECTIVES IN BASIC SCIENCE

Role of vascular endothelial growth factor in the regulation of angiogenesis

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Role of vascular endothelial growth factor in the regulation of angiogenesis. Compelling evidence indicates that vascular endothelial growth factor (VEGF) is a fundamental regulator of normal and abnormal angiogenesis. The loss of a single VEGF allele results in defective vascularization and early embryonic lethality. VEGF plays also a critical role in kidney development, and its inactivation during early postnatal life results in the suppression of glomerular development and kidney failure. Recent evidence indicates that VEGF is also essential for angiogenesis in the female reproductive tract and for morphogenesis of the epiphyseal growth plate and endochondral bone formation. Substantial experimental evidence also implicates VEGF in pathological angiogenesis. Anti-VEGF monoclonal antibodies or other VEGF inhibitors block the growth of several human tumor cell lines in nude mice. Furthermore, the concentrations of VEGF are elevated in the aqueous and vitreous humors of patients with proliferative retinopathies such as the diabetic retinopathy. In addition, VEGF-induced angiogenesis results in a therapeutic benefit in several animal models of myocardial or limb ischemia. Currently, both therapeutic angiogenesis using recombinant VEGF or VEGF gene transfer and inhibition of VEGF-mediated pathological angiogenesis are being pursued clinically.

The cardiovascular system is the first organ system to develop and reach a functional state in an embryo [1]. The initial steps consist of "vasculogenesis," which is the differentiation of endothelial cell precursors, the angioblasts, from the hemangioblasts [2]. The juvenile vascular system evolves from the primary capillary plexus by subsequent pruning and reorganization of endothelial cells in a process called "angiogenesis" [3]. The development of a vascular supply is essential not only for organ development and differentiation during embryogenesis but also for wound healing and reproductive functions in the adult [4]. Angiogenesis is also implicated in the pathogenesis of a variety of disorders: proliferative retinopathies, age-related macular degeneration (AMD), tumors, rheumatoid arthritis (RA), and psoriasis [4, 5].

Several potential regulators of angiogenesis have been identified, including acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), hepatocyte growth factor/scatter factor (HGF/SF), tumor necrosis factor-α (TNF-α), angiogenin, and interleukin (IL)-8, [3, 6], and more recently, the angiopoietins (Angs), the ligands of the Tie-2 receptor [7, 8]. The negative regulators include thrombospondin [9, 10], the 16 kDa N-terminal fragments of prolactin [11] and growth hormone [12], the plasminogen fragment angiostatin [13], the collagen XVIII fragment endostatin [14], and vasostatin, a calreticulin fragment [15].

In 1983, Senger et al reported the partial purification of a protein able to induce vascular leakage in the guinea pig skin [16]. This protein was named tumor vascular permeability factor (VPF) and was thought to be a specific mediator of the high permeability of tumor blood vessels, rather than a growth factor. In 1989, Ferrara and Henzel and Ploüet et al independently reported the purification to homogeneity and NH_2-teminal amino acid sequencing of an endothelial cell-specific mitogen, which they named, respectively, vascular endothelial growth factor (VEGF) [17] and vasculotropin [18]. Subsequent cloning and expression of VEGF [19] and VPF [20] revealed that the activities of VEGF and VPF are mediated by the same molecule. The finding that VEGF is potent, diffusible, and specific for vascular endothelial cells led to the hypothesis that this molecule might play a unique role in the regulation of physiological and pathological growth of blood vessels [17, 19].

Over the last few years, several molecules structurally related to VEGF have been identified, including placenta growth factor (PlGF) [21, 22], VEGF-B [23], VEGF-C [24, 25], and VEGF-D [26, 27]. Recent reviews on these topics have been published by Ferrara and Davis-Smyth [28], Enholm et al [29], Carmeliet and Collen [30], Or-

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tega, Hutchings, and Ploüet [31], or Neufeld et al [32]. The focus of this article is on VEGF. There is compelling evidence that this factor plays an essential role in the development and differentiation of the cardiovascular system, and the loss of a single VEGF allele results in early lethality in mouse embryos [33, 34]. Furthermore, VEGF is a key mediator of pathological angiogenesis associated with tumors or ischemic retinal diseases [28]. VEGF-induced angiogenesis has been shown to result in a therapeutic effect in animal models of coronary or limb ischemia [28]. Presently, both therapeutic angiogenesis using recombinant VEGF or VEGF gene transfer and inhibition of VEGF-mediated pathological angiogenesis are being actively pursued clinically.

BIOLOGICAL EFFECTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR

Vascular endothelial growth factor is a mitogen for vascular endothelial cells derived from arteries, veins, and lymphatics, but it is devoid of consistent and appreciable mitogenic activity for other cell types [28]. VEGF also elicits a pronounced angiogenic response in a variety of *in vivo* models, including the chick chorioallantoic membrane [18, 19]. Also, VEGF induces endothelial sprouting from rat aortic rings embedded in a collagen gel [35]. VEGF and bFGF demonstrate a potent synergism in their ability to promote angiogenesis in an *in vitro* model system, where bovine microvascular endothelial cells invade a tridimensional collagen gel [36]. More recently, the combination of VEGF and Ang-1 or Ang-2 has been shown to have an effect greater than additive in the mouse cornea model [37]. Although neither Ang-1 nor Ang-2 had any angiogenic effect, each of them was able to significantly potentiate the angiogenic response to VEGF in this model [37].

Vascular endothelial growth factor functions as a survival factor for cultured endothelial cells in serum-depleted conditions [38, 39]. Consistent with a prosurvival activity, VEGF induces expression of the antiapoptotic proteins Bcl-2 and A1 in human endothelial cells [38]. Alon et al have provided evidence that VEGF is a survival factor for immature retinal vessels [40]. The same group suggested that pericyte coverage is the critical event that determines whether endothelial cells are dependent on VEGF for survival [41].

Vascular endothelial growth factor induces expression of the serine proteases urokinase-type and tissue-type plasminogen activators (PAs) and also PA inhibitor-1 (PAI-1) in cultured bovine microvascular endothelial cells [42]. Moreover, VEGF increases expression of the metalloproteinase interstitial collagenase in human umbilical vein endothelial cells (HUVECs) but not in dermal fibroblasts [43].

As mentioned earlier in this article, VEGF is known also as vascular permeability factor (VPF) based on its ability to induce vascular leakage in the guinea pig skin [44]. Dvorak proposed that an increase in microvascular permeability to proteins is a crucial step in angiogenesis associated with tumors and wounds [45]. Bates and Curry have shown that VEGF also induces an increase in hydraulic conductivity of isolated microvessels [46] and that such an effect is mediated by increased calcium influx [47]. Other studies have also suggested that VEGF also induces fenestrations in endothelial cells, both *in vivo* [48, 49] and *in vitro* [50].

Melder et al have shown that VEGF promotes expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in endothelial cells [51]. This induction results in the adhesion of activated natural killer (NK) cells to endothelial cells, mediated by specific interaction of endothelial VCAM-1 and ICAM-1 with CD18 and VLA-4 on the surface of NK cells. Transgenic overexpression of VEGF in the skin of mice results not only in increased vascular density but also in enhanced leukocyte adhesion and rolling [52].

Vascular endothelial growth factor has been reported to have regulatory effects on blood cells. Clauss et al reported that VEGF promotes monocyte chemotaxis [53]. Broxmeyer et al have subsequently shown that VEGF induces colony formation by mature subsets of granulocyte-macrophage progenitor cells [54]. Furthermore, Gabrilovich et al have reported that VEGF may have an inhibitory effect on the maturation of host professional antigen-presenting cells such as dendritic cells [55]. VEGF was found to inhibit immature dendritic cells *in vitro*, without having a significant effect on the function of mature cells, suggesting that VEGF may also facilitate tumor growth by allowing the tumor to avoid the induction of an immune response [55]. These findings have been extended to the *in vivo* situation, where VEGF infusion resulted in inhibition of dendritic cell development, associated with an increase in the production of B cells and immature Gr-1(+) myeloid cells [56]. Infusion of VEGF was also found to be associated with inhibition of the activity of the transcription factor nuclear factor-κB in bone marrow progenitor cells [56].

Eichman et al have provided evidence that VEGF is a fundamental regulator of differentiation of the hemangioblast, the common precursor for endothelial and hemopoietic cells [57]. A population of mesodermal cells from chicken embryos at the gastrulation stage was isolated based on the expression of the VEGF receptor Flk-1/KDR (discussed later in this article). In the presence of VEGF, such cells differentiated in endothelial lineage [57]. Hemopoietic differentiation occurred in the absence of VEGF, although it was significantly reduced by soluble Flk-1/KDR, suggesting that this process could be mediated by a second, yet unidentified, Flk-1/KDR ligand. These findings suggest that VEGF is the instruc-

tive signal that results in the differentiation of hemangioblasts into the endothelial lineage.

Vascular endothelial growth factor induces vasodilation *in vitro* in a dose-dependent fashion [58, 59] and produces transient tachicardia, hypotension, and a decrease in cardiac output when injected intravenously in conscious, instrumented rats [59]. Such effects appear to be caused by a decrease in venous return, mediated primarily by endothelial cell-derived nitric oxide (NO) [59]. Accordingly, VEGF has no direct effect on contractility or rate in isolated rat heart *in vitro* [59]. These hemodynamic effects, however, are not unique to VEGF. Other angiogenic factors such as aFGF and bFGF also have the ability to induce NO-mediated vasodilation and hypotension [60, 61].

ORGANIZATION OF THE VEGF GENE AND CHARACTERISTICS OF THE VEGF ISOFORMS

The human VEGF gene is organized in eight exons, separated by seven introns. The coding region spans approximately 14 kb [62, 63]. The human VEGF gene is localized to chromosome 6p21.3 [64]. It is now well established that alternative exon splicing of a single VEGF gene results in the generation of four different molecular species, having, respectively, 121, 165, 189, and 206 amino acids following signal sequence cleavage ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). $VEGF_{165}$ lacks the residues encoded by exon 6, whereas $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Compared with $VEGF_{165}$, $VEGF_{121}$ lacks 44 amino acids; $VEGF_{189}$ has an insertion of 24 amino acids highly enriched in basic residues, and $VEGF_{206}$ has an additional insertion of 17 amino acids [62].

Vascular endothelial growth factor$_{165}$ is the predominant molecular species produced by a variety of normal and transformed cells. Transcripts encoding $VEGF_{121}$ and $VEGF_{189}$ are detected in the majority of cells and tissues expressing the VEGF gene [62]. In contrast, $VEGF_{206}$ is a very rare form [62].

Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45,000 Da [17]. These properties correspond to those of $VEGF_{165}$, the major isoform [65]. $VEGF_{121}$ is a weakly acidic polypeptide that fails to bind to heparin [65]. $VEGF_{189}$ and $VEGF_{206}$ are more basic and bind to heparin with greater affinity than $VEGF_{165}$ [65]. $VEGF_{121}$ is a freely diffusible protein; $VEGF_{165}$ is also secreted, although a significant fraction remains bound to the cell surface and the extracellular matrix (ECM). In contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the ECM [66]. However, these isoforms may be released in a soluble form by heparin or heparinase, suggesting that their binding site is represented by proteoglycans containing heparin-like moieties. The long forms may be released also by plasmin following cleavage at the COOH terminus. This action generates a bioactive proteolytic fragment having molecular weight of approximately 34,000 Da [65]. Plasminogen activation and generation of plasmin have been shown to play an important role in the angiogenesis cascade. Thus, proteolysis of VEGF is likely to occur also *in vivo.* Keyt et al have shown that the bioactive product of plasmin action is comprised of the first 110 NH_2-terminal amino acids of VEGF [67]. These findings suggest that the VEGF proteins may become available to endothelial cells by at least two different mechanisms: as freely soluble proteins ($VEGF_{121}$, $VEGF_{165}$) or following protease activation and cleavage of the longer isoforms. However, the loss of heparin binding, whether it is caused by alternative splicing of RNA or plasmin cleavage, results in substantially decreased mitogenic activity for vascular endothelial cells. Compared with $VEGF_{165}$, $VEGF_{121}$ or $VEGF_{110}$ demonstrate a 50- to 100-fold reduced potency when tested in endothelial mitogenic assay [67]. It is possible that the stability of VEGF-heparan sulfate-receptor complexes contributes to effective signal transduction and stimulation of endothelial cell proliferation [67]. Furthermore, more recent studies have demonstrated that $VEGF_{121}$ fails to bind neuropilin-1, an isoform-specific VEGF receptor that presents $VEGF_{165}$ to its signaling receptors in a manner that enhances the effectiveness of the signal transduction cascade (see section on signal transduction) [68]. Poltorak et al have provided evidence for the existence of an additional alternatively spliced isoform containing exons 1 through 6 and 8 of the VEGF gene, $VEGF_{145}$ [69]. $VEGF_{145}$ is able to promote endothelial cell growth, albeit with a significantly lower potency than $VEGF_{165}$. Ploüet et al have recently proposed a role for urokinase in the generation of bioactive $VEGF_{189}$ [70]. Recombinant $VEGF_{189}$ from insect cells infected with a recombinant baculovirus was purified as a nonmitogenic 50 kDa precursor that binds to the receptor Flt-1 but not to Flk-1/KDR. However, it could be matured by urokinase as a 38 kDa fragment able to bind Flk-1/KDR and promote endothelial cell proliferation [70].

Muller et al have solved the crystal structure of VEGF at a resolution of 2.5 A [71]. Overall, the VEGF monomer resembles that of PDGF, but its N-terminal segment is helical rather than extended. The dimerization mode of VEGF is similar to that of PDGF and unlike that of TGF-β [71].

REGULATION OF VEGF GENE EXPRESSION

Oxygen tension

Oxygen tension has been shown to be a key regulator of VEGF gene expression, both *in vitro* and *in vivo.* VEGF mRNA expression is induced by exposure to low pO_2 in a variety of normal and transformed cultured cell types

[72, 73]. Also, ischemia caused by occlusion of the left anterior descending coronary artery results in a dramatic increase in VEGF RNA levels in the pig and rat myocardium, suggesting that VEGF may be one of the mediators of the spontaneous revascularization that follows myocardial ischemia [74, 75].

Similarities exist between the mechanisms leading to hypoxic regulation of VEGF and erythropoietin (Epo) [76]. Hypoxia inducibility is conferred on both genes by homologous sequences. A 28-base sequence has been identified in the 5′ promoter of the rat and human VEGF gene, which mediates hypoxia-induced transcription [77, 78]. Such a sequence reveals a high degree of homology and similar protein binding characteristics as the hypoxia-inducible factor-1 (HIF-1) binding site within the Epo gene [79]. HIF-1 is a basic, heterodimeric, helix-loop-helix protein consisting of two subunits, HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT), known also as HIF-1β [80]. Evidence for a critical role of HIF-1α has been also provided by gene knockout studies. Inactivation of HIF-1α in mice resulted in developmental arrest and lethality by E11 [81, 82]. Embryos manifested neural tube defects, cardiovascular malformations, and marked cell death within the cephalic mesenchyme. These results suggest that HIF-1α is a key regulator of cellular and developmental O_2 homeostasis [81]. Also, the consequence of HIF-1α inactivation in embryonic stem (ES) cell on *in vitro* proliferation and *in vivo* tumorigenesis has been examined by two groups [82, 83]. Surprisingly, however, very different conclusions were reached. According to Ryan, Lo, and Johnson, HIF-1α inactivation leads to a dramatically decreased tumorigenesis and increased apoptosis, coincident with reduced hypoxia-induced expression of VEGF [82]. In contrast, Carmeliet et al reported that the growth of ES cell-derived tumors is paradoxically enhanced following loss of HIF-1α, possibly because of protection from hypoxia-induced apoptosis [83]. Whether such discrepancies reflect differences in the genotype or clone of ES cells or other experimental variable remains to be established.

However, transcriptional activation is not the only mechanism leading to VEGF up-regulation in response to hypoxia. Increased mRNA stability has been identified as a significant post-transcriptional component [84, 85].

Growth factors and hormones

Several cytokines or growth factors may up-regulate VEGF mRNA expression. EGF, TGF-β, or keratinocyte growth factor 1 (KGF1) result in a marked induction of VEGF mRNA expression [86]. EGF also stimulates VEGF release by cultured glioblastoma cells [87]. In addition, treatment of quiescent cultures of epithelial and fibroblastic cell lines with TGF-β resulted in the induction of VEGF mRNA and the release of VEGF protein in the medium [88]. Both IL-1α and prostaglandin E_2 induce expression of VEGF in cultured synovial fibroblasts, suggesting the participation of such inductive mechanisms in inflammatory angiogenesis [89]. IL-6 has been also shown to induce VEGF expression significantly in several cell types [90]. IGF-1 is able to induce expression of VEGF mRNA and protein release in cultured colorectal carcinoma cells [91]. Thyroid-stimulating hormone has been shown to induce VEGF expression in several thyroid carcinoma cell lines [92]. Also, angiotensin II induces VEGF release from cultured human mesangial cells [93]. Shifren et al have examined the VEGF expression in the midgestation (16 to 20 weeks) human fetal adrenal cortex [94]. Strong cytoplasmic immunostaining for VEGF was detected in clusters of fetal zone cells. In contrast, cells in the outer, less well-vascularized, definitive zone of the cortex stained only weakly for VEGF. Adrenocorticotropic hormone (ACTH) was able to induce VEGF expression in cultured human fetal adrenal cortical cells. These findings suggest that VEGF may be a local regulator of adrenal cortical angiogenesis and a mediator of the tropic action of ACTH.

Cell differentiation and transformation

Cell differentiation has been shown to play an important role in the regulation of VEGF gene expression [95]. The VEGF mRNA is up-regulated during the conversion of 3T3 preadipocytes into adipocytes or during the myogenic differentiation of C2C12 cells.

Specific transforming events also result in induction of VEGF gene expression. Oncogenic mutations or amplification of ras lead to VEGF up-regulation [96–98]. Moreover, the von Hippel-Lindau (VHL) tumor suppressor gene has been recently implicated in the regulation of VEGF gene expression [99–101]. The VHL tumor suppressor gene is inactivated in patients with VHL disease and in most sporadic clear cell renal carcinomas. Although the function of the VHL protein remains to be fully elucidated, it is known that such protein interacts with the elongin BC subunits *in vivo* and regulates RNA polymerase II elongation activity *in vitro* by inhibiting formation of the elongin ABC complex. Renal cell carcinoma cells either lacking endogenous wild-type VHL gene or expressing an inactive mutant demonstrated altered regulation of VEGF gene expression, which was corrected by the introduction of the wild-type VHL gene. Most of the endothelial cells' mitogenic activity released by tumor cells expressing mutant VHL gene was neutralized by anti-VEGF antibodies [99]. These findings suggest that VEGF is a key mediator of the abnormal vascular proliferations and solid tumors characteristic of VHL syndrome. Iliopoulos et al have shown that a function of the VHL protein is to provide a negative regulation of a series of hypoxia-inducible genes, including the VEGF platelet-derived growth factor B chain and the glucose

transporter GLUT1 genes [100]. In the presence of a mutant VHL, mRNAs for such genes were produced both under normoxic and hypoxic conditions. Reintroduction of wild-type VHL resulted in the inhibition of mRNA production under normoxic conditions and restored the characteristic hypoxia-inducibility of those genes [100]. Gnarra et al suggested that VHL regulates VEGF expression at a post-transcriptional level and that VHL inactivation in target cells causes a loss of VEGF suppression [101]. However, Mukhopadhyay et al have provided evidence for an additional mechanism by which the VHL gene product suppresses VEGF: VHL interacts with Sp1 to repress VEGF promoter activity, as assessed by VEGF promoter-luciferase reporter studies [102]. Deletion analysis defined a 144 bp region of the VEGF promoter necessary for VHL repression. This VHL-responsive element is GC-rich and specifically binds the transcription factor Sp1 in crude nuclear extracts.

VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTORS

Initial studies provide evidence for the existence of two classes of high-affinity VEGF binding sites on the surface of endothelial cells, with K_d values of 10 and 100 pM, respectively, and molecular masses in the range of 180 to 220 kDa [103, 104]. Ligand autoradiography studies on fetal and adult rat tissue sections demonstrated that high-affinity VEGF binding sites are localized to the vascular endothelium of large or small vessels *in situ* [105, 106]. VEGF binding was apparent not only on proliferating but also on quiescent endothelial cells [105, 106].

Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) tyrosine kinases

Binding characteristics and structural properties. Two VEGF receptor tyrosine kinases (RTKs) have been identified. The Flt-1 (fms-like-tyrosine kinase) [107] and KDR (kinase domain region) [108] receptors bind VEGF with high affinity. The murine homologue of KDR, Flk-1 (fetal liver kinase-1), shares an 85% sequence identity with human KDR [109]. Both Flt-1 and KDR/Flk-1 have seven immunoglobulin (Ig)-like domains in the extracellular domain (ECD), a single transmembrane region and a consensus tyrosine kinase (TK) sequence, which is interrupted by a kinase-insert domain [109–111]. Flt-1 has the highest affinity for $rhVEGF_{165}$, with a K_d of approximately 10 to 20 pM [107]. KDR has a lower affinity for VEGF, with a K_d of approximately 75 to 125 pM [108].

A cDNA coding an alternatively spliced soluble form of Flt-1 (sFlt-1), lacking the seventh Ig-like domain, transmembrane sequence and the cytoplasmic domain has been identified in HUVECs [112, 113]. This sFlt-1 receptor binds VEGF with high affinity (K_d 10 to 20 pM) and is able to inhibit VEGF-induced mitogenesis [113].

An additional member of the family of RTKs with seven Ig-like domains in the ECD is Flt-4 (VEGFR-3) [114–116], which, however, is not a receptor for VEGF but rather binds a newly identified ligand called VEGF-C or VEGF-related peptide (VRP) [24, 25]. VEGF-C has been shown to be involved in the regulation of lymphatic angiogenesis [117].

Recent studies have mapped the binding site for VEGF to the second Ig-like domain of Flt-1 and KDR [118, 119]. The deletion of the second domain of Flt-1 abolished the binding of VEGF. The introduction of the second domain of KDR into an Flt-1 mutant lacking the homologous domain restored VEGF binding [118]. However, the ligand specificity was characteristic of the KDR receptor because the mutant failed to bind PlGF. Similar conclusions were reached in deletion experiments in KDR [120]. These studies indicate that of the seven IgG-like domains in the ECD, only domains 2 and 3 are needed for tight binding of VEGF to the KDR receptor [120]. Wiesmann et al have solved the crystal structure of a VEGF-Flt-1 domain 2 complex [121]. These studies have shown domain 2 in a predominantly hydrophobic interaction with the poles of the VEGF dimer. However, alanine-scanning mutagenesis analysis of the first three domains of Flt-1 has shown that several charged residues, especially Asp187, are important in maintaining the structural integrity of domain 2, although not directly involved in the ligand–receptor interface [122].

Signal transduction. Vascular endothelial growth factor has been shown to induce the phosphorylation of at least 11 proteins in bovine aortic endothelial cells [123]. Phospholipase Cγ(PLC-γ) and two proteins that associate with PLC-γ were phosphorylated in response to VEGF. Furthermore, immunoblot analysis for mediators of signal transduction that contain SH2 domains demonstrated that VEGF induces phosphorylation of phosphatidylinositol 3-kinase, ras GTPase-activating protein (GAP), and several others. These studies, however, did not identify which VEGF receptor(s) is involved in these events. Recently, it has been suggested that NO mediates, at least in part, the mitogenic effect of VEGF on cultured microvascular endothelium isolated from coronary venules [124].

Several studies have indicated that Flt-1 and KDR have different signal transduction properties [125, 126]. Porcine aortic endothelial cells lacking endogenous VEGF receptors display chemotaxis and mitogenesis in response to VEGF when transfected with a plasmid coding for KDR [125]. In contrast, transfected cells expressing Flt-1 lack such responses [125, 126]. Flk-1/KDR undergoes strong ligand-dependent tyrosine phosphorylation in intact cells, whereas Flt-1 reveals a weak or undetectable

response [125, 126]. Also, VEGF stimulation results in weak tyrosine phosphorylation that does not generate any mitogenic signal in transfected NIH 3T3 cells expressing Flt-1 [126]. These findings agree with other studies showing that PlGF, which binds with high affinity to Flt-1 but not to Flk-1/KDR, lacks direct mitogenic or permeability-enhancing properties or the ability to stimulate tyrosine phosphorylation effectively in endothelial cells [127]. Interestingly, however, high concentrations of PlGF expected to saturate the Flt-1 sites are able to potentiate the activity of VEGF, both *in vivo* and *in vitro* [127]. These findings led to the suggestion that Flt-1 may be not primarily a signaling receptor but rather a "decoy" receptor, able to regulate in a negative fashion the activity of VEGF on the vascular endothelium by sequestering and rendering this ligand less available to Flk-1/KDR [127]. In apparent conflict with this hypothesis, subsequent studies indicated that Flt-1 is indeed able to interact with various signal transducing proteins, including the p85 subunit of the PI3 kinase and the mitogen-activated protein kinase, and generate, under some circumstances, a mitogenic signal, at least in transfected cell lines [125, 128, 129]. Also, constitutively active artificial chimeras of the human leukemia oncoprotein BCR-ABL with the intracellular domain (ICD) of Flt-1, with mutations within the Flt-1 sequence, transform Rat1 fibroblasts, abrogate IL-3 dependence in Ba/F3 cells and induce neurite-like structures in neuroneal PC12 cells, suggesting that the Flt-1 kinase has the potential to be activated and mediate a transforming signal, at least in such an artificial system [130]. However, very recent studies have provided evidence in support of the earlier hypothesis. Hiratsuka et al have demonstrated that a targeted mutation resulting in a Flt-1 receptor lacking the TK domain but able to bind VEGF does not result in lethality or any defect in embryonic development and angiogenesis in mice [131], whereas complete inactivation of the receptor results in early embryonic lethality (discussed later here). Furthermore, endothelial cells isolated from Flt-1($TK^{-/-}$) animals exhibit a normal mitogenic response to VEGF. These results suggest that Flt-1 plays its main role in angiogenesis as a ligand-binding molecule, rather than as a signal-transducing receptor, at least as defined by conventional criteria. Taken together, these studies strongly support the hypothesis that interaction with Flk-1/KDR is essential to induce the full spectrum of VEGF biologic responses. In further support of this conclusion, VEGF mutants, which bind selectively to Flk-1/KDR, are fully active endothelial cell mitogens [132] and anti-idiotypic antibodies that activate Flk-1/KDR promote tumor angiogenesis [133]. Furthermore, Flk-1/KDR activation has been shown to be required for the antiapoptotic effects of VEGF for HUVE cells in serum-free conditions [39]. Such a prosurvival effect of VEGF is mediated by the PI3 kinase/Akt pathway [39]. Most recently, Ogawa et al have isolated a polypeptide with an approximate 25% amino acid identity to mammalian VEGF, encoded by a gene previously identified [134, 135] in the genome of Orf virus, a parapoxvirus that affects sheep and goats and occasionally humans. This polypeptide, named VEGF-E, is a potent mitogen and permeability-enhancing factor. Remarkably, VEGF-E binds KDR/Flk-1 and induces its autophosphorylation to almost the same extent as $VEGF_{165}$ but fails to bind Flt-1 [135]. Nevertheless, at least one biological response, the migration of monocytes in response to VEGF (or PlGF), has been clearly shown to be mediated by Flt-1 [136, 137]. VEGF-induced macrophage migration was suppressed in Flt-1($TK^{-/-}$) mice [131]. Moreover, the ability of VEGF to inhibit maturation of dendritic cells has been associated with activation of Flt-1 [138]. According to these studies, VEGF activation of Flt-1 is able to block the activation of nuclear factor-κB [138]. It is tempting to speculate that Flt-1 is able to mediate a specific regulatory signal in cell types other than endothelial cells, such as macrophages.

Soker et al have demonstrated the existence of an additional VEGF receptor [68] that binds $VEGF_{165}$ but not $VEGF_{121}$. This isoform-specific VEGF binding site is identical to human neuropilin-1, a receptor for the collapsin/semaphorin family that mediates neuroneal cell guidance [139]. When coexpressed in cells with KDR, neuropilin-1 enhanced the binding of $VEGF_{165}$ to KDR and $VEGF_{165}$-mediated chemotaxis. Conversely, inhibition of $VEGF_{165}$ binding to neuropilin-1 inhibits its binding to KDR and its mitogenic activity for endothelial cells. These findings suggest that neuropilin-1 may present $VEGF_{165}$ to the Flk-1/KDR receptor in a manner that enhances the effectiveness of Flk-1/KDR-mediated signal transduction. So far, there is no evidence that neuropilin-1 signals follow VEGF binding. As previously mentioned, these findings may help explain the greater mitogenic potency of $VEGF_{165}$ compared with $VEGF_{121}$. Migdal et al have shown that PlGF-2, a heparin-binding isoform of PlGF [22], also binds to neuropilin-1 [140].

Regulation of vascular endothelial growth factor receptors expression. The expression of Flt-1 and Flk-1/KDR genes is largely restricted to the vascular endothelium. The promoter region of Flt-1 has been cloned and characterized, and a 1 kb fragment of the 5′-flanking region essential for endothelial-specific expression was identified [141]. A 4 kb 5′-flanking sequence has been identified in the promoter of KDR that confers endothelial cell specific activation [142].

Similar to VEGF, hypoxia has been proposed to play an important role in the regulation of VEGF receptor gene expression. Exposure to acute or chronic hypoxia led to pronounced up-regulation of both Flt-1 and Flk-1/KDR genes in the lung vasculature in a rat model [143]. Also, Flk-1/KDR and Flt-1 mRNAs were substantially

up-regulated throughout the heart following myocardial infarction in the rat [144]. However, *in vitro* studies have yielded paradoxical results. Hypoxia increases VEGF receptor number in cultured bovine retinal capillary endothelial cells, but the expression of KDR mRNA is not induced but paradoxically shows an initial down-regulation [145]. Brogi et al have proposed that the hypoxic up-regulation of KDR observed *in vivo* is not direct but requires the release of an unidentified paracrine mediator from ischemic tissues [146]. Recent studies have provided evidence for a differential transcriptional regulation of the Flt-1 and KDR genes by hypoxia [147]. When HUVECs were exposed to hypoxic conditions *in vitro*, increased levels of Flt-1 expression were observed. In contrast, Flk-1/KDR mRNA levels were unchanged or slightly repressed. Promoter deletion analysis demonstrated a 430 bp region of the Flt-1 promoter to be required for transcriptional activation in response to hypoxia. This region includes a heptamer sequence matching the HIF-17α consensus binding site in other hypoxia inducible genes. Such an element was not found in the Flk-1/KDR promoter. These findings indicate that, unlike the KDR/Flk-1 gene, the Flt-1 receptor gene is directly up-regulated by hypoxia via a hypoxia inducible enhancer element located at position −976 to −937 of the Flt-1 promoter [147].

More recent evidence indicates that VEGF itself may up-regulate the expression of KDR in brain tissue slices [148] and in cultured bovine capillary endothelial cells [149]. VEGF-induced KDR expression primarily occurred at the transcriptional level, as assessed by a luciferase reporter assay system. VEGF mutants selective for the KDR receptor induced KDR up-regulation. In contrast, mutants with decreased KDR binding and wild-type Flt-1 binding did not, suggesting that KDR receptor signaling mediated the increase in KDR expression [149]. Barleon et al have also shown that VEGF up-regulates Flt-1 expression in cultured human endothelial cells [150].

Transforming growth factor-β has been reported to regulate in a negative fashion the expression of KDR mRNA in endothelial cells [151]. According to Patterson et al, TNF-α potently reduced mRNA transcripts of both Flt-1 and Flk-1/KDR in venous and arterial endothelial cells in a dose- and time-dependent fashion [152]. These authors concluded that TNF-α down-regulates expression of both VEGF receptors in human endothelial cells and that this effect is transcriptional, at least for KDR. However, Giraudo et al reported that TNF-α up-regulates in a dose- and time-dependent manner the expression and the function of KDR as well as the expression of its coreceptor neuropilin-1 in human endothelium [153].

Role of VEGF and its receptors in physiological angiogenesis

Normal development: (a) Embryonic development. Gene targeting studies have demonstrated that both Flt-1 and Flk-1/KDR are essential for development of the embryonic vasculature in mice [154, 155]. However, their respective roles in endothelial cell proliferation and differentiation appear to be distinct. Mouse embryos homozygous for a targeted mutation in the Flt-1 locus died *in utero* between day 8.5 and 9.5 [154]. Endothelial cells developed in both embryonic and extra embryonic sites but failed to organize in normal vascular channels. As previously noted, more recent studies revealed that normal blood vessel development and survival may occur in the absence of the TK domain of Flt-1 [131]. Flk-$1^{-/-}$ mice lacked vasculogenesis and also failed to develop blood islands. Hematopoietic precursors were severely disrupted, and organized blood vessels failed to develop throughout the embryo or the yolk sac, resulting in death *in utero* between day 8.5 and 9.5 [155]. These findings emphasize the regulatory role of Flk-1/KDR in early hematopoiesis [155, 156]. Also, hematopoietic stem cells, megakaryocytes, and platelets normally express this receptor [157].

Two independent studies [33, 34] have generated direct evidence for the role played by VEGF in embryonic vasculogenesis and angiogenesis. Inactivation of a single VEGF allele in mice resulted in embryonic lethality between day 11 and 12. The $VEGF^{+/-}$ embryos were growth retarded and also exhibited a number of developmental anomalies. The forebrain region appeared significantly underdeveloped. In the heart region, the outflow region was grossly malformed; the dorsal aortae were rudimentary, and the thickness of the ventricular wall was markedly decreased. The yolk sac revealed a markedly reduced number of nucleated red blood cells within the blood islands. Significant defects in the vasculature of other tissues and organs, including placenta and nervous system, were observed. *In situ* hybridization demonstrated the expression of VEGF mRNA in heterozygous embryos. Thus, the $VEGF^{+/-}$ phenotype is due to gene dosage and not to maternal imprinting. These findings indicate that other VEGF-like molecules are unable to compensate for even a partial loss of VEGF. Although several heterozygous phenotypes have been described [158], this may be the first example of embryonic lethality following the loss of a single allele of a gene that is not maternally imprinted, at least in vertebrates.

More recently, Carmeliet et al reported an isoform-specific knockout of the VEGF gene [159]. By Cre-*loxP* technology, they excised exons 6 and 7 of the VEGF gene in ES cells and thus generated mice that express exclusively $VEGF_{120}$. Interestingly, 50% of the $VEGF^{120/120}$ mice died shortly after delivery, whereas the remainder

died within two weeks. The survivors demonstrated impaired myocardial contractility, heart enlargement, and defective angiogenesis leading to ischemic cardiomyopathy. These findings suggest that the action of the heparin-binding isoforms of VEGF cannot be replaced by $VEGF_{120}$. Although it is premature to reach any definitive conclusions on the mechanisms underlying the inability of $VEGF_{120}$ to compensate for the lack of other VEGF isoforms, it is noteworthy that these findings are in agreement with the earlier studies by Keyt et al, which demonstrated the critical role of the heparin-binding domain in determining the mitogenic potency of VEGF [67].

It is interesting to observe that inactivation of the PlGF gene does not result in embryonic lethality, even in the homozygous state [30]. $PlGF^{-/-}$ mice are viable and fertile, although they may have some impairment of wound healing. These findings suggest that PlGF and perhaps some other members of the VEGF gene family are not as critical as VEGF for vascular development and survival. Alternatively, it is possible be that these molecules play so far unidentified functions.

Normal development: (b) Early postnatal development. To determine the role of VEGF in postnatal development, two independent strategies were recently employed. Inducible, Cre-*loxP*–mediated gene targeting or administration of a soluble VEGF receptor chimeric protein [mFlt (1-3)-IgG] were used to inactivate VEGF in early postnatal life [160]. Mice containing the "floxed" VEGF allele were bred to a strain transgenic from Cre recombinase controlled by an interferon-inducible promoter (MX-1) [161]. Partial inhibition of VEGF achieved by this inducible gene targeting system resulted in increased mortality, stunted body growth, and impaired organ development, most notably of the liver [160]. Administration of mFlt (1-3)-IgG, which achieves a considerably higher degree of VEGF inhibition, resulted in nearly complete growth arrest when the treatment was initiated at day 1 or day 8 postnatally. Remarkably, such treatment was also accompanied by rapid lethality. Decreased levels of proliferation of various cell types were detected in all organs studied. Ultrastructural analysis documented alterations in endothelial and other cell types. Interestingly, the liver of mFlt (1-3)-IgG–treated neonates had fewer endothelial cells and focal loss of integrity of the space of Disse. When the endothelial cells were absent, hematopoietic cells could be seen in direct contact with the hepatocyte plasma membrane. The kidneys showed interstitial hemorrhage at the corticomedullary junction. Juxtamedullary and cortical glomeruli were enlarged, hypocellular, and showed accumulation of eosinophillic mesangial matrix. Glomerular capillary loop numbers were reduced compared with controls, and the proximal tubular epithelium contained protein droplets. Ultrastructural analysis of kidneys of treated animals showed that glomerular capillaries were underdeveloped and in some instances entirely missing. Interestingly, these alterations appeared to be mostly restricted to juxtamedullary glomeruli. There was an increased mesangial matrix with granular material and proteinaceous deposits that included fibrin, but no immune complexes were detected. The cytoplasmic foot processes of podocytes were normal in appearance and interacted with a thickened basement membrane. Interestingly, the capillaries in the peritubular regions showed normal fenestration in approximately half the profiles surveyed [160]. Histological and biochemical changes consistent with renal failure were observed. Taken together, these findings are consistent with a critical dependence of glomerular development on VEGF [162–164].

Endothelial cells isolated from the liver of mFlt (1–3)-IgG-treated neonates demonstrated an increased apoptotic index, indicating that VEGF is required not only for proliferation but also for survival of endothelial cells. However, VEGF inhibition resulted in less significant alterations as the animal matured, and the dependence on VEGF was eventually lost some time around the fourth postnatal week. Administration of mFlt (1–3)-IgG to juvenile mice failed to induce apoptosis in liver endothelial cells [160].

Growth plate morphogenesis and endochondral bone formation. Endochondral bone formation is a fundamental mechanism for longitudinal bone growth during vertebrate development [165]. Cartilage, an avascular tissue, is replaced by bone in a process named endochondral ossification. During this process, the epiphyseal growth plate undergoes morphogenesis. A region of resting chondrocytes differentiates into a zone of proliferating chondrocytes that then hypertrophies and finally undergoes apoptotic cell death while being replaced by bone. The net result is lengthening of the bone, whereas the thickness of the growth plate remains relatively constant. Such a sequence of events relies on the precise coupling of chondrogenesis (cartilage production) with osteogenesis (bone formation) [165]. During this process, blood vessel invasion from the metaphysis coincides with mineralization of the extracellular matrix (ECM), apoptosis of hypertrophic chondrocytes, ECM degradation, and bone formation [165]. Although blood vessel invasion is critical, it is unknown whether apoptosis of hypertrophic chondrocytes is the stimulus for recruitment of blood vessels and specialized cells or, rather, if blood vessel invasion is the trigger for death of chondrocytes and for the subsequent steps by conveying cellular and/or humoral regulatory signals [166, 167].

Recently, the role of VEGF in endochondral bone formation has been examined [168]. These studies have shown that the VEGF mRNA is expressed by hypertrophic chondrocytes in the epiphyseal growth plate [168]. To determine the functional significance of VEGF, this factor was inactivated by systemic administration of a

mFlt (1-3)-IgG to 24-day-old mice [168]. Following such treatment, blood vessel invasion was almost completely suppressed, concomitant with impaired trabecular bone formation. Recruitment and/or differentiation of chondroclasts, which express gelatinase B/matrix metalloproteinase-9 [169], and resorption of terminal chondrocytes was substantially decreased. Although proliferation, differentiation, and maturation of chondrocytes were apparently normal, resorption of hypertrophic chondrocytes was inhibited, resulting in a paradoxical threefold to sixfold expansion of the hypertrophic chondrocyte zone [168]. These findings indicate that VEGF-dependent blood vessel invasion is essential for coupling cartilage resorption with bone formation. Following VEGF inactivation, hypertrophic chondrocytes fail to undergo apoptotic cell death [168]. Therefore, the vasculature carries the essential cellular and humoral signals required from correct growth plate morphogenesis. However, cessation of the anti-VEGF treatment was followed by capillary invasion, restoration of bone growth, resorption of the hypertrophic cartilage, and normalization of the growth plate architecture. Interestingly, VEGF receptor expression was localized not only to vascular endothelial cells [168]. Osteoblasts strongly expressed Flt-1 but not Flk-1/KDR. In this context, is it is interesting to point out that an earlier study found VEGF to have chemotactic effects on cultured bovine osteoblasts [170]. It is tempting to speculate that these effects are mediated by Flt-1. These findings indicate that VEGF-mediated capillary invasion is a critical signal that regulates growth plate morphogenesis and triggers cartilage remodeling. Thus, VEGF is a key coordinator of chondrocyte death, chondroclast function, ECM remodeling, angiogenesis, and bone formation in the growth plate. Antiangiogenic strategies targeting VEGF in pediatric patients that have not completed statural growth are expected to result in reversible inhibition of growth plate vascular invasion as an important side-effect.

Female reproductive tract angiogenesis. The development and endocrine function of the ovarian corpus luteum (CL) are dependent on the growth of new capillary vessel [171]. Earlier studies suggested the release of angiogenic factors from the developing CL [172]. Therefore, the identification of the regulators of CL angiogenesis has been the object of intense investigation over the last several decades. Previous studies have shown the VEGF mRNA is temporally and spatially related to the proliferation of blood vessels in the rat, mouse, and primate ovary and in the rat uterus, suggesting that VEGF may be a mediator of the cyclical growth of blood vessels in the female reproductive tract [173–176]. Recently, the availability of an effective inhibitor of rodent VEGF, such as mFlt (1-3)IgG, has made it possible to directly test this hypothesis [118, 177]. Treatment with mFlt (1-3)IgG resulted in virtually complete suppression of CL angiogenesis in a rat model of hormonally induced ovulation [177]. This effect was associated with inhibition of CL development and progesterone release. Also, failure of maturation of the endometrium was observed, probably reflecting suppression of ovarian steroid production plus a direct inhibition of locally produced VEGF. Areas of ischemic necrosis were demonstrated in the CL of treated animals. However, no effect on the pre-existing ovarian vasculature was observed [177]. These findings indicate that VEGF-mediated angiogenesis is essential for CL development and endocrine function. A similar inhibition of CL and uterine angiogenesis has been observed in a toxicological study in normally cycling primates treated with a humanized anti-VEGF monoclonal antibody (rhumAb VEGF) [178].

It is tempting to speculate that these findings will further our understanding of the pathogenesis of several disorders of ovarian function that profoundly impair fertility and may also have therapeutic implications. The hyperplasia and hypervascularity of polycystic ovary syndrome [179] could be due to increased production of VEGF by theca-lutein cells. In this context, up-regulation of VEGF mRNA has been demonstrated in the ovaries of patients affected by this syndrome [180].

Furthermore, inhibition of VEGF-mediated angiogenesis and ovarian growth may have a role in the treatment of ovarian hyperstimulation syndrome (OHSS), a potentially fatal condition characterized by massive ovarian enlargement that may follow medical induction of ovulation with gonadotropins [181]. A previous study suggested that VEGF may be implicated in the pathogenesis of this syndrome by a different mechanism, acting as a direct capillary permeability-enhancing agent [182]. Interestingly, recent studies have suggested that serum VEGF levels in patients undergoing ovulation induction may have a prognostic factor for the development of OHSS [183]. Conversely, it is tempting to speculate that luteal-phase defects [184] may be associated with insufficient VEGF production.

Angiogenesis is also important in the pathogenesis of endometriosis, a well-known condition resulting in infertility and pain, characterized by ectopic endometrium implants in the peritoneal cavity. High levels of VEGF have been measured in the peritoneal fluid of patients with endometriosis [185–187]. Immunohistochemistry indicated that activated peritoneal fluid macrophages, as well as tissue macrophages within the ectopic endometrium, are the major source of VEGF [185, 187]. These findings raise the possibility that VEGF inhibitors may have therapeutic value for the treatment of endometriosis.

Role of vascular endothelial growth factor in pathological angiogenesis

Tumor angiogenesis. In 1945, Algire and Chalkley, based on the observation that the growth of tumor xeno-

grafts in transparent chambers in mice is always preceded by an increase in vascular density, concluded that "the rapid growth of tumor explants is dependent upon the development of a rich vascular supply" [188]. These authors also hypothesized that the ability of tumor cells to induce neovascular proliferation continuously may constitute the single most important factor determining their growth advantage *in vivo* relative to the normal cells from which they arose [188]. In 1968 Greenblatt and Shubi demonstrated that tumor-associated angiogenesis is mediated by a soluble factor(s), which is able to diffuse across a Millipore filter [189]. In 1971, Folkman proposed the novel concept that inhibition of angiogenesis may be a valid strategy for the treatment of solid tumors [190] and initiated experimental work aimed toward the isolation of a tumor angiogenesis factor(s) [191]. Since then, extensive research has been dedicated to the identification and characterization of mediators of tumor angiogenesis [192]. Although several molecules were initially implicated, there was considerable uncertainty as to their role as endogenous mediators of tumor growth and angiogenesis. For example, antibodies against bFGF, the molecule most consistently associated with tumor angiogenesis, did not result in inhibition of growth of several murine tumor cell lines implanted in syngeneic mice [193].

Many tumor cell lines secrete VEGF *in vitro*, suggesting the possibility that this diffusible molecule may be a mediator of tumor angiogenesis [194]. *In situ* hybridization studies have demonstrated that the VEGF mRNA is markedly up-regulated in the vast majority of human tumors examined thus far, including lung [195, 196], breast [197, 198], gastrointestinal tract [199–202], kidney [203–205], bladder [203], ovary [206–208], and endometrial [209] carcinomas and several intracranial tumors including glioblastoma multiforme [210–212] and sporadic, as well as VHL syndrome-associated capillary hemangioblastoma [213, 214]. In glioblastoma multiforme and other tumors with significant necrosis, the expression of VEGF mRNA is highest in hypoxic tumor cells adjacent to necrotic areas [210–212]. Although these studies have shown that tumor cells represent the major source of VEGF, recent studies have indicated that tumor-associated stroma is also an important site of VEGF production [215]. These studies, using transgenic mice expressing green fluorescent protein under the control of the VEGF gene promoter, have shown that the tumor induces activation of the VEGF gene promoter in nontransformed stromal cells, such as fibroblasts [215]. These findings suggest that tumor and stromal cells constitute a highly integrated system that facilitate tumor angiogenesis by releasing VEGF.

A correlation has been noted between VEGF expression and microvessel density in primary breast cancer sections [216]. Postoperative survey indicated that the relapse-free survival rate of patients with VEGF-rich tumors was significantly worse than that of VEGF-poor, suggesting that the expression of VEGF is associated with stimulation of angiogenesis and with early relapse in primary breast cancer [217]. A similar correlation has been described in gastric carcinoma patients [218]. Furthermore, several studies have documented elevations in the VEGF plasma levels in tumor patients compared with tumor-free individuals, and it has been also reported that high VEGF levels prior to chemotherapy may be associated with poor outcome in non-Hodgkin's lymphoma as well as small-cell lung carcinomas [219–222].

The availability of high-affinity anti-VEGF neutralizing monoclonal antibodies (mAb) [223] made it possible to generate direct evidence for a role of VEGF in tumorigenesis. In 1993, Kim et al reported that such mAbs exert a potent inhibitory effect on the growth of three human tumor cell lines injected subcutaneously in nude mice: the SK-LMS-1 leiomyosarcoma, the G55 glioblastoma multiforme, and the A673 rhabdomyosarcoma [224]. The growth inhibition ranged between 70% and more than 95%. These findings provided the first direct demonstration of the concept that inhibition of an endogenous angiogenic factor may result in suppression of tumor growth. Subsequently, other tumor cell lines were found to be inhibited *in vivo* by this treatment [225–229]. Although in most of these studies the treatment was initiated shortly after inoculation of the tumor cells, the anti-VEGF mAb was able to inhibit further growth of already established tumors [230]. Anti-VEGF mAb treatment also inhibits ascites formation in a murine model of ovarian cancer [231]. Anti-VEGF antisense constructs also strongly suppress glioblastoma growth *in vivo* [232].

Other studies have suggested that VEGF also plays a role in the development of hemorrhage associated with brain tumors [233]. Interestingly, overexpression of $VEGF_{121}$ or $VEGF_{165}$ but not of $VEGF_{189}$ resulted in significant intrecerebral bleeding, suggesting an important biological difference among the VEGF isoforms [233].

Intravital videomicroscopy techniques have generated further evidence that anti-VEGF mAb treatment indeed blocks tumor angiogenesis [234]. Noninvasive imaging of the vasculature revealed a nearly complete suppression of tumor angiogenesis in anti-VEGF treated animals as compared with controls, at all time points examined [234]. Intravital fluorescence microscopy and videoimaging analysis have been also applied to address the important issue of the effects of VEGF on permeability and other properties of tumor vessels [235]. Treatment with anti-VEGF mAb was initiated when tumor xenografts were already established and vascularized and resulted in time-dependent reductions in vascular permeability [235]. These effects were accompanied by marked

changes in the morphology of vessels, with reduction in diameter and tortuosity. A regression of blood vessels was observed after repeated administrations of anti-VEGF mAb. Magnetic resonance imaging (MRI) techniques have also documented inhibition of tumor vascular permeability following administration of anti-VEGF mAb [236].

Further evidence that VEGF action is necessary for effective tumor angiogenesis has been provided in an *in vivo* model of ES cell tumorigenesis [33]. ES cells are able to give rise to highly vascularized teratocarcinomas when injected in nude or syngeneic mice [237]. VEGF null ES cells were dramatically impaired in their ability to form tumors in nude mice [33]. Furthermore, transfection with oncogenic *ras* failed to restore an *in vivo* tumorigenic phenotype in $VEGF^{-/-}$ ES cells [238]. These findings strengthen the hypothesis that VEGF-mediated angiogenesis is crucial for effective *in vivo* growth.

An independent verification of the hypothesis that the VEGF action is required for tumor angiogenesis has been provided by the finding that retrovirus-mediated expression of a dominant negative Flk-1 mutant, which inhibits signal transduction through wild-type Flk-1 receptor, suppresses the growth of glioblastoma multiforme as well as other tumor cell lines *in vivo* [239, 240]. Furthermore, two recent studies have demonstrated that high local expression of sFlt-1, achieved by adenoviral-mediated gene transfer or by stable transfection of tumor cells, is able to significantly inhibit tumor growth, metastasis, and mortality rate [241, 242].

A humanized version of a high-affinity anti-VEGF mAb has been generated by site-directed mutagenesis of a human framework [243]. This humanized mAb (rhumAb VEGF) has the same binding characteristics and ability to neutralize VEGF as the original murine mAb. Toxicological studies in primates have shown that rhmAb VEGF is safe even after prolonged treatment, and its effects are limited to inhibition of angiogenesis in the female reproductive tract and induction of growth plate dysplasia in animals that have not completed statural growth [178]. rhumAb VEGF is currently undergoing phase II clinical trials as a treatment for various solid tumors, either as a single agent or in combination with conventional chemotherapy. The results of a phase I study in cancer patients have been recently reported and have shown that rhumAb VEGF is safe and also suggest some activity of the antibody a single agent (abstract; Gordon et al, *34th Annual Meeting of the American Society of Clinical Oncology*, May 16–19, 1998). Small molecules that inhibit Flk-1/KDR signal transduction are also undergoing clinical trials in cancer patients [244].

Other pathological conditions

Diabetes mellitus, occlusion of central retinal vein, or prematurity with subsequent exposure to oxygen can all be associated with intraocular neovascularization [5]. The new blood vessels may lead to vitreous hemorrhage, retinal detachment, neovascular glaucoma, and eventual blindness [5]. Diabetic retinopathy is the leading cause of blindness in the working population [245]. All of these conditions are known to be associated with retinal ischemia [246]. In 1948, Michaelson proposed that a key event in the pathogenesis of these conditions is the release by the ischemic retina into the vitreous diffusible angiogenic factor(s) ("factor X") responsible for retinal and iris neovascularization [247]. Elevations of VEGF levels in the aqueous and vitreous of eyes with proliferative retinopathy have been described [248–250]. In a large series, a strong correlation was found between levels of immunoreactive VEGF in the aqueous and vitreous humors and active proliferative retinopathy associated with diabetes, occlusion of central retinal vein, or prematurity [248]. Furthermore, Okamoto et al recently developed a transgenic mouse model in which the bovine rhodopsin promoter is coupled to the gene for human VEGF [251]. This study demonstrates that over-expression of VEGF in the retina is sufficient to cause intraretinal and subretinal neovascularization.

More direct evidence for a role of VEGF as a mediator of intraocular neovascularization has been generated in a primate model of iris neovascularization and in a murine model of retinopathy of prematurity [252, 253]. In the former, intraocular administration of anti-VEGF antibodies dramatically inhibits the neovascularization that follows occlusion of central retinal veins [254]. Likewise, soluble Flt-1 or Flk-1 fused to an IgG suppresses retinal angiogenesis in the mouse model [255].

Neovascularization is a major cause of visual loss also in AMD, the overall leading cause of blindness [5]. Several studies have demonstrated the immunohistochemical localization of VEGF in surgically resected choroidal neovascular membranes from AMD patients [256, 257]. These findings suggests a role for VEGF in the progression of AMD-related choroidal neovascularization, raising the possibility that a pharmacological treatment with VEGF inhibitors may constitute a therapy for this condition. Currently, anti-VEGF strategies are being explored in clinical trials, using either a recombinant humanized anti-VEGF Fab [243] or 2′-fluoropyrimidine RNA oligonucleotide ligand (aptamers) [258].

Two independent studies have suggested that VEGF is involved in the pathogenesis of RA, an inflammatory disease where angiogenesis plays a significant role [259, 260]. Levels of immunoreactive VEGF were found to be high in the synovial fluid of RA patients, whereas they were very low or undetectable in the synovial fluid of patients affected by other forms of arthritis or by degenerative joint disease [261, 262].

Several studies have suggested that VEGF is implicated in the development of brain edema. VEGF is a

potent mediator of vascular permeability to water [46, 47]. Diffuse VEGF mRNA expression has been observed in adult rat brain but at relatively low abundance [261]. However, as previously noted, hypoxia is a major trigger for VEGF expression, and enhanced levels of VEGF together with its receptors, Flt-1 and Flk-1/KDR, have been reported by several groups in the rat brain following the induction of focal cerebral ischemia [262–264]. Recently, van Bruggen et al tested the hypothesis that VEGF antagonism, achieved by systemic administration of mFlt (1-3)-IgG [118, 177], may have beneficial effects in a murine model of cortical ischemia [265]. Using high-resolution MRI techniques to quantitate the extent of the edematous changes, a significant reduction in the volume of the edematous tissue was observed one day following the onset of ischemia [265]. Furthermore, measurements of the resultant infarct size measured several weeks later revealed a significant sparing of cortical tissue in the mFlt (1-3)-IgG–treated group. These results demonstrate that antagonism of VEGF reduces ischemic-reperfusion related brain edema and injury, implicating VEGF in the pathogenesis of cerebral ischemia.

THERAPEUTIC APPLICATIONS OF VASCULAR ENDOTHELIAL GROWTH FACTOR

Intra-arterial or intramuscular administration of $rhVEGF_{165}$ may significantly augment perfusion and development of collateral vessels in a rabbit model, where chronic hindlimb ischemia was created by surgical removal of the femoral artery [266]. These studies provided angiographic evidence of neovascularization in the ischemic limbs. Arterial gene transfer with cDNA encoding VEGF also led to revascularization in the same rabbit model to an extent comparable to that achieved with the recombinant protein [267, 268]. In addition, several studies have suggested that the angiogenesis initiated by the administration of VEGF improves blood flow and muscle function in ischemic limbs [269, 270]. Other studies have shown that VEGF administration also leads to a recovery of normal endothelial reactivity in dysfunctional endothelium [271]. Furthermore, Mack et al have also shown that an adenovirus vector expressing $VEGF_{165}$ cDNA is capable of stimulating an angiogenic response that protects against acute vascular occlusion in the setting of preexisting ischemia in a rat model of hind limb ischemia, suggesting that VEGF therapy might be useful in the prophylaxis of advancing arterial occlusive disease [272].

Banai et al have shown that VEGF administration results in increased coronary blood flow in a dog model of coronary insufficiency [273]. In addition, Harada et al have demonstrated that extraluminal administration of as little as 2 μg of rhVEGF by an osmotic pump results in a significant increase in coronary blood flow in a pig model of chronic myocardial ischemia created by ameroid occlusion of the left proximal circumflex artery [274]. Also, MRIs provided a noninvasive assessment of the benefits secondary to VEGF administration in the porcine model [275]. Adenoviral-mediated gene transfer of $VEGF_{121}$ has been also shown to result in collateral vessel growth and functional improvement in a porcine model [276].

The hypothesis that VEGF may result in therapeutically significant angiogenesis in humans was initially tested by Isner et al in a gene therapy trial in patients with severe limb ischemia [277]. Arterial gene transfer of naked plasmid DNA encoding $VEGF_{165}$, applied to the hydrogel polymer coating of an angioplasty balloon, resulted in angiographic and histological evidence of angiogenesis in the knee, midtibial, and ankle levels four weeks after the transfer. In a subsequent trial, the $VEGF_{165}$ cDNA was injected intramuscularly [278]. Gene transfer was performed in 10 limbs of nine patients with nonhealing ischemic ulcers and/or rest pain caused by peripheral arterial disease. The ankle-brachial index improved. Newly visible collateral blood vessels were directly documented by contrast angiography in seven limbs, and magnetic resonance angiography showed qualitative evidence of improved distal flow in eight limbs. Additional trials performed by the same group of investigators have indicated that local injection of naked plasmid DNA coding for $VEGF_{165}$ results in an improvement in patients affected by myocardial ischemia [279] or Burger's disease (thromboangiitis obliterans) [280].

Clinical trials using the recombinant $VEGF_{165}$ are also ongoing. A phase I study in patients with coronary ischemia where recombinant human $rhVEGF_{165}$ was administered by intracoronary infusion has been reported [281]. The molecule was safely tolerated at all doses tested. There was overall improvement in nuclear perfusion in 7 out of 15 subjects and improved collateralization in 5 out of 7 who underwent follow-up coronary angiography. Phase II studies with $rhVEGF_{165}$ for the same indication are in progress. However, a relatively large (174 patients) placebo-controlled phase II study in which $rhVEGF_{165}$ was delivered as a single intracoronary infusion, followed by three intravenous injections, has not demonstrated any clinical benefit. The treatment was not superior to the placebo in treadmill time and pain relief, at least at a 60-day view [282]. It is possible that a key difference between animal models and human patients lies in the fact that young and otherwise healthy animals are able to mount an effective endogenous angiogenic response that can be maximized by an additional stimulus provided by a recombinant protein or gene therapy. In contrast, patients with extensive atherosclerotic disease may have a less robust response to endogenous and exogenous factors. Thus, very brief exposures to VEGF such as that achieved in such a trial may be insufficient. It is

possible, however, that a more persistent exposure to an individual growth factor or to a combination of growth factors may be effective. Clinical trials currently ongoing should answer at least in part these questions over the next two to three years.

PERSPECTIVES

There is compelling evidence that the actions of VEGF on the vascular endothelium are complex and by no means limited to the induction of growth. Recent studies have emphasized the role of VEGF in preventing apoptosis of endothelial cells and regression of blood vessels. Such a role, initially suggested by the expression of VEGF mRNA in sites where no angiogenesis occurs and by the localization of high-affinity VEGF binding in quiescent endothelial cells [105, 283], has subsequently received strong support by a variety of studies. Such a "maintenance" function appears to be developmentally regulated, as it is critically dependent on the age of the animal. Therefore, a process of maturation occurs in endothelial cells such that VEGF eventually is no longer essential for survival. This "switch" seems to take place in the mouse around the fourth postnatal week, and in the fully developed animal, VEGF may be required primarily for active angiogenic processes such as CL development or wound healing. The molecular nature of this switch remains largely to be determined. Recent studies have also pointed out the key role of VEGF in growth plate morphogenesis and skeletal growth [168].

The paradoxical finding that, in spite of the redundancy of angiogenic mediators, inactivation of VEGF alone substantially suppresses angiogenesis in a wide variety of physiological and pathological circumstances could be, at least in part, explained if one assumed that VEGF is a mediator of other angiogenic pathways. This hypothesis has recently received direct support by studies on FGF4 [284] and bFGF [285]. Anti-VEGF neutralizing antibodies substantially inhibited the endothelial cell mitogenic of both FGF4 and bFGF. Whether this paradigm applies to growth factors other than bFGF or FGF4 remains to be established.

The high expression of VEGF mRNA in human tumors, the presence of the VEGF protein in ocular fluids of individuals with proliferative retinopathies and in the synovial fluid of RA patients, as well as the localization of VEGF in AMD lesions, all strongly support the hypothesis that VEGF is a key mediator of angiogenesis associated with various disorders. Therefore, anti-VEGF antibodies or other inhibitors of VEGF may be of therapeutic value for a variety of malignancies as well as for other disorders, used alone or in combination with other agents. Several clinical trials in various tumor types and in AMD are currently in progress.

Finally, recent evidence strongly suggests that VEGF is a molecule of major significance for kidney homeostasis, especially during development. It is tempting to speculate that further study of the role of VEGF not only will enhance our understanding of the mechanisms of endothelial repair following renal ischemia, but may also offer new therapeutic avenues for this condition.

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APPENDIX

Abbreviations used in this article are: ACTH, adrenocorticotropic hormone; AMD, age-related macular degeneration; Ang-1, angiopoietin-1; ARNT, aryl hydrocarbon receptor nuclear translocator; bFGF, basic fibroblast growth factor; CL, corpus luteum; ECD, extracellular domain; ECM, extracellular matrix; EGF, epidermal growth factor; ES cells, embryonic stem cells; FGF, fibroblast growth factor; Flk-1, fetal liver kinase-1; Flt-1, fms-like tyrosine kinase; GAP, ras GTPase activating protein; HGF, hepatocyte growth factor; HIF-1, hypoxia inducible factor-1; ICAM, intercellular adhesion molecule; IGF-1, insulin like growth factor-1; IL-1, interleukin-1; KDR, kinase domain region; mAb, monoclonal antibody; mFlt (1-3)-IgG; soluble VEGF receptor chimeric protein; MRI, magnetic resonance imaging; MX-1, interferon-inducible promoter; NF-κB, nuclear factor-κB; NK cells, natural killer cells; OHSS, ovarian hyperstimulation syndrome; PA, plasminogen activator; PAI, plasminogen activator inhibitor; PLC-γ, phospholipase Cγ; PlGF, placenta growth factor; PGE_2, prostaglandin E_2; RA, rheumatoid arthritis; RTK, receptor tyrosine kinase; sFlt-1, spliced soluble form of Flt-1; TGF-β, transforming growth factor-beta; TK, tyrosine kinase; TNF-α, tumor necrosis factor-alpha; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau disease; VPF, vascular permeability factor; VRP, VEGF-related peptide.

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