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Vascular Endothelial Growth Factor: Basic Science and Clinical Progress

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Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen *in vitro* and an angiogenic inducer in a variety of *in vivo* models. Hypoxia has been shown to be a major inducer of VEGF gene transcription. The tyrosine kinases Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) are high-affinity VEGF receptors. The role of VEGF in developmental angiogenesis is emphasized by the finding that loss of a single VEGF allele results in defective vascularization and early embryonic lethality. VEGF is critical also for reproductive and bone angiogenesis. Substantial evidence also implicates VEGF as a mediator of pathological angiogenesis. *In situ* hybridization studies demonstrate expression of VEGF mRNA in the majority of human tumors. Anti-VEGF monoclonal antibodies and other VEGF inhibitors block the growth of several tumor cell lines in nude mice. Clinical trials with various VEGF inhibitors in a variety of malignancies are ongoing. Very recently, an anti-VEGF monoclonal antibody (bevacizumab; Avastin) has been approved by the Food and Drug Administration as a first-line treatment for metastatic colorectal cancer in combination with chemotherapy. Furthermore, VEGF is implicated in intraocular neovascularization associated with diabetic retinopathy and age-related macular degeneration. (*Endocrine Reviews* 25: 581–611, 2004)

Abbreviations: aFGF, Acidic fibroblast growth factor; AMD, age-related macular degeneration; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; CL, corpus luteum; CRC, colorectal carcinoma; ECM, extracellular matrix; EG-VEGF, endocrine gland-derived VEGF; eNOS, endothelial NOS; HIF, hypoxia-inducible factor; HSC, hematopoietic stem cell; IFL, irinotecan, 5-fluorouracil, and leucovorin; LSEC, liver sinusoidal endothelial cell; MMP-9, matrix metalloproteinase 9; NO, nitric oxide; NOS, NO synthase; NP, neuropilin; OHSS, ovarian hyperstimulation syndrome; PCOS, polycystic ovary syndrome; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PI3 kinase, phosphatidylinositol 3-kinase; PlGF, placenta growth factor; RA, rheumatoid arthritis; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; VHL, von Hippel-Lindau; VPF, vascular permeability factor.

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I. Introduction

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," the *in situ* 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). More recent evidence suggests that incorporation of bone marrow-derived endothelial precursor cells contributes to the growing vessels, complementing the sprouting of resident endothelial cells (4), although the precise contribution of these elements in various pathophysiological circumstances has been a matter of debate (5–9).

The development of a vascular supply is essential also for tissue repair and reproductive functions in the adult (10). 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 (10, 11).

In endocrine glands, vascularization serves a unique exchange role for secretory products between interstitial fluid surrounding the parenchymal cells and plasma. Endothelial cells of endocrine glands frequently display fenestrae, which

are highly permeable to fluid and small solutes, thus facilitating bidirectional transport (12).

For more than a decade, the role of vascular endothelial growth factor (VEGF) in the regulation of angiogenesis has been the object of intense investigation (13). Recent evidence indicates that new vessel growth and maturation are highly complex and coordinated processes, requiring the sequential activation of a series of receptors [*e.g.*, Tie1, Tie2, and platelet-derived growth factor (PDGF) receptor-β (PDGFR-β)] by numerous ligands in endothelial and mural cells (for recent reviews see Refs. 14–16). However, VEGF signaling often represents a critical rate-limiting step in physiological angiogenesis. VEGF (referred to also as VEGF-A) belongs to a gene family that includes placenta growth factor (PlGF) (17, 18), VEGF-B (19), VEGF-C (20, 21), and VEGF-D (22, 23). Additionally, homologs of VEGF have been identified in the genome of the parapoxvirus, Orf virus (24), and shown to have VEGF-like activities (25, 26). Importantly, VEGF-C and VEGF-D regulate lymphatic angiogenesis (27, 28), emphasizing the unique role of this gene family in controlling growth and differentiation of multiple anatomic components of the vascular system.

The main focus of this review is the progress in the biology and clinical applications of the prototype member, VEGF-A. For additional reviews on this topic, see Refs. 29–35.

Importantly, very recent data have shown that inhibiting VEGF results in a clinical benefit, including increased survival, in patients with advanced malignancies, providing the first clinical validation of the hypothesis that blocking angiogenesis is a strategy to treat cancer (36, 37).

II. Historical Note on Angiogenic Factors

The observation that tumor growth can be accompanied by increased vascularity was reported more than one century ago (for review, see Ref. 13). In 1939, Ide *et al.* (38) postulated the existence of a tumor-derived blood vessel growth-stimulating factor on the basis of the strong neovascular response induced by tumors transplanted in transparent chambers. These authors proposed that such a factor may be responsible for inducing a neovascularization and thus for delivery of nutrients to the growing tumor (38). In 1945, Algire *et al.* (39) advanced this concept, proposing that "the rapid growth of tumor transplants is dependent upon the development of a rich vascular supply," and speculated that capillary proliferation elicited by tumor cells is mediated by chromatin breakdown products, in agreement with a view prevalent at that time that such products have growth-promoting activity. These investigators also suggested that the acquisition by tumor cells of the ability to promote vascular proliferation is a critical step in tumorigenesis, because it is expected to confer on the tumor cells a growth advantage relative to normal cells (39).

In 1948, Michaelson (40) proposed that a diffusible angiogenic "factor X" produced by the retina is responsible for retinal and iris neovascularization that occurs in proliferative diabetic retinopathy and other retinal disorders, such as central retinal vein occlusion.

In 1968, the first experiments to directly test the hypothesis that tumors produce angiogenic factors were performed. Greenblatt and Shubik (41) and Ehrmann and Knoth (42) demonstrated that transplantation of melanoma or choriocarcinoma cells promoted blood vessel proliferation even when a Millipore filter is interposed between the tumor and the host, thus providing evidence that tumor angiogenesis was mediated by diffusible factor(s) produced by the tumor cells.

In 1971, Folkman (43) proposed that antiangiogenesis might be an effective approach to treat human cancer. Folkman *et al.* (44) initiated initial efforts aimed to isolate a "tumor angiogenesis factor" from human and animal tumors. Subsequently, the angiogenic effects of various factors, including epidermal growth factor, TGF-α, TGF-β, TNF-α, angiogenin, *etc.*, were reported. These molecules all were shown to have activity in angiogenesis bioassays—either directly, by promoting endothelial cell proliferation or indirectly, via recruitment of inflammatory cells that could, in turn, release endothelial mitogens (45). However, much of the attention was directed toward two related potent endothelial cell mitogens and angiogenic factors, acidic and basic fibroblast growth factors (aFGF and bFGF) (46). In 1985, the purification to homogeneity and sequencing of both aFGF (47) and bFGF (48) were reported, and the subsequent year their cDNAs were cloned (49, 50). An unexpected finding was that the genes for both aFGF and bFGF do not encode for a conventional secretory signal peptide. Accordingly, it became clear that these molecules are not efficiently secreted and are mostly cell associated (46). Yet, as previously noted, earlier reports had pointed toward the involvement of diffusible factors in tumor angiogenesis (41, 42). This requirement appeared to be true also for physiological angiogenesis such as that associated with corpus luteum (CL) development (51). Vlodavsky *et al.* (52) suggested that the FGFs are sequestered and stored in the extracellular matrix (ECM) bound to heparan sulfate-containing proteoglycans and can be released in a soluble form when the ECM is degraded. However, several studies suggested that immunoneutralization of bFGF had little or no effect on tumor angiogenesis (53, 54). Furthermore, *bFGF*-null mice, and even double knockout mice with disruptions in *aFGF* and *bFGF* genes, do not develop vascular defects (55, 56). A plausible explanation is that several soluble members of the FGF family compensate for the absence of the cell-associated forms. Thus, the ability of growth factors to promote angiogenesis in *in vitro* or *in vivo* bioassays does not necessarily predict a role for such factors in physiological or pathological angiogenesis (57, 58).

III. Identification of VEGF

Independent and unrelated lines of research converged toward the identification of VEGF (see Ref. 13 for additional details).

In 1983, Senger *et al.* (59) described the partial purification from the conditioned medium of a guinea-pig tumor cell line of a protein able to induce vascular leakage in the skin, which was named "tumor vascular permeability factor" (VPF). The authors proposed that VPF could be a mediator of the high permeability of tumor blood vessels. Because VPF was not

isolated and sequenced, this factor remained molecularly unknown at that time. Senger *et al.* (60) reported the purification and NH_2-terminal amino acid sequencing of guinea pig VPF in 1990.

In 1989, Ferrara and Henzel (61) reported the isolation of a diffusible endothelial cell-specific mitogen from medium conditioned by bovine pituitary follicular cells, which they named "vascular endothelial growth factor" to reflect the restricted target cell specificity of this molecule. NH_2-terminal amino acid sequencing of purified VEGF proved that this protein was distinct from the known endothelial cell mitogens such as aFGF or bFGF and indeed did not match any known protein in available databases (61). Subsequently, Connolly *et al.* (62) followed up on the work by Senger *et al.* and independently reported the isolation and sequencing of human VPF from U937 cells. cDNA cloning of VEGF (63) and VPF (64), reported also in 1989, demonstrated that VEGF and VPF were the same molecule. This was surprising, considering that other endothelial cell mitogens such as FGF do not increase vascular permeability. The finding that VEGF is potent, diffusible, and specific for vascular endothelial cells led to the hypothesis that this molecule might play a role in the regulation of physiological and pathological growth of blood vessels (61, 63, 65).

IV. Activities of VEGF

A. Mitogenesis, angiogenesis, and endothelial survival

A well-documented *in vitro* activity of VEGF is the ability to promote growth of vascular endothelial cells derived from arteries, veins, and lymphatics (for review see Ref. 30). VEGF promotes angiogenesis in tridimensional *in vitro* models, inducing confluent microvascular endothelial cells to invade collagen gels and form capillary-like structures (66, 67). Also, VEGF induces sprouting from rat aortic rings embedded in a collagen gel (68). VEGF also elicits a pronounced angiogenic response in a variety of *in vivo* models including the chick chorioallantoic membrane (63, 69), the rabbit cornea (70), the matrigel plug in mice (71), the primate iris (72), *etc*. VEGF delivery also induces lymphangiogenesis in mice, at least in some circumstances (73). Ergun *et al.* (74) recently proposed that induction of carcinoembryonic antigen-related cell adhesion 1, a membrane glycoprotein expressed in some microvascular endothelial cells, mediates some of the angiogenic effects of VEGF.

VEGF is also a survival factor for endothelial cells, both *in vitro* and *in vivo* (75–79). *In vitro*, VEGF prevents endothelial apoptosis induced by serum starvation. Such activity is mediated by the phosphatidylinositol 3-kinase (PI3 kinase)/Akt pathway (77, 80). Also, VEGF induces expression of the antiapoptotic proteins Bcl-2, A1 (76), XIAP (81), and survivin (82) in endothelial cells. *In vivo*, the prosurvival effects of VEGF are developmentally regulated. VEGF inhibition results in extensive apoptotic changes in the vasculature of neonatal, but not adult, mice (83). Furthermore, a marked VEGF dependence has been demonstrated in endothelial cells of newly formed but not of established vessels within tumors (78, 79). Coverage by pericytes has been proposed to be one of the key events resulting in loss of VEGF dependence (78).

Although endothelial cells are the primary targets of VEGF, several studies have reported mitogenic effects also on certain nonendothelial cell types, such as retinal pigment epithelial cells (84), pancreatic duct cells (85), and Schwann cells (86). Compernolle *et al.* (87) have also shown that VEGF stimulates surfactant production by alveolar type II cells, resulting in a protective effect from respiratory distress syndrome in mice. Recent studies have emphasized the potential role of VEGF as a neuronal protective factor, and a haplotype in the VEGF gene promoter associated with reduced VEGF expression has been reported to be is a risk factor for amyotrophic lateral sclerosis (88).

B. Effects of VEGF on bone marrow cells and hematopoiesis

The earliest evidence that VEGF can affect blood cells came from a report describing its ability to promote monocyte chemotaxis (89). Subsequently, VEGF was reported to have hematopoietic effects, inducing colony formation by mature subsets of granulocyte-macrophage progenitor cells (90). Interestingly, VEGF delivery to adult mice inhibits dendritic cell development (91, 92), leading to the hypothesis that VEGF facilitates tumor growth by allowing escape of tumors from the host immune system. Also, VEGF increased production of B cells and the generation of immature myeloid cells (93). Recently, conditional gene knockout technology has been employed to achieve selective VEGF gene ablation in bone marrow cell isolates and hematopoietic stem cells (HSCs) (94). VEGF-deficient HSCs and bone marrow mononuclear cells failed to repopulate lethally irradiated hosts, despite coadministration of a large excess of wild-type cells. These studies elucidated an internal autocrine loop, not blocked by extracellular inhibitors such as antibodies, whereby VEGF controls HSC survival during hematopoietic repopulation (94).

Interestingly, a VEGF-dependent pathway has been shown to play an important role in hematopoiesis even in *Drosophila*, where it controls migration (95) and proliferation (96) of blood cells. Three VEGF-like ligands and a single receptor, known as PDGF/VEGF receptor or PVR, have been identified in *Drosophila* (97). Because *Drosophila* is devoid of a vascular system, these findings indicate that one ancestral, conserved role of VEGF is indeed the regulation of blood cell function (97).

C. Enhancement of vascular permeability and hemodynamic effects

As previously noted, VEGF is known also as VPF, based on its ability to induce vascular leakage (59, 98). Such permeability-enhancing activity underlies important roles of this molecule in inflammation and other pathological circumstances (see *Section IX.D*). Bates and Curry (99) have shown that VEGF induces an increase in hydraulic conductivity of isolated microvessels, an effect that is mediated by increased calcium influx (100). Consistent with a role in the regulation of vascular permeability, VEGF induces endothe-

lial fenestration in some vascular beds (101) and in cultured adrenal endothelial cells (102).

Several studies have pointed to the critical role of nitric oxide (NO) in VEGF-induced vascular permeability, as well as angiogenesis (103–105). Recently, Fukumura *et al.* (106) assessed the relative contribution of the NO synthase (NOS) isoforms, inducible NOS and endothelial NOS (eNOS) to these processes. Angiogenesis, vessel diameter, blood flow rate, and vascular permeability were proportional to NO levels and were most impaired in $eNOS^{-/-}$ mice. VEGF significantly increased permeability in both wild-type and inducible $NOS^{-/-}$ mice, but not in $eNOS^{-/-}$ mice. VEGF-induced angiogenesis was markedly reduced in $eNOS^{-/-}$ mice, although the mice develop normally and have no apparent defect in the vasculature. These findings suggest that, although eNOS plays a predominant role in angiogenesis and vascular permeability in response to exogenous VEGF, this pathway is dispensable for developmental angiogenesis.

An issue that has been long debated is whether a correlation exists between vascular permeability and angiogenesis. It has been proposed that increase in microvascular permeability is a step necessary and sufficient for angiogenesis, by providing extravasation of fibrin, which represents a scaffold for endothelial cell proliferation and migration (107). However, as previously mentioned, factors such as bFGF are not known to induce vascular permeability and yet potently induce angiogenesis. Furthermore, vascular leakage is not necessarily followed by angiogenesis. For example, in background diabetic retinopathy, vascular leakage and fibrin deposition (108) may occur in the retina for decades before the onset of angiogenesis in the proliferative phase (109, 110). The report by Eliceiri *et al.* (111) that members of the Src family are differentially involved in mediating VEGF-dependent permeability and angiogenesis showed that the permeability-enhancing activity specifically depends on Src, or Yes. Mice lacking src and Yes display a normal angiogenic response to VEGF without any overt defects in the vasculature, suggesting that enhanced vascular permeability is not a requirement for VEGF-dependent angiogenesis, at least in the circumstances examined to date. Recently, Gratton *et al.* (112) have reported that a peptide that prevents the association of eNOS with caveolin inhibits vascular permeability and tumor progression in mice. However, additional studies have emphasized the complexity of the role of eNOS in tumorigenesis, including a role in the recruitment of endothelial progenitor cells (113) as well as a requirement for angiogenesis (114). Clearly, further studies are needed to fully elucidate this complex issue.

VEGF induces vasodilatation *in vitro* in a dose-dependent fashion (115, 116) and produces transient tachycardia, hypotension, and a decrease in cardiac output when injected iv in conscious, instrumented rats (116). Such effects appear to be caused by a decrease in venous return, mediated primarily by endothelial cell-derived NO (116). Hypotension was a dose-limiting side effect in human trials in which VEGF was systemically administered (117). Conversely, administration of anti-VEGF monoclonal antibodies to cancer patients resulted in elevation of blood pressure (36), indicating that VEGF signaling plays a tonic homeostatic role in the regulation of blood pressure. The mechanism is likely to involve eNOS, but remains to be fully elucidated.

V. VEGF Isoforms

VEGF has significant homology to PDGF, and all the eight cysteines found in the A and B chains of PDGF are conserved in VEGF (63, 64). The human VEGF-A gene is organized in eight exons, separated by seven introns (118, 119) and is localized in chromosome 6p21.3 (120). Alternative exon splicing results in the generation of four different isoforms, having 121, 165, 189, and 206 amino acids, respectively, after signal sequence cleavage ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) (118, 119). $VEGF_{165}$, the predominant isoform, lacks the residues encoded by exon 6, whereas $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Less frequent splice variants have been also reported, including $VEGF_{145}$ (121), $VEGF_{183}$ (122), $VEGF_{162}$ (123), and $VEGF_{165b}$, a variant reported to have paradoxically an inhibitory effect on VEGF-induced mitogenesis (124).

VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa (61). Such properties closely correspond to those of $VEGF_{165}$, which is indeed the major VEGF isoform (125).

Solution of the crystal structure of VEGF has shown that VEGF forms an antiparallel homodimer covalently linked by two disulfide bridges between Cys-51 and Cys-60 (126). This mode of dimerization is similar to that of the PDGF monomers. The dominant feature within the VEGF monomer is the cystine knot motif that is found in other growth factors (126). Although the VEGF monomer resembles that of PDGF, its NH_2-terminal segment is helical rather than extended (126).

$VEGF_{121}$ is an acidic polypeptide that fails to bind to heparin (125). $VEGF_{189}$ and $VEGF_{206}$ are highly basic and bind to heparin with high affinity (125). $VEGF_{121}$ is a freely diffusible protein. In contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the ECM. $VEGF_{165}$ has intermediate properties, because it is secreted, but a significant fraction remains bound to the cell surface and ECM (127). The ECM-bound isoforms may be released in a diffusible form by heparin or heparinase, which displaces them from their binding to heparin-like moieties, or by plasmin cleavage at the COOH terminus, which generates a bioactive fragment consisting of the first 110 NH_2-terminal amino acids (125). Given the important role of plasminogen activation during physiological and pathological angiogenesis processes (128), this proteolytic mechanism can be particularly important in regulating locally the activity and bioavailability of VEGF.

Plouet *et al.* (129) have proposed a role for urokinase in the generation of bioactive VEGF. Recombinant $VEGF_{189}$ from insect cells infected with a recombinant baculovirus was purified as a nonmitogenic 50-kDa precursor that binds to the receptor VEGFR-1 but not to VEGFR-2. However, it could be matured by urokinase as a 38-kDa fragment able to promote endothelial cell proliferation (129). Figure 1 illustrates the properties of the VEGF isoforms.

Importantly, loss of the heparin-binding domain results in a reduction in the mitogenic activity of VEGF (130). These findings suggest that $VEGF_{165}$ has optimal characteristics of bioavailability and biological potency. In agreement with

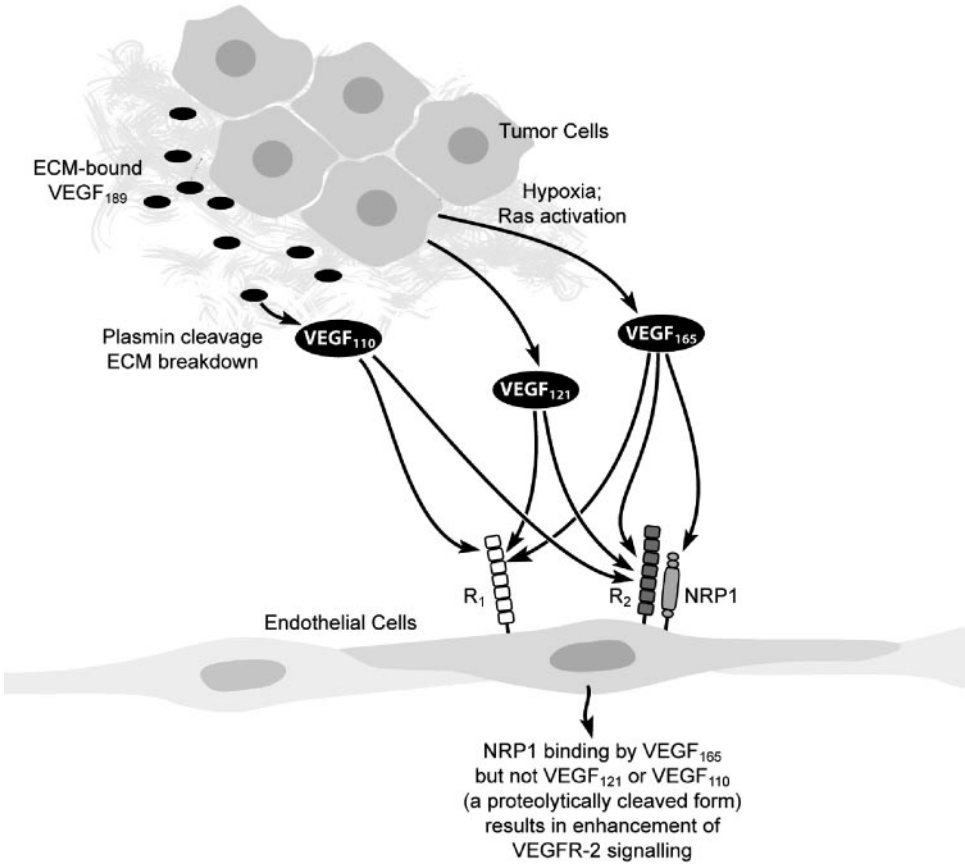

FIG. 1. VEGF isoforms and their interaction with VEGFRs. The diffusible VEGF isoforms, $VEGF_{121}$ and $VEGF_{165}$, are released by a variety of normal and transformed cells (the figure shows tumor cells) and may bind to VEGFR-1 (R1) and VEGFR-2 (R2). $VEGF_{165}$, but not $VEGF_{121}$, interacts also with NP1 and NP2. This binding results in enhancement of VEGFR-2-dependent signaling in endothelial cells. After plasmin generation and ECM breakdown, $VEGF_{189}$ is cleaved at the COOH terminus, and the resulting 110-amino acid NH_2-terminal fragment is diffusible and bioactive.

such conclusions, only $VEGF_{164}$ (murine VEGF is shorter by one amino acid) is able to fully rescue a tumorigenic phenotype in mouse $VEGF^{-/-}$ cells (131). The significance of the heparin-binding VEGF isoform(s) is also emphasized by the finding that 50% of the mice expressing exclusively $VEGF_{120}$ ($VEGF^{120/120}$) die shortly after delivery, whereas the rest die within 2 wk (132). Recent studies have also evidenced a deficit in the distribution of endothelial cells and impaired filopodia extension in $VEGF^{120/120}$ mice, suggesting that the heparin-binding VEGF isoforms provide essential stimulatory cues to initiate vascular branch formation (133).

VI. Regulation of VEGF Gene Expression

A. Oxygen tension

Oxygen tension plays a key role in regulating the expression of a variety of genes (134). VEGF mRNA expression is induced by exposure to low pO_2 in a variety of pathophysiological circumstances (135, 136). Earlier studies indicated similarities between the mechanisms leading to hypoxic regulation of VEGF and erythropoietin (Epo) (137). 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 (138, 139). Such 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 (140). HIF-1 is a basic, heterodimeric, helix-loop-helix protein consisting of two subunits, HIF-1α and aryl hydrocarbon receptor nuclear translocator, known also as HIF-1β (141). It is now well established that HIF-1 is a key mediator of hypoxic responses (142). In response to hypoxia, HIF-1 binds to specific enhancer elements, resulting in increased gene transcription. A gene highly homologous to HIF-1, HIF-2, also forms heterodimers with aryl hydrocarbon receptor nuclear translocator and regulates VEGF expression (143). Recent studies have uncovered the critical role of the product of the von Hippel-Lindau (VHL) tumor suppressor gene in HIF-1-dependent hypoxic responses (for review see Ref. 144). The VHL gene is inactivated in patients with von Hippel-Lindau disease, an autosomal-dominant neoplasia syndrome characterized by capillary hemangioblastomas in retina and cerebellum, and in most sporadic clear cell renal carcinomas (145). Also, the mitogenic activity for endothelial cells in the conditioned medium of renal cell carcinoma cells expressing a mutant VHL was largely neutralized by anti-VEGF antibodies (146). Earlier studies indicated that a function of the VHL protein is to provide negative regulation of VEGF and other hypoxia-inducible genes (147). The spectrum of activities of the VHL protein remains to be fully elucidated, and multiple functions have been proposed, including interaction with fibronectin (148). However, the VHL protein is known to interact with a series of proteins including elongins B and C and CUL2, a member of the Cullin family (149), suggesting homology to yeast ubiquitin ligase complexes known as "SCF complexes." HIF-1 was shown to be consti-

tutively activated in VHL-deficient renal cell carcinoma cell lines (150). More recent studies demonstrated that, indeed, one of the functions of VHL is to be part of a ubiquitin ligase complex that targets HIF subunits for proteasomal degradation after covalent attachment of a polyubiquitin chain (151, 152). Oxygen promotes the hydroxylation of HIF at a proline residue, a requirement for the association with VHL (151, 152). Recently, a family of prolyl hydroxylases related to *Egl-9 Caenorhabditis elegans* gene product were identified as HIF prolyl hydroxylases (134, 153, 154).

Importantly, other studies have implicated the PI3 kinase/Akt pathway in the regulation of HIF-mediated responses in a hypoxia-independent manner. Zundel *et al.* (155) have shown that mutations resulting in loss of function of the tumor suppressor PTEN, which negatively regulates effectors of PI3 kinase/Akt and is mutated in glioblastoma and other tumors (156, 157), result in increased activation of HIF-1 and increased VEGF transcription. Tang and Lasky (158) have recently elucidated the role of the Forkhead transcription factor (FOXO4) in this pathway. Nuclear localization of Forkhead, which is inhibited by PI3 kinase activation, normally down-regulates the HIF-1 protein. These findings emphasize the multiple advantages conferred on tumor cells by PI3 kinase/Akt activation.

B. Growth factors, hormones, and oncogenes

Several major growth factors, including epidermal growth factor, TGF-α, TGF-β, keratinocyte growth factor, IGF-I, FGF, and PDGF, up-regulate VEGF mRNA expression (159–161), suggesting that paracrine or autocrine release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment. Also, inflammatory cytokines such as IL-1-α and IL-6 induce expression of VEGF in several cell types, including synovial fibroblasts, in agreement with the hypothesis that VEGF may be a mediator of angiogenesis/permeability in inflammatory disorders (162, 163).

Hormones are also important regulators of VEGF gene expression. TSH has been shown to induce VEGF expression in several thyroid carcinoma cell lines (164). Shifren *et al.* (165) have also shown that ACTH is able to induce VEGF expression in cultured human fetal adrenal cortical cells, suggesting that VEGF may be a local regulator of adrenal cortical angiogenesis and an important mediator of the tropic action of ACTH. Gonadotropins have been shown to be potent inducers of VEGF transcription in the ovary, both *in vivo* (166, 167) and *in vitro* (168). Also, human chorionic gonadotropin results in increased VEGF mRNA transcription and protein levels in cultured Leydig cells (169). Several studies have implicated sex steroids as an important stimulus for VEGF regulation in hormone-sensitive tissues. *In vitro*, androgen deprivation of LnCaP prostate cancer cells led to decreased VEGF mRNA and protein expression as well as a 5-fold destabilization in VEGF mRNA transcripts. In mice bearing LnCaP tumors, castration resulted in a rapid decrease in mRNA expression and markedly reduced tumor neovascularization (170). Mueller *et al.* (171) have reported that estradiol is a direct transcriptional activator of VEGF, mediated by a variant estrogen response element located 1.5 kb from the transcription start. Progestins have also been reported to induce VEGF gene transcription in endometrial carcinoma cells (172).

Specific transforming events also result in induction of VEGF gene expression. Oncogenic mutations or amplification of ras leads to VEGF up-regulation (173, 174). These studies indicate that mutant ras-dependent VEGF expression is necessary, albeit not sufficient, for progressive tumor growth *in vivo* (173–175). Mutations in the Wnt-signaling pathway, which are frequently associated with premalignant colonic adenomas, result in up-regulation of VEGF (176). K-ras activation appeared to enhance Wnt signaling, which suggests an interaction between these two pathways (176). Interestingly, VEGF is up-regulated in polyps of Apc knockout (Apcδ716) mice, a model for human familial adenomatous polyposis (177). In both benign and malignant mouse intestinal tumors, stromal expression of cyclooxygenase 2 results in elevated PGE2 levels that stimulate, in turn, cell surface receptor EP2, followed by induction of VEGF and angiogenesis (177–179). In this context, Amano *et al.* (180) have recently shown that PGE2-EP3 receptor signaling also plays a significant role in up-regulating VEGF in stromal cells and thus potentially in tumor angiogenesis.

VII. VEGFRs

Initially, VEGF binding sites were identified on the cell surface of vascular endothelial cells *in vitro* (181, 182) and *in vivo* (183, 184). Subsequently, VEGFRs were shown to exist also on bone marrow-derived cells such as monocytes (185). VEGF binds two highly related receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2. Both VEGFR-1 and VEGFR-2 have seven Ig-like domains in the extracellular domain, a single-transmembrane region, and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain (186–188). A member of the same family of RTKs is VEGFR-3 (Flt-4) (189), which, however, is not a receptor for VEGF, but instead binds VEGF-C and VEGF-D (27). In addition to these RTKs, VEGF interacts with a family of coreceptors, the neuropilins (NP). Figure 2 summarizes the interaction of the members of the VEGF gene family with the VEGF RTKs.

A. VEGFR-1 (Flt-1)

Although Flt-1 (fms-like tyrosine kinase) was the first RTK to be identified as a VEGFR more than a decade ago (190), the precise function of this molecule is still the object of debate. Recent evidence indicates that the conflicting reports may be due, at least in part, to the fact that VEGFR-1 functions and signaling properties can be different depending on the developmental stage and the cell type, *e.g.*, endothelial *vs.* hematopoietic cells. VEGFR-1 expression is up-regulated by hypoxia via a HIF-1-dependent mechanism (191). VEGFR-1 binds not only VEGF-A but also PlGF (192) and VEGF-B (193), which fail to bind VEGFR-2. An alternatively spliced soluble form of VEGFR-1 (sFlt-1) has been shown to be an inhibitor of VEGF activity (194). The binding site for VEGF (and PlGF) has been mapped primarily to the second Ig-like domain (195–197). The crystal structure of a VEGF-Flt-1 domain 2 complex has shown the poles of the VEGF dimer to

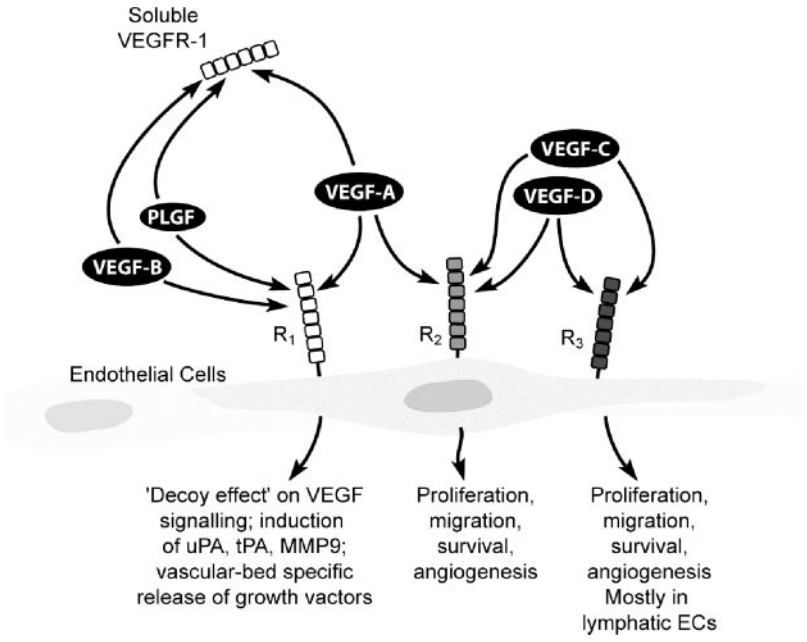

FIG. 2. Role of the VEGFR tyrosine kinases in endothelial cells. VEGFR-1 (R1) and VEGFR-2 (R2) are expressed in the cell surface of most blood endothelial cells. In contrast, VEGFR-3 (R3) is largely restricted to lymphatic endothelial cells. VEGF-A binds both VEGFR-1 and VEGFR-2. In contrast, PlGF and VEGF-B interact only with VEGFR-1. VEGF-C and VEGF-D bind VEGFR-2 and VEGFR-3. There is much evidence that VEGFR-2 is the major mediator of endothelial cell mitogenesis, survival, and microvascular permeability. In contrast, VEGFR-1 does not mediate an effective mitogenic signal in endothelial cells and it may, especially during early embryonic development, perform an inhibitory role by sequestering VEGF and preventing its interaction with VEGFR-2. Such a "decoy" role could be also performed by the alternatively spliced soluble VEGFR-1. EC, Endothelial cell; uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator.

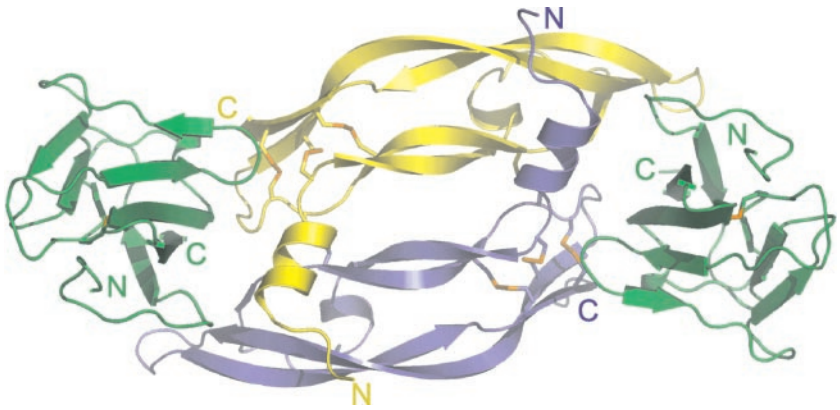

FIG. 3. Ribbon representation of the VEGF-VEGFR1-domain 2 complex. The two VEGF monomers are shown in *blue* and *yellow*, and the two receptor molecules are depicted in *green*. This is a "top down" view and shows the complex looking toward the membrane. The termini are labeled. [Reproduced with permission from C. Wiesmann.]

be in a predominantly hydrophobic interaction with domain 2 (198). Figure 3 illustrates the complex VEGF-VEGFR-1 domain 2 in a ribbon format.

Flt-1 reveals a weak tyrosine autophosphorylation in response to VEGF (190, 199). Park *et al.* (192) initially proposed that VEGFR-1 may be not primarily a receptor transmitting a mitogenic signal, 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 factor less available to VEGFR-2 (see Fig. 2). Thus, the observed potentiation of the action of VEGF by PlGF could be explained, at least in part, by displacement of VEGF from VEGFR-1 binding (192). Not only the full-length membrane-bound form of VEGFR-1, but also sFlt-1, could perform such a decoy function (200). Recent studies have shown that, indeed, a synergism exists between VEGF and PlGF *in vivo*, especially during pathological situations, as evidenced by impaired tumorigenesis and vascular leakage in $Plgf^{-/-}$ mice (200). Gille *et al.* (201) have identified a repressor motif in the juxtamembrane region of VEGFR-1 that impairs PI3 kinase activation and endothelial cell migration in response to VEGF. Zeng *et al.* (202) have proposed that VEGFR-1 activation results in inhibition of VEGFR-2-dependent endothelial cell proliferation and that this inhibitory pathway is PI3 kinase dependent. However, other studies indicated that VEGFR-1 is able to interact with various signal-transducing proteins and generate, in some circumstances, a mitogenic signal (203, 204). Very recently, Autiero *et al.* (205) have proposed that PlGF regulates inter- and intramolecular cross-talk between the VEGF RTKs. Activation of VEGFR-1 by PlGF resulted in transphosphorylation of VEGFR-2, thus amplifying VEGF-driven angiogenesis through VEGFR-2. According to these studies, although VEGF and PlGF both bind VEGFR-1, PlGF uniquely stimulated the phosphorylation of specific VEGFR-1 tyrosine residues, and this results in the expression of distinct target genes (205). This finding is somewhat surprising, considering that PlGF and VEGF bind to the same binding interface of VEGFR-1 in a very similar fashion (206).

Irrespective of the conflicting evidence on the role of VEGFR-1 as a signaling receptor, gene-targeting studies have demonstrated the essential role of this molecule during embryogenesis. $Flt\text{-}1^{-/-}$ mice die *in utero* between d 8.5 and d 9.5 (207, 208). Endothelial cells develop but fail to organize

in vascular channels. Excessive proliferation of angioblasts has been reported to be responsible for such disorganization and lethality (208), indicating that, at least during early development, VEGFR-1 is a negative regulator of VEGF action. More compelling evidence in support of this view stems from the report that a targeted mutation resulting in a VEGFR-1 lacking the tyrosine kinase (TK) domain, but able to bind VEGF, does not result in lethality or any overt defect in vascular development (209). Nevertheless, one specific biological response, the migration of monocytes in response to VEGF (or PlGF) has been shown to require the tyrosine kinase domain of VEGFR-1 (209, 210) **(**Fig. 4**).** Selvaraj *et al.* (211) have shown recently that PlGF binding to VEGFR-1 in monocytes results in activation of PI3 kinase/AKT and ERK-1/2 pathways, leading to chemotaxis as well as to the induction of a series of inflammatory cytokines. Furthermore, Lewis lung carcinoma cells overexpressing PlGF grow in wild-type mice faster than in VEGFR-1 tyrosine kinase-deficient mice, suggesting that VEGFR-1 may be a positive regulator under pathological conditions when a VEGFR-1-specific ligand is highly expressed (212). These findings suggest that VEGFR-1 has a dual function in angiogenesis, acting in a positive or negative manner in different circumstances. Recently, VEGFR-1 signaling has been also linked to the induction of matrix metalloproteinase 9 (MMP-9) in lung endothelial cells and to the facilitation of lung metastases (213). Recent studies have emphasized the effects of VEGFR-1 in hematopoiesis and recruitment of endothelial progenitors. Hattori *et al.* (214) have shown that VEGFR-1 activation by PlGF is able to reconstitute hematopoiesis by recruiting VEGFR-1$^+$ HSC. In addition, Gerber *et al.* (94) have shown that VEGFR-1 activation by enforced expression of PlGF rescues survival and ability to repopulate in VEGF$^{-/-}$ HSC. Furthermore, PlGF can promote collateral vessel growth and arteriogenesis in models of myocardial and limb ischemia through the recruitment of bone marrow cells such as monocytes (215, 216).

LeCouter *et al.* (217) recently provided evidence for a novel function of VEGFR-1 in liver sinusoidal endothelial cells (LSECs). VEGFR-1 activation achieved with a receptor-selective VEGF mutant or PlGF resulted in the paracrine release of hepatocyte growth factor, IL-6, and other hepatotrophic molecules by LSECs, to the extent that hepatocytes were stimulated to proliferate when cocultured with LSECs. VEGF had no direct mitogenic effect on hepatocytes. A VEGFR-1 agonist protected the liver from CCl4-induced damage, in spite of the inability to induce LSEC proliferation **(**Fig. 5). These findings suggest that a key function of VEGFR-1 signaling in the vascular endothelium is not the regulation of angiogenesis but, rather, the paracrine release of tissue-specific growth/survival factors, possibly in a vascular bed-specific fashion (217). In this context, liver and pancreas morphogenesis is induced by endothelial cells before the establishment of a blood flow, indicating that a paracrine function of gut endothelial cells plays a critical role during organogenesis (218, 219).

B. VEGFR-2 (KDR, human; Flk-1, mouse)

VEGFR-2 binds VEGF, albeit with lower affinity relative to VEGFR-1 [dissociation constant (K_d) 75–250 pM *vs.* 25 pM] (220–222). The key role of this receptor in developmental angiogenesis and hematopoiesis is evidenced by lack of vasculogenesis and failure to develop blood islands and organized blood vessels in Flk-1 null mice, resulting in death *in utero* between d 8.5 and d 9.5 (223). Consistent with a role in hematopoiesis, VEGFR-2 has been identified on a subset of multipotent human HSCs (224). There is now general agree-

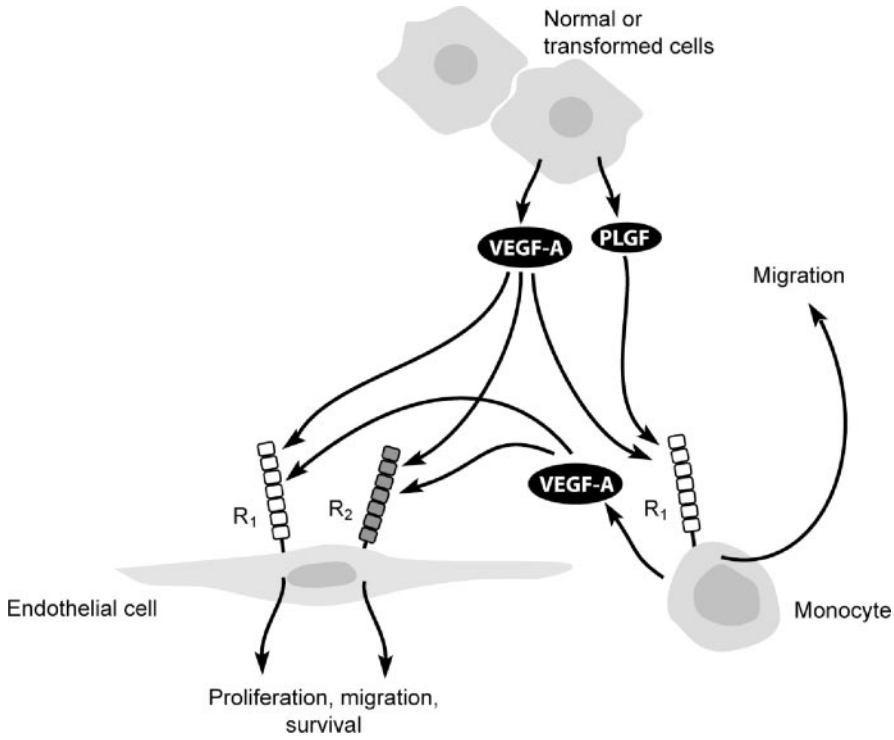

FIG. 4. Role of VEGFR-1 (R1) in monocyte chemotaxis. VEGFR-1 activation by VEGF-A, PlGF, or VEGF-B (data not shown) results in monocyte recruitment in normal or transformed tissues. Monocytes may, in turn, produce VEGF-A and other angiogenic factors, which may amplify the angiogenesis cascade.

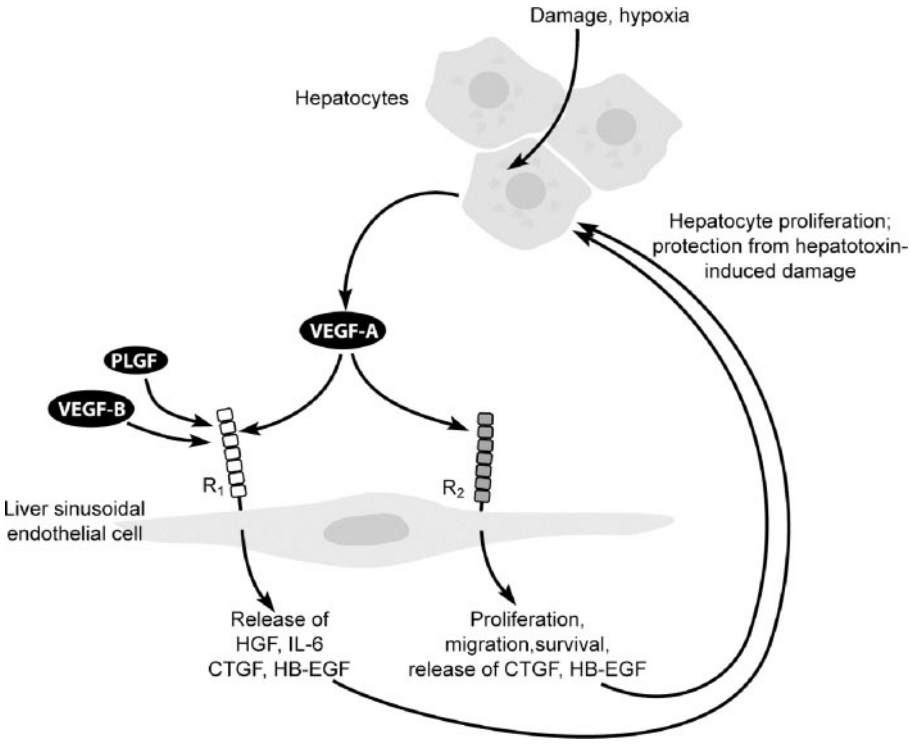

FIG. 5. Differential effects of VEGFR-1 and VEGFR-2 in LSECs. In response to VEGFR-1 activation, LSECs are not stimulated to proliferate but are instructed to up-regulate a series of hepatotrophic genes, including hepatocyte growth factor (HGF), IL-6, and heparin-binding epidermal growth factor (HB-EGF). Thus, VEGFR-1 agonists may result in significant hepatocellular protection from hepatotoxins, without stimulation of angiogenesis. VEGFR-2 activation not only mediates LSEC proliferation, migration, and survival, but also results in induction of a subset of hepatotrophic genes. CTGF, Connective tissue growth factor.

ment that VEGFR-2 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF.

The binding site for VEGF has been mapped to the second and third Ig-like domains (225). VEGFR-2 undergoes dimerization and strong ligand-dependent tyrosine phosphorylation in intact cells and results in a mitogenic, chemotactic, and prosurvival signal. Several tyrosine residues have been shown to be phosphorylated (for review see Ref. 226). Takahashi *et al.* (227) have shown that Y1175 and Y1214 are the two major VEGF-A-dependent autophosphorylation sites in VEGFR-2. However, only autophosphorylation of Y1175 is crucial for VEGF-dependent endothelial cell proliferation. Also, VEGF has been shown to induce the phosphorylation of at least 11 proteins in bovine aortic endothelial cells (228). Among these, VEGF induces phosphorylation of phospholipases Cγ, PI3-kinase, ras GTPase activating protein (228), src family (111), and several other signal transduction molecules (226). Byzova *et al.* (229) have reported that VEGFR-2 activation by VEGF results in PI3 kinase/Akt-dependent activation of several integrins. VEGF enhanced cell adhesion, migration, soluble ligand binding, and adenovirus gene transfer mediated by $\alpha v\beta 3$ and also activated other integrins known to be involved in angiogenesis, $\alpha v\beta 5$, $\alpha 5\beta 1$, and $\alpha 2\beta 1$ (229). VEGFR-2 activation induces endothelial cell growth by activating the Raf-Mek-Erk pathway. An unusual feature of VEGFR-2 activation of this pathway is the requirement for protein kinase C but not ras (230, 231). VEGF mutants that bind selectively to VEGFR-2 are fully active endothelial cell mitogens, chemoattractants, and permeability-enhancing agents, whereas mutants specific for VEGFR-1 are devoid of all three activities (232). Also, VEGF-E, a homolog of VEGF identified in the genome of the parapoxvirus Orf virus (24), which shows VEGF-like mitogenic and permeability-enhancing effects, binds and activates VEGFR-2 but fails to bind VEGFR-1 (25, 26). Interestingly, similar biological effects and receptor selectivity have been recently reported with snake-derived VEGF (233). Furthermore, VEGFR-2 (but not VEGFR-1) activation has been shown to be required for the antiapoptotic effects of VEGF for human umbilical vein endothelial cells (77). As previously noted, such a prosurvival effect of VEGF is mediated by the PI3 kinase/Akt pathway (77). This pathway is critical also for VEGF-dependent endothelial chemotaxis (201, 232, 234). Recent studies suggest, however, that at least in some circumstances, VEGFR-1 may transmit a prosurvival signal in endothelial cells, possibly mediated by induction of the antiapoptotic gene survivin (235).

C. Neuropilin (NP)1 and NP2

Earlier studies indicated that certain tumor and endothelial cells express cell surface VEGF-binding sites distinct in affinity and molecular mass from the two known VEGF RTKs (236). Interestingly, $VEGF_{121}$ failed to bind these sites, indicating that exon 7-encoded basic sequences are required for binding to this putative receptor (236). Subsequently, Soker *et al.* (237) identified such isoform-specific VEGF receptor as NP1, a molecule that had been previously shown to bind the collapsin/semaphorin family and was implicated in neuronal guidance (for review see Ref. 238). When coexpressed in cells with VEGFR-2, NP1 enhanced the binding of $VEGF_{165}$ to VEGFR-2 and $VEGF_{165}$-mediated chemotaxis (237). NP1 appears to present $VEGF_{165}$ to the VEGFR-2 in a manner that enhances the effectiveness of VEGFR-2-mediated signal transduction (237). Fuh *et al.* (239) have shown that NP1 is able to directly bind VEGFR-1, suggesting that one of the

mechanisms by which VEGFR-1 functions as a negative regulator of VEGF activity is competing for NP1 binding. Binding to NP1 may help to explain the greater mitogenic potency of $VEGF_{165}$ relative to $VEGF_{121}$. So far, there is no clear evidence that NP1 or the related NP2 signals after VEGF binding (238). In contrast, in response to semaphorin binding, NP1 and NP2 signals axon repulsion. Interestingly, collapsin 1 is able to inhibit the motility of porcine aortic endothelial cells expressing NP1 (240). Recent evidence indicates that the formation of complexes with plexins is a requirement for NP signaling in neurons (241, 242). The role of NP1 in the development of the vascular system has been demonstrated by gene-targeting studies, documenting embryonic lethality in null mice (243). Furthermore, Lee *et al.* (244) have shown that, in the zebrafish, NP1 is required for vascular development and mediates VEGF-dependent angiogenesis. Interestingly, recent studies have linked NP2 to lymphatic vessel development (245).

VIII. Role of VEGF in Physiological Angiogenesis

A. Embryonic and postnatal development

In 1996, two studies demonstrated an essential role of VEGF in embryonic vasculogenesis and angiogenesis in the mouse (246, 247). Inactivation of a single VEGF allele resulted in embryonic lethality between d 11 and d 12. The $vegf^{+/-}$ embryos exhibited a number of developmental anomalies, defective vascularization in several organs, and a markedly reduced number of nucleated red blood cells within the blood islands in the yolk sac, indicating that VEGF regulates both vasculogenesis and early hematopoiesis. Conditional VEGF gene inactivation in VEGF *loxP* mice, using a Nestin promoter-driven Cre-recombinase, has shown that the dosage of VEGF from neural progenitor cells is a critical determinant in the development and density of vascular plexus in the developing nervous system, to the extent that severe reductions in VEGF led to decreases in vascularity and subsequent hypoxia, resulting in the specific degeneration of the cerebral cortex and neonatal lethality (248, 249). Conversely, even modest increases in VEGF gene expression, achieved by the insertion of a LacZ cassette in the 3′-untranslated region of the VEGF gene, result in severe abnormalities in heart development and embryonic lethality at embryonic d 12.5 (E12.5)–E14 (250). These findings indicate a critical VEGF gene-dosage dependence during development. In contrast, inactivation of PlGF (200) or VEGF-B (251) genes did not result in any major development abnormalities, although VEGF-B inactivation in mice results in reduced heart size and impaired recovery from experimentally induced myocardial ischemia (251). So far, it appears that, among the other members of the VEGF gene family, only VEGF-C plays an essential role in development, because its inactivation results in embryonic lethality due to defective lymphatic development and fluid accumulation in tissues (252).

To determine the role of VEGF in early postnatal life, several strategies have been employed (83). Partial inhibition of VEGF achieved by Cre-*loxP*-mediated gene targeting resulted in increased mortality, stunted body growth, and impaired organ development. Administration of a soluble VEGFR-1 chimeric protein, which achieves a nearly complete VEGF inhibition, results in almost complete growth arrest, when the treatment is initiated at d 1 or d 8 postnatally. Endothelial cells isolated from the liver of VEGFR1-IgG-treated neonates demonstrated increased apoptotic index, indicating that VEGF is required not only for proliferation but also for survival of endothelial cells (83). Such treatment is also accompanied by rapid lethality, primarily due to inhibition of glomerular development leading to kidney failure (83). Defective glomerular endothelial development in neonates was also observed in studies using anti-VEGF antibodies (253). The pivotal role of VEGF in kidney development was also demonstrated by a very recent study showing that selective VEGF deletion in podocytes, using a Nephin promoter-driven Cre recombinase, leads to glomerular disease in a gene dosage-dependent fashion (254). Heterozygous mice developed renal disease by 2.5 wk of age, characterized by proteinuria and endotheliosis. Homozygosity resulted in perinatal lethality (254). However, VEGF neutralization in fully developed normal mice (83) or rats (255) had no significant effects on glomerular function. In contrast, VEGF inhibition in adult rats with mesangioproliferative nephritis led to a reduction of glomerular endothelial regeneration and an increase in endothelial cell death, indicating that VEGF may be important for glomerular endothelial cell repair after injury, but not for endothelial survival in a healthy animal (255). In apparent conflict with these conclusions, Sugimoto *et al.* (256) have recently reported that the administration of anti-VEGF antibodies or sFlt-1 to adult mice results in proteinuria accompanied by glomerular endothelial cell detachment and hypertrophy, in association with down-regulation of nephrin. The reason for such discrepancies is unclear.

Importantly, VEGF neutralization in juvenile primates using a humanized anti-VEGF monoclonal antibody (bevacizumab) did not result in any renal or other significant abnormalities, except the suppression of growth plate and ovarian angiogenesis, as described below (257). Furthermore, as discussed in *Section IX.A*, long-term administration of bevacizumab to cancer patients resulted in minimal kidney toxicity (36, 258).

B. Skeletal growth and endochondral bone formation

Endochondral bone formation is a fundamental mechanism for longitudinal bone growth. Cartilage, an avascular tissue, is replaced by bone in a process named endochondral ossification (259). VEGF mRNA is expressed by hypertrophic chondrocytes in the epiphyseal growth plate, suggesting that a VEGF gradient is needed for directional growth and cartilage invasion by metaphyseal blood vessels (260, 261). After VEGF blockade with a soluble VEGFR-1 chimeric protein or an anti-VEGF monoclonal antibody, blood vessel invasion is almost completely suppressed, concomitant with impaired trabecular bone formation, in developing mice and primates (257, 260). Although proliferation, differentiation, and maturation of chondrocytes were apparently normal, resorption of hypertrophic chondrocytes was inhibited, resulting in a marked expansion of the hypertrophic chondrocyte zone. Importantly, cessation of the anti-VEGF treatment is fol-

lowed by capillary invasion, restoration of bone growth, and normalization of the growth plate architecture. Recent studies indicate that VEGF mRNA in osteoblasts is induced by bone morphogenetic proteins (BMPs), suggesting that VEGF produced by osteoblasts in response to BMPs may couple angiogenesis to bone formation (262). Conversely, VEGF may induce BMP-2 expression in endothelial cells, suggesting that endothelial cells may play also an osteogenic role by a BMP-2-dependent stimulation of osteoblasts (263).

Interestingly, a growth plate abnormality similar to that induced by VEGF inhibitors was observed in MMP-$9^{-/-}$ mice (264). Recent evidence indicates that a function of MMP-9 is to render VEGF bioavailable to its receptors (265). VEGF blockade inhibits bone repair (266); MMP-$9^{-/-}$ mice have delayed healing of fractures, and administration of exogenous VEGF corrects this defect (267). Furthermore, VEGF has direct chemotactic and other effects on osteoblasts (268) and osteoclasts (269). These findings indicate not only that VEGF-dependent blood vessel recruitment is essential for coupling cartilage resorption with bone formation, but also that the effects of VEGF on bone homeostasis are complex and involve direct effects on bone cells (270).

A similar, although less dramatic, phenotype was obtained, when VEGF was deleted in the cartilage of developing mice by means of Cre-*loxP*-mediated, tissue-specific gene ablation (271). Furthermore, examination of $VEGF^{120/120}$ mice not only revealed a delayed recruitment of blood vessels into the perichondrium but also showed delayed invasion of vessels into the primary ossification center, demonstrating a significant role of heparin-binding VEGF isoform at both an early and later stage of cartilage vascularization (272).

C. Angiogenesis in endocrine glands

Angiogenesis is a key aspect of normal cyclical ovarian function. Follicular growth and the development of the CL are dependent on the proliferation of new capillary vessels (273). The process of selection of a dominant follicle in monovular species has been also associated with angiogenesis, as there is evidence that selected follicles possess a more elaborate microvascular network than other follicles (274). The angiogenesis that accompanies CL development also plays a key role in the delivery of cholesterol to luteal cells for progesterone biosynthesis (275). Subsequently, the blood vessels regress, suggesting the coordinated action of inducers as well as inhibitors of angiogenesis in the course of the ovarian cycle (276, 277).

Previous studies have shown that the VEGF mRNA expression is temporally and spatially related to the proliferation of blood vessels in the ovary (278, 279). Administration of VEGF inhibitors delays follicular development (280) and suppresses luteal angiogenesis in rodents (167, 281) as well as in primates (257, 282–284). These studies have established that VEGF is indeed the principal regulator of ovarian angiogenesis and that blockade of the VEGF pathway is sufficient to disrupt angiogenesis. Figure 6 illustrates the experiments that demonstrated, for the first time, such a key role of VEGF, using VEGF-soluble receptors in a rat model of hormonally induced ovulation (167).

More recent studies have indicated that endocrine gland-derived VEGF (EG-VEGF), a novel angiogenic factor that is selectively expressed in steroidogenic tissues, plays a cooperative role with VEGF in the regulation of angiogenesis in the human ovary (285). EG-VEGF is not structurally related

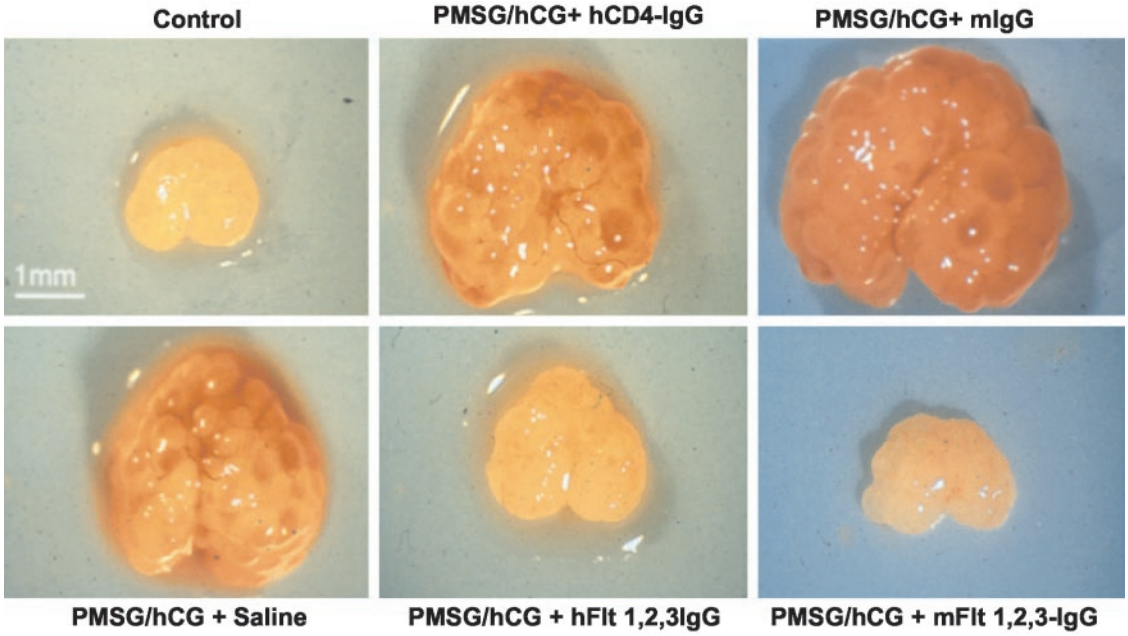

FIG. 6. VEGF is essential for CL angiogenesis (167). Ovulation was hormonally induced in rats using PMSG, followed by human chorionic gonadotropin (hCG), and this treatment resulted in a dramatic increase in ovarian weight and vascularity 5 d after PMSG administration. Animals were given human (h) or mouse (m) Flt(1–3)-IgG, which potently inhibits VEGF, or control proteins (CD4-IgG or mIgG). Note the complete suppression of ovarian angiogenesis and growth after administration of the VEGF inhibitors. PMSG, Pregnant mare's serum gonadotropin.

to VEGF but belongs to a unique gene family having distant homology to Dickopf, an inhibitor of Wnt signaling (286, 287). A sequential activation of the two genes occurs in the human ovary (288). Whereas VEGF mRNA is strongly expressed in early-stage CL, coincident with the initial development of a capillary plexus, its expression is markedly reduced by midluteal phase. In contrast, EG-VEGF starts being expressed later than VEGF but persists throughout mid- and early-late luteal phase, suggesting that EG-VEGF may be important for the persistence and adequacy of luteal function (288). Thus, the ovary has apparently developed a highly specific local mechanism to complement the action of VEGF. Interestingly, such an acquisition seems to be, at least in part, a late event in evolution and may reflect a greater functional/morphological complexity of organs like the ovary. Although association of human EG-VEGF expression with steroidogenic cells is compelling, the mouse ortholog of this gene has a different expression pattern (289). In this context, a consensus binding site for the NR5A1 orphan nuclear receptor is present within the human EG-VEGF promoter (289). NR5A1, considered to be a key regulator of endocrine development and function (290, 291), regulates multiple target genes involved in gonadal and adrenal determination and development, steroidogenesis, and reproduction (for review see Ref. 292). Although rodents have served as models for endocrinology and ovarian physiology, clear differences exist between the rodent and human ovary. The length of the ovarian cycle also distinguishes the human or primate from the rodent. In humans the cycle is 28 d, and in rodents the cycle is completed every 4 d (293). The primate CL is functional for 2 wk before its regression in the infertile cycle, whereas the rodent CL is active for less than 1 d (294).

Recently, the role of VEGF in the development of pancreatic islets has been investigated (295). Deletion of VEGF in the mouse pancreas reveals that endocrine cells signal back to the adjacent endothelial cells to induce the formation of a dense network of fenestrated capillaries in islets. Interestingly, VEGF is not required for the development of all islet capillaries. However, the remaining capillaries found in the VEGF-deficient islets were not fenestrated and contained an unusual number of caveolae. In addition, glucose tolerance tests reveal that the VEGF-induced capillary network is not strictly required for blood glucose control but is essential for fine tuning blood glucose regulation (295).

IX. Role of VEGF in Pathological Conditions

A. Solid tumors

Many tumor cell lines secrete VEGF *in vitro*, suggesting the possibility that this diffusible molecule may be a mediator of tumor angiogenesis (29). *In situ* hybridization studies have demonstrated that the VEGF mRNA is expressed in the vast majority of human tumors so far examined, including carcinoma of the lung (296, 297), breast (298, 299), gastrointestinal tract (300–303), kidney (304–306), bladder (304), ovary (307–309), and endometrium (310) and several intracranial tumors including glioblastoma multiforme (311–313) and sporadic, as well as VHL syndrome-associated, capillary hemangioblastoma (314, 315). In glioblastoma multiforme and other tumors with significant necrosis, the expression of VEGF mRNA is highest in hypoxic tumor cells adjacent to necrotic areas (311–313).

VEGF mRNA is also expressed in endocrine tumors. Expression of VEGF has been demonstrated in a variety of pituitary tumors. Lloyd *et al.* (316) examined a series of 148 tumors and found that VEGF expression, as assessed by immunohistochemistry, although in general less intense than in normal pituitary tissue, is more prominent in certain adenoma subtypes, especially GH adenomas. Furthermore, carcinomas show increased VEGF expression relative to adenomas, suggesting an up-regulation of VEGF during pituitary tumor progression (316). Soh *et al.* (317) found the VEGF mRNA to be higher in thyroid cancer cell lines compared with primary cultures of normal thyroid cells and higher in thyroid cancers of follicular than those of parafollicular cell origin. Furthermore, Klein *et al.* (318) have shown that expression of VEGF by immunohistochemistry is a negative prognostic marker in papillary thyroid carcinoma. The distribution of VEGF and other angiogenic factors in endocrine tumors has been recently reviewed by Turner *et al.* (319).

In 1993, Kim *et al.* (320) reported that anti-VEGF monoclonal antibodies exert a potent inhibitory effect on the growth of several tumor cell lines in nude mice, whereas the antibody had no effect on the tumor cells *in vitro*. Subsequently, many other tumor cell lines were found to be inhibited *in vivo* by anti-VEGF monoclonal antibodies (321–327). Tumor growth inhibition was demonstrated also with other anti-VEGF treatments, including a retrovirus-delivered dominant negative Flk-1 mutant (328), small molecule inhibitors of VEGFR-2 signaling (329–331), antisense oligonucleotides (332, 333), anti-VEGFR-2 antibodies (334), and soluble VEGF receptors (335–339).

Tumors of endocrine origin are also substantially growth inhibited by anti-VEGF treatment. Treatment with an antihuman VEGF monoclonal antibody resulted in more than 90% inhibition of tumor growth in a model of thyroid cancer (340). Also, administration of PTK787, a small molecule VEGFR-2 kinase inhibitor, led to a 41% reduction of tumor volume in a nude mouse model of poorly differentiated thyroid carcinoma (341).

Although tumor cells usually represent the major source of VEGF, tumor-associated stroma is also an important site of VEGF production (337, 342–344). As illustrated in Fig. **7**, chemotactic signals from tumor cells recruit stromals cells, which also produce VEGF and other angiogenic factors. The growth of a variety of human tumor cell lines transplanted in nude mice is substantially reduced, but not completely suppressed, by antihuman VEGF monoclonal antibodies (320). Administration of mFlt (1–3)-IgG, a chimeric receptor containing the first three Ig-like domains of VEGFR-1, that binds both human and mouse VEGF, results in a nearly complete suppression of tumor growth, accompanied by dramatic tumor cells necrosis, in a nude mouse model of human rhabdomyosarcoma **(**Fig. 8**)** (337). Similar results were obtained using a chimeric soluble receptor consisting of domain 2 of VEGFR-1 fused with domain 3 of VEGFR-2, referred to as "VEGF-trap" (339). Therefore, the use of VEGF inhibitors that only target human VEGF in human xenograft models

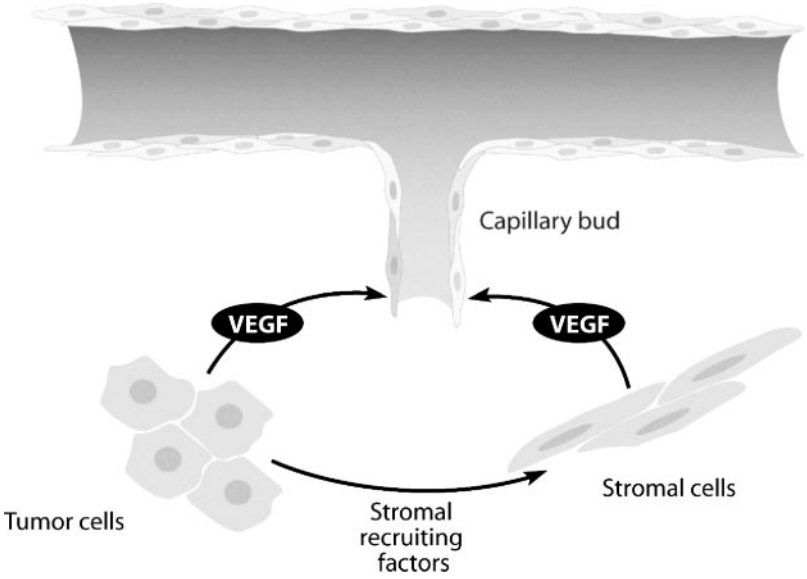

FIG. 7. Both tumor and stromal VEGF contribute to tumor angiogenesis. In response to chemotactic stimuli, stromal cells are recruited into the tumor and produce VEGF and other angiogenic factors.

frequently results in underestimating the contribution of VEGF to the process of tumor angiogenesis.

Cre-*LoxP*-mediated gene targeting has been used to show that VEGF inactivation suppresses tumor angiogenesis in the Rip-Tag model, a well-established genetic model of insulinoma (345). Furthermore, at least in the Rip-Tag model, MMP-9-mediated proteolytic events have been shown to determine an "angiogenic switch," mediated by enhancement of the activity of low constitutive levels of VEGF that become available to bind VEGFR-2 (265, 346).

Several studies have shown that combining anti-VEGF treatment with chemotherapy (347) or radiation therapy (348, 349) results in greater antitumor effects than either treatment alone. An issue that is being debated is the mechanism of such potentiation, and various hypotheses, not mutually exclusive, have been proposed. Klement *et al.* (347) proposed that chemotherapy, especially when delivered at low doses, preferentially damages endothelial cells, and the blockade of VEGF blunts a key survival signal for endothelial cells, thus amplifying the antitumor cell effects of chemotherapy. Jain (350) proposed that antiangiogenic therapy "normalizes" the tumor vasculature, leading to pruning of excessive endothelial cells and perivascular cells, reduction in vessel tortuosity, and drop in interstitial pressure and improved delivery of chemotherapy to tumor cells. These effects would provide an explanation for the apparent paradox that administration of VEGF inhibitors leads to a reduction in vascular permeability (79, 351), which would be expected to reduce the delivery of protein-bound chemotherapy into the tumor cells.

Most recently, Willett *et al*. (352) have shown that VEGF blockade using an anti-VEGF monoclonal antibody (bevacizumab) decreases tumor perfusion, vascular volume, microvascular density, interstitial fluid pressure, and the number of viable, circulating endothelial and progenitor cells in colorectal cancer patients, providing direct evidence for antivascular effects after VEGF blockade.

Clinical trials in cancer patients are ongoing with several VEGF inhibitors, including a humanized anti-VEGF monoclonal antibody (rhuMab VEGF; bevacizumab; Avastin, Genentech, South San Francisco, CA) (353), an anti-VEGFR-2 antibody (334), small molecules inhibiting VEGFR-2 signal transduction (330, 331), and a VEGFR chimeric protein (339). Phase II clinical data provided initial evidence that bevacizumab, in combination with 5-fluorouracil/leucovorin, results in increase in time to progression and survival in patients with metastatic colorectal carcinoma (CRC) (258). Thrombosis and increased blood pressure, as well as some proteinuria, were among the side effects of treatment observed in such a trial.

A double-blind placebo-controlled phase II trial demonstrated a significant increase in time to progression in renal cell carcinoma patients treated with bevacizumab as a single agent (36). Interestingly, the toxicity of the treatment was very modest and consisted of asymptomatic proteinuria and hypertension. In light of the fact that many renal cell carcinoma patients harbor mutations in the VHL gene, which result in altered regulation of VEGF (145), these results are particularly significant.

Recently, Hurwitz *et al.* (37) presented the results of a large randomized placebo-controlled phase III trial in which bevacizumab was tested in combination with chemotherapy as first-line therapy for previously untreated metastatic CRC. Patients were randomized to receive weekly bolus irinotecan, 5-fluorouracil, and leucovorin (IFL) plus bevacizumab (5 mg/kg every 2 wk), or IFL plus bevacizumab placebo. Survival was significantly increased in the IFL/bevacizumab arm compared with the IFL/placebo arm. Progression-free survival, response rate, and duration of response were also significantly increased in the bevacizumab group. Hypertension was more common in the IFL/ bevacizumab-treated group but was readily managed in all cases with oral antihypersensitive agents (37). Interestingly, the increased incidence of thrombosis and proteinuria, which was observed in phase II, was not observed in this phase III study. The prolongation of survival and improvement in other markers of clinical benefit observed with the addition of bevacizumab to standard chemotherapy confirms the importance of angio-

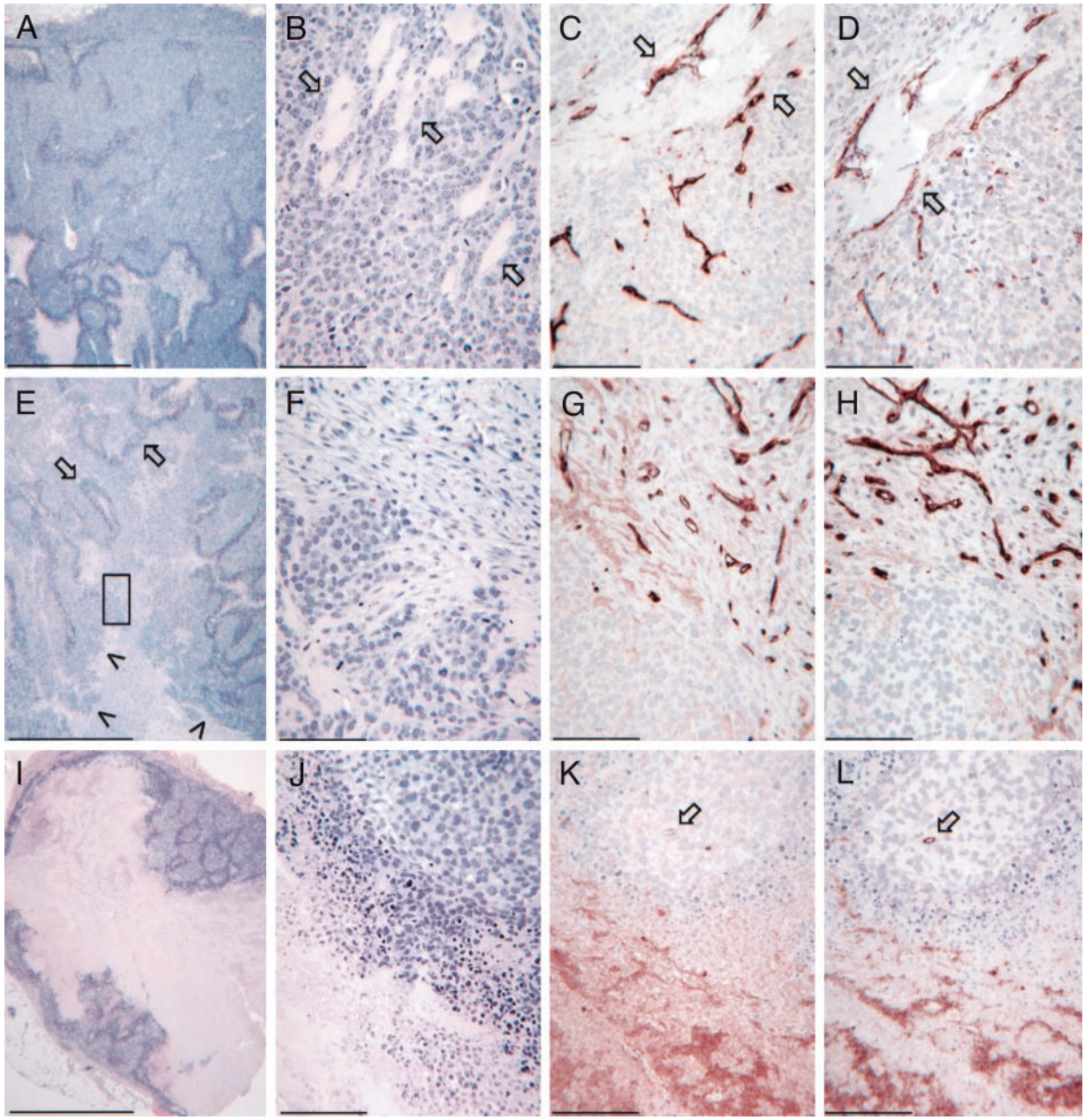

FIG. 8. Complete suppression of tumor angiogenesis requires inhibition of both tumor and stromal VEGF in a nude mouse model of human A673 rhabdomyosarcoma. To test whether the incomplete inhibition of tumor growth achieved with systemic administration of an antihuman VEGF monoclonal antibody is due to incomplete tumor penetration of the antibody or up-regulation of host-derived murine VEGF, systemic administration of the antibody was performed in conjunction with intratumoral administration of an antihuman VEGF Fab (E–H) or mFlt(1–3)-IgG (I–L), which blocks both human and mouse VEGF. A–D, Control antibody. Note the extensive tumor cell necrosis in the mFlt(1–3)-IgG-treated animals (panels I and J), which is not observed in the other groups, although the monoclonal antibody treatment resulted in more than 90% inhibition of tumor growth. Systemic administration of mFlt(1–3)-IgG as a single agent resulted in tumor cell necrosis indistinguishable from that induced by intratumoral mFlt(1–3)-IgG. Note also the nearly complete suppression of vascularization in the mFlt(1–3)-IgG-treated group, as assessed by two vascular-specific markers, Flk-1 (C, G, and K) and CD31 (D, H, and L). *Arrows* in B point to entrapped host-derived elements such as skeletal muscle fibers. *Arrows* in C and D point to microvessels, often present in greater number around host-entrapped elements. *Arrows* in E point to pyknotic areas, staining more deeply than the viable tumor, at the interface between viable and necrotic regions. However, other regions (*box* and *arrowheads*) in E lack such pyknotic changes. *Arrows* in K and L point to a single blood vessel visible within viable tumor, verifying the dramatic reduction in vascularization. [Reproduced with permission from H. P. Gerber *et al.*: *Cancer Res*. 60:6253–6258, 2000 (337).]

genesis in the clinical outcome of patients with CRC. Based on these results, Avastin was approved by the Food and Drug Administration on February 26, 2004 as a first-line treatment for metastatic CRC.

Several additional phase III studies are currently ongoing to fully assess the benefit of bevacizumab and other anti-VEGF therapies, such as small-molecule kinase inhibitors, in patients with advanced cancer.

B. Hematological malignancies

VEGF is expressed in a wide variety of cell lines derived from various hematological malignancies, including T cell lymphoma, acute lymphoblastic leukemia, Burkitt's lymphoma, acute lymphocytic leukemia, histiocytic lymphoma, promyelocytic leukemia, *etc*. (for review see Ref. 354). Expression of both VEGFRs has been detected in some, but not all, leukemia cell lines, and VEGFR-1 was found to be more frequently expressed than VEGFR-2. These findings suggest that the production of VEGF by malignant myeloid precursors might serve both as an autocrine growth stimulus and a diffusible, paracrine, signal-mediating angiogenesis within the bone marrow. The inhibitory effects of small molecule inhibitors targeting VEGFR-1 and VEGFR-2 on the growth of human myeloid leukemia cell lines have been documented (355). Further evidence for a functional role of VEGFR-2 in leukemic cell growth was provided by experiments showing that an anti-VEGFR-2 antibody inhibits proliferation of xenotransplanted human leukemia cells and significantly increased survival of nude mice (356). Recently, Fiedler *et al.* (357) reported a multicenter phase II trial of SU5416, a small-molecule inhibitor of phosphorylation of VEGF receptors, c-kit, the SCF receptor, and FLT3 in patients with advanced acute myelogenous leukemia, and some preliminary evidence of efficacy was evidenced. One patient had a morphological remission lasting for 2 months, and seven patients achieved a partial response. Interestingly, patients with acute myelogenous leukemia blasts expressing high levels of VEGF had a higher response rate and reduction of bone marrow microvessel density than patients with low VEGF expression, consistent with the antiangiogenic effects of SU5416. Taken together, these findings suggest that inhibition of VEGF or VEGFR signaling may be effective in the treatment of hematological malignancies. Currently, several clinical trials are testing this hypothesis.

C. Intraocular neovascular syndromes

Diabetes mellitus, occlusion of central retinal vein, or prematurity with subsequent exposure to oxygen can all be associated with retinal ischemia and intraocular neovascularization, which may result in vitreous hemorrhages, retinal detachment, neovascular glaucoma, and blindness (11, 358). As previously mentioned, in 1948 Michaelson (40) postulated the existence of a diffusible angiogenic factor, released by the ischemic retina. Given its hypoxia inducibility, VEGF became an attractive candidate as a mediator of pathological intraocular neovascularization.

Hypoxia-regulated VEGF release likely plays a key role in the normal development of the retinal vasculature. Stone *et al.* (359) proposed that hypoxia caused by the onset of neuronal activity leads to the release of VEGF by populations of astrocytes, resulting in induction of the superficial and deep layers of retinal vessels. As vessels become patent and perfusion begins, the hypoxic stimulus recedes. Retinal vessels are initially dependent on VEGF as a survival factor (75), but such dependence is lost as soon as capillaries are covered by pericytes, a process mediated by endothelial cell-derived PDGF-BB acting through PDGFRβ (360, 361).

Expression of VEGF mRNA spatially and temporally correlates with neovascularization in several animal models of retinal ischemia (75, 362, 363). Interestingly, down-regulation of VEGF expression by hyperoxia is likely to be, at least in part, responsible for the vasoobliteration and cessation of normal retinal blood vessel growth observed in premature infants in whom retinopathy of prematurity develops (75, 363). The subsequent retinal hypoxia leads to VEGF up-regulation and neovascularization, when normoxia is restored. Administration of VEGF during the hyperoxic phase significantly prevented the vascular regressive changes and the neovascularization (75, 363).

Elevations of VEGF levels in the aqueous and vitreous humor of human eyes with proliferative retinopathy secondary to diabetes and other conditions have been previously described (364, 365). Similar to the animal models, these studies demonstrated a temporal correlation between VEGF elevations and active proliferative retinopathy (364). Subsequently, animal studies using various VEGF inhibitors, including soluble VEGF receptor chimeric proteins (366), monoclonal antibodies (367), antisense oligonucleotides (368), and small molecule VEGFR-2 kinase inhibitors (369), have directly demonstrated the role of VEGF as a key mediator of ischemia-induced intraocular neovascularization. Activation of protein kinase C β 2 isoform has been reported to be important for VEGF-dependent retinal neovascularization (370).

Neovascularization and vascular leakage are major causes of visual loss also in the wet form of AMD, the overall leading cause of blindness (11). Earlier studies demonstrated the immunohistochemical localization of VEGF in surgically resected choroidal neovascular membranes from AMD patients (371), suggesting a role for VEGF in the progression of AMD-related choroidal neovascularization. Whether such VEGF up-regulation is hypoxia related is unclear (372). Currently, anti-VEGF strategies are being explored in clinical trials in AMD patients, using either a recombinant humanized anti-VEGF Fab (rhuFab VEGF) (373) or 2′-fluoropyrimidine RNA oligonucleotide ligand (aptamers) (374). rhuFab VEGF has been recently found to reduce angiogenesis and vascular leakage in a primate model of AMD (375). Both the aptamer and rhuFab VEGF, administered intravitreally, are currently in phase III trials. Most recently, preliminary results of a phase III study with Macugen (Eyetech, Boston, MA) (aptamer) in patients with wet AMD indicate reduced vision loss compared with placebo (C. Puliafito: Abstract Proc. American Academy of Ophthalmology Subspecialty Day - Retina The Retina Debates 2003: New Technology & Controversies from the Posterior Segment). However, the magnitude of the effect did not appear markedly greater than that achieved by photodynamic therapy (Visudyne, QLT, Vancouver, British Columbia, Canada), the only approved treatment for wet AMD. However, Macugen neutralizes only intact $VEGF_{165}$ and does not bind $VEGF_{121}$ or bioactive proteolytic fragments of $VEGF_{165}$ lacking the heparin-binding domain that may be generated after plasminogen activation (374). In contrast, rhuFab VEGF neutralizes all VEGF isoforms and bioactive fragments (373). Whether a more complete VEGF neutralization will translate in greater clinical efficacy remains to be established. For review of anti-VEGF approaches in AMD clinical trials, see Ref. 372.

VEGF is implicated also in the corneal angiogenesis associated with herpes simplex infection (376). After viral infection in mice, VEGF was expressed by stromal as well as by corneal epithelial cells. Administration of a soluble VEGF receptor markedly reduces angiogenesis and severity of lesions associated with the viral infection, suggesting that the control of angiogenesis represents a useful adjunct to therapy of herpetic ocular disease, an important cause of human blindness (376).

D. Inflammatory disorders and brain edema

VEGF up-regulation has been implicated in various inflammatory disorders (for review see Ref. 34). VEGF is strongly expressed by epidermal keratinocytes in wound healing and psoriasis, conditions that are characterized by increased microvascular permeability and angiogenesis (377). Transgenic overexpression of VEGF in the skin results in increased density of tortuous cutaneous blood capillaries and enhanced leukocyte rolling and adhesion in postcapillary skin venules, suggesting that overexpression of VEGF in the epidermis is sufficient to induce features of chronic skin inflammation. Interestingly, no changes in lymphatic vessels were detected in these studies (378). Very recent studies have shown, however, that myeloid cell activation and infiltration, key aspects of acute inflammatory responses, require HIF-1α but are largely independent of VEGF (379).

VEGF has been implicated also in the pathogenesis of RA, an inflammatory disease in which angiogenesis plays a significant role (380, 381). 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 (380, 381). Interestingly, administration of VEGF inhibitors significantly delayed the development of arthritis and decreased clinical score and paw thickness as well as histological severity (382–384). Recent studies have also emphasized the importance of VEGFR-1 signaling in the development of an inflammatory exudate in RA (385–387).

Recently, Reinders *et al.* (388) provided evidence for a role of VEGF as a proinflammatory mediator in allograft rejection. VEGF was found to be functional in the trafficking of human T cells into skin allografts *in vivo* in the humanized SCID mouse. *In vitro*, VEGF enhanced endothelial cell expression of several chemokines and, in combination with interferon-γ (IFN-γ), synergistically induced endothelial cell production of the potent T cell chemoattractant IFN-γ-inducible protein-10 (IP-10). Treatment of BALB/c recipients of fully major histocompatibility complex-mismatched C57BL/6 donor hearts with an anti-VEGF antibody markedly reduced T cell infiltration of allografts and acute rejection (388).

VEGF up-regulation has been also implicated in the development of brain edema. Diffuse, low-abundance, VEGF mRNA expression has been observed in the adult rat brain (389). However, as previously noted, hypoxia is a major trigger for VEGF expression, and enhanced levels of VEGF, together with VEGFR-1 and VEGFR-2, have been reported by several groups in the rat brain after the induction of focal cerebral ischemia (390–392). Because brain edema is a major determinant of morbidity in patients with cerebral ischemia, the hypothesis that VEGF blockade may be beneficial was tested. van Bruggen *et al.* (393) have shown that administration of a soluble VEGF receptor has beneficial effects in a murine model of cortical ischemia, resulting in a significant reduction in the volume of the edematous tissue shortly after the onset of ischemia and in the infarct size measured several weeks later. As previously noted, some members of the src family may mediate VEGF-dependent vascular permeability (111). In this context, Paul *et al.* (394) reported that $src^{-/-}$ mice have reduced brain damage after induction of cortical ischemia and that a src inhibitor has protective effects in wild-type mice in a similar brain injury model. However, other studies have shown that infusion of VEGF itself may have protective effects in similar models, reducing infarct size (395) and even brain edema formation (396) by virtue of its endothelial and possibly also neuronal protective effects. Such conflicting results likely reflect a "double-edged sword" role of VEGF in stroke, such that the timing of administration and the dose of VEGF (or the VEGF inhibitor) will determine whether the treatment will be beneficial or detrimental.

E. Pathology of the female reproductive tract

Angiogenesis is a prominent feature of polycystic ovary syndrome (PCOS), a leading cause of infertility affecting as many as 5–10% of women of reproductive age. PCOS was originally described as a disorder characterized by the association of hirsutism, obesity, reduced fertility, and enlarged, polycystic ovaries (397). Hyperplasia of the theca interna and stroma and excessive production of androgens are hallmarks of PCOS (for review see Ref. 398). Indeed, ultrasonographic assessment of stromal area (399) and blood flow (400) is currently used as a diagnostic test. Although PCOS was described more than 50 yr ago, its etiology has remained largely unclear. However, increased LH/FSH ratio, defective selection of a dominant follicle, and anovulation are considered to be key aspects of the pathogenesis. Recent evidence also indicates that PCOS is part of a complex endocrine/metabolic disorder in which insulin resistance plays a major role (401).

Interestingly, VEGF levels have been reported to be elevated in the serum of PCOS patients compared with normal controls, although the degree of increase varied among different studies, being as little as 25% (402) or approximately 2- to 3-fold (403). *In situ* analysis indicated that both VEGF and EG-VEGF are expressed in all PCOS ovaries examined, but with an almost mutually exclusive expression pattern (288, 404). Somewhat surprisingly, expression of VEGF mRNA is largely limited to the cyst walls, with little or no expression in the stroma. Cysts appear to express VEGF only, EG-VEGF only, or to express VEGF in an inner rim surrounded by an outer rim of EG-VEGF expression. Some VEGF expression was seen in theca interna, although not as consistently as in granulosa cells (Fig. 9). EG-VEGF expression was strongest in theca interna of follicles in various stages of atresia. Importantly, thecal and stromal tissue expressing EG-VEGF maintain an abundant vascular supply, despite lacking significant VEGF expression. Endothelial immunostaining with anti-CD34 demonstrates persistent vas-

cularity in these areas (288). These findings raise the possibility that, whereas VEGF is an essential player in normal cycling ovaries, EG-VEGF might be particularly significant for the acyclical angiogenesis occurring during chronic anovulation. However, tumor studies have shown that even a low, constitutive VEGF expression may be of great pathophysiological significance as assessed by loss of function studies (343). Therefore, studies with specific inhibitors will be crucial to determine the respective roles of VEGF and EG-VEGF in models of ovarian dysfunction.

Previous studies (405, 406) have implicated VEGF also in the pathogenesis of ovarian hyperstimulation syndrome (OHSS), a potentially fatal condition characterized by ovarian enlargement, with multiple follicular cysts and increased vascular permeability (407, 408). PCOS is a well-established risk factor for OHSS (409). However, other studies have cast doubt on the hypothesis that VEGF may be the causative factor in the vascular permeability associated with OHSS and suggested that the kallikrein system is of greater importance (410). Such discrepancies may be due, at least in part, to the fact that although VEGF may be an important mediator in OHSS, it is by itself insufficient, and the symptoms reflect the contribution of other factors, such as EG-VEGF.

Angiogenesis is important in the pathogenesis of endometriosis, a condition characterized by ectopic endometrium implants in the peritoneal cavity. High levels of VEGF have been measured in the peritoneal fluid of patients with endometriosis (411–413). Recently, a model in which human endometrium is implanted into nude mice was used to test the effects of two VEGF inhibitors, a soluble truncated Flt-1 receptor and an antibody to human VEGF. Both agents significantly inhibited the growth of nude mouse explants, suggesting that antiangiogenic agents may provide a novel therapeutic approach for the treatment of endometriosis (413).

According to Maynard *et al.* (414), circulating levels of sFlt-1 derived from placenta are increased in preeclampsia, resulting in reduced free VEGF and PlGF. Thus, endothelial dysfunction of preeclampsia may be due to excess VEGF/PlGF neutralization by circulating sFlt-1. Furthermore, recent studies have indicated that increased levels of sFlt-1 and reduced levels of PlGF predict the subsequent development of preeclampsia (415) .

X. VEGF and Therapeutic Angiogenesis

The development of pharmacological treatments for disorders characterized by inadequate tissue perfusion would fulfill an unmet medical need, as there are no effective alternatives to surgical reconstruction procedures. For example, chronic limb ischemia, most frequently caused by obstructive atherosclerosis affecting the superficial femoral artery, is associated with a high rate of morbidity and mortality, and treatment is currently limited to surgical revascularization or endovascular interventional therapy (416). The hypothesis that "therapeutic angiogenesis" may be beneficial for these conditions generated a high level of enthusiasm in the field of cardiovascular medicine over the last several years and led to several clinical trials.

Early studies indicated that intraarterial or im administration of $VEGF_{165}$ may significantly augment perfusion and development of collateral vessels in a rabbit model of chronic hindlimb ischemia (417). 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 (418, 419). Other studies have shown that VEGF administration also leads to a recovery of normal endothelial reactivity in dysfunctional endothelium (420). Furthermore, adenovirus-delivered $VEGF_{165}$ stimulated an angiogenic response that protected against acute vascular occlusion in the setting of preexisting limb ischemia in a rat model (421). VEGF gene transfer was also reported to prevent the ischemic peripheral neuropathy associated with lower extremity vascular insufficiency in a rabbit model (422). In addition, extraluminal administration of as little as 2 μg of recombinant human VEGF was reported to result in a significant increase in coronary blood flow in a pig model of chronic myocardial ischemia (423). Adenoviral-mediated gene transfer of $VEGF_{121}$ has also been found to result in collateral vessel growth and functional improvement in a porcine model (424).

The hypothesis that VEGF may result in therapeutically significant angiogenesis in humans was initially tested by Isner *et al.* (425) using a gene therapy approach. Arterial gene transfer of naked plasmid DNA encoding $VEGF_{165}$ was reported to result in angiographic and histological evidence of angiogenesis in the knee midtibial and ankle levels 4 wk after the transfer in a single patient with severe limb ischemia. In a subsequent study, the $VEGF_{165}$ cDNA was injected im in 10 limbs of nine patients with nonhealing ischemic ulcers and/or rest pain due to peripheral arterial disease and was reported to improve distal blood flow in several patients (426). The same group also reported that local injection of naked plasmid DNA encoding $VEGF_{165}$ results in a therapeutic effect in patients with myocardial ischemia (427). However, none of these studies were placebo controlled. A relatively large (174 patients) placebo-controlled phase II study in which recombinant human $VEGF_{165}$ was delivered as a single intracoronary infusion, followed by three iv injections, did not demonstrate clinical benefit. The treatment was not superior to the placebo in treadmill time and pain relief, at least at a 60-d assessment, although some improvement in angina class was measured at a later time point (117). This study indicated that the placebo effect is considerably greater than initially suspected and that even patients with markedly compromised myocardial function may show, at least initially, a significant improvement in response to placebo. A major difference between animal models and human patients may lie 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 recombinant protein or gene therapy, whereas patients with extensive atherosclerotic disease may have an impaired response to endogenous and exogenous factors. However, an increase in vascularity was recently reported in a controlled trial with adenovirus-mediated delivery of $VEGF_{165}$ in limb ischemia patients (428).

Currently, several laboratories are exploring the possibility that a more persistent exposure than that achieved in the early trials may achieve better results. In this context, recent

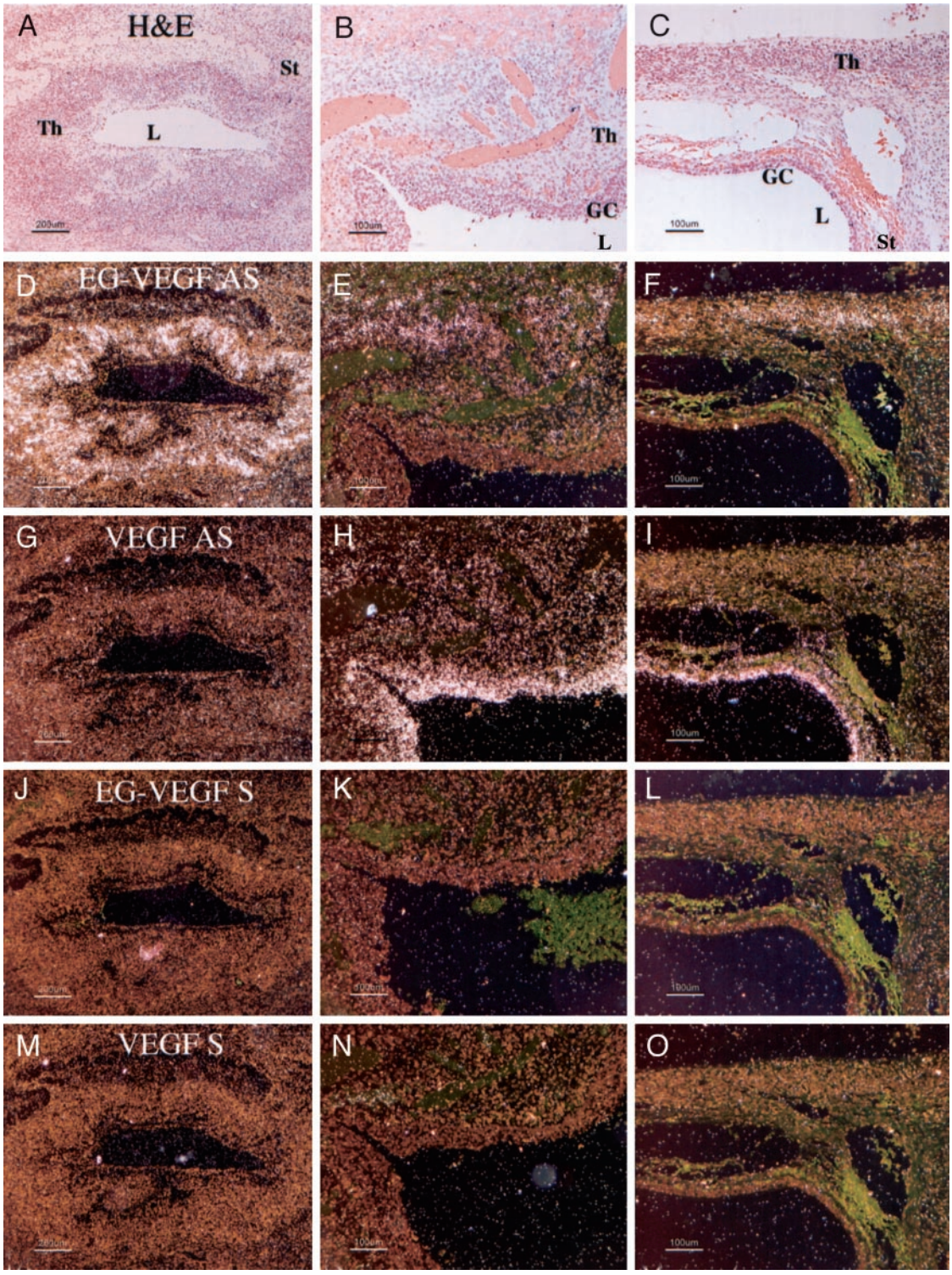

FIG. 9. Distribution of VEGF and EG-VEGF mRNA in parallel sections of cysts in individual PCOS ovaries. Parallel sections were hybridized with EG-VEGF antisense (D–F), VEGF antisense (G–I), EG-VEGF sense (J—L), and VEGF sense (M–O) riboprobes. Hematoxylin and eosin (H&E) images (A–C) are shown for reference. Panels A, D, G, J, and M, Detail of late-stage atretic follicle; EG-VEGF (D) is strongly expressed in theca cells surrounding follicle lumen in which the granulosa cell layer has degenerated. Panels B, E, H, K, and N, Detail of early-stage atretic follicle; VEGF (H) is strongly expressed in granulosa cells surrounding the follicle lumen; some surrounding thecal cells are weakly VEGF positive; EG-VEGF (C) is expressed in clusters of surrounding thecal cells. VEGF (I) is strongly expressed in granulosa cells surrounding lower follicle lumen; the surrounding thin layer of thecal cells are weakly VEGF positive, and EG-VEGF negative; EG-VEGF (F) is expressed in the thecal cells of the upper follicle in which the granulosa cell layer has degenerated. GC, Granulosa cells; Th, theca; St, stroma; L, lumen. *Scale bars* are 200 μm (A, D, G, J, and M) and 100 μm (B, C, E, F, H, I, K, L, N, and O). [Reproduced with permission from N. Ferrara *et al.*: *Am J Pathol* 162:1881–1893, 2003 (288).]

studies using a conditional VEGF switch have shown that early cessation of the VEGF stimulus results in regression of newly formed vessels in the heart or in the liver. However, after a critical duration of exposure, the vessels persisted for months after VEGF withdrawal and resulted in an improvement in organ perfusion (429). Furthermore, a combination of growth factors offers the theoretical advantage of recapitulating at least some of the events leading to the correct assembly of the vessel wall. Coadministration of VEGF and angiopoietin-1 has been proposed to result in more normal and less leaky vessels than those induced by VEGF alone (430). Likewise, delivery of both bFGF and PDGF-BB has been reported to result in the induction of stable vascular networks in a model of limb ischemia (431).

A greater understanding of the differential role of VEGF receptors may open additional avenues. In particular, recent studies have emphasized that VEGFR-1, a molecule with highly complex and apparently conflicting roles, possesses important activities in hematopoiesis and in the recruitment of mononuclear cells. The fact that VEGFR-1 activation is associated with fewer side effects relative to VEGF makes it a particularly attractive target. Furthermore, the recent report that a VEGFR-1 agonist protects the liver from toxic damage, by instructing the quiescent endothelium to produce a series of tissue-specific growth factors, extends the potential clinical applications of VEGFR-1 agonists (217).

Other activities of VEGF may have interesting clinical implications. For example, on the basis of the key role played by VEGF in angiogenesis and endochondral bone formation (258), the application of this factor might be useful to enhance revascularization and healing of fractures and other skeletal conditions (for review, see Ref. 270). Recent studies have shown that both recombinant (266) and adenovirus-delivered (432) VEGF leads to enhanced blood vessel formation and ossification in models of bone damage. Figure 10 illustrates the effects of recombinant VEGF in a rabbit model of segmental gap defect.

XI. Perspectives

The VEGF family clearly plays an essential role in the regulation of embryonic and postnatal physiological angiogenesis processes, such as normal growth processes (83, 260) and cyclical ovarian function (167). Furthermore, VEGF inhibition has been shown to suppress pathological angiogenesis in a wide variety of models, including genetic models of cancer, leading to the clinical development of a variety of VEGF inhibitors. An important question is what impact VEGF inhibition will have in human patients, especially those with highly advanced malignancies. Initial encouraging phase II results were followed by setbacks, such as the lack of efficacy of SU5416 in a phase III study in metastatic CRC in combination with chemotherapy, or the lack of survival benefit in patients with refractory metastatic breast cancer treated with bevacizumab plus chemotherapy as a third-line therapy (433). Most recently, however, a large phase III study has provided unequivocal evidence that VEGF inhibition, using bevacizumab in combination with chemotherapy, may provide a substantial clinical benefit, including increased survival, in patients with previously untreated metastatic CRC (37). Bevacizumab is the first anti-angiogenic agent to be approved by the Food and Drug Administration as a cancer therapy.

It is unclear at present whether such differences in response to bevacizumab reflect a different biology/angiogenic profile between breast and CRC or simply a reduced response in more advanced disease. However, progression eventually occurs in many CRC patients, raising the question of what might mediate angiogenic escape after VEGF inhibition, although one cannot rule out the possibility that a different dosage/regimen of bevacizumab might achieve even greater efficacy. Different angiogenic mechanisms might be differentially important at various stages of the neoplastic progression, and some data suggest that VEGF may be especially important in the initial stages (434). Such a notion may be useful for the design of further clinical trials. The existence of organ-specific angiogenic pathways may be particularly important in some endocrine tumors, in which both VEGF and EG-VEGF are highly expressed (286).

Recent studies have proposed that pericyte recruitment into tumor vasculature, a process dependent on PDGFRβ signaling, is a mechanism of resistance in late-stage tumors to therapies that only target VEGF (435). These findings suggest that combination therapies that target both VEGF

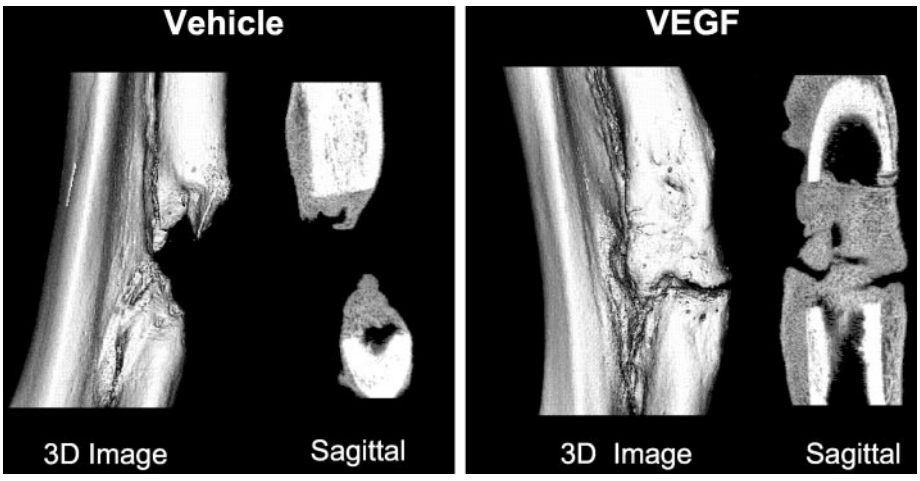

FIG. 10. VEGF promotes bone repair. Three-dimensional (3D) renderings of CT images of radius critical defects at 28 d in vehicle and VEGF (250 μg)-treated rabbits. Segmental defects in rabbit radii were created, and animals were implanted with a pump with various doses of VEGF (0, 50, 100, 250, and 1000 μg) continuously released over the first 7 d after surgery. Consistent with the critical size (10 mm) of these defects, vehicle-treated defects were not able to create a bony bridge across the gap. In contrast, VEGF treatment caused significant filling with bone. VEGF, at 250 μg, caused a 91% increase ($P = 0.02$) in total callus volume and a 95% increase ($P = 0.02$) in calcified callus volume. [Adapted with permission from Street *et al.*: *Proc Natl Acad Sci USA* 99:9656–9661, 2002 (266). © National Academy of Sciences, U.S.A.]

and PDGF may be promising. Furthermore, reliable markers that can predict which patients are more likely to respond to anti-VEGF therapy (or other antiangiogenic treatments) would be of utmost importance, but so far they have been elusive.

The potential clinical utility of VEGF inhibition is not limited to cancer. Trials in AMD patients are already in phase III and, as already noted, initial evidence indicates that the treatment has efficacy. Furthermore, gynecological conditions such as endometriosis or PCOS might also benefit from this approach.

Finally, the recent progress in the molecular and biological understanding of blood vessel growth and differentiation raises hope that a return to human trials for therapeutic angiogenesis may be more rewarding than the early attempts.

Acknowledgments

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