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The biology of VEGF and its receptors

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Vascular endothelial growth factor (VEGF) is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth and reproductive functions. VEGF has also been implicated in pathological angiogenesis associated with tumors, intraocular neovascular disorders and other conditions. The biological effects of VEGF are mediated by two receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2, which differ considerably in signaling properties. Non-signaling co-receptors also modulate VEGF RTK signaling. Currently, several VEGF inhibitors are undergoing clinical testing in several malignancies. VEGF inhibition is also being tested as a strategy for the prevention of angiogenesis, vascular leakage and visual loss in age-related macular degeneration.

The existence of angiogenic factors was initially postulated on the basis of the strong neovascular response induced by transplanted tumors. Subsequently, it was shown that normal tissues are also a source of angiogenic activity. Many molecules have been implicated as positive regulators of angiogenesis, including acidic fibroblast growth factor (FGF), basic FGF, transforming growth factor (TGF)-α, TGF-β, hepatocyte growth factor (HGF, or scatter factor), tumor necrosis factor-α, angiogenin, interleukin (IL)-8 and the angiopoietins[1,2].

For over a decade, the role of VEGF (also referred to as VEGFA) in the regulation of angiogenesis was the object of intense investigation (see ref. 3 for a historic overview of the VEGF field). Recent evidence indicates that new vessel growth and maturation are highly complex and coordinated processes, requiring the sequential activation of a series of receptors by numerous ligands (reviewed in refs. 2,4,5), but VEGF signaling often represents a critical rate-limiting step in physiological angiogenesis. VEGF also seems to be important in pathological angiogenesis, such as that associated with tumor growth[6]. *VEGF* belongs to a gene family that includes placental growth factor (*PLGF*), *VEGFB*, *VEGFC* and *VEGFD*. Homologs of *VEGF* have also been identified in the genome of the parapoxvirus Orf virus and shown to have VEGF-like activities[6,7]. This review focuses on the biology of the prototype member, VEGFA, a key regulator of blood vessel growth. VEGFC and VEGFD regulate lymphatic angiogenesis[8], emphasizing the unique role of this family in controlling growth and differentiation of multiple anatomic components of the vascular system.

Activities of VEGF

A well-documented *in vitro* activity of VEGF is the ability to promote growth of vascular endothelial cells (ECs) derived from arteries, veins and lymphatics (reviewed in ref. 6). VEGF induces a potent angiogenic response in a variety of *in vivo* models[9,10]. VEGF delivery also induces lymphangiogenesis in mice[11]. Although ECs are the primary target of VEGF, several studies have reported mitogenic effects on certain non-EC types (reviewed in ref. 12). Recent studies have also shown that VEGF stimulates surfactant production by alveolar type II cells[13].

VEGF is a survival factor for ECs, both *in vitro* and *in vivo*[14–17]. *In vitro*, VEGF prevents apoptosis induced by serum starvation. Gerber *et al.* have shown that such activity is mediated by the phosphatidylinositol (PI)-3 kinase–Akt pathway[15]. VEGF also induces expression of the anti-apoptotic proteins Bcl-2 and A1 in endothelial cells[14].

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[18]. Furthermore, a marked VEGF dependence has been shown in endothelial cells of newly formed but not of established vessels within tumors[16,17]. Coverage by pericytes has been proposed to be one of the key events resulting in loss of VEGF dependence[16].

VEGF has also effects on bone marrow–derived cells. It promotes monocyte chemotaxis[19] and induces colony formation by mature subsets of granulocyte-macrophage progenitor cells[20]. VEGF delivery to adult mice inhibits dendritic cell development[21] and increases production of B cells and generation of immature myeloid cells[22]. Conditional gene knock-out technology has been used to achieve selective *VEGF* gene ablation in bone-marrow cell isolates and hematopoietic stem cells (HSCs)[23]. VEGF-deficient HSCs and bone-marrow mononuclear cells did not repopulate lethally irradiated hosts, despite the coadministration of a large excess of wild-type cells. These studies also pointed to an internal autocrine loop, not blocked by extracellular inhibitors such as antibodies, by which VEGF controls HSC survival during hematopoietic repopulation[23].

VEGF is known also as vascular permeability factor, based on its ability to induce vascular leakage[24,25]. It is now well established that such permeability-enhancing activity underlies significant roles of this molecule in inflammation and other pathological circumstances. VEGF induces an increase in hydraulic conductivity of isolated microvessels; this effect is mediated by increased calcium influx[26]. Consistent with a role in the regulation of vascular permeability, VEGF induces endothelial fenestration in some vascular beds[27].

VEGF induces vasodilatation *in vitro* in a dose-dependent fashion as a result of endothelial cell–derived nitric oxide[28], and produces tran-

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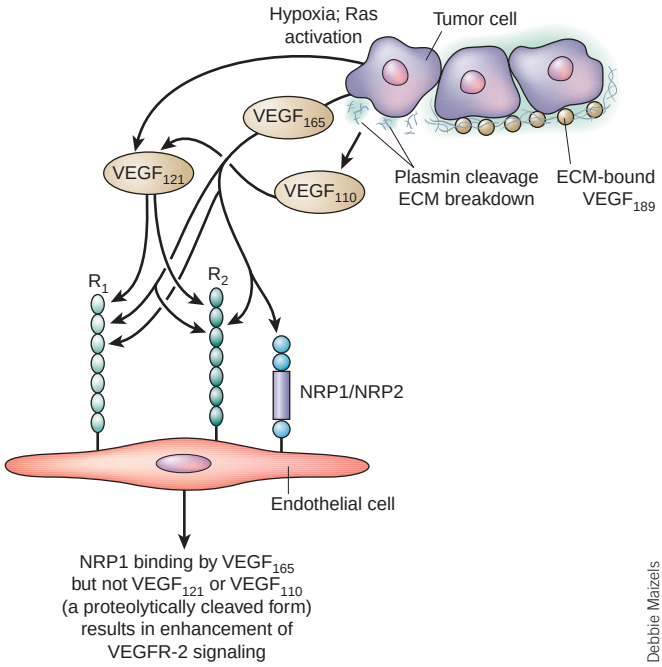

Figure 1 The VEGF isoforms and their interaction with VEGF receptors. In response to a variety of stimuli, the diffusible VEGF isoforms, $VEGF_{121}$ and $VEGF_{165}$, are released by a variety of normal and transformed cells (tumors cells shown) and may bind to VEGFR-1 (R_1) and VEGFR-2 (R_2). $VEGF_{165}$, but not $VEGF_{121}$, also interacts with NRP1 and NRP2. This binding results in enhancement of VEGFR-2-dependent signaling in endothelial cells (EC). Following plasmin generation and extracellular matrix (ECM) breakdown, $VEGF_{189}$ is cleaved at the COOH-terminus and and the resulting 110-amino acid NH_2-terminal fragment is diffusible and bioactive.

sient tachycardia, hypotension and a decrease in cardiac output when injected intravenously in conscious, instrumented rats[29].

VEGF isoforms

The human *VEGFA* gene is organized as eight exons separated by seven introns[30,31]. Alternative exon splicing was initially shown to result in the generation of four different isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$), having respectively 121, 165, 189 and 206 amino acids, after signal sequence cleavage[30,31]. $VEGF_{165}$, 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, such as $VEGF_{145}$ and $VEGF_{183}$ (ref. 7).

Native VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa (ref. 32). The properties of native VEGF closely correspond to those of $VEGF_{165}$ (ref. 33). $VEGF_{121}$ is an acidic polypeptide that does not bind heparin[33]. $VEGF_{189}$ and $VEGF_{206}$ are highly basic and bind to heparin with high affinity[33]. Whereas $VEGF_{121}$ is a freely diffusible protein, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the extracellular matrix (ECM). $VEGF_{165}$ has intermediary properties, as it is secreted but a significant fraction remains bound to the cell surface and ECM[34]. The ECM-bound isoforms may be released in a diffusible form by plasmin cleavage at the C terminus, which generates a bioactive fragment[33]. **Figure 1** summarizes the properties of the VEGF isoforms. Loss of the heparin-binding domain results in a significant loss of the mitogenic activity of VEGF[35]. These findings suggest that $VEGF_{165}$ has optimal characteristics of bioavailability and biological potency. The significance of the heparin-binding VEGF isoform(s) is also emphasized by the finding that 50% of the mice expressing exclusively $VEGF_{120}$ (mouse VEGF is shorter by one amino acid) die shortly after delivery, whereas the remainder die within two weeks[36]. Recent studies have also shown a deficit in the distribution of ECs 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[37].

Regulation of *VEGF* gene expression

Oxygen tension. Oxygen tension has a key role in regulating the expression of a variety of genes. *VEGF* mRNA expression is induced by exposure to low oxygen tension under a variety of pathophysiological circumstances[38]. It is now well established that hypoxia-inducible factor (HIF)-1 is a key mediator of hypoxic responses[39]. Recent studies have uncovered the crucial role of the product of the von Hippel–Lindau (VHL) tumor suppressor gene in HIF-1-dependent hypoxic responses (reviewed in ref. 40). The VHL gene is inactivated in patients with VHL disease, a condition characterized by capillary hemangioblastomas in retina and cerebellum, and in most sporadic clear-cell renal carcinomas. Most of the mitogenic activity of endothelial cells released by renal-cell carcinoma cells expressing a VHL mutant was neutralized by antibodies to VEGF[41]. A function of the VHL protein is to provide negative regulation of VEGF and other hypoxia-inducible genes[42]. HIF-1 is constitutively activated in VHL-deficient renal carcinoma cell lines (43). Other studies showed that VHL is part of a ubiquitin-ligase complex that targets HIF-1 subunits for proteasomal degradation after covalent attachment of a polyubiquitin chain (reviewed in ref. 44). Oxygen promotes the hydroxylation of HIF-1 at a proline residue, a requirement for the association with VHL. Recently, a family of prolyl hydroxylases related to the *Caenorhabditis elegans Egl-9* gene product was identified as the HIF prolyl hydroxylases[44,45].

Growth factors and oncogenes. Several major growth factors, including epidermal growth factor, TGF-α, TGF-β, keratinocyte growth factor, insulin-like growth factor-1, FGF and platelet-derived growth factor, upregulate *VEGF* mRNA expression, suggesting that paracrine or autocrine release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment[6,7]. In addition, inflammatory cytokines such as IL-1α and IL-6 induce expression of VEGF in several cell types, including synovial fibroblasts; this observation is in agreement with the hypothesis that VEGF may be a mediator of angiogenesis and permeability in inflammatory disorders[7]. Specific transforming events also result in induction of VEGF gene expression. Oncogenic mutations or amplification of Ras lead to VEGF upregulation[46,47]. These studies indicate that mutant Ras–dependent VEGF expression is necessary, albeit insufficient, for progressive tumor growth *in vivo*.

The VEGF receptors

Initially, VEGF binding sites were identified on the cell surface of vascular ECs *in vitro* and *in vivo*. Subsequently, it became apparent that receptors for VEGF also occur on bone marrow–derived cells[6]. VEGF binds two related receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2. Both VEGFR-1 and VEGFR-2 have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain[48,49]. VEGFR-3 (fms-like-tyrosine kinase (Flt)-4) is a member of the same family of RTKs but is not a receptor for VEGF, binding instead to VEGFC and VEGFD[8]. In addition to these RTKs, VEGF interacts with a family of coreceptors, the neuropilins. **Figure 2** summarizes the interaction of the members of the VEGF family with their various receptors.

VEGFR-1 (Flt-1). Although Flt-1 was the first RTK to be identified as a VEGF receptor a decade ago[50], the precise function of this molecule is still under debate. Recent evidence indicates that the conflicting reports may be due, at least in part, to the fact that the functions and signaling properties of VEGFR-1 can be different depending on the developmental stage of the animal and the cell type (for example, ECs versus bone marrow cells). VEGFR-1 expression is upregulated by hypoxia by a HIF-1 dependent mechanism[51]. VEGFR-1 binds not only VEGFA but also PLGF[52] and VEGFB[53], which do not bind VEGFR-2. An alternatively spliced, soluble form of VEGFR-1 (soluble Flt-1) is an inhibitor of VEGF activity[54]. The binding site for VEGF (and PLGF) has been mapped primarily to the second immunoglobulin-like domain[55]. Flt-1 undergoes weak tyrosine autophosphorylation in response to VEGF[50,56]. Park *et al.* initially proposed that VEGFR-1 may not be 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 preventing VEGF binding to VEGFR-2 (ref. 52). Thus, the observed potentiation of the action of VEGF by PGF could be explained, at least in part, by displacement of VEGF from VEGFR-1 binding[52]. Such a 'decoy' function could be performed by not only the full-length, membrane-bound form of VEGFR-1, but also by soluble Flt-1[57]. Recent studies have shown that a synergism exists between VEGF and PLGF *in vivo*, especially during pathological situations, as evidenced by impaired tumorigenesis and vascular leakage in $Pgf^{-/-}$ mice[57]. Gille *et al.* identified a repressor motif in the juxtamembrane region of VEGFR-1 that impairs PI-3 kinase activation in response to VEGF[58]. Other studies indicated that VEGFR-1 is able to interact with various signal-transducing proteins and generate, under some circumstances, a mitogenic signal[59].

Gene targeting studies have demonstrated that $Flt1^{-/-}$ mice die *in utero* between days 8.5 and 9.5 (refs. 60,61). ECs develop but do not organize into vascular channels. Excessive proliferation of angioblasts is responsible for the lethality[61], indicating that, at least during early development, VEGFR-1 is a negative regulator of VEGF action. Support for this view also stems from the report that a targeted mutation resulting in a VEGFR-1 lacking the tyrosine kinase domain, but still able to bind VEGF, does not result in lethality or any overt defect in vascular development[62]. The migration of monocytes in response to VEGF, however, requires the tyrosine kinase domain of VEGFR-1 (refs. 62,63). Recently, VEGFR-1 signaling has been linked to the induction of matrix metalloproteinase-9 in lung ECs and to the facilitation of lung metastases[64]. Recent studies have also emphasized the role of VEGFR-1 in hematopoiesis and recruitment of endothelial progenitors. VEGFR-1 activation by PLGF is able to reconstitute hematopoiesis by recruiting VEGFR-1-positive HSCs[65]. In addition, VEGFR-1 activation rescues the ability of $VEGF^{-/-}$ HSCs to repopulate[23]. Luttun *et al.* have shown that PGF promotes collateral vessel growth in a model of myocardial ischemia, through the recruitment of monocytes[66]. LeCouter *et al.* provided evidence for a new function of VEGFR-1 in liver sinusoidal endothelial cells (LSECs). VEGFR-1 activation resulted in the paracrine release of HGF, IL-6 and other hepatotrophic molecules by LSECs, such that hepatocytes were stimulated to proliferate when cultured with LSECs[67]. Such a mechanism protected the liver from toxic damage, in spite of the inability of a VEGFR-1 agonist to induce LSEC proliferation. These findings suggest that a function of VEGFR-1 signaling in the vascular endothelium is to release tissue-specific growth factors, possibly in a vascular bed–specific fashion[67]. **Figure 3** summarizes the differential effects of VEGFR-1 and VEGFR-2 activation in sinusoidal ECs.

VEGFR-2 (also known as kinase domain region (KDR) or Flk-1). VEGFR-2 binds VEGF with a K_d of approximately 75–125 pM (ref. 68). The key role of this receptor in developmental angiogenesis and hematopoiesis is evidenced by a lack of vasculogenesis and failure to develop blood islands and organized blood vessels in *Flk1*-null mice, resulting in death *in utero* between days 8.5 and 9.5 (ref. 69). There is now agreement that VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF.

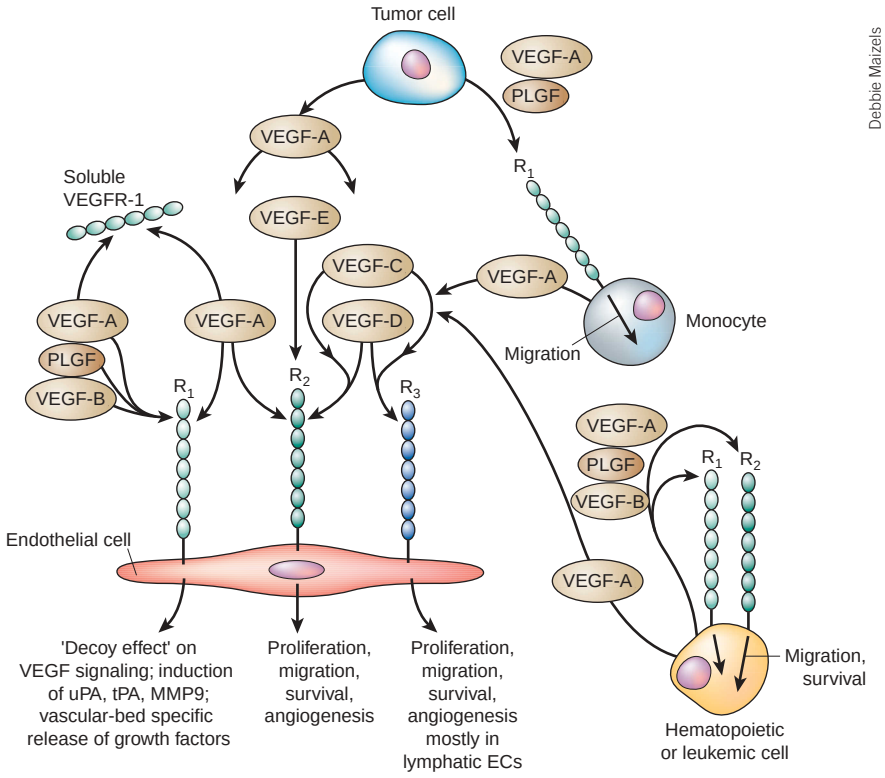

Figure 2 Role of the VEGF receptor tyrosine kinases in different cell types. VEGFR-1 and VEGFR-2 are expressed in the cell surface of most blood ECs. Instead, VEGFR-3 is largely restricted to lymphatic EC. VEGFA binds both VEGFR-1 and VEGFR-2. In contrast, PLGF and VEGFB interact only with VEGFR-1. The orf-virus-derived VEGF-E is a selective VEGFR-2 agonist. VEGFC and VEGFD bind VEGFR-2 and VEGFR-3. There is much evidence that VEGFR-2 is the major mediator of EC mitogenesis and survival, as well as angiogenesis and microvascular permeability. In contrast, VEGFR-1 does not mediate an effective mitogenic signal in EC 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. However, VEGFR-1 has an established signaling role in mediating monocyte chemotaxis. Also, in hematopoietic stem cells (HSC) or leukemic cells, both VEGFR-1 and VEGFR-2 may mediate a chemotactic and a survival signal. R_1, VEGFR-1, R_2, VEGFR-2; R_3, VEGFR-3.

VEGFR-2 undergoes dimerization and ligand-dependent tyrosine phosphorylation in intact cells and results in a mitogenic, chemotactic and prosurvival signal. Several tyrosine residues are phosphorylated (reviewed in ref. 12). VEGF induces the phosphorylation of several proteins in ECs[70]. Among these are phospholipase C-γ, PI-3 kinase, Ras GTPase-activating protein[70] and the Src family[71]. VEGF induces EC 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[72]. VEGF mutants that bind selectively to VEGFR-2 are fully active EC mitogens and permeability-enhancing agents, whereas mutants specific for VEGFR-1 are devoid of both activities[72]. In addition, VEGFR-2 (but not VEGFR-1) activation is required for the antiapoptotic effects of VEGF for human umbilical-vein ECs[15]. As previously noted, such a prosurvival effect of VEGF is mediated by the PI-3 kinase–Akt pathway[15]. Recent studies suggest, however, that at least under some circumstances, VEGFR-1 may transmit a prosurvival signal in ECs, possibly mediated by induction of the antiapoptotic gene survivin[74].

Neuropilin (NRP1 and NRP2). 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[75]. $VEGF_{121}$ did not bind these sites, indicating that exon 7–encoded basic sequences are required for binding to this putative receptor[75]. Subsequently, Soker *et al.*[76] identified this isoform-specific VEGF receptor as NRP1, a molecule that binds the collapsin-semaphorin family and is implicated in neuronal guidance (reviewed in ref. 77). When coexpressed in cells with VEGFR-2, NRP1 enhanced the binding of $VEGF_{165}$ to VEGFR-2 and $VEGF_{165}$-mediated chemotaxis[76]. It has been proposed that NRP1 presents $VEGF_{165}$ to VEGFR-2 in a manner that enhances the effectiveness of VEGFR-2-mediated signal transduction[76]. Binding to NRP1 explains, in part, the greater mitogenic potency of $VEGF_{165}$ relative to $VEGF_{121}$. There is no evidence that NRP1 or NRP2 signal after VEGF binding[77]. In contrast, NRP1 and NRP2 signal axon repulsion in response to semaphorin binding[77]. The role of NRP1 in the development of the vascular system has been shown by gene targeting studies documenting embryonic lethality in *Nrp1*-null mice[78]. NRP1 is also required for vascular development in the zebrafish, where it mediates VEGF-dependent angiogenesis[79].

Role of VEGF in physiological angiogenesis

Embryonic and early postnatal development. In 1996, two studies showed an essential role of VEGF in embryonic vasculogenesis and angiogenesis[80,81]. Inactivation of a single *Vegf* allele in mice resulted in embryonic lethality between days 11 and 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. In contrast, inactivation of *Pgf*[57] or *Vegfb*[82] did not result in any obvious development abnormalities.

VEGF plays an important role also in early postnatal life[18]. Partial inhibition of VEGF achieved by Cre-*lox*P–mediated gene targeting results in increased mortality, stunted body growth and impaired organ development. Administration of a soluble VEGFR-1 chimeric protein, which achieved nearly complete VEGF inhibition, resulted in growth arrest when the treatment was initiated at days 1 or 8 postnatally. Such treatment was also accompanied by rapid lethality, primarily a result of kidney failure[18]. Defective glomerular development in neonates was also observed in studies using antibodies to VEGF[83]. The pivotal role of VEGF in kidney development is also demonstrated in recent studies showing that selective VEGF deletion in podocytes leads to glomerular disease in a manner dependent on gene dosage[84]. Heterozygous mice developed renal disease, characterized by proteinuria and endotheliosis, by 2.5 weeks of age. Homozygosity resulted in perinatal lethality[84].

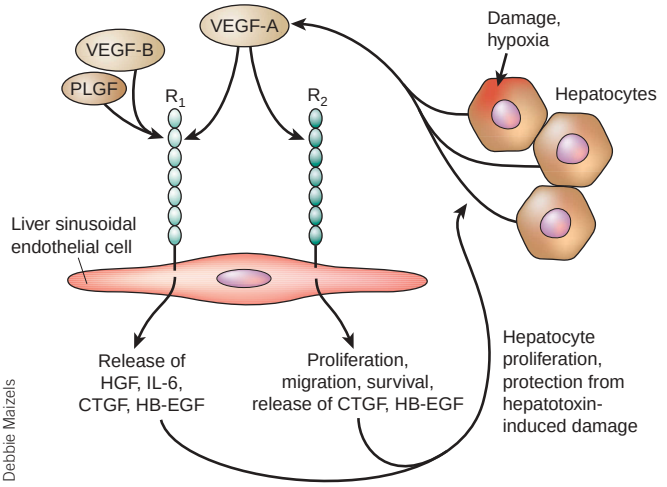

Figure 3 Differential effects of VEGFR-1 (R_1) and VEGFR-2 (R_2) in LSECs. In response to VEGFR-1 activation, LSECs are not stimulate to proliferate but are instructed to up-regulate a series of hepatotrophic genes, including HGF, IL-6 and HB-EGF. Thus VEGFR-1 agonists may result in significant hepatocellular protection from hepatotoxins, without stimulation of angiogenesis. R_2 activation not only mediates LSEC proliferation, migration and survival, but also results in induction of a subset of hepatotrophic genes.

VEGF neutralization in juvenile primates does not result in any significant abnormalities except the suppression of growth-plate and ovarian angiogenesis, as described below[85].

Skeletal growth and endochondral bone formation. Endochondral bone formation is a fundamental mechanism for longitudinal bone growth[86]. *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[87]. After VEGF blockade with a soluble VEGFR-1 chimeric protein or a monoclonal antibody against VEGF, blood vessel invasion is almost completely suppressed, concomitant with impaired trabecular bone formation, in mice and primates[85,87]. Although the 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. Cessation of the anti-VEGF treatment was followed by capillary invasion, restoration of bone growth and normalization of the growth-plate architecture. A similar, though less dramatic, phenotype was obtained when *Vegf* was deleted in the cartilage of developing mice by means of Cre-*lox*P–mediated, tissue-specific gene ablation[88]. Furthermore, examination of $VEGF^{120/120}$ mice showed not only delayed recruitment of blood vessels into the perichondrium, but also delayed invasion of vessels into the primary ossification center, indicating a substantial role of heparin-binding VEGF isoforms at both early and later stages of cartilage vascularization[89].

Ovarian angiogenesis. Follicular growth and the development of the corpus luteum are dependent on the proliferation of new capillary vessels. After these events, the blood vessels regress, suggesting the coordinated action of inducers and inhibitors of angiogenesis[90]. Previous

studies have shown that *VEGF* mRNA expression is temporally and spatially related to the proliferation of blood vessels in the ovary[91]. Administration of VEGF inhibitors suppresses luteal angiogenesis[85,92,93] and delays follicular development[94] in rodents and primates. Recent studies have indicated that endocrine gland–derived VEGF (EG-VEGF), a new selective angiogenic factor, has a cooperative role with VEGF in the regulation of angiogenesis in the human ovary[95]. EG-VEGF is not structurally related to VEGF, but belongs to a unique gene family[96]. A sequential activation of the two genes occurs in the corpus luteum[97]. Although VEGF is strongly expressed in early-stage corpus luteum, its expression is reduced by mid–luteal phase. In contrast, EG-VEGF starts being expressed later than VEGF but persists at least throughout the mid-luteal phases[97].

Role of VEGF in pathologic conditions

Solid tumors and hematologic malignancies. *In situ* hybridization studies have shown that *VEGF* mRNA is upregulated in many human tumors (reviewed in refs. 6,25). In 1993, Kim *et al.* reported that antibodies to VEGF exert a potent inhibitory effect on the growth of several tumor cell lines in nude mice[98]. Subsequently, many other tumor cell lines were found to be inhibited *in vivo* by this and other anti-VEGF treatments, including small-molecule inhibitors of VEGFR signaling, antisense oligonucleotides and antibodies to VEGFR-2 (reviewed in ref. 6). Although tumor cells represent the major source of VEGF, tumor-associated stroma is also an important site of VEGF production[99–101]. Cre-*lox*P–mediated gene targeting has been used to show that VEGF inactivation suppresses tumour angiogenesis in the Rip-Tag model, a well-established genetic model of insulinoma[102]. Furthermore, at least in this model, proteolytic events mediated by matrix metalloproteinase-9 result in enhancement of the activity of low, constitutive VEGF, by making it available to bind VEGFR-2 (ref. 103).

Several studies have shown that combining anti-VEGF treatments with chemotherapy[104] or radiation therapy[105] results in a greater anti-tumor effect than either treatment alone.

Clinical trials in cancer patients are ongoing with several VEGF inhibitors, including a humanized monoclonal antibody to VEGF (rhuMab VEGF)[106], an anti-VEGFR-2 antibody[107], small molecules inhibiting VEGFR-2 signal transduction[108] and a soluble VEGF receptor[109]. Phase 2 clinical data have provided initial evidence that rhuMab VEGF, in combination with conventional chemotherapy, results in increase in time to progression and even survival in patients with metastatic colorectal carcinoma[110]. Thrombosis, hypertension and some proteinuria were among the side effects of such treatment. A randomized, double-blind, placebo-controlled phase 2 trial has shown a highly significant increase in time to progression in renal-cell carcinoma patients treated with rhuMab VEGF as a single agent[111]. In light of the fact that many renal-cell carcinoma patients harbor mutations in the VHL gene, these results are particularly intriguing. Treatment with rhuMab VEGF had modest toxicity in this study, primarily hypertension and asymptomatic proteinuria[111]. Phase 3 studies are currently under way to confirm and fully assess the benefit of these anti-VEGF treatments in patients with advanced cancer.

VEGF is expressed in a variety of cell lines derived from various hematologic malignancies, including multiple myeloma, T-cell lymphoma, acute lymphoblastic leukemia, Burkitt lymphoma, histiocytic lymphoma and chronic myelocytic leukemia (reviewed in ref. 112). Expression of both VEGF receptors has been detected in some leukemia cell lines, and VEGFR-1 is more frequently expressed than VEGFR-2. The inhibitory effects of small molecules targeting VEGFR-1 and VEGFR-2 on human myeloid leukemia cell lines have been documented[113]. In addition, an antibody to VEGFR-2 inhibits proliferation of xenotransplanted human leukemia cells and increases survival of nude mice[114]. Taken together, these findings suggest that VEGF inhibitors may be effective for the treatment of hematological malignancies, and several clinical trials are currently testing this hypothesis.

Intraocular neovascular syndromes. Diabetes mellitus, occlusion of the central retinal vein or prematurity with subsequent exposure to oxygen can all be associated with intraocular neovascularization, which may result in vitreous hemorrhages, retinal detachment, neovascular glaucoma and blindness[115]. All of these conditions are associated with retinal ischemia. Increases in VEGF in the aqueous and vitreous humor of the eyes, with proliferative retinopathy secondary to diabetes and other conditions, have been previously described[116,117]. Subsequent animal studies using various VEGF inhibitors have directly shown the role of VEGF as a mediator of ischemia-induced intraocular neovascularization[118,119].

Neovascularization and vascular leakage are also a major cause of visual loss in age-related macular degeneration (AMD), the overall leading cause of blindness[115]. Earlier studies have shown the immunohistochemical localization of VEGF in choroidal neovascular membranes from AMD patients[120]. Currently, anti-VEGF strategies are being explored in clinical trials in AMD patients, using either a recombinant humanized VEGF-specific Fab (rhuFab VEGF)[121] or 2′-fluoropyrimidine RNA oligonucleotide ligand (aptamer)[122]. Treatment with rhuFab VEGF reduces angiogenesis and vascular leakage in a primate model of AMD[123]. Both the aptamer and rhuFab VEGF are currently in phase 3 trials.

Inflammation and brain edema. VEGF has been implicated in various inflammatory disorders (reviewed in ref. 25). VEGF is strongly expressed by epidermal keratinocytes in wound healing and psoriasis, conditions that are characterized by increased microvascular permeability and angiogenesis[124]. 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. Notably, no changes in lymphatic vessels were detected in these studies[125]. 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[126].

VEGF upregulation has been implicated in the development of brain edema. Enhanced levels of VEGF and its receptors have been reported in the rat brain after induction of focal cerebral ischemia[127]. van Bruggen *et al.* have shown that VEGF antagonism has beneficial effects in a mouse 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[128]. It has been proposed that members of the Src family mediate VEGF-dependent vascular permeability (71). Accordingly, Paul *et al.* have 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[129].

Pathology of the female reproductive tract. Hyperplasia and hypervascularity are features of polycystic ovary syndrome, a leading cause of infertility[130]. Recent studies suggest that VEGF and EG-VEGF may cooperate in the induction of angiogenesis in this condition[97]. Angiogenesis is also important in the pathogenesis of endometriosis, a

condition characterized by ectopic endometrial implants in the peritoneal cavity. Large amounts of VEGF have been measured in the peritoneal fluid of patients with endometriosis[131].

According to recent studies, circulating levels of soluble Flt-1 derived from the placenta are increased in pre-eclampsia, resulting in reduced free VEGF and PGF[132]. Thus, endothelial dysfunction of pre-eclampsia may be a result of excess VEGF or PLGF neutralization by circulating soluble Flt-1.

Therapeutic implications and perspectives

There is now little doubt that the VEGF family has an essential role in the regulation of embryonic and postnatal physiologic angiogenic processes, including normal growth processes[18,87] and cyclical ovarian function[92]. VEGF inhibition inhibits pathological angiogenesis in a wide variety of tumor models, a phenomenon that has led to the clinical development of a variety of VEGF inhibitors. A major question is what impact VEGF inhibition will have in human patients, especially those with highly advanced malignancies. This question will be answered by the various phase 3 clinical trials, targeting colorectal, lung and renal-cell carcinomas, that are currently under way. Initial results indicate that there is at least some reason for optimism. As mentioned, phase 2 clinical trials with VEGF inhibitors have provided evidence of clinical efficacy[110,111]. One study represents the first randomized, placebo-controlled trial to show clinical benefit from an antiangiogenic agent[111]. Progression eventually occurs in many patients, however, raising the issue that there may exist pathways that mediate angiogenic escape after VEGF inhibition. Different angiogenic mechanisms might be differentially important in different tumor types and at various stages of the neoplastic progression[3,96]. Some evidence suggests that VEGF may be especially critical during the initial stages[133]. Such a notion may be crucial for the design of further clinical trials. Furthermore, the identification of reliable markers that can predict which patients are more likely to respond to anti-VEGF therapy (or other antiangiogenic treatments) is of utmost importance.

The potential clinical usefulness of VEGF inhibition is not limited to cancer. Phase 3 trials in AMD patients are already under way. As already noted, gynecologic conditions such as endometriosis or polycystic ovary syndrome might also benefit from this treatment. Indeed, it is conceivable that non-neoplastic conditions will show a greater clinical response, given the reduced likelihood of nontransformed cells to activate alternative angiogenic pathways and thus develop resistance.

The ability of VEGF and other angiogenic factors to promote collateral vessel growth in various animal models of ischemia generated much enthusiasm and led to several clinical trials in patients with coronary or limb ischemia[4]. Thus far, clinical results with VEGF (or basic FGF) have been somewhat disappointing because the treatment did not significantly increase exercise treadmill time, although some improvement in angina class was measured in a placebo-controlled trial with recombinant $VEGF_{165}$ in coronary ischemia patients[134]. An increase in vascularity was reported in a controlled trial with adenovirus-mediated delivery of $VEGF_{165}$ in limb ischemia patients[135]. Currently, several laboratories are exploring the possibility that better results may be achieved by a more persistent exposure than that achieved in an early trial, when only bolus and brief infusions were administered[134]. In this context, recent studies using a conditional VEGF switch have shown that early cessation of the VEGF stimulus results in regression of newly formed vessels. A critical duration of exposure, however, resulted in persistence of vessels for months after VEGF withdrawal, and improved organ perfusion[136]. In addition, a greater understanding of the different roles of the VEGF receptors may open additional avenues. In particular, recent studies have emphasized that VEGFR-1, a protein with complex and apparently conflicting functions, has important roles in hematopoiesis and recruitment of mononuclear cells. This is interesting in light of the fact that most of the side effects of VEGF are related to VEGFR-2 activation. Furthermore, the recent report that a VEGFR-1-selective mutant may protect the liver from toxic damage extends the potential clinical applications of VEGFR-1 agonists[67].

Other activities of VEGF may have interesting clinical implications. For example, on the basis of the key role of VEGF in bone angiogenesis and endochondral bone formation, the application of this factor might be useful to enhance revascularization in non-healing fractures and other conditions. A recent study has shown that VEGF administration leads to enhanced blood vessel formation and ossification in models of bone damage[137].

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

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