Roles for VEGF in the adult

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Roles for VEGF in adult

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Abstract

The role of VEGF during development and in pathology is well known, but its function in normal adult tissues is poorly understood. Adverse effects associated with the use of anti-angiogenic therapies targeting VEGF in human pathologies have begun to reveal potential functions of VEGF in quiescent vasculature. Further clues from expression studies of VEGF and its receptors in the adult, from the disease preeclampsia, and from experimental neutralization studies, have suggested that VEGF is involved in endothelial cell survival and fenestration, as well as in the signaling and maintenance of non-endothelial cells. The various biochemical properties of VEGF, and its interaction with other growth factors, may be an important point in determining whether VEGF functions as a maintenance factor versus an angiogenic factor. A thorough understanding of the function of VEGF in the adult may lead to more efficacious pro- and anti-angiogenic therapies.

Keywords

VEGF; angiogenesis; preeclampsia; anti-angiogenic therapy; bevacizumab; endothelial cells; Avastin; fenestrations

Introduction

Vascular endothelial growth factor (VEGF, VEGF-A or VPF) was first described as a tumor-derived factor with potent ability to induce endothelial cell permeability [1], proliferation and angiogenesis [2,3]. Since its initial discovery, VEGF's action on endothelial cells (EC) has been expanded to include migration and invasion into the basement membrane, proliferation, survival and the formation of fenestrations, which has largely been elucidated using in vitro and in vivo tumor studies. VEGF is biologically active as a homodimer of approximately 40kD, belonging to a family of secreted glycoproteins, including VEGF- B, C, D and placenta growth factor (PlGF). During development, VEGF expression initiates prior to gastrulation [4], and is important in both vasculogenesis, the process by which blood vessels develop de novo from endothelial cell precursors, and angiogenesis, in which blood vessels sprout from existing blood vessels [5,6]. Deletion of either a single or both alleles of the *VEGF* gene in mice results in

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embryonic lethality by E9.5 and E10.5 with severe vascular abnormalities [7,8]. Furthermore, overexpression of VEGF also results in embryonic lethality [9]. These observations underscore the importance of proper regulation of VEGF expression for normal development to occur. VEGF signaling is mediated via two receptors: VEGFR1/Flt1 and VEGFR2/Flk1; homozygous mutation of either of these receptors results in embryonic lethality [10–12]. In addition, two co-receptors for VEGF, neuropilin-1 and 2 (Nrp-1 and Nrp-2) [13,14], are also required embryonically [15,16]. The relative contributions of the various VEGF receptors on VEGF signaling in different endothelial cell beds are still not well understood.

While extensive research has shown that VEGF is crucial for developmental, physiologic and pathologic angiogenesis, whether it is required in the adult is not well understood. Early studies of genetic *VEGF* targeting [8,17] and neutralization studies in tumorigenic mice [18] suggested that VEGF is not required in the adult as these adult mice showed no obvious phenotype, making it an attractive target in many diseases. As a result, a wealth of pharmacologic agents have been designed to target either VEGF or its receptors. Though beneficial effects have been observed, the presence of consistent significant side effects suggests that VEGF is important in the maintenance of quiescent vasculature and non-vascular tissues. This review discusses the expression of VEGF and its receptors in adult tissue, VEGF association with fenestrated vasculature, VEGF effects on non-vascular cells, conditions and factors that may affect VEGF action, and implications for manipulating VEGF in disease treatment.

VEGF expression in the adult

VEGF is robustly expressed embryologically, and is critical for proper blood vessel formation, but its expression is also important in the adult in mediating physiologic angiogenesis during the female reproductive cycle in the uterus, ovary and breast [19,20], in wound healing [21, 22], in bone repair [23] and in skeletal muscle in response to exercise [24]. VEGF is also upregulated and involved in pathophysiologic processes such as in rheumatoid arthritis [25, 26], psoriasis [27], atherosclerosis [28], amyloid lateral sclerosis (ALS) [29], age-related macular degeneration (AMD) [30,31], diabetic retinopathy [32,33], retinopathy of prematurity [34], sepsis [35] and tumor angiogenesis [36,37]. Although VEGF is required for postnatal processes that involve angiogenesis, little is know about its potential role in quiescent vascular beds.

Some insight into VEGF's potential role in the adult comes from the analysis of its expression pattern. VEGF is expressed in virtually every tissue in the adult [38–47]. A systematic study of VEGF expression in the adult using *VEGF-lacZ* mice [46], revealed that specific subsets of cells in each tissue express VEGF. Three general patterns of expression were observed, which may provide information to the role of VEGF in those tissues (Table 1). In regions with sparse cellular VEGF expression, VEGF was expressed primarily by pericytes and vascular stromal cells. During blood vessel maturation, pericytes are differentiated from mesenchyme and become tightly associated with EC, an event associated with blood vessel maturation. Coincident with their differentiation, pericytes begin to synthesize VEGF, which mediates, at least in part, capillary stabilization [48]. In tissues including the retina (Figure 1a), brain and testes, the vasculature is characterized as having barrier function (retinal-blood barrier, blood-brain barrier, testis-blood barrier); interestingly, relatively few cells express VEGF in these organs, and it is possible therefore that the relatively low expression of VEGF contributes to the impermeability of these vessels. In addition to its putative role as an endothelial survival factor, there is recent evidence to support a role for VEGF as a tropic factor for retinal neurons [49].

In the tissues with an intermediate number of cells expressing VEGF, such as in cardiac and skeletal muscles, most myocytes appeared to express VEGF (Figure 1b). As these tissues are

physiologically dynamic and have the capacity to hypertrophy with training, VEGF may act to support the rich vasculature of the muscle [50, 51], depending on the physiologic requirement of the muscle.

The highest density of VEGF expressing cells was found in tissues with fenestrated vasculature and was localized to the epithelia in contact with fenestrated vessels. Tissues, whose microvasculature is fenestrated, have secretory and/or filtration functions, for example the glomerulus (urine filtrate), choroid plexus (Figure 1d) (cerebrospinal fluid production), pancreas (exocrine and endocrine secretions) and liver (filtration and secretion). Thus, VEGF in these tissues may mediate both survival and maintenance of the fenestrated state of the EC.

Though ECs are the main target of VEGF, they do not generally express VEGF [52], but instead respond to VEGF secreted in a paracrine manner. It is speculated that expression of both the receptor and ligand in EC would lead to a dangerous positive feedback, resulting in overproliferation, such as is observed in hemangiomas [53] and in tumorigenesis [54]. One exception to this appears to be in the largest blood vessel, where aortic EC, but not inferior vena cava EC, express VEGF (Figure 1e, f, respectively)[46]. The mechanism or function of VEGF expression in the aortic EC is currently unknown.

Although VEGF binds to VEGFR1, VEGFR2, Nrp-1 and Nrp-2, its main signaling receptor in the endothelium is VEGFR2. VEGFR2 belongs to the family of receptor tyrosine kinases [55] (reviewed in [56]), and upon VEGF binding, there is dimerization and activation of the tyrosine kinase, resulting in phosphorylation of specific tyrosine residues on the cytoplasmic tail, which in turn promotes docking of signal transducing molecules. Several key tyrosine residues have been identified, and activation of specific residues leads to different downstream effects. Of these residues, Tyr 1175 is implicated in endothelial survival and permeability, though not exclusively [57], through the PI3-K/AKT pathway [56,58]. Signaling via residue Tyr 951 is also involved in vascular permeability [59]. Although VEGFR1 is also expressed by EC, it is believed to act primarily to modulate VEGFR2 signaling. Evidence for this concept comes from observations that although targeted disruption of VEGFR1 signaling results in EC overgrowth and vascular disorganization [10,60], mice in which the intracellular kinase domains of VEGFR1 was deleted have no obvious vascular phenotype [61]. Recent evidence has suggested that VEGFR1 can anchor VEGF to the cell-surface, allowing increased interaction with VEGFR2 [62].

For VEGF to exert an effect in the adult, its receptor also has to be expressed in close proximity to the VEGF source. Several recent reports have demonstrated that VEGFR2 is expressed and activated in adult tissue [46,47,63,64] (Figure 1g, h), giving further evidence that VEGF plays a biologic role in the adult.

Implications of the role of VEGF in the adult from clinical observations

A. Side effects from clinical systemic VEGF neutralization

All cells require oxygen, and due to the limits of oxygen diffusion, cells more than 100 μm away from blood vessels become oxygen deprived. For this reason, solid tumors are restricted to a size of 3–4 mm in diameter without neovascularization. The discovery of a tumor-secreted, angiogenic factor [65], which was later identifed as VEGF [3], and was hypoxia-inducible [66], led to the concept that neutralization of VEGF could block tumor-induced blood vessel growth, thus inhibiting tumor growth and metastasis [67]. Early reports suggested that VEGF was not important in microvascular homeostasis [8,17], and thus VEGF became an attractive target for anti-angiogenesis; it was believed that VEGF neutralization would specifically target the tumor microvasculature without affecting non-diseased vascular beds, thus circumventing side effects associated with standard chemotherapy. This model has now been also applied to

other VEGF-mediated diseases, and has led to the implementation of therapies for ocular vascular proliferative diseases as well [68].

As an anti-tumor agent, clinical development of many agents that target either VEGF or its receptors (see [69] for a detailed list of agents) are in progress. The first FDA-approved anti-VEGF therapy, bevacizumab (Avastin™, Genentech) was introduced into the clinics in 2004 in combination with Irinotecan, fluorouracil, or leucovorin for the treatment of metastatic colorectal cancer (mCRC) [70] and more recently for the combination treatment of advanced non-small-cell lung cancer [71]. Subsequently, Avastin is now being clinically tested as a mono- or combination therapy agent for the treatment of cancers in almost every organ system [72], with many in either Phase III or IV. Clinical positive effects of Avastin treatment in mCRC and lung cancer include a marked tumor regression and increase in median survival as well as improvement in long-term survival in some patients [73].

Common side effects of Avastin treatment have been noted including hypertension, proteinuria, bleeding and impaired surgical wound healing [70,71,74]. Infrequent, but life-threatening complications have been reported as well, including, arterial thrombosis [75,76], gastrointestinal perforation [77], and reversible focal posterior leukoencephalopathy (RFLP) [78–80] (Figure 2).

In addition to targeting the VEGF ligand, another approach of anti-VEGF therapy is to target its receptors [81]. Several drugs targeting VEGF receptors have been FDA approved, including sunitinib (Sutent™, Pfizer) for the treatment of advanced renal cell carcinoma [82,83] and gastrointestinal stromal tumors [84] and sorafenib (Nexavar, Bayer/Onyx Pharmaceuticals) for the treatment of clear-cell renal cell carcinoma [85]. Side effects similar to those noted with Avastin have been reported; however, these drugs have several additional side effects probably due to the effects on other receptor tyrosine kinases. Although these drugs selectively block VEGFR2 and VEGFR1, there is significant inhibition of other receptors as well, due to the homology of these receptors, including VEGFR3, PDGFRs, and receptor kinases associated with KIT, and RET receptor kinases [84,85].

The reproducible side effects related with these drugs indicate that VEGF neutralization in quiescent tissue does in fact influence tissue function. Furthermore, the incidence and severity of side effects associated with VEGF blockage may provide insight to VEGF-sensitive tissues and VEGF-dependent processes in the adult.

B. VEGF neutralization in the pathogenesis of preeclampsia

Recent evidence from the disease preeclampsia has also begun to provide evidence concerning the role of VEGF in the adult. Preeclampsia (reviewed in [86]), a disease of pregnancy, affects 5–7% of women in their 2nd and 3rd trimester, and is marked by hypertension, edema and proteinuria. The pathology is caused by systemic endothelial dysfunction, and if left untreated, ascites, pleural edema, thrombocytopenia, headaches, disseminated intravascular coagulation, and blindness can occur. In severe cases, there is progression to eclampsia, characterized by seizures, and other neurologic manifestations including strokes.

Because delivery of the placenta results in immediate improvement and cessation of most symptoms, it was hypothesized that the placenta was the source of the insulting agent. Several factors were found to be over expressed by preeclamptic placentas, including soluble VEGFR1/sFlt1, which is found at high levels in the blood of preeclamptic mothers [87–89]. sFlt1 is produced by alternative splicing of the *Flt1* transcript, resulting in a deletion of the intracellular and transmembrane domains of Flt1, rendering the truncated protein soluble. sFlt includes 85% of the extracellular domain, plus 30 amino acids from the 13th intron [90]. sFlt1 binds VEGF with an affinity 10-fold greater than VEGFR2, thus acting as a soluble trap of VEGF [91,92].

sFlt1 can also heterodimerize with VEGFR2 acting in a dominant negative manner [93]. Systemic administration of sFlt1 to pregnant rats recapitulates several, but not all symptoms of preeclampsia, including hypertension and proteinuria [87]. In addition to proteinuria and hypertension, reversible posterior leukoencephalopathy, a severe complication of preeclampsia, has also been seen in patients on Avastin [94]. The parallel between these hallmarks of preeclampsia and the side effects of Avastin support the concept that VEGF is involved in vasculature maintenance. Although hypertension, which is common to both preeclamptic and Avastin patients, has been suggested to be the basis of the leukoencephalopathy, it has also been suggested that primary insult to brain endothelium may also contribute to this condition. However, this has not been studied, and remains an interesting, unanswered question.

Role of VEGF in the adult

A. Vascular stability

While therapeutic VEGF neutralization and preeclamspia have revealed potential roles for VEGF in the adult, experimental studies in rodents have begun to unravel the nature of VEGF function in quiescent tissues. Inhibition of VEGF or its receptors has been shown to lead to alteration of the microvasculature as well as vessel regression in a number of tissues. In the kidney this resulted in glomerular endotheliosis and proteinuria (Figure 3c, f) [87, 95], whereas in the lung alveolar apoptosis and enlarged airspaces were observed (Figure 3a, d) [96, 97]. Vessel regression was also noted in the pancreas [98, 99], trachea [98, 100], thyroid (Figure 3b, e) [98], and small intestine [98]. Interestingly, there were no apparent alterations in the microvasculature of heart, musculature of the tongue, or brain [98]. In addition to microvascular regression, hypertension was a common finding, and may be the direct result of disrupting vascular NO-mediated tone [87, 101]. Alternatively, it may be secondary to kidney injury or to destruction of microvasculature (rarefaction), resulting in increased systemic resistance.

The blood vessel regression and tissue dysfunction observed in these experiments are presumably due to interference with the trophic effect of VEGF on endothelial survival [100, 102]. The cascade of events likely begins with local thrombosis and decreased vascular perfusion, leading to endothelial cell apoptosis [102]. Basement membrane "sleeves" with pericytes devoid of EC [98] persist for a period of time, and can be recanalized by EC if neutralization is reversed. Neutralization of VEGF for seven days followed by seven days of reversal resulted in almost complete revascularization [103]. That short inhibition of VEGF may result in regrowth of vessels following cessation of VEGF inhibition, may provide a challenge in anti-VEGF therapies. Conversely, the vestigial presence of the basement membrane ghost in tissues such as infarcted brain and heart tissue, may allow targeted delivery of VEGF to efficiently stimulate new blood vessels.

B. Effects of VEGF on non-vascular cells

VEGF, like other growth factors (e.g. hepatocyte growth factor and fibroblast growth factor) was so named because of initial observations demonstrating EC as its target. However, VEGF may act on other cell types based on the presence of either VEGFR1 or VEGFR2 on those cells. In addition, several reports have demonstrated the action of VEGF on monocytes [104, 105], macrophages [106], mast cells [107], eosinophils [108], dendritic cells [109], megakaryocytes [110], lymphocytes [111], hematopoeitic stem cells/bone marrow-derived circulating cells [112–115], type II lung alveolar epithelial cells [116] and lens epithelium [117]. VEGF has been shown to mediate the survival of hematopoeitic stem cells, neuronal cells, neuronal stem cells and lymphocytes; the differentiation of megakaryocytes and dentritic cells; and, to mobilize bone marrow precursor cells. In addition to its direct effects on

endothelial cells during angiogenesis, VEGF also influences angiogenesis via recruitment of bone marrow endothelial cell precursors and inflammatory cells.

Observations from the therapeutic use of anti-VEGF, as well as from preeclampsia, also provide evidence regarding the action of VEGF on non-endothelial cells. Findings of thrombocytopenia and neutropenia [69,74,118] are consistent with a role for VEGF in the regulation of platelet and leukocyte production. Gastroperforation and impaired wound healing likely result from impaired immune cell function and angiogenesis.

VEGF is also involved in lymphangiogenesis [119–121]. Although its function in lymphatic maintenance is unknown, edema in preeclamptic patients [86] may be due, at least in part, to lymphatic destruction besides decreased osmotic pressure, and warrants further study.

In addition to its action on vascular and immune cells, increasing evidence points to a role for VEGF in neuronal growth and survival. VEGF acts on neuronal cells [49,122,123] and neuronal stem cells [124] in vitro and in vivo. In the eye, both neuronal and glial cells express VEGF receptors [125]; addition of VEGF120 following ischemic/reperfusion injury resulted in neuroprotection of retinal ganglion cells and of cells in the inner nuclear layer (Figure 4) [126]. Pathologically, low levels of VEGF are associated with motor neuron degeneration [127], and are implicated as a modifier in amyotrophic lateral sclerosis in humans and mice [29,128] and Alzheimer's disease in humans [129]; experimental delivery of VEGF acts to prolong neuron survival in the ALS mice [130]. Although not examined experimentally, peripheral neuropathy associated with Avastin, reversible focal posterior leukoencephalopathy in both Avastin-treated and preeclamptic patients, as well as seizures in eclamptic patients, are consistent with a neuro-protective role for VEGF [131].

C. VEGF and fenestrations

VEGF was first identified as a potent vascular permeability factor [1]. Permeability is important in many physiologic processes such as in glomerular filtration, cerebrospinal fluid production, liver blood filtration, and endocrine secretion into the blood stream [132], is central to wound healing [21], and exacerbates tumors by promoting ascites and edema. Endothelial cell permeability is mediated via both VEGFR1 and VEGFR2 [56], resulting in the formation of transcellular gaps, vesiculovacuolar organelle formation, and fenestrations [133]. Fenestrae are endothelial cell plasma membrane specializations that appear as circular discontinuities approximately 60 nm in diameter and facilitate movement of particles in and out of the circulation. Fenestrated EC are found in most endocrine tissues [132] as well as in the kidney glomerulus, choroid of the eye, choroid plexus, gastrointestinal tract and in tumor vessels. In each of these tissues VEGF expressing cells are found in close juxtaposition to the underlying fenestrated microvasculature (Figure 1d) [46].

VEGF has been shown to be involved in the induction of fenestrae formation in vitro [134–136], but the mechanism of fenestrae formation and maintenance in the adult is not well understood. One link that has been made between VEGF and fenestrae is the regulation of the caveolar protein plasmalemmal vesicle-associated PV-1 protein [137] [138]. However, not all fenestrations are diaphragmmed, so PV-1 may not be the only mediator of VEGF and fenestrations.

Although all fenestrated tissue contain VEGF expressing cells, not all tissue with VEGF expressing cells are fenestrated. There are several possible explanations for this observation. Although VEGF may be important for inducing and maintaining fenestration, other factors may be required along with VEGF for fenestrations. For instance, leptin has been shown to act synergistically with VEGF to induce fenestrations [139]. Whether leptin is expressed in all fenestrated tissues, is to be determined. These 'co-factors' may not be present in all tissue, even

if VEGF is present, thus not allowing fenestrations. Conversely, some tissues may even produce factors to prevent permeability [140]. Another likely possibility is that the induction of fenestrations may simply be a dosage effect, with tissues that display fenestrated capillaries expressing higher levels of VEGF than tissues without fenestrated endothelium.

Several studies have demonstrated that VEGF neutralization in tumors in vivo leads to a diminution of fenestrations [102]. Tumors are often edematous, and it has been postulated that high interstitial pressure may make the delivery of chemotherapy to tumor cells inefficient. Therefore, reducing tumor vessel permeability may have therapeutic advantages in cancer treatment, allowing normalization of tumor vasculature and more efficient delivery of chemotherapy [69,141]. However, systemic VEGF neutralization has also been reported to affect non-tumor fenestrated vasculature, including the thyroid, pancreas and glomerulus [98].

Biochemical, molecular and cellular factors affecting VEGF signaling in vivo

Although VEGF is constitutively expressed in the adult, and is a potent angiogenic factor, it does not result in widespread angiogenesis in resting tissue. The discovery of multiple pro-angiogenic and endogenous anti-angiogenic factors [142] with overlapping expression patterns indicates that angiogenesis is dependent on a balance between pro- and anti- angiogenic factors. In addition to countering the action of local angiogenic factors in quiescent tissue, endogenous factors maintain the avascularity, for example, chondromodulin-I acting in cardiac valves [143] and sFlt-1 in the cornea [144,145]. VEGF signals via several receptors and co-receptors, and via multiple signaling pathways [56,146]. Thus, whether VEGF signaling leads to permeability, proliferation, migration, or survival, may therefore depend on the signaling pathway components, effective tissue concentration as well as on the action of other factors.

VEGF expression in the adult is cell-type specific [45,46] and is controlled at many levels from transcription [147] to translation [148], and is upregulated in tumors and in various pathologic states. One of the best-characterized stimuli of VEGF transcription is hypoxia, which acts by stabilization of the hypoxia-inducible factor-1 alpha (HIF1α) transcription factor [149]. Hypoxic regulation of VEGF also takes place post-transcriptionally via mRNA stabilization [150](reviewed in [151]). VEGF expression is induced by other growth factors and cytokines including IGF-1, Il-6, Il-1, PDGF, TNF-α, TGF-β and FGF-4 [152,153]. In addition, VEGF expression is also stimulated by physical forces, including stretch [50,154], with one putative transcription factor being the Kruppel like factor-2 [155]. Analysis of the *VEGF* promoter reveals many other potential transcription factor responsive elements [149], of which several pathways have been elucidated, for example EGF [156] and HGF signaling [157] via the SP1 responsive element (for an extensive list see [149]).

Alternative splicing of *VEGF* mRNA results in various isoforms, which include VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206, in humans and VEGF120, VEGF164 and VEGF188 in mice. These isoforms display tissue-specific patterns of expression [45]. Studies of genetically engineered mice expressing only one VEGF isoform indicate that VEGF isoforms have distinct yet some overlapping roles in vascular development and function as evidenced by tissue-specific vascular defects in these mice [158]. The VEGF isoforms display differences in their biochemical properties, including receptor binding with VEGF165 and VEGF188 but not VEGF120 binding to neuropilins and heparan sulfate. The differential affinity to heparan sulfate is important in their binding to VEGFR1 and VEGF2 as heparan sulfate can mediate the binding and transactivation of these receptors [159]. Furthermore, differential binding to heparan sulfate is reported to lead to different VEGF actions, including endothelial cell survival, adhesion and vascular branch formation [160,161]. Both VEGF164 and VEGF188 bind heparan sulfate, making them partially or fully cell-bound, respectively,

whereas VEGF120 does not bind heparan sulfate, and is freely diffusible [162]. A newly identified splice variant of VEGF, VEGF165b, is postulated to have an inhibitory effect on angiogenesis [163].

VEGF, a secreted glycoprotein, is subject to a variety of extracellular modifications that have direct effects on its biochemistry and bioavailability. In addition to alternative splicing, VEGF variants arise from proteolytic processing (reviewed in [21]), which leads to differences in biologic effects. For instance, cleavage of VEGF189 by urokinase, allows it to more potently bind to VEGFR2 [148]; plasmin digestion of VEGF165 results in two fragments, one with and one without a heparin binding domain [164], while cleavage of VEGF by various metalloproteinases results in different cleavage products with different outcomes on vascular morphogenesis [165].

VEGF exerts its action via binding to VEGFR1, VEGFR2, Nrp-1 and Nrp-2. Whereas binding of VEGF to Nrps enhance VEGF's action [166], binding to VEGFR1 results in a diminution of VEGF availability. Therefore, it is possible that the tissue ratios of VEGFR1:VEGFR2 determine the tissue's angiogenic status. For instance, under hypoxic conditions, when VEGF is upregulated, concomitant upregulation of VEGFR1 [167], may act to modulate VEGF signaling.

Implications for therapeutic manipulation of VEGF

The role of VEGF in development and pathology is well known and emerging evidence points to a role for VEGF in the maintenance of quiescent tissues. Knowledge of adverse-effects associated with anti-VEGF therapy, observations from preeclampsia, and experimental blockade of VEGF in animal models have exposed tissues and vascular beds sensitive to VEGF neutralization and revealed potential limitations of current anti- therapy. One of the most frequently observed side effects of VEGF neutralization is hypertension [74,87]. However, this issue can be pharmacologically managed, allowing continued patient treatment. Similarly, the knowledge of increased risks for other side effects such as impaired wound healing, gastrointestinal perforation, and arterial thrombosis allows careful monitoring of the patient to avoid serious consequences.

In addition to observations from experimental and clinical observations of VEGF, important information about additional roles for VEGF can be garnered from clinical observations in which low VEGF levels are associated with tissue dysfunction, including observation of alopecia in humans with loss of VEGF in hair follicles [168], decreased VEGF in the aged cochlea [169], and reduced VEGF associated with thyroid cartilage ossification [170]. Another interesting recent report that warrants careful clinical monitoring is an association of increased erythropoiesis with VEGF neutralization [171], as polycythaemia can lead to high blood viscosity and an increased risk for thrombosis. As studies reveal other functions of VEGF in the adult, clinicians should be able to incorporate this information into their clinical follow-up.

Understanding side effects and biologic effects of VEGF neutralization can also provide useful surrogate markers of therapeutic efficacy. Some of the markers currently being investigated include protein levels in plasma (e.g. VEGF) [172,173] and urine [174], circulating endothelial progenitor cells [175,176], and wound healing time [177].

Systemic side effects may also be minimized by local or targeted delivery of anti-VEGF therapies. VEGF-A is a major mediator of pathologic angiogenesis in the eye and is associated with diseases such as age-related macular degeneration (AMD) [31] and proliferative diabetic retinopathy [33]. As in tumors, VEGF is upregulated in these ocular diseases and is, thus, a useful potential therapeutic target (reviewed in [30,68]). Two VEGF inhibitors, pegaptanib

(Macugen™; OSI/Pfizer) [178] and ranibizumab (Lucentis™, Genentech) [179] have been approved by the FDA for intra-ocular use in AMD, and Avastin has been utilized intraocularly off-label for the treatment of AMD as well [180]. For this approach however, a primary consideration will be potential effects that may arise from high local delivery. As stated in Section B, VEGF is involved in lens signaling [117], choroid maintenance [47] and retinal neuronal maintenance [125].

As these drugs are delivered intravitreally, the potential for systemic side effects should be minimized, and have indeed resulted in minimal systemic side effects [68]. However, these medications appear to have the potential to inhibit VEGF systemically as intraocular injections of Lucentis in Rhesus monkeys have resulted in systemic serum levels of approximately 150 ng/ml following bilateral injection of 500 μl of the drug with a half life of 3.5 days [181]. This circulating level may be able to sequester plasma VEGF and act locally in tissue, and may be causal for increased incidences of arterial thromboembolic events in patients treated with Lucentis [182].

A better understanding of the role of VEGF in the adult may also lead to therapies aimed at stimulating blood vessel growth or tissue growth in damaged tissue. Pro-angiogenic therapy using VEGF may be useful in ischemic diseases such as stroke [183], myocardial ischemia and coronary artery disease [184–186], peripheral neuropathy [187], wound [21] and fracture healing [188], Alzheimer's disease [129] and ALS [122,123]. However, delivery of VEGF will have to be approached carefully to avoid a systemic VEGF increase [189] because shifting the balance between endogenous angiogenesis inhibitors and stimulators may lead to aberrant vessel growth and/or exacerbation of existing diseases. Support for this comes from studies in which overexpression of VEGF in mouse podocytes leads to collapsing glomerulopathy [95], and in which VEGF overexpression in the skin results in inflammation [163]. In clinical trials using VEGF therapy, lower extremity edema and hypotension was observed [190], likely attributable to increased vascular permeability.

The manipulation of VEGF has allowed for therapies that lead to significant improvement in survival in cancer, vision in AMD, cardiac function in heart disease, and is currently in clinical and experimental trials in the treatment of many more diseases. Elucidating the role of VEGF in the adult should permit the development of strategies that effectively target the disease, while minimizing side effects and morbidity.

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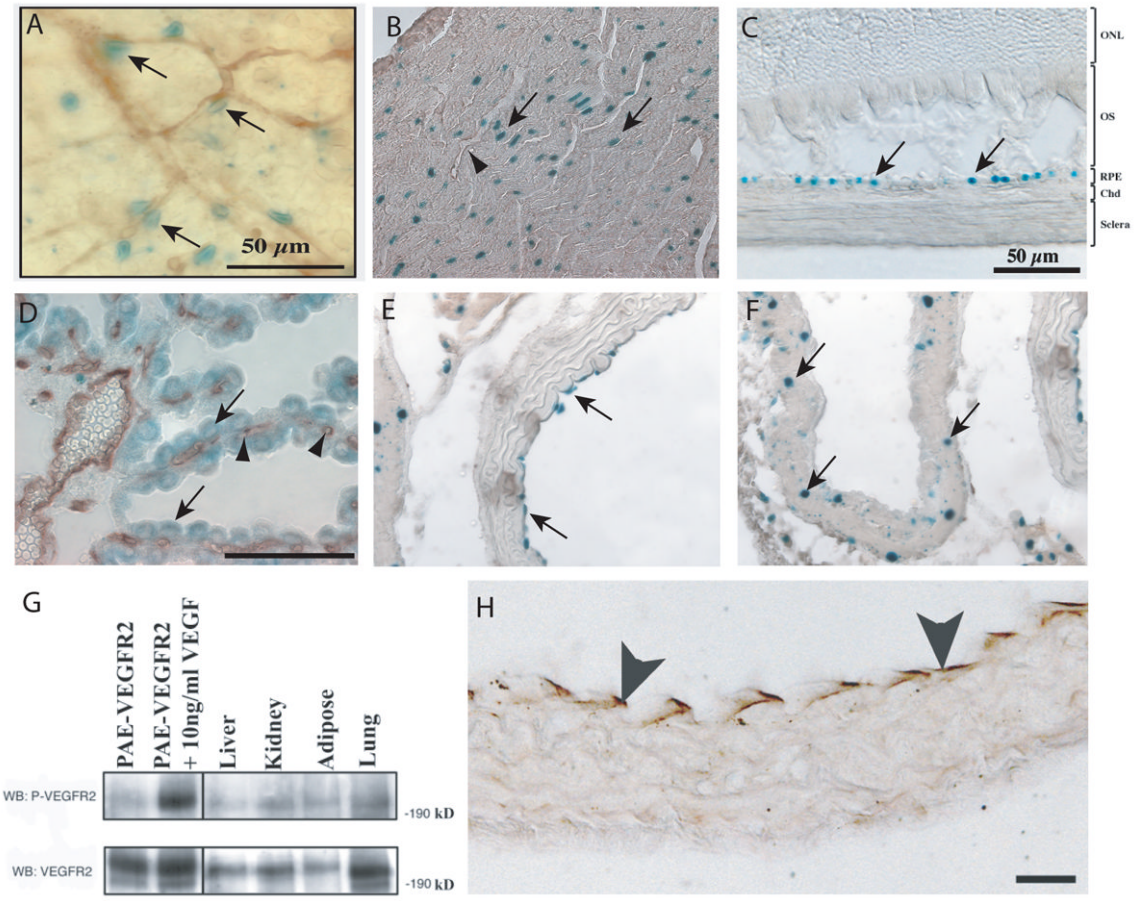

Figure 1. Expression of VEGF and its receptors in the adult

VEGF expression is shown in adult *VEGF-lacZ* mice, which make a nuclear localized β-galacotosidase (β-gal) protein wherever VEGF is expressed. Blood vessels, where shown, were identified by immunohistochemistry using the pan-endothelial cell marker- CD31 (arrowheads). VEGF expression in the pericytes in retina (A, arrows), cardiac myocytes (B), choroid RPE layer (C, arrows), choroid plexus epithelium (D, arrows) overlying fenestrated blood vessels (D, arrowheads), aortic endothelium (E, arrows), inferior vena cava medial layer cells (F, arrows). VEGFR2 expression and activation in adult tissues as identified by (G) western blot for VEGFR2 of protein lysates from liver, kidney, adipose and lung (bottom panel) and for phosphorylated VEGFR2 (top panel) and by (H) immunohistochemistry of aorta for phosphorylated VEGFR2 (arrows). Abbreviations (C): ONL, outer nuclear layer, OS, outer segment, RPE, retinal pigmented epithelium, Chd, choroid. Reprinted from IOVS 2006, 47: 3135–3142 (C), Am J Pathol 2006, 168: 639–658 with permission from the American Society of Investigative Pathology (B, D–H), unpublished data D'Amore lab (A).

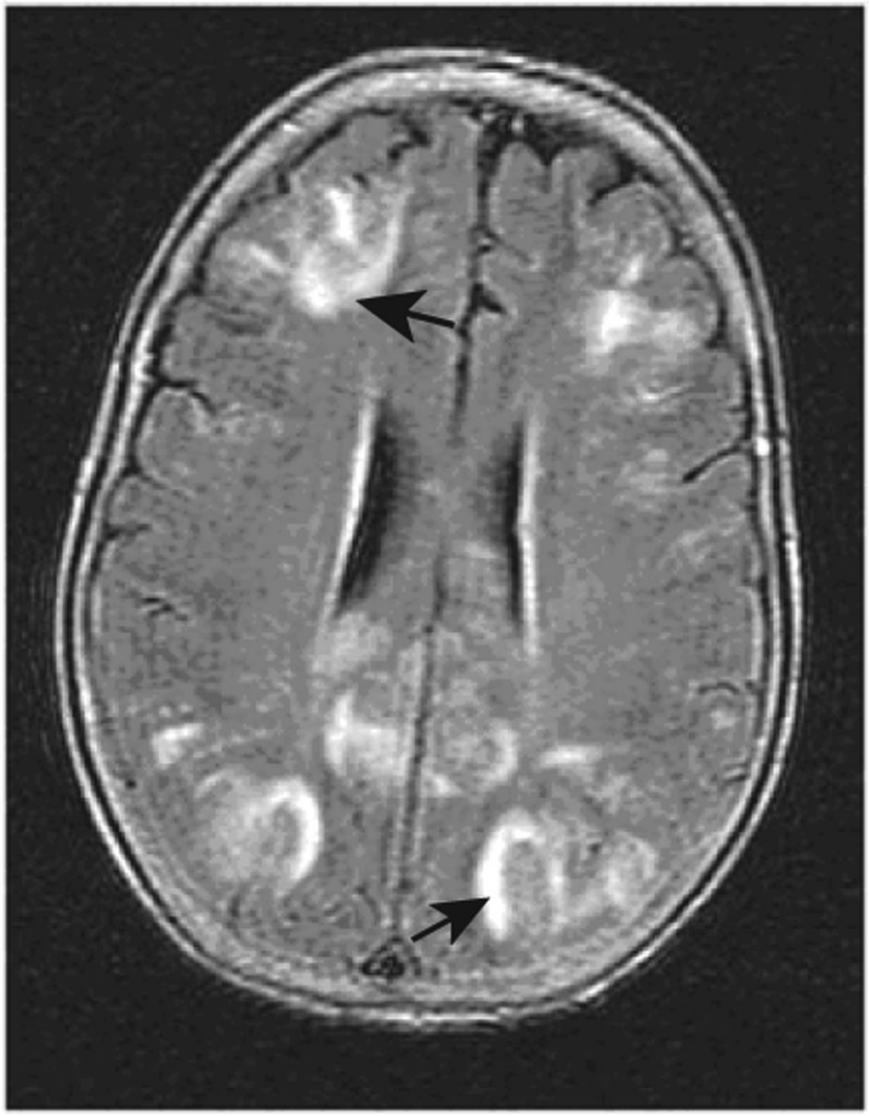

Figure 2.
MRI FLAIR image showing focal lesions (arrows) associated with reversible focal leukoencephalopathy in a patient on Avastin. Taken with permission from N Engl J Med (2006) 354; 980–982, Copyright © 2006 Massachusetts Medical Society

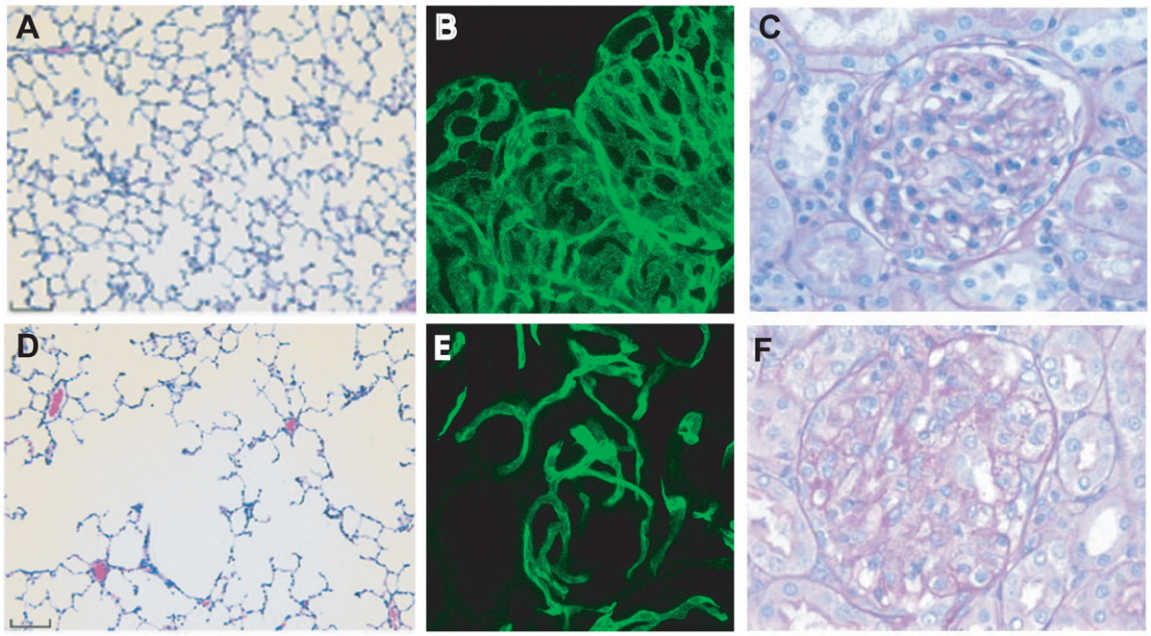

Figure 3. Role of VEGF in vascular stability

Administration of SU5416, a tyrosine kinase inhibitor with selective inhibition of VEGFR, to rats by bronchiolar delivery results in enlargement of air spaces, indicative of emphysema (D) compared to vehicle (A). Adenoviral delivery of sVEGFR1 to adult mice (B, E) results in decreased vascular density in the thyroid (E) compared to vehicle delivery (B), and endotheliosis in the glomerulus in the kidney in pregnant rats (F) compared to vehicle delivery (C). Taken with permission from J Clin Invest (2000) 106; 1311–1319 (A, D), Am J Physiol Heart Circ Physiol (2006): H560-H576 (B, E), and J Clin Invest (2003) 11; 649–658 (C, F).

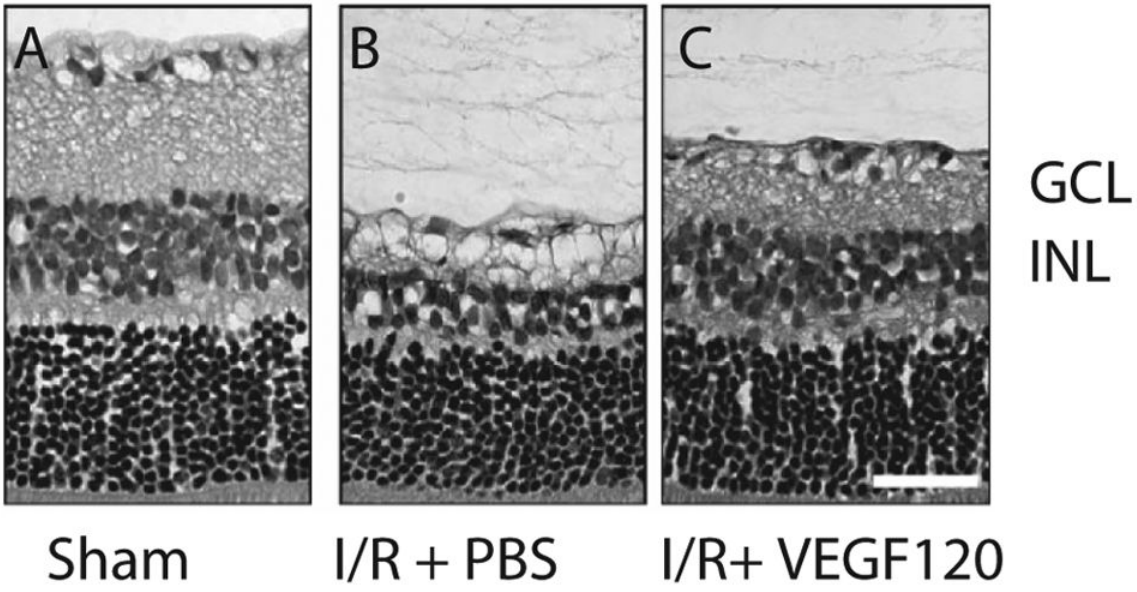

Figure 4. Neuroprotective role of VEGF in the eye

Addition of VEGF120 after 60 min of ischemic/reperfusion injury resulted in decreased cell death in the ganglion cell layer (GCL), and inner nuclear layer (INL) after 14 days (C) compared to addition of PBS only (B). Taken from Nishijima *et al*. AJP 2007 (IN PRESS) with permission from the American Society of Investigative Pathology.

Table 1

Pattern of VEGF expression in the adult

	Sparse cellular expression	**Intermediate cellular**
Cellular Source	Vascular stromal cells: pericytes and smooth muscle cells	Non-vascular mesench
Examples	Brain parenchymal cells, pericytes, retinal pericytes, Leydig cells	Skeletal myocytes, car
Microvasculature Characteristics	High resistance endothelium (blood-brain-barrier, retinal-blood-barrier, testis-blood-barrier)	Dynamic capillaries re
Postulated Function of VEGF on Endothelial cells	Endothelial cell survival	EC survival, dynamic a