Commentary

Vascular Endothelial Cell Growth Factor-A

Not Just for Endothelial Cells Anymore

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When vascular endothelial cell growth factor-A (VEGF-A) was first identified as an endothelial cell mitogen and vascular permeability factor, it was thought to be a specific mitogen for endothelial cells, and its main signaling receptors, VEGFR2/Flk1/KDR and VEGFR1/Flt-1, were reported to be expressed solely by the endothelium. On what was this claim based? First, early studies with newly described growth factors are usually focused almost exclusively in the initial field of discovery (eg, VEGF-A and the vasculature). Second, in the early days of a growth factor's characterization, reagents are limiting—they may not be very accessible or of particularly good quality. Once a growth factor is better characterized and reagents improve in quality and sensitivity (eg, antisera for immunohistochemical localization of receptors and development of gene-reporter transgenic mice), more comprehensive and reliable analyses of expression and actions are possible. Finally, and perhaps more importantly, growth factors are often named for the activity on which they were first identified: for VEGF-A, its ability to induce vascular endothelial cell proliferation. The naming of a factor to reflect its identifying activity is an historical pattern in the designation of peptide growth factors and cytokines including, for example, fibroblast growth factor, epidermal growth factor, and hepatocyte growth factor. It seems then that the simple labeling of a factor limits, at least initially, consideration of activities outside of that implied by its name. For VEGF-A, the belief that its activities were specific to the vasculature were likely to have been further enforced by the observation of its critical and central role during vascular development: targeted disruption of even a single VEGF-A allele leads to the total disruption of vascular development and early embryologic lethality.^{1,2}

Perhaps, then, it was just a matter of time before the pluripotent nature of VEGF-A began to be revealed. The most significant findings outside of the vasculature re-

lates to the role of VEGF-A in the neural system. VEGF-A has been previously demonstrated to be neuroprotective in models of ischemic/hypoxic injury in the central nervous system (see reviews^{3,4}) and now in the retina, as described in the article by Nishijima et al⁵ in this issue of *The American Journal of Pathology*. A variety of activities have been suggested to account for the observed neuroprotection, including enhancement of axonal regeneration,⁶ inhibition of caspase-3, and enhancement of neurogenesis in the subventricular and subgranular zones⁷ as well as activation of Akt.⁸ In addition, decreased VEGF-A levels via deletion of the hypoxia-response element in the VEGF-A promoter led to progressive degeneration of motor neurons due, at least in part, to reduced action on VEGFR2-expressing motor neurons.⁹ Despite this growing body of data demonstrating significant effects of VEGF-A on neural cells, very little is known about the role of VEGF-A in neural retina.

Considering the widespread use of VEGF-A-neutralizing therapies in the treatment of macular edema and choroidal neovascularization associated with the wet form of age-related macular degeneration (AMD),¹⁰ the lack of knowledge regarding the role of VEGF-A on neural retina represents a particularly serious information gap. VEGF-A clearly plays a role in the developmental vascularization of the retina, where it both directs angiogenic ingrowth¹¹ and acts as a survival factor.¹² A few early reports also suggested a nonvascular role for VEGF-A in the retina. Retinal progenitors express VEGFR2,¹³ and exogenously added VEGF-A induces the differentiation of photoreceptor cells *in vitro*¹⁴ as well as neurite outgrowth from postnatal retinal explants.¹⁵ In the adult retina, VEGF-A is produced by pericytes and astrocytes,¹⁶ Müller glial cells, amacrine cells, and ganglion cells¹⁷ as well as by the retinal pigment epithelial cells.¹⁸ However, data

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regarding a function for VEGF-A in the adult—on either vascular or nonvascular cells—are lacking.

In the article by Nishijima et al⁵ in this issue of the *AJP*, this topic is addressed with the demonstration of a very compelling and timely role for VEGF-A in neural retinal survival. In a retinal model of ischemia-reperfusion injury, the authors demonstrate a dose-dependent ability of VEGF-A to prevent neural cell death. They further show that ischemic preconditioning leads to elevated VEGF-A, which in turn decreases neural retinal cell death, and that blocking VEGF-A in this model leads to increased neural cell death. Finally, the authors report that neutralization of VEGF-A by both systemic and local delivery of inhibitors for 8 weeks leads to retinal ganglion cell death. The report by Nishijima et al⁵ highlights two evolving stories that relate to the biological activity of VEGF-A: first, that it acts on cells outside of the vasculature, and second, that it plays a significant role in the adult.

Effect of VEGF-A on Nonvascular Cells

A growing number of reports point to a major role for VEGF-A on nonvascular cells. VEGFR1 and/or VEGFR2 have been reported to be expressed by a variety of nonvascular cell types, including type 2 pneumocytes, monocytes, mast cells, megakaryocytes, dendritic cells, hematopoietic stem cells, lens epithelial cells, and a variety of neural cells. Despite these observations, data indicating a direct biological effect of VEGF-A on nonvascular cells have been relatively slow to accumulate. The exception is the nervous system, where there is now significant evidence indicating both neurotrophic and neuroprotective effects on neural cells both *in vitro* and *in vivo* (see review³). VEGF-A has been shown to act on astrocytes and microglia, as well as a variety of neurons (sensory, cortical, hippocampal, etc), with neurogenic, neuroprotective, and/or neurotrophic effects. In addition, VEGF-A induces the proliferation of neural progenitors in parts of the brain capable of neurogenesis (hippocampus, olfactory, and subventricular zone). Despite quite a large body of literature describing the neuroprotective and neurotrophic effects in the brain, parallel studies regarding VEGF-A effects in the retina had not been reported. However, the fact that the retina is an extension of the central nervous system supports the idea that neural retina might also be a target for VEGF-A.

Interpreting an effect of VEGF-A manipulation (either addition or subtraction) as being directly on the nonvascular cell is complicated by the role of VEGF-A in the vasculature of the tissue of interest. Thus, two specific criteria should be met before such a conclusion can be drawn. First, it is important to demonstrate the expression of VEGF-A receptors (VEGFR1 and/or VEGFR2) on the putative target cells. Although *in vitro* studies providing such evidence would be suggestive, it is also the case that establishment of cells in culture often leads to the expression of genes not observed *in vivo*.¹⁹ That said, direct demonstration of VEGF-A receptor expression by immunohistochemistry in the cells of interest *in vivo* would be critical to conclude convincingly that VEGF-A could

act directly on the nonvascular cells. Nishijima and coworkers⁵ demonstrate the presence of VEGFR2 by both indirect and direct methods. Administration of VEGF-E, a ligand for VEGFR2, reduced cell death in a model of ischemia-reperfusion, whereas placental-derived growth factor, which does not bind VEGFR2, did not prevent cell death, providing evidence of a neuroprotective role for VEGFR2 signaling. In a more direct demonstration, they used immunohistochemistry to demonstrate the presence of VEGFR2, not only on endothelial cells but also on a population of nonendothelial cells. Although they did not investigate the identity of these cells further by immunohistochemistry, some of them are probably neuronal cells of the retina.

A second requirement is the demonstration, in the absence of a vascular supply, of a relevant biological effect on the target nonvascular cells. Nishijima and his coworkers⁵ addressed the second of these criteria by culturing a fragment of the neonatal (and as yet unvascularized) rat retina in culture. In the absence of added factors, there was significant retinal ganglion cell death, which was prevented by the addition of exogenous VEGF-A. This definitive effect, in the absence of a vascular supply, provides strong evidence for a direct role of VEGF-A on neural retinal cells.

Role of VEGF-A in the Adult

Early reports suggested that the actions of VEGF-A were limited to developmental and postnatal periods. One report used a mouse model of inducible Cre to systemically delete VEGF-A as well as systemic VEGF-A neutralization and concluded that “the dependence on VEGF-A was lost sometime after the 4th postnatal week.”²⁰ However, the continued expression of VEGF-A in virtually all adult tissues, in the absence of active angiogenesis, suggests otherwise.²¹ Indeed, a series of studies using a variety of approaches to block the action of VEGF-A in adult mice has revealed a dramatic dependence of the microvasculature on local exogenous VEGF-A. The neutralization of VEGF-A led to the disappearance of fenestrations, specializations that characterize the microvasculature of secretory and filtering organs, as well as to regression of capillaries.^{22,23}

The consequences of systemic VEGF-A neutralization in the adult human can be seen in preeclampsia, a pathology of pregnancy that is characterized by elevated levels of soluble VEGFR1 (sFlt-1) (see review²⁴). The hallmarks of preeclampsia, hypertension and proteinuria, are at least partially the result of the blockade of VEGF-A on vascular endothelial cells, the hypertension resulting from diminished nitric oxide, and the proteinuria from the disruption of the paracrine interactions between podocyte-derived VEGF-A and its target glomerular endothelium. Although it is tempting to extrapolate these findings to what might be observed in therapeutic VEGF-A neutralization, it is important to keep in mind that preeclampsia is distinct in several ways, including the complex physiological effects of pregnancy, the fact that sFlt1 is not specific for VEGF-A (sFlt1 also binds placental-de-

rived growth factor), and the recent finding that preeclampsia is also associated with elevated soluble endoglin.²⁵ In contrast, the therapeutic inhibition of VEGF-A for antitumor angiogenesis is achieved with systemic infusion of bevacizumab (Avastin; Genentech, Inc., South San Francisco, CA), a humanized recombinant VEGF-A-neutralizing monoclonal antibody, not complicated by these caveats. Interestingly, however, the two most common side effects reported with bevacizumab administration are hypertension and proteinuria, the hallmarks of preeclampsia.

Clinical Significance

The observations of Nishijima and coworkers⁵ combine these two new evolving fields of VEGF-A actions—that of its effects outside of the vasculature and of its effects in the adult. So what do the observations of Nishijima et al⁵ imply for the therapeutic manipulation of VEGF-A? The studies of the effect of VEGF-A neutralization on neural retinal response to ischemic injury and survival are clearly quite relevant to the current widespread use of anti-VEGF-A therapy for the treatment of the wet form of AMD. AMD, a pathology of the retina that affects central vision, is the leading cause of vision loss in Americans older than 55. Current estimates indicate that there are close to 2 million US residents with significant symptoms related to AMD. The disease is diagnosed either as dry (or non-neovascular) or wet macular degeneration. The dry form accounts for a majority (90%) of the diagnoses and is associated with deposits of complex insoluble materials beneath the retinal pigment epithelium (RPE) or within Bruch's membrane, the elastic lamina that separates the RPE from the underlying choroidal circulation. The wet (or exudative) form accounts for only 10% of the cases but nearly 90% of the vision loss and is characterized by the growth of new blood vessels beneath the retina, particularly in the macula, the site of central vision. As is the case for virtually all nascent vessels associated with pathologies, these vessels are highly permeable, and the fluid that accumulates under the retina can cause retinal detachments, photoreceptor atrophy, and vision loss.

Prior to the introduction of ocular anti-VEGF-A therapies such as pegaptanib sodium (Macugen; OSI/Eyetechnology, Melville, NY), an aptamer reportedly specific for VEGF-A164, and ranibizumab (Lucentis; Genentech, Inc.), a Fab fragment of a humanized monoclonal anti-VEGF-A antibody, the most common form of treatment for wet AMD involved photodynamic therapy in which a photosensitizer (verteporfin, Visudyne; Novartis, Basel, Switzerland) is injected and then activated with a laser. Although some response was observed with this treatment, the effects were short-term and there was no significant effect on disease progression. The Food and Drug Administration approval in December 2004 of pegaptanib sodium marked the first therapeutic for AMD with a "rational" target. Patient response to pegaptanib sodium was mixed, and the treatment was shown to slow the progression of vision loss from wet AMD. The introduction of pegaptanib sodium was fol-

lowed about one and a half years later with the approval of ranibizumab. Intravitreal administration of ranibizumab has been shown to reduce disease progression in 95% of patients (versus 62% of sham-injected controls) and to lead to increased visual acuity in more than one-third of patients.¹⁰ The treatment has been appropriately hailed as "miraculous."²⁶

Are there reasons to be wary of the potential long-term effects of intraocular VEGF-A neutralization? The observations of Nishijima et al⁵ as well as those emerging from other groups suggest that there may be. Evidence pointing to a role for VEGF-A as an endogenous neuroprotectant indicate that continued neutralization of retinal VEGF-A may have unintended consequences, including loss²⁷ of neural retina cells. Furthermore, demonstration that the benefits of preconditioning are due, at least in part, to the up-regulation of VEGF-A indicate that VEGF-A blockage may interfere with physiological mechanisms that are intended to attenuate ischemic injury. Although the pathogenesis of AMD is not known, ischemia is often postulated as a component and the results of Nishijima et al⁵ suggest that VEGF-A inhibition may exacerbate ischemia-induced neural damage.

The existence of splice variants of VEGF (three in mice and at least five in humans) raises the possibility that the isoforms might serve distinct functions. In fact, an observation of mice that were engineered to express single isoforms supports the conclusion that the isoforms are not functionally equivalent; for instance, mice expressing only VEGF120 develop to term (although not in the predicted ratios) and die perinatally due to pulmonary and cardiac insufficiency.²⁸ More recently, based on results using a mouse model of ocular angiogenesis, it was suggested that VEGF164 mediates pathological but not physiological neovascularization.²⁷ In light of these observations, one very promising observation from the work of Nishijima and colleagues⁵ is that VEGF120 was as effective as VEGF164 in protecting the retina against hypoxic injury. This finding suggests the possibility that targeting specific VEGF isoforms block vessel growth while sparing the neuroprotective effects of VEGF. Because so little is known about the relative actions of the various VEGF isoforms *in vitro* or *in vivo*, the cellular, biochemical, and molecular basis of these observations is not clear. Clearly, additional work is necessary to determine whether this suggestion will be sustained in clinical settings.

Furthermore, although Nishijima and colleagues⁵ did not detect an effect of VEGF-A neutralization on the retinal vessels within the 8-week time course of their study, it is possible that extended inhibition may have revealed an effect. This possibility is supported by the observation that VEGF-A acts as a survival factor on developing retinal vessels¹² and that VEGF-A continues to be produced by retinal pericytes and astrocytes in the vicinity of the inner retinal vessels in the adult retina.¹⁶ Finally, a fact that was not discussed by Nishijima et al⁵ but which must be given serious consideration is the potential effect of VEGF-A neutralization on the choroidal circulation. The choroidal vasculature underlies the RPE and nourishes the outer retina, in-

cluding the photoreceptor cells. Development of the choroidal circulation is dependent on RPE-produced VEGF-A.²⁹ More importantly, the fact that RPE continues to express VEGF-A in the adult and that the endothelial cells of the choriocapillaris (the capillary plexus of the choroids) express VEGFR2³⁰ strongly support a role for VEGF-A in the maintenance of the adult choriocapillaris, including its fenestrated phenotype. Results of animal studies have revealed fenestrated vascular beds to be uniquely vulnerable to VEGF-A blockage, exhibiting not just loss of fenestrations but outright vessel regression.^{22,23} Thus, long-term therapeutic VEGF-A neutralization may lead to the unexpected and unintentional degeneration of the choroidal circulation.

Despite these dire predictions, the availability of a treatment that is effective in not only slowing the progress of AMD but in restoring lost vision can be considered nothing short of a major breakthrough. That said, a more thorough understanding of the source of VEGF-A, its regulation, and the pathways through which it signals in AMD should permit the development of a more specific treatment. The ideal therapeutic will be one that targets only that component of VEGF-A associated with disease pathogenesis while sparing the VEGF-A that participates in normal tissue and vessel integrity.

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