VEGF₁₆₄(₁₆₅) as the Pathological Isoform: Differential Leukocyte and Endothelial Responses through VEGFR₁ and VEGFR₂

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PURPOSE. Vascular endothelial growth factor (VEGF) induces angiogenesis and vascular permeability and is thought to be operative in several ocular vascular diseases. The VEGF isoforms are highly conserved among species; however, little is known about their differential biological functions in adult tissue. In the current study, the inflammatory potential of two prevalent VEGF isoform splice variants, VEGF₁₂₀(₁₂₁) and VEGF₁₆₄(₁₆₅), was studied in the transparent and avascular adult mouse cornea.

METHODS. Controlled-release pellets containing equimolar amounts of VEGF₁₂₀ and VEGF₁₆₄ were implanted in corneas. The mechanisms underlying this differential response of VEGF isoforms were explored. The response of VEGF in cultured endothelial cells was determined by Western blot analysis. The response of VEGF isoforms in leukocytes was also investigated.

RESULTS. VEGF₁₆₄ was found to be significantly more potent at inducing inflammation. In vivo blockade of VEGF receptor (VEGFR)-1 significantly suppressed VEGF₁₆₄-induced corneal inflammation. In vitro, VEGF₁₆₄ more potently stimulated intracellular adhesion molecule (ICAM)-1 expression on endothelial cells, an effect that was mediated by VEGFR₂. VEGF₁₆₄ was also more potent at inducing the chemotaxis of monocytes, an effect that was mediated by VEGFR₁. In an immortalized human leukocyte cell line, VEGF₁₆₄ was found to induce tyrosine phosphorylation of VEGFR₁ more efficiently.

CONCLUSIONS. Taken together, these data identify VEGF₁₆₄(₁₆₅) as a proinflammatory isoform and identify multiple mechanisms underlying its proinflammatory biology. (Invest Ophthalmol Vis Sci. 2004;45:368–374) DOI:10.1167/iovs.03-0106

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Vascular endothelial growth factor (VEGF) is an endothelial-cell-specific mitogen and angiogenic factor, as well as a potent mediator of vascular permeability.¹ The biological effects of VEGF are mediated by at least two tyrosine kinase receptors, Flt-1 (VEGFR-1) and KDR (VEGFR-2).¹ VEGF is essential for vascular development and has been strongly implicated in the pathogenesis of diabetic retinopathy,²–⁴ retinopathy of prematurity,⁵,⁶ age-related macular degeneration,⁷,⁸ and corneal neovascularization.⁹–¹⁰

The VEGF gene produces alternatively spliced mRNA variants, leading to at least three distinct major VEGF isoforms.¹ They encode 120-, 164-, and 188-amino-acid-containing murine proteins (the human proteins are one residue longer, and thus produce isoforms of 121, 165, and 189 amino acids, respectively).¹¹–¹³ The isoforms are highly conserved among species; however, their differential biology is poorly understood. Biochemically, the VEGF isoforms differ in their affinity for heparin, with the larger isoforms binding to heparin more avidly.¹⁴–¹⁶ The isoforms also vary in their affinity for their cognate receptors. For example, VEGF₁₂₁ binds to VEGFR₁ up to 20 times less efficiently than VEGF₁₆₅ (Kₛᵣ = 200 pM vs. 10 pM for 121 and 165, respectively) in vitro.¹⁷ Finally, VEGF₁₆₄ is unique among the various VEGF isoforms in its ability to bind to the receptor neuropilin-1,¹⁰ a coreceptor for VEGFR₂ (flk-1 or KDR). These studies were restricted to isoform-specific in vitro mitogenesis.

Studies in VEGF isoform-specific knockout mice have shed some light on the differential biology of the VEGF isoforms during development. Homozygous mice expressing only the VEGF₁₂₀ isoform, but normal absolute VEGF levels, die shortly after birth and exhibit an abnormal coronary microvasculature and defective vascular patterning.¹⁹ Mice expressing only the VEGF₁₆₄ isoform display impaired arterial development.²⁰ It is also known that relative and absolute VEGF isoform expression levels vary among normal tissues and that they are altered in disease.¹¹,¹²,¹³,¹⁴,¹⁵,¹⁶ For example, the selective upregulation of VEGF₁₆₄ is observed in rheumatoid arthritis and diabetic retinopathy.¹¹,¹³,¹⁴,¹⁵,¹⁶ A similar pattern is observed in injured cornea undergoing inflammation and neovascularization.⁹ Finally, others have demonstrated the inflammatory potential of VEGF₁₆₄ in vivo,²⁴,²⁵ but isoform-specific comparisons have never been attempted.

In the present study, we compared and contrasted the biology of two prevalent VEGF isoforms: VEGF₁₂₀(₁₂₁) and VEGF₁₆₄(₁₆₅). The cornea, owing to its optical transparency and avascular state, was used to study the differential effects of these isoforms on inflammation, and the mechanisms underlying any variances were explored.

MATERIALS AND METHODS

Corneal Micropocket Assay

Age-matched (8-week-old) C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME) were used for the murine corneal micropocket assay.
as previously described.²⁵ Micropockets were created with a modified von Graefe cataract knife. Into each pocket, a 0.34 × 0.34-mm pellet of 12% Hydron polymer type NCC (Interferon Science, New Brunswick, NJ) containing 10 mg sucrose aluminum sulfate (Sigma-Aldrich, St. Louis, MO) and approximately 150 ng of either mouse recombinant VEGF₁₂₀ or VEGF₁₆₅ (R&D Systems, Minneapolis, MN) was implanted. The pellets were positioned 1.0 mm from the corneal limbus. After implantation, erythromycin ophthalmic ointment was applied to each eye.

To assess the effect of VEGF₁ in a mouse corneal pocket assay, animals were randomized to receive intraperitoneal injections of 1 mg/kg anti-VEGF₁–neutralizing antibody (AF471; R&D Systems) 2 hours before pellet implantation. Control animals received an equivalent amount of a preimmune control goat IgG (Alpha Diagnostic International, San Antonio, TX).

VEGF ELISA

On days 2 and 7 after corneal pellet implantation, the eyes were enucleated and the pellets removed. The corneal samples were placed into 150 mL of lysis buffer (20 mM imidazole HCl, 10 mM KCl, 1 mM MgCl₂, 10 mM EDTA, 1% Triton, 1 mM sodium molybdate, and 1 mM EDTA [pH 6.8]) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and sonicated. The lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C, and the VEGF levels in the supernatant were determined with a mouse VEGF kit (QuantiKine; R&D Systems), according to the manufacturer’s protocol. The assay recognized all VEGF isoforms. Total protein was determined with a bicinchoninic acid (BCA) assay kit (Bio-Rad, Hercules, CA) and was used for the normalization of corneal VEGF protein levels.

Leukocyte Counts

Eyes were enucleated 2 days after pellet implantation, embedded in optimal cutting temperature (OCT) compound, snap frozen in liquid nitrogen, and cut into 7-μm-thick sections. After fixation with ice-cold acetone and blocking with normal goat serum, the sections were stained with a monoclonal rat anti-mouse CD45 (leukocyte common antigen, clone 30-F11; BD Pharmingen, San Diego, CA) antibody to detect infiltrating leukocytes, followed by staining with FITC-conjugated anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The cells were visualized by fluorescence microscopy and counted in a masked fashion. Eight consecutive serial sections extending through the pellet and limbus were studied per cornea. The number of CD45 cells was determined by fluorescence microscopy and counted in a masked fashion. Eight consecutive serial sections extending through the pellet and limbus were studied per cornea. The number of CD45 cells was determined by counting in a masked fashion.
and then incubated for 1 hour with an anti-phosphotyrosine monoclonal antibody 4G10 (10 μg/mL; UBI, Salt Lake City, UT). The immune complexes were recovered on rabbit anti-mouse IgG-agarose. Immunoreactivity was determined by the enhanced chemiluminescence reaction (Amersham Pharmacia Biotech).

**Lectin Angiography and Neovascularization Quantitation**

Corneal neovascularization was imaged through lectin angiography. Mice received intravenous isoelectin B4 conjugated with FITC (500 μg; Vector Laboratories, Burlingame, CA) and were killed 30 minutes later. The eyes were enucleated and fixed with 1% paraformaldehyde for 15 minutes. After fixation, the corneas were placed on glass slides and studied through fluorescence microscopy (Leica, Deerfield, IL). Images were captured using a CD-330 charged-coupled device camera (Dage-MTI Inc., Michigan City, IN; controlled by Openlab software; Improvision, Lexington, MA), as described previously.23 Briefly, NIH Image 1.62 (available by ftp at zippy.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) was used for image analysis. The neovascularization was quantified by setting a threshold level of fluorescence, above which only vessels were captured. The entire flatmounted cornea was analyzed to minimize sampling bias. The neovascularization quantitation was performed in a masked manner. The vascularized area was outlined using the innermost vessel of the limbal arcade as the border.

Concanavalin A (ConA) lectin (Vector Laboratories) was used for imaging adherent leukocytes and the vasculature, as described previously.24 Briefly, after anesthesia with xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (35 mg/kg), the mouse’s chest cavity was carefully opened and a 24-gauge cannula was introduced into the left ventricle. The animals were perfused with 10 mL PBS to remove erythrocytes and nonadherent leukocytes, after which, 10 mL FITC-conjugated ConA lectin (20 μg/mL) was perfused. Residual unbound lectin was removed with 10 mL PBS solution. After the eyes were enucleated, the corneas were excised and fixed with 1% paraformaldehyde for 15 minutes. The corneas were flatmounted on glass slides and observed by fluorescence microscopy (Leica).

**Statistical Analysis**

All results are expressed as the mean ± SD. The data were analyzed with the Mann-Whitney test, and post hoc comparisons were tested with the Fisher protected least significance procedure. Differences were considered statistically significant at P < 0.05.

**RESULTS**

**VEGF Isoform Clearance**

Corneal VEGF protein levels were determined by ELISA after removal of the pellet. VEGF120 and VEGF164 levels did not differ on day 2 after pellet implantation (1.00 ± 0.09 vs. 0.97 ± 0.14 pg/μg cornea, n = 4, P > 0.05). However, the levels differed on day 7 after VEGF120 and VEGF164 pellet implantation (1.66 ± 0.14 vs. 3.09 ± 0.40 pg/μg cornea, n = 4, P < 0.01).

**VEGF-Mediated Leukocyte Adhesion and Inflammation**

Leukocyte adhesion to the limbal vascular endothelium was examined after VEGF pellet implantation. ConA lectin perfusion was performed to highlight the adherent leukocytes in the limbal vasculature (A, B, arrows). The mean number of adherent leukocytes increased significantly in the corneas implanted with VEGF164 pellets compared with those implanted with VEGF120 pellets (C, n = 5, *P < 0.01). Error bars, SD. Treatment with the anti-VEGFR1-neutralizing antibody did not affect the number of adherent leukocytes.

![Figure 1. Leukocyte adhesion to the limbal vasculature after VEGF pellet implantation. On the second day after pellet implantation (VEGF120, VEGF164), ConA lectin perfusion was performed to highlight the adherent leukocytes in the limbal vasculature (A, B, arrows). The mean number of adherent leukocytes increased significantly in the corneas implanted with VEGF164 pellets compared with those implanted with VEGF120 pellets (C, n = 5, *P < 0.01). Error bars, SD. Treatment with the anti-VEGFR1-neutralizing antibody did not affect the number of adherent leukocytes.](https://example.com/image)
VEGF Induction of Leukocyte VEGFR1 Tyrosine Phosphorylation

VEGF receptor signaling is mediated by tyrosine kinases. The tyrosine phosphorylation of VEGFR1 was studied in Jurkat cells after stimulation with VEGF. VEGFR1 is expressed in Jurkat cells. The expression of VEGFR1 was confirmed at the mRNA level; however, VEGFR2 was not detected by RT-PCR (data not shown). Both VEGF₁₂₀ and VEGF₁₆₄ induced tyrosine phosphorylation of VEGFR1 in a dose-dependent manner (Fig. 6). Notably, the phosphorylation of VEGFR1 was greater after the addition of VEGF₁₆₄ versus VEGF₁₂₀.

VEGF Induction of Neovascularization

Corneal inflammation is causally linked to the subsequent development of corneal neovascularization. To evaluate the differential angiogenic responses induced by VEGF₁₂₀ and VEGF₁₆₄, we implanted controlled-release pellets containing VEGF₁₂₀ or VEGF₁₆₄ in the corneal stroma (Fig. 7A). The VEGF₁₆₄ pellet elicited more corneal neovascularization on days 4 and 7 than did the VEGF₁₂₀ pellet (Fig. 7B, n = 6 per condition, P < 0.01) than did pellets containing VEGF₁₂₀.

DISCUSSION

The present study focused on two major VEGF isoforms, VEGF₁₆₄(165) and VEGF₁₂₀(121) and determined that they have...
differential effects on inflammation and neovascularization. Although studies have revealed biochemical differences between the VEGF isoforms in vitro, functional differences between the two isoforms have not been demonstrated in adult animals, nor have the mechanisms underlying their differential effects on inflammation been delineated. The current data demonstrate that VEGF$_{164}$ is more potent at inducing corneal inflammation and neovascularization in vivo and identify several of the mechanisms underlying these responses.
VEGF<sub>120(121)</sub> is a non–heparin-binding acidic protein that is freely diffusible. In contrast, VEGF<sub>164(165)</sub> binds to heparin-containing proteoglycan, suggesting that extracellular matrix may represent a major repository of VEGF. Although it is has been speculated that the differential affinity for heparin among the VEGF isoforms may provide for spatial targeting, this has not been demonstrated in vivo. The current data demonstrate that VEGF<sub>164</sub> resides in the heparin proteoglycan-rich cornea for a longer period than VEGF<sub>120</sub>. Therefore, one potential explanation for the superior potency of VEGF<sub>165</sub> is its prolonged corneal clearance and hence bioavailability.

VEGF can upregulate ICAM-1 expression on cultured endothelial cells as well as retinal endothelial cells in vivo. The current data demonstrate that VEGF<sub>165</sub> is more potent at inducing ICAM-1 expression than VEGF<sub>121</sub> (Fig. 3). This effect was largely abrogated with an anti-VEGFR2 antibody in vitro. In vivo, the antibody blockade of VEGFR1 did not inhibit VEGF-induced leukocyte adhesion in the limbal vasculature. A VEGFR1 antibody was used because a suitable anti-mouse VEGFR2 antibody was not available for in vivo use. However, the in vivo result is consistent with the VEGFR2-mediated ICAM-1 increases observed in vitro. The more pronounced VEGF<sub>165</sub>-induced ICAM-1 increases are also consistent with the differential VEGFR2 tyrosine phosphorylation activities observed. Previous work has shown that the EC<sub>50</sub> for VEGFR2 phosphorylation is lower with VEGF<sub>165</sub> than with VEGF<sub>121</sub> (34.9 pM and 2.09 nM for VEGF<sub>165</sub> and VEGF<sub>121</sub>, respectively).

Unlike VEGFR1 on endothelial cells, VEGFR1 on leukocytes is active and serves to trigger chemotactic migration. Early studies using radiolabeled VEGF revealed that VEGF bound almost exclusively to the vasculature. However, subsequent studies have confirmed the presence of high-affinity VEGF receptors on monocyte-macrophage subpopulations and neutrophils. The identification of these receptors further highlights the intertwined nature of the angiogenic and inflammatory pathways. It is also notable that the T lymphocytes expressed neuropilin-1, which was also recently suggested by Tordjman et al. We speculate that T-lymphocyte–expressed neuropilin-1 is not involved in mediating lymphocyte migration, because administration of VEGF did not show chemotaxis in vitro (Fig. 5). Instead, neuropilin-1 may be involved in the cellular immune response, and VEGF<sub>164(165)</sub> may be involved in this process. More work is needed to delineate more clearly the effect of VEGF stimulation on T lymphocytes.

The migration of monocytes appeared to be mediated in large part by VEGF<sub>121</sub>. The administration of an anti-VEGFR1-neutralizing antibody blocked both inflammation in vivo and leukocyte chemotaxis in vitro (Figs. 2, 5). These results confirm and extend previous work in this area. Hiratsuka et al. showed that VEGF<sub>165</sub> tyrosine-kinase–deficient mice produced macrophages deficient in VEGF-dependent migration. The mechanisms underlying the selective migration of leukocytes to VEGF<sub>164(165)</sub> may lie in the greater affinity of VEGF<sub>164(165)</sub> for VEGFR1. Keyt et al. demonstrated that VEGF<sub>165</sub> binds VEGFR1 with greater affinity. Other mechanisms may apply; however, the fact that leukocyte VEGFR1 undergoes greater phosphorylation on binding VEGF<sub>164(165)</sub> suggests that the differential affinity for VEGFR1 may underlie the differential activation observed in this study.

Because inflammatory cells enhance the angiogenic process, it is not surprising that VEGF<sub>164(165)</sub> was also more potent at inducing angiogenesis. The mechanisms underlying the enhanced angiogenic response are probably complex; however, leukocytes make and release VEGF into the extracellular milieu. The recruitment of leukocytes to sites of angiogenesis may produce an amplification of local VEGF production and release. In addition, some leukocytes recruited to sites of angiogenesis may in fact be endothelial progenitor cells, exhibiting features of both leukocytes and endothelial cells. Some of these endothelial progenitor cells may be directly incorporated into the growing corneal neovascularure (Usui et al., manuscript submitted). VEGF<sub>164(165)</sub> may serve to recruit these cells to sites of angiogenesis differentially, thereby contributing to the VEGF<sub>164(165)</sub>–induced”inflammation.” Last, others have shown that VEGF<sub>165</sub> is a more potent endothelial cell mitogen, an effect that may also be operative, in part, in the differential responses observed in the present study.

Finally, the expression pattern of the VEGF isoforms appears to be altered in ocular disease. RT-PCR analysis has been used in severe cases of human proliferative diabetic retinopathy to show preferential expression of VEGF<sub>165</sub>. In an animal model of diabetes, the expression VEGF<sub>164</sub> greatly predominated over VEGF<sub>120</sub>. Diabetic blood–retinal barrier breakdown was also more potently induced by VEGF<sub>164</sub> than VEGF<sub>120</sub>. VEGF<sub>164</sub> was also selectively upregulated in the ischemic retina in a rat model of retinopathy of prematurity. These data suggest that targeting VEGF<sub>164(165)</sub> which predominates in ocular vascular disease and is more potent at inducing disease, may represent a more targeted therapeutic strategy in ocular neovascular disease.

In summary, the current data further define the differential bioactivities of two major VEGF isoforms and demonstrate that they have differential effects on inflammation and angiogenesis. VEGF<sub>164(165)</sub> was more potent at inducing endothelial ICAM-1 expression by VEGFR-2, leukocyte migration by VEGFR-1, and inflammation and angiogenesis in the adult cornea. These data provide additional insights into the unique biology of the VEGF isoforms.

References