

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, VEGF₁₆₅ 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, VEGF₁₆₅ 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 ($K_d = 200$ pM vs. 10 pM for 121 and 165, respectively) in vitro.¹⁷ Finally, VEGF₁₆₅ is unique among the various VEGF isoforms in its ability to bind to the receptor neuropilin-1,¹⁸ 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 VEGF₁₈₈ 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.^{15,21–23} For example, the selective upregulation of VEGF₁₆₄ is observed in rheumatoid arthritis and diabetic retinopathy.^{21–23} 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/6J male mice (Jackson Laboratories, Bar Harbor, ME) were used for the murine corneal micropocket assay,

as previously described.²⁶ 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 VEGF₁₆₄ (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 VEGFR-1 inhibition in a mouse corneal pocket assay, animals were randomized to receive intraperitoneal injections of 1 mg/kg anti-VEGFR1-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 EGTA, 1% Triton, 10 mM NaF, 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 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- μ m-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 between the limbus and pellet was determined at 50 \times magnification.

Cell Culture

Single-donor human umbilical venous endothelial cells (HUVECs) were purchased from Cascade Biologics Inc. (Portland, OR) and cultured in Medium 200 with low serum growth supplement (Cascade Biologics Inc.). The cells were used within five passages. The human leukocyte cell line (Jurkat cells) was kindly provided by Nicholas Mitsiades (Dana Farber Cancer Institute, Boston, MA) and cultured in RPMI 1640 with 10% fetal bovine serum (FBS).

Western Blot Analysis

Intracellular adhesion molecule (ICAM)-1 expression in the HUVECs was studied by Western blot analysis. After exposure to medium 200 with 1% FBS for 24 hours, recombinant human VEGF₁₂₁ or VEGF₁₆₅ (R&D Systems) was added to the HUVEC cultures. Some HUVECs were preincubated with 30 ng/mL of anti-VEGFR2-neutralizing antibody (clone CUE04; R&D Systems) 1 hour before administration of VEGF. After incubation for 6 hours with recombinant VEGF, the cells were washed with PBS and suspended in sample buffer. The samples were boiled for 5 minutes, separated by SDS-polyacrylamide gel electrophoresis under denaturing conditions, and electroblotted to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were incubated in blocking buffer, followed by the monoclonal anti-ICAM-1

antibody (Santa Cruz Biotechnology), washed, and incubated with a horseradish-peroxidase-conjugated secondary antibody. The blot was visualized with a chemiluminescence kit (ECL Plus; Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instructions.

Cell Preparation and Sorting

Splenocytes from C57/B6 mice were prepared by teasing the spleen through a nylon screen. Erythrocytes were lysed by osmotic shock, and single-cell suspensions were prepared. The cells were first incubated with 50 μ g normal rat immunoglobulin and anti-mouse immunoglobulin Fc γ R antibody (clone 2.4G2; BD Pharmingen) before the addition of the appropriate fluorescein-conjugated antibodies against cell surface markers for leukocyte subpopulations. The following antibodies were used for flow cytometry: anti-CD3 for T cells (BD Pharmingen), anti-F4/80 for monocytes and macrophages (Serotec, Raleigh, NC), and 7/4 for neutrophils (Serotec). The fluorescein-conjugated reagents used for the second-stage were FITC-avidin and phycoerythrin (PE)-avidin. Flow cytometry was then performed (model XLEPICS XL flow cytometer; Beckman Coulter, Miami, FL). Dead cells were excluded based on low forward light scattering.

Reverse Transcription-Polymerase Chain Reaction

The expression of VEGF receptors on mouse leukocytes at the mRNA level was investigated by reverse transcription-polymerase chain reaction (RT-PCR), which was performed on subpopulations of leukocytes after cell sorting. Total RNA was isolated from each leukocyte subpopulation (1×10^6 cells; TRIzol; Invitrogen-Gibco, Grand Island, NY), and cDNA was produced with reverse transcriptase (SuperScript II; Invitrogen, San Diego, CA). The PCR conditions were as follows: 30 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C, with an initial 5-minute denaturation step and a final 7-minute elongation step. The oligonucleotide primers for VEGFR1 and VEGFR2 were purchased from R&D Systems (human/mouse VEGF R1 and VEGF R2 Primer Pair). The primers for NP-1 were based on the sequences of mouse NP-1. The primers were 5'-TCA GGA CCA TAC AGG AGA TGG-3' (sense), corresponding to nucleotides 2396-2416 in the mouse NP1 mRNA, and 5'-TGA CAT CCC ATT GTG CCA AC-3' (antisense), corresponding to nucleotides 2995-3014 in the mouse NP1 mRNA. The fragments amplified by RT-PCR were subcloned into the TA vector (Invitrogen).

Cell Migration Assay

VEGF isoform-induced chemotaxis was evaluated with a 48-well microchemotaxis chamber (Neuro Probe, Bethesda, MD), as previously described.²⁷ Briefly, serum-free RPMI 1640 medium was added, with or without mouse VEGF₁₂₀ and VEGF₁₆₄ (R&D Systems) at a concentration of 10 ng/mL. A 5- μ m pore size PVP(-) polycarbonate filter, (Neuro Probe) was overlaid, and 50 μ L of cell suspension per well (1×10^6 cells/mL) was seeded in the upper compartment and incubated at 37°C for 2 (monocytes) or 4 (T lymphocytes) hours in a 5% CO₂ incubator. Some cells were preincubated with an anti-VEGFR1-neutralizing antibody (10 ng/mL) at 37°C for 1 hour. Migrated cells were counted in a masked fashion.

VEGFR1 Activation

Jurkat cells (1×10^7) were incubated in 1% FBS in RPMI 1640 with human recombinant VEGF₁₂₁ or VEGF₁₆₅ (R&D Systems) for 2 minutes at 37°C after incubation in 1% FBS in RPMI 1640 for 12 hours. The cells were then centrifuged, lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 1% NP40, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM Na₃VO₄) supplemented with a protease inhibitor cocktail (Roche Diagnostics) and sonicated. The lysates were cleared by centrifugation at 14,000 rpm for 15 minutes at 4°C. The supernatants were then precleared by incubation for 1 hour with rabbit anti-mouse IgG-agarose

and then incubated for 1 hour with an anti-phosphotyrosine monoclonal antibody 4G10 (10 $\mu\text{g}/\text{mL}$; UBI, Salt Lake City, UT). The immune complexes were recovered on rabbit anti-mouse IgG-agarose. Immunoprecipitates were washed four times with lysis buffer, twice with the same buffer without detergent, and once with Tris-buffered saline. The proteins were separated by 7.5% SDS-PAGE, blotted onto PVDF membrane, and probed with polyclonal anti-VEGFR1 antibody (C-17; Santa Cruz Biotechnology). 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 isolectin 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.²⁸ Briefly, NIH Image 1.62 (available by ftp at zippy.nimh.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.²⁹ 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 $\mu\text{g}/\text{mL}$) 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 \pm 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 was used to image the limbal vasculature and adherent leukocytes. At day 2,

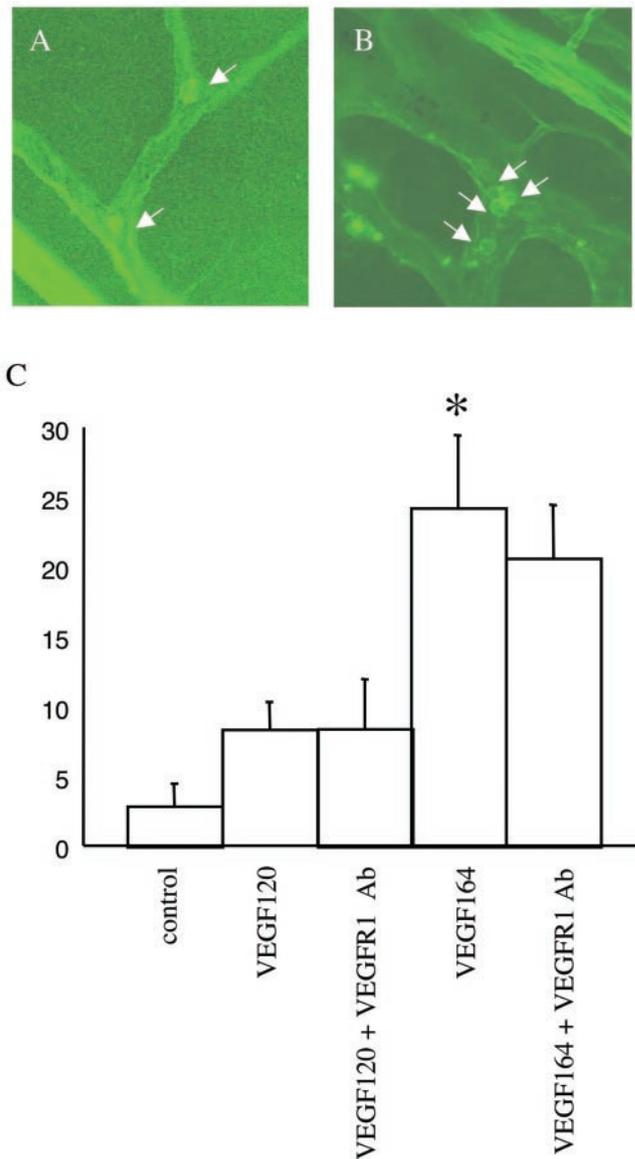


FIGURE 1. Leukocyte adhesion to the limbal vasculature after VEGF pellet implantation. On the second day after pellet implantation (VEGF₁₂₀ or VEGF₁₆₄), ConA lectin perfusion was performed to highlight the adherent leukocytes in the limbal vasculature (A, B, arrows). The mean number of adherent leukocytes 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.

the number of adherent leukocytes in the limbal vasculature of the VEGF₁₆₄-implanted corneas was significantly greater than in the VEGF₁₂₀-implanted corneas (Fig. 1, 21.4 ± 3.1 VEGF₁₆₄ vs. 8.4 ± 2.1 VEGF₁₂₀ leukocytes/cornea, $n = 6$ to 8 /each condition, $P < 0.05$). However, leukocyte adhesion to the limbal vasculature was not inhibited by the systemic administration of a neutralizing antibody against VEGFR1 (Fig. 1).

The number of CD45-positive corneal leukocytes was assessed 2 days after the implantation of VEGF pellets into the corneal stroma. At this point, corneal VEGF levels were comparable between the two treatment groups, as noted earlier. Light microscopy revealed significantly more leukocytes in the VEGF₁₆₄-implanted corneas than in VEGF₁₂₀-implanted corneas or control (empty pellet) corneas (Fig. 2, 62.5 ± 7.0 VEGF₁₆₄

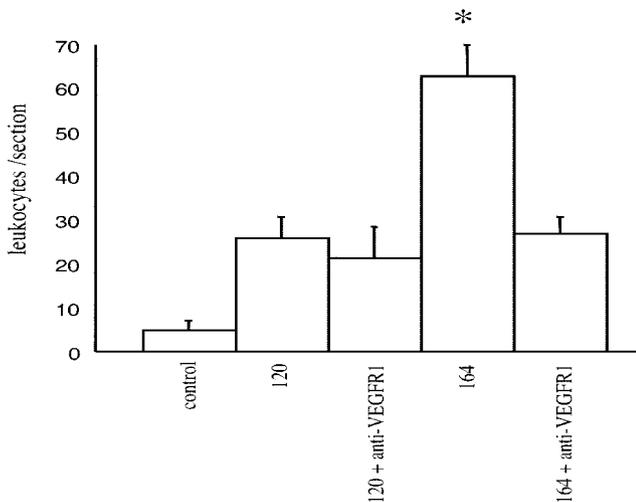


FIGURE 2. Corneal inflammation after VEGF pellet implantation. Two days after VEGF pellet implantation, the corneas were examined to quantify the mean number of leukocytes in the corneal stroma. Empty (sucralfate) pellets were used as the control. Immunohistochemical staining was performed with antibodies against CD45 (leukocyte common antigen), an inflammatory cell marker. Cells were counted from the limbus to the pellet in a standardized fashion. Although both VEGF isoforms produced an inflammatory cell infiltrate, VEGF₁₆₄ was more potent (P). The recruitment of inflammatory cells was suppressed in vivo through the administration of a VEGFR1-neutralizing antibody. Error bars, SD ($n = 6$ to 8 per condition, $*P < 0.01$).

vs. 25.3 ± 5.2 VEGF₁₂₀ vs. 4.5 ± 2.4 control, $n = 8$ per condition, $P < 0.01$). Systemic treatment with a neutralizing antibody against VEGFR1 significantly reduced the number of corneal leukocytes after VEGF₁₆₄ pellet implantation (Fig. 2, 62.5 ± 7.0 VEGF₁₆₄ vs. 26.5 ± 3.8 VEGF+anti-VEGFR1 antibody, $P < 0.01$).

VEGF Induction of CAM-1

In vitro studies were performed to delineate the mechanisms underlying the differential in vivo responses. Human recombinant VEGF₁₂₁ or VEGF₁₆₅ (1–10 ng/mL) was applied to cultured HUVECs. On Western blot, VEGF₁₆₅ stimulated ICAM-1 expression in HUVECs more potently than VEGF₁₂₁ (Fig. 3). The upregulation of ICAM-1 by VEGF was inhibited with a neutralizing antibody directed against VEGFR2 (30 ng/mL).

Expression of VEGF Receptors on Leukocytes

The expression of VEGF receptors on mouse leukocytes at the mRNA level was investigated by RT-PCR (Fig. 4). Pure leukocyte subpopulations were isolated from normal C57BL/6 mice. VEGFR1 was expressed in monocytes and neutrophils, but not in T lymphocytes at the mRNA level. However, only T lymphocytes expressed neuropilin-1. VEGFR2 mRNA was not detected in any of the mouse leukocyte subpopulations examined.

VEGF Induction of Monocyte Migration

To observe the chemotactic activities of the VEGF isoforms, in vitro migration assays were performed with mouse monocytes and T lymphocytes using 48-well microchemotaxis chambers (i.e., Boyden chambers; Neuro Probe). As shown in Figure 5, VEGF₁₆₄ was more effective than VEGF₁₂₀ at inducing the migration of monocytes, but not that of T lymphocytes (data not shown). Monocyte migration was inhibited after preincubation with a mouse VEGFR1-neutralizing antibody (Fig. 5).

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.³⁰ The expression of VEGFR1 was confirmed at the mRNA level; however, VEGFR2 was not detected by RT-PCR (data not shown). Both VEGF₁₂₁ and VEGF₁₆₅ induced tyrosine phosphorylation of VEGFR1 in a dose-dependent manner (Fig. 6). Notably, the phosphorylation of VEGFR1 was greater after the addition of VEGF₁₆₅ versus VEGF₁₂₁.

VEGF Induction of Neovascularization

Corneal inflammation is causally linked to the subsequent development of corneal neovascularization.²⁸ 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, VEGF_{164(165)}} and VEGF_{120(121)}} and determined that they have

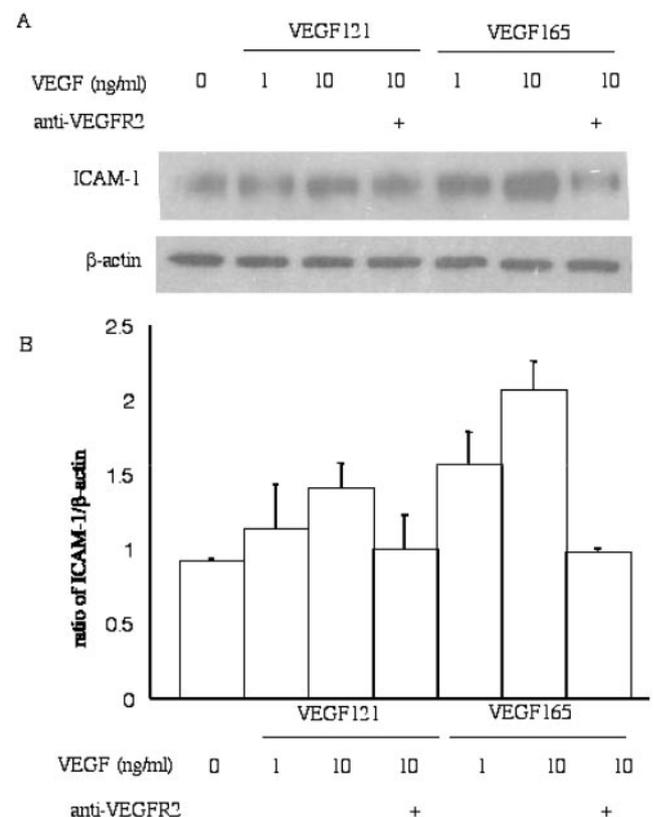


FIGURE 3. ICAM-1 protein levels in VEGF stimulated HUVEC cells. (A) HUVECs were incubated for 6 hours with 1 to 10 ng/mL human recombinant VEGF₁₂₁ or VEGF₁₆₅ after serum starvation for 24 hours. Each lane contained 35 μg of total cellular protein. VEGF₁₆₅ stimulated HUVEC ICAM-1 expression more potently than VEGF₁₂₁. Pretreatment with a neutralizing antibody against VEGFR2 attenuated the VEGF-induced ICAM-1 increases. The results were comparable in three independent experiments. (B) ICAM-1 protein levels were normalized to an internal control (β-actin).

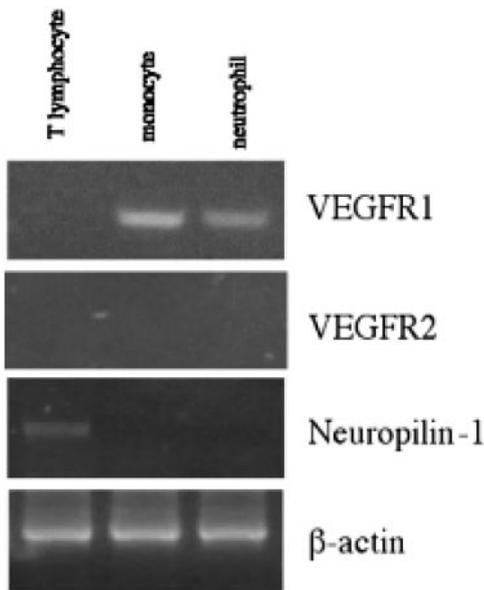


FIGURE 4. VEGF receptor expression in various mouse leukocyte subpopulations. Mouse leukocyte subpopulations were isolated by flow cytometry, with specific antibody markers (CD3 for T lymphocytes, F4/80 for monocytes, and 7/4 for granulocytes), and were examined by RT-PCR. VEGFR1 was found to be expressed in monocytes and neutrophils; however, neuropilin-1 was expressed in only T lymphocytes. None of the mouse leukocyte subpopulations expressed VEGFR2.

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

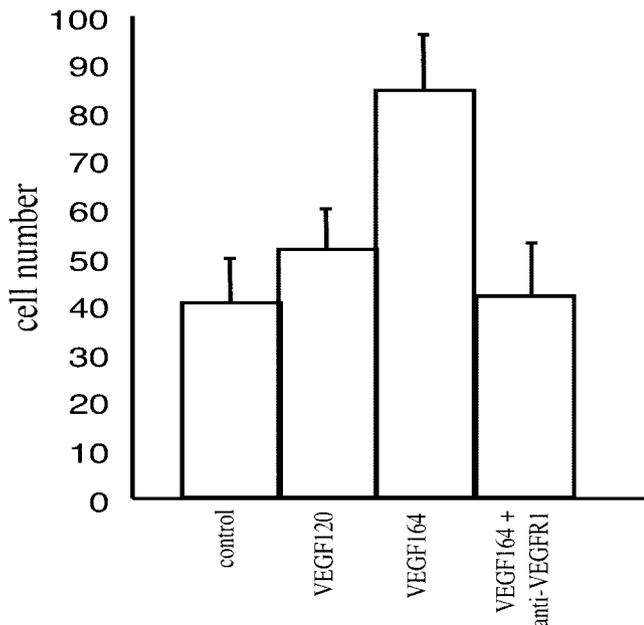


FIGURE 5. VEGF-induced chemotactic migration of peripheral murine leukocytes. VEGF-dependent cell migration was analyzed in murine monocytes and T lymphocytes, which were isolated by flow cytometry, using specific cell surface markers (F4/80 for monocytes or CD3 for T lymphocytes). The cells were exposed to 10 ng/mL of VEGF₁₂₀ or VEGF₁₆₄. In some wells, an anti-VEGFR1-neutralizing antibody (30 μ g/mL) was applied. Error bars, SD.

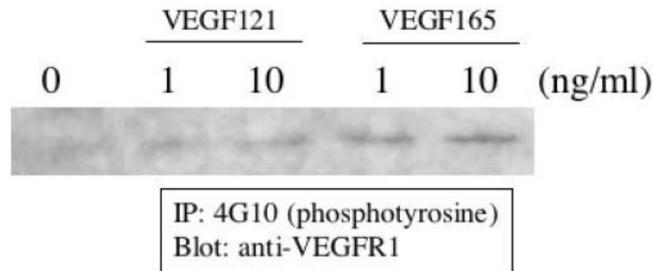


FIGURE 6. VEGFR1 tyrosine phosphorylation in a human T cell line. Induction of tyrosine phosphorylation by the two VEGF isoforms in Jurkat cells was analyzed by immunoprecipitation followed by Western blot analysis. VEGFR1 expressing Jurkat cells (1×10^7 cells/each condition) were stimulated with 1 to 10 ng/mL VEGF₁₂₁ or VEGF₁₆₅ for 2 minutes.

inflammation been delineated. The current data demonstrate that VEGF₁₆₄₍₁₆₅₎ is more potent at inducing corneal inflammation and neovascularization in vivo and identify several of the mechanisms underlying these responses.

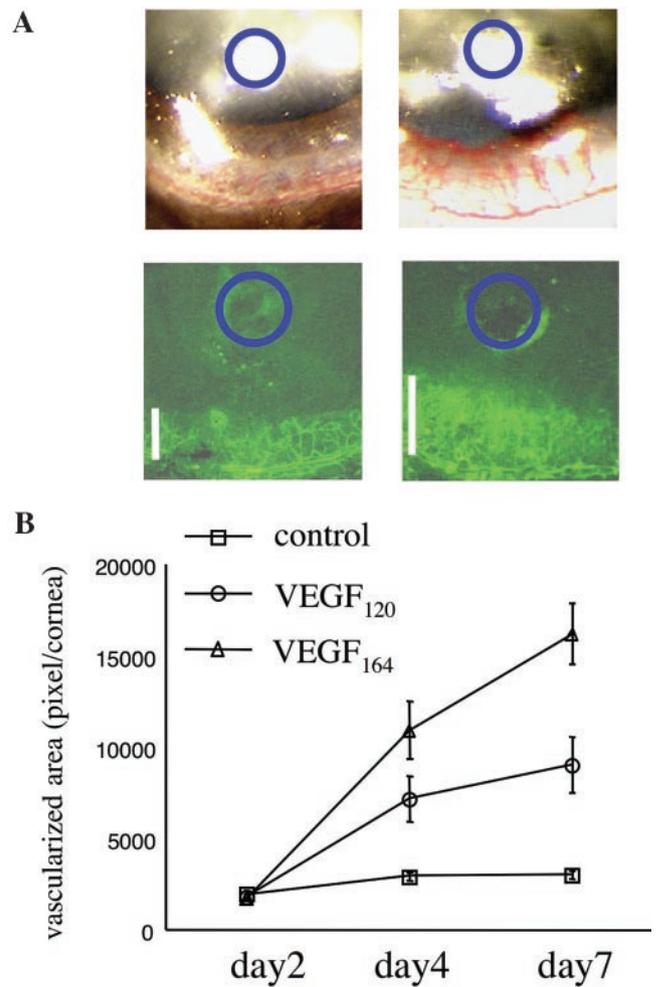


FIGURE 7. Corneal neovascularization after VEGF pellet implantation. (A) Top: representative micrographs of mouse corneas day 4 after the implantation of pellets (circle) containing either mouse VEGF₁₂₀ (left) or VEGF₁₆₄ (right). Bottom: representative corneas studied with isolectin B4 angiography after implantation of either mouse VEGF₁₂₀ (left) or VEGF₁₆₄ (right). (B) Surface area (in pixels) of corneal neovascularization 2, 4, and 7 days after pellet implantation. Error bars, SD.

VEGF₁₂₀₍₁₂₁₎ is a non-heparin-binding acidic protein that is freely diffusible. In contrast, VEGF₁₆₄₍₁₆₅₎ binds to heparin-containing proteoglycan, suggesting that extracellular matrix may represent a major repository of VEGF.³¹ Although it 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 VEGF₁₆₄ resides in the heparin proteoglycan-rich cornea for a longer period than VEGF₁₂₀. Therefore, one potential explanation for the superior potency of VEGF₁₆₄ is its prolonged corneal clearance and hence bioavailability.

VEGF can upregulate ICAM-1 expression on cultured endothelial cells³² as well as retinal endothelial cells in vivo.³³ The current data demonstrate that VEGF₁₆₅ is more potent at inducing ICAM-1 expression than VEGF₁₂₁ (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 VEGF₁₆₅-induced ICAM-1 increases are also consistent with the differential VEGFR2 tyrosine phosphorylation activities observed. Previous work has shown that the EC₅₀ for VEGFR2 phosphorylation is lower with VEGF₁₆₅ than with VEGF₁₂₁ (34.9 pM and 2.09 nM for VEGF₁₆₅ and VEGF₁₂₁, respectively).³⁴

Unlike VEGFR1 on endothelial cells, VEGFR1 on leukocytes is active and serves to trigger chemotactic migration.^{27,35-37} Early studies using radiolabeled VEGF revealed that VEGF bound almost exclusively to the vasculature.³⁸ However, subsequent studies have confirmed the presence of high-affinity VEGF receptors on monocyte-macrophage subpopulations^{27,35-37} and neutrophils.²⁷ 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.³⁹ 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 VEGF₁₆₄₍₁₆₅₎ 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 VEGFR1. 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.³⁶ showed that VEGFR1 tyrosine-kinase-deficient mice produced macrophages deficient in VEGF-dependent migration. The mechanisms underlying the selective migration of leukocytes to VEGF₁₆₄₍₁₆₅₎ may lie in the greater affinity of VEGF₁₆₄₍₁₆₅₎ for VEGFR1. Keyt et al.¹⁷ demonstrated that VEGF₁₆₄₍₁₆₅₎ binds VEGFR1 with greater affinity. Other mechanisms may apply; however, the fact that leukocyte VEGFR1 undergoes greater phosphorylation on binding VEGF₁₆₄₍₁₆₅₎ suggests that the differential affinity for VEGFR1 may underlie the differential activation observed in this study.

Because inflammatory cells enhance the angiogenic process,²⁸ it is not surprising that VEGF₁₆₄₍₁₆₅₎ 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.⁴⁰⁻⁴³ The recruitment of leukocytes to sites of angiogenesis may produce an amplification of local VEGF pro-

duction 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.⁴⁴ Some of these endothelial progenitor cells can be directly incorporated into the growing corneal neovasculature (Usui et al., manuscript submitted). VEGF₁₆₄₍₁₆₅₎ may serve to recruit these cells to sites of angiogenesis differentially, thereby contributing to the VEGF₁₆₄₍₁₆₅₎ induced "inflammation." Last, others have shown that VEGF₁₆₄₍₁₆₅₎ 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.⁴⁵

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 VEGF₁₆₅.²² In an animal model of diabetes, the expression VEGF₁₆₄ greatly predominated over VEGF₁₂₀.²³ Diabetic blood-retinal barrier breakdown was also more potently induced by VEGF₁₆₄ than VEGF₁₂₀.⁴⁶ VEGF₁₆₄ was also selectively upregulated in the ischemic retina in a rat model of rat retinopathy of prematurity.⁴⁶ These data suggest that targeting VEGF₁₆₄₍₁₆₅₎, 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. VEGF₁₆₄₍₁₆₅₎ 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.

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