Growth Factors, Cytokines, Cell Cycle Molecules

Vascular Endothelial Growth Factor-A Is a Survival Factor for Retinal Neurons and a Critical Neuroprotectant during the Adaptive Response to Ischemic Injury

Kazuaki Nishijima, Yin-Shan Ng, Lichun Zhong, John Bradley, William Schubert, Nobuo Jo, Jo Akita, Steven J. Samuelsson, Gregory S. Robinson, Anthony P. Adamis, and David T. Shima

From (OSI) Eyetech, Incorporated, Lexington, Massachusetts

Vascular endothelial growth factor-A (VEGF-A) has recently been recognized as an important neuroprotectant in the central nervous system. Given its position as an anti-angiogenic target in the treatment of human diseases, understanding the extent of VEGF's role in neural cell survival is paramount. Here, we used a model of ischemia-reperfusion injury and found that VEGF-A exposure resulted in a dose-dependent reduction in retinal neuron apoptosis. Although mechanistic studies suggested that VEGF-A-induced volumetric blood flow to the retina may be partially responsible for the neuroprotection, *ex vivo* retinal culture demonstrated a direct neuroprotective effect for VEGF-A. VEGF receptor-2 (VEGFR2) expression was detected in several neuronal cell layers of the retina, and functional analyses showed that VEGFR2 was involved in retinal neuroprotection. VEGF-A was also shown to be involved in the adaptive response to retinal ischemia. Ischemic preconditioning 24 hours before ischemia-reperfusion injury increased VEGF-A levels and substantially decreased the number of apoptotic retinal cells. The protective effect of ischemic preconditioning was reversed after VEGF-A inhibition. Finally, chronic inhibition of VEGF-A function in normal adult animals led to a significant loss of retinal ganglion cells yet had no observable effect on several vascular parameters. These findings have implications for both neural pathologies and ocular vascular diseases, such as diabetic retinopathy and age-related macular degeneration. (*Am J Pathol* 2007, 171:53–67; DOI: 10.2353/ajpath.2007.061237)

Vascular endothelial growth factor-A (VEGF-A), a protein initially identified as an endothelial cell mitogen and vascular permeability factor, has recently been shown to influence neuronal growth, differentiation, and survival. *In vitro*, VEGF-A stimulates axonal outgrowth, improves the survival of superior cervical and dorsal route ganglion neurons, enhances the survival of mesencephalic neurons in organotypic explant cultures, and can rescue HN33 hippocampal cells from apoptosis induced by serum withdrawal.^{1–3} *In vivo*, VEGF-A coordinates the migration of motor neuron soma,⁴ whereas local delivery of VEGF-A prolongs motor neuron survival.⁵ Conversely, low VEGF-A levels have been linked to motor neuron degeneration in both animal models and human disease.^{6,7} Taken together, these results suggest an important role for VEGF-A in neuronal development and maintenance within the central nervous system.

A neuroprotective role for VEGF-A has not been well established; however, previous reports have shown that the receptors for VEGF-A are present in normal retinal neuronal cells,^{8–10} indicating a possible functional role for VEGF-A in the neural retina. Moreover, gene expression studies in the brain, myocardium, and retina also suggest that VEGF-A, as well as other hypoxia-inducible proteins such as erythropoietin, are up-regulated by ischemic preconditioning, a brief ischemic episode that protects various tissues, including neurons, against subsequent prolonged ischemia-reperfusion (I/R)-related damage.^{11–13} Thus, we hypothesized that treatment with VEGF-A might provide neuroprotection in the retina, particularly during ischemic eye disease. In the present study, we investigated both the potential benefit and

K.N. and Y.-S.N. contributed equally to this work.

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Address reprint requests to David T. Shima, R&D Consultants, 75 Lancaster Ave., Barnet, Herts EN4 0ES, UK. E-mail: davesfenestra@yahoo.com.

mode of action of VEGF-A after exposure of the retina to ischemia and explored its potential role as a maintenance factor for retinal neurons. The findings described herein have implications both for the potential use of VEGF-A as a therapeutic in neural pathologies and, importantly, for VEGF-A blockade within the context of ocular vascular diseases such as diabetic retinopathy and age-related macular degeneration.

Materials and Methods

Retinal I/R Model

All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Transient retinal ischemia was induced in the right eye of anesthetized (0.5 mg/kg xylazine hydrochloride and 50 mg/kg ketamine hydrochloride) male pigmented Long-Evans rats (Charles River Laboratory, Wilmington, MA). Pupils were dilated with 0.5% tropicamide (Alcon Laboratories, Fort Worth, TX) and 2.5% phenylephrine hydrochloride (Akorn, Buffalo Grove, IL). After lateral conjunctival peritomy and disinsertion of the lateral rectus muscle, the optic sheath was exposed by blunt dissection and ligated with a 6-0 nylon suture until retinal vessel blood flow ceased as visualized by the operating microscope. After 60 minutes, the suture was removed. Controls underwent a sham surgery without tightening the suture.

After the I/R injury, VEGF120, VEGF164, and placental growth factor-1 (PlGF-1; R&D Systems, Inc., Minneapolis, MN), and VEGF-E (Cell Sciences, Norwood, MA) were diluted in 5 μ l of phosphate-buffered saline (PBS, pH 7.4) and administered intravitreally. The inducible nitric-oxide synthase (iNOS) inhibitor 1400W (3 mg/kg; Cayman Chemical, Ann Arbor, MI) was injected subcutaneously 24 hours before I/R injury. To visualize retinal vascular leakage, anesthetized rats were intravenously injected with 0.3 ml of 1% sodium fluorescein. Images were obtained 5 minutes later with a scanning laser ophthalmoscope (Rodenstock Instruments, Munich, Germany).

For ischemic preconditioning experiments, a 5-minute ischemic episode was induced 24 hours before I/R followed immediately by intravitreal injection of either 5 μ l of VEGFR1/Fc protein (5 μ g, 12.5 pmol; R&D Systems, Inc.) or PBS. For neutralization experiments, 5 μ l (5 pmol) of anti-VEGF antibody (AF-493-NA; R&D Systems Inc.) was injected intravitreally immediately after ischemic preconditioning or I/R.

Detection and Quantification of Apoptotic Cells

At various time points up to 48 hours after I/R, eyes were enucleated and cut into two pieces along the limbus, and the iris and lens were removed. Eyecups were immersed overnight in 4% paraformaldehyde in PBS at 4°C, in 30% sucrose overnight at 4°C, embedded in Tissue-Tek (Miles, Inc., Elkhart, IN), and were frozen on dry ice. Ten- μ m serial sections of the optic nerve were subjected to terminal de-

oxynucleotide transferase dUTP nick-end labeling (TUNEL) staining (DeadEnd Fluorometric TUNEL System; Promega Corp., Madison, WI), according to the manufacturer's instructions but with minor modifications. Tissues were counterstained using 4,6-diamidino-2-phenylindole (DAPI).

Using an epifluorescence microscope (model DMRA2; Leica Microsystems, Bannockburn, IL), images of the stained tissues were captured at $\times 200$ and digitized using a three-color charge-coupled device video camera (Hamamatsu ORCA-ER; Meyer Instruments, Houston, TX). The number of apoptotic cells in the inner nuclear layer (INL) at 24 hours was counted in each 200- μ m-width section at a distance of 1.5 mm from the center of the optic nerve head and was averaged from 12 measurements of six sections per eye. Because of fewer apoptotic cells in the ganglion cell layer (GCL), the number of apoptotic cells in the GCL at 12 hours was counted from each entire retinal section, and the number of apoptotic retinal ganglion cells (RGCs) per retina was averaged from six retinal sections per eye.

Immunohistochemical Analysis

Frozen sections of retinas were obtained along the horizontal meridian through the optic nerve head, TUNEL-stained, and labeled with biotinylated *Griffonia simplicifolia* isolectin B4 (1:100; Vector Laboratories, Burlingame, CA), mouse anti-glutamine synthetase (1:500; Chemicon International Inc., Temecula, CA), rabbit anti-glial fibrillary acidic protein (1:200; DakoCytomation, Carpinteria, CA), or mouse anti-neuronal nuclei (NeuN, MAB377, 1:100; Chemicon International Inc.) or were double-labeled with biotinylated GSL I isolectin B4 and rabbit anti-VEGFR2/flk-1 (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A peptide inhibitor supplied by the manufacturer was used to confirm antibody specificity (data not shown). Fluorescein isothiocyanate-conjugated avidin (1:500; Molecular Probes, Carlsbad, CA) was used to detect the *G. simplicifolia* isolectin B4; anti-mouse secondary antibodies conjugated to Cy3 (1:1000; Jackson ImmunoResearch Laboratories, Inc., Philadelphia, PA), Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 633 (all at 1:500; Molecular Probes) were used to visualize GS and NeuN; and anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (1:500; Molecular Probes) were used to detect glial fibrillary acidic protein and VEGFR2. Images from immunostaining were acquired using a Hamamatsu charge-coupled device camera on a Leica DMRA2 upright microscope with Metamorph software (Universal Imaging Corp., Downingtown, PA).

Histological Evaluation of Retinas after I/R

Fourteen days after I/R and injection of PBS or VEGF120 (20 pmol), rats were sacrificed, and their eyes were enucleated, fixed (1.48% formaldehyde/1% glutaraldehyde in PBS followed by 3.7% formaldehyde), dehydrated, and embedded in paraffin. Eyes were sectioned (2 μ m) along the horizontal meridian through the optic nerve head, stained with hematoxylin and eosin, and examined microscopically ($\times 400$) by a masked investigator. Images were digitized using a

charge-coupled device camera. The average thickness of the inner plexiform layer (IPL), the INL, and the outer nuclear layer (ONL) and the overall retina thickness from the outer to the inner limiting membranes were determined from 10 measurements of five sections from each eye taken 1.5 mm from the optic nerve head center.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for VEGF

Total RNA was extracted from isolated retinas and cDNA was produced by RT-PCR using standard methodology. The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and VEGF were 5'-CCATGG-AGAAGGCTGGGG-3' (sense) and 5'-CAAAGTTGTCAT-GGATGACC-3' (anti-sense); and 5'-ACCTCCACCATGC-CAAGT-3' (sense) and 5'-TAGTTCCTCCGAAACCCTGA-3' (anti-sense), respectively. The size of the amplified cDNA fragments of GAPDH, VEGF120, VEGF164, and VEGF188 were 0.20, 0.43, 0.57, and 0.69 kb, respectively.

Enzyme-Linked Immunosorbent Assay for VEGF

The retina-vitreous-lens capsule complex from enucleated eyes was isolated and homogenized in 150 μ l of lysis buffer (20 mmol/L imidazole HCl, 10 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1% Triton, 10 mmol/L NaF, 1 mmol/L sodium molybdate, and 1 mmol/L ethylenediaminetetraacetic acid, pH 6.8, supplemented with protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). Lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C, and the VEGF levels in the supernatant were determined by enzyme-linked immunosorbent assay (Quantikine; R&D Systems Inc.) normalized to total protein levels (bicinchoninic acid assay; Bio-Rad, Hercules, CA).

Volumetric Blood Flow

Volumetric blood flow was estimated by evaluating the maximum velocity of fluorescein isothiocyanate-labeled erythrocytes through fundus vessels. Whole rat blood was harvested from the abdominal artery, collected in phosphate-buffered physiological salt solution (PBSS, pH = 7.4) with glucose (0.5 mg/ml), and washed repeatedly in PBSS. Erythrocytes were incubated in fluorescein isothiocyanate (50 mg; Sigma-Aldrich, St. Louis, MO) in 0.5 ml of dimethyl sulfoxide for 3 hours at room temperature¹⁴ followed by two washes. At 24 hours after I/R, 5×10^6 erythrocytes in 0.2 ml of PBSS were infused intravenously into control, PBSS-treated, and VEGF120-treated rats. The fundus was observed under scanning laser ophthalmoscope in the 40° field for 3 minutes while images were recorded digitally at a rate of 30 frames/second. Volumetric blood flow in individual veins was estimated from red blood cell velocity (*V*) and microvascular cross-sectional area (πr^2) according to the equation of Gross and Aroesty¹⁵ (volumetric blood flow = $V\pi r^2$), assuming a cylindrical shape for the microvessel.

Retinal Explanation

Retinal explants (1 mm²) without pigmented epithelium were dissected 1.5 mm from the optic nerve head of postnatal day 2 Long-Evans rats (Charles River Laboratory) and placed on culture membranes (24-mm Transwell with 0.4- μ m pore polycarbonate membrane inserts; Corning Inc., Acton, MA) with the GCL facing down. Explants were cultured at 34°C in 5% CO₂ in medium (Neurobasal; Life Technologies, Inc., Gaithersburg, MD) supplemented with 0.5 mmol/L L-glutamine, 2% B27 (Life Technologies, Inc.), 100 U/ml penicillin G, potassium, and 100 μ g/ml streptomycin sulfate,¹⁶ with or without VEGF120 (100 ng/ml). After 24 hours, the explants were harvested and processed for TUNEL staining.

Systemic and Local VEGF Blockade

For analysis of RGC viability after systemic VEGF blockade, recombinant soluble human VEGFR1 (shVEGFR1, 1.33 pmol; R&D Systems, Inc.), goat polyclonal anti-mouse VEGF-A neutralizing antibody (0.133 pmol and 1.33 pmol, AF-493-NA; R&D Systems Inc.), and control (nonimmune goat) IgG (1.33 pmol, AB-108C; R&D Systems, Inc.) were diluted in PBS. These solutions, as well as a PBS control, were injected into the tail vein of normal adult C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) three times per week (Monday, Wednesday, and Friday) for 8 weeks.

For local intraocular VEGF blockade, neutralizing antibody against VEGF-A (1 pmol or 5 pmol, AF-493-NA; R&D Systems Inc.), control IgG (5 pmol, AB-108C; R&D Systems Inc.), and the VEGF165-specific antagonist pegaptanib sodium [1 pmol or 5 pmol; OSI Eyetech, New York, NY] were diluted in PBS. These solutions, as well as a PBS control, were injected intravitreally (3 μ l volume) into eyes of anesthetized (0.5 mg/kg xylazine HCl and 50 mg/kg ketamine HCl) male pigmented Long-Evans rats once per week for 6 weeks.

Labeling and Quantification of Viable RGCs

After 8 weeks of systemic VEGF blockade, or 6 weeks of local VEGF blockade, numbers of viable RGCs were determined by retrograde fluorogold labeling as previously reported.¹⁷ Briefly, mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight) and the skull exposed. The point of injection in the superior colliculus was designated using a stereotaxic device (Stoelting Co., Wood Dale, IL). For systemically treated mice, the point of injection was designated at a depth of 2.0 mm from the brain surface, 2.5 mm behind the bregma in the anteroposterior axis, and 0.5 mm lateral to the midline. For locally treated mice, the point of injection was designated at a depth of 3.5 mm from the brain surface, 6.5 mm behind the bregma in the anteroposterior axis, and 2.0 mm lateral to the midline. A hole was drilled in the skull above the designated coordinates in the right or left hemisphere using a high-speed microdrill (Fine Science

Figure 1. I/R in the rat induces temporally and spatially defined apoptosis of retinal cells. TUNEL staining of retinal sections at 3, 6, 12, 24, and 48 hours after I/R. In sham-operated eyes (depicted here 24 hours after the procedure), there was no observable TUNEL staining. In I/R injured eyes, small numbers of TUNEL-positive cells (green) could be detected in the GCL and the INL at 3 hours (I/R 3 hours) and 6 hours (I/R 6 hours) after injury. The number of TUNEL-positive cells was greatest at 12 hours (I/R 12 hours) and 24 hours (I/R 24 hours) after I/R in the GCL and INL, respectively. At 48 hours (I/R 48 hours), TUNEL-positive cells were detected mainly in the ONL. Cell nuclei are stained blue. Scale bar = 20 μ m.

Tools, Foster City, CA). The superior colliculi were injected with 2.0 μ l (systemic treatment) or 2.5 μ l (local treatment) of 4% Fluoro-Gold (Fluorochrome LLC, Denver, CO) solution in double-distilled water at an injection rate of 0.5 μ l/minute using a Hamilton modified microliter syringe (Fisher Scientific, Palatine, IL) positioned 2 mm below the surface of the brain using the stereotaxic device.

Retrograde labeling of RGCs was allowed to proceed for 3 days on which animals were euthanized with an overdose of carbon dioxide. Eyes were enucleated and fixed with 4% paraformaldehyde, and the retinas were dissected from the ora serrata. Four radial incisions were made in each retina, and the retinas were placed on silane-coated slides. Images of fluorogold-labeled (viable) RGCs were acquired (two images per quadrant) using the $\times 20$ objective of an epifluorescence microscope (Leica DMIRB, Leica) equipped with a Chroma A filter cube (530 nmol/L to 600 nmol/L). Digital images were collected using a charge-coupled device camera (Retiga EXi; Qimaging, Burnaby, Canada). To quantify automatically viable RGCs, images were processed using ImageJ (National Institutes of Health, Bethesda, MD) and Metamorph software. The background of images was subtracted, and they were converted to grayscale and binarized using a threshold value derived by: threshold = (average intensity of image + 1 SD of the intensity of image). Background noise and debris were removed using a one-step erosion procedure, and then all remaining objects were counted. The number of RGCs per image was expressed per mm², and the average number per retinal flat mount was determined.

Assessment of p-Akt in Mouse Retinas

For analysis of p-Akt levels after systemic VEGF blockade, mice were injected every other day through the tail

vein with PBS, goat anti-VEGF-A neutralizing antibody (66.67 pmol, AF-493-NA; R&D Systems Inc.) or goat IgG control (66.67 pmol, AB-108C; R&D Systems, Inc.) for 2 weeks. Retinas were fixed in 4% paraformaldehyde in PBS overnight at 4°C and then incubated with anti-mouse phosphorylated AKT (p-AKT) antibody (1:100; Cell Signaling Technology, Danvers, MA) overnight at 4°C followed by incubation with anti-goat secondary antibody conjugated to Alexa Fluor 488 (1:500, Molecular Probes). Retinas were flat-mounted using Prolong Anti-fade (Invitrogen, Carlsbad, CA). Images at $\times 40$ magnification were obtained as described above. Three to four different images from three different retinas per treatment group generated from two different experiments were collected and quantified using Metamorph software.

Statistical Analysis

Values are presented as mean $\pm$ SEM. Student's *t*-test was used to compare two groups. Analysis of variance was used to compare three or more conditions, with post hoc comparisons tested using the Fisher-protected least significant difference procedure. Differences were considered statistically significant when the probability values were less than 0.05.

Results

Transient Ischemia Induces Neuronal Cell Apoptosis in the Retina

To examine the neuroprotective effects of VEGF-A, we used the retinal I/R model. The time course of apoptosis in the retina was first characterized after 60 minutes of transient ischemia. As shown in Figure 1, there were only a few apoptotic cells per section in both the GCL and INL

at 3 and 6 hours after I/R. The number of apoptotic cells peaked at 12 and 24 hours after reperfusion in the GCL and INL, respectively. Few apoptotic cells were observed in the ONL, an expected finding because cells of the ONL are primarily served by a distinct blood supply, the choroidal vasculature. At 48 hours after I/R, TUNEL-positive apoptotic cells in the GCL and INL diminished, and a few apoptotic cells were detectable in the ONL. In sham-operated retinas, apoptotic cells were absent from all retinal layers.

To identify the TUNEL-positive cells, double-immunofluorescent staining with TUNEL and cell-specific markers was used. Double staining by TUNEL and isolectin B4, a vascular endothelial cell marker, indicated that the apoptotic cells at 12 and 24 hours after I/R were not vascular endothelial cells (Figure 2A). Furthermore, TUNEL-positive cells did not co-localize with cells expressing glutamine synthetase, a Müller cell marker (Figure 2B), or glial fibrillary acidic protein, an astrocyte marker (data not shown). To confirm that neuronal cells were among the apoptotic population, we used an anti-NeuN antibody, which labels a subset of RGCs and various neuronal cells in the INL but does not label photoreceptors.¹⁸ In contrast to endothelial and glial cell markers, numerous TUNEL-positive cells co-localized with cells expressing NeuN in both the GCL and INL at 24 hours after I/R (Figure 2C); however, not all apoptotic cells were NeuN-positive, and not all NeuN-positive neuronal cells were apoptotic in the I/R retina. Together, these results suggest that a significant number of the apoptotic cells resulting from I/R are neuronal cells of the retina and are consistent with reported data.¹⁹

Exogenous VEGF-A Rescues Retinal Cell Apoptosis Induced by Ischemic Injury

Intraocular injection of recombinant murine VEGF-A just after reperfusion reduced neuronal cell apoptosis in the retina in a dose-dependent manner. At 24 hours after reperfusion, the total number of apoptotic cells was reduced compared with the vehicle-injected group by 51.2% ($P < 0.01$, $n = 6$) and 84.6% ($P < 0.01$, $n = 5$) with 20 pmol and 40 pmol of the VEGF120 isoform, respectively (Figure 3, A and B). In the GCL, 20 pmol of VEGF120 also showed a protective effect 12 hours after ischemic insult (Figure 3, D and E; $P < 0.01$, $n = 5$). Injection of 20 pmol and 40 pmol of the VEGF164 reduced the total number of apoptotic neuronal cells in the retina by 46.7% ($P < 0.01$, $n = 6$) and 65.0% ($P < 0.01$, $n = 4$), respectively (Figure 3, A and C). The slightly diminished potency of VEGF164 as a neuroprotectant at the higher dose could be related to the accompanying increase in edema and hemorrhage observed (see below). At 48 hours after reperfusion, when apoptosis is greatest in the ONL, neither VEGF120 nor VEGF164 had a significant protective effect (data not shown). Together, these data demonstrate that exposure to either of the two most prevalent VEGF-A isoforms is effective in protecting neuronal cells in both the GCL and the INL after retinal I/R injury. Still, VEGF164-treated eyes after ischemia showed

obvious signs of disseminated intraretinal hemorrhages, suggesting an increase in vascular leakage caused by the VEGF164 treatment whereas no retinal hemorrhage was detected in the VEGF120-treated eyes (Supplemental Figure S1, see <http://ajp.amjpathol.org>). These findings are consistent with previously published work demonstrating significant differences in the potency of the VEGF-A isoforms in terms of vascular permeability and ability to induce retinal inflammation and damage.²⁰ Thus, because we were interested in the role of VEGF in neuroprotection, only VEGF120 was used for the remaining experiments in this study.

Exogenous VEGF-A Reduces Long-Term Ischemia-Induced Retinal Damage

Histological changes in the retina were examined at 14 days after the 60-minute retinal I/R injury. I/R caused destruction of retinal structures, resulting in decreased retinal thickness, tissue edema in the IPL, and reduction in the number of retinal cells in the GCL and INL (Figure 4A). Furthermore, the retinal thickness from inner limiting membrane (ILM) to INL was $39.1 \pm 3.6 \mu\text{m}$ ($n = 5$) in PBS-treated retinas after ischemia compared with $88.0 \pm 5.3 \mu\text{m}$ ($n = 5$) in sham-operated retinas (Figure 4B), suggesting that substantial tissue damage in the retina after I/R injury occurs between the ILM and INL. Importantly, treatment with 20 pmol of VEGF-A immediately after the ischemic injury resulted in ILM to INL thickness of $56.0 \pm 5.9 \mu\text{m}$ ($n = 5$) at 14 days, a significant improvement compared with PBS-treated retinas ($P < 0.05$) (Figure 4B).

The Role of Volumetric Blood Flow in VEGF-Mediated Neuroprotection

Having demonstrated the potent neuroprotective properties of VEGF-A within the neural retina, we sought to investigate the potential mechanisms of these effects. It is well established that increasing volumetric blood flow to neuronal tissues can enhance neuroprotection.^{21,22} Because VEGF-A can increase blood flow in tissues by inducing vessel dilation, we next sought to determine whether an increase in blood flow to the retina might contribute to the VEGF-A neuroprotective effects. Because blood circulating in the retinal microvasculature exits the retina via the major retinal veins, the total amount of blood in the retina correlates with the diameter of these veins and the velocity of blood flow within. The change in vein diameter was measured at 24 hours after I/R injury. In the normal retina, the average vein diameter was $40.6 \pm 0.4 \mu\text{m}$ ($n = 9$) (Figure 5, A and B). The major veins were significantly dilated to $58.5 \pm 1.9 \mu\text{m}$ ($n = 6$, $P < 0.01$) in vehicle-treated retinas after ischemia whereas treatment with 40 pmol VEGF-A in the retina after ischemia further dilated the major retinal veins to $62.7 \pm 1.4 \mu\text{m}$ ($n = 7$, $P < 0.05$) (Figure 5, A and B). Thus, both I/R and VEGF-A treatment caused dilation of major veins in the retina, probably increasing blood flow.

A Merge: Isolectin B4 / TUNEL / DAPI

B Merge: glutamine synthetase / TUNEL / DAPI

C NeuN

Merge: NeuN / TUNEL

Merge: NeuN / TUNEL / DAPI

Merge: NeuN / TUNEL / DAPI

Figure 2. I/R injury in the rat induces retinal neuronal cell apoptosis, as demonstrated by co-localization of TUNEL staining with a neuronal cell marker. **A:** At 12 hours (I/R 12 hours) and 24 hours (I/R 24 hours) after injury, TUNEL staining (green) did not co-localize with isolectin B4 (red), an endothelial cell marker. **B:** At 12 hours (I/R 12 hours) and 24 hours (I/R 24 hours) after injury, TUNEL staining (green) did not co-localize with glutamine synthetase (red), a marker of retinal glial cells (Müller cells). **C:** At 24 hours (I/R 24 hours) after injury, TUNEL staining (green) co-localized with NeuN, a neuronal cell marker (red) in both the GCL and the INL. Cell nuclei are stained blue (DAPI). Scale bars = 50 μ m.

Figure 3. Both VEGF120 and VEGF164 prevented retinal cell apoptosis resulting from I/R injury in the rat. **A:** Eyes injected immediately after I/R injury with either VEGF120 or VEGF164 had far fewer TUNEL-positive cells (green) in the INL 24 hours later compared with eyes injected with PBS. Quantification of TUNEL-positive cells per 200 μm of retina revealed that both VEGF120 (**B**) and VEGF164 (**C**) produce a significant and dose-dependent reduction in TUNEL-positive cells in the INL 24 hours after I/R. VEGF120 also significantly reduced the number of TUNEL-positive cells in the GCL at 12 hours (**D, E**). At least five animals were used per treatment condition. Cell nuclei are stained blue. * $P < 0.05$ and ** $P < 0.01$. Scale bars = 20 μm .

To estimate the blood volume flow of retinal veins, the mean velocity of erythrocytes was determined. Erythrocytes were fluorescently labeled and then immediately infused into the tail vein of recipient rats. Labeled erythrocytes were visible as distinct fluorescent dots circulating in the retinal vessels. Calculation of the change in blood flow of each vein demonstrated that the total blood volume flow of vehicle-treated retinas after ischemia increased to 145% of sham-operated retinas ($P < 0.01$) (Figure 5C). In addition, total volumetric blood flow of VEGF-treated retinas after ischemia increased to 169% of sham-operated retinas and to 117% of vehicle-treated retinas ($P < 0.05$) (Figure 5C). Together, these data

suggest that I/R causes a significant increase in volumetric blood flow to the retina and that VEGF-A treatment further increases blood flow, although by a modest amount.

In an additional examination of the role of blood flow in protecting retinas following ischemia-induced apoptosis, we blocked the function of iNOS, a downstream mediator of VEGF-A signaling that is a potent vasodilator.²³ Treatment with 1400W, an inhibitor of iNOS, reduced the protective effect of VEGF-A in the retina after ischemia by ~50% (Figure 5D). These data further suggest that an increase in blood flow may be partially responsible for VEGF-mediated neuroprotection in the retina.

Figure 4. VEGF120 provides partial protection against retinal thinning after I/R injury in the rat. **A:** Eyes in which I/R was induced showed a decrease in retinal thickness 14 days after injury compared with sham-operated eyes. The INL decreased dramatically in thickness, and edema was apparent in the IPL, which is located between the INL and the GCL. **B:** Quantification of the thickness of the retina (measured from the border of the ILM to the INL) revealed that administration of VEGF120 significantly reduced thinning of the retina when compared with PBS. At least five animals were used per treatment condition. ** $P < 0.01$. Scale bar = 20 μm .

Figure 5. Retinal blood flow in the rat retina is elevated after I/R and further increased on treatment with VEGF120. **A:** Representative images of the major retinal vessels. Both arteries and veins were dilated 24 hours after I/R compared with the sham-operated eyes. Treatment with VEGF120 (40 pmol) seemed to increase the vasodilation when compared with PBPSS. **B:** Quantification of the diameter of major veins revealed a significant increase in vein diameter after I/R compared with sham; treatment with VEGF120 (40 pmol) further increased vein diameter. **C:** I/R and treatment with VEGF120 (40 pmol) significantly increase volumetric blood flow (calculated from vein diameter and velocity of flowing erythrocytes; see Materials and Methods). **D:** VEGF120 (40 pmol) reduced the number of apoptotic cells in the INL 24 hours after I/R injury; administration of the iNOS inhibitor 1400W reduced the protective effect of VEGF120 from ~84 to ~50% ($P < 0.05$). All treatment groups included at least five animals. One-way analysis of variance with a post hoc Bonferroni test was applied to all groups in each experiment. * $P < 0.05$ and ** $P < 0.01$. Scale bar = 200 μm.

Direct Neuroprotection by VEGF-A in Retinal Explant Culture

VEGF-mediated neuroprotection in the central nervous system has been attributed to both blood flow effects and the direct effect of VEGF-A on neuron survival. To determine whether VEGF-A has direct effects on neurons independent of blood flow, we used a retinal explant culture model. The retina of a postnatal day 0 (P0) rat is avascular. The superficial vascular layer of the retina first starts to spread outward from the optic nerve disk and parallel to the retinal surface at P0, and the developing vessels reach the edge of the retina at around P9 (Figure 6A).²⁴ At P2, the peripheral region of the retina still lacks vasculature; a portion of this region was used for *ex vivo* culture studies (Figure 6A, square box). In retinal explants fixed immediately after dissection, only a few apoptotic cells were seen in the GCL (Figure 6B, top). The number of TUNEL-positive apoptotic cells as well as the total number of DAPI-stained cells in the GCL were counted in 22 sections from four explants (four rats); only a few apoptotic cells were detected in the fresh retinal explants ($n = 2248$ cells examined) (Figure 6C, control). After 24 hours of culture, 14 sections from four explants (four rats) were examined ($n = 1342$ counted cells). The proportion of apoptotic cells increased significantly ($P <$

0.01) [Figure 6, B (middle) and C (no treatment)]. This effect was probably attributable to the loss of neurotrophic factors after disturbance of axonal transport from axon terminals to neuronal cell bodies in the explants. The addition of 100 ng/ml VEGF120 in the culture medium significantly rescued the apoptotic neuronal cells in the GCL at 24 hours (four rats, four explants, 11 sections, $n = 1366$ total cells counted) when compared with the no-treatment control ($P < 0.01$) [Figure 6, B (bottom) and C]. These results suggest that VEGF-A has a direct neuroprotective effect on retinal neurons independent of changes in retinal blood flow.

VEGFR2 Involvement in VEGF-Mediated Neuroprotection

To determine which VEGF receptor(s) mediates the neuroprotection effect observed in the I/R model, we used two additional members of the VEGF family of proteins that are specific agonists of the two VEGF receptor tyrosine kinases. PlGF-1 and VEGF-E, which selectively stimulate VEGFR1 and VEGFR2, respectively, were administered to the retina immediately after reperfusion, and the number of apoptotic cells was determined after 24 hours. VEGF-E, although not as potent as VEGF120 or

Figure 6. VEGF120 inhibits apoptosis in rat retinal explant cultures. **A:** Lectin staining of the developing retinal vasculature [postnatal day 1 to postnatal day 9 (P1 to P9)]. **Arrows** indicate the leading edge of the developing vasculature. The **inset** indicates the 1-mm² region of avascular retina used for the explant culture. **B:** TUNEL-positive cells (green) in the GCL of the retina immediately after explant from the retinal flatmount (top, control) and after culturing for 24 hours with no treatment (middle), or with the addition of 100 ng/ml VEGF120 (bottom). Cell nuclei are stained blue. **C:** Quantification of the percentage of apoptotic cells in the GCL as determined by dividing the number of TUNEL-positive cells by the number of DAPI-stained cells. All treatment groups included at least four animals. ** $P < 0.01$. Scale bars: 1 mm (**A**); 10 μ m (**B**).

VEGF164, significantly suppressed apoptosis in the retinal neurons (49.9%, $P < 0.05$) whereas PlGF-1 had no neuroprotective effect (Figure 7, A and B). These results demonstrate that VEGFR2, not VEGFR1, is involved in VEGF-mediated neuroprotection in the retina. Next, the expression of VEGFR2 in retinas after ischemia was determined by immunolocalization. VEGFR2 was highly expressed in vascular endothelial cells, which were also isolectin B4-positive (Figure 7C). Interestingly, isolectin B4-negative cells, including neuronal cells in both the GCL and the INL of retinas after ischemia, also expressed VEGFR2 whereas photoreceptor cells in the ONL were negative for VEGFR2 expression (Figure 7C). This VEGFR2 expression pattern is consistent with the observation that VEGF-A treatment did not rescue cell death in the ONL of the retina after

ischemia and further suggests that VEGF-A may have a direct neuroprotective effect on VEGFR2-expressing neuronal cells in the GCL and INL.

Neuroprotective Effect of Ischemic Preconditioning in the Retina and the Role of VEGF-A

Finally, given both the potency of VEGF-A as an exogenously administered neuroprotectant and its well-characterized regulation by hypoxia and retinal ischemia,²⁵ we investigated a potential role for VEGF-A as an endogenous neuroprotectant in the setting of ischemic preconditioning, a brief, sublethal ischemic insult, which protects neuronal cells from a subsequent ischemic event. First, to determine the expression levels of VEGF-A after preconditioning and I/R, semiquantitative RT-PCR and VEGF-A protein enzyme-linked immunosorbent assay assays were used. The mRNA expression levels of both VEGF120 and VEGF164 were increased at 3 hours after ischemic preconditioning, and the up-regulation lasted for at least 24 hours (Figure 8A, lanes 1 to 3). The VEGF188 isoform was not detectable in this experiment, probably attributable to its low levels of expression in the retina. Ischemic preconditioning followed by I/R also increased both VEGF120 and VEGF164 mRNA levels at 3 hours after I/R, and the up-regulation lasted for 12 to 24 hours (Figure 8A, lanes 4 to 7). Enzyme-linked immunosorbent assay data confirmed that VEGF-A protein levels were increased in the ischemic preconditioned retinas, in the I/R retinas, and most dramatically in the ischemic preconditioned retinas with subsequent I/R as compared with sham-operated retinas ($P < 0.05$) (Figure 8B). These results indicate that ischemic preconditioning and I/R can both induce VEGF-A expression in the retina and demonstrate that VEGF-A levels are highest after I/R follows ischemic preconditioning.

A role of VEGF-A in ischemic preconditioning-mediated neuroprotection was also examined after I/R injury. Ischemic preconditioning reduced apoptosis of neuronal cells at 24 hours after I/R by 75.1% ($P < 0.01$) compared with the ischemic group without ischemic preconditioning (Figure 8, C and D). The anti-apoptotic effect of ischemic preconditioning was reversed by VEGF-A inhibition using a soluble receptor antagonist, VEGFR-1/Fc ($P < 0.05$) (Figure 8, C and D), at 12.5 pmol immediately after ischemic preconditioning. Because VEGFR1/Fc also binds to PlGF-1 and VEGF-B, an anti-VEGF-A neutralizing antibody that blocks all VEGF isoforms was used in a similar experiment. Injection of even low levels (5 pmol, a dose limited by the low concentration of the antibody preparation) of the VEGF-A neutralizing antibody right after ischemic preconditioning significantly reduced the protective effects ($P < 0.01$) (Figure 8E), whereas injection of the antibody right after I/R, when enzyme-linked immunosorbent assay data demonstrate peak VEGF-A levels, abolished the protective effects ($P < 0.01$) (Figure 8E). Taken together, these results indicate that VEGF-A is

Figure 7. VEGF receptors are involved in inhibiting rat retinal apoptosis after I/R injury. **A:** VEGF-E (40 pmol), a VEGFR2 ligand, but not PlGF-1 (40 pmol), which does not bind VEGFR2, reduced the number of TUNEL-positive cells (green) in the INL of eyes 24 hours after I/R. **B:** Quantification of the number of TUNEL-positive cells in the INL of eyes 24 hours after I/R. Only VEGF-E showed a statistically significant decrease in TUNEL-positive cells ($P < 0.01$). All treatment groups included at least five animals. **C:** Double labeling of VEGFR2 and isolectin B4, an endothelial cell marker, in the retina after ischemia showing isolectin B4-labeled vessels in the GCL, IPL, and INL (red) and VEGFR2 (green). Most retinal cells in the GCL and INL expressed VEGFR2 whereas neuronal cells in the ONL did not. VEGFR2-positive cells were also observed in the layer above the cells in the GCL, which may correspond to nerve fibers of the retina. Cell nuclei are stained blue. $**P < 0.01$. Scale bars = 20 μm .

required for ischemic preconditioning-mediated retinal neuroprotection.

A Maintenance Role for VEGF-A in Normal RGC Survival

Our findings on the neuroprotective role of VEGF-A in the adaptive response to ischemia raise the possibility that VEGF-A could be involved in the maintenance and survival of normal retinal neurons. Indeed, within the central nervous system it has already been shown that a chronic decrease in endogenous VEGF-A levels is linked to an increased risk of motor neuron degeneration in amyotrophic lateral sclerosis, or Lou Gehrig's disease.^{6,7} Moreover, significant levels of VEGF-A and its cellular receptors have been detected in the normal adult retina, with the latter being present on RGCs.^{26–28} To investigate a role for VEGF-A in the maintenance of retinal neurons, animals were exposed

to several different antagonists, and the numbers of viable RGCs were determined by fluorogold retrograde labeling. Significant RGC loss was observed after systemic blockade of VEGF-A in adult mice for 8 weeks using soluble human VEGFR1 (shVEGFR1) (Figure 9A). This antagonist was devoid of an Ig Fc region and thus was unlikely to elicit an immune cell effector response. To additionally substantiate that the loss of RGCs was VEGF-specific, two different neutralizing anti-VEGF antibodies were used and led to similar results as that with the soluble receptor (Figure 9B and data not shown). Examination of optic nerve segments and quantitation of dead axons using paraphenylenediamine staining confirmed the findings provided by direct RGC counts (Supplemental Figure S2, see <http://ajp.amjpathol.org>).

To exclude further the possibility that the loss of RGCs was attributable to a systemic anti-VEGF epiphenomena, we performed several additional experiments. The effective doses of the antagonists used in this

Figure 8. Increased expression of VEGF120 and VEGF164 plays a direct role in the anti-apoptotic effects of ischemic preconditioning in the rat retina. **A:** Retinal VEGF mRNA expression after induction of I/R with (top row, labeled IP) or without (second row, labeled I/R) ischemic preconditioning; mRNA samples for semiquantitative RT-PCR analysis were obtained from retinas from sham-operated eyes (column 1), at 3 hours after 5-minute ischemic preconditioning (column 2), at 24 hours after ischemic preconditioning (column 3), at 3 hours after 60-minute ischemia with ischemic preconditioning (column 4), at 6 hours after 60-minute I/R with ischemic preconditioning (column 5), at 12 hours after 60-minute I/R with ischemic preconditioning (column 6), and at 24 hours after 60-minute ischemia with ischemic preconditioning (column 7). Two bands show the expression of VEGF120 and VEGF164 mRNA. Up-regulation of VEGF120 and VEGF164 mRNA by ischemic preconditioning was observed at 3 hours and lasted until the 24-hour time point. **B:** Retinal VEGF protein levels after I/R injury were significantly up-regulated by ischemic preconditioning ($P < 0.05$). **C:** TUNEL staining (green) in retinal cross sections 24 hours after ischemic injury with or without ischemic preconditioning. Mice were injected intravitreally with PBS or with VEGFR1/Fc (5 μ g, 12.5 pmol), a VEGF antagonist. Cell nuclei are stained blue. **D:** Ischemic preconditioning dramatically reduced induced apoptosis subsequent to I/R injury ($P < 0.01$). Blockade with VEGFR1/Fc protein significantly reduced the benefit of ischemic preconditioning ($P < 0.05$). Three to six animals were used per group. **E:** Intravitreal injection of an anti-VEGF-A neutralizing antibody (anti-VEGF, 5 pmol) after ischemic preconditioning (IP + anti-VEGF + I/R) significantly reduced the protective effect of ischemic preconditioning, whereas antibody treatment after I/R (IP + I/R + anti-VEGF) nearly abolished the protective effect of ischemic preconditioning ($P < 0.01$). At least three rats were used per group; all data represent mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$.

study were significantly less than those previously reported to cause loss of capillaries and endothelial fenestrae in highly VEGF-dependent vascular beds.²⁹ Histological and ultrastructural examination of kidney, adrenal cortex, thyroid, and pancreas confirmed no deleterious action of the systemic VEGF-A blockade on capillary density and differentiation (Supplemental Figure S3, see <http://ajp.amjpathol.org>). In addition, systemic VEGF-A antagonists did not alter intraocular pressure (Supplemental Figure S4, see <http://ajp.amjpathol.org>), suggesting that RGC loss was not the result of a treatment-induced glaucoma. Next, in support of a local role for VEGF-A in RGC survival, the levels of activated p-Akt, a downstream target of VEGF-A signaling involved in cell survival, were shown to be

significantly reduced in the retina after the administration of a systemic VEGF-A antagonist (Figure 9C). Finally, repeated intravitreal injection of low-dose neutralizing VEGF antibodies in the adult rat throughout a 6-week period caused a similar decrease in the number of viable RGCs. In contrast, no RGC loss was observed after administration of a control IgG or the selective VEGF-A inhibitor, pegaptanib sodium (Figure 9D). This antagonist inhibits the pathological VEGF165 isoform and spares VEGF120,³⁰ which is presumably sufficient to support normal RGC survival. Taken together, these results strongly suggest that endogenous retinal VEGF-A plays an important role in maintaining the viability of RGCs, potentially through the Akt pathway.

Figure 9. VEGF-A is required for the maintenance of the normal RGCs in the retina. **A:** Mice treated systemically three times per week for 8 weeks with shVEGFR1 had a reduction in the number of viable RGCs (to ~50% of PBS or IgG controls), as determined by retrograde fluorogold labeling. The number of mice used per group is indicated within each bar; data represent mean $\pm$ SEM. ****P** < 0.01. **B:** Mice treated systemically with a polyclonal anti-VEGF antibody for three times per week for 8 weeks had a dose-dependent reduction in the number of viable RGCs (to ~50% of IgG and PBS controls with 1.33 pmol anti-VEGF), as determined by retrograde fluorogold transport. The number of mice used per group is indicated within each bar; data represent mean $\pm$ SEM. ****P** < 0.01. **C:** Systemic administration of a polyclonal anti-VEGF antibody on alternate days for 14 days caused a significant decrease in the number of cells expressing p-Akt in the mouse retina compared with IgG controls, as determined by immunostaining. The mean ($\pm$ SEM) number of p-Akt-positive cells in the retina was quantified from at least 10 images from three different eyes per group; administration of anti-VEGF antibody reduced the number of p-Akt-positive cells by ~50% in the anti-VEGF antibody-treated mice as compared with IgG controls. ****P** < 0.01. **D:** Intravitreal injection of rats once weekly with a polyclonal anti-VEGF antibody at 1 pmol or 5 pmol for 6 weeks resulted in a dose-dependent loss of viable RGCs (of ~60% in the high-dose group) compared with IgG and PBS controls, as determined by retrograde fluorogold transport. Pegaptanib injection at both 1 pmol and 5 pmol did not cause any detectable loss of viable RGCs in the rat retina. Number of rat eyes analyzed per group is shown within each bar; data represent mean $\pm$ SEM. Each comparison line represents **P** < 0.01.

Discussion

VEGF-A has long been known to be a survival factor for vascular endothelial cells under duress,³¹ during development,³² and more recently for fenestrated vascular beds in adult animals.²⁹ In addition, investigators have demonstrated that VEGF-A also has direct protective effects on cultured neuronal cells in situations such as *N*-methyl-D-aspartic acid-induced toxicity³³ or ischemic insult.¹ The neuroprotective function of VEGF-A in the retina has not been characterized, despite the fact that VEGF antagonists are being applied widely to combat retinal vascular disease.^{25,34}

Using a model of retinal I/R, we have demonstrated that exogenously administered VEGF-A was a potent anti-apoptotic agent for retinal neurons. In addition, a single VEGF-A administration reduced ischemia-induced alterations to the cellular architecture of the GCL, IPL,

and INL in the retina at 2 weeks after reperfusion injury. We also investigated a model of ischemic preconditioning in the retina, an adaptive protective response triggered by brief ischemia before a prolonged ischemic event. VEGF-A had previously been implicated in ischemic preconditioning associated with myocardial protection and also in cultured central nervous system neurons. We found that ischemic preconditioning up-regulated VEGF-A in the retina and that the elevated VEGF-A played a significant role in the neuroprotective effect of ischemic preconditioning against subsequent ischemic injury.

Although both the 120- and 164-amino acid VEGF isoforms were similarly effective in protecting the retina after ischemia, retinas injected with VEGF164 developed dotted hemorrhage and a significant increase in vascular permeability that were not detected in the retinas treated with VEGF120 (Supplemental Figure S1, see <http://ajp.amjpathol.org>). Previously, Ishida and colleagues²⁰ showed that VEGF164 is significantly more potent than VEGF120 at inducing intercellular adhesion molecule-1-mediated retinal leukostasis and blood-retinal barrier breakdown *in vivo*, and Abumiya and colleagues³⁵ have recently reported that intra-arterial infusion of exogenous VEGF aggravates hemorrhagic transformation at a very early point after reperfusion. Together, these results highlight the prohemorrhagic activity of VEGF164 and indicate that VEGF120 might be the most suitable form of VEGF-A for therapeutic neuroprotection, especially in the context of ischemic disease.

In terms of mechanism, it has been well established that VEGF-A can increase tissue blood flow via nitric oxide induction³⁶ and that increased blood supply in many different ischemic settings has been shown to preserve tissue integrity and function.^{22,37} Indeed, our results confirmed that VEGF-A injection can increase volumetric blood flow in the retina, although only moderately over that induced by I/R alone. The importance of the incremental increase in blood flow on neuroprotection was assessed by inhibiting the activity of iNOS in the I/R injury model, which led to a significant reduction in the effects of VEGF-A. Because VEGF induced only a slight increase in flow, however, and the iNOS inhibitor did not completely abrogate VEGF-mediated neuroprotection, it is possible that VEGF-A has a direct survival effect on neuronal cells of the retina, independent of blood flow. Using *ex vivo* retinal explants, we were able to confirm a direct neuroprotective effect of exogenous VEGF-A in the absence of vessels and blood flow. Together, these observations strongly suggest that VEGF-A is capable of protecting neurons from I/R injury both by increasing the blood flow to assist the damaged tissue and by directly promoting survival of neuronal cells.

Similar conclusions have been reached concerning VEGF-A's role in the setting of motor neuron degeneration. Jin and colleagues¹ reported the first direct neuroprotective effects of VEGF-A *in vitro* and provided evidence for the importance of VEGFR2 signaling in the isolated hippocampal neuron model. Strong expression of VEGFR2 by neuronal cells in the retina provides a plausible mechanism by which VEGF-A could directly

protect neurons against I/R injury. Furthermore, our use of the receptor-specific ligands PIGF-1 and VEGF-E confirmed that VEGFR2 activation is sufficient to trigger retinal neuroprotection. Investigations into VEGF-mediated neuronal survival in the central nervous system have also suggested a role for the VEGF co-receptor neuropilin-1 in mediating neuroprotection effects, although whether VEGF-mediated neuropilin-1 signaling affects vessels, neurons, or both has not been clarified.^{38,39} Although VEGF164 can bind to neuropilin-1-VEGFR2 complexes, an action that is thought to amplify VEGFR2 signaling, VEGF120 does not. Because VEGF120 has neuroprotective effects similar to those of VEGF164 in our model, it is unlikely that neuropilin-1 is required for this effect.

Although exogenous sources of VEGF-A may prove therapeutically useful for promoting neural survival, endogenous VEGF-A too clearly has a role in neuroprotection. Ischemic preconditioning is a well-recognized phenomenon characterized by a strong intrinsic protective effect against a subsequent prolonged I/R in the retina.^{13,40} Various investigators have suggested that ischemic preconditioning triggers hypoxia inducible factor-1-induced target genes, including VEGF and erythropoietin.^{41,42} Moreover, erythropoietin, a protein with erythrogenic, angiogenic, and neuroprotective properties, has been shown to promote retinal cell survival after light-induced damage as well as in a murine model of glaucoma.^{43,44} We found that brief retinal ischemia increased VEGF-A expression and that potent inhibitors of all VEGF-A isoforms, the VEGFR1/Fc fusion protein and an anti-VEGF-A neutralizing antibody, significantly diminished the protective effects of ischemic preconditioning on neurons. Interestingly, I/R alone was also effective in up-regulating VEGF-A protein in the retina yet massive neuronal cell apoptosis was still observed. One potential explanation is that effective neuroprotection requires that VEGF-A levels attain a threshold concentration only observed after ischemic preconditioning and I/R treatments. An alternative explanation is that VEGF-A must be present at sufficiently high levels before an as yet ill-defined critical time point, after which I/R-induced apoptosis is inevitable. Although it is well documented that I/R-related injury occurs within minutes after reperfusion,⁴⁵ our intervention with VEGF-A administered immediately after I/R demonstrates that the critical window for cell rescue extends beyond the I/R event. Clearly, further kinetic studies will be important for identifying the period of time after the onset of I/R-related damage during which VEGF-A can still provide a neuroprotective benefit.

As a protein that is exquisitely regulated in response to changes in oxygen tension, VEGF-A would seem perfectly suited to play a dual role during the adaptive protective responses to hypoxia. Increased VEGF-A levels trigger acute and more prolonged vascular changes, such as increased blood flow and angiogenesis, respectively, to increase blood provision to ischemic tissue. However, in addition to these more indirect tissue protective effects, data from the current study would suggest that increased VEGF-A would also directly trigger survival signals in neurons, thus increasing the probability of tissue preservation.

Perhaps the most surprising finding in this study concerned the reliance of normal RGCs on VEGF-A for survival. Through both direct quantification of RGC numbers and assessment of optic nerve axon viability, we observed a dose-dependent decrease in neuron numbers after VEGF depletion with an antibody that blocks all VEGF isoforms. The requirement of VEGF-A for the maintenance of the normal vasculature and more recently for neurons of the central nervous system has been established.^{46–49} Now within the context of the retina, our results suggest that RGCs may be exquisitely sensitive to VEGF-A depletion compared with other tissues, including the vasculature. This observation is intriguing and perhaps may be explained by the high metabolic demands of the retina compared with other tissues.⁵⁰ Hence, VEGF-A reduction strategies in the retina may be a double-edged sword: inhibition of VEGF-A will probably reduce the edema, inflammation, hemorrhage, and neovascularization associated with retinal vascular diseases such as diabetic retinopathy and age-related macular degeneration; however, depressed VEGF-A levels could also reduce the innate neuroprotective capabilities that directly impact neural cell survival. Interestingly, when the effects of VEGF were blocked with pegaptanib, which does not bind to VEGF120, there was no decrease in retinal RGC viability. If these animal data are predictive of the outcome of the chronic anti-VEGF treatment that may be required to combat ocular vascular disease, future therapeutic strategies may need to refocus on the challenge of normalizing rather than abrogating VEGF-A responses if we are to preserve neurons in the long term. Further studies using ocular models that facilitate the simultaneous study of neovascular pathology and neuronal cell survival seem warranted.

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