Effect of Intravitreal Injection of Bevacizumab on Optic Nerve Head Leakage and Retinal Ganglion Cell Survival in a Mouse Model of Optic Nerve Crush

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Purpose. To evaluate the effect of bevacizumab, a VEGF inhibitor, on optic nerve edema and retinal ganglion cell (RGC) loss in a mouse model of optic nerve crush (ONC).

Methods. Two hundred C57BL/6 wild-type mice were anesthetized. Right ONC was induced in 150 mice, of which half ($n = 75$) received an intravitreal injection of bevacizumab immediately thereafter and half ($n = 75$) did not. The remaining 50 received only bevacizumab. The left eyes served as a control. Findings were analyzed by fluorescein angiography (days 0, 1, 3), histologic and immunohistochemical tests (days 1, 3, 4, 21), and quantitative real-time PCR.

Results. Angiography revealed a reduction in postinjury disc leakage following bevacizumab injection (days 1, 3), confirmed with IgG staining. On PCR, expression of HO-1 and SOD-1 mRNA increased following ONC and further increased with bevacizumab. VEGF gene expression decreased following bevacizumab injection without ONC, remained at baseline after ONC, and increased slightly after ONC+bevacizumab. Histologically, there was a 38% RGC loss 21 days after ONC alone, which dropped to 14% with bevacizumab treatment; it was close to 15% with bevacizumab alone. Mean (SEM) microvascular perfusion in the optic nerve 4 days after ONC was significantly higher in the bevacizumab-treated (85% ± 10%) than the vehicle-treated (33% ± 13%) animals.

Conclusions. Bevacizumab treatment following ONC induction exerts a protective effect, manifested by reduced optic nerve head edema. The underlying mechanism probably involves a lesser interruption of axonal transport. Reduced expression of antioxidative and ischemic genes may contribute to RGC preservation.

Keywords: bevacizumab, optic nerve crush, mouse model, neuroprotection

Nonarteritic anterior ischemic optic neuropathy (NAION) is the most common clinical presentation of acute ischemic damage to the optic nerve.[1] It is associated with edema of the optic nerve head, which compresses the optic nerve axons, disturbs axonal flow, and increases neuronal damage. Most treatments are empirical and presumed to act on the thrombosis or inflammation of the optic head microvasculature or to have a neuroprotective effect via unknown mechanisms.[1–6] In the only class I multicenter prospective study of NAION treatment to date, surgical release of optic nerve head compression proved ineffective.[7–16] There are no class I studies showing benefit from any of the medical treatments attempted, and most of the data derive from retrospective or prospective case series and case reports. The benefit of therapies aimed at secondary prevention of fellow eye involvement also remains unproven.[15,17,18]

Several studies have suggested that agents that inhibit VEGF, a signaling protein that stimulates angiogenesis and increases microvascular permeability, have the potential to reduce vasogenic edema.[19] Accordingly, anti-VEGF agents, such as bevacizumab (Avastin) or ranibizumab (Lucentis), both humanized monoclonal antibodies, are routinely used to treat neovascular macular degeneration, diabetic macular edema, and macular edema associated with other retinal vascular disorders. However, their effect on visual function in NAION is unclear. One patient showed considerable improvement in visual acuity (counting fingers to 20/70) and visual field defects and reduced optic nerve head edema 9 days after treatment with 1.25 mg/0.05 mL bevacizumab, although residual swelling remained after 8 weeks,[20] and four patients experienced visual gain after a single injection of ranibizumab delivered within 2 weeks of NAION onset.[21] By contrast, among five patients treated with intravitreal bevacizumab, visual acuity improved in one and decreased in four, and visual field defects improved slightly in one, remained stable in one, and worsened in three[22]; in one case, NAION developed in the fellow eye. This clinical outcome was consistent with studies of the natural history of the disease showing that the optic edema resolves spontaneously after a

median (25th–75th percentile) of 7.9 weeks (range, 5.8–11.4)[23,24] (longer in diabetic patients). It is also noteworthy that intravitreal injection of bevacizumab for the treatment of choroidal neovascularization[25,26] or diabetic macular edema[27] has itself been associated with acute onset of NAION.

The recent introduction of animal models of NAION has greatly improved the ability of researchers to study the effect of potential therapeutic agents.[28–33] The aim of the present study was to measure the effect of intravitreal injection of bevacizumab on optic nerve edema and retinal ganglion cell (RGC) survival in a mouse model of optic nerve crush (ONC).

Methods

Animal Model

Two hundred C57BL/6 male wild-type mice aged 6 to 8 weeks were obtained from Harlan Laboratories (Jerusalem, Israel). The mice were maintained and handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health guidelines. All animal protocols used in the study were approved by the local Animal Research Committee.

The mice were placed under general anesthesia by intramuscular injection of combined ketamine/xylazine (80 mg/kg and 8 mg/kg, respectively) supplemented by topical ophthalmic anesthesia (proparacaine hydrochloride 0.5%). In 150 of the 200 mice, the right optic nerve was crushed by applying forceps at 2.5 to 3.0 mm posterior to the globe for 7 seconds; this procedure was repeated three times. The procedure is not under direct visualization, but macroscopically the distance was confirmed after enucleation and optic nerve removal.[4]

In half these mice ($n = 75$), ONC was followed immediately by a single intravitreal injection of bevacizumab (75 μg/3 μL; into the right eye) using a Hamilton syringe with a 30-gauge tip (Hamilton, Reno, NV). The other half ($n = 75$) received a saline injection or remained untreated. The remaining 50 mice were injected intravitreally with bevacizumab without undergoing ONC. The left eye of each animal served as a control. Mice were euthanized by carbon dioxide asphyxiation at 1, 3, and 21 days after injury.

The intravitreal injections of bevacizumab were performed in a standardized manner under direct ophthalmoscopic control with an operating microscope (Zeiss Opmi 6S Microscope; Carl Zeiss Microscopy GmbH, Oberkochen, Germany). After penetration of the sclera immediately behind the limbus in a slightly oblique direction, the beveled needle tip of a microsyringe (30-gauge; Hamilton) was placed intravitreally in the retrolental space of the eye. Earlier studies using intravitreal mouse eye injections showed that during placement of the needle, some vitreous outflow occurs due to the beveled configuration, greatly reducing the risk of subsequent drug reflux.[34–36] In our hands, 3 μL can be reliably injected without reflux or apparent postoperative inflammation.[37] Therefore, we injected one eye with up to 3 μL bevacizumab and either injected the contralateral eye with up to 3 μL physiological 0.9% sterile NaCl solution (sham injection) or left it untreated. A search of the literature revealed that the standard vitreous volume of the mouse eye ranges from 7 to 20 μL.[34–36] Thus, because of the preinjection vitreous outflow, the injected volume replaced, but did not add to, the fluid in the eye. When there was any doubt as to the quality or quantity or volume of a particular intravitreal bevacizumab injection, the mouse was excluded from the analysis.

Fluorescein Angiography

To test for vascular leakage in the optic nerve head, fluorescein angiography (FA) was performed immediately after ONC induction and 1 and 3 days later (5 mice in each group at each time point; total 15 mice). The mice were injected intraperitoneally with 0.04 mL 25% sodium fluorescein (AK-Fluor 25% AMP; Akorn, Decatur, IL), and photos were taken with a digital fundus camera (TRC ×50; Topcon, Farmingdale, NY) using a plastic contact lens specialized for mice. To confirm the findings, India ink (Higgins #4418; Sanford Corp., Bellwood, IL) was injected transcardially to 10 of the mice, which were then euthanized by intraperitoneal injection of pentobarbital-based euthanasia solution. The globes were enucleated and prepared for retinal flat-mount study under a light microscope (Fluoview X; Olympus, Tokyo, Japan).

Histological Studies

Hematoxylin and Eosin. Hematoxylin and eosin (H&E) staining was done on day 21 after the intervention. Fifteen mice (five from each ONC group) were terminally anesthetized by CO_2 inhalation. The eyes were enucleated, fixed in 4% formaldehyde, placed overnight in 30% sucrose dissolved in PBS (1×; Biological Industries, Beit HaEmek, Israel) at 4°C, and embedded in optimum cutting temperature compound (Sakura Tissue-Tek, Tokyo, Japan). Cryosections of the globes and optic nerve (6 μm) were mounted on slides and stained with H&E for light microscopy assessment (Fluoview X; Olympus), three consecutive sections on each slide. RGCs were counted in a ×40 magnification field of three consecutive sections of every 7 to 10 slides of each eye, and the mean number was calculated. The counts for the outer and inner nuclear layers were recorded for the same areas to exclude central artery occlusion or total destruction of the retinal layers.

2,3,5-Triphenyltetrazolium Chloride. To evaluate optic nerve infarct size, we used a slightly modified version of a reported technique.[38] The 2,3,5-triphenyltetrazolium chloride (TTC) staining was performed on day 21. Fifteen mice (five in each group) were injected intravitreally with 3 μL TTC and then terminally anesthetized. The eyes and optic nerve were removed and embedded in TTC for 10 minutes at 37°C. TTC is chemically reduced by most dehydrogenase enzymes, precipitating into a bright red water-insoluble compound. Ischemic tissue lacking these enzymes remains a whitish (unstained) color with a clear border with the normal red tissue. Cryosections of the stained optic nerves were obtained and photographed at ×10 magnification (Fluoview X; Olympus).

Luxol Fast Blue. Luxol Fast Blue (LFB) staining of the optic nerves was done on days 1 and 3 after the intervention. Five sections of each nerve were prepared and stained with LFB (for myelin) or LFB combined with neutral red (for cell nuclei) (Edward Gurr, Ltd., London, UK). In brief, longitudinal optic nerve cryosections were washed in water (2 minutes) followed by 100% ethanol (2 minutes), incubated in 0.1% LFB (wt/vol), dissolved in 95% ethanol, and acidified with 0.5 mL glacial acetic acid for 4 hours at 65°C in a sealed container. They were then washed in 100% ethanol (2 minutes), differentiated in 0.05% lithium carbonate solution in water (2–3 minutes), cleared using 100% ethanol followed by xylene, and mounted in a nonaqueous medium. Slides were imaged using a ×10 objective (Fluoview X; Olympus) and then composited (ICE; Microsoft, Redmond, CA) before cropping. LFB stains myelinated axons a bright blue color; pale staining indicates myelin damage or loss.[39,40] The degree of myelin damage was evaluated semiquantitatively and classified as follows: 0, normal LFB intensity; 1, decreased LFB intensity compared with controls (mild damage); 2, decreased LFB intensity and vacuole formation (moderate damage).

Table 1. Primer Sequences Used for the RT-PCR Analysis

Gene	Forward	Reverse
HO-1	CAGGTGTCCAGAGAAGGCTTT	TCTTCCAGGGCCGTGTAGAT
SOD-1	GCCCGGCGGATGAAGA	CGTCCTTTCAGCAGTCACA
VEGF	CACGACAGAAGGAGAGCAGAA	CGCTGGTAGACGTCCATGA
ACTB	TAGGCACCAGGGTGTGATGGT	CATGTCGTCCCAGTTGGTAACA

Apoptosis Assays

Three cryosections of retina and optic nerves, 6 μm each from each eye, were used for TdT-mediated dUTP nick end-labeling assay (TUNEL) (Roche Diagnostics, Mannheim, Germany). TUNEL staining was performed with the fluorescein-tagged apoptosis detection system. Results were analyzed with a fluorescence microscope (Fluoview X; Olympus) at 580 nm wavelength.

Perfusion With Fluorescent Gelatin and Tissue Processing for Optic Nerve Imaging

Fifty mice (20 on day 1 and 30 on day 3, from the ONC-only and ONC+bevacizumab groups) were perfused with fluorescent gelatin using a technique applied in rats,[41] with appropriately reduced volumes and omission of adenosine (apparently unnecessary for optic nerve infusion) from the perfusion solutions. Rhodamine-conjugated bovine serum albumin (BSA) (fraction V; Sigma, St. Louis, MO) was produced using the same technique as for fluorescein-conjugated BSA.[41] Briefly, animals were terminally anesthetized by pentobarbital overdose, and transcardially perfused with a series of solutions: 20 mL heparinized 0.9% saline, 10 mL 2% type A gelatin in saline with rhodamine-conjugated BSA (approximately 0.01 mg/mL), and 2 mL 4% type A gelatin in saline with rhodamine-conjugated BSA. At the end of the perfusion, the ascending aorta was clamped with a hemostat while under pressure, and the animal was immersed in an ice-water bath to congeal the gelatin. The eyes were carefully enucleated to avoid disturbing the gelatin and fixed in cold 4% PBS-buffered paraformaldehyde overnight at 4°C. The gelatin-perfused and embedded optic nerves were cryosectioned in slices of 40 μm thickness and imaged with a Zeiss LSM510 (Carl Zeiss Microscopy GmbH) at a ×40 objective. A vascular filament model of the optic nerve capillaries was constructed using Imaris 7.1 software (Bitplane Corp. AG, Zurich, Switzerland), as previously described.[37] For quantification, optic nerve sections measuring less than 1 mm were selected from behind the globe of both eyes of each animal. Sections from the left optic nerve were used as a control to ensure that the gelatin perfusion was successful and as a reference for the normal vascular density of the animal. The relative fractional vessel volume of the damaged right optic nerve was calculated for each animal according to the following formula: (capillary volume with ONC/volume of tissue imaged with ONC)/(capillary volume without ONC/volume of tissue imaged without ONC).

Immunohistochemistry

Retinal sections were placed on slides and washed with PBS prior to blocking with 2% BSA 0.5% Triton X-100 for 15 minutes. The sections were then incubated with the primary anti-VEGF antibody (1:100; Chemicon International Biotest, Temecula, CA) at 4°C overnight. The slides were again washed with PBS and incubated with the secondary antibodies, goat anti-mouse Alexa fluor 594 (diluted 1:200; Molecular Probes Invitrogen, Carlsbad, CA), at room temperature for 1 hour. The retinal sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes Invitrogen) to reveal cell nuclei. Images were obtained using a conventional fluorescence microscope (Fluoview X; Olympus).

Bevacizumab Detection in Mouse Tissue

To detect bevacizumab in mouse tissue, sections were washed in PBST (PBS with 0.2% Triton X-100; Sigma) for 1 hour, incubated in 1:2000 Cy5-conjugated donkey anti-human IgG (H+L) antibody (minimally cross-reactive, Cat #709-175-149; Jackson Immunoresearch, West Grove, PA) in PBST for 2 hours, washed again in PBST for 1 hour, then mounted using Vectashield with DAPI (Vector Labs, Burlingame, CA), all performed at room temperature. Cy5 labeling was selected to avoid the intense autofluorescence of pigmented mouse retina and optic nerve. Positive and negative control sections were taken from an uninjured companion eye of a randomly selected vehicle-treated animal that had never been injected with bevacizumab. The positive control was created by soaking the optic nerve section in commercially available bevacizumab at room temperature for 30 minutes, followed by fixing without washing in 4% paraformaldehyde at room temperature for 1 hour. The negative control was derived

Table 2. RGC Loss in the Study Groups

	ONC Only, $n = 5$		Bevacizumab Only, $n = 5$		ONC+Bevacizumab, $n = 5$	
	Right Eye	Left Eye	Right Eye	Left Eye	Right Eye	Left Eye
Average no. cells in RGC layer	21.6	35.1	27.6*	32.7	26.9*	30.9
SD	2.7	4.4	2.1	3.0	1.3	1.2
Difference %	38.3		15.6		13.0	

Cryosections of the globes (6 μm) were mounted on slides and stained with H&E for light microscopy assessment (Fluoview X), three consecutive sections on each slide. RGCs were counted in a ×40 magnification field of three consecutive sections of every 7 to 10 slides of each eye, and the mean number was calculated. The counts for the outer nuclear layer and the inner nuclear layer were recorded for the same areas to exclude central artery occlusion or total destruction of the retinal layers. Calculation of % difference = ([average LE − average RE]/average LE) × 100. We corrected the miscalculation in the table.

* Right eyes of bevacizumab-only and ONC+bevacizumab groups had significant preservation of cells in the RGC layer compared with the ONC-only group ($P = 0.014$ and $P = 0.02$, respectively). There was no significant difference between eyes injected with bevacizumab only and the left control eyes of the same animals.

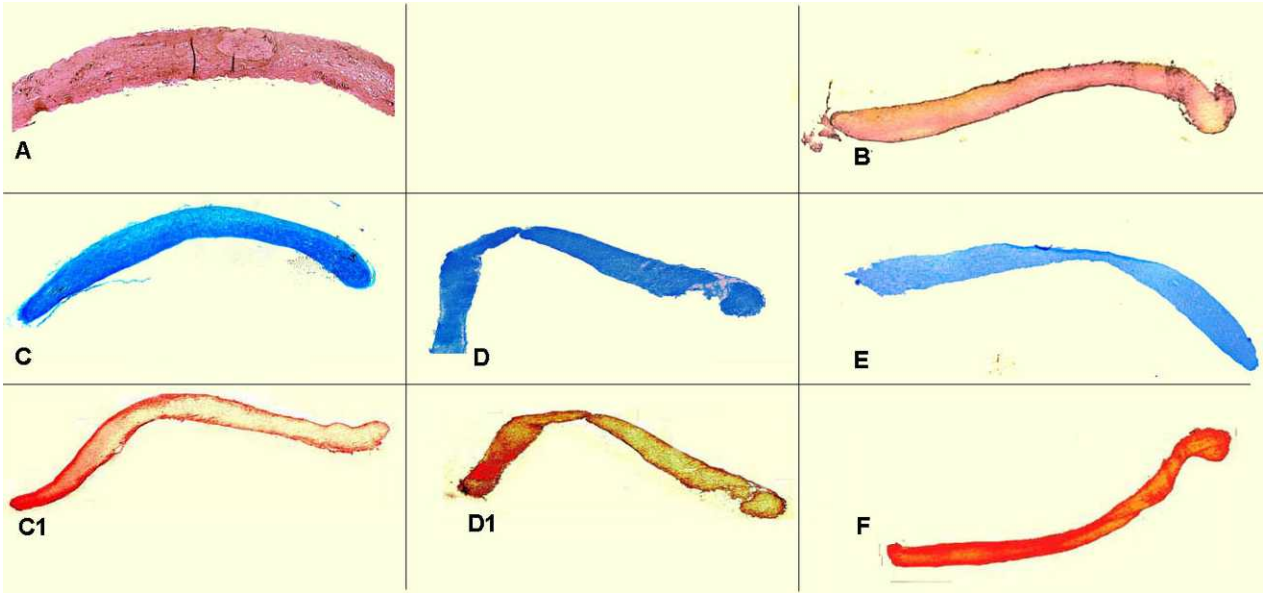

FIGURE 1. Optic nerve infarction and demyelination 21 days after ONC induction. H&E: Sample from a mouse after ONC only showing vacuolization and disruption of the optic nerve structure (**A**) relative to normal nerve in the left (control) eye (**B**). LFB: Sample from a mouse after ONC only showing moderate loss of myelin (**C**) and a mouse after ONC+bevacizumab showing mild loss of myelin (**D**), relative to normal nerve in left (control) eye (**E**). TTC: Sample from a mouse after ONC only showing severe damage along the nerve (**C1**) and a mouse after ONC+bevacizumab similar damage (**D1**), relative to control (**F**).

from the same area of the optic nerve and was incubated in PBS before fixation. The positive and negative controls were subsequently immunostained for human antibody as described above.

Imaging and Image Processing

Imaging was performed on a Zeiss LSM510 confocal microscope (Carl Zeiss Microscopy GmbH) using the standard ×40 objective. Excitation wavelengths were 405 nm for DAPI, 594 nm for rhodamine, and 633 nm for Cy5. For Cy5, illumination and other instrument settings were identical for all sections and retinas imaged. For DAPI and rhodamine, laser illumination was optimized for each section for viewing the cell nuclei and vasculature, which are not compared for staining intensity. Positive and negative control sections were imaged at a lower illumination with the pinhole set to 1 Airy unit, as calculated by the LSM software (Carl Zeiss Microscopy GmbH). Scale bars and color saturation adjustments for non-Cy5 color channels were added using Imaris software (Bitplane Corp. AG). Cropping and color separations were performed using Irfanview software (www.irfanview.com), and ×4 linear palette stretching was performed on the Cy5 channel for ON sections showing the presence of bevacizumab. Palette stretching was not necessary for color separation in the positive and negative controls because of the very large signal.

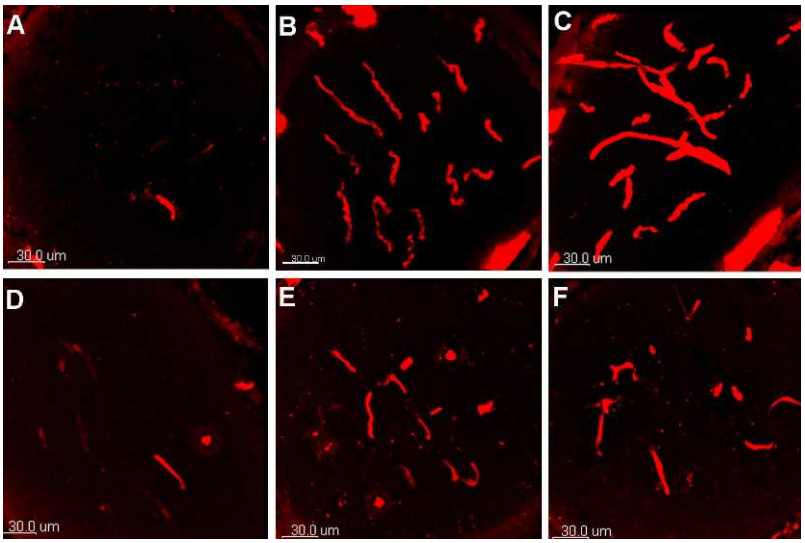

FIGURE 2. Vasculature of the optic nerve head. Samples from two mice after ONC showing loss of optic head vasculature (**A**, **D**) and two mice after ONC+bevacizumab showing preservation of the patent optic head vasculature (**C**, **F**), relative to normal optic nerve head in left (control) eye ([**B**, **E**], respectively).

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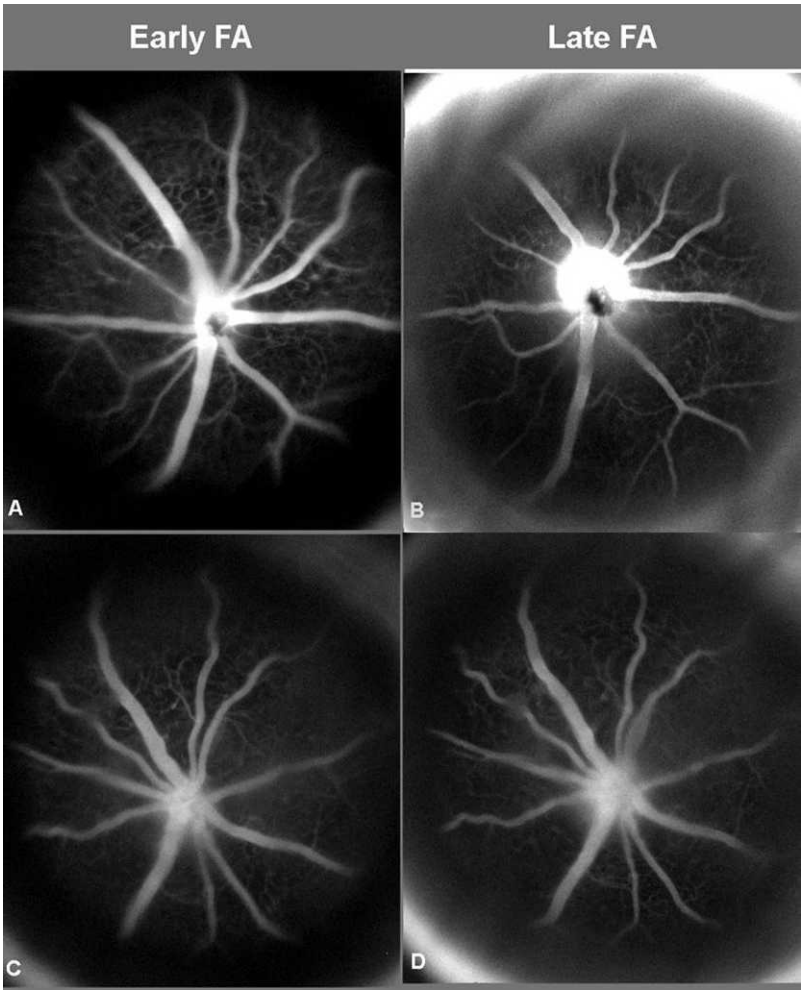

FIGURE 3. Optic nerve head leakage 1 day after ONC: FA study. Samples from two mice on day 1 after ONC showing significant leakage from the optic nerve head (*blurred disc margin*) on early FA (**A**, **C**) and late FA ([**B**, **D**], respectively). Of note, early and late FA are in respect to the time from the fluorescein injection.

Molecular Analysis

Molecular analysis was performed on days 1 and 3. Sixty mice were euthanized (five at each time point from each group, repeated twice), and retinas were dissected and snap-frozen in liquid nitrogen. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol, and then reverse-transcribed into cDNA using random hexamers (Bioline, London, UK) and Moloney murine leukemia virus (M-MLV)-reverse transcriptase (Promega, Madison, WI). Two-stage real-time quantitative polymerase chain reaction (PCR; Sequence Detection System, Prism 7900; Applied Biosystems, Foster City, CA) was applied to evaluate the genes encoding *VEGF*, heme-oxygenase 1 (*HO-1*), superoxide dismutase 1 (*SOD-1*), and mouse beta-actin (*ACTB*; to normalize cDNA input levels) in each group (for primer list, see Table 1). Reactions were performed in a 20-μL volume containing 4 μL cDNA, 0.5 μM each of forward and reverse primers, and buffer included in the Master Mix (SYBR Green I; Applied Biosystems). Duplicate reactions were performed for each gene to minimize individual tube variability, and an average was taken for each time point. Threshold cycle efficiency corrections were calculated, and melting curves were obtained using cDNA for each individual gene assay. PCR cycling conditions consisted of an initial denaturation step of 95°C for 10 minutes followed by 40 cycles of 15-second denaturation at 95°C and 1 minute of annealing and extension at 60°C. Standard curves were obtained using untreated mouse cDNA for each gene assay. The results were quantified using a comparative threshold cycle (Ct) method, also known as the $2^{-\Delta\Delta Ct}$ method,[42] where: $\Delta\Delta Ct = \Delta Ct$ (sample) $- \Delta Ct$ (reference gene). For each treatment, the levels of expression in the cauterized right eye were compared with the untreated eye.

Statistical Analysis

Differences between groups were analyzed by unpaired Student's *t*-test. Significance was set at $P < 0.05$.

RESULTS

Bevacizumab Protection of RGCs After Optic Nerve Injury

ONC caused a 38% RGC loss 21 days after injury. Acute treatment of ONC injury with intravitreal bevacizumab injection led to a significant reduction in RGC loss of 13.0% ($P < 0.05$ Student's *t*-test) (Table 2). Bevacizumab injection to the vitreous without induction of ONC had no significant effect on RGC count, with a mean RGC loss of 15.6% (Table 2).

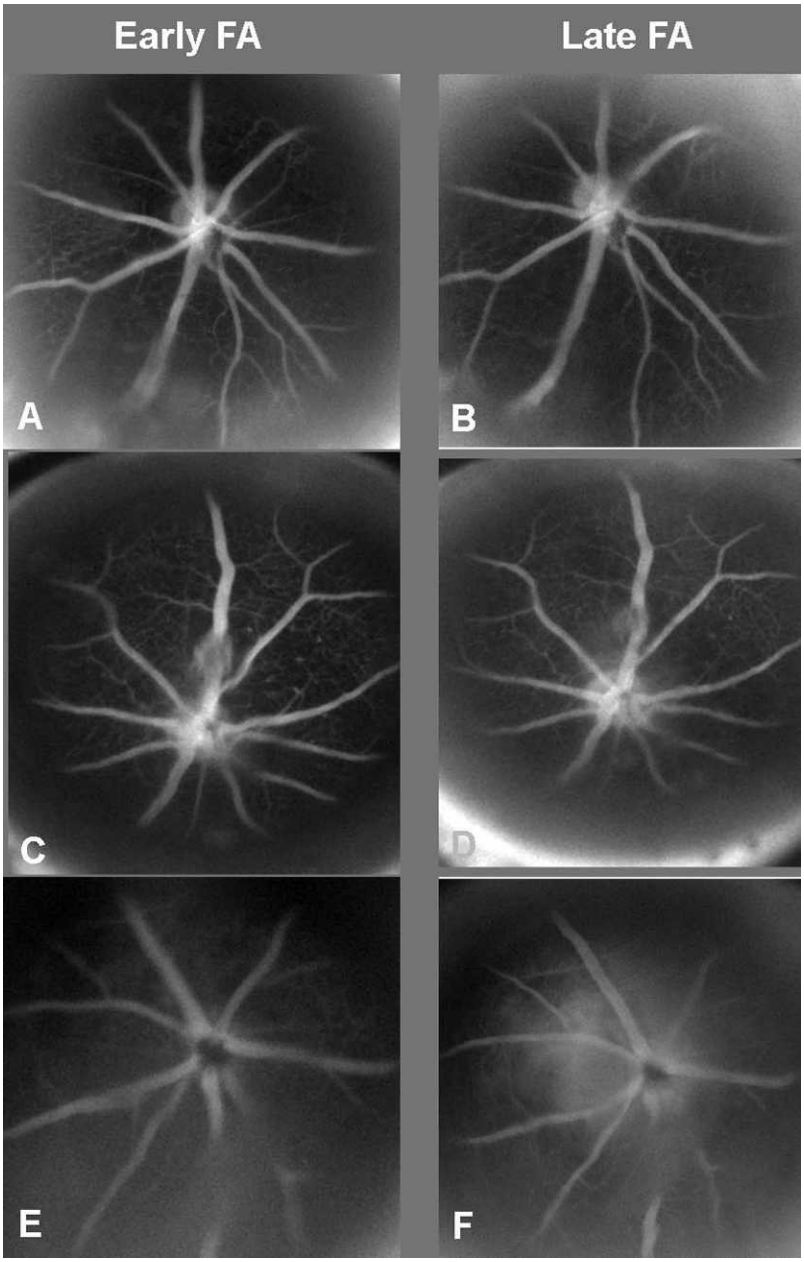

FIGURE 4. Optic nerve head leakage 3 days after ONC+bevacizumab: FA study. Samples from three mice on day 3 after ONC+bevacizumab showing minimal leakage from the optic nerve head on early FA (**A**, **C**, **E**) and late FA ([**B**, **D**, **F**], respectively) following fluorescein injection.

TUNEL staining of the retinas revealed an increased number of apoptotic cells in the RGC layer at 1 and 3 days after ONC compared with the control (left) eyes. On day 1, 43% of the cells in the retinal ganglion cell layer were positive for apoptotic staining in the ONC model, 42% in the bevacizumab-treated ONC, and 6% in the bevacizumab only. On day 3: percentages were 64%, 45%, and 8%, respectively. A small number of apoptotic cells was found in the outer and inner nuclear cell layers in all groups.

Bevacizumab Reduction of Optic Nerve Lesion Area After ONC

At 21 days after ONC, optic nerves showed a large infarct starting in the anterior retrobulbar area and extending toward the chiasm (Fig. 1A, as compared to normal control nerve, Fig. 1B), viewed by TTC staining (Fig. 1C1, as compared to the control, Fig. 1F). Average infarct size (lack of mitochondrial staining) was similar in the ONC mice with or without bevacizumab injection (Figs. 1C, 1C1, 1D, 1D1).

Bevacizumab injection alone, without ONC, did not cause optic nerve infarction or damage. LFB staining revealed moderate myelin loss following ONC induction (Fig. 1C) compared with the normal control nerve (Fig. 1E), and only mild loss of myelin in nerves after ONC+bevacizumab (Fig. 1D). No myelin loss was detected in the bevacizumab-only group. TTC staining showed the presence of infarcts in nerves after ONC without bevacizumab injection (Fig. 1C1) and after ONC+bevacizumab (Fig. 1D1).

TUNEL staining of the optic nerve showed an increase in apoptotic cells in the anterior segment on day 1 after ONC (data not shown). There was less of an increase with

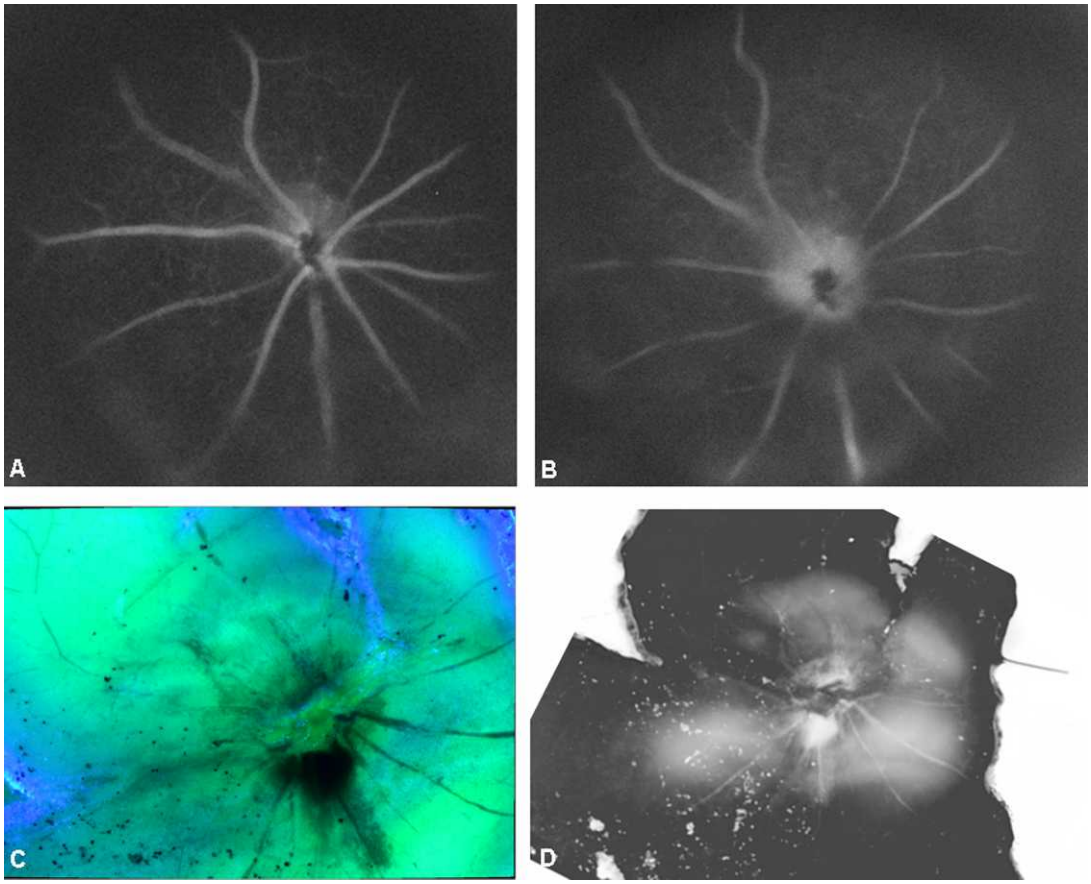

FIGURE 5. Optic nerve head leakage 3 days after ONC: FA study and flat mount retinas. Samples from a mouse on day 3 after ONC showing significant leakage from the optic nerve head (*blurred disc margin*) on early FA (**A**) and late FA (**B**) following fluorescein injection. Flat-mount retina study in the same mouse shows similar findings of leakage at ×20 (**C**) and ×10 (**D**) magnification. For comparison with ONC+bevacizumab, see Figure 4.

bevacizumab injection and no change after bevacizumab injection without ONC.

Quantitative microvascular analysis of the retrobulbar optic nerve (<1 mm from the globe) demonstrated a significant loss of perfusion 1 day after ONC relative to the contralateral eye and significant preservation of vascular perfusion with bevacizumab treatment (Fig. 2). Calculating the fractional microvascular filling of the injured nerve normalized to that of the uninjured (contralateral) nerve yielded a dimensionless quantity on which basis the loss of perfusion in the injured nerves could be estimated. The mean (SEM) ratio of injured to control optic nerve at 4 days after ONC induction was 85% ± 10% for bevacizumab-treated animals ($n = 7$) and 33% ± 13% for vehicle-treated animals ($n = 6$). The difference was statistically significant ($P = 0.011$).

Bevacizumab Attenuation of Optic Nerve Head Leakage

On FA, no optic nerve head edema was apparent immediately after induction of ONC in either the bevacizumab-treated or untreated mice. One day after ONC (Fig. 3), leakage was detected in both groups, but less in the mice treated with bevacizumab. On day 3, maximal leakage was detected, although the level was still lower in the bevacizumab-treated mice (Figs. 4A–F) compared to ONC without treatment (Figs. 5A–D). As expected, there was no leakage in the left (control) eyes or in the mice treated with bevacizumab without ONC. IgG staining revealed a reduction in optic nerve head leakage in mice after ONC+bevacizumab relative to mice after ONC only (data not shown).

Analysis of the flat-mounted fluorescein-injected retinas revealed significant leakage in the ONC-only group and reduced or no leakage in the ONC+bevacizumab (data not shown) and control groups, respectively. Similar to the immunohistochemistry results, perfusion occurred in both groups before euthanization. The findings using India ink supported the results of the flat-mount studies.

Bevacizumab Presence After 4 Days

On immunohistochemistry study, bevacizumab, a humanized antibody, was detected in the retina and optic nerve 4 days after ONC. We examined three randomly selected injured optic nerves from bevacizumab-treated eyes and one optic nerve from a vehicle-treated eye 4 days after ONC. Bevacizumab was found on the optic nerve head (ONH) and appears to track with retinal vessels (Figs. 6A, 6A1). There was a slight drop in the amount of bevacizumab found in the lamina (Figs. 6B, 6B1), but staining was positive in the retrolaminar nerve (Figs. 6C, 6C1, 6D, 6D1). In the positive control, bevacizumab was distributed around the optic nerve fascicles and other extracellular spaces (Fig. 6, positive control). However, the bevacizumab-treated nerves showed a slightly elevated and evenly dispersed signal behind the globe that did not track with blood vessels (Figs. 6B–D), suggesting the presence of

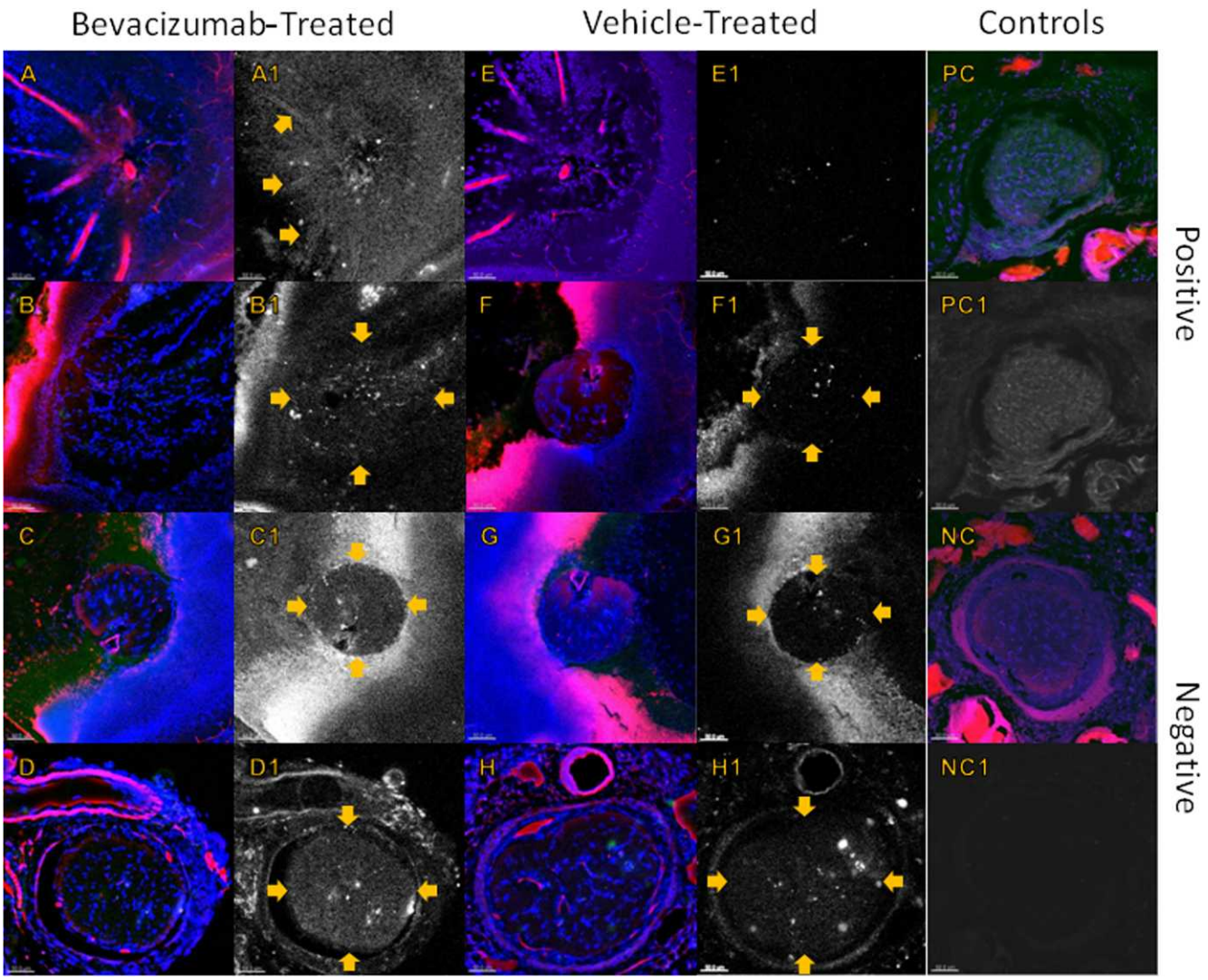

Figure 6. Bevacizumab is present in the optic nerve 4 days post-ONC. Each column of images (**A–H**) represents 50-μm optic nerve sections taken in order from the ONH (**A**, **A1**, **E**, **E1**) to the initial optic nerve segment (**D**, **D1**, **H**, **H1**). Color images show nuclear stain (*DAPI/blue*), vascular filling (*rhodamine/red*), and bevacizumab (*Cy5/green*), whereas grayscale images (**A1–D1**, **E1–H1**) show the bevacizumab Cy5 channel extracted from the corresponding color figure with palette stretching to show contrast. Grayscale images (**A1–H1**) were processed equivalently to allow comparison. Immunostaining for bevacizumab showed increased staining in bevacizumab-treated optic nerves (outlined by *orange arrows*) 4 days post-ONC (**A1–D1**), as compared with background autofluorescence in vehicle-treated (**E1–H1**). Bright dots and the band of photoreceptor cells (see [**B**, **B1**, **C**, **C1**, **F**, **F1**, **G**, **G1**]) represent autofluorescent artifacts present in the pigmented mouse independent of staining. Positive staining of the nerve behind the globe demonstrates the presence of bevacizumab in the optic nerve during the period of vascular protection shown in Figure 2. Bevacizumab appeared to colocalize with ONH vasculature (see *orange arrows* in [**A1**]) but appeared more diffuse farther back in the optic nerve. Bevacizumab-positive control (**PC**, **PC1**) and -negative control (**NC**, **NC1**) were performed to demonstrate the specificity of the antibody. The positive control (**PC1**) also shows a different distribution of applied bevacizumab than intravitreal administered bevacizumab (**D1**).

some sort of transport process for bevacizumab in the retrolaminar optic nerve. The retinas show strong bevacizumab staining in the innermost layers and also in the vascularized plexiform layers (Figs. 7B, 7B1) compared with background fluorescence of vehicle-treated control (Figs. 7A, 7A1). Bevacizumab could be seen colocalized with vessels in the choriocapillaris (Figs. 7C, 7C1), as well as in the optic nerve sections (Figs. 6A, 6A1).

Effect of Bevacizumab on VEGF Gene Expression

On day 1, the expression of both *HO-1* and *SOD-1* genes was elevated in the right eyes of mice in all study groups relative to the left (control) eyes. It increased from day 1 to 3 only in the mice after ONC+bevacizumab. VEGF gene expression remained at baseline 1 day after ONC regardless of bevacizumab treatment. On day 3, VEGF gene expression increased in the ONC+bevacizumab group and was reduced or unchanged in the mice after ONC alone or bevacizumab injection alone (Table 3).

Discussion

This study shows that the intravitreal injection of bevacizumab following the induction of ONC in a mouse model reduces optic nerve head leakage and edema and preserves the RGCs. Although the size of the optic nerve infarct on histological study was similar in the bevacizumab-injected mice with and without ONC, the RGC preservation may have been a consequence of the reduction in optic nerve head edema

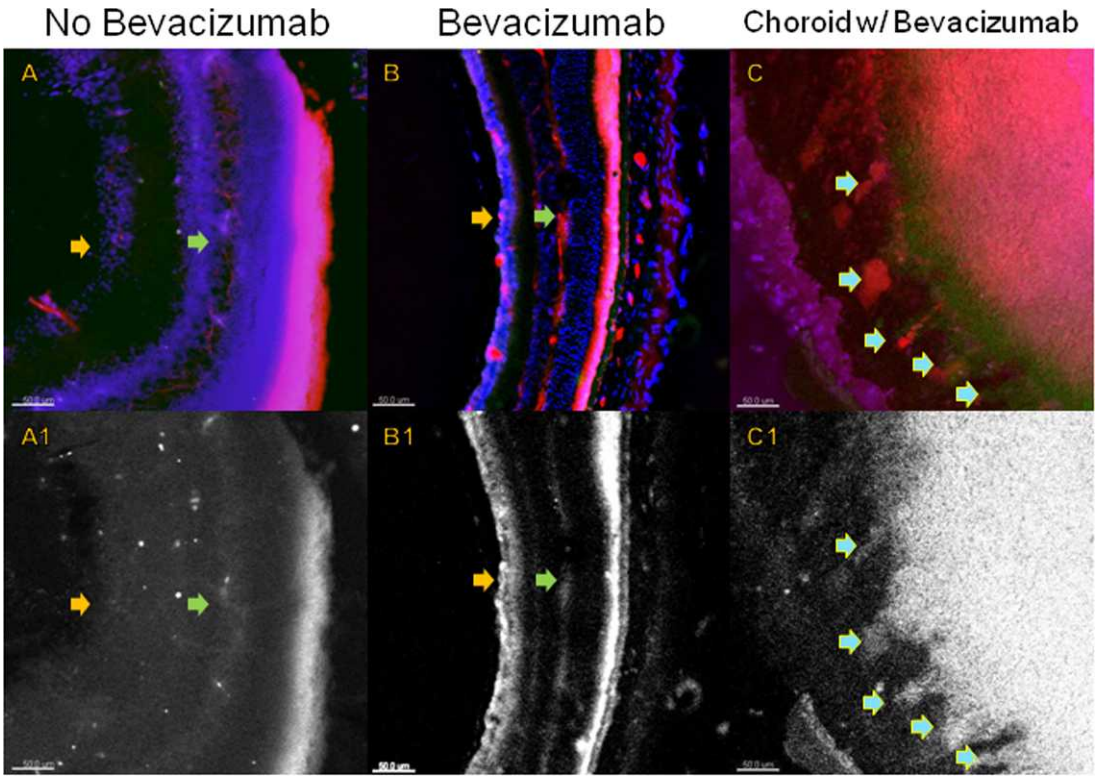

Figure 7. Bevacizumab is present in the retina 4 days post-ONC. Immunohistostaining shows positive staining in bevacizumab-treated retina 4 days post-ONC (**B**, **B1**) with staining in the RGC and nerve fiber layer (*orange arrows*, [**B**, **B1**]) and inner and outer plexiform layers (*green arrows*, [**B**, **B1**]) with background autofluorescence in untreated retina (**A**, **A1**). (**C**, **C1**) show the choroid with bevacizumab (*blue arrows with orange border*) apparently colocalizing with rhodamine-BSA–filled vessels. This pattern of staining (see also Figs. 6A, 6A1) suggests that, in the retina, bevacizumab may be moving along blood vessels. Color images (**A**, **B**, **C**) show nuclear stain (*DAPI/blue*), vascular filling (*rhodamine/red*), and bevacizumab (*Cy5/green*). Grayscale images (**A1**, **B1**, **C1**) were extracted from their corresponding color panels with palette stretching to show contrast and equivalent processing to allow comparison. It should be noted that (**C**, **C1**) show a large amount of autofluorescence toward the upper right corner from the retinal photoreceptor layer due to the oblique cut of the tissue section.

and lesser interruption of axonal transport. This explanation is in line with the notion that edema causes axonal stress by "compartment-like" pressure.[20] Our findings suggest that bevacizumab may exert the same action in optic stroke as reported for diabetes, retinal vein occlusion, and AMD.[43–45]

So far, clinical studies of bevacizumab injections for NAION have included only small groups of patients and yielded inconclusive results. Thus, the primary aim of our study was to determine if bevacizumab is a relevant option for the treatment of NAION. We therefore selected a mouse model of severe optic injury (ONC that simulates traumatic optic neuropathy) and optimal treatment conditions (treatment administered immediately after injury). We found that although the ONC damage to the RGCs was more severe than in the mouse model of AION,[46] bevacizumab treatment still proved effective. It is also noteworthy that we measured the final outcome of NAION at 21 days, which is considered long-term in mice. Given the gap usually found in clinical NAION between onset of the episodes and administration of treatment, further studies using a mouse model of AION are needed to determine the optimal time of intervention after the damage and to corroborate the apparent neuroprotective effect of bevacizumab and its therapeutic potential in patients with NAION.

Optic Nerve Head Edema and Leakage

A decrease in acute optic nerve head leakage was noted on days 1 and 3 following intravitreal injection of bevacizumab, by FA, histological flat-mount retina study, and staining for IgG, with preservation of the optic nerve head vasculature on gelatin perfusion study. These findings are consistent with reports in patients with NAION, which described a bevacizumab-induced decrease in optic nerve head edema and leakage.[20,21] In previous studies in mice, disc edema and

Table 3. Retinal Expressions of Ischemia-Related Genes in the Different Groups

	Day 1			Day 3		
Gene	ONC Only, $n = 5$	Bevacizumab Only, $n = 5$	ONC + Bevacizumab, $n = 5$	ONC Only, $n = 5$	Bevacizumab Only, $n = 5$	ONC + Bevacizumab, $n = 5$
HO-1	2.0 ± 1.4	5.7 ± 6.4	3.9 ± 3.7	1.0 ± 0.3	3.6 ± 0.04	5.5 ± 1.2
SOD-1	1.9 ± 0.7	1.9 ± 1.0	1.5 ± 0.9	0.4 ± 0.2	1.9 ± 1.1	2.5 ± 1.2
VEGF	1.1 ± 0.3	0.9 ± 0.2	1.1 ± 0.2	0.5 ± 0.1	0.8 ± 0.1	1.8 ± 0.1

leakage occurred within hours of the insult, and the effect of bevacizumab injected immediately after injury induction could be detected only after the edema appeared.[28,29] The present study shows that maximal leakage occurs on day 3, which may account for the delayed effect of a single bevacizumab injection.

Optic Nerve Structure

ONC leads to apoptosis of the RGCs and proliferation of optic nerve glial cells, with damage to the oligodendrocytes. Several weeks later, Wallerian degeneration causes axonal loss and demyelination.[29,30,46] In the present study, analysis by TTC, a mitochondrial stain, revealed the presence of infarcts. As TTC may not have been sufficiently sensitive to detect minor changes in infarct size between the groups, but rather axonal transport interruption, we followed this procedure with the LFB and TUNEL assays. Both showed myelin loss and a higher rate of apoptosis in the anterior segment of the optic nerve in the ONC group not treated by bevacizumab. Accordingly, an evaluation to rule out a possible inductive effect of bevacizumab on macular edema and stroke[25–27] revealed no damage to the optic nerves following injection.

Levin and Danesh-Meyer[47] proposed that NAION results from closure of the tributary venules through which blood reaches the optic nerve head and then drains to the central retinal vein. In the acute phase, cytotoxic edema is rapidly followed by vasogenic edema, which can further damage the optic nerve and surrounding retina. This hypothesis might explain cases of NAION that present with asymptomatic optic nerve head edema and subsequent progressive visual loss. The vasogenic edema may be increased by capillary leakage. Although the vasculature of the optic nerve head differs slightly between humans and mice, we found decreased leakage after bevacizumab injection. Future use of an AION mouse model, with analysis of the vascular system, may yield more information on the mechanisms leading to the visual loss in NAION and provide researchers with tools to improve treatment in patients.

RGC Preservation

ONC caused the loss of approximately half the RGCs, with maximal apoptosis on day 3, as reported also in previous studies.[3,4,48] The bevacizumab-treated mice had both less apoptosis and better preservation of the RGCs than the untreated ONC mice. These findings are consistent with the mechanism suggested by Bennett et al.[20] in humans. The 13% RGC loss after bevacizumab in the mice without ONC is within the SD of normal variability.

Our molecular analysis supported the suggestion that bevacizumab prevents RGC damage by reducing vasogenic edema in the ONH and surrounding retina. Partial recovery of ischemic tissue is possible if blood flow is restored before most of the tissue has been destroyed, with preservation/restoration of physiological function.[49] However, oxidative stress response genes are significantly upregulated after ONC. We found that the expression of the *HO-1* and *SOD-1* genes increased in the ONC mice on day 1, and further increased only in the bevacizumab-treated ONC mice on day 3. The elevated expression of these genes has been associated with a protective effect in ischemic conditions.[50,51] An ex vivo study of rat hearts showed that ischemic reperfusion-induced HO-1 activity decreases infarct size and greatly enhances postischemia recovery,[50] and a study in genetically altered mice reported that SOD-1, the major intracellular SOD isoform, plays an important role in endothelial function by protecting nitric oxide release from endothelium.[51] Thus, the increased expression of these genes toward day 3 in the bevacizumab-treated mice in our study probably indicates that bevacizumab stimulated their expression, thereby decreasing cell damage.

Our results also support reports of a positive effect of bevacizumab on mouse VEGF, using doses as low as 0.625 μg.[37,52–57] On molecular analysis, VEGF gene expression was initially unchanged in all groups, but later increased only in the ONC+bevacizumab group. We assume that this elevation was due to VEGF inhibition by bevacizumab, leading to continuous feedback signaling to express VEGF as a response to the ischemia.

Bevacizumab Pharmacokinetics

Immunohistochemistry study on day 4 after ONC showed that bevacizumab appears to track well with the vasculature in the retina, as previously reported in rabbits.[58] Some vascular colocalization in the choriocapillaris was also noted. This was not evident in the optic nerve. Thus, it is not clear how bevacizumab was transported in the initial segment of the optic nerve. It is noteworthy that the distribution in the optic nerve is unlike that in the interstitial spaces of the nerve, suggesting some sort of active trafficking. It has been reported that bevacizumab injected intravitreally into one eye may be found later in the companion eye.[59] However, our immunohistochemical technique was not sensitive enough to establish whether this is true for our system, as well owing to the low signal to noise in the pigmented C57Bl/6 mouse strain.

Safety

Electrophysiological and histological studies have shown that intravitreal bevacizumab lacks ocular toxicity in cell cultures, animal (specifically rodent) eyes, and human eyes, at least in the short term.[35] Accordingly, we found no bevacizumab toxicity in undamaged RGCs, similar to the published literature in mice and rabbits.[34–36,59–61] It did not induce optic nerve damage or leakage. Although it is possible that we prevented bevacizumab toxicity by injecting the drug immediately after ONC, our results indicated that in the ONC mice, the selective damage to the RGCs was reduced after treatment. Plasma bevacizumab is elevated systemically,[59] but there were also no adverse effects in the contralateral (left) eyes in the ONC+bevacizumab and bevacizumab-only groups.

The 13% RCG loss with bevacizumab alone is not significant given the size of our group. The possibility that bevacizumab toxicity may have been due to the high concentration of the drug (75 μg/3 μL) might be ruled out by earlier reports of an absence of evidence of toxicity after multiple intravitreal injections of bevacizumab at dosages of 1.25, 2.5, or 5.0 mg. However, larger doses may induce transient inflammation.[58,62] The current widespread use of bevacizumab in humans for a variety of indications supports its safety as well.

Limitations of the Study

The study was limited by the small sample and lack of consistency among the mice. Additionally, ONC represents a combination of mechanical traumatic and ischemic injury and thus may not completely simulate NAION, a purely ischemic insult, or completely reflect its pathogenesis. Therefore, we are planning to explore the effect of bevacizumab in a large mouse model of AION to determine its potential applicability in human NAION. Further studies of the timing and dose of injections are needed as well.

Conclusions

A single intravitreal injection of bevacizumab has a neuroprotective effect in the ONC mouse model, as shown by clinical, angiographic, histologic, vascular imaging, immunohistochemical, and molecular analysis. This effect is manifested by a decrease in optic nerve head leakage and edema, increased optic nerve microvasculature perfusion, and decreased cell loss and reduced apoptosis in the RGC layer. Ischemia-related genes, such as *HO-1* and *SOD-1*, may play a contributory role. Bevacizumab at the dose used has no demonstrable toxic effect in the mouse eye. Bevacizumab is a human-specific anti-VEGF antibody and its effect on mouse VEGF is controversial.[52,59] Nevertheless, there is as yet no effective treatment of NAION, and these promising results should prompt further studies of bevacizumab or other anti-VEGF substances in mouse models of AION, to determine the feasibility of small-group clinical trials of anti-VEGF drugs for the treatment of NAION.

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