Erythropoietin Promotes Survival of Retinal Ganglion Cells in DBA/2J Glaucoma Mice

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PURPOSE. Retinal ganglion cell (RGC) loss occurs in response to increased intraocular pressure (IOP) and/or retinal ischemia in glaucoma and leads to impairment of vision. This study was undertaken to test the efficacy of erythropoietin (EPO) in providing neuroprotection to RGCs in vivo.

METHODS. The neuroprotective effects of EPO were studied in the DBA/2J mouse model of glaucoma. Mice were intraperitoneally injected with control substances or various doses of EPO, starting at the age of 6 months and continuing for an additional 2, 4, or 6 months. RGCs were labeled retrogradely by a gold tracer. IOP was measured with a microelectro-mechanical system, and EPO receptor (EPOR) expression was detected by immunohistochemistry. Axonal death in the optic nerve was quantified by para-phenylenediamine staining, and a complete blood count system was used to measure the number of erythrocytes.

RESULTS. In DBA/2J mice, the average number of viable RGCs significantly decreased from 4 months to 10 months, with an inverse correlation between the number of dead optic nerve axons and viable RGCs. Treatment with EPO at doses of 3000, 6000, and 12,000 U/kg body weight per week all prevented significant RGC loss, compared with untreated DBA/2J control animals. EPO effects were similar to those of memantine, a known neuroprotective agent. IOP, in contrast, was unchanged by both EPO and memantine. Finally, EPOR was expressed in the RGC layer in both DBA/2J and C57BL/6J mice.

CONCLUSIONS. EPO promoted RGC survival in DBA/2J glaucomatous mice without affecting IOP. These results suggest that EPO may be a potential therapeutic neuroprotectant in glaucoma. (Invest Ophthalmol Vis Sci. 2007;48:1212–1218) DOI:10.1167/iovs.06-0757

Glaucoma is a major cause of preventable blindness with more than 2 million people in the United States currently affected and more than 80,000 are legally blind from the disease.1,2 Glaucoma is often associated with high intraocular pressure (IOP) and/or retinal ischemia in glaucoma and leads to impairment of vision. This study was undertaken to test the efficacy of erythropoietin (EPO) in providing neuroprotection to RGCs in vivo.

METHODS

Animals and Drug Administration

Two age-matched mouse strains, DBA/2J and C57BL/6J (Jackson Laboratories, Bar Harbor, ME), were used at age 4 to 12 months for all experiments. All mice were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. From the age of 6 months, DBA/2J mice were treated intraperitoneally three times per week for a further 2, 4, or 6 months with bovine serum albumin (BSA, 0.1%); Sigma-Aldrich, St. Louis, MO), memantine (70

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mg/kg body weight/wk; Sigma-Aldrich), or rhEPO-α (3000, 6000, and 12,000 U/kg body weight per week; BayBiotec Inc., Norcross, GA). The doses were centered around 6000 U/kg body weight per week because similar doses have been used for neuroprotection and treatment of anemia.\textsuperscript{29–32}

RGC Labeling

Retrograde labeling of RGCs was conducted as previously reported.\textsuperscript{33} Briefly, RGCs were retrogradely labeled with a neuronal tracer (Fluoro-Gold; Fluorochrome LLC, Denver, CO) by injection of the superior colliculi performed with a stereotaxic device (Stoelting Co., Wood Dale, IL). Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg body weight; LLOYD Laboratories, Shenandoah, IA). Under sterile conditions, a 2-cm incision was made along the midline of the scalp over the cranium, to expose the skull. The point of injection into the superior colliculi was designated and the bregma identified and marked at a depth of 2 mm from the brain surface, 2.50 mm behind the bregma in the anteriorposterior axis, and 0.5 mm lateral to the midline. A hole was drilled in the skull above the designated coordinates in the right and left hemispheres with a high-speed micro-drill (Fine Science Tools, Foster City, CA). The superior colliculi were injected with 2 μL of 4% fluorogold solution in double distilled (dd)H\textsubscript{2}O at an injection rate of 0.5 μL/min with a Hamilton (Renov, NV) modified microliter syringe (Fisher Scientific, Palatine, IL) positioned 2 mm below the surface of the brain. The skin was then sutured and the mice monitored carefully until they had fully recovered from anesthe-sia.

Retinal Flatmount Preparation and Imaging

Retrograde labeling of RGCs was allowed to proceed for 3 days, and then the mice were euthanatized with an overdose of carbon dioxide. The eyes were immediately enucleated and fixed with 4% paraformaldehyde at 4°C for 4 hours, and the retinas were dissected from the ora serrata. Retinal flatmounts were prepared by making four radial incisions and then carefully placing the retinas on silane-coated slides. Images of fluorogold-labeled (viable) RGCs were acquired immediately after flatmount preparation (two images per quadrant) using the 20× objective of an epifluorescence microscope (DMIRB; Leica, Bannock-burn, IL) equipped with a chroma A filter cube (530–600 nm). For each quadrant, an image was acquired from each side of an imaginary line between the optic nerve and the ora serrata, approximately two-thirds the distance from the optic nerve (two images per quadrant). Digital images were collected with a CCD camera (Hamamatsu, Hamamatsu City, Japan). Three to 10 images (20×) per optic nerve were captured for quantification of dead optic nerve axons; dead optic nerve axons were expressed as the number per square millimeter of the optic nerve area.

IOP Measurement

Applanation tonometry was used to measure IOP, using a silicone microelectric-mechanical systems (MEMS)-based fiber optic pressure sensor (Fiso Technologies, Quebec, QC, Canada). The applanating surface consists of a silicone diaphragm bonded to a photolithographically etched Pyrex glass substrate enclosing a vacuum space that was attached to an optical fiber. The perpendicular distance of the vacuum space between the silicone diaphragm and the glass substrate varied inversely with the pressure. The two semireflective surfaces of the silicone and the glass acted as a Fabry-Perot interferometer, allowing the distance to be ascerained by analyzing their reflectance properties from a multiple-frequency light source. Two IOP readings per second were recorded by an optical signal conditioner (FTI-10; Fiso Technolo-gies, Quebec, QC, Canada) and transferred to a computer, and the average IOP data (means ± SD) from a total of 120 readings/min per eye were used for each analysis. The optical interferometer was cali-brated by cannulating the mouse eye and manometrically measuring the IOP between the pressure range 10 to 80 mm Hg. IOP was measured noninvasively in both C57BL/6j and DBA/2j mice at the ages of 6, 8, 10, and 12 months, both before and after administration of the therapeutic agents.

EPO Receptor Immunohistochemistry

Eyes from 6-month-old DBA and C57/bl6 mice were enucleated and snap frozen in OCT compound (Ted Pella, Inc., Redding, CA) and stored at −80°C. Using a cryostat, 10-μm-thick sections were placed onto 3-aminopropyl triethoxysilane-coated slides (CM3050S; Leica) and stored at −20°C until stained. Before immunostaining, retinal sections were dried for 30 to 60 minutes at room temperature, washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde solution in PBS for 30 minutes at room temperature, and washed again with PBS. The sections were blocked using 10% donkey serum in PBS with 0.3% Triton X-100 for 1 hour at room temperature and washed with PBS. Sections were stained with a combination of both anti-EPOR (goat polyclonal; R&D systems, Minneapolis, MN) and anti-NeuN (mAb; Chemicon, Temecula, CA) or anti-EPOR and anti-GFAP (rabbit polyclonal; Abcam, Cambridge, MA) antibodies. The primary antibodies or normal goat serum (10 μg/mL; negative control; R&D Systems) were incubated on the sections overnight at 4°C followed by a PBS wash. The NeuN antibody was used at a concentration of 1:250 and the EPO antibody at 1:100. The GFAP antibody was used at the prediluted concentration provided by the manufacturer. All antibodies were di-luted into blocking buffer containing 10% donkey serum and 2% BSA in 1× PBS. All secondary antibodies were used at a dilution factor of 1:500 in blocking buffer. The EPO antibody was detected with a donkey anti-goat AlexaFluor 488 antibody (Invitrogen-Molecular Probes, Carlsbad, CA). The NeuN antibody was detected with a donkey anti-mouse Cy3 antibody (Jackson ImmunoResearch, West Grove, PA). The GFAP antibody was detected with a donkey anti-rabbit Cy3 antibody (Jackson ImmunoResearch). The secondary antibodies were in-cubated for 45 minutes at room temperature. The sections were then coverslipped with Antifade medium (Vectorshiel; Vector Laboratories, Burlingame, CA) with 4′,6-diamino-2-phenylindole (DAPI) and using a
flourescence microscope (DMRA2; Hamamatsu) to capture the images.

**Erythrocyte Counting**

Blood from the heart (~300–500 μL) was taken from 10-month-old DBA/2J mice that had received 4 months of treatment with intraperitoneal injections of either 0.1% BSA or EPO at 6000 U/kg body weight per week. The number of erythrocytes was counted by using a basic complete blood count (CBC) system (IDEXX Laboratories, North Grafton, MA).

**Statistical Analysis**

The mean number of RGCs per square millimeter in all treatment groups at all time points (each group contained at least 11 mice) was analyzed by one-way ANOVA. A pair-wise Bonferroni post hoc test was then performed with significance set at \( P < 0.05 \) and \( P < 0.01 \) (Analyze-It Plug-In for Microsoft Excel; Analyze-It Software, Leeds, UK). The same approach was also applied to all groups of mean IOPs (each group contained at least 11 mice). A Student’s unpaired 2-t test was used to compare the number of erythrocytes in mice treated with either BSA or EPO. Correlations between automatically and manually counted RGCs and between RGCs and dead axons were made using a least-squares fit analysis (Excel; Microsoft, Redmond, WA).

**RESULTS**

**RGC Loss and Optic Nerve Axon Death in DBA/2J Mice**

We analyzed RGC death in 385 C57BL/6J (\( n = 91 \)) and DBA/2J (\( n = 294 \)) mice (male \( n = 194 \), female \( n = 191 \)) by fluoroagold injections into the superior colliculi (SC) and retrograde transport. Viable RGCs were successfully labeled in 90.91% of mice, with only 2.77% resulting in labeling failure and 6.49% resulting in death after surgery for injecting of SC with the tracer (data not shown). Viable RGCs in DBA/2J mice at the ages of 4 months (\( n = 11 \), six males and five females) and 10 months (\( n = 12 \), five males and seven females) were determined. Three days after injection, we found accumulation of gold in the cell bodies of RGCs, as observed by fluorescence microscopy of retinal flatmounts (Fig. 1A). To count the number of RGCs reproducibly and objectively, we developed an automated image analysis routine (see Methods and Supplementary Fig. S1, online at http://www.iovs.org/cgi/content/full/48/3/1212/DC1). Quantification of RGCs in retinas of DBA/2J mice revealed a significant reduction in RGCs from 4 to 10 months, compared with retinas of control animals (Fig. 1B). Although not statistically significant, we found that female DBA/2J mice showed a trend for greater RGC loss than did male mice (Fig. 1B).

Because our measure of RGC death was indirect, as we measured a change in the number of viable RGCs as detected by uptake of fluoroagold, we validated our approach by an alternative method. Using the dye PPD, which specifically binds to membranes of dead cells, we quantified the number of dead axons in optic nerves collected from the eyes used for RGC quantification. We observed an inverse correlation between the number of gold dye–positive, viable RGCs and the number of dead axons in the optic nerve (Fig. 1C). Therefore, we believe that the death of RGCs prevents retrograde labeling of these cells with the dye, and that a loss of gold dye–positive RGCs is a valid measure of RGC death.

**RGC Survival in EPO-Treated DBA/2J Mice**

Having established a method for precisely quantifying viable RGCs in large numbers in retinal flatmounts, we next tested the hypothesis that systemic administration of EPO exerts a neuroprotective effect on RGCs. We first characterized the progressive loss of RGCs in the retinas of DBA/2J mice at 6, 8, 10, and 12 months. At 8, 10, and 12 months, the number of RGCs was reduced by 15.84%, 33.87%, and 54.77%, respectively, compared to the RGC number at 6 months (Fig. 2A). A comparison with the retinas of C57/Bl6 control mice at 6 and 12 months revealed that RGC number at 12 months in DBA/2J mice was significantly lower than that in C57/Bl6 mice at 12 months (Fig. 2A).

To evaluate the potential neuroprotective effect of EPO, DBA/2J mice were treated with various doses of EPO. BSA was used as a vehicle control and memantine as a positive control. Memantine is known to protect neurons by blocking pathologic activation of NMDA receptors and glutamate toxicity in animal models. Relative to untreated DBA/2J mice, the number of viable RGCs increased by 23.90% and 41.15% after 4 and 6 months of EPO treatment, respectively, at a dose of 3000 U/kg body weight per week (Fig. 2B). Similar results were observed for EPO at 6,000 and 12,000 U/kg body weight per week (Fig. 2B). DBA/2J mice treated with the vehicle control showed a similar profile of RGC loss as was observed for untreated DBA/2J mice (Figs. 2A–B). These results with EPO were similar to the results of memantine treatment in which the viable RGCs increased by 21.19% and 40.93% after 4 and 6 months (Figs. 2A–B). These data reveal that systemic administration of EPO prevents the loss of RGCs in a mouse model of glaucoma.

**IOP in EPO-Treated DBA/2J Mice**

Because elevated IOP is a common risk factor in glaucoma and has been previously observed in the DBA/2J model, we monitored IOP in all mice. We confirmed elevated IOP averaging 15.53 to 16.26 mm Hg in nontreated DBA/2J mice 6 to 12 months of age, whereas the age-matched control C57/Bl6 mice had normal IOP averaging 9.78 to 9.88 mm Hg (Fig. 2C). Neither EPO (Fig. 2D) nor memantine (Fig. 2C) treatments changed the IOP significantly in treated mice compared with control animals. Therefore, we conclude that EPO has a neuroprotective effect on RGCs without affecting IOP.

To confirm the bioactivity of EPO, we assessed the effect of intraperitoneally injected EPO on erythrocyte counts. We found that 10-month-old DBA/2J mice treated with EPO at 6000 U/kg body weight per week for 4 months showed a statistically significant increase in erythrocytes (\( n = 5 \); 16.10 million cells/μL) compared with 0.1% BSA treatment (\( n = 7 \); 9.87 million cells/μL; Fig. 2E).

**Colocalization of EPOR with RGCs**

To determine whether EPO might exert its neuroprotective effect by directly stimulating RGCs, we immunostained for EPOR in retinal cross sections from untreated 6-month-old DBA/2J mice and C57/Bl6 mice. In both strains of mice, EPOR was highly expressed in the RGC layer of the retina, appearing as cell surface immunoreactivity surrounding the NeuN-positive nuclei of RGCs (Figs. 3, top). No staining was detected in control experiments without the primary antibody (data not shown). The retinal expression patterns for EPOR in the DBA/2J and C57/Bl6 mice appeared to be similar, in that the RGC layer, INL, and the photoreceptor layer were positive for EPOR quantification (compare Figs. 3A and 3B), in good agreement with previously published results. In addition to RGCs, a few GFAP-positive astrocytes displayed EPOR immunoreactivity, although most of the EPOR-expressing cells were located below the astrocytes in the ganglion cell layer of the retina (Figs. 3, bottom). These results suggest that many of the RGCs express EPOR and therefore may have the potential to respond directly to EPO.
Neuroprotective Effects of EPO

In the present study, systemic EPO treatments nearly abolished RGC loss in the DBA/2J glaucoma model, without lowering the IOP of the animals. RGC loss is a critical component of the neurodegeneration observed with glaucoma. In DBA/2J mice, first the axons and dendrites of RGCs are compromised, then retrograde transport is generically impaired, and finally the cell bodies of RGCs shrink and are lost.39 In the mouse retina, specific synaptic connections identified by silver staining techniques were used to identify several different types of RGCs.40

EPO has been reported to protect neuronal cells in different models of neurodegeneration by inhibiting neuronal apoptosis.

Neuroprotective Signal Transduction of EPO

Expression of EPOR has been detected in many different cells and tissues, providing evidence for autocrine, para-
EPO and IOP

The aqueous production and aqueous humor turnover rate of the mouse eye are similar to those observed in the human eye. DBA/2J mice develop age-dependent progressive eye abnormalities that closely resemble the pigment glaucoma disease in humans, including increase in IOP.23 We confirmed that IOP of the DBA/2J mice was indeed elevated to a level significantly greater than that of C57/Bl6 control mice. Of note, we observed no significant change in IOP in mice that received EPO or memantine compared with vehicle control. Furthermore, EPO treatment did not rescue any of the structural abnormalities in the anterior segment of the DBA/2J eyes (data not shown); thus, it is unlikely that it improved the outflow of aqueous humor dynamics in these mice. These results suggest that the protective effects of EPO and memantine are independent of IOP in this model.

CONCLUSIONS

Systemic treatment with EPO reduces RGC loss in the DBA/2J model of glaucoma. EPO treatments were as effective as memantine, an agent previously shown to protect neurons in the DBA/2J glaucoma model.36 These results further support our hypothesis that the neuroprotective effect of EPO is mediated by inhibition of RGC apoptosis. Because EPO has been reported to be a retinal angiogenic factor in proliferative diabetic retinopathy,51 this neuroprotective role of EPO also highlights some potential problems in targeting EPO for antiangiogenesis therapy in the retina. It should be noted that EPO was also effective in increasing the number of circulating erythrocytes in the treated DBA/2J mice. This effect may indirectly improve the survival of the RGCs,52,53 as it does to central nervous system (CNS) neurons,54 and we are currently inves-
tigating the effects of increased blood flow and erythrocyte count on RGC survival in the DBA/2J model. Increased blood provision to tissues, induced by another ischemia-induced protein, VEGF, has also been linked to neuroprotection in the CNS\(^{37}\) and the retina (Y.-S. Ng, manuscript in preparation). Whether the effect of EPO is direct or indirect, however, our results suggest that systemic EPO treatment is sufficient for preventing RGC loss and may represent a potential adjunct therapy with IOP lowering medications for patients with glaucoma.

References


