Gene Therapy with Brain-Derived Neurotrophic Factor
As a Protection: Retinal Ganglion Cells in a Rat Glaucoma Model

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PURPOSE. To develop a modified adenoassociated viral (AAV) vector capable of efficient transfection of retinal ganglion cells (RGCs) and to test the hypothesis that use of this vector to express brain-derived neurotrophic factor (BDNF) could be protective in experimental glaucoma.

METHODS. Ninety-three rats received one unilateral, intravitreal injection of either normal saline (n = 30), AAV-BDNF-woodchuck hepatitis postranscriptional regulatory element (WPRE; n = 30), or AAV-green fluorescent protein (GFP)-WPRE (n = 33). Two weeks later, experimental glaucoma was induced in the injected eye by laser application to the trabecular meshwork. Survival of RGCs was estimated by counting axons in optic nerve cross sections after 4 weeks of glaucoma. Transgene expression was assessed by immunohistochemistry, Western blot analysis, and direct visualization of GFP.

RESULTS. The density of GFP-positive cells in retinal whole-mounts was 1,828 ± 299 cells/mm² (72,273 ± 11,814 cells/retina). Exposure to elevated intraocular pressure was similar in all groups. Four weeks after initial laser treatment, axon loss was 52.5% ± 27.1% in the saline-treated group (n = 25) and 52.3% ± 24.2% in the AAV-GFP-WPRE group (n = 30), but only 32.3% ± 23.0% in the AAV-BDNF-WPRE group (n = 27). Survival in AAV-BDNF-WPRE animals increased markedly and the difference was significant compared with those receiving either AAV-GFP-WPRE (P = 0.002, t-test) or saline (P = 0.006, t-test).

CONCLUSIONS. Overexpression of the BDNF gene protects RGCs as estimated by axon counts in a rat glaucoma model, further supporting the potential feasibility of neurotrophic therapy as a complement to the lowering of IOP in the treatment of glaucoma. (Invest Ophthalmol Vis Sci. 2005;44:4357–4365) DOI:10.1167/iovs.02-1332

Glaucoma is the second most common cause of blindness in the world and is becoming increasingly prevalent as people survive longer.1,2 Death of retinal ganglion cells (RGCs) is the principal pathologic finding in glaucoma, and RGC apoptosis has been detected in experimental glaucoma in rats, monkeys, and humans.3–5 Elevated intraocular pressure (IOP) is a major risk factor for glaucomatous RGC loss.6–8 Three recent clinical trials demonstrate that lowering of IOP significantly reduces the rate of glaucoma progression.9–11 However, glaucoma worsens in a minority of patients, despite presently prescribed IOP reduction.12 This may result from IOP targets that are too high, from poor compliance with intended therapy, or from the limited ability of lowering IOP to slow progression. It is the intention of current research to devise methods to slow the progression of glaucomatous optic neuropathy independent of, or in addition to, IOP reduction.13–17

Brain-derived neurotrophic factor (BDNF) is an important survival factor for RGCs, both during development18,19 and in adult life.20–22 BDNF produced by the superior colliculus (or the lateral geniculate nucleus in higher mammals) binds to the trkB receptor and is retrogradely transported from RGC axons to cell bodies in microsomal vesicles.23,24 When IOP is acutely elevated in rats and monkeys, retrograde transport of the trkB-BDNF receptor complex is obstructed at the optic nerve head.25 It is therefore possible that BDNF deprivation plays a role in glaucomatous RGC death. This hypothesis is supported by experiments showing that in experimental rat glaucoma, BDNF injected into the vitreous can temporarily slow RGC loss.26 However, multiple intravitreal injections of BDNF were necessary for significant beneficial effect, reducing the clinical usefulness of intraocular delivery of BDNF protein as an adjunct to IOP reduction in glaucoma therapy.

Gene delivery to the retina by viral vectors has been shown to be effective in reducing structural and functional damage in several models of ocular diseases.27–32 In addition, intravitreal treatment with a BDNF-expressing adenoviral vector temporarily delays RGC death after optic nerve transection.33 However, vectors based on adenovirus are limited by the relatively short duration of efficient transgene expression and by their propensity to induce inflammation. Adenoassociated virus (AAV) vectors are capable of long-term transgene expression in the retina and cause minimal ocular inflammation in a broad range of host species.34–38

Our hypothesis was that a modified AAV incorporating cDNA for BDNF would be more protective in experimental glaucoma than injections of the neurotrophic factor alone. We tested this hypothesis in a rat model of glaucoma, in which 532-nm diode laser treatment of the trabecular meshwork induces moderate, chronic IOP elevation with consequent specific loss of RGCs without damage to other retinal layers.39 We used a novel AAV-BDNF incorporating the woodchuck hepatitis post-transcriptional regulatory element (WPRE), a construct that we have shown to facilitate highly efficient transection of neurons in the rat RGC layer within 2 weeks of a single

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intravitreal virus injection. We report a marked and statistically significant increase in RGC survival as estimated by axon counting after 4 weeks of experimental glaucoma in rats treated with intravitreal AAV-BDNF-WPRE compared with intravitreal saline or a control virus without BDNF.

METHODS

Plasmid Preparation

The pGFP plasmid, previously described in detail, was used to create both AAV-GFP-WPRE and AAV-BDNF-WPRE (Fig. 1). Flanked by AAV terminal repeats, the expression cassette of pGFP included a 1.7-kb sequence containing the hybrid CMV immediate early enhancer/β-actin (CBA) promoter. The pGFP construct had GFP cDNA downstream of the CBA promoter and incorporated the WPRE. For AAV-BDNF-WPRE, GFP was replaced by BDNFmyc cDNA, shown previously to produce biologically active BDNF.

Vector Packaging and Titering

Plasmids were packaged in rAAV by the streamlined method of Zolotukhin et al. Briefly, human embryonic kidney 293 cells were transfected by the calcium-phosphate method with an AAV terminal repeat-containing plasmid (pTR-UF12 or pSyn30) in a 1:3 molar ratio with the plasmid pDG. After 3 days, cells and media were harvested and centrifuged at 3000g. The pellet was resuspended, freeze thawed three times, incubated with endonuclease, and centrifuged at 3700 rpm. A discontinuous gradient of iodixanol (OptiPrep; Nycomed Pharma, Oslo, Norway) was added to the supernatant in four layers (60%, 40%, 25%, and 15% iodixanol). The tubes were heat sealed and centrifuged at 69,000 rpm for 1 hour at 18°C. AAV was extracted and added to a 5-mL heparin-agarose type I affinity column (Sigma-Aldrich, St. Louis, MO). After washing with 1× TD buffer (phosphate buffer plus 1 mM MgCl2 and 5 mM KCl) and eluting with 15 mL of 1× TD/1 M NaCl, the sample was concentrated by passage through membranes (Biomax 100 Ultrafree-15 units; Millipore, Bedford, MA). The sample was concentrated to 1 mL before adding 9 mL of lactated Ringer’s solution. This 10-mL volume was again concentrated to 1 mL, and 9 mL of lactated Ringer’s solution was added. The 10-mL volume was concentrated to produce a 400-μL stock of rAAV. We used an AAV concentration of 2 × 10^{13} particles per milliliter, titered by a previously described method.

Animals Used

A total of 138 adult Wistar rats (375–425 g) were used in the study. Experimental glaucoma was induced in 93 rats 2 weeks after a single unilateral intravitreal injection of normal saline (n = 30), AAV-BDNF-WPRE (n = 30), or AAV-GFP-WPRE (n = 33). Investigators were masked to the contents of the intravitreal injections. The number of remaining axons was counted after 4 weeks of experimental glaucoma by observers masked to the protocol for each rat. A further control group of 35 rats that did not undergo experimental IOP elevation was randomized to receive a unilateral intravitreal injection of normal saline (n = 10), AAV-BDNF-WPRE (n = 10), or AAV-GFP-WPRE (n = 15). Surviving axons were counted 6 weeks after injection. Retinal BDNF expression was quantified by Western blot analysis 6 weeks after unilateral intravitreal injection of: normal saline (n = 3), AAV-BDNF-WPRE (n = 6), or AAV-GFP-WPRE (n = 3). In each of the animals to be examined by Western blot analysis, the injection was followed 2 weeks later by 4 weeks of experimental IOP elevation. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved and monitored by the Animal Care Committee of the Johns Hopkins University School of Medicine.

Intravitreal Injection Technique

Animals were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg) and topical 1% proparacaine eye drops. Pupillary dilation was achieved with 1% tropicamide and 2.5% phenylephrine eye drops. Guided by an operating microscope, a superotemporal conjunctival incision was performed, large enough to expose the sclera posterior to the lens. A partial-thickness scleral pilot hole was made with a 30-gauge needle to facilitate penetration of the underlying sclera, choroid, and retina by a fine glass micropipette with a tip diameter of 30 μm and a tip length of 2.5 mm. Glass micropipettes were found to be preferable to metal needles; because the tip diameter is smaller, the depth of injection is easier to standardize, and the tapered pipette tip effectively seals the injection site during injection, reducing leakage of the injected fluid from the eye. The micropipette
Retinal cross sections were examined to demonstrate the histologic localization of GFP-positive cells within the retina. Before sectioning, isolated optic cups were fixed in 4% paraformaldehyde for 45 minutes followed by serial exposure to 2% paraformaldehyde/5% sucrose and 5%, 10%, 12.5%, and 15% sucrose for 30 minutes each, then 20% sucrose overnight (all sucrose solutions were 0.2 M phosphate buffered, pH 7.2). Optic cups were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA) and sectioned at 8 μm. GFP fluorescence was directly visualized using the GFP filter set. Digital images of all sections were then captured (Axioskop; Carl Zeiss Meditec).

Immunohistochemistry of GFP-Expressing Cells

Sections from eyes that had been exposed to AAV-GFP injection underwent immunofluorescence labeling with the following primary antibodies: rabbit anti-Thy1 (Santa Cruz Biotechnology) to identify RGCs, rabbit anti-calbindin (Calbiochem/Oncogene Research Products, San Diego, CA) to detect amacrine cells, or rabbit anti-GFAP (Sigma-Aldrich) to localize glial cells. After the sections were washed in TBS, nonspecific binding was blocked with 2% normal goat serum in TBS for 20 minutes. Blocking of endogenous avidin and biotin sites was performed using the avidin-biotin blocker kit (Vector Laboratories) with TBS washes after each 15-minute blocking step. Incubation of primary antibodies diluted in 1% BSA/TBS (1:200 for Thy1, 1:100 for calbindin, or 1:100 for glial fibrillary acidic protein [GFAP]) was performed overnight at 4°C in a humid chamber. After thorough washing, goat anti-rabbit secondary (KPL, Gaithersburg, MD) and goat anti-chicken secondary (Promega Corp., Madison, WI), at dilutions of 1:25 and 1:50, respectively. Slides were rinsed with 20 ml/min for 20 minutes. Both eyes were incubated, and their anterior segments were cryopreserved in sucrose/OCT (Sakura Finetek USA, Inc.). Cryosections 8 μm thick were collected onto slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) and stored at −80°C before immunolabeling. Sections were fixed in methanol at −20°C for 5 minutes and washed in Tris-buffered saline (TBS) containing 0.3% Triton X-100. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide for 30 minutes. Nonspecific binding was blocked with 10% normal goat serum in TBS and 0.3% Triton X-100 for 1 hour before incubation with primary antibody for 16 hours at 4°C. Sections were incubated with biotinylated secondary antibodies, either goat anti-rabbit or rabbit anti-chicken (Promega) at dilutions of 1:250 and 1:200, respectively. Slides were washed and incubated with the secondary reagent from the avidin-biotin complex (ABC) kit (Vectastain Elite; Vector Laboratories), using 3-amino-9-ethylcarbazole as the chromogen. Negative control experiments included nonimmune serum of the same species as the primary antibody at the same protein concentration in incubation buffer alone. Labeled sections were mounted in Kaiser’s glycerol jelly and viewed by Nomarski optics. Images of all slides were captured digitally using standardized microscope and camera settings (Axioskop and AxioCam with Axiosvision ver. 3 software; Carl Zeiss Meditec).

Western Blot Analysis

Eyes were enucleated under deep ketamine/xylazine anesthesia, anterior segments were removed, and retinal wholemounts were isolated.
and shock frozen at −80°C within approximately 2 minutes of enucleation. Retinas were later homogenized into 300 μL of a solution containing 20 mM Tris (pH 7.4), 10% sucrose, 1 mM EDTA and protease inhibitors at 4°C. Whole cell homogenates were prepared ultrasonically and protein concentration was assayed using a protein assay (Bio-Rad, Hercules, CA). Proteins were separated by 15% sodium dodecylsulfate–polyacrylamide gel electrophoresis, with 30 μg of protein loaded in each lane and two duplicate lanes per eye to assess variability. Human recombinant BDNF was used as a positive control (1–2.5 mg). Proteins were transferred to nitrocellulose membrane with 0.2 μm pore size (Sigma-Aldrich). Effective protein transfer was verified by Coomassie protein staining. Mammaries were blocked and probed overnight at 4°C with rabbit polyclonal anti-BDNF N-20 (Santa Cruz Biotechnology) at 1:200 dilution. A peroxidase-conjugated donkey anti-rabbit secondary antibody (Amersham Pharmacia Biotech UK, Ltd., Amersham, UK) was used at a dilution of 1:2500. Immunoblots were visualized by chemiluminescence (ECL Plus; Amersham Pharma- cia Biotech UK, Ltd.), with the exposure time to autoradiograph film (XOMAT AR film; Eastman Kodak, Rochester, NY) adjusted to avoid over- or undersaturation. Image-analysis software (OptiQuant, ver. 3.1; Packard Bioscience, Meriden, CT) was used to quantify the intensity of the specific bands.

**RGC Axon Counting Procedure**

Rats were killed by exsanguination under deep ketamine/xylazine anesthesia before intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at a rate of 20 ml/min for 5 minutes followed by 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for an additional 20 minutes at 20 ml/min. A cross section of the optic nerve from both eyes of each animal was removed 1.5 mm posterior to the eye and 1 mm in thickness. This was post-fixed in 1% osmium tetroxide in phosphate buffer, processed into epoxy resin, sectioned at 1 μm thickness, and stained with 1% toluidine blue. The total nerve fiber count for treated eyes was estimated for each animal from these sections. To accomplish this, the area of the optic nerve in cross section was measured by outlining its outer border at 10× magnification on an image-analysis system with a digital camera and software (Sensys with Metamorph software; Universal Imaging Corp., West Chester, PA). The mean of three optic nerve area measurements was used for each nerve. To measure the density of axons, we captured 10 randomly selected, nonoverlapping images with a 100× phase contrast objective from each nerve. These were edited to eliminate nonneural objects and density in axons per square millimeter was calculated for each image and averaged for all images to derive a mean density for each nerve. The mean density was multiplied by the total nerve area to estimate the total fiber number. The number of axons counted per nerve was approximately an 11% sample (9500 axons) of the total number for a normal rat nerve. Examination of the nerves was performed by a person who was masked to the procedures that the eye had undergone. For this study, the number of axons in treated eyes was compared with the mean axon estimate from 205 normal Wistar rat nerves collected as part of other studies and counted in identical fashion. We counted 20 of the fellow normal eyes from this study and found them indistinguishable from the normal control number of axons recorded in a previous study (87,518 ± 4,955 cells/retina).  

### RESULTS

Of the 138 rats that were initially treated, 124 completed the study. The 14 exclusions included nine deaths related to anesthesia, two rats killed due to severe weight loss, and one rat with hypoxemia as a complication of experimental IOP elevation. Exclusions did not differ significantly between treatment groups.

### IOP Exposure

There was no significant difference in mean IOP among the three treatment groups (P > 0.05, ANOVA; Table 1). In addition, there was no significant difference in any measure of IOP exposure between saline and AAV-BDNF-WPRE-injected eyes (P > 0.05, ANOVA). A subset of 11 rats in the AAV-GFP-WPRE treatment group had peak IOP of more than 44 mm Hg, higher than the peak IOP of any rat in the other two groups. The reason for the higher peak IOP in these animals was unexplained and considered to be due to random variation. To evaluate the effect this small difference might have on the conclusions, we performed three analyses with respect to IOP. The first analysis compared all 30 AAV-GFP-WPRE rats with those in the other groups, whereas a second analysis included only the 19 AAV-GFP-WPRE rats with IOP less than 43.6 mm Hg. The third and final analysis was a multivariate regression of RGC survival, adjusting for IOP variation among animals (described in detail later).

### Assessment of Transgene Expression

The efficiency of transgene expression could most easily be assessed for AAV-GFP-WPRE because GFP-positive cells could be directly quantified by examination of retinal wholemounts. Transfection using AAV-GFP-WPRE was highly efficient among cells of the RGC layer, many of which had the size and dendritic morphology of RGCs and possessed an axon (Figs. 2 and 3). Many of the GFP-positive neurons were also positive for antibodies to Thy1, an antigen found predominately in RGCs (Fig. 5B). A small number of GFP-positive cells were identified by antibodies to calbindin, a molecule found in amacrine cells (Fig. 3D). None of the GFP-positive cells were labeled by antibodies to GFAP (data not shown).

The density of GFP-positive cells was 1.828 ± 299 cells/mm², or an estimated total of 72,273 ± 11,814 cells/retina. If every transfected cell were an RGC, the transfection efficiency would be 84.5% ± 13.8% of all RGCs (this comparison is derived by dividing the estimated total of GFP-positive cells by the estimated number of RGCs from axon counts). Because some proportion of the transfected cells were amacrine cells, the true efficiency would be lower than this upper estimate. Similarly, if the density of GFP-positive cells were divided by the number of RGCs back labeled after fluorescent gold injection into the superior colliculus, the upper limit of RGC transfection would be 74.7% ± 12.2% (values for both estimates use the mean ± SD for 13 retinas per group). In retinal cross sections (Figs. 3A, 3C), 40% transfected cells were found almost

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**Table 1. IOP Exposure in Glaucoma and Control Eyes**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean IOP (mm Hg)</th>
<th>Glaucoma</th>
<th>Control</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n=25)</td>
<td>21.4 ± 2.5</td>
<td>16.1 ± 1.1</td>
<td>5.2 ± 0.2</td>
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<td>AAV-BDNF (n=27)</td>
<td>21.2 ± 2.1</td>
<td>16.1 ± 1.2</td>
<td>5.1 ± 0.3</td>
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<td>AAV-GFP (all n=30)</td>
<td>22.7 ± 2.5</td>
<td>17.5 ± 1.1</td>
<td>5.2 ± 0.2</td>
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<tr>
<td>AAV-GFP (peak IOP &lt; 43.6 n=19)</td>
<td>21.8 ± 2.0</td>
<td>17.1 ± 1.0</td>
<td>4.7 ± 0.2</td>
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</table>

<table>
<thead>
<tr>
<th>Positive G-C Integral IOP (mm Hg-days)</th>
<th>Glaucoma</th>
<th>Control</th>
<th>Difference</th>
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</thead>
<tbody>
<tr>
<td>Saline (n=25)</td>
<td>34.5 ± 4.7</td>
<td>15.5 ± 2.4</td>
<td>18.5 ± 5.6</td>
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<tr>
<td>AAV-BDNF (n=27)</td>
<td>34.5 ± 5.5</td>
<td>16.5 ± 2.1</td>
<td>17.8 ± 5.5</td>
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<tr>
<td>AAV-GFP (all n=30)</td>
<td>40.4 ± 4.9</td>
<td>16.6 ± 1.8</td>
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<tr>
<td>AAV-GFP (peak IOP &lt; 43.6 n=19)</td>
<td>37.7 ± 4.2</td>
<td>16.0 ± 1.6</td>
<td>21.7 ± 4.0</td>
</tr>
</tbody>
</table>

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*IOVS*, October 2003, Vol. 44, No. 10
RGC layer (data not shown). The labeling in eyes exposed to AAV-BDNF-WPRE and AAV-GFP-WPRE was similarly localized to the RGC layer, but the differences from controls that were quantified by Western blot analysis could not be distinguished by masked grading of the less sensitive histologic method.

Estimation of Surviving RGCs by Axon Counting

In groups that did not have experimental glaucoma, differences in axon survival after 4 weeks of IOP elevation were not significant (P > 0.2, one-way ANOVA; Table 3). Compared with un.injected eyes, axon loss was 0.0% ± 12% after saline injection (n = 10), and 8.7% ± 12.3% after AAV-GFP-WPRE injection (n = 13; mean ± SD, P = 0.26, one-way ANOVA). In glaucomatous eyes, injection of AAV-BDNF-WPRE resulted in a highly significant increase in axon survival compared with injection of either AAV-GFP-WPRE (P = 0.002, T-test) or saline (P = 0.006, T-test; Table 3). Axon loss was 52.3% ± 27.1% in the saline-treated group (n = 25), 52.3% ± 24.2% in the AAV-GFP-WPRE-treated group (n = 30), but only 32.3 ± 23.0% in the AAV-BDNF-WPRE-treated group (n = 27). To exclude the possibility that differences in survival were due to differences in IOP between the groups, multivariate regression analysis was conducted using axon count as the dependent variable, treatment group as an independent variable and either mean IOP, peak IOP, or positive integral IOP as an additional independent variable. Adjusting for mean IOP or positive integral IOP differences between treatment groups, AAV-BDNF-WPRE-treated eyes had significantly higher axonal survival than saline or AAV-GFP-WPRE-treated eyes (all P < 0.05, Table 4). Adjusting for peak IOP differences between the groups, AAV-BDNF-WPRE-treated eyes had significantly greater axon survival than saline-treated eyes (P = 0.0059), and the increase in survival compared with AAV-GFP-WPRE-treated eyes nearly reached significance (P = 0.065).

Discussion

In an experimental rat glaucoma model, gene therapy with AAV-BDNF-WPRE had a beneficial effect on estimated RGC survival that was highly significant. Axon loss after 4 weeks of experimental IOP elevation was 52% in eyes treated with either saline or AAV-GFP-WPRE compared with only 32% in eyes treated with AAV-BDNF-WPRE. Considerable evidence suggests that this protective effect was mediated by BDNF. First, no protective effect was observed after injection of saline, excluding the possibility that the differential survival was mediated by events related to manipulation or penetration of the eye. Second, no protective effect was seen after injection of a virus that was identical with AAV-BDNF-WPRE, with GFP in place of BDNF. Similarly, we previously found no protective effect from an AAV vector incorporating both GFP and BDNF without WPRE, a construct that expressed very poorly in cells of the RGC layer (data not shown). Thus, viral expression of the BDNF transgene was necessary for the protective effect. Finally, increased BDNF expression in the inner retina by RGCs was necessary for the protective effect. Furthermore, many axons were labeled with GFP (Fig. 2, inset) leading from the RGCs to the optic disc (seen at left in A). Scale bars: (A, B), 100 μm; (insets) 50 μm.

Immunohistochemistry for BDNF showed substantial labeling in normal control retinas that was most prominent in the
produced in the normal adult retina, and even by RGCs themselves, it is likely that the glaucoma process disturbs the normal levels of BDNF. Our past reports show that retrograde transport of BDNF from outside the eye to RGC is obstructed with IOP elevation. Thus, transgene overexpression may compensate for loss of BDNF during the disease process, allowing greater RGC survival. There is evidence that neuronal injury downregulates responsiveness to BDNF, potentially decreasing the effect of existing levels of BDNF. Cheng et al., using AAV-mediated TrkB gene transfer into RGCs combined with exogenous BDNF administration, reported transiently increased neuronal survival after optic nerve transaction. Similarly, BDNF overexpression, as produced in our experiment, may balance a loss of receptor number and improve survival.

**Table 2.** Ratio of BDNF in Treated Eye to Fellow, Control Eyes by Quantitative Western Blot Analysis

<table>
<thead>
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<th></th>
<th>AAV-BDNF</th>
<th>AAV-GFP</th>
<th>Saline</th>
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<tbody>
<tr>
<td>Mean</td>
<td>4.92</td>
<td>1.24</td>
<td>1.28</td>
</tr>
<tr>
<td>SD</td>
<td>7.29</td>
<td>0.17</td>
<td>0.17</td>
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</table>

**Figure 3.** Immunolabeling of GFP-expressing neurons with specific antibodies. In (A) and (C), the retinal cross section shows that GFP-expressing neurons (green) were localized to the RGC layer, and had the morphology of RGCs. (B) and (D) are the same section as (A) and (C), respectively, reacted with specific antibodies (arrows). In (B), anti-Thy1 antibodies show that many of the GFP-positive neurons expressed this antigen, which is specific for RGCs (arrows). (D) Antibodies to calbindin, a molecule found in amacrine cells, were also identified in some cells that were GFP positive (arrows). (E) A section through the optic nerve head showing the large number of GFP-positive axons of RGCs that enter and pass through the optic disc. Scale bars, 50 μm.

**Figure 4.** BDNF Western blot. Increased BDNF expression relative to control was seen in a subset of glaucomatous eyes that received AAV-BDNF-WPRE (lanes B) but not saline (lanes S) or AAV-GFP-WPRE (not shown). Duplicate samples from each eye were run in adjacent lanes. Untreated fellow eyes are shown for comparison (lanes C). Human recombinant BDNF (2.5 ng) was used as a positive control (lane P).
We performed statistical analyses to exclude the possibility that differences in survival resulted from lower IOP exposure in the group expressing the BDNF transgene. The AAV-BDNF-WPRE- and saline-treated groups had no statistically significant difference in any IOP measure. The AAV-GFP-WPRE-treated group had a subset of eyes with higher peak IOP than in the other groups. The difference between this viral control group and the BDNF transgene expression group remained when these outliers were removed in a subgroup analysis. Furthermore, multivariate analysis adjusting for differences in IOP between groups showed that significant benefit remained for the BDNF transgene group.

Another study has reported a beneficial effect of gene therapy in a glaucoma model. McKinnon et al.54 injected an AAV vector coding for human baculoviral IAP repeat-containing protein-4 (BIRC4), an inhibitor of the cysteine protease enzymes, into one eye of rats. Experimental glaucoma was induced by sclerosis of aqueous humor outflow channels and the transgene-expressing eyes had significantly greater RGC survival than control glaucoma eyes. BIRC4 promotes RGC survival, presumably by inhibiting the enzymatic completion of apoptosis. There were differences in IOP exposure among treatment groups that were significant and greater than those observed in our study. As further experiments are undertaken to inhibit glaucomatous RGC death, it may be advantageous to block neuronal dysfunction at the earliest possible stage. In this regard, inhibition of neurotrophin deprivation is an upstream block neuronal dysfunction at the earliest possible stage. In this regard, inhibition of neurotrophin deprivation is an upstream block neuronal dysfunction at the earliest possible stage. In this regard, inhibition of neurotrophin deprivation is an upstream block neuronal dysfunction at the earliest possible stage. In this regard, inhibition of neurotrophin deprivation is an upstream block neuronal dysfunction at the earliest possible stage. In this regard, inhibition of neurotrophin deprivation is an upstream block neuronal dysfunction at the earliest possible stage. In this regard, inhibition of neurotrophin deprivation is an upstream block neuronal dysfunction at the earliest possible stage.

BDNF Gene Therapy in a Rat Glaucoma Model

expression.59 Some wild-type viruses exploit a similar effect to increase production of viral protein in host cells. The WPRE induces high level expression of native mRNA transcripts, acting to enhance mRNA processing and gene transport.60 Incorporation of WPRE into AAV improves GFP expression in cerebral neurons42 and RGCs.49 The results presented show that a large number of neurons were transected in the RGC layer. It is known that amacrine cells may represent as many as 50% of the RGC layer nuclei in the rat. We show clear evidence that many of the cells expressing transgenes with our vector are RGCs. Their somal size is larger than the 7-μm diameter of amacrine cells, whereas their dendritic morphology and the presence of an axon shows that they are RGCs. In addition, they frequently expressed Thy1, an antigen typically found in RGCs. A few neurons in the RGC layer that expressed GFP were positive for calbindin, indicating that they were amacrine cells. However, it may be that overexpression of BDNF by amacrine or other cells of the inner retina is equally effective as a mechanism for increased survival of RGCs. Although we believe that most of the cells expressing the transgene were RGCs, our conclusion that BDNF overexpression is protective does not depend on exclusive transfection of RGCs.

Estimation of the number of RGCs can be accomplished by several methods: axon counting, backfilling with dyes, labeling of RGCs with antibodies, and morphologic identification by light microscopy. None of these methods is ideal, but axon counts were the best choice for our experiment for several reasons. First, axon counting provides an accurate representation of the number of RGCs and has been used by many laboratories in vision science in exactly this setting.61–66 In mammalian optic nerves, there are no known fibers other than RGC axons. Severe injury to these axons (e.g., transaction) causes loss of all recognizable RGCs by retrograde degeneration in a shorter time than in the present experiment.67 Some reports suggest that a small proportion of RGCs survive without an axon after transaction for prolonged periods, but this was an artifact based on the backfilling method.68,69 Only 2 weeks after backfilling with dye, RGCs leak dye to glia and other cells of the RGC layer, producing inaccurate counts. This is especially a problem when RGCs degenerate, and their dye-filled contents are phagocytosed by neighboring cells. In a chronic glaucoma experiment, one might try to circumvent this problem by waiting to backfill with dye until just before death. However, the dye passage could be differentially obstructed from entering the eye at the nerve head by high IOP and by degeneration and derangement of architecture at the nerve head.69 Antibodies to identify RGCs have not been devised that are reproducible and that recognize all the RGC subtypes. Hence, antibody labeling is not a better alternative, because it would be subject to bias if certain RGC types were more susceptible to injury than others or died at different rates. Simply counting RGCs by morphology is imperfect, as the marginal difference between small RGCs and amacrine cells can be problematic.

Table 3. Percent Axon Loss Relative to Control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glaucoma n</th>
<th>No Glaucoma n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>52.3 ± 27.1</td>
<td>0.0 ± 12.0</td>
</tr>
<tr>
<td>AAV-BDNF</td>
<td>32.3 ± 23.0</td>
<td>7.9 ± 13.8</td>
</tr>
<tr>
<td>AAV-GFP (all)</td>
<td>52.3 ± 24.2</td>
<td>8.7 ± 12.3</td>
</tr>
<tr>
<td>AAV-GFP (peak IOP &lt; 43.6)</td>
<td>45.1 ± 24.7</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Data are the mean percentage ± SD

For retinal gene therapy to be successful, gene expression efficiency must be optimized. RGC transfection densities in published studies have often been low or unreported, although Cheng et al.53 have recently reported transduction of 68% of rat RGC after intravitreal AAV injection. We achieved high levels of RGC transfection after single intravitreal injections of AAV vectors with two important elements. First, we used a hybrid CMV/CBA promoter that gives more efficient transfection of retinal neurons, particularly RGCs, compared with the CMV promoter alone. Second, we added the posttranscriptional regulatory element from the woodchuck hepatitis virus (WPRE) to increase viral protein translation. Transduction requires not only gene delivery, but also efficient transfection of transfecting genes into functional protein. The presence of introns associated with a gene of interest can increase expression.59 Some wild-type viruses exploit a similar effect to increase production of viral protein in host cells. The WPRE induces high level expression of native mRNA transcripts, acting to enhance mRNA processing and gene transport. Incorporation of WPRE into AAV improves GFP expression in cerebral neurons and RGCs. The results presented show that a large number of neurons were transected in the RGC layer. It is known that amacrine cells may represent as many as 50% of the RGC layer nuclei in the rat. We show clear evidence that many of the cells expressing transgenes with our vector are RGCs. Their somal size is larger than the 7-μm diameter of amacrine cells, whereas their dendritic morphology and the presence of an axon shows that they are RGCs. In addition, they frequently expressed Thy1, an antigen typically found in RGCs. A few neurons in the RGC layer that expressed GFP were positive for calbindin, indicating that they were amacrine cells. However, it may be that overexpression of BDNF by amacrine or other cells of the inner retina is equally effective as a mechanism for increased survival of RGCs. Although we believe that most of the cells expressing the transgene were RGCs, our conclusion that BDNF overexpression is protective does not depend on exclusive transfection of RGCs.

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Table 4. Comparison of Differences in Axon Loss between Treatment Groups by Multiple Regression Analysis Adjusting for IOP

<table>
<thead>
<tr>
<th>RGC Loss</th>
<th>Mean G-C IOP Difference</th>
<th>Peak Glaucoma IOP</th>
<th>Positive G-C Integral IOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV-BDNF vs. saline</td>
<td>0.0066</td>
<td>0.0059</td>
<td>0.0087</td>
</tr>
<tr>
<td>AAV-BDNF vs. AAV-GFP</td>
<td>0.0027</td>
<td>0.0047</td>
<td>0.0211</td>
</tr>
<tr>
<td>AV-GFP vs. saline</td>
<td>0.9857</td>
<td>0.1048</td>
<td>0.7981</td>
</tr>
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Hence, axon counting allows a survey of the complement of RGC axons in one section, whereas the other methods require production of a whole mount specimen with difficulty in differential preservation and shrinkage across the tissue. We have counted the number of axons and the number of morphologically identifiable RGC bodies in a large number of human and monkey specimens and have compared the human tissues in this work with functional testing by visual field perimeter (Carl Zeiss Meditec-Humphrey Systems, San Leandro, CA) in the same eyes. The fact that the methods are highly correlated shows validity for the axon counts. A large group of investigators have found that axon counting is a useful and valid method to estimate the number of RGCS. The method that we use samples a high proportion of the axons, giving the most valid estimate of any published method for axon counting.

Although we found a protective effect of BDNF transgene overexpression after 4 weeks of IOP elevation, it is important to demonstrate that the benefit continues over longer periods. We also plan to investigate whether other neurotrophic factors, such as ciliary-derived neurotrophic factor, give similar or additive protection. Demonstration of a similar protective effect in nonhuman primate eyes with experimental glaucoma is an important step that would support the concept of gene therapy in human glaucoma.

In summary, we report a highly significant increase in estimated survival of RGCs after 4 weeks of experimental glaucoma in rats overexpressing a BDNF transgene. This study is a successful demonstration of a major neuroprotective effect of gene therapy in a glaucoma model. Development of gene therapeutic strategies may provide new options for the treatment of patients with glaucoma.

References


