Recurrent Injections of a Ciliary Neurotrophic Factor Analogue Leading to Long-term Photoreceptor Survival in Hereditary Retinal Degeneration

N. H. Victor Chong,1,2 Robert A. Alexander,1 Lorraine Waters,1 Keith C. Barnett,1 Alan C. Bird,2 and Philip J. Luthert1

Purpose. To determine whether ciliary neurotrophic factor (CNTF) or brain-derived neurotrophic factor (BDNF) treatment leads to long-term photoreceptor survival in hereditary retinal degeneration.

Methods. An autosomal dominant feline model of rod-cone dystrophy was used throughout the study with two normal animals. In the first experiment, intravitreal injections of a human CNTF analogue (Axokine; Regeneron Pharmaceuticals, Tarrytown, NY) were administered to one eye of each animal (n = 10) beginning on postnatal day 10 and were repeated every 4 weeks. Clinical and histopathologic examinations were performed at 5.5, 9.5, and 13.5 weeks. The second experiment, animals (n = 17) were randomly assigned to receive intravitreal injections of either Axokine (at half the initial dose), human BDNF, or the vehicle for Axokine to one eye at 5.5 weeks. The same therapy was repeated every 4 weeks in each group. Clinical and histopathologic examinations were performed at 9.5, 13.5, and 17.5 weeks. Photoreceptor survival was assessed by cell counting. Apoptotic cells were identified by morphology and a modified TdT-dUTP terminal nick-end labeling (TUNEL) technique. In the third experiment, two normal animals were treated with Axokine as in the first experiment. Glial fibrillary acidic protein (GFAP) immunohistochemistry was performed to assess glial cell reaction.

Results. In the first two experiments, Axokine significantly prolonged photoreceptor survival (P < 0.01) and reduced the presence of apoptotic cells (P < 0.05) and TUNEL-positive cells (P < 0.05). In the second experiment, results in the the BDNF- and sham-injected eyes were not significantly different from those in the untreated eyes. Minimal posterior subcapsular cataract and mild retinal folds were found in all Axokine-treated eyes in both dystrophic and normal animals. These complications were milder in the second experiment when injections were started later and at a reduced dose. GFAP immunolabeling was also increased in all Axokine-treated eyes.


Retinitis pigmentosa (RP) is a group of hereditary disorders involving retinal degeneration characterized by poor night vision and restricted visual field. It affects approximately 1 in 3500 people. There is currently no effective treatment, and it is one of the leading causes of working-age blindness in the developed world. Despite the increasing number of disease-causing mutations that have been identified, the pathophysiology of photoreceptor death in this group of conditions is largely unknown. There is convincing evidence that the genetic defects can lead indirectly to cell death. This is evident, for example, in cone loss in patients with retinitis pigmentosa due to mutations in the rhodopsin gene, which is expressed only in rods. It is of interest that in these and other retinal degenerations photoreceptor cell death occurs by apoptosis. These observations imply that therapeutic modification of the process leading to apoptosis or the apoptotic pathway itself may modify the course of these disorders.
### TABLE 1. Apoptotic Cell Count and TUNEL-Positive Cell Count

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<th>9.5 (n = 4)</th>
<th>13.5 (n = 3)</th>
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<td><strong>First experiment: Axokine-treated versus untreated eyes</strong></td>
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<td>Apoptotic cell count</td>
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<td>Axokine</td>
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<td>1.08 ± 1.02†</td>
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<td>Untreated</td>
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<td>TUNEL count</td>
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<td>Untreated</td>
<td>0.64 ± 0.60</td>
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<table>
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<th></th>
<th>9.5*</th>
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<td><strong>Second experiment: Axokine versus BDNF versus vehicle versus no treatment</strong></td>
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<tr>
<td>Axokine†</td>
<td>2.76 (1)</td>
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<td>TUNEL count</td>
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<td>2.52 ± 1.54 (6)</td>
<td>1.78 ± 0.60 (7)</td>
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All data are means ± SD cell counts in 1 mm of retina with sample number in parentheses.

* Age in weeks.
† P < 0.05, treated versus untreated eyes.

An unexpected finding was observed during the early attempts at retinal transplantation for photoreceptor rescue. Focal injury to the retina appears to protect nearby photoreceptors from degeneration. This was clearly illustrated in the Royal College of Surgeons (RCS) rat in which mechanical injury produced by an injection of saline into the subretinal space or into the vitreous or even insertion of a needle without injection led to protection of photoreceptors near the wound. This protection is not restricted to genetically determined retinal degeneration. Similar photoreceptor rescue by mechanical injury was observed in light-induced retinal damage in the rat. Injury-induced photoreceptor rescue extends beyond the immediate vicinity of the lesion, suggesting that diffusible factors may be involved. Because mechanical injury to the eye increases the expression of basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF) in the rat retina, it is logical to assume that these agents may be responsible, at least in part, for this protection.

A single intravitreal injection of bFGF delayed photoreceptor loss in the RCS rat. Subsequently, bFGF, CNTF, and brain-derived neurotrophic factor (BDNF) were found to protect photoreceptors from light-induced retinal damage in the rat and the mouse. In addition, CNTF appears to be protective in the retinal degeneration (rd) mouse and the transgenic mouse with the Q344ter rhodopsin mutation.

To date, neurotrophic factors have been shown to be effective only in rodents in which a single injection is used for short-term rescue. To determine whether this phenomenon is specific to rodents and whether these factors can achieve long-term rescue, we studied the capacity of multiple intravitreal injections of a human CNTF analogue (Axokine) and human BDNF to provide long-term protection of photoreceptors in an autosomal dominant feline model of rod-cone dystrophy (Rdy cats). Axokine (Regeneron Pharmaceuticals, Tarrytown, NY) is a modified form of human CNTF to enhance its specific activity.

### METHODS

The Rdy cat was used throughout the study with two normal animals. In brief, the features are those of an autosomal dominant rod-cone dystrophy with photoreceptor degeneration beginning at 5 weeks of age. The peak of apoptosis occurs at approximately 9 weeks. The inner retina is relatively well preserved until end-stage disease.

The animals were bred and studied under the regulation of the UK Animals (Scientific Procedures) Act 1986 and all animal procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

All the intravitreal injections were performed by one of the authors (NHVC). Animals were anesthetized by a gas mixture of halothane, nitrous oxide, and oxygen. A disposable 0.3-ml insulin syringe with a fixed 29-gauge needle was inserted 2 mm to 4 mm behind the limbus (depending on the age of the animal) and directed toward the optic nerve. When the tip of needle reached the midvitreous, the injection was administered in a single, swift action. A gentle ocular massage was performed for 2 minutes to reduce the intraocular pressure.

In the first experiment (early treatment), Axokine (5 µg in 50 µl vehicle) was injected intravitreally in one eye at postnatal day 10 (n = 10). The other eye acted as an untreated control. The injection was repeated every 4 weeks. After each intravitreal injection a 5-day course of topical prednisolone were given to both eyes. Clinical (ophthalmoscopy) and histopathologic
examinations were performed in cats at 5.5, 9.5, and 13.5 weeks of age.

In the second experiment (delayed treatment), we investigated the effects of the Axokine (2.5 μg in 25 μl), human BDNF (250 μg in 25 μl) or sham injection with 25 μl phosphate-buffered solution (PBS) at pH 8 (vehicle of Axokine) in one eye, with the other eye untreated. Animals (n = 17) were randomly assigned to one of the three injection groups. Only one eye was injected, the other eye remained untreated. The injections were started at 5.5 weeks and repeated every 4 weeks. For 5 days after each intravitreal injection, topical prednisolone was applied to both eyes. Clinical (ophthalmoscopy) and histopathologic examinations were performed in cats at 9.5, 13.5, and 17.5 weeks of age.

In the third experiment, we injected a small number (n = 2) of normal animals with Axokine to evaluate complications using identical protocol as the first experiment.

All animals were killed with an overdose of systemic phenobarbital. The eyes were immersed in 10% formalin in the first experiment and in 4% paraformaldehyde with PBS in the second and third experiments. After 24 to 48 hours of fixation, the eyes were hemisectioned circumferentially at the equator. The posterior eyecup was then divided horizontally and vertically through the optic nerve head. The superonasal quadrant was embedded in Araldite resin using a standard protocol. Photoreceptor cell and apoptotic cell counts of the dystrophic animals were performed in 1-μm-thick toluidine blue-stained Araldite sections. The same portions of the eye were counted in all cases. The inferonasal quadrant was embedded in paraffin wax for the modified Terminal deoxynucleotidyl transferase, Uridine triphosphate, Nick End Labeling technique and immunohistochemistry. The same portions of the eye were counted in all cases. The superior and inferior quadrants were oriented in such a way that the sections counted were only separated by the cut edge.

An antibody against glial fibrillary acidic protein (GFAP) (Dako, UK) was used to assess Müller cell activity using a standard biotin-streptavidin peroxidase method. Antigen retrieval pretreatment with trypsin was performed before primary antibody incubation. Appropriate positive and negative controls were used.

**Photoreceptor Cell Count**

A random location near the center of the section was chosen at low magnification. The section was then moved 550 μm from the random spot. An eyepiece graticule (Graticules, Tonbridge, UK) was used to obtain a 25-μm-wide retinal strip. The number of cells within this strip was counted. The section was then
moved 250 μm toward the center, and another 25-μm-wide retinal strip was counted. This was repeated twice to obtain a total of four readings. One hundred micrometers of retinal width was counted. The number of photoreceptor cells counted was then multiplied by 10 and expressed as the number of cells per 1 mm of retina.

**Identification of Apoptosis**

Apoptotic cells were identified on morphologic grounds as cells with condensed pyknotic nuclei. The whole section was counted. The result was expressed as the number of apoptotic cells per 1 mm of retina.

An in situ 3'-tailing reaction was used to detect double-strand breaks in DNA. In this modification of the TdT-dUTP terminal nick-end labeling (TUNEL) technique, dUTP was replaced with deoxyadenosine triphosphate (dATP). Sections of rat duodenum were used as positive control specimens, and omission of the transferase enzyme from the reaction mixture served as a negative control. Cells in the entire section were counted. The result was expressed as the number of TUNEL-positive cells per 1 mm of retina.

Counts of photoreceptor cells, apoptotic cells, and TUNEL-positive cells were performed using the microscope setting of ×40 objective with a ×10 eyepiece, providing a 0.5-mm diameter field. All apoptotic cell counts and TUNEL-positive cell counts were performed independently by two observers. The difference in results between the two observers was less than 10%, and the mean was taken as the final result.

**Retinal Fold Counting**

The number of retinal folds was counted in each section of each animal. The results were expressed as the number of folds per 1 mm of retina.

**Statistical Analysis**

All three parameters (photoreceptor cell count, apoptotic cell count, TUNEL-positive cell count) in each treatment group were compared with those in the untreated group using paired Student's t-test at individual time points and then the groups as a whole. All statistical analysis was performed using commercial software (Excel 95; Microsoft, Redmond, WA).

**RESULTS**

During the study, the animals tolerated the procedure well and appeared to be pain free. Topical prednisolone was applied prophylactically and no active inflammation was observed clinically, even in the early postoperative period.
FIGURE 2. Histopathology of Rdy cats. (A) Retina of the untreated eye in a cat 17 weeks of age showing apoptotic cells (arrows); (B) retina of the Axokine-treated eye at 17 weeks of age; (C) lens of the Axokine-treated eye at 17 weeks of age showing posterior subcapsular cataract (arrows); (D) retina of the Axokine-treated eye at 17 weeks of age showing retinal fold.

Cell Counts in Axokine-Treated Versus Untreated Eyes

In the first experiment, we assessed whether Axokine had a protective effect. The results of photoreceptor cell counts, apoptotic positive cell counts, and TUNEL cell counts in the initial experiment are summarized in Table 1A and Figure 1. Photoreceptor cell counts were significantly higher in the Axokine-treated group at all time points (P = 0.02 at 5.5 weeks; P < 0.0001 at 9.5 weeks; P = 0.003 at 13.5 weeks). Conversely, apoptotic cell counts (P = 0.01) and TUNEL cell counts (P = 0.04) were significantly reduced at 9.5 weeks, and the TUNEL count was significantly lower at 13.5 weeks (P = 0.04).

Cell Counts in Axokine-Treated Versus BDNF-Treated Versus Sham-Treated Versus Untreated Eyes

After the initial success with Axokine, in the second experiment, we delayed the beginning of therapy to 5.5 weeks and compared the effects of Axokine (at half the dosage of the initial experiment), human BDNF, and vehicle of Axokine. The results of photoreceptor cell counts, apoptotic cell counts, and TUNEL positive cell counts are summarized in Table 1B and Figure 1B. The difference in photoreceptor cell counts was statistically significant in the Axokine-treated group at 13.5 (P = 0.03) and 17.5 weeks (P = 0.02; Figs. 2A, 2B). Although the indices of apoptosis were not statistically significant in the untreated eyes at individual time points, the entire Axokine-treated group had significantly fewer apoptotic cells (P = 0.03) and TUNEL-positive cells (P = 0.006) than did the untreated controls. The difference between results in the BDNF- and vehicle-treated groups and those in the untreated eyes were not statistically significant in any of the parameters measured.

Other Clinical and Histopathologic Findings

Minimal posterior subcapsular cataracts were observed clinically and histopathologically in all Axokine-treated eyes in dystrophic and normal animals. The lens opacities were less than 5% of the surface area of the lens in all Axokine-treated
FIGURE 3. GFAP immunohistochemistry in cat retina. (A) Untreated normal eye in a cat 13 weeks of age; (B) Axokine-treated normal eye at 13 weeks of age; (C) untreated eye of Rdy cat at 13 weeks of age; (D) Axokine-treated eye of Rdy cat at 13 weeks of age.

animals. They affected the posterior subcapsular area only, and there was no extension into the nucleus of the lens. None of the cataracts obscured the fundus examination. In light microscopy, there were more cells present posterior to the equator of the lens with the associated opacities (Fig. 2C). Although quantification was not performed, treated animals in the second experiment (delayed and lower dose) appeared to have fewer lens opacities.

In all Axokine-treated eyes in dystrophic and normal animals, there were small retinal folds seen in histopathologic analyses (Fig. 2D). These folds were not detected in clinical examination by ophthalmoscopy. No vitritis or inflammatory response was observed either clinically or histopathologically. In Axokine-treated eyes, the mean number of retinal folds per millimeter of retina in the first and second experiments was 1.802 ± 0.419 and 0.588 ± 0.366, respectively. The difference was statistically significant (P = 0.0006). Neither cataract nor retinal folds were seen in any of the BDNF-injected, vehicle-injected, or untreated eyes.

Although no formal quantification of the ophthalmoscopic findings was obtained, the overall difference between treated and untreated eyes appeared to be small.

GFAP Immunohistochemistry

In the untreated normal animals, astrocytes were immunopositive for GFAP (Fig. 3A). In the Axokine-treated normal animals, there was a marked increase of GFAP immunolabeling in the Müller cells (Fig. 3B). In the untreated Rdy cats, an increase of GFAP immunolabeling of Müller cells was found (Fig. 3C), and there was a suggestion of a further increase of GFAP immunolabeling of Müller cells in the Axokine-treated Rdy cats (Fig. 3D).

DISCUSSION

In the present study, Axokine (a human CNTF analogue) reduced apoptotic cell death of photoreceptors in the Rdy cat, and multiple injections provided long-term protection. However, human BDNF was not effective. These results are similar to those reported in the mouse models. In the light damage model of retinal degeneration, both CNTF and BDNF delay photoreceptor degeneration. The ineffectiveness of BDNF in this cat model may be explained by the fact that we were using human BDNF instead of feline BDNF. However, the same preparation at a slightly lower dose (100 µg) of human BDNF
promotes outer segment regeneration in an acquired feline retinal degeneration model. Although we cannot exclude the possibility that different dosages of BDNF may have an effect in the Rdy cats, it seems unlikely, because the difference in dosage was only 2.5-fold. It is possible that BDNF can protect the retina from acute injury, as occurs in light damage and retinal detachment, but that it is ineffective in hereditary retinal degeneration. 

The difference in response may be explained by the probable existence of more than one route leading to apoptosis, the final common pathway of retinal degeneration. The synergistic effect of CNTF and BDNF in enhancing the rate of functional recovery after peripheral nerve transection also suggests that the two neurotrophic factors may act on different pathways. Furthermore, they are known to activate different signal transduction pathways.

Cataract formation and retinal folds were relatively minor, and have not been reported after CNTF therapy. These complications are unlikely to be related to surgical trauma, because they were present only in Axokine-treated eyes. Both complications were less severe in the second, lower dose experimental group, suggesting that the response may be dose dependent.

CNTF is known to increase expression of glial fibrillary acidic protein (GFAP) in Müller cells. It is also possible that Axokine increases GFAP expression in the treated animals and may therefore, directly or indirectly, constitute other components of a glial injury response, including the release of endogenous growth factors.

In principle, Axokine may cause cataract formation directly or through the secondary release of other growth factors. Although the CNTF alpha receptor has not been shown in the human lens epithelium, a direct effect cannot be excluded. The receptors of other growth factors, including bFGF are present in lens epithelial cells. Because bFGF can induce proliferation of lens epithelial cells in vitro, it is therefore a possible candidate for an intermediate agent.

Similarly, the retinal folds may also be secondary to bFGF, because intravitreal injection of this growth factor causes retinal folds with severe inflammation in the rabbit. This effect is also dose dependent. The absence of inflammation in the Rdy cats may reflect a species difference. It is, however, possible that Axokine acts on the Müller cells, leading directly to retinal swelling and then folds. CNTF is known to produce swelling of astrocytes in vitro.

Based on our data, repeated intravitreal injections of Axokine, but not human BDNF, provide long-term photoreceptor survival in hereditary feline retinal degeneration and may be a therapy for human retinitis pigmentosa.

Acknowledgment

The authors thank Regeneron Pharmaceuticals, Tarrytown, New York, for generously donating Axokine and human recombinant brain-derived neurotrophic factor.

References

Retinopathy Associated with Enterococcus Enteropathy in the Neonatal Rat

Shuichen Zhang,1 David A. Leske,1 James R. Ubl,2 Franklin R. Cockerill III,2 William L. Lanier,3 and Jonathan M. Holmes1

Purpose. Preretal neovascularization has been previously observed in neonatal rats with spontaneously occurring diarrhea. This neovascularization appears analogous to retinopathy of prematurity (ROP), which occurs in human neontates. A new enterococcus species, designated Enterococcus rattus, has been isolated from the duodenal contents of these rats, and it was determined to be a species new to the Enterococcus genus. In this report, we describe a controlled study of Enterococcus rattus in vivo to determine whether it induces preretal neovascularization similar to ROP in the neonatal rat.

Methods. One hundred fifty newborn Sprague-Dawley rats were randomly assigned to six expanded litters (n = 25). On the second day of life, animals were gavaged with either 100 μl of E. rattus suspension (1.0 × 107 colony forming units, inoculated group, n = 100 rats) or 100 μl saline (control group, n = 50 rats). All rats were raised in room air and were killed on day 13 of life. Duodenal and blood samples were cultured. The retinal vasculature was assessed using fluorescent microscopy and ADPase staining in a masked manner. Two additional inoculated litters and one control litter were studied for evaluation of arterial blood gases and validation of the grading method for preretal neovascularization.

Results. One hundred percent of rats in the inoculated group developed severe diarrhea and had duodenal cultures positive for E. rattus compared with 0% in the control group. Preretal neovascularization similar to ROP occurred in 55% of rats in the inoculated group compared with 2% in the control group (P = 0.001). Retinal vascular areas were reduced in the inoculated group (mean ± SD, 89% ± 5% versus 96% ± 2%; P < 0.001). Rats in the inoculated group demonstrated severe growth retardation (final weight, 9.7 ± 2.2 versus 16.7 ± 2.7 g, P < 0.001). Inoculated animals also experienced acidosis (pH 7.31 ± 0.06 versus 7.39 ± 0.06 control, P = 0.04).

Conclusions. A previously undescribed enterococcal enteropathy was associated with preretal neovascularization similar to ROP in the neonatal rat. This supports an independent role for factors other than inspired oxygen in the development of ROP. (Invest Ophthalmol Vis Sci. 1999;40:1305-1309)

Although hyperoxia is considered to be a major risk factor in the development of retinopathy of prematurity (ROP) in human neonates, the incidence of ROP is increasing despite the judicious use of oxygen.1 ROP occurs more frequently in the sickest and smallest of premature infants.1 Clinical and experimental studies have suggested that other factors, such as sepsis, carbon dioxide, acidosis, and postnatal growth retardation, may play a role in the pathogenesis of ROP.2-6

The analysis of factors by retrospective clinical studies is limited by potentially confounding variables. The use of animal models of ROP may help evaluate the contributions of a single factor in pathogenesis. Severe preretal neovascularization analogous to ROP is observed in the rat model of oxygen-induced retinopathy (OIR) after exposure to cyclic hyperoxia and hypoxia. Room air controls in this model do not develop neovascularization.4

We have previously reported the observation of preretal neovascularization in neonatal rats with spontaneously occurring diarrhea.7 These rats were never exposed to hyperoxia or hypoxia. We isolated the causative bacterium from the duodenal content of these rats, and it was determined to be a previously undescribed Enterococcus by analysis of the DNA sequence of the 16S ribosomal RNA gene.8 This bacterium has been designated Enterococcus rattus. In this report, we describe a controlled study of E. rattus gavage on the retinal vasculature of the neonatal rat.

Methods

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.