Protection of Ganglion Cells in Experimental Glaucoma by Retinal Laser Photocoagulation

T. Michael Nork, MD; Gretchen L. Poulsen; Robert W. Nickells, PhD; James N. Ver Hoeve, PhD; Nam-Chun Cho, MD; Leonard A. Levin, MD, PhD; Mark J. Lucarelli, MD

**Objective:** To test a hypothesis of photoreceptor involvement in retinal ganglion cell (RGC) death in chronic glaucoma.

**Methods:** Laser spots were applied to 6 eyes of 3 rhesus monkeys, causing focal destruction of the outer retina, including the photoreceptors. After 3 to 4 weeks, experimental glaucoma was induced in the right eyes of each monkey using argon laser trabecular destruction (ALTD). The intraocular pressures in these eyes were elevated for 3 to 7 months. As a control, 1 additional monkey underwent retinal laser photocoagulation followed by optic nerve transection instead of ALTD. Following enucleation, the retinas were embedded and sectioned for histologic evaluation.

**Results:** There was extensive loss of RGCs in the eyes with ALTD except over the large retinal laser spots, where there was an increased survival of RGCs. The RGC protection was not observed in the monkey that had undergone optic nerve transection.

**Conclusion:** Photocoagulation of the outer retina that completely destroys the photoreceptors results in survival of the overlying RGCs in experimental glaucoma in monkey eyes.

**Clinical Relevance:** Although this is an experimental model and not a therapeutic option, these results suggest that treatments other than lowering intraocular pressure may be potential therapies for preventing RGC death in glaucomatous eyes.


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Daniel M. Albert, MD, MS

**C**HRONIC GLAUCOMA is one of the leading causes of blindness despite advances in pharmacologic and surgical treatment to lower intraocular pressure (IOP). Elevated IOP is traditionally thought to injure the retinal ganglion cell (RGC) axons as they pass through the optic nerve head, either by mechanical deformation of the lamina cribrosa or by reducing the blood supply to the nerve. The RGCs are then presumed to die by retrograde degeneration owing to interruption of axonal transport that brings neurotrophins from the brain to the eye. However, this hypothesis of RGC death in glaucoma, referred to in this article as the retrograde hypothesis, does not explain several observations.

For example, it has long been known that glaucoma results in a blue-yellow color confusion—a type of color vision deficit that is more characteristic of outer retinal disease than optic nerve injury. Findings from some electrophysiological studies have suggested photoreceptor involvement, including a recent finding of increased latency of the early multifocal electroretinographic response in an arcuate distribution in human glaucoma. Furthermore, Yancey and Linsenmeier found decreased choroidal PO2 and changes seen in the C wave and standing potential of the full-field electroretinogram (which they ascribed to outer retinal ischemia) with even moderate short-term elevations of IOP in cat eyes.

Photoreceptor involvement in glaucoma has also been observed at the histopathologic and molecular levels. In hu-
MATERIALS AND METHODS

RETINAL LASER PHOTOCOAGULATION FOLLOWED BY INDUCTION OF EXPERIMENTAL GLAUCOMA

All animal procedures adhered to the Association for Research in Vision and Ophthalmology, Rockville, Md, statement on the use of animals in vision research. Eight eyes of 4 rhesus monkeys underwent retinal laser photocoagulation. The laser spots, using the argon green wavelength (514.5 nm) (Coherent, Santa Clara, Calif), were applied to the region of the posterior pole that corresponds to early visual field loss in humans with glaucoma, ie, 5 to 20 arc degrees eccentric to fixation. Various spot sizes were created, from 100 to 4000 µm (spots more than 1000 µm were made by applying confluent 200-µm individual burns) with energies from 50 to 130 mW and exposure times from 0.1 to 0.5 seconds.

After about 1 month (enough time to permit the debris of photocoagulation to be cleared by the retina), argon laser trabecular destruction (ALTD) was performed in 1 eye of 3 animals.21,22 Briefly, a Kaufman-Wallow single-mirror monkey goniolens (Ocular Instruments Inc, Bellevue, Wash)23 was used to deliver the laser to the trabecular meshwork. During each treatment session, either 270° or 360° of the anterior (nonpigmented) meshwork was photocoagulated with 514.5 nm of argon green laser using either 50- or 100-µm spots of 1.0-W intensity and a 0.5-seconds’ duration. Seventy-five to 200 such spots were applied per session. The IOP, which was measured twice weekly with a handheld digital tonometer (Tono-Pen XL; Mentor O & O, Nowell, Mass), usually took 3 weeks to rise, and the elevation lasted variable lengths of time. (This device was found to underestimates the IOP at higher pressures in the cynomolgus monkey.23 A similar study has not been done for the rhesus, although its eye being intermediate in size between the human and the cynomolgus monkey, it might be expected to have a smaller error than that found in the cynomolgus monkey.) Additional laser treatments were carried out as needed to maintain elevated pressures. Two sessions of ALTD were administered to monkeys No. 1 and No. 3, and 4 sessions were used for monkey No. 2. In 1 animal (monkey No. 1), 1 drop of 0.5% timolol maleate (Timoptic; Merck & Co, Whitehouse Station, NJ) was applied to the cornea of the treated eye on 2 occasions to lower the IOP.

OPTIC NERVE TRANSECTION

To control for the possibility that retinal laser photocoagulation may promote RGC survival by mechanisms other than photoreceptor removal, the optic nerve was transected (instead of inducing experimental glaucoma) in 1 eye of 1 animal. As with the other monkeys, the fellow eye underwent retinal laser photocoagulation only. The optic nerve transection (ONT) was accomplished using the technique described by Gonnering et al.20 Briefly, a lateral orbitotomy was first performed. The optic nerve was then transected 6 to 8 mm behind the globe posterior to the entry of the retinal artery and vein. On recovery (approximately 1 week), a fluorescein angiogram with the animal receiving ketamine anesthesia was obtained to demonstrate patency of the retinal artery.

HISTOLOGIC PREPARATION

The animals were euthanized and the eyes were removed within 10 minutes and promptly chilled in 0.1-mol/L sodium phosphate buffer (pH 7.4) at 4°C. Within 5 minutes, the eyes were removed from the buffer and cut coronally just anterior to the equator. The posterior pole was fixed in 4% paraformaldehyde at 4°C for 24 hours and then stored in 0.1-mol/L sodium phosphate buffer (pH 7.4) at 4°C. Segments of retina containing individual laser spots were removed and embedded in glycol methacrylate, sectioned at a thickness of 1.9 µm, and stained with thionin.

TWO-DIMENSIONAL RECONSTRUCTIONS

Serial sections were cut through some of the retinal laser spots. For each histologic section, the nuclei of cells in the ganglion cell layer that had the anatomical characteristics of RGCs (ie, large round nucleus, prominent nuclei, abundant cytoplasm, and metachromatic thionin staining consistent with Nissl substance) were identified. The position of each nucleolus of cells meeting these criteria was measured with respect to a given point (either the mid point of the smaller laser burns or the edge of the large, confluent burns) using a micrometer in the oculus of the microscope. Two-dimensional (D) reconstructions of the laser spots were then plotted.

mans with glaucoma and in experimental glaucoma in monkeys, we found that the photoreceptors, especially the red and green cones, were swollen.19 Tezel et al19 reported that heat shock protein HSP 60 is elevated in the photoreceptors of human eyes with glaucomatous damage. Preliminary work in our laboratory also suggests that the messenger RNA for red- and green-cone opsin is selectively reduced in humans with glaucoma as well as in experimental glaucoma in monkeys.20

Photoreceptor injury in glaucoma might be an epiphenomenon or a response to dying ganglion cells. Alternatively, it could be that the photoreceptors play an important role in either causing or exacerbating ganglion cell injury in this disease. We will refer to this latter possibility as the anterograde hypothesis of RGC death in glaucomatous eyes.

Retinal ganglion cells do not depend on anterograde neurotrophic stimuli, as evidenced by their survival in such conditions as retinal laser photocoagulation,21,22 retinitis pigmentosa,23 and the Royal College of Surgeons rat.24 Therefore, the anterograde hypothesis requires that glaucoma cause some specific malfunction of viable photoreceptors that in turn destroys the RGCs. If the hypothesis is correct, local ablation of photoreceptors before induction of experimental glaucoma should protect the overlying RGCs, an effect that would not be predicted by the retrograde hypothesis (Figure 1). We present the results of such an experiment in which rhesus monkey photoreceptors were focally destroyed by retinal laser photocoagulation prior to the induction of experimental glaucoma. This treatment pro-
 spots. The locations of the putative RGC nucleoli were epithelium [RPE] cells, or macrophages), which were clei or contained cytoplasmic pigment granules (char-

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layer and in some cases the inner nuclear layer were intact. Few cells were present.

In the RGC layers of the 3 monkeys treated with ALTD, increased densities of cells were seen over the large laser spots (Figure 2) relative to the surrounding retina. (The effect was not seen over the small laser spots.) These cells were located in a layer contiguous with the adjacent RGC layer. The inner plexiform layer and in some cases the inner nuclear layer were intact. Few cells were present in the inner plexiform layer. The cells in the ganglion cell layer over the laser spots had large round nuclei, prominent nucleoli, abundant cytoplasm, and metachromatic thionin staining that was consistent with Nissl substance. Only a few cells in this layer had spindle-shaped nuclei or contained cytoplasmic pigment granules (characteristic of activated glial cells, retinal pigment epithelium [RPE] cells, or macrophages), which were readily distinguishable from the putative RGCs.

To better illustrate the phenomenon, serial sections were cut 1.9 μm thick through some of the laser spots. The locations of the putative RGC nucleoli were then measured and plotted on 2-D graphs. Figure 3 shows such a reconstruction comparing sections of retina from the same location of the temporal maculae in the left (control) and right (glaucomatous) eyes of monkey No. 1. Marked reduction in numbers of ganglion cells in the glaucomatous eye were seen everywhere except over the laser spot. Within the region where the photoreceptors had been ablated by previous retinal laser photoocoagulation, the overlying putative RGCs are present in densities approaching that of the control eye (Figure 4).

At somewhat greater eccentricities than the spot shown in Figure 2, there are fewer RGCs normally present. Even so, the effect was still present, as shown in Figure 5. Unlike the more intense laser spot shown in Figure 2, the cells in the inner nuclear layer over these spots were present in apparently normal numbers; however, a downward stretching of this layer was seen. In addition, a few macrophages and/or activated RPE cells were present.

A similar effect was also found over the large, confluent laser spot in monkey No. 3 (Figure 6) where the average density of cells in the RGC layer over the laser-damaged area in the glaucomatous eye was greater than the adjacent unlasered region (Figure 7). Unlike the more intense laser spot shown in Figure 2, the bipolar cells over these spots were present in normal numbers (not shown).

The mean densities as a function of foveal eccentricity for the sampled areas shown in Figure 7 were plotted in Figure 8. The densities were greater everywhere in the control eye (note the different vertical scales). Since the specimens were sampled from regions near the foveas (about 1 mm inferotemporal to the foveal centers, where ganglion cell density changes rapidly with eccentricity), these greater apparent densities in the control eye could be the result of the samples not being taken from corresponding areas. However, a generalized reduction in ganglion cell density in the eye with glaucoma could not be ruled out. The transition of densities across the edge of the laser spot was a smooth one for the control, with a gradual increase in ganglion cell density with decreasing foveal distances. By contrast, the glaucomatous eye showed a marked reduction in densities from the lasered to the unlasered portions of the retina.

Figure 1. Design of experiment to discriminate between the retrograde and anterograde hypotheses of ganglion cell death in chronic glaucoma. First, a retinal laser is applied (light gray), which is absorbed by the pigment epithelium and converted to heat (dark gray) that kills the photoreceptors (upper right). Experimental glaucoma is then produced by argon laser trabecular destruction. The 2 hypotheses predict different outcomes with respect to survival of the subset of ganglion cells overlying the healed laser spot (lower half). NF indicates nerve fiber layer; GC, ganglion cell layer; IN, inner nuclear layer; ON, outer nuclear layer; R/C, rod/cone layer; BM, basement membrane; and Ch, choroid.

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Figure 5. Unlike the more intense laser spot shown in Figure 2, the cells in the inner nuclear layer over these spots were present in apparently normal numbers; however, a downward stretching of this layer was seen. In addition, a few macrophages and/or activated RPE cells were present.

Table. The mean IOP ± SD, IOP Range, mm Hg‡.
One large spot (≥200-µm diameter of ablated photoreceptors) was quantitatively analyzed by measuring nucleolar positions and creating 2-D reconstructions for each of 3 eyes with glaucoma. All of these spots showed greater densities of ganglion cells than the surrounding retinas. In addition, a fourth large spot in the glaucomatous eye of monkey No. 1 was examined that subjectively appeared to have a greater RGC density than the surrounding retina (Figure 5). Several small spots (<100 µm) were visually inspected in all of the glaucomatous eyes. There was no qualitative increase in ganglion cells over these spots. Two-dimensional reconstructions were not made for the small spots.

Three large spots were also examined by nucleolar measurement and 2-D reconstruction in nonglaucomatous eyes. No difference in ganglion cell density was apparent compared with the surrounding retina (Figure 7 and Figure 9).

**TYPE OF CELLS OVER LASER SPOTS**

Although a few cells had features consistent with macrophages, activated RPE cells, or glial cells, most of the cells present over the retinal laser spots were probably RGCs. They were located at the level of and were continuous with the adjacent RGC layer. Few cells were seen in the inner plexiform layer, which would not have been the case had a great number of other cells (eg, glial or RPE) migrated in from elsewhere. Furthermore, a similar concentration of cells was not found either over the laser spots in the control eyes or over the retinal laser
spots in the eye with ONT—again suggesting that most of the cells over the laser spots in the glaucomatous eyes were not other cell types that might have formed or congregated as part of the wound-healing process.

Morphologically, the cells resembled RGCs in that they had large round nuclei, prominent nucleoli, abundant cytoplasm, and metachromatic thionin staining consistent with Nissl substance. Although anatomically similar in appearance, displaced amacrines are far too rare at these eccentricities to account for the density of cells found. Only a few cells in this layer had the spindle-shaped nuclei or contained cytoplasmic pigment granules characteristic of activated glial cells, RPE cells, or macrophages. Also, the distribution of these cells seen on 2-D reconstruction was similar to (albeit of greater density than) the surrounding RGC layer (see the large laser patch in Figure 7).

OTHER POTENTIAL CAUSES OF ALTERED RGC DENSITY OVER LASER SPOTS

We cannot completely rule out some effect on the RGCs over the laser spots in the nonglaucomatous eyes. A few histologic sections of some of the spots appeared to have a subtle increase in RGC-like cells. However, when the positions of all nucleoli in all the cells with morphologic characteristics of RGCs were measured and plotted, no average increase in density was apparent (see controls in Figures 5, 7, and 9). The cause of this apparent increase in RGC concentration in some of the sections may have been owing to normal random fluctuation, which is also seen over the nonlasered areas, or to slight shrinkage of the tissue over the laser spots. Increased RGC density has not been noted in the numerous publications describing the histologic features of laser spots in the eyes of either humans or monkeys. In any event, the effect, if it exists, is small compared with the markedly higher RGC concentrations over the laser spots relative to the surrounding retinas seen in the glaucomatous eyes.
POSSIBLE MECHANISMS FOR THE PROTECTIVE EFFECT OF RETINAL LASER PHOTOCOAGULATION IN GLAUCOMA

Nonspecific Protective Effect

Although, these results are predicted by the anterograde hypothesis, it is also possible that they could be

owing to some as yet unknown nonspecific effect not necessarily related to photoreceptor injury per se, such as thinning of the retina, which would allow more oxygen to reach the RGC layer from the choroid; releasing of neurotrophic or neuroprotective factors, such as basic fibroblastic growth factor, or activating synthesis of heat shock proteins in the RGCs. Activated retinal pigment epithelial cells, macrophages, or Müller cells could serve

Figure 5. Comparison of 2 retinal laser spots of similar intensity and eccentricity in monkey No. 1. The arrows indicate the border between viable and absent photoreceptors. A, Control eye. Spot is 3 mm superior to foveal center. One or 2 layers of nuclei are present in the ganglion cell layer. The density of cells in this layer over the ablated photoreceptors is similar to the surrounding, normal retina (toluidine blue, bar = 50 µm). B, Glaucomatous eye. Spot is 3 mm superotemporal to foveal center (indicated by white arrow in Figure 2, A). As in part A, 1 or 2 layers of nuclei are present in the ganglion cell layer over the ablated photoreceptors. However, the density of cells in this layer are greatly reduced in the surrounding, untreated retina.

Figure 6. Fundus photographs of both eyes for monkey No. 3 immediately following retinal laser photocoagulation. Note the confluent patches in the inferior maculae. A, Experimental glaucoma was subsequently produced in the right eye. The white rectangles represent the areas used for 2-dimensional reconstruction (Figure 7). B, Note that the sampled area in the control left eye is somewhat closer to the fovea than the treated right eye.
as mediators for these products. However, no protective effect was seen in the case of retinal laser photocoagulation preceding ONT. Therefore, a nonspecific effect, if there is one, is not sufficient to permit ganglion cell survival under these circumstances.

Owing to heat dissipation at the edges of the laser spots, the area of absent photoreceptors is smaller than the area of disturbed pigment in the RPE cells. This effect can be seen in Figure 2, where the diameter of whitening of the original spot is about 500 µm compared with a diameter of only about 250 µm of photoreceptor destruction (this disparity is notable even considering the approximately 15% tissue shrinkage on embedment in glycol methacrylate). Yet the extent of this protection seems to be limited precisely to the region of photoreceptor destruction. This, too, is consistent with the hypothesis that RGC death is mediated specifically by photoreceptor injury and not the result of laser damage to the RPE. Similarly, we believe that the small laser spots killed too few photoreceptors to produce a measurable protective effect.

The presence or absence of bipolar cells in the laser spots does not seem to be critical in affecting protection of the RGCs. Furthermore, as noted previously, simply damaging the RPE cells without killing the photoreceptors is insufficient to protect the RGCs. Therefore, it may be that elimination of the photoreceptors is a critical aspect of the protective effect.

Figure 7. Two-dimensional reconstruction of ganglion cells over the edge of 2 symmetrically placed, large, confluent laser spots in monkey No. 3. The positive numbers on the left axis denote distances over the burns. The thick black lines mark the edges of the burns—no photoreceptors are present over the burns (positive numbers). A distinct reduction in ganglion cell density is evident in the eye with glaucoma but not in the control eye. Larger distances along ordinates indicate decreasing foveal eccentricities.

Figure 8. Plots of average ganglion cell density as a function of foveal eccentricity. Positive numbers (to the right of the dotted lines) indicate ganglion cells overlying the laser spots (destroyed photoreceptors). The larger positive distances represent decreasing foveal eccentricity. Bars denote SEMs.

Figure 9. Two-dimensional reconstruction of ganglion cells over laser spots (no photoreceptors are present between the thick black lines) in a control eye and one that had undergone optic nerve transection (ONT) approximately 1 month following the laser application. The 2 eyes are from monkey No. 4. Both spots are located about 2.5 mm supranasal to the foveal centers. The plots are oriented such that the larger negative distances on the ordinates are closest to the foveas. There is no apparent change in density of ganglion cells seen over the laser spots in either eye, although the putative retinal ganglion cell density in the eye with the ONT is only about 10% of that in the control eye. The bipolar cells are present in normal numbers over the laser spots (not shown). The linear strip of lower density just above the upper thick black line in the control eye corresponds to a retinal vein.
Finally, nonspecific forms of RGC neuroprotection have been observed in a rat model of ONT. Yip et al. reported a preliminary study showing that a short period of low-level laser applied to the retina enhances RGC survival after optic nerve axotomy, and Di Polo et al. showed that RGC death could be delayed by prolonged delivery of brain-derived neurotrophic factor. In both of these studies, the effects were transient, while our laser treatment provides a long-term (at least for the several months’ duration of these experiments) rescue in primates with experimental glaucoma. The differences between these studies could be accounted for by several factors. First, axotomy may be too damaging for any nonspecific neuroprotective environment to overcome. This is evidenced in part by failure of the retinal laser photocoagulation to prevent RGC death in the axotomized primate eye. Alternatively, the loss of photoreceptors would provide a permanent break in the anterograde chain of events that leads to RGC death in glaucomatous eyes (see following section), and we would predict that the protective effect would be permanent. Both these models are consistent with our results and warrant further study.

Interruption of the Transsynaptic Glutamate Cascade

The protective effect on RGCs described here could be specific to a mechanism of RGC death in glaucomatous eyes (ie, consistent with the anterograde hypothesis). One possible mechanism by which this could occur might be by interruption of the transsynaptic glutamate cascade between the photoreceptors and the RGCs. Elevated levels of glutamate have been found in the vitreous body of eyes of humans and monkeys with glaucoma. The source of this excess glutamate has not been demonstrated in glaucomatous eyes, although Yoles and Schwartz showed of this excess glutamate has not been demonstrated in primates with experimental glaucoma. The differences between these studies could be accounted for by several factors. First, axotomy may be too damaging for any nonspecific neuroprotective environment to overcome. This is evidenced in part by failure of the retinal laser photocoagulation to prevent RGC death in the axotomized primate eye. Alternatively, the loss of photoreceptors would provide a permanent break in the anterograde chain of events that leads to RGC death in glaucomatous eyes (see following section), and we would predict that the protective effect would be permanent. Both these models are consistent with our results and warrant further study.


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