Retinal Damage after 3 to 4 Months of Elevated Intraocular Pressure in a Rat Glaucoma Model

Thom W. Mittag, John Danias, Geralyn Poborenc, Hong–Mei Yuan, Evren Burakgazi, Ruth Chalmers–Redman, Steven M. Podos, and William G. Tatton

PURPOSE. To characterize a long-term elevated intraocular pressure (IOP) glaucoma model in the rat with respect to electroretinographic (ERG) changes and the pattern and mechanism of retinal ganglion cell (RGC) death.

METHODS. An approximate doubling of IOP was induced in one eye (G) of female Wistar rats (150–180 g) by cautery of 3 episcleral/limbal veins. At intervals over 3 to 4 months, measurements of IOP and ERG changes (contact-lens electrode) were made in both the G and contralateral normal (N) eyes. At the end of 3 to 4 months of elevated IOP, RGCs were fluorescently labeled with Fluorogold (retrogradely from the superior colliculus), or retinas were labeled by intravitreal injection of a mitochondrial potential indicator dye and stained for apoptotic nuclei with a DNA dye. Flatmounts of fixed, dye-labeled retinas were examined by epifluorescence, confocal, or interference contrast microscopy.

RESULTS. Elevated IOP was consistently maintained for up to 4 months in G eyes, but ERG a- and b-waves showed a statistically significant decline, of 30% to 40% in amplitude, after 3 months. Loss of RGCs in G retinas was primarily focal with no statistically significant loss demonstrable outside of the focal areas when assessed by an area sampling method for counting RGCs, which totaled 2% to 3% of the entire retinal area. Mitochondrial membrane potential of cells in the RGC layer was reduced by 17.5% (P < 0.05) in regions surrounding areas of focal loss compared with comparable locations in control N eyes. After 3.5 months' elevated IOP the G retinas showed cell nuclei at various stages of apoptosis, from initial DNA condensation to fragmentation.

CONCLUSIONS. The three-vein episcleral/limbal vein occlusion model for inducing glaucomatous pathology in the rat eye gives a consistent long-term elevation of IOP. After 3 to 4 months of ~100% increased IOP, the ERG responses begin to decline, there is a variable focal loss of RGCs, and some of the remaining RGCs show characteristics of stress and apoptosis. These changes seem consistent with retinal damage in human glaucoma (focal field defects), and this rat model appears to mimic some features of primary open-angle glaucoma. (Invest Ophthalmol Vis Sci. 2000;41:3451–3459)

The current pharmacological therapy for open-angle glaucoma relies almost exclusively on drugs that lower intraocular pressure (IOP). Recently the clinical introduction of newer agents has provided a range of mechanisms to lower IOP. Yet, in many cases, despite adequate IOP control, damage to the retina continues, as it also does in normal-pressure (low-tension) glaucoma. For these reasons it is desirable for the next generation of anti-glaucoma agents to have, in addition to the ability to maintain IOP in the normal range, a neuroprotective activity that prevents or delays the loss of retinal ganglion cells (RGCs). The possibility of developing such agents is becoming increasingly clear from experimental drug studies in various ocular neuropathology model systems, such as the optic nerve-crush model or the acute ocular hyperpressure ischemia model. However, to discover such agents for chronic in vivo anti-glaucoma therapy requires a practical and consistent animal model, preferably in rodents, because unlike models for testing IOP-active drugs (such as the lasered monkey eye), determining RGC survival or optic nerve axon counts is terminal for the experimental animal.

Several investigators have devised various ways to induce elevated IOP in the rat eye with the objective of impeding the flow of aqueous humor out of the eye. The first one, the Moore–Morrison model, involves injection of hypertonic saline into limbal aqueous humor collecting veins, and the Shareef–Sharma model uses cautery of 2 or 3 of the episcleral/extraorbital veins to block outflow of the aqueous humor. The most recent rat glaucoma model involves trabecular laser photocoagulation after injection of India ink into the anterior chamber.

Each of the aforementioned rat glaucoma models has advantages and disadvantages. Hypertonic saline injection has the advantage of impeding aqueous outflow close to Schlemm’s canal. Potential disadvantages of this model are that multiple...
saline injections may be needed, the level of IOP elevation is variable for individual rats, and optic nerve damage does not show gradation or correlate completely with time and degree of IOP elevation. The laser photocoagulation model has the advantage that the initial pathology is localized to the trabecular meshwork. However, multiple lasering is required to achieve ~60% elevation of IOP and peripheral anterior synechiae develop, which contribute to the IOP elevation. The Shareef-Sharma model has the advantage that it is technically easier than the two models discussed above and that it gives a more consistent long-term IOP elevation in groups of animals. The disadvantage of this procedure is that blood flow out of the eye is impeded by the occlusion of veins leaving the globe, which can cause congestion in the intraocular vasculature. Thus, the elevation of IOP may be caused in part by the vascular congestion and by partial block of aqueous humor outflow. Although the retinal blood vessels appear normal by funduscopy examination, it is uncertain to what degree retinal pathology may be affected by vascular congestion and/or decreased blood flow separate from the elevation of IOP in this model.

In a recently published study to assess the effects of high IOP on retinal pathology in the vein-occlusion model, the RGC cells were retrogradely dye-labeled from the superior colliculus before induction of elevated IOP. Thus, the laser photocoagulation procedure the RGCs may be subjected to two concurrent insults for the duration of the experiment: presence of the intracellular dye and elevated IOP. The objective of the present investigation was to achieve and characterize a rat glaucoma model with consistently elevated IOP to facilitate evaluation of potential neuroprotective agents administered on a long-term basis. The present study differs from similar previous studies, which used the 2-vein Shareef-Sharma procedure, because 3-vein occlusion was used and because a high IOP was maintained for a longer time, 12 to 15 weeks. Both IOP and ERG changes were assessed at intervals over this period. A most important difference from previous studies is that the labeling of RGCs was performed only at the end of the period of elevated IOP, at which time the glaucomatous retinas were assessed for the presence of apoptotic markers (condensed nuclear DNA and loss of mitochondrial membrane potential) in cells located in the RGC layer.

METHODS

All experiments were performed in compliance with the ARVO Statement on the Use of animals in Ophthalmic and Vision Research.

Induction of Elevated IOP

Fifteen female Wistar rats (180–200 g) were anesthetized with an intramuscular injection of 0.1 to 0.15 ml of a mixture of acepromazine maleate, xylazine, and ketamine (AXK; 1, 9, and 45 mg/ml, respectively). The surgery was performed on one eye (G), essentially as described by Shareef et al., by severing two dorsal episcleral veins located near the superior rectus muscle and one temporal episcleral vein near the lateral rectus muscle with a standard disposable ophthalmic cautery. After surgical isolation, the veins were lifted up and away from adjacent tissues with a 3-mm-wide wooden spatula (made from a tongue depressor) before applying the cautery. Great care was taken to avoid thermal damage and touching the surrounding tissues with surgical instruments. The contralateral control eye (N eye) was sham-operated by similarly isolating the veins but not cauterizing them. By the end of the surgery (30 minutes) the IOP was already elevated to >30 mm Hg in the cauterized eye. The eyes were flushed with saline, then treated with antibiotic ointment. In one group of rats, after the conjunctival incision had healed (~4–5 days), a subconjunctival injection of 50 μl of Adrucil (Pharmacia, Kalamazoo, MI), which is a solution of 5-fluorouracil (5-FU) 50 mg/ml, was given under AXK anesthesia to both eyes. Injections of 5-FU were repeated 4 to 5 more times at 3- to 4-day intervals in both eyes. In a second group of 10 rats not treated with 5-FU the IOP was determined at 5-day intervals for 15 days after surgery, and their eyes were not further evaluated.

Measurement of IOP

IOP was determined on both eyes using the Tono-Pen XL (Mentor, Norwell, MA) immediately after surgery and then at 5-day or ~2-week intervals in AXK-anesthetized rats with 0.5% proparacaine topical local anesthesia. The Tonopen probe was applied multiple times to the cornea to activate the instruments’ built-in microprocessor averaging analysis of 4 consecutive signals. Probe applications were made until 4 to 5 averages were obtained, each with a coefficient of variation <5%. These 4 or 5 average values from each rat were again averaged, and the resultant mean value was used to compute the group mean IOP ± SD.

Measurement of ERG Changes

Scotopic ERG changes were measured at approximately 2-week intervals beginning 8 weeks after glaucoma surgery on AXK-anesthetized rats. After at least 16 hours’ dark-adapta tion, eyes were dilated with a topical application of 1% tropicamide and 2.5% phenylephrine. Custom-made contact lenses with a gold-wire ring electrode on the concave surface and coated with 2.5% hydroxy propyl methyl cellulose were applied to both eyes. Stimuli consisted of 10-μsec stroboscopic flashes of unattenuated white light (two, 1 minute apart) generated by a Grass PS-22 stimulus generator (Grass Instruments, Quincy, MA; irradiance = 236 lux/s). Data from both eyes were accumulated simultaneously and analyzed using the LKC Technologies (Gaithersburg, MD) electrodiagnostic recording instrument (model UTAS-2000) and software (LKC Technologies Advanced Analysis). Differences between the G and contralateral N eyes of each rat were calculated for the primary response parameter (a- and b-wave amplitudes in negative microvolt and positive microvolt units, respectively) and were analyzed by ANOVA and by paired t-test.

RGC Labeling and Counting

RGCs were retrogradely labeled by injection of 1 μl volumes of the amidine dye Fluorogold (Fluorochrome, Denver, CO) fluorescent tracer (5% in H2O) into the superior colliculus at 2 locations on each side using a Hamilton (Reno, NV) syringe with a 33-gauge needle. Injections were performed by placing the head of AXK-anesthetized rats on a stereotaxic apparatus using coordinates from the rat brain atlas, essentially as previously described. Rats were AXK-anesthetized 5 days after the
Fluorogold injections, perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS), and the eyes enucleated. Eyecups were made by cutting off the anterior segment at the level of the limbus, and the eyecups were immersion-fixed for 1 hour in 4% paraformaldehyde in PBS. Cuts were made through the sclera to form a Maltese cross pattern, and the retinas detached from the eyecup at the optic nerve head and fixed overnight in 1% paraformaldehyde in PBS. Both retinas from one rat were flatmounted, vitreous side up, on a glass slide, air-dried, and cover-slipped with Advantage mounting medium (Axell). The retinas were visualized under epifluorescence microscopy (Zeiss Axiomat with Omega Optical XF05 filter set) and scanned for the areas in which RGCs were absent (patches). Also, in 8 of the rats 4 adjacent micrographs were taken in the peripheral region of each quadrant of the G and N retinas (superior nasal and temporal, inferior nasal and temporal) along the centerline of each quadrant from eccentricity 2- to 4-mm distant from the center of the optic nerve head using a 16× objective. Microscope fields (400 × 300 μm) were selected to avoid obvious patches in which RGCs were absent by moving the field laterally from the midline of the quadrant at the same eccentricity. Some fields were photographed with both epifluorescence and interference contrast (Nomarski) optics. The RGCs in 16 fields of each retina were counted using a computer-based image analysis system (ImageJ; NIH) and totaled 2500 to 3000 RGCs in G, N, or control (no surgery) retinas. RGC counts for each quadrant of the G and N retinas were calculated as percent of the counts ± SEM in the corresponding quadrant of the contralateral N retina. Significance between the mean cell counts in corresponding quadrants of G and N retinas was determined by Student’s t-test.

**N-Methyl-d-Aspartate Injections**

Six rats were injected in one eye with 5 μl of sterile (0.22 μl filter, Millipore Corp., Bedford, MA) N-methyl-d-aspartate solution (NMDA; 10 mM in PBS) via a 33-gauge microsyringe needle into the center of the vitreous.12 Contralateral control eyes received a 5 μl injection of sterile PBS. After 10 days the RGCs were retrogradely labeled with Fluorogold, and the retinas were prepared as above and statistically analyzed as above.

**Determination of Mitochondrial Membrane Potential and Nuclear DNA Staining**

Fifteen weeks after surgery the glaucomatous and contralateral N eyes of 4 rats anesthetized with AXK were injected with the dye CMTMR (MitoTracker orange; Molecular Probes, Eugene, OR), 5 μl of a 125-μM solution in aqueous 20% dimethyl sulfoxide.13 After 30 minutes, the rats were perfused with 4% paraformaldehyde in PBS. Retinas were isolated as described above and immersed for 2 hours in the nuclear acid–staining dye YOYO-1 (Molecular Probes), 3 μM in PBS, and then washed in PBS. Flatmounts of the retinas were examined for fluorescence of CMTMR and YOYO-1 by dual-channel confocal fluorescence microscopy. A Leica TCS-4D confocal scanning microscope coupled to an argon-krypton laser (Omnichrome; Wessling, Germany) was used to resolve 3000 to 4000 individual mitochondria labeled with CMTMR in the RGC layer of each retina. A pinhole setting of 50 was used with an excitation wavelength of 488 nm and a long-pass emission filter of 590 nm. Images were scanned using an oil immersion, 40×, 1.0 NA objective at 512 × 512 × 8 bits per pixel resolution, background offset at −1, and averaged 32 times in line-average scan mode. The images were saved in tagged image file format (TIFF) and transferred to a personal computer–run Metamorph software program (Universal Imaging) to threshold individual mitochondrial outlines and then to measure the mean intensity within each mitochondrion. The value for each mitochondrion was normalized against mean intensity for the immediately adjacent cytoplasm, and the values were presented as frequency distributions. In an additional 2 rats, the retinas were isolated without CMTMR injection as above and labeled with YOYO-1 alone.

**RESULTS**

After gaining experience of the surgery, the vein-occlusion procedure with 5-FU resulted in a stably elevated IOP (~100% increase over the contralateral eye) for > 12 weeks with a high degree of group consistency (Fig. 1A). There was however also a much smaller but statistically significant increase in IOP in the sham-operated control eyes relative to the baseline over the first 8 weeks. When 5-FU treatment was omitted, the IOP of the
vein-occluded eyes returned to the same level as that in the contralateral N eyes by 15 days post surgery in the majority of rats (Fig. 1B).

Figure 2 illustrates the ERG differences between glaucomatous eyes relative to the contralateral N eyes measured from 8 to 15 weeks after the glaucoma surgery. Mean values for both a- and b-wave amplitudes showed a decline after 12 weeks of elevated IOP when compared with the contralateral N eyes but became statistically significant (30% to 40% decrease) only at the 15-week point because of the inherent variability in ERG measurements.

After 12 weeks of elevated IOP the RGCs were visualized by retrograde labeling with bilateral injections of Fluorogold into the superior colliculus. Examination of the retinal flat-mounts by fluorescence photomicroscopy revealed a significant number of patches in the glaucomatous eyes in which the density of RGCs was less or labeled cells were absent.

Figures 3 and 4 show two representative fields from glaucomatous retinas, each taken with both fluorescence and Nomarski optics, as can be seen by coincidence of the retinal blood vessel pattern in each pair. The images show, respectively, 2 small patches and the edge of a larger patch in which RGCs are not detectable. The corresponding Nomarski images show that the patches are not holes or tears resulting from damage during preparation of the flatmounts. Occasionally, no fluorescent RGCs are visible in a field because patches can be larger than the field at this magnification, as shown in the low-magnification micrograph taken with a 4× objective (Fig. 5). Scanning of six glaucomatous rat retinas with patches gave an average of 5 to 6 such patches per retina with a size greater than 100 μm in the smallest dimension. Occasionally, areas without RGCs were evident in contralateral control retinas, but they appear to be mostly artifact caused by damage during preparation of flatmounts, because they occur near tears or the cuts made in the retina.

To determine whether there was, in addition to the focal loss, a more evenly distributed loss of RGCs, fluorescent micrographs avoiding patches were taken in the center of each quadrant of the retina from eccentricity 2 to 4 mm distant from the optic nerve head in both retinas from 6 unilateral glaucomatous rats. All fluorescent RGCs in the 16 fields of each retina were counted (2500–3500 cells/retina), which is 2% to 3% of the total RGCs in the normal rat retina, and the counts in the G eyes compared with those from contralateral N eyes. As a positive control experiment for this sampling and counting procedure we also injected one eye of 6 rats with 50 nmol NMDA, which causes RGC death by excitotoxicity with a more evenly distributed loss of RGCs in all areas of the retina. The other eye was injected with saline as the control. Retrograde fluorescent labeling and cell counts were performed on the NMDA-injected retinas as described for the glaucoma retinas.

The comparison of glaucoma- and NMDA-mediated loss of RGCs relative to the respective contralateral N control eye is shown in Figure 6. This dose of intravitreous NMDA caused a 60% or greater loss of RGCs in all quadrants (P < 0.001), whereas in the glaucoma retinas only one quadrant (inferior temporal) showed a small but statistically significant loss of RGCs (P < 0.05).

We examined the retinas of six rats with high IOP in one eye for 15 weeks by staining with the nucleic acid dye YOYO-1, which has a high fluorescence intensity when bound to DNA of condensed nuclei in apoptotic cells compared with the low binding of the dye to the DNA of normal nuclei. We found by confocal fluorescence (Fig. 7A) and interference contrast microscopy (Fig. 7B) that nuclei with DNA condensation typical of various stages of apoptosis are present in the G retinas, but cells with apparent condensed nuclei were extremely rare in the contralateral N eyes. Various transient forms of apoptotic nuclei were also observed, including "walnut-shaped" nuclei showing DNA condensation typical of various stages of apoptosis. We found by confocal fluorescence (Fig. 7C) and interference contrast microscopy (Fig. 7D) that nuclei about to fragment (Fig. 7D), nuclei showing the first cleft leading to fragmentation (Fig. 7C), and clusters of fragments (Fig. 7D).

Relative mitochondrial membrane potential (ΔΨm) was also determined in the RGC layer of four of the rats using the fluorescence intensity of CMTMR accumulated by mitochondria of cells in the RGC layer as a relative marker of membrane potential (Fig. 8). The retinas subjected to high IOP for 15 weeks showed a lower mean level of mitochondrial CMTMR fluorescence in regions surrounding the patches (82.5% ± 7.3%, P < 0.05) relative to mitochondria in randomly chosen areas of the RGC layer at the same approximate eccentricity in the contralateral N eyes.
DISCUSSION

The successful preparation of the vein–occlusion rat glaucoma model requires experience. Our first attempts resulted in an initial high IOP that gradually returned to that of the contralateral eye over 2 to 4 weeks in ~75% of the surgeries as shown in Figure 1B, possibly because of restoration of venous patency by growth of new vessels. This can be avoided by careful surgical technique and, particularly, by subconjunctival 5-FU injections. A few animals develop complications (lens or cor}

**Figure 3.** Microscope fields of Fluorogold-labeled RGCs in glaucomatous retinas taken with both epifluorescence (A) and Nomarski (B) optics showing the type of focal loss of RGCs found in various regions of the retina. Patches without labeled RGCs marked by asterisks.

**Figure 4.** Microscope fields of Fluorogold-labeled RGCs in glaucomatous retinas taken with both epifluorescence (A) and Nomarski (B) optics showing some larger-sized RGCs surviving at the edge of a patch without labeled RGCs (marked by an asterisk).
nea opacities), but survival to 15 weeks with elevated IOP and otherwise apparently normal eyes is achieved in ~80% of surgeries that are followed by 5-FU treatment. The small elevation of IOP that occurs in the contralateral N eyes may be a centrally mediated response to the rise of IOP in the G eye, or may be a result of the sham surgery plus 5-FU treatments. The ERG response measurements suggest a decline in the amplitude of both a- and b-waves beginning after approximately 3 months and, thus, becoming a significant feature in this glaucoma model only after a prolonged time of elevated IOP (Fig. 2). These results are similar to reports on ERG findings in subjects with advanced open-angle glaucoma.\textsuperscript{15,16} However, as in human glaucoma, more detailed analysis of ERG parameters at lower light intensities, such as oscillatory potentials or pattern ERG, may indicate glaucomatous retinal pathology at earlier times in this rat glaucoma model.

**Figure 5.** Low-magnification field of Fluorogold-labeled RGCs in a glaucomatous retina showing some larger patches of missing RGCs, marked by asterisks, and also some of the smaller patches, marked by arrows (A), and a normal retina for comparison (B).

**Figure 6.** Comparison of the percent loss of RGCs in the four quadrants of each retina (relative to the respective contralateral retina of the control N eye) in eyes with glaucoma (G; solid columns) and in eyes 1 week after injection of 50 nmol NMDA into the vitreous (hatched columns). * $P < 0.05$; ** $P < 0.001$. 

Rat Retinal Ganglion Cell: Glaucoma vs NMDA (50nmoles)
The RGC loss that has occurred after 12 weeks of elevated IOP in this model appears to be primarily focal. Additionally, the RGCs in areas bordering a patch of missing cells showed a somewhat more disordered spatial distribution when compared visually with the more regular columnar arrangement of RGCs seen in N or control retinas (see Fig. 4). All the glaucomatous retinas showed obvious patches of missing RGCs, but the number of such patches in individual G retinas was variable, ranging from 3 to 12, and occurred in all quadrants of the retina. Thus, the counting of the number of such patches is too subjective and variable to be a reliable quantitative measure of RGC loss after 3 to 4 months of elevated IOP in this glaucoma model. Even if we could determine the aggregate number of RGCs missing in the focal areas this would likely be too small a fraction of the total RGCs to be a useful quantitative measure. Most of the patches found were smaller than the field size (0.12 mm²), and even if the maximum number of patches observed were all this size, the number of missing RGCs (~4000) represents less than 4% of the total RGCs in a rat retina.

The formation of a patch is most probably caused by the dead cells having been removed by phagocytic microglia before the time of Fluorogold labeling. It is also possible that RGC cell bodies are still present in a patch but do not label with Fluorogold because of axon pathology. However, if a patch gradually enlarges over time, the adjacent cells at the periphery might be under stress or in the initial stages of the death

**FIGURE 7.** Apoptotic cell nuclei in the RGC layer of glaucomatous retinas stained with YOYO-1 dye and visualized by confocal fluorescence microscopy (A, C, D) and by interference contrast microscopy (B; same field as in panel A). Nucleus in center of (A), brightly stained with YOYO-1 (arrow), is a condensed nucleus, indicated by arrow in (B). A small condensed nucleus in “walnut” stage of apoptosis is shown in (C, arrow), and a nucleus about to fragment in (D), marked by arrow. Condensing nuclei and a cluster of fragments are also present in (D).

**FIGURE 8.** Distribution of CMTMR fluorescence intensity (a relative measure of transmembrane potential, ΔΨₘ) in mitochondria of the RGC layer in the retinas from two rats (1 and 2) with glaucomatous (G) left eyes and their contralateral control (N) right eyes. The mean fluorescence intensity ± SD is given for the frequency distribution determined for each eye. Representative of similar distribution experiments done in 4 rats.
process and might exhibit characteristics of apoptosis. In fact, cells with condensed nuclei that stain strongly with YOYO-1 were most often found in regions near patches (see Fig. 7 and below). We believe that the patches result from the death of RGCs in a pattern comparable to the loss in human glaucoma and that this focal loss of RGCs might be sufficient to result in field defects if this could be measured in the rat eye.

We also evaluated whether there was a more evenly distributed loss of RGCs in areas of the retina outside the patches. Sampling of ~3% of the total RGCs in representative fields in the peripheral retina in all quadrants but outside patchy areas showed no significant loss within the limits of the sampling technique. The same procedure was able to determine the more uniformly distributed loss of ~60% of the RGCs resulting from the intravitreal injection of NMDA. In a recent report on a rat glaucoma model with 2-vein occlusion similar to the model in this study, but with prelabeling of RGCs using Fast Blue (another amidine dye), a uniformly distributed loss of ~50% of the RGCs was found in the peripheral retina after 10 weeks of elevated IOP. The sampling method, the number of RGCs counted, and the retinal area counted (3%–3.5% of RGCs) was comparable to those used in the present study. As shown by the positive control NMDA experiment (Fig. 5), this level of uniform RGC loss in the glaucoma retinas would have been detected by the sampling method used in this study, but was not found. Thus, the pattern of RGC loss seems to be shifted toward a more uniform loss when RGCs are subjected to both dye-labeling and high IOP together over a period compared with the more focal damage found in the present experiments when the prolonged insult is elevated ocular pressure alone. One possible explanation of the discrepancy between the present findings and previously published results is the difference in marking of the RGCs, either by post-labeling (present results) or prelabeling, with amidine dyes, such as Fluorogold and Fast Blue, which are known to be toxic to some neurons in long-term experiments. In a recent article, Neufeld et al. reported on a very similar glaucoma model in male Wistar rats after 6 months of elevated IOP and found a uniform loss of RGCs of ~5% in the peripheral retina after post-labeling with Fluorogold, a result also considerably less than the 50% loss after 3 months reported with prelabeling. In this case ~15% of the total retinal area was sampled for RGC counts. These findings taken together with the present results suggest that prelabeling with amidine dyes that are potentially toxic to RGCs should be avoided, that a uniform loss of RGCs becomes more apparent after 6 months of elevated IOP, and that at least 15% of the retinal area needs to be counted to quantitate RGC loss. Our findings after 3 to 4 months of glaucoma are in general agreement with the results of Morrison and coworkers on the patterns of axon loss in the optic nerves of rats with chronically elevated IOP induced by the saline injection method.

The mode of RGC death in glaucoma is thought to be mainly via an apoptotic mechanism. If RGC death is an ongoing process in the glaucomatous rat retina there should have been some cells in various stages of apoptosis at the time the retinas were isolated. YOYO-1–stained condensed nuclei, representing late stages of cell death by apoptosis, were consistently present in all the glaucomatous retinas. However, a relatively early stage in cellular stress that can proceed to apoptosis is a decrease in mitochondrial membrane potential occurring before changes in nuclear DNA. These markers, in mitochondria and in nuclei, can be assessed in the same retina by dual-channel fluorescence confocal microscopy after labeling with both the CMTMR and a DNA-binding dye such as YOYO-1. The intensity of the CMTMR fluorescent label in mitochondria, a relative measure of mitochondrial membrane potential, showed a significant downward shift in distribution in the RGC layer (Fig. 8), indicating a larger number of cells with reduced mitochondrial potential in glaucomatous retinas relative to N retinas. However, because ~45% of cells in the RGC layer are displaced amacrine cells, which will also have their mitochondria labeled with CMTMR, the loss of mitochondrial potential specifically in RGCs could be larger than the overall 17.5% mean decrease actually measured, or the proportion of RGC cells affected could be greater. These findings indicate that a significant number of functional RGCs may exhibit markers of cellular stress when subjected to a prolonged period of elevated pressure and that it is most likely from this population that individual cells proceed over time to apoptotic cell death.

In conclusion, we found that the 3-vein occlusion model for inducing glaucoma in the rat eye provides a consistent long-term pressure elevation. After 12 to 15 weeks of high IOP there is a variable focal loss of RGCs, and some of the remaining cells show changes characteristic of stress and apoptosis. By 16 weeks of high IOP there is a significant decline in amplitude of the scotopic ERG a- and b-waves. These changes seem consistent with retinal damage that causes field defects in human glaucoma, and, thus, this rat model appears to mimic some features of primary open-angle glaucoma.

References


