
Retinal Ganglion Cell Death in Experimental Glaucoma and After Axotomy Occurs by Apoptosis

Harry A. Quigley,* Robert W. Nickells,* Lisa A. Kerrigan,* Mary E. Pease,*
Diane J. Thibault,* and Donald J. Zack*

Purpose. To investigate whether retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis.

Methods. Chronic elevated eye pressure was produced in 20 monkey eyes, and the optic nerve was transected unilaterally in the orbit of 10 monkeys and 14 rabbits. Sixteen monkey and 14 rabbit eyes were studied as normal controls. Analytic methods included light and electron microscopy, histochemistry for DNA fragmentation (TUNEL method), and DNA electrophoresis in agarose gels.

Results. Dying ganglion cells in the experimental retinas exhibited morphologic features of apoptosis, including chromatin condensation and formation of apoptotic bodies. Cells with a positive reaction for DNA fragmentation were observed in eyes subjected to axotomy and experimental glaucoma but were only rarely encountered in control eyes. No evidence of internucleosomal fragmentation was detected electrophoretically, possibly because of the small proportion of cells that were dying at any given time.

Conclusion. Some retinal ganglion cells injured by glaucoma and by axotomy die by apoptosis. *Invest Ophthalmol Vis Sci.* 1995;36:774-786.

Glaucoma is characterized by a typical excavated appearance of the optic nerve head and by death of retinal ganglion cells. The mechanisms mediating ganglion cell death in glaucoma are not well understood. Neuronal cell death is not limited to pathologic conditions such as glaucoma. Programmed cell death plays an important role in vertebrate nervous system development.¹ In the eye, there is marked cell death involving a number of different cell types during retinal differentiation.² A large excess of retinal ganglion cells is generated during fetal life, but those that do

not establish appropriate synaptic connections generally die.³⁻⁶

The neurotrophic hypothesis suggests that factors secreted by target tissues are important for the survival of the innervating neurons.^{1,7} In part, ganglion cells may die during development because, in failing to contact their target neurons, they fail to receive appropriate trophic factors. Consistent with this hypothesis, retinal ganglion cells express neurotrophin receptors and can respond to exogenous trophic factors. The trkB receptor for a neurotrophin, brain-derived neurotrophic factor, has been localized to rat ganglion cells using *in situ* hybridization and immunohistochemistry.⁸ Brain-derived neurotrophic factor has been shown capable of promoting ganglion cell survival *in vitro* and *in vivo*.⁹⁻¹² Other factors that have been reported to promote ganglion cell survival after injury include fibroblast growth factor, ciliary neurotrophic factor, and nerve growth factor.¹³⁻¹⁷

Cell death in glaucoma may also be related to the loss of trophic influences caused by the inhibition of transmission of neurotrophic signals from axon terminals to neuronal cell bodies. In human eyes with glau-

From the *Department of Ophthalmology, Wilmer Institute, and the †Departments of Molecular Biology and Genetics and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland.

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Reprint requests: Harry Quigley, Johns Hopkins University School of Medicine, 120 Wilmer, 600 N. Wolfe Street, Baltimore, MD 21287.

coma and in experimental model systems with increased eye pressure, ganglion cells exhibit obstruction of orthograde and retrograde axonal transport at the optic nerve head, where their axons exit the eye.¹⁸⁻²¹ This reduces the communication between ganglion cells and their target neurons in the brain, possibly inhibiting the transmission of trophic signals.

Because neuronal cell death from neurotrophin withdrawal can be mediated by apoptosis,²² we decided to test the hypothesis that apoptosis is also involved in retinal ganglion cell death in glaucoma. Apoptosis is a genetically programmed process in which cells commit suicide. It is characterized by chromatin condensation, intracellular fragmentation associated with membrane-enclosed cellular fragments (apoptotic bodies), and internucleosomal DNA fragmentation.²³⁻²⁵ Phagocytosis of cells undergoing apoptosis is typically performed by cells of the surrounding tissue. There is a conspicuous absence of inflammatory reaction. The process is active and requires new RNA and protein synthesis. At the molecular level, significant progress has been made in understanding the genetic basis of apoptosis.²⁶

As a model system for human glaucoma, we used monkeys in which intraocular pressure was elevated in one eye either by argon laser treatment of the trabecular meshwork²⁷ or by injection of glutaraldehyde-fixed red blood cells into the anterior chamber of the eye.²⁸ These chronic glaucomatous models mimic the specific ganglion cell loss pattern of human glaucoma.²⁹⁻³⁴ Because optic nerve transection also interrupts communication from nerve terminal to cell body, we investigated the effect of axotomy on apoptosis in monkey and rabbit retinas. Recent work has implicated apoptosis in ganglion cell death in the rat after axotomy.^{35,36} Furthermore, in our previous research with nerve transection in monkeys, the rapidity of cell disappearance and the absence of inflammation were noteworthy and compatible with apoptosis.³⁷

To identify apoptotic cells, we used light and electron microscopy and two analytic techniques designed to detect the internucleosomal DNA fragmentation that is a characteristic of apoptosis: agarose gel electrophoresis of retinal DNA³⁸ and TdT-mediated biotin-dUDP nick-end labeling (TUNEL method).³⁹

METHODS

Animal Models

Male and female cynomolgus monkeys (*Macaca fascicularis*) and New Zealand White rabbits (each weighing approximately 5 kg) were used; both groups consisted of juvenile and adult animals. The procedures used

were approved and supervised by the Animal Care Committee of the Johns Hopkins University School of Medicine, and they adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For procedures that might produce discomfort, monkeys were anesthetized with intravenous pentobarbital, and rabbits were anesthetized with intramuscular ketamine and xylazine.

Red blood cells fixed in glutaraldehyde and rinsed in phosphate buffer were injected into the anterior chamber of one eye as previously described.²⁸ Argon laser treatment to the trabecular meshwork was also performed as previously described.²⁷ Intraocular pressure was measured under topical anesthesia (proparacaine hydrochloride) with a pneumatonograph manometrically calibrated for rabbit and monkey eyes. Elevated eye pressure lasted from 4 days to 11 months.

In monkeys, the optic nerve was transected by a lateral approach through the skin and bone of the orbit approximately 6 mm posterior to the globe,³⁷ and, in rabbits, through the conjunctiva approximately 4 mm posterior to the globe. Ophthalmoscopic examination demonstrated the patency of the retinal and choroidal blood circulation after surgery. Animals survived from 1 to 12 weeks and were killed under deep anesthesia by exsanguination or pentobarbital overdose.

Tissue Preparation

For histologic studies, monkeys were killed and perfused with 4% paraformaldehyde and 5% glutaraldehyde in phosphate buffer through the abdominal aorta. Specimens of retina and cross-sections of the optic nerve were embedded in epoxy resin and sectioned at 1 μ m for phase-contrast microscopy. Thin sections were stained with uranyl acetate and lead citrate and examined using a JEOL (Tokyo, Japan) 100 CX transmission electron microscope. The monkey tissue included 12 specimens with chronic glaucoma, 9 after transection, and 9 controls. Ten transection and 10 normal rabbit retinas and their optic nerves were fixed by immersion in 4% paraformaldehyde, embedded in epoxy resin, and examined similarly. The degree of loss of optic nerve axons was judged from cross-sections viewed by light microscopy on a semiquantitative scale (mild, moderate, or severe).

³H-Leucine Incorporation and Autoradiography

Anterograde axonal transport was measured by injection of tritiated leucine intravitreally.²⁹ Systemic blood pressure was monitored by means of a femoral artery catheter connected to a pressure transducer. Eye pressure was set at the prevailing level for that eye during the preceding 24 hours with an anterior chamber needle connected to a variable height reservoir. An intrav-

itrete injection of 0.1 ml (100 μ Ci) of ^3H -leucine (L-leucine-4,5- ^3H (N); 30 Ci/mmol to 50 Ci/mmol; New England Nuclear, Boston, MA) was administered with a 25-gauge needle, and the entry site was sealed with epoxy glue. The eye pressure was kept constant at the prescribed level for 4 hours, after which the animals were killed. Autoradiography was performed on 1- μ m thick sections of epoxy resin-embedded material from the retinas by a reported method.¹⁹

TUNEL Staining

Portions of retina approximately 15 mm \times 15 mm were dissected after fixation in 4% paraformaldehyde, and they were left attached to the choroid and sclera with the vitreous removed. Relaxing incisions were made with a razor to allow a flat position for embedding in paraffin. Sections were cut at 6- μ m thickness parallel to the retina surface to maximize the number of ganglion cells visible per slide using differential interference contrast microscopy. TUNEL staining was performed according to the method of Gavrieli et al.³⁰ with the following modifications. Endogenous peroxidase was inactivated with 3% hydrogen peroxide (instead of 2%). The peroxidase reaction was terminated with a buffer containing 150 mM sodium chloride and 15 mM sodium citrate (instead of the buffer reported). Peroxidase staining was performed with the VectaStain ABC kit (Vector Laboratories, Burlingame, CA).

TUNEL staining was performed on tissues from 9 chronic glaucomatous and 7 control monkey eyes and 10 transected and 10 control rabbit eyes. Three observers, masked to the treatment of each eye, evaluated the number of ganglion cells that exhibited positive staining in an approximately equal number of slides from experimental and control eyes. TUNEL-labeled cells were classified as either definite or probable. Definite cells were defined as those with a labeled, round, or oval nucleus surrounded by cytoplasm. Probable cells were defined as those cells with a condensed and irregularly shaped nucleus and little or no surrounding cytoplasm. Probable cells may have represented cells partially included in a section or cells in the latter stages of apoptosis. The coordinate position of all positive cells was identified, and adjudication of definite and probable cells was carried out by consensus. The total number of ganglion cells per section was enumerated in several representative slides stained with hematoxylin.

Electrophoretic Analysis of DNA Fragmentation

For the study of DNA fragmentation by electrophoresis, whole or half retinas were separated from vitreous humor, retinal pigment epithelium, choroid, and sclera, and they were frozen at -85°C without fixation.

Genomic DNA was extracted as has been described,⁴⁰ and agarose electrophoresis was performed in 1% gels by standard methods. End-labeling of the genomic DNA with gamma ^{32}P -ATP and polynucleotide kinase and Southern transfer, followed by hybridization with random hexamer-labeled genomic DNA probe, were also performed by standard methods.⁴¹

RESULTS

Light and Electron Microscopy

We studied 12 monkey eyes with experimental glaucoma, and all were found to have ganglion cells with histologic features compatible with apoptosis. By phase-contrast microscopy, there were two patterns of abnormal morphology that could be detected. In the first pattern, the nucleus was so dense that no detail was visible (Fig. 1A). In the second pattern, multiple dense cytoplasmic bodies smaller than a nucleus were found without a nucleus (apoptotic bodies) (Fig. 1B). These were sometimes found to be metachromatic with toluidine blue staining. There was no associated inflammatory reaction. In each monkey eye, several hundred ganglion cells were inspected at $\times 1200$ magnification, and the frequency of detection of these two abnormal cell types was quantified. The comparison of abnormal cell frequency to duration of elevated eye pressure and degree of ganglion cell loss indicated that only a modest proportion of cells had the two abnormal appearances in any specimen (Table 1). Abnormal cells were seen at early and late stages of glaucoma injury.

We confirmed that the abnormal cells were indeed dying cells (and not, for example, active macrophages or misidentified, nonneuronal cell types), by assaying their ability to synthesize protein. Tritiated leucine was injected into the vitreous cavity of several eyes 4 hours before sacrifice. Protein synthesis was demonstrated autoradiographically by the dense accumulation of silver grains over normal cells. Apoptotic bodies had only background levels of silver grains (Fig. 2), indicating that the cells were not actively synthesizing protein at these later stages of cell degeneration.

A small but significant proportion of detectably abnormal cells was seen in all the glaucomatous specimens (Table 1). However, in nine control monkey eyes, only one apoptotic cell was detected among more than 2,400 ganglion cells examined. Hence, the experimental eyes dramatically exceeded the control eyes in the presence of cells that were abnormal by light microscopy.

The appearance of the abnormal cells with dense nuclei and apoptotic bodies was examined in detail by transmission electron microscopy in the monkey

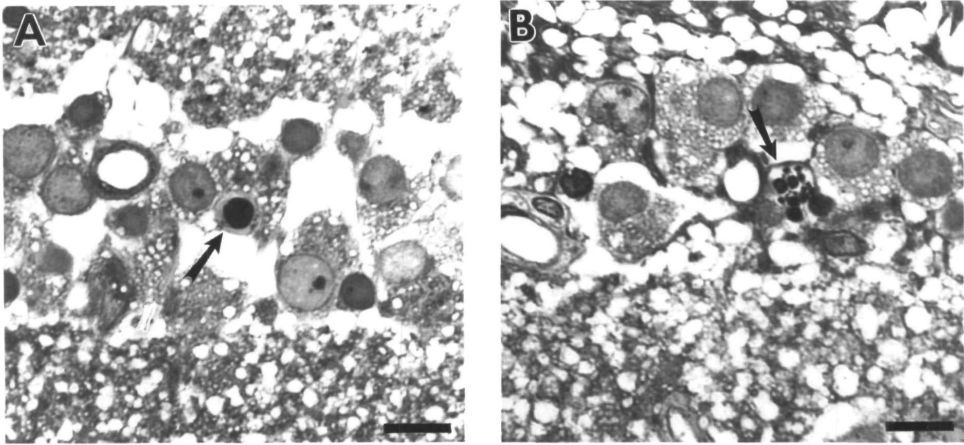


FIGURE 1. Light micrographs of retinal ganglion cells in monkeys with experimental glaucoma that demonstrate features of apoptosis. **(A)** The arrow indicates a cell with a dense nucleus and contracted cytoplasm. **(B)** The arrow indicates a presumed ganglion cell represented by multiple, dense inclusion (apoptotic) bodies. Bars = 10 μm; phase-contrast, paraphenylenediamine stain.

eyes with experimental glaucoma (Fig. 3). They displayed features in common with other cell types that undergo apoptosis.^{23,24} Among the features present were condensation of nuclear chromatin at the edge of the nucleus and, in some cells, frank absence of

nuclear membrane with a dense residuum of nuclear material. The cytoplasmic contents were condensed, but organelles remained generally intact even when the cell was represented by 2 or 3 apoptotic bodies that were smaller than a whole cell and contained one or more dense nuclear fragments.

Eyes from monkeys that had undergone unilateral optic nerve transection were also studied. Five retinas were analyzed at 2 to 4 weeks after axotomy, two at 10 weeks and two at 12 weeks. Abnormal ganglion cells of the dense nucleus type or apoptotic bodies were visible in every one of the 2- and 4-week postinjury specimens (Table 2). Their ultrastructural features were identical to those of the eyes with chronic glaucoma (Fig. 4). In the 10- to 12-week animals, few ganglion cells remained in the retina for examination; no abnormal cells were detected. The lack of any residual sign of ganglion cell nuclear or cytoplasmic contents was remarkable in these retinas. Occasional cellular remnants were visible, largely confined to the Müller cell cytoplasm. The space formerly occupied by ganglion cells and their axons had largely been obliterated.

To complement the monkey studies, we studied 14 rabbits with optic nerve transection in one eye: two each at 1, 2, 3, and 4 weeks after axotomy, four at 1.5 weeks, and one each at 6 and 12 weeks. (The 1.5 week posttransection animals were used only for DNA analysis; see DNA Fragmentation.) Light microscopic

TABLE 1. Histologic Study of Monkey Glaucomatous Eyes

<i>Duration of IOP Elevation</i>	<i>IOP Mean (mm Hg)</i>	<i>% Positive Cells</i>	<i>Degree of Axon Loss</i>
2 (5)	50	13	Mild
2 (31)	42	4	Mild
4 (35)	45	5	Mild
25 (31)	41	2	Mild
42 (42)	24	1	Mild
10 (11)	72	4	Moderate
7 (18)	66	9	Moderate
15 (15)	64	4	Moderate
15 (25)	45	13	Moderate
17 (30)	48	5	Moderate
22 (22)	58	5	Severe
210 (210)	43	1	Severe

Duration: The first number is the number of days the intraocular pressure (IOP) was elevated. The number in parentheses is the number of days from first elevation to sacrifice. IOP mean: mean eye pressure during period of elevation in millimeters of mercury. % Positive cells: percent of ganglion cells with morphology compatible with apoptosis by light microscopy (see text). Degree of axon loss: semiquantitative estimate of amount of retinal ganglion cell axon loss based on analysis of optic nerve cross-sections. Twelve monkey eyes were analyzed.

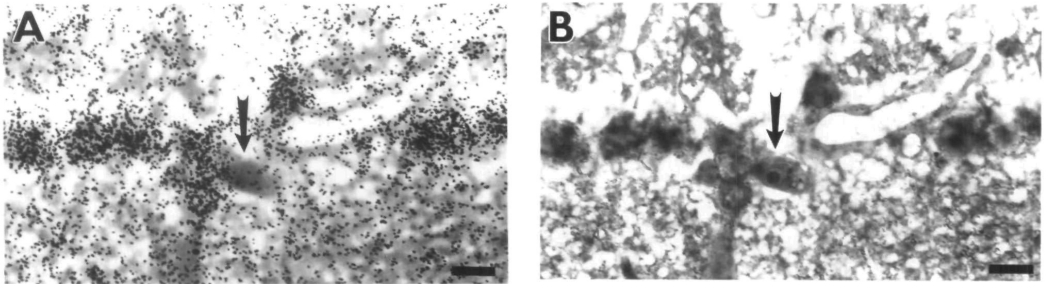


FIGURE 2. Autoradiograph of retinal ganglion cell layer from a monkey with experimental glaucoma. Ganglion cells have incorporated ^3H -leucine that was injected into the vitreous cavity. In (A), which is focused on the emulsion, dense silver grains overlie the cell bodies. Note that one cell (*arrow*) appears to have only a background level of grains overlying it. In (B), which is focused on the tissue section, the cell with reduced protein synthesis is shown to have a dense nucleus characteristic of apoptosis. Bars = 10 μm ; phase-contrast, paraphenylenediamine stain.

evaluation of the rabbit retinas after transection disclosed dense nucleus cells and apoptotic bodies in each of the specimens obtained within 4 weeks of transection. Similar cells were not seen in the normal control eyes. At 1 week after transection, there was a normal density of ganglion cell bodies in the retina (approximately 100 ganglion cells in a 4-mm section of posterior retina), and the optic nerve cross-sections showed only a few axonal profiles with abnormal myelin sheaths that suggested early degeneration. Among several hundred ganglion cells, 5% exhibited one of the two abnormal patterns at 1 week. At 2 weeks, the retinal density of ganglion cells was decreased by 25% to 50%, and there was myelin disruption in at least half the axons in the optic nerve. At this time, 20% of the ganglion cells exhibited an abnormal appearance. At 3 weeks, both specimens had only 25% of the normal ganglion cell body density in the retina, and nearly every axonal profile in the nerve was abnormal. An abnormal ganglion cell appearance was found in 8% of the remaining cell bodies, nearly all of the apoptotic body type. At 4 weeks, only scattered cells recognizable as ganglion cells were found in the retina. One or two abnormal cells were still found. The ultrastructural appearance of the abnormal cells was similar to that in both monkey models (Fig. 5). In 6- and 12-week specimens, there were too few remaining ganglion cells to make any substantive observations.

DNA Fragmentation

Because one of the earliest events in apoptosis is the fragmentation of nuclear DNA,^{24,42} we examined monkey glaucomatous retinas and retinas from monkey and rabbit eyes that had undergone nerve transection

for evidence of DNA fragmentation. Conventional analysis of DNA fragmentation was performed with the transected rabbit retinas by isolating genomic DNA and subjecting it to electrophoresis on 1% agarose gels. There was no evidence of internucleosomal DNA fragmentation (data not shown). To detect low levels of internucleosomal cleavage that might not be evident by ethidium bromide staining, we tried to increase the sensitivity of the method in two ways. First, we Southern blotted the cold DNA and then hybridized the transferred DNA with high-specificity, ^{32}P -labeled genomic DNA as a probe. Second, we labeled the initial retinal genomic DNA by ^{32}P end-labeling. Both methods failed to show evidence of internucleosomal DNA fragmentation.

As an alternative approach to electrophoresis, we used the TUNEL technique.³⁹ Seventy-six slides were evaluated from glaucomatous and control tissues in monkeys (Table 3). There were significantly more positive ganglion cells in the glaucomatous tissue than in fellow, control eyes, and this was particularly well demonstrated in the moderately glaucomatous eyes (Fig. 6). Of nine glaucomatous eyes studied, six had at least one positive cell. Of the three glaucomatous eyes in which we found no positive cells, two were mildly damaged and one was a moderately damaged.

The total number of ganglion cells per slide was counted in six specimens, three from normal eyes and three from moderately glaucomatous eyes. When the frequency of positive cells was estimated from these specimens as a proportion of the total ganglion cell population, the percentages were 0.14% in controls and 0.98% in glaucoma (total number of cells counted

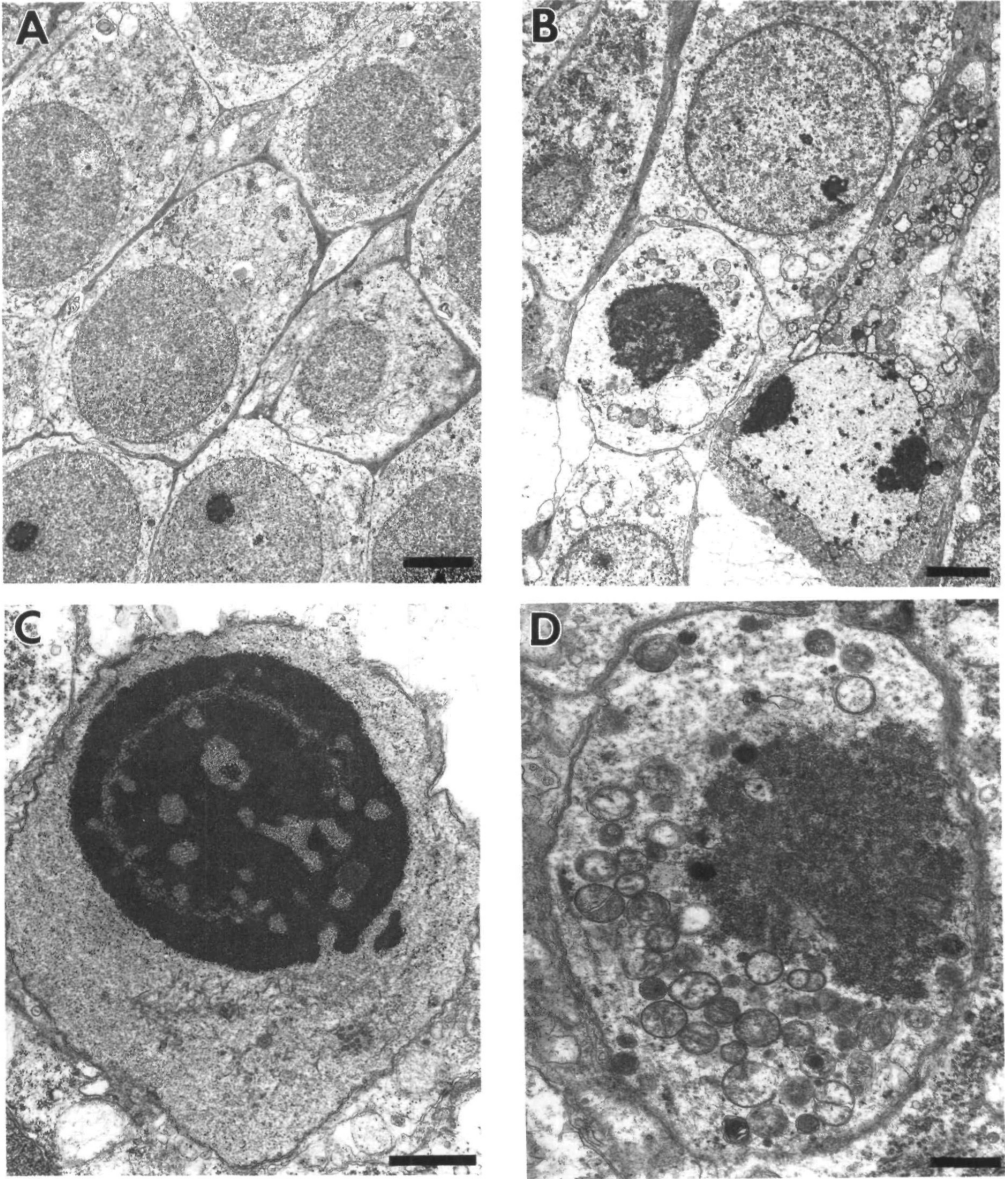


FIGURE 3. Electron micrographs of retinal ganglion cells from the monkey retina. **(A)** Normal-appearing ganglion cells. **(B)** Ganglion cells in a retina with experimental glaucoma, with two cells illustrating abnormal nuclear morphology. Chromatin is eccentrically clumped into two dense masses in one cell, whereas it is clumped centrally in the other nucleus. **(C)** Monkey glaucoma. An apoptotic cell with dense residual nuclear chromatin and absent nuclear envelope (similar to the cell with a dense nucleus in **A**). **(D)** An apoptotic body with intact cytoplasmic organelles and cell membrane, and a residual fragment of nuclear material. **(A,B)** Bars = 3 μm . **(C,D)** Bars = 1 μm .

TABLE 2. Histologic Study of Monkey Eyes After Transection

<i>Time After- Transection (weeks)</i>	<i>% Positive Cells</i>	<i>Degree of Axon Loss</i>
2	7	Mild
3	3	Mild
3	13	Mild
3	1	Moderate
4	8	Moderate
10	0	Severe
10	0	Severe
12	0	Severe
12	0	Severe

Time post-transection: time from optic nerve transection until sacrifice. % Positive cells: percent of ganglion cells with morphology compatible with apoptosis by light microscopy (see Light and Electron Microscopy). Degree of axon loss: semiquantitative estimate of amount of retinal ganglion cell axon loss based on analysis of optic nerve cross-sections. One of the 10 eyes, sacrificed 3 weeks after transection, was poorly preserved by fixation and is not included.

in three normal eyes = 2,071; in three glaucomatous eyes = 716).

Although some apparently positive cells were detected in controls, six of the eight positive cells were from only two of the control eyes. These had high levels of melanin pigment from the retinal pigment epithelium and choroid scattered over the specimens. This melanin has a color similar to that of the reaction product of the peroxidase enzyme in the TUNEL method.

We also studied 10 rabbits that underwent nerve transection by the TUNEL technique. TUNEL-positive cells were detected in every rabbit eye whose optic nerve had been transected 1 to 4 weeks before sacrifice (Table 4). The relative number of positive cells per slide was 10 times higher in the transected eyes than in their fellow, normal eyes. Control specimens had an average of 1,250 cells identified as ganglion cells per slide. At 1 to 2 weeks after transection, three slides averaged 955 ganglion cells, and, at 3 to 4 weeks after transection, a mean of 500 ganglion cells was present in three slides. By 6 weeks, there were fewer than 100 cells per slide. The relative number of positive cells was, then, approximately 1 in 200 to 400 cells in the month after transection. This compares to the background rate in controls of 1 in 3,600 cells. By 6 weeks after axotomy, few ganglion cells remained in the retina, and none were identified as TUNEL positive.

DISCUSSION

We have presented evidence that retinal ganglion cells die by apoptosis in experimental glaucoma and after

axotomy. The changes in ganglion cell morphology after both types of injury resemble those reported with apoptosis. A dramatically increased rate of cell nuclei with DNA cleavage was indicated by the TUNEL method. Two research groups recently reported^{35,36} TUNEL-positive ganglion cells in rat retina after axotomy. Our report in rabbit and monkey confirms these findings and points to apoptotic death as a generalized mammalian response to some injuries of ganglion cell axons. Our electron microscopic observations and quantitative histomorphometry lend further support for apoptosis as the mode of cell death for many ganglion cells after severe axonal injury.

During periods of active cell loss in our model systems, up to 10% of ganglion cells underwent apoptosis as defined by light microscopy and up to 1% of ganglion cells underwent apoptosis as defined by TUNEL analysis. The identification of fewer cells by the TUNEL method than by light microscopy may reflect the brief time window during which dying cells have a positive TUNEL reaction. This interval has been estimated to be as short as 1 to 3 hours in other cell types,³⁹ and it has been estimated at 8 to 11 hours in rat retinal ganglion cells.³⁵

We did not detect internucleosomal DNA cleavage by electrophoretic methods. Only one of the two

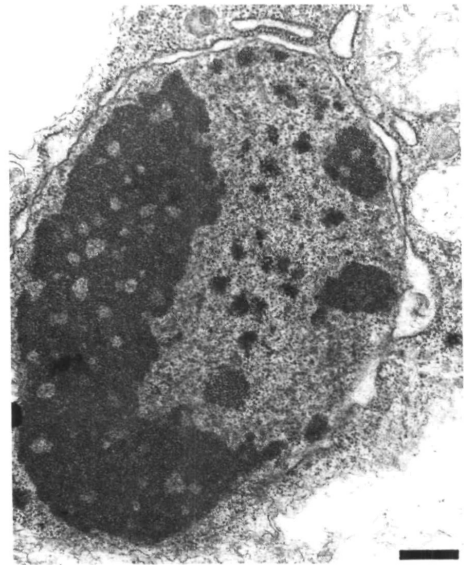


FIGURE 4. Electron micrograph of apoptotic cell morphology in a retinal ganglion cell from a monkey after optic nerve transection. Note the eccentric clump of dense chromatin in the nucleus. Bar = 0.5 μ m.

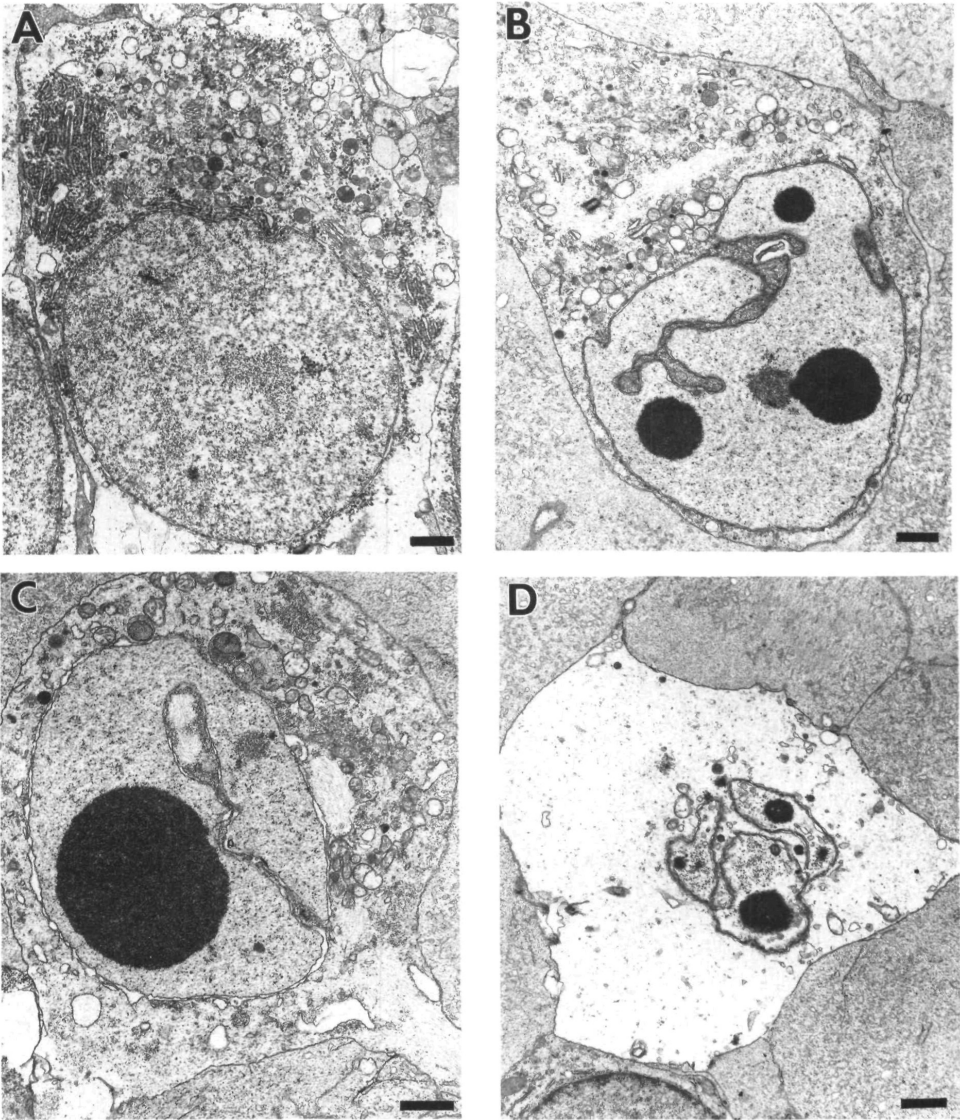


FIGURE 5. Electron micrographs of retinal ganglion cells from the normal rabbit retina (**A**) and 1 or 2 weeks after optic nerve transection (**B,C,D**). Cells after transection exhibit features characteristic of apoptosis in other cell systems. (**B**) The nucleus has three clumps of dense chromatin and lacks nuclear detail in the remainder of the nucleus. (**C**) One large dense clump of nuclear chromatin in the ganglion cell. (**D**) The ganglion cell is reduced to three apoptotic bodies, each with a dense clump of presumed chromatin and intact surrounding membranes. Note the lack of free, electron-dense cellular contents. Bars = 1 μ m.

recent reports on rat retina after axotomy claims to provide some evidence for DNA fragmentation by electrophoresis.³⁵ Some forms of programmed cell death have been reported to use a mechanism that does not involve internucleosomal DNA cleavage.⁴³ However, the inability to detect a ladder-like electrophoretic pattern is more likely to result from the proportionately tiny amount of DNA undergoing fragmentation at any time in our models. Ganglion cells represent only a fraction of the cell nuclei in the retina, and only a minority of the ganglion cells undergo apoptosis at any given time. Furthermore, probably only a fraction of the cells undergoing apoptosis contains fragmented DNA that can be detected by electrophoresis. This proportion is unknown but is probably small based on analogy with the TUNEL procedure in which, as noted, the time window during which cells have a positive reaction is brief.³⁹

The expected frequency of ganglion cells undergoing apoptosis as defined by the TUNEL method can be estimated by a crude calculation. In the monkey, there are approximately one million ganglion cells.⁴⁴ Moderately damaged glaucomatous eyes, as defined here, have approximately 50% ganglion cell loss.

A reasonable estimate of the rate of ganglion cell loss in the monkey model is 100,000 cells per month. If we assume that the number of ganglion cells lost per unit time is constant and if we use 10 hours as the time window during which apoptotic cells are TUNEL positive,³⁹ then, at any given time, 1,200 TUNEL-positive ganglion cells are present in a monkey retina. In a moderately damaged glaucomatous retina with 500,000 remaining cells, the predicted frequency of positive cells is 0.3%. This is within an order of magnitude of the 1% value we measured experimentally. Given the many assumptions and estimates used in the calculation, this level of agreement seems reasonable.

The finding of rare TUNEL-positive cells in the

control eyes may either reflect false positives or may indicate that apoptosis takes place at a low level in the normal adult retina. We suspect that some of these were artifacts because they were found most often in eyes with the highest amount of choroidal melanin pigment debris, which is of similar color to the peroxidase reaction product. Some investigations indicate that there may be modest ganglion cell loss in the adult retina.⁴⁴⁻⁴⁷ It is feasible that some apoptosis occurs as part of normal aging.

The finding of apoptosis in retinal neurons is not unprecedented. Apoptosis has been implicated in photoreceptor cell death, both during development and with the pathologic degeneration associated with the retinal dystrophies.^{40,48,49} As described above, two research groups recently reported apoptosis in retinal ganglion cells after axotomy in the rat.^{35,36} Previous investigations of severe ganglion cell injury have demonstrated some histologic features compatible with apoptosis after ocular ischemia,⁵⁰ after optic nerve crush injury,⁵¹ and after loss of postsynaptic target cells.^{52,53} We think our findings suggesting apoptotic death in experimental glaucoma are the first involvement of this mechanism in a ganglion cell disease process.

It has been suggested that programmed death of retinal ganglion cells is a routine part of normal ocular development.^{2,4} Hence, the genetic program for apoptosis is, presumably, present in ganglion cells. The continued survival of neurons after attainment of their normal adult connections may depend on continued arrival at the cell body of trophic factors from the target cells. Mammalian ganglion cells do not successfully regenerate after significant injury, though some survive and regenerate when they are provided with such trophic factors experimentally.⁵⁴⁻⁵⁶

We hypothesize that transection and glaucoma initiate apoptotic cell death by interfering with the

TABLE 3. TUNEL Staining of Monkey Glaucomatous Eyes

	Number of Eyes/Slides	% Positive Cells/Slide			% Positive
		Definite	Probable	All	
Control	7/34	0.18	0.06	0.24	0.1
Total glaucoma	9/42	0.50	0.14	0.64	
Mild glaucoma	3/13	0.31	0.23	0.54	
Moderate glaucoma	5/25	0.64	0.12	0.76	1.0
Severe glaucoma	1/4	0.25	0.00	0.25	

All is the sum of definite plus probable cells. Mild, moderate, and severe glaucoma are defined by degree of axon loss based on optic nerve cross-sections (see Methods). % Positive is an estimation of the number of positive ganglion cells among all ganglion cells, based on counts of total ganglion cells from three representative normal and three representative moderate glaucoma slides. The difference between all glaucoma and control eyes in all positive ganglion cells is statistically significant ($P = 0.046$, chi-square analysis), as are the differences between moderate glaucoma and control in all and definite positive cell numbers (both $P = 0.029$).

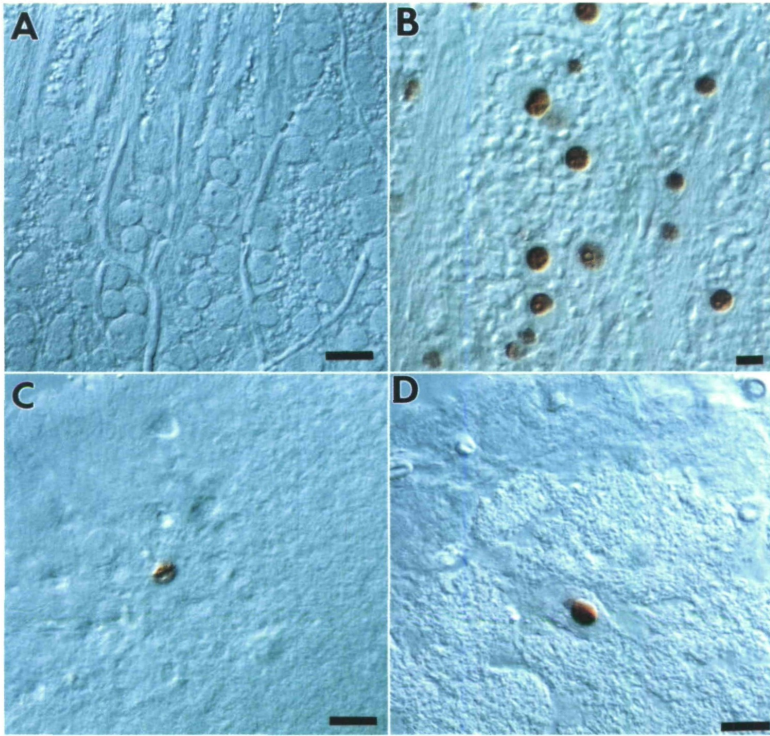


FIGURE 6. TUNEL-treated retinal ganglion cells viewed by differential interference contrast microscopy in sections cut parallel to the retinal surface. (A) The appearance of normal retina with round ganglion cell nuclei, bundles of axons, and retinal capillaries. No TUNEL-positive cells are present. (B) The positive control specimen is a normal retina that was reacted with DNase I before the TUNEL reaction. The expected uniform red-brown positive reaction product is seen in all cell nuclei of the ganglion cell layer. (C) A positive cell present in a retina from a monkey with experimental glaucoma. (D) A rabbit retina with a positive ganglion cell, 1 week after optic nerve transection. Bar = 10 μ m.

TABLE 4. Tunal Staining of Rabbit Retina After Transection

	Number of Eyes/Slides	Positive Cells		% Positive Cells
		Number Positive	Cells/Slide	
Control	10/10	3	0.3	0.03
All Transection	8/27	62	2.3	
1-2 Weeks After	3/14	48	3.4	0.36
3-4 Weeks After	4/11	14	1.3	0.25
6 Weeks After	1/2	0	0.0	0.0

% Positive cells is an estimation based on counts of total ganglion cells from representative cross-sections. No ganglion cells were present after 6 weeks post-transection. The difference between control and all transection in the proportion of positive cells per slide is significant ($P = 0.003$, chi square analysis).

transmission of neurotrophic signals that normally arrive by retrograde axonal transport from target cells. Experimental and human glaucoma lead to the interruption of both anterograde and retrograde axonal transport in ganglion cell axons at the level of the optic nerve head.^{18-21,29,33} At this time, we have no direct evidence to support this mechanism with respect to glaucoma. However, it is logical that the pathogenic insult to the axons in glaucoma may act to interrupt the flow of trophic signals to the cell body, just as transection does.

The demonstration that chronic experimental glaucoma leads to apoptotic ganglion cell death suggests future research to understand the mechanisms underlying glaucomatous ganglion cell death and leads to potential new approaches to therapy. Patients with glaucoma differ in their susceptibility to optic nerve damage. Perhaps susceptibility is determined in part by differences in the sensitivity to activation of the apoptotic mechanism in ganglion cells. Intraocular administration of neurotrophins may reduce the activation of apoptosis.¹¹ Manipulation of the expression level of gene products known to be involved in apoptosis is known to alter cell survival. For example, increased expression of *bcl-2* or *crmA* (an inhibitor of interleukin-1 β converting enzyme) prolongs the survival of neurotrophin-deprived neurons.⁵⁷⁻⁵⁹ Eventually, it may be possible to alter resistance to glaucoma damage by modulating gene expression in retinal cells.

There may be more than one pathway to cell death for ganglion cells in glaucoma. Although we have suggested that apoptosis is one mode of death, there is preliminary evidence that release of excitotoxins, perhaps glutamate, may lead to cell injury as well.⁶⁰ It is possible that the pathogenetic factors in glaucoma lead to more than one mode of cell death, depending on local tissue circumstances, ganglion cell subtype, or magnitude of insult.

Key Words

glaucoma, axotomy, apoptosis, retinal ganglion cells, optic nerve

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