

Apoptosis in Retinal Ganglion Cell Decrease in Human Glaucomatous Eyes

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Abstract: Hematoxylin-eosin staining, the TUNEL method for in situ detection of the intranuclear DNA fragmentation, which indicates apoptosis, and electron microscopy were used to study the morphologic changes in specimens from the eyes of 8 patients with secondary glaucoma and 2 normal control eyes to evaluate our hypothesis that apoptosis causes a decrease in retinal ganglion cells in human glaucomatous eyes. The TUNEL method permits identification of intranuclear DNA fragmentation. Apoptosis was found in the ganglion cells of 2 glaucomatous eyes with recent sight loss, and in the ganglion cells of a control eye from a 95-year-old subject, taken at autopsy. Results of our study indicate that a decrease in retinal ganglion cells in glaucomatous eyes is caused by apoptosis. In addition, apoptosis resulting from aging must be considered in order to understand the reduction of retinal ganglion cells in the glaucomatous eyes of elderly patients. *Jpn J Ophthalmol* 1997;41:84-88 © 1997 Japanese Ophthalmological Society

Key Words: Aging, apoptosis, electron microscopy, human glaucomatous eyes, retinal ganglion cells, TUNEL method.

Introduction

Histopathologic changes in glaucomatous eyes are characterized by a specific excavation of the optic disc (glaucomatous cup) and a reduction in the number of retinal ganglion cells. It is believed that this reduction is caused by the ischemia resulting from the direct effect of high intraocular pressure (IOP) on the nerve fiber,^{1,2} although the pathologic mechanism is not yet clearly understood. Various explanations of the simultaneous disappearance of retinal ganglion cells and impairment of the nerve fiber axon have been suggested: intracellular electrolyte imbalance, phagocytosis of the glia cells, and blockage of retrograde alimentation.^{1,3,4} Since the death of nerve cells due to neurotrophin withdrawal is believed to be mediated by apoptosis,⁵ it has also been proposed that the disappearance of retinal ganglion cells in glaucomatous eyes can be induced by apoptosis. There have been recent reports of apoptotic reti-

nal ganglion cell decrease produced by experimental glaucoma and ablation of the optic nerve fiber.^{2,6}

To substantiate the hypothesis that a decrease in retinal ganglion cells is induced by apoptosis in the human glaucomatous eye, we studied the cells of enucleated eyeballs from glaucoma patients, using hematoxylin-eosin staining, the TUNEL method with which intranuclear DNA fragmentation can be identified in situ, and electron microscopy.

Materials and Methods

Eyeballs were enucleated from 8 secondary glaucoma patients 41 to 86 years old in order to alleviate severe eye pain (Table 1). All patients had given their written, informed consent. All of these patients had lost light perception from 2 months to 15 years prior to enucleation; intraocular pressure of the 8 specimen eyes had been 52-68 mm Hg. One symblepharon eye, taken for cosmetic reasons from a 59-year-old patient who also gave his written, informed consent to participation in the study, and an eye from a 95-year-old subject obtained at autopsy were used as controls. The autopsy eye was fixed 6 hours postmortem; all others were fixed immediately on

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Table 1. Specimens

| | Patient No. | Age | Sex | Reason for Enucleation | Duration of Blindness | Apoptosis |
|-------------------------|-------------|-----|-----|-------------------------------|-----------------------|------------------------------|
| Control eyes | 1 | 95 | F | Autopsy | | Positive (Figures 1C, 1D) |
| | 2 | 59 | M | Symblepharon | | Negative |
| Secondary glaucoma eyes | 3 | 87 | F | Neovascular glaucoma | 2 months | Positive (Figures 1A, 1B, 2) |
| | 4 | 79 | M | Neovascular glaucoma | 2 months | Positive |
| | 5 | 88 | F | Neovascular glaucoma | 2 months | Negative |
| | 6 | 79 | M | Neovascular glaucoma | 4 months | Negative |
| | 7 | 41 | F | Neovascular glaucoma | 1 year | Negative |
| | 8 | 52 | F | Secondary open-angle glaucoma | 3 years | Negative |
| | 9 | 62 | F | Secondary open-angle glaucoma | 9 years | Negative |
| | 10 | 71 | F | Secondary open-angle glaucoma | 15 years | Negative |

enucleation in 1.0% glutaraldehyde and 2.5% formalin in 0.15 mol phosphate buffer solution (pH 7.2) for 1–7 days. A block 4 mm wide, which included the pupil and optic nerve, and an adjacent block were cut, embedded in celloidin/paraffin, and then cut serially into 5 μ m sections. The sections were stained with hematoxylin eosin and examined by light microscopy for any retinal changes. We followed the basic TUNEL procedure described by Gavrieli et al⁷ using the ApoptagTM *In Situ* Apoptosis Detection Kit, with some modifications: proteinase K was used for 1 hour at room temperature; intrinsic peroxidase was deactivated by 0.3% H₂O₂ for 30 minutes at room temperature; and diaminobenzidine was used as a chromagen for the reaction of the antibody (30 minutes at 37°C). A rat intestine was used as the positive control. The retinochoroidal tissues adjacent to the pupil-optic nerve block at the posterior region was cut into 5–7 small pieces, postfixed in 1.0% osmic acid, and embedded in epoxy resin. Ultrathin sections were double-stained with uranium-lead, and the retinal ganglion cells were examined by electron microscope.

Results

The middle and external retinal layers of the specimen glaucomatous eyes were generally well maintained, but atrophy of the nerve fiber layer and a decrease in the number of ganglion cells were frequently seen. This damage was greater in blindness of longer duration: atrophy of the synapse and reduction in amacrine, bipolar, and horizontal cells were also observed in the inner plexiform and inner nuclear layers of the retina. Pyknosis of retinal ganglion cells was found by hematoxylin-eosin staining at the posterior region in the 2 cases (patients 3 and

4) who had relatively recent loss of sight. There were one to three cells per section in 10 random samples. TUNEL-positive cells were also found in 5 random sample sections (Figures 1A,B).

Pyknosis of retinal ganglion cells (1–2 per section), and positive TUNEL staining were found in the control autopsy eye from the 95-year-old woman (Figures 1C, 1D). The enucleated symblepharon control eye from the 59-year-old man had no pyknosis or TUNEL-positive cells in the ganglion cell layer.

Electron microscopic examination of the pyknotic retinal ganglion cells of the 5 random sample blocks were grouped into 4 stages of cell activity. In the first stage (Figure 2A), the nuclear membrane was difficult to distinguish and chromatin was abnormally concentrated in the nucleus and surrounding areas. At this stage, there was no decrease in staining ability of the cytoplasm, but there was a reduction in the number of organelles.

In the second stage (Figure 2B), the nucleus was malformed and segmented; chromatin was still concentrated in the nucleus and its surroundings. There was less cytoplasm and the organelles were significantly reduced, but there was an occasional well-developed Golgi apparatus.

In stage 3 (Figure 2C), most of the nucleus was strongly and uniformly stained; the double-layered nuclear membrane was unidentifiable. There were significant microfilament structures in the cytoplasm, but the granular matrix was thin and organelles were difficult to distinguish.

In the final stage (Figure 2D), the cytoplasm was stained almost uniformly; it had become granular and most of the membrane structure was lost. The nucleus, enclosed in a single-layered membrane, was strongly stained in the center and surrounded by a granular substance similar to the cytoplasm.

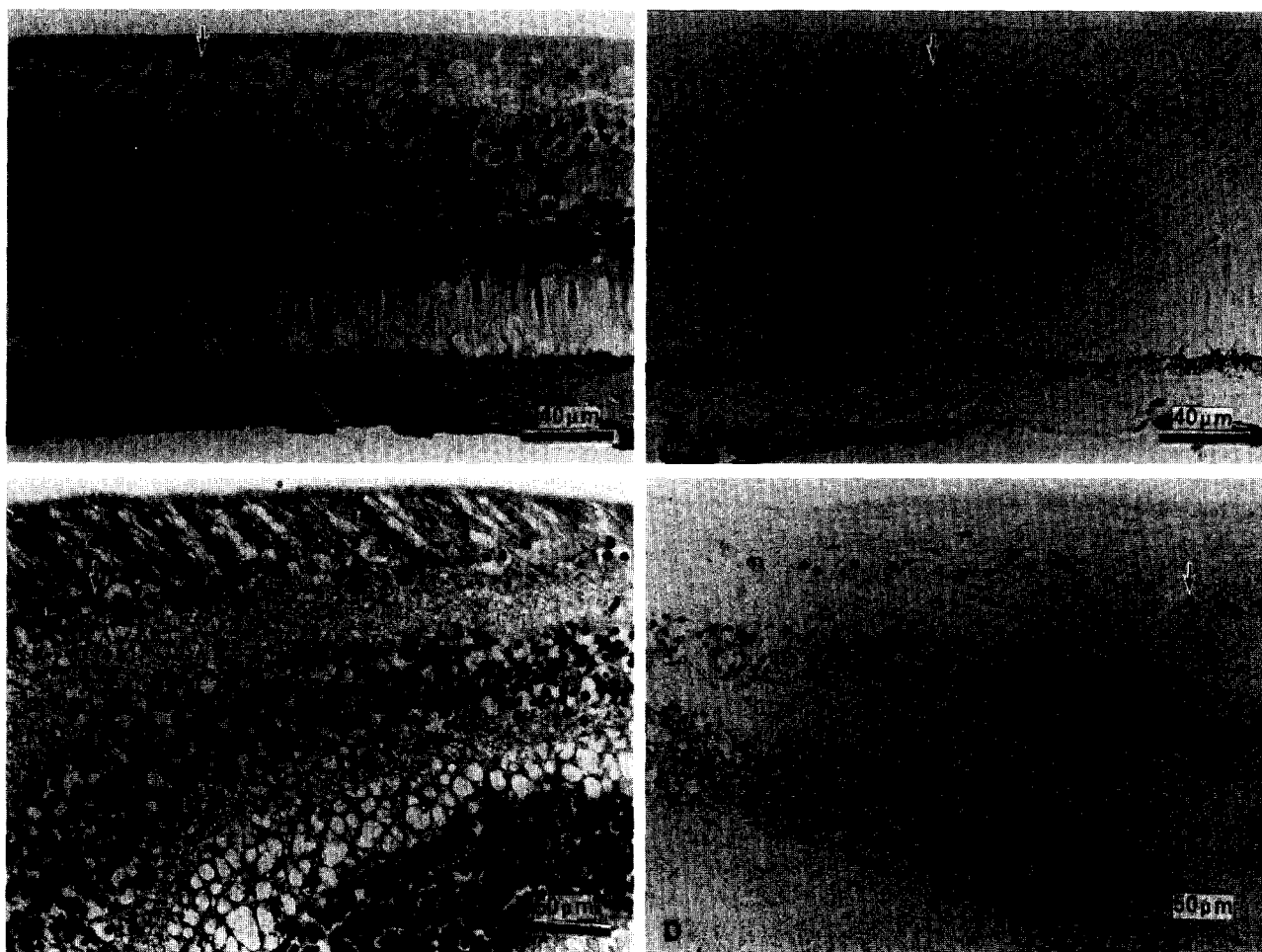


Figure 1. (A): Light micrograph (hematoxylin-eosin staining) of retina and choroid of neovascular glaucomatous eye. Pyknosis of retinal ganglion cell (arrow). (B): Light micrograph (TUNEL method): retina and choroid of neovascular glaucomatous eye. TUNEL-positive cell (arrow) in ganglion cell layer. (C): Light micrograph (hematoxylin-eosin staining): retina and choroid of 95-year-old control eye. Pyknosis of retinal ganglion cell (arrow). (D): Light micrograph (TUNEL method): retina and choroid of 95-year-old control eye. TUNEL-positive cell (arrow) in ganglion cell layer.

Discussion

Light microscopic observation of hematoxylin-eosin stained sections revealed pyknosis in the retinal ganglion cells from the control eye of an elderly subject and the secondary glaucoma specimen eyes that had only a 2-month history of sight loss. The TUNEL technique (for detection of intranuclear DNA fragmentation) and electron microscopy implicated apoptosis in the development of pyknotic cells. It has recently been reported that experimentally induced glaucoma and ablation of optic nerve fibers in rabbits, monkeys, and rats result in a positive TUNEL response in the retinal ganglion cells.^{2,6} Since we also observed similar apoptosis, it appears

that apoptotic cell death in mammals is a response to a disorder in the axon of the retinal ganglion cells.

Epidemiologic study results also indicate that higher intraocular pressure (IOP) is the most frequently observed risk factor in glaucoma.⁸ Quigley et al² reported that experimentally induced glaucoma in monkeys, producing an IOP of > 43 mm Hg for 2-22 consecutive days resulted in 4-13% of the retinal ganglion cells becoming TUNEL-positive, with slight to moderate disappearance of the nerve fiber axon. With higher IOP (mean: 43 mm Hg) lasting for 210 consecutive days, 1% of the retinal ganglion cells were TUNEL-positive, but a significant number of the nerve fiber axons had disappeared.² This indicates that in experimentally induced glau-

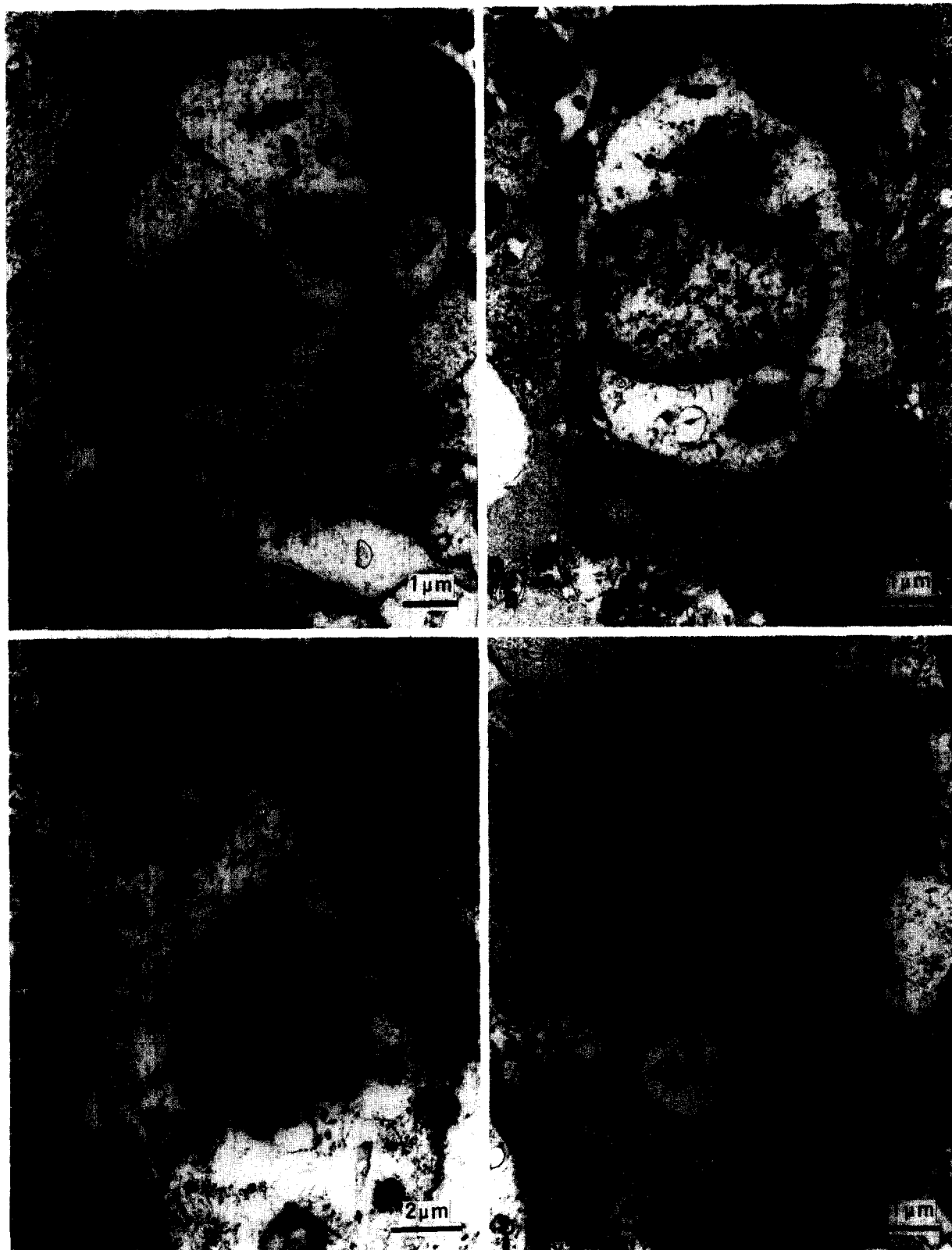


Figure 2. Electron micrographs of retina (ganglion cell layer); neovascular glaucomatous eye. (A) Nuclear membrane is difficult to recognize; chromatin is highly concentrated. (B) Nucleus is malformed and segmented; little cytoplasm; few organelles. (C) Nucleus strongly and uniformly stained; double-layer structure of nuclear membrane recognizable. (D) Nucleus is strongly stained; surrounded by granular substance similar to cytoplasm.

coma, a short period (2–22 days) of high IOP can result in apoptosis of retinal ganglion cells and elimination of some nerve fiber axons. A longer period (210 days) of higher IOP gradually reduces the retinal ganglion cell death controlled by genes, but induces significant loss of nerve fiber axons from cumulative retinal ganglion cell death. We also found that, in the enucleated secondary glaucomatous eyes, a few TUNEL-positive cells were observed, but most of the nerve fibers were still intact in the eyes having blindness of a shorter duration. In eyes with a longer history of blindness, no TUNEL-positive cells were observed and a significant amount of nerve fibers had disappeared. These results appear to be consistent with those of Quigley et al.²

Retinal disorders related to the primary disease need to be further studied since we used the eyes of secondary glaucoma patients for this study. It seems reasonable, however, to conclude that similar cell death would be involved in the reduction of ganglion cells in primary open-angle glaucoma because higher IOP is the most frequently observed characteristic.

The few TUNEL-positive cells found in the control autopsy eye from the elderly woman may indicate either a false-positive response to the staining or an incidence of a lower level of apoptosis in the normal human retina with aging. The reduction in the number of retinal ganglion cells and the nerve fibers with aging is reported by many investigators.^{9–12} In addition, TUNEL-positive cells were observed only in the retina of the 95-year-old control subject; there were none in the eye of the 59-year-old control subject. This suggests that apoptosis may be part of the normal aging process of the retinal ganglion cells.

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