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LABORATORY INVESTIGATIONS

Experimental Glaucoma Model in the Rat Induced by Laser Trabecular Photocoagulation After an Intracameral Injection of India Ink

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Abstract: A simple and reproducible rodent glaucoma model is required to elucidate the pathophysiology of damage to the optic nerve. We developed chronically elevated intraocular pressure (IOP) unilaterally in rats by injecting india ink into the anterior chamber of one eye using a 30-gauge needle. One week later, trapped carbon particles in the chamber angle formed a black band along the corneal limbus in the injected eyes. We performed direct laser photocoagulation without a gonio lens, and selectively burned the trabecular meshwork. Intraocular pressure was measured every week and laser photocoagulation was repeated until mean IOP in the experimental eyes rose above 25 mmHg. Unilateral IOP elevation was attained in all rats within 4 weeks. Twelve weeks after ink injection, we sacrificed the rats and excised the eyes for histologic analysis. The anterior chamber angle showed peripheral anterior synechia caused by laser photocoagulation, and carbon particles were engulfed by macrophages that infiltrated the ciliary cleft. In the optic nerve head, a remarkable decrease in the nerve fiber layer and cavernous degeneration were observed, suggesting glaucomatous optic nerve damage. This experimental rodent model should facilitate the study of the complex mechanisms involved in glaucoma. **Jpn J Ophthalmol 1998;42:337–344** © 1998 Japanese Ophthalmological Society

Key Words: Experimental model, glaucoma, histology, india ink, laser photocoagulation, optic nerve damage, rats.

Introduction

Monkey eyes have historically provided experimental models of glaucomatous optic nerve damage,[1] because their lamina cribrosa morphologically resemble those of human eyes. Numerous reports[2–5] describe glaucomatous changes in the composition of extracellular matrices of the monkey optic nerve head. Recent investigations have attempted to determine the mechanisms involved in neuropathy by means of molecular biology. These include the cellular responses to elevated intraocular pressure (IOP) in the optic nerve head[6] and the regulation of lamina cribrosa remodeling mediated by cytokines and growth factors.[7] On the other hand, current neurologic studies are rapidly clarifying the mechanism of apoptosis. The mechanism of ganglion cell death,[8,9] is of ophthalmologic interest, and "neuroprotection"[10] shows promise as a therapy for glaucoma. To relate these latest findings to glaucoma, a rodent glaucoma model is required. Johansson[11] reported that the retrograde axoplasmic transport in the rat optic nerve is inhibited during IOP elevation. Morrison et al[12] demonstrated that the rat optic nerve head possesses an identifiable lamina cribrosa with structural proteins that is nearly identical to that of the primate. Johnson et al[13] immunohistochemically examined the optic nerve head in the rat glaucoma model, and showed extracellular matrix changes replicating those found in human glaucomatous eyes

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and monkey eyes with experimentally elevated IOP. The rat lamina cribrosa consists of radially oriented laminar beams instead of cribriform plates. Further histologic studies are required to clarify whether the mechanism of rat optic nerve damage is identical to that in human glaucomatous eyes. The rodent model is considered to be a useful alternative model in cell biology studies, because human ganglion cells similarly die as a result of axonal injury caused by elevated IOP. Furthermore, the rodent lamina cribrosa is remodeled with a matrix alteration similar to that occurring in human glaucomatous eyes.

Glaucoma has previously been induced in monkey eyes using laser treatments. This procedure cannot be applied to rodent eyes, which are too small to accommodate a conventional gonio lens, and the anterior chamber angle is too narrow to aim precisely toward the trabecular meshwork. In this study, we designed a means of selectively burning the trabecular meshwork in rodents by laser photocoagulation. We describe this experimental rodent model and discuss IOP elevation and the histologic findings in the anterior chamber angle and optic nerve head.

Materials and Methods

Animals

Ten Wistar Kyoto rats were kept in accordance with the ARVO Statements for the Use of Animals in Ophthalmic and Vision Research and used in this study at the age of 8 months. India ink marketed as writing material was injected into the anterior chamber of one eye of each animal, and the contralateral eye was retained untouched as control. Seven rats underwent repeated laser photocoagulation after an intracameral injection of ink, and unilateral IOP became elevated as described below. Laser photocoagulation was not performed on the 3 other rats, one of which was sacrificed for histological analysis of the anterior chamber angle 1 week after ink injection.

Procedures to Develop IOP Elevation

Rats were anesthetized by diethyl ether inhalation and intraperitoneal injection of 0.3 mL/kg sodium pentobarbiturate and 0.6 mL/kg ketamine hydrochloride. Before surgery, we measured the IOP in each eye three times with a pneumatonometer and the mean value was taken as baseline IOP. First, we injected approximately 0.05 mL of india ink into the anterior chamber of one eye with a 30-gauge needle. We inserted the needle obliquely into the peripheral cornea, with its bevel down, so that the iris and lens would not be injured and the aqueous humor would not flow out. Three to 7 days later, carbon particles had been deposited in the trabecular meshwork by the aqueous flow, forming a black band about 0.2 mm wide along the corneal limbus (Figure 1). One week later, we performed direct argon laser photocoagulation to the pigmented target without a gonio lens in the seven treated eyes. Sixty to 100 laser burns were delivered around the black band at the setting of 0.5 mm, 250 mW, and 0.2 seconds. We measured IOP every week and repeated laser treatments until the mean IOP rose to >25 mmHg. After that, laser

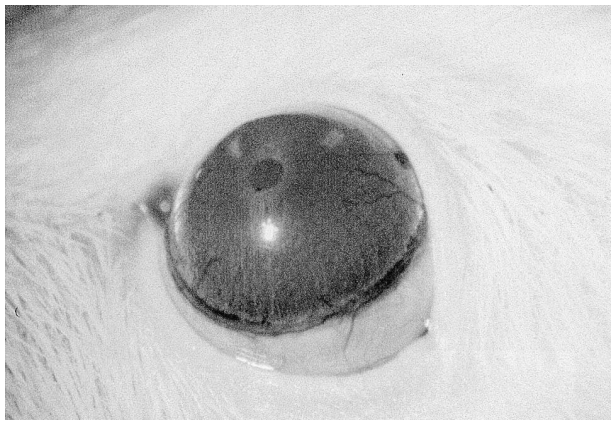


Figure 1. One week after intracameral ink injection. Carbon particles have collected in trabecular meshwork forming black band about 0.2 mm wide along corneal limbus.

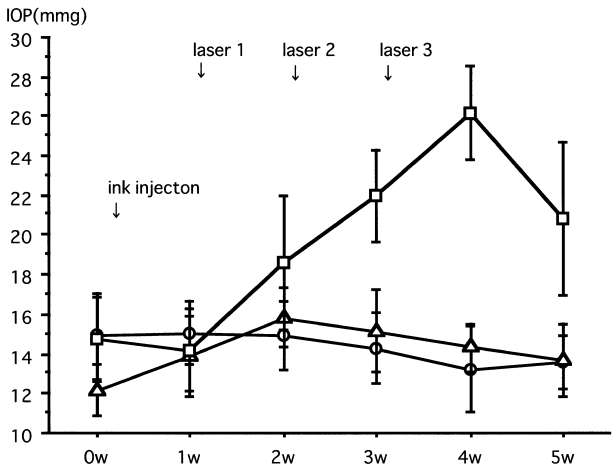
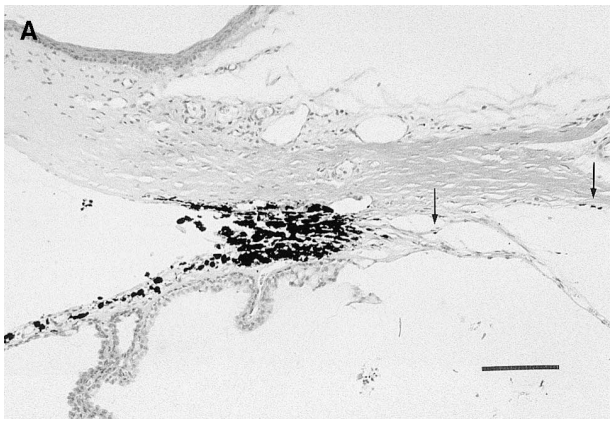
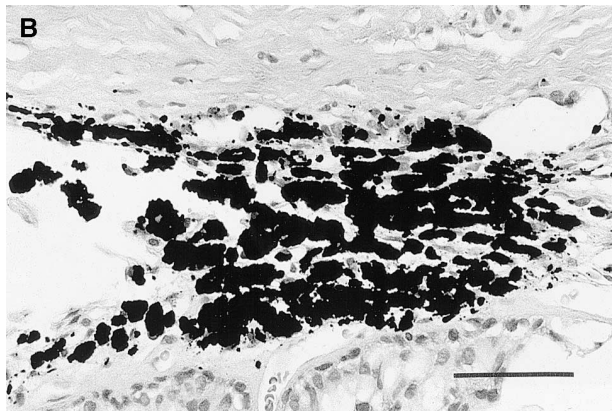


Figure 2. Progress of IOP elevation (○: control, △: ink injection alone, □: laser treatments after ink injection.) Mean IOP of eyes given laser treatments after ink injection was elevated after each photocoagulation.





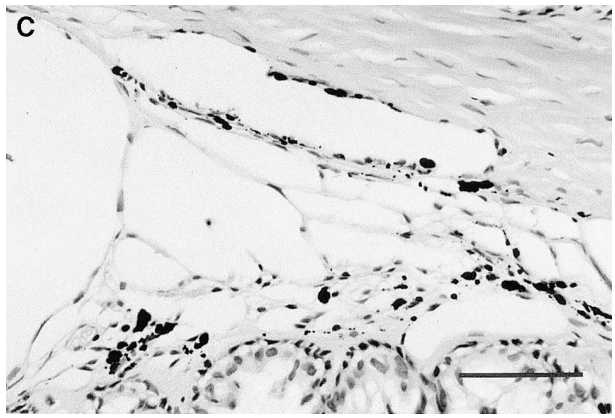


Figure 3. (**A**) Anterior chamber angle in eye 1 week after ink injection. Carbon particles are trapped mainly in intertrabecular spaces. Some are located in choroid (arrows) seeming to reflect route of uveoscleral outflow. (Lower magnification. Hematoxylin and eosin stained. Bar = 100 μm). (**B**) Anterior chamber angle in eye 1 week after ink injection. Large particles have occupied ciliary cleft, and small particles are trapped along trabecular beams. (Higher magnification. Hematoxylin and eosin stained. Bar = 50 μm). (**C**) Anterior chamber angle in eye excised 12 weeks after ink injection without laser treatments. Most particles have dispersed from eye. (Hematoxylin and eosin stained. Bar = 50 μm).

treatment was regulated according to the degree of IOP elevation. When IOP was >25 mmHg, we added no irradiation; around 20 mmHg, 10–30 exposures; <15 mmHg, 60–100 exposures around the pigmented band. We three-dimensionally observed cupping of the optic disc using a Topcon stereo fundus camera.

Tissue Preparation

Twelve weeks after ink injection, we sacrificed five of the seven rats treated with laser photocoagulation and two rats without laser treatments for histologic analysis. Rats were perfused with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (PB) (pH7.4). The eyeballs were dissected and further immersed in the same fixative for 3 hours, then rinsed with PB. Specimens for light microscopic observation were dehydrated through a graded ethanol series, embedded in paraffin, and sliced to a thickness of 4 μm using a sliding microtome. Sections from the anterior chamber angle were stained by hematoxylin and eosin, from the optic nerve head by azan; then observed by light microscopy. Tissue of a rat sacrificed 1 week after ink injection was prepared in the same manner. Some specimens from the anterior chamber angle were prepared for transmission electron microscopy (TEM), by dissection and postfixation in 1% aqueous osmium

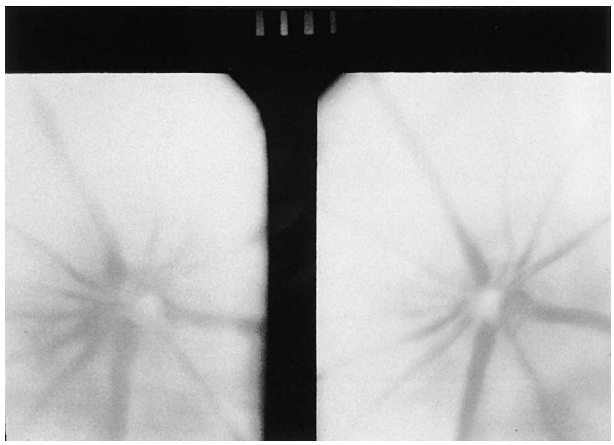


Figure 4. Stereo fundus photographs of experimental glaucomatous eye (12 weeks after ink injection). Unilateral large cup in optic nerve head.

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tetroxide for 3 hours. After dehydration through a graded ethanol series, they were embedded in Poly/Bed 812 Resin. Ultrathin sections were stained with uranyl acetate and lead citrate, then examined under a transmission electron microscope (JEM-100CX II; JEOL, Tokyo). Five months after ink injection, we sacrificed the other two rats and dissected the retrobulbar optic nerve 1 and 2 mm posterior to the globe. The specimens were prepared in the same way as those for TEM study and were embedded in Poly/Bed 812 Resin. Semiultrathin sections were stained by toluidine blue and were observed by light microscopy.

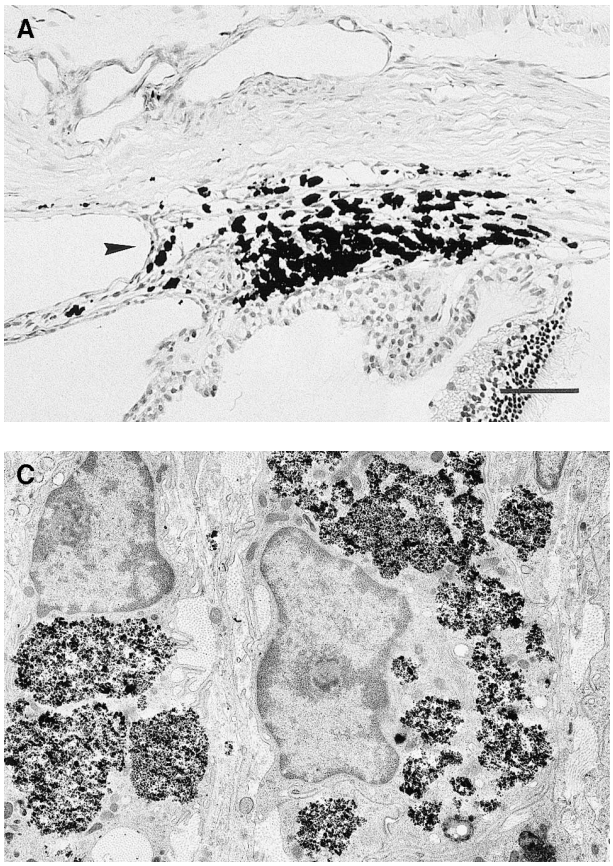
Results

Intraocular pressure elevation during the 5 weeks after ink injection is shown in Figure 2. The IOP of the eyes that received only an ink injection normalized within 1 week, and the difference was not statistically significant as compared with the control eyes throughout the course of the study. On the other hand, the mean IOP of the eyes that received laser treatments after the ink injection elevated after each laser photocoagulation. After three laser treatments, the mean IOP in 6 of the treated 7 eyes was >25 mmHg. After that, the IOP in 30–60% of the treated eyes fell <20 mmHg without further laser photocoagulation. In these eyes, laser treatments were repeated according to individual IOP levels, whenever required to maintain IOP >20 mmHg during the course of the study.

Figure 3A shows light microscopic findings of the anterior chamber angle in an eye 1 week after ink injection. Carbon particles are trapped mainly in the trabecular meshwork. Some are observed in the choroid (arrows), which seem to reflect the route of uveoscleral outflow. Higher magnification shows that large particles fill the ciliary cleft, and that small particles are trapped along trabecular beams (Figure 3B).

Figure 3C shows the anterior chamber angle of the eye excised 12 weeks after ink injection without laser treatment. Most of the carbon particles disappeared and only a few small particles are observed, which are caught in trabecular beams.

Figure 4 demonstrates an evident large cup in the optic disc observed unilaterally in the eyes of the group given an ink injection and laser treatments.



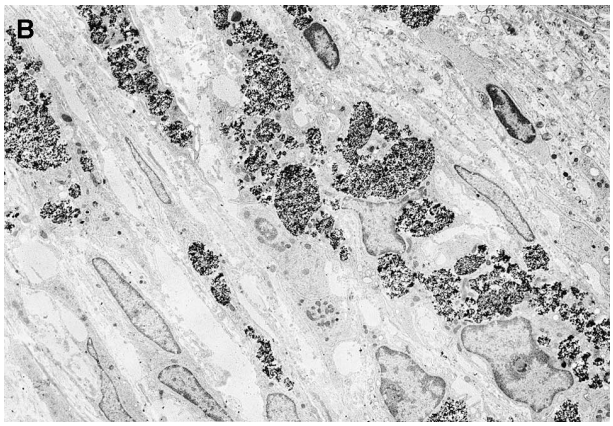


Figure 5. (**A**) Anterior chamber angle in the experimental glaucomatous eye (12 weeks after ink injection). Large amount of particles remain in trabecular meshwork. Anterior chamber angle is closed with extensive peripheral anterior synechia (arrowhead). (Hematoxylin and eosin stained. Bar = 50 μm). (**B**) Transmission electron microscopy findings of anterior chamber angle in experimental glaucomatous eye (12 weeks after ink injection). Macrophages with engulfed carbon particles fill decreased intertrabecular spaces. (×8,000, ×16,000).

Figure 5A shows the anterior chamber angle in the treated eyes excised 12 weeks after ink injection. A large amount of carbon particles remain in the trabecular meshwork in contrast to the eyes given ink alone (Figure 3C). A structural angle closure is also formed with peripheral anterior synechia (arrowhead). Transmission electron microscopy reveals extremely reduced intertrabecular spaces filled with macrophages with engulfed carbon particles (Figures 5B, C).

Light microscopic findings compare the optic nerve head in the control eyes (Figure 6A) with that in experimental glaucomatous eyes (Figure 6B). The thickness of the nerve fiber layer (arrows) in the glaucomatous eyes decreases, and the surface nerve fiber layer and prelaminar region in the optic nerve head are considerably atrophic. Laminar beams chiefly composed of collagen fibers, which can be detected by azan stain, bow posteriorly (arrowheads). At higher magnification, cavernous degenerations caused by axonal loss are observed in the prelaminar region (Figure 6C). A cross-sectional view of the retrobulbar optic nerve in the treated eyes at 5 months after ink injection (Figure 7B, left) shows decreased diameter compared to controls (Figure 7A, left) suggesting optic atrophy. At higher magnification, the number of myelinated nerve fibers decreases in the experimental glaucomatous eyes (Figure 7B, right) in contrast to controls (Figure 7A, right), and the atrophic lesions are replaced with glia and connective tissues.

Discussion

Pressure loading with cannulation into the anterior chamber[14] can produce an excellent acute model of experimental glaucoma in terms of the accurate regulation of IOP. Several subacute or chronic models, such as those produced by the intracameral injection of α-chymotrypsin,[15] ghost cells,[2] or pigment particles,[16] or by the administration of steroids,[17] or laser trabeculoplasty (LTP)[18–20] have been described. The laser-induced glaucoma model is routinely employed, because the extent of the exposure can be altered according to the degree of IOP elevation, the elevated IOP can be sustained with high reproducibility, and the procedure causes less inflammation than the intraocular injection of foreign bodies.

Monkey eyes are the experimental model of

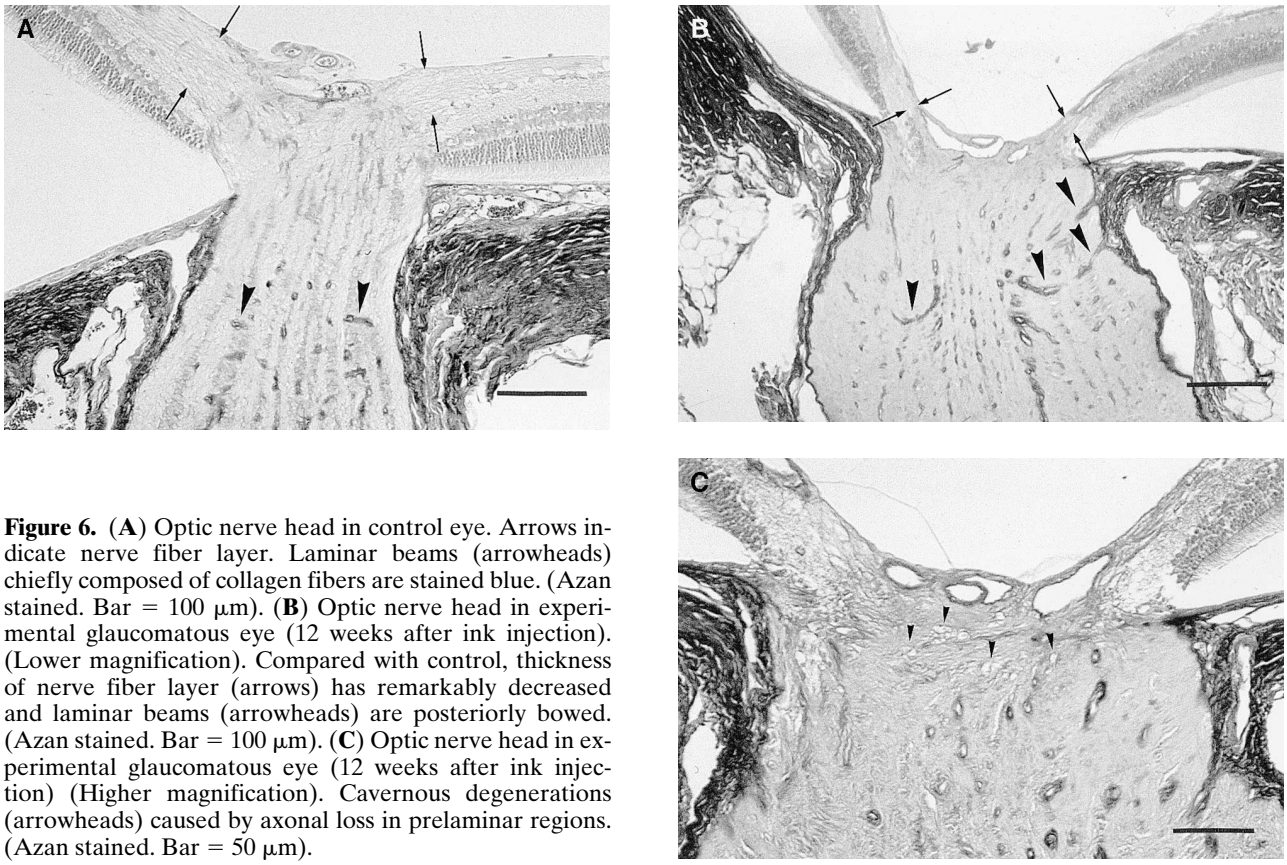


Figure 6. (**A**) Optic nerve head in control eye. Arrows indicate nerve fiber layer. Laminar beams (arrowheads) chiefly composed of collagen fibers are stained blue. (Azan stained. Bar = 100 μm). (**B**) Optic nerve head in experimental glaucomatous eye (12 weeks after ink injection). (Lower magnification). Compared with control, thickness of nerve fiber layer (arrows) has remarkably decreased and laminar beams (arrowheads) are posteriorly bowed. (Azan stained. Bar = 100 μm). (**C**) Optic nerve head in experimental glaucomatous eye (12 weeks after ink injection) (Higher magnification). Cavernous degenerations (arrowheads) caused by axonal loss in prelaminar regions. (Azan stained. Bar = 50 μm).

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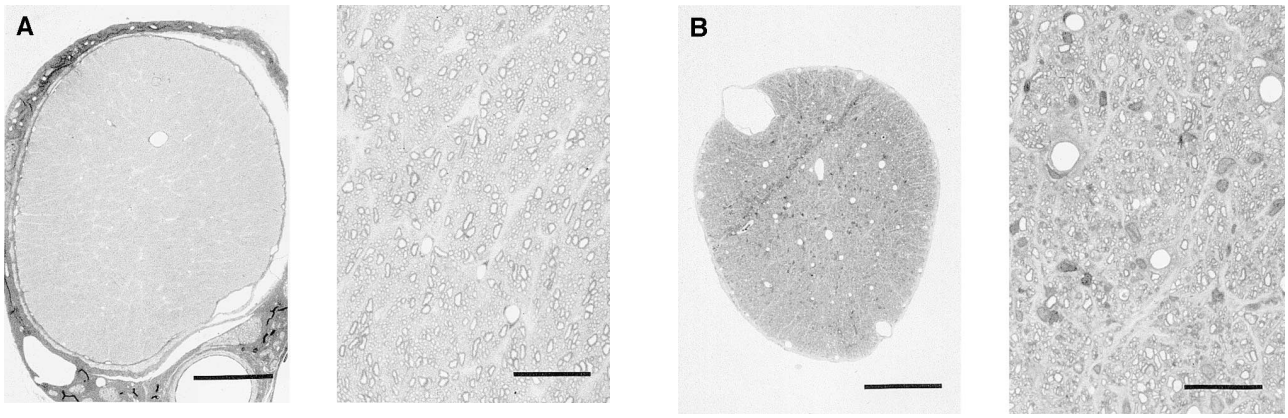

Figure 7. (**A**) Cross-sectional view of retrobulbar optic nerve in control eye. Myelin sheaths are stained blue and nerve fibers of various sizes are regularly arranged. (Left: lower magnification. Bar = 100 μm. Right: higher magnification. Bar = 20 μm. Toluidine blue stained). (**B**) Cross-sectional view of retrobulbar optic nerve in experimental glaucomatous eye (5 months after ink injection). At lower magnification, diameter of optic nerve decreases due to optic atrophy. At higher magnification, myelinated nerve fibers decrease in number compared with controls. Atrophic lesions are replaced with glia and connective tissues. (Left: lower magnification. Bar = 100 μm. Right: higher magnification. Bar = 20 μm. Toluidine blue stained.)

choice for many glaucoma studies, because their lamina cribrosa morphologically resemble those of human eyes. This similarity is essential for morphologic and immunohistochemical studies of the optic nerve head. However, there are many problems associated with the use of monkeys. For example, they are very expensive, so experiments requiring large numbers of animals are costly. Monkeys are difficult to procure, handle, and maintain, and they can harbor a virus that can infect humans.

A cell biology approach is required to elucidate the mechanism of optic nerve damage, and an alternative model is increasingly being sought. However, laser techniques cannot be directly applied to small animals such as rodents. Moore et al[21] and Morrison et al[22] injected hypertonic saline into episcleral veins in rats with microneedles. Among 20 consecutive animals, IOP was elevated in 9 following a single injection; subsequent injections raised IOP in 7 others. Garcia-Valenzuela et al[8] incised the conjunctiva of rats to expose limbus-draining veins and cauterized two of the major venous trunks to increase IOP. The procedure involved in tapering glass microneedles is troublesome, and the intravenous injection, exposure, and selective cauterization of limbus-draining veins require extremely fine manipulations. In addition, it is difficult to sustain the IOP at the level attainable in laser-induced models. The intracameral injection of foreign bodies such as microspheres or cyanoacrylates is another approach, although it is difficult to chronically elevate IOP by a single injection. The laser model is also superior in terms of the low risk of inflammation and high reproducibility. However, a novel approach was required to make this model feasible in rodents.

For this study, we designed a new means of selectively burning the trabecular meshwork. The method is based on the fact that carbon particles efficiently absorb laser energy to generate focal heat. With the laser setting used in our study, a single exposure sufficient to burn carbon paper does not scar the cornea or sclera in eyes without ink injection, thus the target can be exclusively photocoagulated. If the ink is less traumatically injected, inflammation is limited to the burn in the trabecular meshwork, and once a pigmented band is formed, frequent laser treatments can be applied without a gonio lens. This method can be used for other small animals, such as mice, guinea pigs, and others.

We speculate that the chronic IOP elevation in this model is mainly caused by two mechanisms. One is formation of extensive peripheral anterior synechia (PAS) or endothelialization, as shown in Figure 5A; the other is reduced intertrabecular spaces because of the accumulation of extracellular matrix, debris, and macrophages engulfing carbon particles, as shown in Figures 5B and 5C. Pederson and Gaasterland[18] who developed the laser-induced glaucoma model in monkey eyes reported gonioscopic findings of irregular pigmentation in the trabecular meshwork with several low PAS. In the same model, Radius and Pederson[19] histopathologi-

cally described that the trabecular beams and Schlemm's canal appeared unremarkable, although the intertrabecular spaces were collapsed. We consider that the laser treatment after ink injection, as in this study, produces results equivalent to those achieved by excessive laser photocoagulation upon pigmented trabecular meshwork.

The histologic findings of the optic nerve head in this study were similar to those reported by Morrison et al[22]: cavernous degeneration and a decrease in the nerve fiber layer, which are observed as glaucomatous changes in human eyes. Morrison et al[22] demonstrated by a TEM study that axonal swelling and increased cellularity were greatest at the level of the lamina cribrosa in eyes with early damage. Axonal swelling was also suggested by our light microscopic study; the thickness of the laminar portion was similar to that of the retrolaminar portion that originally had a bottleneck shape, and the intensity of staining had decreased in the swollen portion. The posterior bowing of the laminar beams shown in Figure 6B is a morphologic change similar to the glaucomatous cupping of the human lamina cribrosa. This finding confirmed that the remodeling in rodent laminar beams was consistent with the extracellular matrix changes reported by Johnson et al.[13] Lamina cribrosa in rats consist of two or three layers of approximately 30 radially oriented laminar beams bridged by loose collagen fibrils,[23] whereas in human eyes they are made up of piles of laminar sheets with longitudinally aligned pores. Accordingly, it is not clear whether nerve fibers in rats are compressed by laminar beams directly, or whether the mesh-like web of astrocyte fibrous processes surrounding axons participates in the pathogenesis of the nerve damage.[24] Further morphologic studies, including autoradiography, are required before the mechanisms of axonal transport inhibition in rats can be fully assessed.

This experimental rodent model should contribute to molecular and cell biological studies of glaucomatous optic nerve damage, and help contribute to the development of new therapies, such as gene transfection as a drug delivery system and neuroprotection against ganglion cell death.

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