Early Glial Responses after Acute Elevated Intraocular Pressure in Rats

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Purpose. To study the responses of glial cells to a short-term elevation in intraocular pressure (IOP) in rats.

Methods. Adult Sprague-Dawley albino rats, 45 to 55 days old, were given India ink intracameraly. After 7 days, 200 spots of laser burn over 560° were delivered by an Argon laser (620–657 nm; 200 mW; 200 mm; 0.2 seconds) aimed at the ink deposits in the trabecular meshwork. IOP was recorded and eye tissues at 12 hours and 1, 3, 5, 7, or 14 days after laser were examined by immunohistochemistry with antibodies against glial fibrillary acidic protein (GFAP), vimentin, S-100, ED1, and OX42. To evaluate neuronal loss, the number of cells in the retinal ganglion cell layer (RGCL) in response to the elevation of IOP was counted on flat preparations of retinas at various times after elevation of IOP.

Results. Significant elevation of IOP from 1 to 7 days and loss of cells in the RGCL from 3 days onward were noted after trabecular laser photocoagulation. In the inner retina, there was a gradual and sustained increase in GFAP and S-100 immunoreactivity, but only a transient increase in vimentin immunoreactivity. No remarkable changes in GFAP, vimentin, and S-100 immunoreactivity were noted at the optic nerve head (ONH). ED1- and OX42-labeled cells were noted in the choroidal plexus, the parapapillary region of the optic nerve, and the ONH from 3 days onward, whereas expression in the retina was unremarkable.

Conclusions. There is differential expression of glial cell markers in the retina and the ONH, with early loss of cells in the RGCL in response to the elevation of IOP. Macroglia such as astrocytes and Müller cells may be involved in the pathophysiology of retinal ganglion cell death or retinal repair, and activated microglial/phagocytic cells may play an important role in modulating the changes in the ONH that occur with the elevation of IOP. (Invest Ophthalmol Vis Sci. 2003;44:658–645) DOI:10.1167/iovs.02-0255

Glaucma is a heterogeneous group of disorders with optic disc cupping, loss of visual fields, and loss of retinal ganglion cells (RGCs) and their axons. Elevated intraocular pressure (IOP) is a major risk factor, but the pathophysiological processes leading to the loss of RGCs remain unclear. Astrocytes and Müller cells are the major glial cells in the retina, and astrocytes and oligodendrocytes are the major ones in the optic nerve head (ONH) and the optic nerve. These glial cells are responsible for modulating the microenvironment of individual neurons by maintaining tissue integrity, ion homeostasis, transports, uptake and metabolism of neurotransmitters. They may also involve in immunomodulation and neuromodulation. Because of their multiple functions, there is increasing evidence to suggest that glial cells may play pivotal roles in the development, injury, repair and regeneration of the nervous system. Many reports have shown the activation of glial cells in both the ONH and the retina in experimental glaucoma models and in humans with glaucoma. For example, in glaucomatous optic neuropathy, increased protein and gene expression of glial fibrillary acidic protein (GFAP), a glia-specific intermediate filament, in astrocytes is commonly observed in the retina and the ONH. These changes in GFAP may be associated with cytoprotective effects in reactive astrocytes, by increasing the synthesis of small heat-shock proteins, or with cytotoxic effects on the RGCs and their axons, by expressing tumor necrosis factor (TNF). The glial changes were also noted in the response of the glutamate transporter in the retinas of rats and monkeys with experimentally induced glaucoma and in the extensive remodeling of extracellular matrix (ECM) and recognition molecules in the ONHs of humans and monkeys with experimental glaucoma. Detailed descriptions of these changes are lacking, and how these glial changes contribute to the loss of RGCs in glaucoma remains to be elucidated.

Microglia are known as rapid sensors in neuronal damage and play important roles in the defense of the central nervous system (CNS). Activated microglia not only act as scavengers but also are responsible for tissue repair and neural regeneration. However, cytotoxic substances such as reactive oxygen radicals, nitric oxide, proteases, arachidonic-acid derivatives, excitotoxins, and cytokines may be released by these cells during neurodegeneration. Neufeld and Lin and Neufeld observed the presence of activated microglia in the ONH and the parapapillary chorioretinal region of the ONH in tissue specimens from humans with glaucoma. Whether this is an early or subsequent persistent involvement of microglia in glaucoma has not been determined. Wang et al. also reported the presence of microglia in rat retinas exposed to chronically elevated IOP, but there was no mention of these cells in the ONH.

In this study, we examined the early temporal responses of astrocytes, Müller cells, and phagocytic/microglial cells in the retina and the ONH in adult rats in response to a mild to moderate and short-term elevation of IOP. We used a procedure previously described by Ueda et al. to induce a mild to moderate increase in IOP in rats with laser photocoagulation to the trabecular meshwork after injection of India ink. IOPs and the number of cells in the retinal ganglion cell layer (RGCL)
were measured at various time points. Immunohistochemistry with antibodies against GFAP (astrocyte), vimentin (Müller cell), S-100 (Müller cell), ED1 (phagocytic cells), and OX-42 (activated microglia) was performed on tissues obtained at various times after induction of IOP elevation.

**METHODS**

**Induction of Elevated IOP**

Male Sprague-Dawley (SD) albino rats (45–55 days old, Animal House of The Chinese University of Hong Kong, weight ranging 200 to 250 g, were used and kept under 12-hour light (at 46–55 ft-c from 6 AM to 6 PM) and dark (from 6 PM to 6 AM) cycles. A pneumotachygraph (Model 30; Mentor, Norwalk, MA) was calibrated on a rat eye with hydrostatic pressure generated by adjusting the height of a saline column. The hydrostatic pressure was monitored by a computerized pressure transducer unit consisted of a pressure transducer (model ML1050), bridge amplifier (model ML110), recorder (model ML201), and a personal computer with software (all from AD Instruments, Castle Hill, New South Wales, Australia). Awake IOP in rats was measured between 9 and 11 AM (during the light phase) throughout the study. Topical proparacaine was administered, and the probe was placed so that it touched the cornea gently along the visual axis. A reading was recorded when there was a standard deviation lower than 1.0 mm Hg among readings for 3 seconds. Three readings were taken in each eye and the mean was used for analysis.

The procedure by Ueda et al. was modified to induce elevation of IOP and loss of RGCs in rat retinas. Briefly, animals were anesthetized with intraperitoneal injections of 400 mg/kg chloral hydrate. Proparacaine was administered topically and 10 μL aqueous humor was withdrawn with a syringe (Hamilton, Reno, NV) through a 30-gauge needle. The same volume of autoclaved India ink was injected into the anterior chamber. The needle was kept in place for 30 seconds during withdrawal of aqueous humor and injection of India ink to ensure minimal leakage. Only the right eye was injected with India ink; the contralateral eye served as the normal control. Prophylactic tobramycin-dexamethasone ointment (0.3%; Alcon, Puurs, Belgium) was applied, and the animals were allowed to recover.

At 7 days after injection of ink, 200 laser spots were delivered to the black band (ink deposit) on the trabecular meshwork around the limbus of anesthetized animals, with an argon laser delivery system (620–647 nm; spot size of 200 μm, 200 mW, 0.2 seconds; Novus Omni laser; Coherent, Palo Alto, CA). To eliminate the effects resulting from the procedures of intracameral injection and/or laser photocoagulation and possible retinal damage by the laser photocoagulation, two groups of control animals were used in addition to the untouched contralateral eyes, which served as the normal control: (1) eyes with India ink injection but without laser photocoagulation and (2) eyes with India ink injection followed by laser photocoagulation at midiris instead of trabecular laser photocoagulation, at 7 days after ink injection. All data from these three control groups (normal, ink injected only, and ink injected plus iris laser photocoagulation) were collected to compare with those obtained from eyes with elevated IOP after trabecular laser photocoagulation and injection of India ink. Animals with cataract or possible retinal damage by the laser photocoagulation, two groups

**RESULTS**

Compared with the normal control eyes, IOP at 1, 5, and 7 days after trabecular laser photocoagulation showed significant elevation (P < 0.05; n = 61; ANOVA, Tukey’s test; Fig. 1A). The increase was up to 56% over the normal average value (13.37 ± 3.34 mm Hg vs. 8.55 ± 1.31 mm Hg) at day 3 after laser photocoagulation. By 14 days after laser treatment, IOP had returned to normal. Intracameral injection of India ink itself (Fig. 1A) or in combination with irido-laser photocoagulation did not cause any change in IOP (not shown).

In retinal sections obtained after trabecular laser photocoagulation, TUNEL-positive cells were noted in the RGCL of each retina counted by light microscopy in a masked fashion, and the data were expressed as number of TUNEL-positive nuclei per section.

**Western Blot Analysis for GFAP**

A previously described standard method was used for Western blot analysis for GFAP, using the same rat anti-GFAP antibody as described earlier.

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RGCL was modified. Cresyl violet stain was reliable for studying the quantitative changes of neurons and avoided the damage introduced by retrograde labeling after optic nerve transection. Counting was performed with an eyepiece reticle of a microscope at 400× magnification. Each area (superior, temporal, and inferior) of each retina was sampled in two regions: 1.5 mm (central) and 3.5 mm (peripheral) from the center of the ONH. Three microscopic fields (0.25 × 0.25 mm²) were sampled from each of the six resultant regions, for a total of 18 microscopic fields, representing approximately 3.0% to 3.8% of the flatmounted (half) retinal area (corresponding to 1.5%-1.9% of the total retinal area). Morphologically distinguishable glial cells and vascular endothelial cells were not counted. There was no attempt to distinguish the type of RGCs and amacrine cells, and the number of amacrine cells was assumed to be constant. Cells with cytoplasm rich in Nissl substance and with irregular outlines were counted and referred to as neurons.

**Immunohistochemistry**

The bisected eyeballs were postfixed in 10% neutral buffered formalin for 24 hours, processed, and embedded in paraffin. Paraffinized retinal sections (4 μm thick) with ONBs were selected for immunohistochemistry using the DAB detection method. The primary antibodies were rat polyclonal anti-GFAP (1.5 μg/mL; Calbiochem, La Jolla, CA), mouse polyclonal anti-vimentin (predilute; Serotec, Oxford, UK), rabbit polyclonal anti-iNOS (predilute; Serotec), mouse monoclonal anti-OX-42 (1:100; Pharmingen, San Diego, CA) and mouse monoclonal anti-ED1 (5 μg/mL; Serotec). Immunoreactivity was examined with light microscopy. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline or serum. Specimens from all control groups were included and compared with the experimental ones. For all immunohistochemical staining, a minimum of six eyes at each time point was graded by two independent examiners in a masked fashion (using a score of 1 to 5, with 5 being the strongest in immunoreactivity) and the scores were averaged.

**In Situ Labeling with TUNEL Method and Morphometry**

An apoptosis detection kit (ApopTag Peroxidase In Situ Apoptosis Detection; catalog number S7100; Intergen Co., Purchase, NY) was used according to the accompanying instructions and diamobenzidine (DAB) was used as color substrate. For quantitative analysis, the number of TUNEL-positive nuclei in the RGCL of each retina was counted by light microscopy in a masked fashion, and the data were expressed as number of TUNEL-positive nuclei per section.
retinal sections (n = 6 at each time point) showed a maximum at 5 days after laser treatment (Fig. 1C). No detectable TUNEL-positive cells were noted in sections from all control groups. Concomitant with the appearance of TUNEL-positive cells, cell counts in the RGCL with flat preparations of retinas showed a time-dependent loss of cells in the central (Fig. 1D; upper panel) and the peripheral (Fig. 1D; lower panel) retina after trabecular laser photocoagulation reaching a statistical significance at 3, 5, 7 and 14 days (P < 0.05). At 14 days, a decrease of 24% was registered in the central and the peripheral retinas. No significant loss of cells in the RGCL was noted at all time points in the two control groups: intracameral injection of India ink and irido-laser photocoagulation after ink injection (data not shown; n = 6 for each time point per control group).

Immunohistochemical study of these retinas showed an obvious increase (compared with normal retinas; Fig. 2A) in GFAP immunoreactivity in the inner limiting membrane (ILM) as early as 12 hours (Fig. 2B) after trabecular laser photocoagulation. Immunoreactivity of GFAP gradually increased and spread to the glial processes from the ILM to the outer retina, as seen at 7 and 14 days (Figs. 2F, 2G). The two control groups showed no changes in GFAP immunoreactivity in the retina at all time points (not shown). No remarkable changes in GFAP immunoreactivity were noted in the ONH at all time points, either in the experimental (Fig. 3) or control eyes. Western blot analysis of retinal proteins (Fig. 2H) showed an increase in band intensity corresponding to GFAP at 3 (lane 2) and 14 (lane 3) days after trabecular laser photocoagulation when compared with the normal (lane 1) and the ink-injected irido-laser-treated control (lane 4) confirming the immunohistochemical finding.

A substantial increase (compared with normal retina; Fig. 4A) in vimentin immunoreactivity was noted in the Müller cell bodies and their processes throughout the whole retina at 5 (Fig. 4B) and 5 days (not shown). In contrast to GFAP, by 7 or 14 days, vimentin immunoreactivity returned to near normal levels. S-100 immunoreactivity also showed a marked increase in some Müller cell bodies and their processes at 3 days (Fig. 4E). However, contrary to vimentin but similar to GFAP, S-100 immunoreactivity persisted at 5 days (Fig. 4F). In the ONH, similar to GFAP, no changes in vimentin and S-100 immunoreactivity were detected throughout the observation period (not shown). In the control eyes, there was no change in vimentin...
and S-100 immunoreactivity in the retina and the ONH at all time points examined (not shown).

There was no detectable ED1 immunoreactivity in the retina at all time points examined (Fig. 5A; retina at 14 days). Although there were absent or a few scattered ED1-positive cells in the choroid, ONH, and parapapillary region, of normal and control animals (Fig. 5D; ONH at 14 days after irido-laser photocoagulation), there was a gradual increase in the number of ED1-labeled cells in the choroid (Fig. 5B; choroid at 3 days), the ONH (Fig. 5C; ONH at 3 days), and parapapillary vessels from 12 hours to 14 days after trabecular laser photocoagulation (Table 1).

In contrast to ED1, there were scattered OX42-positive cells in the inner retina (Fig. 6A; 3 days) of trabecular laser-treated eyes. Between 3 and 14 days, the choroid (Fig. 6B; 3 days) and the ONHs (Fig. 6C; 3 days) of trabecular laser-treated eyes also had OX42 positive cells but not the normal and the control eyes (Fig. 6D; ONH at 14 days after irido-laser photocoagulation).

**DISCUSSION**

This study recorded the temporal sequence of the loss of cells from the RGCL and the macroglial and the phagocytic/microglial responses in the retina, the choroid, and the ONH of eyes exposed to a short-term (up to 7–12 days) mild to moderate elevation of IOP induced by laser photocoagulation to the trabecular meshwork after a single intracameral injection of India ink in rats. We noted a gradual loss of cells from the RGCL, with a significantly reduced number at 3 days and onward after laser photocoagulation. The three macroglial cell markers showed different patterns of change. GFAP immunoreactivity in the Müller cell end feet was increased as early as 12 hours after trabecular laser photocoagulation and remained elevated throughout the observation period, whereas S-100 immunoreactivity was increased in the Müller cell bodies at 3 and 14 days. In contrast, vimentin immunoreactivity showed only a transient increase in the Müller cell bodies and their processes between 3 and 5 days. However, no notable changes...
FIGURE 3. Immunohistochemical staining of ONH for GFAP. (A) Normal control and (B) 14 days after trabecular laser photocoagulation. Positive staining of GFAP was noted in the ONH (∗) and the ILM of the retina. No detectable increase in staining was noted in the ONH after trabecular laser photocoagulation when compared with the normal control. A substantial increase in staining in the IPL (arrows) and ONL (∗) was detected after trabecular laser photocoagulation. Arrowheads: level of the sclera.

FIGURE 4. (A–C) Immunolocalization of vimentin in retinas after trabecular laser photocoagulation. (A) Normal rat retina; retinas at (B) 3 and (C) 14 days after trabecular laser photocoagulation. A transient increase in immunoreactivity was noted in Müller cell bodies in the INL (B, arrows) and their processes in the ILM, IPL, INL, OPL, and ONL at 3 and 5 days. (D–F) Immunolocalization of S-100 in retinas after trabecular laser photocoagulation. (D) Normal rat retina; retinas at (E) 3 and (F) 14 days after trabecular laser photocoagulation. S-100 immunoreactivity was increased in Müller cell bodies in the INL (arrows) at 3 (E) and 14 (F) days.
of these markers were observed in the ONH, whereas the phagocytic markers ED1 and OX42 showed sustained phagocytic/microglial responses in the choroid, the parapapillary vessels, and the ONH. These differential responses of increased macroglial changes in the retina versus increased microglial/phagocytic changes in the ONH and parapapillary vessels may represent a difference in the roles of these cells in the pathophysiology of the glaucomatous changes in IOP. The macroglia may have a major role in modulating the early changes in the retina, whereas the microglial/phagocytic cells may play a major role in modulating the early changes in the ONH.

Although our study focused on the early changes in glial markers after elevation of IOP, our findings shared some similarities with the report by Wang et al., which focused on chronic changes. These similarities include the absence of ED1 immunoreactivity and the increases in GFAP and OX42 immunoreactivity in the retina. These similarities suggest that the early glial changes after elevation of IOP in the retina may persist and that the early glial responses may play important roles in early as well as chronic phases in the pathophysiology of retinal changes in elevated IOP and probably in glaucoma. Unfortunately, their study did not mention any of these changes in the ONH, the choroid, or the parapapillary region.

GFAP is known to be a sensitive marker of astrocytic activation in response to injury in the CNS. Increased expression of GFAP has been found in astrocytes and Müller cells in retinas with various degenerative changes such as those in retinal ischemia, retinal detachment, diabetic retinopathy, and chronic experimental glaucoma in monkeys and rats. In addition, Varela and Hernandez showed an increased expression of GFAP in astrocytes at the prelaminar region and lamina cribrosa in ONHs in humans with open-angle glaucoma, whereas Johnson et al. demonstrated a transient decrease at 13 days after elevated IOP, followed by its recovery in the ONHs of experimental glaucoma rats. The latter findings were similar to those of optic nerve crush injury in rats. These changes in the ONH are believed to be related to the sites of nerve injury in glaucoma. In contrast, our study showed no detectable early changes at the ONHs. In Varela and Hernandez, the ONH tissues were collected from patients with glaucoma who were aged from 58 to 91 years, and most of them had diagnoses of advanced glaucomatous damage (cup-to-disc ratios in 9 of 15 patients were above 0.8). It is possible that a more chronic and extensive loss of RGCs and their axons, such as in those human specimens studied, is needed to validate a reactive astrogliosis in the ONH. The specific functions of GFAP in astroglial cells are not clear, but recent studies in transgenic and knockout mice and in vitro studies suggest a crucial role of GFAP in maintaining the blood-brain barrier, the glial differentiated state, and the number of processes and in the suppression of glial cell growth and proliferation. The sustained elevated GFAP levels we observed may represent the effort of these astroglial cells to maintain the retinal architecture and functions after an increase in IOP.

The differential variation in the changes of vimentin and S-100 (both are Müller cell markers) immunoreactivity—a transient increase in vimentin versus a persistent increase in S-100—suggest that these two proteins may play very different roles in the activated Müller cells after elevation of IOP. Increased expression of vimentin was reported to be associated with increased immunoreactivity of glutamine, an indicator of glutamate metabolism, in Müller cells of monkey eyes with experimental glaucoma. Whether this reaction may be beneficial or deleterious to the survival of RGCs remains controversial. It is suggested that Müller cells may increase their uptake of vitreous glutamate in glaucoma, which was reported to be increased in human and experimental glaucoma. The transiently increased expression of vimentin may therefore indicate the compensatory response of Müller cells in inhibiting the sudden increase in vitreous glutamate released from the degenerating RGCs after elevation of IOP. Other studies have linked vimentin to dedifferentiation of the glia and their proliferation. Activated glial cells have been reported to play a role in damaging the neurons in the CNS by releasing cytotoxic substances. The reduction of the glial source of nitric oxide by using a nitric oxide synthase inhibitor provided protection for RGCs in vitro. More recently, Tezel and Wax showed evidence that there was an increased amount of TNF-α and nitrite, a breakdown product of nitric oxide, in culture medium of incubating RGCs and astrocytes after ischemia-like insult or elevated hydrostatic pressure, and those substances were released from the activated glial cells. Neuroprotection under these noxious conditions by inhibition of TNF-α and nitric oxide synthase further suggests that reactive astrocytes may take part in neuronal damage in glaucoma.

**Table 1. Grading of ED1 Immunoreactivity after Trabecular Laser Photocoagulation**

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<th>Normal</th>
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<th>5</th>
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<th>14</th>
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<td>Choroid</td>
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<td>ONH</td>
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<td>Parapapillary vessels</td>
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There was an increasing number of ED1-labeled cells in the choroid and the ONH at 12 hours to 14 days after laser trabecular photocoagulation and a detectable number of ED1-labeled cells in parapapillary vessels from 3 to 7 days. Six specimens were examined for each time point. +, Absent or very mild; ++, mild; ++++, moderate; +++++, strong.
In the present study, we used an antibody that recognized both S-100A and S-100B, to examine the overall expression of S-100 protein, a calcium-binding guanylate cyclase activator, as a specific marker of retinal Müller cells in adult mammals. S-100 has not only been implicated in a variety of intracellular activities such as protein phosphorylation, enzyme activities, dynamics of cytoskeleton constituents, and protection from oxidative cell damage, but it is also known to be secreted into the extracellular space to exert trophic or toxic effects, such as chemotraction for leukocytes and regulation of macrophage activation. Nanomolar concentrations of brain extracellular S-100B may be beneficial, whereas micromolar levels of the protein may be deleterious and have been linked to Down syndrome and Alzheimer disease. The persistent increase in S-100 in the retina may represent a chronic response and change in cell behavior after an elevation of IOP. Its pathophysiological roles remain to be examined.

Although OX42 has been shown not only to exist in activated and phagocytic/microglial cells but also in resting microglia, it is not conclusive that OX42 is expressed exclusively in microglia. The ED1 antibody is directed against a single-chain glycoprotein that is present predominately on the lysosomal membrane of myeloid cells and hence is a marker for phagocytic cells. The persistent presence of these cells in the retina, choroid, and ONH suggests a major role in the pathophysiological responses to elevated IOP. However, determination of their exact roles necessitates further studies.

Enlarged and activated microglial cells were reported to appear in the parapapillary chorioretinal region in the ONHs of humans with glaucoma. Neufeld proposed that these microglial cells migrate from the disorganized prelaminar and laminar region of the ONH and may be responsible for the parapapillary chorioretinal atrophy seen in patients with glaucoma. This is supported by in vitro studies showing the release of cytotoxic factors such as free radicals, nitric oxide, proteases, excitatory amino acids, and cytokines by activated microglial cells. On the other hand, microglial cells may be neuroprotective to the axons of the optic nerve against the possibly compromised blood–brain barrier in glaucoma. However, contrary to Neufeld’s finding in humans, our study demonstrated clusters of phagocytic cells at parapapillary vessels and the ONH but not at the parapapillary chorioretinal region. Whether our findings represent the early changes, whereas those noted in humans represent chronic changes remains to be clarified. In addition, the protective or cytotoxic roles of microglial cells remain controversial.

References


23. Pena JDO, Taylor AW, Richard CS, Vidal I, Hernandez MR. Trans-...