Immunohistologic Evidence for Retinal Glial Cell Changes in Human Glaucoma

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PURPOSE. Glial cells are structurally and functionally linked to neuronal tissues. Pathologically, the cells may be activated and characterized by increased size and number and altered cellular properties. In glaucoma, pathologic mechanisms within the anterior optic nerve may include glial activation. This study examines morphologic changes of glial cells in the retinas of glaucomatous eyes compared with age-matched control retinas.

METHODS. Paraffin-processed or flatmounted retinas from 17 human donor eyes [7 normal (donor age, 87.5 ± 8.3 years) and 10 glaucomatous (donor age, 87.1 ± 6.9 years)] were examined. With immunohistochemical methods, retinal glial cells were stained with an antibody to glial fibrillary acidic protein (GFAP). The morphology of the glial cells in normal and glaucomatous retinas was evaluated with fluorescence microscopy.

RESULTS. Three types of glial cells were identified in flatmounted retinas with differing distributions in the peripapillary region, the nerve fiber layer (NFL), and along the capillaries. Compared with normal eyes, in glaucomatous retinas the glial cells in the peripapillary region showed an increase in density and exhibited a deformation of the end feet. The astrocytes distributed among the NFL showed little difference from normal. The astrocytes accompanying the capillary bed showed a redistribution in the glaucomatous retinas. The quantification of glial cells in paraffin-processed glaucomatous retinas exhibited a profound increase in density and a significant increase of GFAP immunoreactivity in contrast to the lightly stained glial cells in normal retinas.

CONCLUSIONS. The activation of glial cells in the glaucomatous retina was characterized in changes of intensity of GFAP immunoreactivity and morphology around the larger blood vessels, compared with age-matched normal retinas. The relationships between glial cells, neuronal cells, and the vasculature, as well as the potential role of glial cells in pathologic mechanisms during different stages of neuronal damage in glaucoma, are discussed. (Invest Ophthalmol Vis Sci. 2002;43:1088–1094)

Glial cells are important structural and functional components of the nervous system, including the optic nerve and retina. There are several types of glial cells in human ocular tissues. In the optic nerve, glial cells include astrocytes, oligodendrocytes, and microglia. In the retina, there are mainly Müller cells and astrocytes. Müller cells span radially across the retina, with their end feet forming the inner and outer limiting membranes. Astrocytes appear to originate, embryologically, in the optic nerve and subsequently migrate to their final location in the retina. Human retinal astrocytes can be morphologically divided into two major subgroups. One type of astrocyte is elongated, whereas the other is star shaped. Both types of astrocytes are distributed throughout the entire retina, except in avascular areas such as the foveal avascular zone, with lower densities found in the periphery. The processes of the elongated astrocytes form densely packed bundles that codistribute along with the ganglion cell axons in the nerve fiber layer (NFL), the star-shaped astrocytes form a honeycomb-shaped plexus that distributes slightly deeper than the former, around the level of the ganglion cell layer.

These basic anatomic features suggest that glial cells in the retina may be functionally correlated with neuronal tissues. Indeed, there is growing evidence that glial cells are integral components of and intimately involved in maintaining normal neuronal activity through bidirectional communication in the central nervous system (CNS). This system is believed to have considerable plasticity and a wide range of properties, such as production of neurotrophic factors, provision of basic metabolic support for neurons, regulation of the local concentration of potentially neurotoxic molecules, and modulation of the ionic balance of the extracellular space surrounding neurons. Many of these important glial cell functions have been demonstrated in the retina as well.

Glucomatous optic neuropathy is characterized by a progressive loss of retinal ganglion cells associated with visual functional deficits. As the ganglion cells die in glaucoma, there is a progressive thinning of the NFL. The underlying pathophysiologic mechanisms, however, remain unclear. Many mechanisms are involved in the initiation and process of irreversible ganglion cell death in glaucoma. These include reduced axoplasmic transport, accumulation of toxic levels of neurotransmitter, increased nitric oxide and endothelin synthesis, and remodeling of the extracellular matrix, among others.

Increasingly, glial cells in the CNS have been cited as participants in the pathologic course of neuronal damage after mechanical, ischemic, and various other insults. The mechanisms by which glial cells are involved in CNS neuronal damage may vary, but many of the mechanisms are surprisingly similar to those proposed in glaucoma. Glial cell activation is a hallmark of CNS injury, characterized by an increase in size and number of glial cells and upregulation of glial fibrillary acidic protein (GFAP), with additional cellular changes that may cause or relieve neuronal impairment. Such activation of glial cells has been demonstrated in the glaucomatous optic nerve and in retinas in glaucoma secondary to anterior segment tumors, and in experimental glaucoma models.

These potential associations between glial cells and neuronal tissues in health and disease have lead to new hypotheses that the retinal glial cells may also be involved in ganglion cell dysfunction in diseases such as primary glaucoma. To date, the status of retinal astrocytes and Müller cells in human glaucoma has not been studied in detail. In this study, the morphologic patterns and the activation status of retinal glial cells are examined with immunohistochemical methods in human eyes of...
donors with a clinical history of glaucoma and in age-matched control donor eyes.

**Materials and Methods**

**Donors**

Seventeen eyes from 7 normal donors (four male and three female) and 10 donors with glaucoma (six male and four female) were obtained from the local branch of the Lions Eye Bank (Lions Sight & Hearing Foundation, Portland, OR) and included in this study. One eye from each individual was chosen randomly. The average (±SD) age of the normal subject group was 87.3 ± 8.5 years and of the glaucoma group was 87.1 ± 6.9 years. Clinical history was obtained from the most recent available records. Nine donors had primary open-angle glaucoma (POAG) and one had normal-tension glaucoma. The range of disease duration (i.e., from initial diagnosis) was 2.5 to 10 years. Visual field damage varied among the donors (−5 to −18 dB). Visual acuity in this group ranged from 20/30 to 20/100. Clinical cup-to-disc ratio ranged from 0.4 to 0.9. Highest recorded intraocular pressure ranged from 22 to 30 mm Hg (treated). Donor eyes with diabetic retinopathy, retinal detachment, or other diseases that may affect the retina, were excluded from the study.

**Tissue Preparation**

All eyes were fixed in 4% formaldehyde-paraformaldehyde within approximately 3 hours after enucleation. The eyes used for paraffin sections were cut into 6-μm sections. Four retinas were dissected from the eye cups, flatmounted, and used for floating stain with immunohistochemistry. After thoroughly removing the vitreous body, the retinas were stored in 0.1 M phosphate-buffered saline (PBS).

**GFAP Immunohistochemistry**

**Avidin-Biotin Method.** Paraffin-embedded sections were used for this method. The sections were dewaxed, put in 0.3% H2O2 in methanol for 30 minutes for antigen retrieval, and incubated with 1% horse serum and 1% bovine serum albumin mixture for 30 minutes to reduce nonspecific binding. Monoclonal mouse anti-human primary antibody to GFAP (Novocastra Laboratories, Ltd., Newcastle-upon-Tyne, UK), was applied at a concentration of 1:200 and incubated at room temperature for 90 minutes. After three washes in 0.01 M PBS for 10 minutes each, biotinylated horse anti-mouse secondary antibody (1:100, Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA) was applied for 30 minutes and followed by avidin-biotin complex (1:200, Vector Laboratories, Inc.) was incubated for another 12 to 24 hours at 4°C to form the complex. Monoclonal mouse anti-human primary antibody to GFAP (Novocastra Laboratories, Ltd., Newcastle-upon-Tyne, UK), was applied at a concentration of 1:200 and incubated at room temperature for 90 minutes. After three washes in 0.01 M PBS for 10 minutes each, biotinylated horse anti-mouse secondary antibody (1:100, Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA) was applied for 30 minutes and followed by avidin-biotin complex for another 30 minutes. 3,3′-Diaminobenzidine (DAB kit; Vector Laboratories, Inc.) was used for substrate chromogen staining for 2 to 5 minutes. The sections were counterstained with 0.1% Mayer hematoxylin (Sigma Diagnostics, St. Louis, MO) and mounted.

**Fluorescence Immunohistochemistry on Flatmounted Retina.** For flatmounted preparations, either the whole retina (one normal and three glaucomatous eyes) or a wedge with the vertex at the optic nerve (three normal and four glaucomatous) was used. The peripheral area of the retina was not measured, because this area was too small for fluorescent or confocal microscopy. Three areas in each flatmounted section were used for the statistical method and borderline for the acceptance of the statistical hypothesis was used to evaluate the difference of the counts between normal and glaucomatous eyes.

**Quantification of GFAP and Astrocytes**

The intensity of GFAP immunoreactivity (GFAP-IR) integrated with the area of the GFAP-IR was quantified with an imaging system (Bioquant; R&M Biometrics, Inc., Nashville, TN). Two areas in a paraffin-embedded section of each eye (three normal and four glaucomatous) at approximately 2 mm and 6 to 8 mm from the optic disc were measured. The peripheral area of the retina was not measured, because this fraction was absent in many of the sections. Each image was 315 μm long with a width of the full thickness of the NFL. The images under the light microscope at ×20 were stored digitally in a computer. After a background correction, an arbitrary intensity value representing the GFAP-IR integrated with the corresponding stained area was generated with the image analysis system.

**Results**

**Characteristics and Distribution of Astrocytes in the Normal Retina**

**Paraffin-Embedded Tissues.** In paraffin-embedded control retinas, very few astrocytes were faintly stained with GFAP. In transverse sections, these weakly stained astrocytes were found only in the NFL and ganglion cell layer (Fig. 1A). No GFAP-IR Müller cells were observed in control eyes. In the anterior optic nerve, similar to the remaining retina, GFAP-IR was weak in the NFL, with only a slight increase in the prelaminar region (Fig. 2A). In contrast, astrocytes with stronger GFAP-IR were observed in the laminar and retrolaminar regions of control eyes.

**Flatmounted Retinas.** Unlike paraffin-embedded normal retinas in which astrocytes stained only weakly with GFAP-IR, most, if not all, astrocytes showed GFAP-IR in the flatmounted normal retinas. These astrocytes were of two types, according to their shapes: star-shaped and elongated, as has been described previously. In addition, another group of GFAP-IR glial cells was observed in normal retinas in the present study. They were distributed near the inner limiting membrane, close to the surface of the peripapillary retina (central area). Unlike the star-shaped and elongated astrocytes, the slim processes of these cells extended randomly from the cell bodies and had
little contact with each other (Fig. 3A). The distribution of these cells was limited to the area adjacent to the optic disc corresponding approximately to the area of radial peripapillary capillaries.

As previously published, the elongated astrocytes were distributed in the NFL mostly in the peripapillary and midperipheral retina. This type of astrocyte had two major processes that paralleled the course of ganglion cell axons and also accompanied radial peripapillary capillaries.

Star-shaped astrocytes were located at approximately the level of the ganglion cell layer, distal to the elongated astrocytes (Figs. 3C, 3E). The star-shaped astrocytes were more numerous at midperipheral and peripheral eccentricities than in the peripapillary retina.

The star-shaped astrocytes and their processes formed a honeycomb pattern that closely followed the distribution of retinal blood vessels and the two layers of retinal capillaries. On the surface of the vessels, the processes of the star-shaped astrocytes terminated and divided further into numerous fibers and formed a sheath (Fig. 4).

**Glial Cell Changes in Glaucomatous Retina**

**Paraffin-Embedded Tissues.** In all glaucomatous retinas, stronger GFAP-IR within a larger area was observed in glial cells within the NFL and ganglion cell layer at all eccentricities. The integrated GFAP-IR intensities in the glaucomatous and normal eyes in the peripapillary area were 8656 ± 2710 (mean ± SD; \( n = 4 \)) and 603 ± 430 (\( n = 3 \)), respectively. The intensities in the midperipheral area were 1928 ± 1154 and 298 ± 193, respectively. The differences between the normal and the glaucomatous retinas in both the peripapillary and the midperipheral areas were statistically significant (\( P < 0.05 \), Mann-Whitney test).

The mean number of glial cells in the counted areas in the glaucomatous retinas (52 ± 29; \( n = 6 \)) was twice as many as that in normal retinas (25 ± 9, \( n = 4 \)). However, the difference was not statistically significant (\( P = 0.10 \)).

Figure 1B shows a section from an eye with POAG of 9 years' duration. The donor's history showed visual field loss of −13 dB (by the Humphrey 24-2 program; Humphrey Systems, Dublin, CA), visual acuity of 20/30, and maximum recorded intraocular pressure of 22 mm Hg (under treatment). Note that GFAP-IR was particularly strong around the blood vessels. Figure 2B shows the anterior optic nerve from the same glaucomatous eye as shown in Figure 1B. The same preparation from a control eye is shown in Figure 2A for comparison. Note that theNFL in the peripapillary area of the glaucomatous retina is thinned and that GFAP-IR is very strong throughout this area. In contrast, only the lamina and retrolaminar areas expressed GFAP-IR in control eyes (e.g., Fig. 2A). Similar changes were observed in the peripapillary region of all glaucomatous retinas prepared in this manner.

Müller cells that stained positively for GFAP were also observed in some glaucomatous retinas. However, there were fewer GFAP-IR Müller cells than GFAP-positive astrocytes in glaucomatous eyes. Figure 2 shows an example of GFAP-IR Müller cells in the peripapillary area of a glaucomatous retina. Characteristically, these cells spanned the retina from the inner

**FIGURE 1.** A normal (A) and glaucomatous (B) paraffin-embedded retina stained immunohistochemically with GFAP and counterstained with hematoxylin. The GFAP-IR glial cells stained brown. In the normal retina, only a few astrocytes were faintly stained with GFAP. In contrast, GFAP-IR in the glaucomatous retina increased significantly. Note also the Müller cells (arrows) and the stronger GFAP-IR in the astrocytes around the blood vessel (♦) in the glaucomatous retina.

**FIGURE 2.** Glial cells in the optic nerve and peripapillary area of a normal (A) and a glaucomatous (B) retina stained with GFAP. The astrocytes in the laminar region (LC) of normal and glaucomatous eyes were GFAP-IR positive. In the prelaminar (PL) and NFL in the glaucomatous eye, the GFAP-IR was much stronger than in the normal eye. Note that the Müller cells in the peripapillary area of the glaucomatous eye were also stained with GFAP (arrowhead).
The elongated astrocytes that were typically found in the normal eyes adjacent to the nerve fibers appeared to be normal in shape in the glaucomatous retinas. However, in at least one glaucomatous retina, there were far fewer of these astrocytes, which may reflect loss of retinal ganglion cell axons.

**Midperipheral Retina.** Compared with normal retinas (Fig. 3C), the GFAP-IR elongated astrocytes that ran parallel to the nerve fibers in glaucomatous retinas appeared decreased in density overall (Fig. 3D); however, in some individual astrocytic bundles, there was increased GFAP-IR (Fig. 3D, arrowheads). The star-shaped astrocytes appeared to be redistributed. Compared with the normal retinas, the density of the honeycomb-shaped net of astrocytes in capillary beds was decreased, whereas, astrocyte density along larger blood vessels seemed increased. The processes looked straighter, and the normal integrity of the network was more deteriorated than in normal retinas (Fig. 3C versus Fig. 3D).

**Peripheral Retina.** The distribution of elongated astrocytes in glaucomatous retinas was sparser than in normal retinas (e.g., Fig. 3F). The density of star-shaped astrocytes in the capillary beds between the larger blood vessels was also decreased. Similar to the midperiphery, the astrocyte processes on the surface of larger vessels viewed under the microscope appeared to be thicker, and GFAP-IR was stronger than in normal retinas (Fig. 5).

**DISCUSSION**

In the present study, a significant increase of GFAP-IR and a tendency toward glial cell proliferation were demonstrated in limiting membrane to the outer limiting membrane or the outer plexus.

**Flatmounted Retina.** Peripapillary Retina. The most dramatic changes were observed in glial cells on the surface of the retina in the peripapillary area of the glaucomatous eyes. By comparison with the glial cells in the corresponding area of the control retinas, GFAP-IR was much stronger in glaucomatous eyes. There appeared to be an increase in the number of cells and thickened processes. Figure 3B shows an example of a flatmounted retina (peripapillary region) of a POAG-affected eye with disease of only 3 year’s duration between diagnosis and the donor’s death. The age of the donor at death was 88 years, visual acuity was 20/25, cup-to-disc ratio was 0.4, and maximum recorded intraocular pressure was 30 mm Hg. Note the finding of a patch of lattice-shaped alterations at the processes (Fig. 3B, asterisk). These changes were most commonly observed along the large peripapillary vessels but in some eyes extended farther along the blood vessels into the midperipheral retina.

**FIGURE 3.** Astrocytes in the superior quadrant of flatmounted normal (A, C, E) and glaucomatous (B, D, F) retinas: peripapillary (A, B), midperipheral (C, D), and peripheral areas (E, F). In the innermost retinal layer of the peripapillary region, a group of astrocytes were distributed with slim and randomly arranged processes (A, top left). In the glaucomatous retina (B), these astrocytes showed strongly increased GFAP-IR in the thickened processes (arrowhead) and weblike deformed end feet (>). The elongated astrocytes (B, top left to bottom right) were located slightly deeper and are out of focus in this micrograph. In the midperipheral area of the normal retina (C), both elongated and star-shaped astrocytes were distributed uniformly and had equal GFAP-IR. The glaucomatous retina (D) showed enhanced GFAP-IR in the elongated astrocytes (arrowhead) and fewer star-shaped astrocytes. The processes of both types of astrocytes became straighter than in the normal retina (C). In the peripheral area, there were far fewer star-shaped astrocytes in glaucomatous (F) than in normal (E) retina. In both, there were fewer elongated astrocytes, possibly because there were fewer axons in the area. Note the strong GFAP-IR on the surface of a vessel in the glaucomatous retina (E, *, bottom right) in contrast to that indicated in the normal retina (E, arrowheads). Magnification, ×10.

**FIGURE 4.** The processes of the star-shaped astrocyte gave off numerous fine fibers on the surface of a blood vessel (V) and formed a thin layer of sheath in a normal flatmounted retina. Magnification, ×10.
Astrocytes stained with GFAP in flatmounted normal (A) and glaucomatous (B) retinas. In the glaucomatous retina, the intensity of GFAP-IR appeared stronger around the blood vessels (V, arrowheads), which agreed with the quantification of GFAP-IR in the paraffin-embedded sections.

Vascular disorders have been proposed as one of the important factors in the development of glaucomatous optic neuropathy. Although several studies have shown a high occurrence of hemodynamic circulatory disturbances in patients with glaucoma and direct evidence of pathologic changes in the blood vessels serving the optic nerve and retina has yet to be convincingly demonstrated. In the present study, the prominent changes in glial cells throughout most of the retina in eyes with a clinical history of glaucoma. Most of the GFAP-positive cells with morphologic changes were astrocytes. However, under pathologic conditions Müller cells, with nuclei normally located in the inner nuclear layer, as demonstrated in Figure 2, may migrate onto the surface of the retina. When considering the present results, it cannot be excluded that the glial cells with morphologic changes in the peripapillary region were migrating Müller cells.

In addition to maintaining structural stability and to helping in wound healing in neural tissues, glial cells are critical in maintenance of ionic balance, in neurotransmitter uptake, and in cell surface adhesion systems, among many other functions. In response to neural injury, glial cells may be activated. One important marker of activation is enhanced expression of GFAP, among many other molecular responses that have been linked to various types of neuronal damage in the CNS. These responses may include changes in membrane electrical properties and expression of ionic channels and currents, expression of glutamate receptors, activation of endothelin and endothelin receptor systems, and changes in cell surface adhesion systems, among others. In glaucoma, glial cell function in the retina may be involved in many of these mechanisms.

Upregulation of GFAP in astrocytes of the optic nerve, accompanied by changes in the expression of different types of extracellular matrix proteins and increased nitric oxide synthase, has been demonstrated in human glaucoma. The present study demonstrated a significant increase in GFAP expression in glial cells in the retina. However, the functional correlates that may be associated with the increased GFAP-IR in glial cells of glaucomatous retina remain to be elucidated. Further, it is unclear what impact these glial cell changes have on the mechanisms of glaucomatous neural damage and during what stage of the disease process they occur. Reactive astrocytes in the retina may or may not perform the same role as those in the optic nerve, because the astrocytes may function differently due to regional heterogeneity. However, because retinal astrocytes originate from one type of astrocytes in the optic nerve in lower animals, the astrocytes in the two locations may share common properties in normal and disease states. It should be noted that although immunohistochemical staining of GFAP has been used as a specific marker to identify glial cells in the retina, the quantity of the staining may vary, depending on the type of cells and their status of activation. Additional factors affecting the staining may include species differences, antibodies used, and types of fixatives used.

The hypothesis that retinal glial cells are involved in neural damage is also supported by animal models and in vitro experiments that are designed to mimic possible glaucomatous pathogenic factors. For example, in response to raised intraocular pressure, ischemia, or other insults, retinal astrocytes and Müller cells highly express GFAP and alter neurotrophic factor expression. Vascular disorders have been proposed as one of the important factors in the development of glaucomatous optic neuropathy. Although several studies have shown a high occurrence of hemodynamic circulatory disturbances in patients with glaucoma and direct evidence of pathologic changes in the blood vessels serving the optic nerve and retina has yet to be convincingly demonstrated.

In previous studies, investigators have reported morphologic changes and increased GFAP-IR in astrocytes in the optic nerve in human POAG and in retina in secondary glaucoma. Changes in glial cells have been noted as well in animals with experimental glaucoma. The present results demonstrated a profound morphologic change, significant increase of GFAP-IR, and potential glial cell proliferation throughout most of the retina in eyes with a clinical history of glaucoma. Most of the GFAP-positive cells with morphologic changes were astrocytes. However, under pathologic conditions Müller cells, with nuclei normally located in the inner nuclear layer, as demonstrated in Figure 2, may migrate onto the surface of the retina. When considering the present results, it cannot be excluded that the glial cells with morphologic changes in the peripapillary region were migrating Müller cells.

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growth factor\textsuperscript{61,62} and nitric oxide\textsuperscript{15,63} are present in the astrocytes. Alteration of the factors in astrocytes has been demonstrated and correlated to many vascular and nonvascular cerebral diseases\textsuperscript{41,64} and experimental neural insults.\textsuperscript{80,65} Based on studies of astrocytes in the retina\textsuperscript{66} and CNS, it may be speculated that the changes of glial cells around the blood vessels in the glaucomatous retinas, rather than vasculature changes per se, are a response to glaucomatous insults, which in turn are linked to neuronal damage. For example, in a separate study in our laboratory, using CD34 (an immunohistochemical marker for vascular endothelium), there was no qualitative difference in retinal capillary density between six pairs of age-matched normal and glaucomatous eyes (Wang et al., unpublished results, 2001).

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**References**