Astrocyte Processes Label for Filamentous Actin and Reorient Early Within the Optic Nerve Head in a Rat Glaucoma Model

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PURPOSE. To determine if astrocyte processes label for actin and to quantify the orientation of astrocytic processes within the optic nerve head (ONH) in a rat glaucoma model.

METHODS. Chronic intraocular pressure (IOP) elevation was produced by episcleral hypertonic saline injection and tissues were collected after 5 weeks. For comparison, eyes with optic nerve transection were collected at 2 weeks. Fellow eyes served as controls. Axonal degeneration in retrobulbar optic nerves was graded on a scale of 1 to 5. Optic nerve head sections ($n \geq 4$ eyes per group) were colabeled with phalloidin (actin marker) and antibodies to astrocytic glial fibrillary acidic protein and aquaporin 4, or axonal tubulin β III. Confocal microscopy and Fiji software were used to quantify the orientation of actin bundles.

RESULTS. Control ONHs showed stereotypically arranged actin bundles within astrocyte processes. Optic nerve head actin bundle orientation was nearly perpendicular to axons ($82.9^\circ \pm 6.3^\circ$ relative to axonal axis), unlike the retrobulbar optic nerve ($45.4^\circ \pm 28.7^\circ$, $P < 0.05$). With IOP elevation, ONH actin bundle orientation became less perpendicular to axons, even in eyes with no perceivable axonal injury (i.e., $38.8^\circ \pm 15.1^\circ$ in grade 1, $P < 0.05$ in comparison to control ONHs). With severe injury, ONH actin bundle orientation became more parallel to the axonal axis ($24.1^\circ \pm 28.4^\circ$, $P < 0.05$ in comparison to control ONHs). Optic nerve head actin bundle orientation in transected optic nerves was unchanged.

CONCLUSIONS. Actin labeling identifies fine astrocyte processes within the ONH. Optic nerve head astrocyte process reorientation occurs early in response to elevated IOP.

Keywords: astrocyte, optic nerve head, actin, intraocular pressure, glaucoma

Glaucoma is the leading cause of irreversible blindness in the world, involving axonal injury within the optic nerve head (ONH) and corresponding visual field deficits.^{1–4} While lowering intraocular pressure (IOP) is the mainstay of current treatment for glaucoma, many patients continue to lose vision despite apparently satisfactory pressure control.^{5,6} The inciting events, the cellular and molecular responses, and the final cause of axonal degeneration in glaucoma remain unclear. A better understanding of these will be crucial to the development of novel treatments that protect optic nerve fibers and augment the beneficial effects of reducing IOP in glaucoma.

Within the ONH, axons receive mechanical and biochemical support from astrocytes that ensheath and envelop axon bundles with numerous processes.⁷ Astrocyte processes extend radially from the cell body and terminate in end-feet at the pial surface of the optic nerve,⁸ coupling the meningeal vasculature to axons.⁹ In rodent models, astrocyte processes often span the entirety of the optic nerve diameter,^{10,11} allowing for contact with multiple axon bundles. Astrocytes are mechanosensitive to stretch,¹² and elevated IOP has been shown to activate astrocytes and cause various intracellular responses that may be incompatible with axonal support.^{13–15} These include changes in gene expression,^{16,17} retraction of astrocytic processes,¹⁸ and increased astrocyte migration.^{19,20} The astrocyte actin cytoskeleton is crucial to these changes in morphology,¹⁹ process length,¹⁹ and migration²¹; and more than 70 ONH actin

cytoskeletal genes are significantly altered with early glaucomatous injury in rodent models of glaucoma.^{16,17} Proteomic analysis of the optic nerve after IOP elevation has also demonstrated upregulation of actin cytoskeletal regulators in a nonhuman primate model of glaucoma (Zhang L, et al. *IOVS* 2014;55:ARVO E-Abstract 4519; Burgoyne CE, et al. *IOVS* 2014;55:ARVO E-Abstract 4555).

The actin cytoskeleton is controlled by extracellular cues, including alterations in tissue stress and strain,²² which are then amplified through cell surface receptors (i.e., integrins), small guanosine triphosphatases (GTPases), and kinases that modify downstream regulators of actin polymerization.²³ Indeed, modulation of the actin cytoskeleton via RhoA GTPase, the RhoA-dependent kinase ROCK, and the actin nucleator Arp2/3 has been shown to be critical to astrocyte process formation and morphology in culture.^{21,24} Protein levels of the actin nucleator Arp2/3 are also upregulated within the optic nerve after IOP elevation in a nonhuman primate model of glaucoma (Zhang L, et al. *IOVS* 2014;55:ARVO E-Abstract 4519). Thus, the ONH actin cytoskeleton is a strong candidate for providing a mechanistic link between elevated IOP, myriad astrocyte responses, and ultimately, axonal degeneration.

Understanding the structural and molecular mechanisms of actin cytoskeleton dynamics in astrocytes may lead to novel targets for stabilizing astrocytic processes, which may ultimately enhance axon survival. We hypothesized that astrocyte

processes are actin rich, that the orientation of actin-rich astrocytic processes within the ONH may be a sensitive marker for astrocyte response to elevated IOP, and that IOP elevation evokes early ONH actin cytoskeletal changes in astrocytes that precede axonal degeneration. To test these propositions, the source of actin bundles and the structural actin cytoskeletal changes within the optic nerve were measured in response to elevated IOP in a rat model of glaucoma.

MATERIALS AND METHODS

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all experimental methods were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee.

Glaucoma Model

Twenty-one 8-month-old Brown Norway rats (350–400 g) were housed in constant low-level light to reduce diurnal IOP fluctuations and underwent unilateral injection with hypertonic saline through episcleral veins to sclerose and obstruct aqueous outflow.²⁵ Controls included fellow uninjected eyes, as well as eyes from naïve (uninjected) animals. Intraocular pressure was measured in awake animals at least three times a week with a handheld tonometer (TonoLab; Icare Finland Oy, Espoo, Finland) and monitored until euthanasia. Tissues were collected after 5 weeks. For comparison, three additional animals underwent unilateral retrobulbar optic nerve transection without IOP elevation, followed by tissue collection after 2 weeks (Jia L, et al. *IOVS* 2005;46:ARVO E-Abstract 1251). Optic nerve injury was evaluated as previously described.^{26,27} Briefly, eyes were perfusion fixed with freshly prepared buffered 4% formaldehyde solution. The retrobulbar optic nerves were removed, postfixed in 5% glutaraldehyde, embedded in plastic, sectioned, and stained with toluidine blue (Electron Microscopy Sciences, Hatfield, PA, USA), followed by light microscopy grading on a scale of 1 (no axonal injury) to 5 (axonal degeneration involving the entire nerve area). Sections were graded by five masked observers, and the grades were averaged. We have previously determined that each unit of grade corresponds to approximately a 12% increase in the number of degenerating axons in the optic nerve.^{26,27}

Actin Labeling and Immunolabeling

Perfusion-fixed optic nerve tissue was cryopreserved in 30% sucrose, positioned for vertical longitudinal sectioning in optimal cutting temperature support medium, frozen in liquid nitrogen, and cryosectioned (5 μ m) onto glass slides. Tissue sections were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Actin bundles were labeled with tetramethylrhodamine (TRITC)-labeled phalloidin (Sigma-Aldrich Corp., St. Louis, MO, USA) at a concentration of 1 μ g/mL in PBS at room temperature for 30 minutes.²⁸ Sections underwent three cycles of wash with PBS at room temperature for 5 minutes. Tissue sections were colabeled with primary antibodies at 1:500 dilution in 1% BSA/PBS at 4°C overnight, using antibodies against astrocytic markers glial fibrillary acidic protein (GFAP, rabbit polyclonal; Dako, Carpinteria, CA, USA),²⁹ or aquaporin 4 (Aqp4, rabbit polyclonal; Sigma-Aldrich Corp.),³⁰ or axonal marker anti-tubulin β III (Tuj1, mouse monoclonal; Covance, Seattle, WA, USA)³¹ to delineate ONH astrocytes and axons, respectively. Sections were washed as above, followed by incubation with secondary fluorescent-labeled goat anti-mouse or goat anti-rabbit monoclonal

antibodies (Alexa 488; Life Technologies, Grand Island, NY, USA) at 1:500 dilution in 1% BSA/PBS at 4°C overnight. After washing as above, cell nuclei were stained with the mounting medium (Prolong Gold with 4',6-diamidino-2-phenylindole [DAPI]; Life Technologies).

Microscopy and Image Analysis

Confocal images were obtained with an Olympus FV1000 microscope (Center Valley, PA, USA), using an UplanFLN $\times$ 40/NA1.30 oil objective and $\times$ 3.8 optical zoom. Images were captured using FV10-ASW version 4.0 software (Olympus) with laser wavelength settings of 405, 488, and 559 nm at 1 μ m/slice. FIJI image analysis software (<http://fiji.sc/Fiji> [in the public domain]; an open source image processing package based on the National Institutes of Health software ImageJ, <http://imagej.nih.gov/ij/> [in the public domain]) was used to project a Z-stack of all acquired images per tissue section. The Directionality plug-in feature of FIJI³² was used to produce a quantitative histogram of the orientation and distribution of actin and axon bundles at different regions of the optic nerve, relative to the axonal axis and Bruch's membrane. In addition, the FIJI plug-in fitted the orientation output histograms using a Gaussian function. The axonal axis was used to establish the anterior-posterior (A-P) axis. Area under the curve (AUC) calculations were performed using Microsoft Excel (Redmond, WA, USA). Mean fluorescence pixel intensity was calculated using the Color Histogram plug-in feature of FIJI software. Statistical analysis was performed by analysis of variance (ANOVA; GraphPad Software, La Jolla, CA, USA).

RESULTS

Astrocytic Processes Label for Actin and Are Stereotypically Arranged Within the Normal ONH

We first determined the architecture and source of the actin cytoskeleton in normal, naïve rat optic nerves. Low-magnification imaging of optic nerve demonstrated intense actin labeling of the ONH region of the nerve relative to the posterior, myelinated optic nerve (Fig. 1A). High-magnification actin labeling of ONH tissue demonstrated nearly linear actin bundles, which were arranged in a stereotypical fashion, perpendicular to the longitudinal axis of axons and the A-P axis of the rat eye (Fig. 1B). In contrast to the ONH, the arrangement of the actin cytoskeleton in the posterior, myelinated optic nerve appeared more random (Fig. 1C). As the orientation of actin bundles within the ONH appeared to follow previously described orientation of astrocyte processes,^{10,18} and as phalloidin did not significantly label axonal actin bundles (Figs. 1B, 1C), we asked if astrocytes were the primary source of the observed ONH actin cytoskeleton. Within the ONH, actin bundles localized parallel to known astrocytic markers GFAP and Aqp4 positivity, suggesting that actin-rich astrocytic processes are the dominant source of ONH actin bundles (Figs. 1D, 1E). However, actin labeling with phalloidin appeared to delineate astrocytic processes with higher sensitivity, as more processes were defined by phalloidin labeling relative to GFAP or Aqp4 labeling (Figs. 1D, 1E).

Orientation Analysis of Astrocytic Processes Within Normal ONHs

Given the qualitative difference between astrocyte actin bundle orientation within the ONH and the myelinated optic nerve, we quantified the orientation of actin bundles in both regions of the normal, naïve optic nerve using FIJI image

FIGURE 1. Actin bundle orientation in normal rat optic nerves. (A) Optic nerve, including optic nerve head (ONH) and the myelinated optic nerve, labeled with TRITC-phalloidin (actin bundle marker), Tuj1 (axonal tubulin marker), and DAPI. (B) Optic nerve head at the level of Bruch's membrane labeled with TRITC-phalloidin, Tuj1, and DAPI. Note that actin bundle orientation is perpendicular to the A-P and the axonal axis. (C) The posterior, myelinated optic nerve, labeled with TRITC-phalloidin, Tuj1, and DAPI, indicating less stereotypically arranged actin bundles. (D, E) The ONH at the level of Bruch's membrane, labeled with TRITC-phalloidin, astrocyte marker glial fibrillary acidic protein (GFAP) or aquaporin 4 (Aqp4), and DAPI. Note that actin labeling delineates a larger portion of the astrocyte processes than either GFAP or Aqp4. A-P, anterior-posterior.

analysis software. Optic nerve actin bundle orientation within individual ONHs was nearly perpendicular to axons, with a relatively narrow distribution of orientation (Fig. 2A). In contrast, actin bundle orientation within the myelinated optic nerve was less stereotypic, with a relatively broader distribution of orientation (Fig. 2B). In a group of normal eyes ($n = 4$ animals), actin bundle orientation within the anterior ONH was significantly more ordered and stereotypic ($82.9^\circ \pm 6.3^\circ$ relative to axonal axis), in contrast to the myelinated optic

nerve ($45.4^\circ \pm 28.7^\circ$, $P < 0.05$ in comparison to the ONH) (Fig. 2C).

We next determined the anatomical location of the transition between the actin bundle orientation within the ONH and the myelinated optic nerve. Actin bundle orientation was measured at various locations along the optic nerve as a function of distance from Bruch's membrane. The transition between actin bundle orientation within the ONH and the myelinated optic nerve occurred at approximately 250 μm from Bruch's membrane, which corresponded to the transition zone between the unmyelinated ONH and the posterior, myelinated optic nerve (Figs. 2C, 2D).

ONH Astrocytic Processes Reorient in Response to Elevated IOP

As ONH astrocytes have been observed to retract with IOP elevation,¹⁸ we asked if the stereotypical orientation of ONH astrocyte actin bundles is altered by IOP elevation in a chronic model of glaucoma. Optic nerve head astrocytic process orientation became highly disorganized in response to experimentally elevated IOP, even in eyes with little or no perceivable axonal injury (Fig. 3A, grade 1 and grade 1.17). In eyes with significant axonal injury after IOP elevation, ONH actin bundles appeared thicker and assumed a highly ordered arrangement along the A-P axis (Fig. 3A, grade 3.73; compare to Fig. 1B). In contrast, in eyes that underwent retrobulbar transection (grade 5 axonal injury) without IOP elevation, ONH astrocyte process orientation was not significantly changed from fellow or naïve eyes (Fig. 3A, transected). Increasing axonal injury was noted in glaucoma model eyes with increasing injury grades above 1, as well as in eyes with transected optic nerves, as demonstrated by reduced tubulin staining (Fig. 3A). Thus, astrocytic processes reorient specifically in response to IOP elevation, and not simply due to axon loss as illustrated by the lack of similar changes in ONHs after optic nerve transection.

To assess the response of previously described markers of astrocytes to IOP elevation and axon injury, actin and GFAP or Aqp4 colabeling was performed in glaucoma model eyes and eyes with optic nerve transection. The labeling pattern of GFAP within ONH astrocytes in grade 1 injured eyes appeared similar to that of controls (Fig. 3B, grade 1; compare to Fig. 1D). However, with increasing injury grade, GFAP labeling became less pronounced and less consistent (Fig. 3B, grade 1.17 and grade 3.73). Optic nerve head transection did not qualitatively alter GFAP labeling pattern (Fig. 3B, transected). The labeling pattern of Aqp4 within ONH astrocytes in grade 1 injured eyes appeared less linear and more punctate relative to controls (Fig. 3C, grade 1; compare to Fig. 1E). In glaucoma model and optic nerve transection eyes with notable axon loss, Aqp4 labeling became less pronounced (Fig. 3B, grade 1.17, grade 3.73, and transected; compare to Fig. 1E).

Astrocytic Process Reorientation in Response to Elevated IOP Is Specific for the ONH and Occurs Prior to Axonal Injury

We quantitatively assessed the orientation of optic nerve astrocyte processes as a function of axonal injury grade after experimental IOP elevation. The IOP histories after episcleral venous injection are listed in the Table. With IOP elevation but no observable axonal injury (grade 1), ONH actin bundle orientation distribution became left-shifted with a wider distribution, and the corresponding AUC became significantly reduced relative to controls (Figs. 4A–C). In eyes with moderate ($1 < \text{grade} < 3$) axonal injury, ONH actin bundle orientation

FIGURE 2. Actin bundle orientation analysis in normal rat optic nerves. (A) Fiji Directionality software analysis of actin orientation within the anterior optic nerve head (ONH) adjacent to Bruch's membrane, relative to axonal orientation for image in Figure 1B. Mean actin orientation is strongly perpendicular to axon bundle orientation ($82.9^{\circ} \pm 6.3^{\circ}$ relative to axonal and A-P axis). Best-fit Gaussian curves are included as *solid lines*. (B) Fiji Directionality software analysis of posterior, myelinated optic nerve actin bundle orientation relative to axonal orientation for image in Figure 1C. Mean actin orientation is less stereotypic with a broader distribution ($45.4^{\circ} \pm 28.7^{\circ}$ relative to axonal and A-P axis). Best-fit Gaussian curves are included as *solid lines*. (C) Actin bundle and axonal orientation in the optic nerve as a function of distance from Bruch's membrane. $N = 4$ animals in each group. $*P < 0.05$ by ANOVA; indicates statistically significant difference from bundle orientation at 0- to 100- μ m levels. (D) Simple schematic of actin bundle orientation within the ONH and myelinated optic nerve, relative to axonal orientation.

distribution became further left-shifted with a wider distribution, and the corresponding AUC became further reduced relative to controls (Figs. 4A-C). In eyes with severe ($3 < \text{grade} < 5$) axonal injury, ONH actin bundle orientation became significantly more parallel to the A-P axis, but maintained a similar AUC, relative to controls (Figs. 4A-C). Actin bundle orientation in the retrobulbar optic nerve was not significantly altered despite IOP elevation for

any grade of axonal injury (Fig. 4C). Optic nerve head and retrobulbar optic nerve actin bundle orientation and AUC in transected optic nerves without IOP elevation (with grade 5 axonal injury) were unchanged from normal ONHs (Figs. 4A-C). When plotted against mean and peak IOP measurements in the glaucoma model eyes, ONH actin bundle orientation was clearly less stereotypic with elevated IOP itself, irrespective of axonal

FIGURE 3. Optic nerve head (ONH) actin, GFAP, and Aqp4 rearrangement in response to elevated intraocular pressure (IOP). (A) Optic nerve head at the level of Bruch's membrane labeled with TRITC-phalloidin (actin marker), Tuj1 (axonal tubulin marker), and DAPI in the setting of elevated IOP and various axonal injury grades. Note the rearrangement in actin bundle orientation compared to control in Figure 1B. Optic nerve head after optic nerve transection, without IOP elevation, shows no observable change in actin bundle orientation compared to control in Figure 1A. (B) Optic nerve head at the level of Bruch's membrane labeled with TRITC-phalloidin, GFAP (astrocyte marker), and DAPI in the setting of elevated IOP and various axonal injury grades. (C) Optic nerve head at the level of Bruch's membrane labeled with TRITC-phalloidin, Aqp4 (astrocyte marker), and DAPI in the setting of elevated IOP and various axonal injury grades. A-P, anterior-posterior.

TABLE. Intraocular Pressure Histories of Rat Eyes in a Chronic Model of Glaucoma

Injury Grade	<i>n</i>	IOP _{Mean} ± SD, mm Hg	IOP _{Max} ± SD, mm Hg
Control	6	21.4 ± 1.4	28.3 ± 2.8
1	4	24.6 ± 2.4	43.0 ± 6.6
1 < grade < 3	7	24.7 ± 5.5	44.3 ± 8.9
3 < grade < 5	10	28.2 ± 4.9	48.8 ± 7.4

n, number of experimental animals; SD, standard deviation.

injury grade (Fig. 4D). Thus, ONH astrocytic processes reorient early in response to IOP elevation, prior to observable axonal injury, and specifically at the anatomical site of axonal injury (i.e., within the ONH). While a qualitative reduction in the actin-labeled fluorescence intensity was noted in ONHs with moderate injury (1 < grade < 3), no statistically significant change in ONH actin fluorescence intensity was noted in glaucoma eyes and optic nerve transected eyes, relative to control eyes (Fig. 4E). Thus, over- or undersampling of actin bundle orientation in the glaucoma eyes relative to control eyes using Fiji software is unlikely to be a significant factor.

DISCUSSION

Our results suggest that actin labeling with phalloidin highlights fine astrocytic processes not necessarily reflected by traditional astrocyte markers GFAP or Aqp4. This may be explained by a number of reasons. First, GFAP is an intermediate filament marker with an expression pattern that is highly dependent on the developmental stage of the tissue.³³ Second, the cellular distribution of GFAP is restricted and does not occupy the entirety of the cell.³⁴ Third, Aqp4 is a water channel that localizes to the end-feet of astrocytic junctions with pial blood vessels, which are not found throughout the entirety of astrocyte processes. Lastly, GFAP and Aqp4 expression levels have been shown to vary significantly with tissue injury.^{35–40} Indeed, in our study, GFAP and Aqp4 labeling did not fully represent all actin-labeled astrocytic extensions in normal ONHs (Figs. 1D, 1E). In addition, the labeling pattern of GFAP and Aqp4 with early glaucoma injury became sparse (Figs. 3B, 3C), with poor identification of actin-rich processes. Thus, astrocyte process delineation using actin bundle labeling may be more consistent and sensitive than GFAP or Aqp4 labeling.

Our results indicate that in the normal rat optic nerve, actin-rich astrocyte processes are oriented stereotypically within the ONH, essentially perpendicular to the axonal (and A-P) axis. This is in agreement with the observed morphology of ONH astrocytes using non-actin-based labeling.^{10,41–43} We further show that actin-rich astrocyte processes are more randomly and widely oriented in the posterior optic nerve, starting at approximately 250 μm posterior to Bruch's membrane. Using intracellular injection of dyes within intact tissue, Butt et al.⁸ previously described the morphology of rat astrocytes within the posterior optic nerve and found that the majority (approximately 60%) of astrocytes were randomly oriented, consistent with the findings in this study. While prior techniques using individual green fluorescent protein-expressing astrocytes (Lye-Barthel MH, et al. *IOVS* 2011;52:ARVO E-Abstract 2672)^{10,11} and intracellular dye injection^{8,44,45} allow for excellent imaging and study of individual astrocytes, our results indicate a complementary, consistent, and sensitive method to detect the location and orientation of a large population of astrocytes within normal and injured tissue. One limitation of actin labeling to highlight astrocyte processes,

particularly in the posterior myelinated optic nerve (which is more rich in oligodendrocytes and oligodendrocyte precursor cells, relative to the ONH), is that multiple cell types may be contributing to the actin labeling signal. Although the Gaussian fit of the actin bundle orientation within the posterior myelinated optic nerve is a unimodal curve (Fig. 2B), the actual distribution of actin bundle orientation was clearly wider relative to the ONH (Fig. 2A). Nonparametric bootstrapping with dip testing⁴⁶ of the posterior myelinated actin bundle distribution in Figure 2B showed no strong evidence against unimodality. While no multimodal fit of the actin bundle distribution of the posterior optic nerve was achieved, the wide distribution of the actin bundle orientation may argue for a more random astrocyte process orientation and/or the presence of multiple cell types. Thus, colabeling for actin as well as specific cell makers may be more useful in the analysis of cellular structure in the posterior myelinated optic nerve.

The strikingly stereotypic arrangement of ONH astrocyte processes (as opposed to the more random distribution of astrocyte processes within the posterior optic nerve) may be due a combination of mechanical and functional factors. The load forces (mathematically described as stress and strain)⁴⁷ within the ONH may be unique. In eyes, IOP fluctuations induce radial, transverse force vectors within the scleral canal surrounding the ONH.^{1,48,49} In cultured cells, external mechanical forces conveyed through interactions between the extracellular matrix (ECM) and integrins induce rapid and localized alterations of the actin cytoskeleton and result in the reorientation of actin bundles in the direction of the force vector.^{22,50,51} Thus, actin-rich astrocyte processes, through interactions with the ECM, may sense and align along mechanical force vectors induced by physiologic IOP fluctuations within the normal ONH tissue. This alignment would produce a stereotypic arrangement of actin-based astrocyte processes within the ONH along the radial force vectors, as observed in this study.

With further distance from the ONH scleral opening, the ECM and astrocytes within the posterior optic nerve are less likely to experience force vectors from natural IOP fluctuations. This reduction in mechanical force vectors would allow astrocyte processes to orient based on nonmechanical requirements and may result in a more random orientation, as observed in this study. The function of astrocytes within the ONH may be unique relative to astrocytes in the posterior retrobulbar optic nerve. For example, astrocyte phagocytosis is predominantly observed in the myelination transition zone in the optic nerve.⁵² The unique astrocytic process orientation within the ONH may be the result of potentially different metabolic needs of the unmyelinated axons in this region. If so, astrocyte process orientation perpendicular to the axonal axis would maximize the total number of axons any given astrocyte may be in contact with, thus enhancing the ability of astrocytic processes to support multiple axon bundles.

With chronic IOP elevation, we observed a dramatic alteration in the orientation of ONH astrocyte processes relative to fellow and naïve control eyes. In early-moderate glaucoma, ONH astrocyte processes became more random in orientation, even prior to observable axon degeneration. With moderate-late glaucoma, ONH astrocytes assumed a more ordered orientation along the axonal (A-P) axis, perpendicular to the orientation seen in control ONHs. With chronic IOP elevation, the longitudinal mechanical force vectors within the ONH may compete with the radial (i.e., transverse) mechanical force vectors in the ONH and sclera,^{1,53} which may explain the realignment of ONH astrocyte processes in experimental eyes. Alternatively, chronic IOP elevation may result in changes in the nonmechanical environment of astrocytes within the ONH, which may outweigh the mechanical and biological factors that

FIGURE 4. Analysis of optic nerve head (ONH) actin rearrangement in a rat model of glaucoma. **(A)** Fiji Directionality software analysis of actin bundle orientation distribution in ONHs as a function of axonal injury grade. **(B)** Mean area under the curve analysis of actin bundle orientation distribution curves as a function of axonal injury grade. **(C)** Mean ONH actin orientation as a function of axonal injury grade. Note that even prior to any observable axonal injury (grade 1), IOP elevation results in significant ONH actin bundle rearrangement. Actin bundle arrangement within the myelinated optic nerve is unchanged despite IOP elevation. Optic nerve transection does not significantly alter actin bundle orientation within the ONH or the myelinated optic nerve. **(D)** Mean ONH actin bundle orientation as a function of mean or peak IOP. **(E)** Mean ONH actin fluorescence intensity as a function of axonal injury grade. * $P < 0.05$ by ANOVA; indicates statistically significant difference between control and experimental groups. Number of animals in each x -axis group ([B, C, and E] left to right) = 6, 4, 7, 10, and 3, respectively.

necessitate astrocyte alignment perpendicular to the A-P axis. Optic nerve head astrocyte process orientation did not change after optic nerve transection, which induced severe axonal degeneration. Thus, axon degeneration alone in the absence of elevated IOP does not result in ONH astrocyte rearrangement.

In addition, astrocyte process orientation within the posterior optic nerve did not change despite IOP elevation or optic nerve transection. Therefore, alterations in ONH astrocyte process orientation are specific to IOP elevation and to the ONH, occur prior to perceivable axonal injury in the setting of

elevated IOP, and do not occur as a simple passive response to axonal death. Given these findings, ONH astrocyte process orientation appears to be a sensitive marker for astrocyte response to elevated IOP. While we did not find a significant change in ONH actin fluorescence intensity in the glaucoma eyes relative to control eyes, future studies using the Fiji Directionality software to determine bundle orientation should include this important control, as the software may be limited by inherent oversampling of brighter bundles.

Although we did not observe any significant difference in astrocytic ONH actin-rich process orientation between normal (naïve) eyes and fellow eyes of animals undergoing contralateral IOP elevation, retinal activation of retinal glial cells in fellow eyes of a unilateral laser-induced ocular hypertension rodent model,^{54,55} as well as a unilateral episcleral vein cauterization rodent model,⁴⁰ has been reported. While our model uses episcleral venous injection of hypertonic saline to induce chronic IOP elevation (which may cause less generalized inflammation), the observations from the laser-induced ocular hypertension model and the episcleral vein cauterization model may be important in future studies of the ipsilateral and contralateral effects of IOP elevation on molecular signaling, gene regulation, and inflammation using our model.

In addition to the morphologic changes of astrocytes within the ONH with IOP elevation, as reported here and elsewhere,¹⁸ significant retinal astrocyte structural changes have also been reported in glaucoma models. In a trabecular meshwork-targeted laser-induced rat glaucoma model, the area of the retina occupied by astrocytes was significantly reduced after IOP elevation.⁵⁴ Similarly, in an episcleral vein-targeted laser-induced rat glaucoma model, a significant reduction in the retinal astrocytes as judged by GFAP labeling was noted.⁵⁶ Thus, significant astrocyte structural changes occur in both the retina and ONH as a result of IOP elevation, which is likely to impact the capacity of astrocytes to provide support to ganglion cell axons.

In conclusion, ONH astrocyte response to elevated IOP has been proposed as a mechanism for axonal injury.^{2,13-15} Thus, identifying early astrocyte changes prior to axonal injury may provide clues to axonal susceptibility to injury. Here, we show one such early structural change in astrocyte process orientation that is specific to chronic IOP elevation and to the ONH. Rearrangement of ONH astrocyte process orientation in response to elevated IOP preceded any observable axonal injury, suggesting the possibility that such changes may be an early event in development of axonal injury. Our results are consistent with a mouse model of acute IOP elevation that resulted in changes in astrocyte process extension prior to axonal injury.¹⁸ Although our study does not show a causal relationship between ONH astrocyte process rearrangement and axonal degeneration, it does support a possible link between elevated IOP, structural astrocytic changes, and axonal loss. Future studies aimed at altering the ONH actin cytoskeleton (to stabilize or destabilize astrocyte actin processes), with and without IOP elevation, may elucidate any causal relationships between early astrocyte structural changes and eventual axonal injury. If a causal relationship is found, potential future modalities aimed at stabilizing the astrocytic architecture may lead to novel treatments for glaucomatous optic neuropathy.

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