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Altered F-actin Distribution in Retinal Nerve Fiber Layer of a Rat Model of Glaucoma

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Abstract

Glaucoma damages retinal nerve fiber layer (RNFL). The purpose of this study was to investigate the distribution in RNFL of axonal F-actin, a cytoskeletal component, under the development of glaucoma.

Intraocular hypertension was induced in a rat model by translimbal laser photocoagulation of the trabecular meshwork. The retinas of control and treated eyes were obtained after different exposures to elevated IOP. Nerve fiber bundles were identified by fluorescent phalloidin staining of F-actin. Nuclei of cell bodies were identified by DAPI fluorescent counterstain. F-actin distribution in whole-mounted retinas was examined by confocal microscopy. En face and cross-sectional images of RNFL were collected around the optic nerve head (ONH).

F-actin in normal RNFL was intensely and uniformly stained. In glaucomatous retina, F-actin staining was not uniform within bundles and total loss of F-actin staining was found in severely damaged areas. Altered F-actin often occurred near the ONH in bundles that appeared normal more peripherally. Both alteration and total loss of F-actin were found most often in dorsal retina.

In normal RNFL, F-actin is rich and approximately uniformly distributed within nerve fiber bundles. Elevated IOP changes F-actin distribution in RNFL. Topographic features of F-actin alteration suggest that F-actin near the ONH is more sensitive to glaucomatous damage. The alteration pattern also suggests an ONH location for the glaucomatous insult in this rat model.

Keywords

Retinal Nerve Fiber Layer; Animal Model of Glaucoma; Cytoskeleton; F-actin; Phalloidin Staining; Confocal Laser Scanning Microscopy

Introduction

Glaucoma is a progressive optic neuropathy. Glaucoma damage to retinal nerve fiber layer (RNFL) usually precedes detectable visual field loss and, therefore, direct assessment of RNFL is more sensitive to predict disease progression. Current clinical assessment of RNFL depends on the optical properties of the RNFL, such as the reflectance of RNFL detected by optical

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coherence tomography (OCT) and birefringence of RNFL measured by scanning laser polarimetry (SLP) (Bagga and Greenfield, 2004; Medeiros et al., 2007; Trick et al., 2006; Wollstein et al., 2005). The optical properties of the RNFL, in turn, depend on the cylindrical structure of axons, including cytoskeletal components (Huang and Knighton, 2005; Huang et al., 2006a; Knighton and Huang, 1999a; Knighton et al., 1998; Zhou and Knighton, 1997). Elucidation of the precise subcellular components of axons preferentially affected by glaucoma could lead to the design of diagnostic methods that are sensitive to the earliest structural changes, methods that may open a therapeutic window during which damage might be prevented.

The axonal cytoskeleton is made up of three major protein filaments: actin filaments (F-actin), intermediate filaments, and microtubules (MTs) (Darnell et al., 1990). They are responsible for maintaining cell shape, positioning cellular organelles and transporting intracellular proteins. F-actin and MTs closely coordinate in both function and structural organization (Fath and Lasek, 1988; Goode et al., 2000; Zhou et al., 2002). F-actin is assembled in two general types: bundles and networks. Bundled F-actin in axons is concentrated in the region of and runs parallel to MTs. It provides a substrate for MT transport and affects the structural organization of MTs. Neurofilaments (NFs), a type of intermediate filament, are the most abundant filaments along axons and, therefore, are frequently studied immunohistochemically to understand axonal damage mechanisms (Balaratnasingam et al., 2007; Hoffman et al., 1987; Jakobs et al., 2005; McKerracher et al., 1993; Vickers et al., 1995; Villegas-Perez et al., 1988). Such studies have shown regional loss of NFs in glaucoma (Jakobs et al., 2005; Vickers et al., 1995; Villegas-Perez et al., 1988). Microtubules within axons contribute significantly to the optical property of RNFL birefringence (Fortune et al., 2008; Huang and Knighton, 2005; Huang et al., 2006a; Knighton et al., 1998). Clinical assessment shows that glaucoma causes a decrease of RNFL birefringence, suggesting loss of MTs (Bagga et al., 2003; Greaney et al., 2002; Huang and Knighton, 2005; Medeiros et al., 2007). Because of the close coordination of F-actin and MTs, F-actin in RNFL also might be expected to change. Little is known, however, about the architecture of axonal F-actin in glaucoma (Hoffman et al., 1987).

Features of human glaucoma can be observed in animal models of glaucoma, making them a valuable tool to understand mechanisms of glaucomatous damage. Many animal models have shown spatially selective glaucomatous damage, although there is disagreement between models on the pattern of loss. For example, optic nerve axon counting in rat glaucoma models shows preferential axon loss in the dorsal/superior quadrants of the optic nerve (Johnson et al., 2000; Mabuchi et al., 2004; Morrison et al., 1997), whereas monkey and mouse models have shown loss of RGCs in peripheral retina (Filippopoulos et al., 2006; Garcia-Valenzuela et al., 1995; Reichstein et al., 2007; Sawada and Neufeld, 1999; Vickers et al., 1995). Spatially selective damage requires quantifying RNFL structure in the same retina over a large area. Although conventional histology is a powerful way to study axonal damage, it becomes extremely labor intensive when quantifying a large area of RNFL. Confocal laser scanning microscopy (cLSM) provides detection of fluorescence stained subcellular structures and is a useful tool to study RNFL distribution in a whole-mounted retina (Huang et al., 2006b; Jakobs et al., 2005; Villegas-Perez et al., 1988).

In the present study, we used phalloidin labeling of F-actin to study structural changes of the RNFL during the development of glaucoma in a rat model.

Methods and Materials

Rat Model of Glaucoma

Female Wistar rats weighting 250 – 350 g were used in this study. Animals were housed under a 12-hour light – 12-hour dark cycle with standard food and water provided ad libitum.

Experimental glaucoma was induced by translimbal laser photocoagulation of the trabecular meshwork (Levkovitch-Verbin et al., 2002; Ueda et al., 1998). Animals were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg) and topical proparacaine 1% eye drops. The laser treatment (a diode laser with wavelength of 532nm, 500mW power, 0.5 second duration, 50µm diameter spot size) was administered to the left eye of each rat. Around 55 – 60 trabecular burns were evenly distributed. The treatment was repeated after a week for all rats.

A Tonopen XL tonometer was used to monitor the intraocular pressure (IOP) after the animals were deeply anesthetized (Moore et al., 1993). The tonometer was held perpendicular to the center of the cornea. Ten valid measurements were obtained in both eyes; from these measurements, the mean was calculated. IOP was measured just before treatment and one, three, five and seven days after each treatment and then once a week until enucleation or the IOP returned to its baseline value. For each animal, a graph of IOP (IOP in mm Hg vs. days after treatment) for treated and fellow eyes was constructed. Cumulative IOP (cIOP), the area between the two curves in units of mmHg-days, was calculated (Levkovitch-Verbin et al., 2002).

All experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol for the use of animals was approved by the Animal Care and Use Committee of the University of Miami.

Retinal Tissue Preparation and Fluorescence Staining

After treated eyes were exposed to elevated IOP for a certain period of time (2 to 13 weeks), both eyes of each animal were taken and prepared for immunohistologic study. Tissue preparation followed previously developed procedures (Huang et al., 2006b; Knighton and Huang, 1999b). Briefly, an eye of an anesthetized animal was removed and the animal was euthanized. An eye cup of 5 mm diameter that included the optic nerve was excised with a razor blade. The excised tissue was placed in a dish of warm (33 – 35°C) oxygenated physiologic solution. The retina was dissected free of the retinal pigment epithelium and choroid with a fine glass probe and then draped across a slit in a membrane with the photoreceptor side against the membrane. A second, thinner membrane with a matching slit was put on the RNFL surface to gently stretch the retina and eliminate wrinkles. The mounted retina was fixed in 3% glutaraldehyde for 25 min at room temperature and rinsed thoroughly in phosphate buffered saline (PBS).

Orientation of retinas was documented by first marking the rostral side of the eye in situ with a skin marker and then, after dissection, cutting a notch into the eyecup and retinal edge at the marked position. The notch position relative to the pattern of retinal blood vessels subsequently provided orientation information in the confocal images.

Retinal nerve fiber bundles in whole-mounted retinas were identified by using phalloidin labeling specific for F-actin (Huang et al., 2006b). Ganglion cell bodies in the inner retina were identified by 4',6-Diamidino-2-phenylindole (DAPI) fluorescent counterstain of nuclei. To reduce autofluorescent background in the retinas fixed with glutaraldehyde, the tissue was incubated in freshly prepared 1% NaBH₄ for 20 minutes before the staining procedures. The tissue was permeabilized in PBS containing 0.4% TritonX-100 for 30 minutes followed by incubation in blocking serum (5% horse serum and 0.4% TritonX-100) for 60 minutes at room

temperature. The tissue was washed in PBS (three changes of ten minutes each) and transferred into a solution of phalloidin (diluted 1:100 in PBS, Alexa Fluor 488 Phalloidin, Molecular Probes) for 20 minutes at room temperature. After rinsing in PBS containing 0.2% TritonX-100, the tissue was incubated in DAPI (FluoroPure grade, Molecular Probes) for 20 minutes in subdued lighting. The stained retina was rinsed again and mounted in an imaging chamber (Coverwell™, Electron Microscopy Sciences) with PBS as a mounting medium. The retina was preserved at 4°C until confocal microscopy imaging.

Confocal laser scanning imaging

A cLSM (cLSM510 Carl Zeiss Microimaging, Inc.) was used to provide both en face and cross sectional images of the whole-mounted fluorescence stained retina. Detail of the method has been published (Huang et al., 2006b). Briefly, a 10× or 20× objective was used to take en face images providing a large view of the retina (1300 μm × 1300 μm or 460 μm × 460 μm) with the optic nerve head (ONH) centered in the images. A 40× water objective was then switched in and en face subimages were taken with a full field of view of 230 μm × 230 μm. At least eight subimages around the ONH, at approximately the same distance, were taken to cover most of the bundles that merged into the ONH (see, for example, Fig. 7 in Results). For each subimage, cross-sectional images were collected through the retina to a depth at least covering the ganglion cell layer. At least three cross-sectional images were collected at evenly spaced positions along nerve fiber bundles. The same confocal parameters were applied to all experiments. The z-scaling (Z step size) and axial resolution (optical slice size) were read from the system with no additional calibration. No deconvolution was applied to the images.

With the above imaging scheme, experiments in this study investigated glaucomatous change of RNFL identified by F-actin, located within a circular region centered on the ONH with the diameters of inner and outer circles approximately of 210 μm and 550 μm, respectively. F-actin staining was evaluated for each subimage and its cross-sectional images.

Results

Twelve Wistar rats were treated unilaterally and two rats were not treated with both eyes serving as controls. The baseline IOP (mean ± sd) before treatment and under general anesthesia was 26.7 ± 2.3 mm Hg ($n = 12$) and 26.0 ± 2.6 mm Hg ($n = 16$) for treated and control eyes, respectively. With the first laser treatment, the IOP of all treated eyes increased at least 4 mmHg from baseline IOP. However, the IOP of five eyes returned to normal after 4 – 5 days. After one week, the second treatment was applied to all eyes. The resultant IOP was elevated again to at least 6 mmHg higher than the baseline and remained elevated, but gradually decreased. Fig. 1 demonstrates the time courses of IOP in a treated eye and its control eye for one animal. For unknown reasons, the baseline IOP was about 6 mmHg higher than the values obtained in similar conditions by other groups (Levkovitch-Verbin et al., 2002). However, this difference does not affect the calculated values of cIOP.

Nerve fiber bundles were identified by phalloidin labeled F-actin as bright stripes in en face images (Fig. 2A). Blood vessels were distinguished from bundles by strong phalloidin staining on the blood vessel walls and/or as hollow structures. In cross sectional images, bundles lay just under the retinal surface and were separated from deeper layers by a single layer of nuclei with DAPI counterstain (Fig. 2B). Bundles were thick near the ONH and became thinner with distance away from the ONH. In normal RNFL, phalloidin staining was strong and uniform along bundles and across bundle cross sections. For the four retinas of the two control rats, all cross sectional images, with distances from the ONH center ranging from 210 μm to 510 μm, showed well-defined bundle boundaries and strong staining of F-actin. For ten control eyes of the 12 treated rats, the confocal images are similar to that of the normal control animals. Two control eyes, however, each had one subimage area that showed altered F-actin staining in the

cross sectional images near the ONH, which was similar to the images of treated eyes shown in Fig. 5A and B (see details below). These two cross sections were located at a distance of about 220 μm from the ONH and in the dorsal and ventral regions, respectively.

Structural organization of F-actin in RNFL was altered in ocular hypertensive eyes, as demonstrated in the severely changed retina of Fig. 3. Nerve fiber bundles were still discernible in the en face image (Fig. 3A), but phalloidin staining was much less dense within bundles than in normal retina. Other weak phalloidin staining formed a less-oriented network across the entire image. A closer look at bundles (Fig. 3B) revealed strands of F-actin with widths varying from 0.44 μm to 0.88 μm (2 to 4 pixels wide with a resolution of 0.22 $\mu\text{m}/\text{pixel}$) oriented along the bundle direction. In the cross sectional images (Fig. 3C), RNFL identified by phalloidin stain appeared as a thin layer of bundles that was still well separated from deeper layers. Abundant nuclei were distributed across the retina (Fig. 3A and B) and embedded in the RNFL near the retinal surface (Fig. 3C).

Elevated IOP also can cause total loss of F-actin in RNFL as shown in Fig. 4. Weakly stained F-actin distributed diffusely across the entire image formed a network without an apparent trace of nerve fiber bundles (Fig. 4A). The cross sectional images demonstrate no RNFL identified by F-actin, but do show a single layer of nuclei under the retinal surface.

In some retinas with cIOP around 85 mmHg-days, a cIOP lower than the two examples above, a change of F-actin in RNFL occurred in bundles near the ONH while more distally the same bundles retained normal appearance. Fig. 5B shows bundles that were well defined and uniformly stained for the portion away from the ONH (the middle and lower cross sectional images). For the same bundles closer to the ONH, however, F-actin staining was not uniform and the deep RNFL boundary was hardly distinguishable. Note also in the en face image (Fig. 5A) bundles near the top (closer to the ONH) were less intensely stained. Fig. 5C, D gives another example in which there were no clearly defined deep boundaries for the RNFL in the cross sectional images closest to the ONH (Fig. 5D, upper and middle images) while the RNFL boundaries were easily identified in a cross section 112 μm further along the bundles (Fig. 5D, lower image). In the en face image, diffusely distributed F-actin was also found between bundles. Compared with adjacent subimages of normal appearing bundles (not shown), F-actin staining in Fig. 5C was much less intense over the entire image.

The alteration in F-actin occurred significantly more often in the eyes with elevated IOP. In the 16 control eyes 96 cross sectional images near the ONH were evaluated and only two were found to have change in F-actin staining. In contrast, in a total of 84 cross sectional images of the 12 treated retinas 32 images showed F-actin alteration near the ONH. This difference tested by a chi-square test was significant with $p < 0.001$. Furthermore, the degree of F-actin alteration in treated eyes seemed related to the exposure to elevated IOP. Fig. 6 groups the 12 treated eyes based on the degree of F-actin alteration; the mean cIOP of each group is also displayed. Table 1 gives the mean of IOP in the 1st and 2nd weeks, i.e. mean IOP during the week after each laser treatment, for the treated and fellow control eyes of each group. Group 1 showed no apparent change of F-actin staining in any of imaged regions, each retina in Group 2 had one or two subimages located in dorsal retina that showed non-uniform staining or change of F-actin near the ONH, as shown in Fig. 5, and each retina in Group 3 had regions of severe change, as shown in the examples of Figs. 3 and 4. The mean cIOP between groups tested by a homoscedastic t-test was significantly different with $p = 0.02$ between Groups 1 and 2 and $p < 0.001$ between Groups 2 and 3.

In retinas with severe change, the approximate extent of change was quantified as illustrated in Fig. 7. Fig. 7A shows the entire retina around the ONH for one treated eye. Several high-power cLSM subimages have been superimposed on a low-power image to show their relation

to one another. Bundles in a fan-shaped dorsal region were much less dense and appeared as thin stripes in the en face images (Fig. 7A). The change was confirmed in cross-sectional images. Fig. 7B shows six cross-sectional images obtained at a distance of about 460 μm from the ONH center. The region of altered RNFL was identified as a layer of thin bundles that extended from the right end of image (b), through image (c) to the left end of image (d). In both rostral and caudal regions, however, bundles appeared normal and were well defined in both en face and cross sectional images [Fig. 7B, images (a) and (e)]. In this treated eye, a focal loss of F-actin was also found near the ventral retina [Fig. 7B, image (f)]. To quantify change, angles converging to the ONH that encompassed the altered bundles were determined (dashed lines in Fig. 7A) and expressed as a percentage of 360°.

In most retinas with severe alteration, the subimages adjacent to the severely changed region showed the pattern of differential nerve fiber bundle alteration illustrated in Fig. 5. In the retina with 90% F-actin change, only one ventral subimage had any normal appearing bundles, but these showed altered F-actin stain near the ONH. In the retinas with 50% and 60% change the area of severe alteration covered the dorsal half of the retina, flanked by two subimages that showed alteration near the ONH. In the retina with 20% change, nerve fiber bundles adjacent to the severe changed areas appeared intact over their observed length, but the sub-images in this retina were somewhat farther from the ONH than in others.

Discussion

F-actin, a filamentous protein polymer, is composed of globular-actin subunits. Phalloidin binds specifically at the interface between subunits, locking adjacent subunits together (Barden et al., 1987). Change identified by phalloidin binding, therefore, is associated with ultrastructural change of the F-actin polymer. Fluorescent phalloidin in saturating quantities provides a measure of the amount of F-actin in tissue (Cooper, 1987). Decreased phalloidin stain indicates loss or depolymerization of F-actin.

In normal retina, RNFL was found to be intensely stained with phalloidin, indicating that normal nerve fiber bundles are rich in F-actin. Uniform staining along and across nerve fiber bundles suggests an approximately uniform distribution of F-actin within bundles. In normal RNFL, F-actin staining between bundles is weak. In contrast, F-actin staining of glaucomatous retinas is not uniform along bundles (Fig. 3A) or across bundle cross sections (Fig. 5B). In addition, a diffuse distribution of F-actin across the imaged area becomes apparent (Figs. 3 and 4). The result suggests that glaucoma alters F-actin distribution in the RNFL. Compared with nearby normal-looking bundles, bundles with altered F-actin have less intense phalloidin staining, which indicates a loss of F-actin in axons. In severely changed retinas, strands of F-actin staining oriented along bundles are visible (Fig. 3B), which may represent a few remaining axons or thin bundles of axons and also suggests a large reduction of F-actin in the RNFL.

F-actin is intimately involved in the regulation of neuron excitotoxicity, receptor activity and synaptic function (Cristofanilli and Akopian, 2006; Goode et al., 2000). Change of axonal F-actin found in this study may suggest that F-actin alteration contributes to axonal transport obstruction and axonal degeneration as found in eyes with elevated IOP (Johnson et al., 2000; Pease et al., 2000; Quigley et al., 2000; Villegas-Perez et al., 2005).

Many studies have investigated topographic change of RNFL and RGCs with different animal models, with various results. Loss of RGCs follows different topographic patterns, including RGC loss occurring first near the ONH (Yu et al., 2006) and RGC apoptosis or loss starting peripherally and moving toward the ONH (Filippopoulos et al., 2006; Reichstein et al., 2007; Sawada and Neufeld, 1999; Vorwerk et al., 1999). Loss of RGC and nerve fiber bundles can be either regionally selective (Johnson et al., 2000; Mabuchi et al., 2004; Morrison et al.,

1997; Villegas-Perez et al., 2005) or not (Danias et al., 2006; Mittag et al., 2000; Urcola et al., 2006; Yu et al., 2006). Immunohistochemistry of neurofilament protein in a monkey model of glaucoma shows more severe damage of NFs in peripheral than in central RNFL (Vickers et al., 1995). These discrepancies in damage features between studies could be due to different animal species and glaucoma models and suggest that cellular and subcellular structures respond differently to axonal injury depending on circumstances (Hoffman et al., 1987; McKerracher et al., 1993). Understanding these differences, therefore, may enhance our knowledge of glaucomatous damage mechanisms.

Two topographical features of the alteration of F-actin in the RNFL are evident from our study of a rat model of glaucoma. First, F-actin in the portion of a bundle near the ONH is more sensitive to damage by intraocular hypertension than in the same bundle more peripherally. In the four retinas that only showed change near the ONH (Group 2 in Fig. 6), altered F-actin distribution was observed in cross sectional images at distances of 220 – 380 μm from the ONH center, but in corresponding cross sections located more peripherally bundles were approximately uniformly stained with well defined boundaries (Fig. 5). This result suggests that alteration of F-actin in a nerve fiber bundle occurs first near the ONH. Evaluating F-actin distribution near the ONH, therefore, provides early detection of F-actin change in this rat glaucoma model. Second, RNFL in dorsal retina seems more sensitive to F-actin change than RNFL in other regions. In the four retinas with change near the ONH and one retina with only 20% severe alteration, the altered bundles were all in dorsal retina. In the three other retinas with severe alteration, normal looking bundles were found only in rostral-ventral-caudal retina; severely altered bundles were situated in dorsal regions; areas adjacent to the severe alteration contained bundles with altered F-actin near the ONH. This spatially progressive structural change seen in individual retinas supports the idea of temporally progressive F-actin alteration spreading out from dorsal retina toward rostral and caudal regions. Selective change of axonal F-actin in dorsal retina is consistent with selective loss of RGCs or axons found in rodent models of glaucoma by other studies (Johnson et al., 2000; Mabuchi et al., 2004; Morrison et al., 1997; Villegas-Perez et al., 2005). This pattern, however, is different than that found in primate eyes, in which early damage of RNFL appears in both superior and inferior regions (Pederson and Gassterland, 1984; Quigley and Addicks, 1982; Quigley et al., 1981; Sommer et al., 1991). The difference has been suggested to be due to anatomic differences between rodents and primates (Mabuchi et al., 2004; Morrison et al., 1997).

Because early alteration and later loss of F-actin were observed in the same topographic sectors, our study suggests that within affected regions F-actin undergoes a progression of structural changes that precedes total loss of the structure. Change of F-actin within axons may start first in the portion of axons near the ONH and then propagate along bundles to the peripheral retina. Alternatively, the axons of RGCs near the ONH may be more vulnerable to elevated IOP, while axons of peripheral RGCs may be affected in the later stages of damage; in this view, the observed pattern of change represents regional damage to entire ganglion cells. Regardless of which mechanism underlies the change of F-actin caused by elevated IOP, we propose that topographic change in this rat model of glaucoma progresses as follows: 1) change of F-actin occurs first in the portion of nerve fiber bundles near the ONH, then develops in peripheral retinas, and 2) loss or change of F-actin usually occurs first in dorsal retina, then spreads to rostral and caudal retina. These two features are consistent with the idea that axonal degeneration induced by ocular hypertension results from focal insult to groups of neighboring axons within the optic nerve (Jakobs et al., 2005) and, hence, support an ONH location for the insult.

This study suggests that F-actin alteration results from exposure to elevated IOP. As shown in Fig. 6, the overall change of F-actin is likely related to cIOP (Danias et al., 2006; Levkovitch-Verbin et al., 2002). In eyes with cIOP around 50 mmHg-days, no apparent F-actin alteration

was found. When cIOP reached above 70 mmHg-days, F-actin alteration was often found near the ONH. Further increase of cIOP led to loss of F-actin in a fan-shaped region centered on dorsal retinas. Based on these limited data, however, the relation between cIOP and F-actin alteration is not perfect; the cIOP of Groups 1 and 2 overlaps and the extent of F-actin alteration within Group 3 did not correlate with cIOP. A likely explanation is that susceptibility to elevated IOP varies among individual rats, similar to the variable response of human eyes to IOP elevation. Variability of response to glaucomatous damage, which is also found in RGC density maps in glaucomatous eyes (Danias et al., 2006), may result from variable RGC distribution in rat retinas.

In this study whole-mounted retinas were used to provide a full view of nerve fiber bundle distribution around the ONH, which allowed detection of focal changes of cytoskeletal components in axons across the retina. The confocal imaging method is a powerful tool to reveal structural distribution across the retina, although it has been mostly used only to display en face images (Jakobs et al., 2005; Lafuente et al., 2002; Villegas-Perez et al., 1988; Villegas-Perez et al., 2005). As demonstrated in Figs. 3–5 and 7, cross-sectional images of retina provide further and enhanced assessment of cytoskeletal change in tissues. To the authors' best knowledge, axonal F-actin distribution across the retina and its change in the context of glaucoma has not been shown yet. The results presented here provide a map for guiding future studies of F-actin alteration at the ultrastructural level.

The present study investigates glaucomatous damage to RNFL identified by axonal F-actin and shows that glaucoma alters the topographic distribution of F-actin during disease development. Axonal F-actin, as an abundant cylindrical structure in the RNFL, might contribute to its optical properties (Knighton and Huang, 1999a; Zhou and Knighton, 1997). Future studies should evaluate a possible role for F-actin in the optical detection of glaucomatous change.

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Figure 1. Intraocular pressure (IOP) of a treated eye and its control eye. IOP was measured with a Tonopen XL tonometer. Vertical lines: standard error of the mean IOP calculated from 10 repeat readings of the tonometer. Arrows: days of the 1st and 2nd laser treatments. The 4th retina in Group 3, Fig. 6.

Figure 2.

RNFL assessment by confocal microscopy. A: an *en face* image of a control retina shows nerve fiber bundles identified by phalloidin labeled F-actin (green) as bright stripes. B: cross-sectional images of bundles, located on the lines in A, show uniform staining across bundle cross sections. The RNFL is separated from deeper layers by a single layer of nuclei with DAPI counterstain (blue). Nerve fiber bundles are thick near the ONH and get thinner with distance away from the ONH. Distances of the cross sections (from top to bottom) are 208, 320, and 432 μm from the ONH center. BV: blood vessels. The scale bar applies to both horizontal and vertical directions of all images.

Figure 3.

Disorganization of F-actin in RNFL. A: alteration of F-actin distribution across an entire subimage; F-actin along bundles is much less dense than in normal retinas. B: magnified bundles in A (rectangular area) show strands of F-actin along bundles (arrows); less oriented F-actin also becomes apparent. C: cross sectional images of bundles along the lines in A show thinning of RNFL. The cross sections (from top to bottom) are at a distance of 425, 537 and 649 μm from the ONH center. BV: blood vessels. The 2nd retina in Group 3, Fig. 6.

Figure 4.

Total loss of axonal F-actin. A: en face image of a severely altered retinal region showing weakly stained F-actin network without apparent nerve fiber bundles. B: a single layer of nuclei lying under the retinal surface. The cross sections (from top to bottom) are at a distance of 460, 572, 684 μm from the ONH center. BV and arrows in B indicate blood vessels. The 1st retina in Group 3, Fig. 6.

Figure 5.

F-actin alteration near the ONH in mildly damaged retinas. A: F-actin staining diminishes as bundles near the ONH. B: cross sectional images of bundles along the lines in A. Bundles near the ONH (upper image, 300 μm from the ONH center) were not well defined by F-actin stain while bundles away from the ONH (middle and lower images, 412 and 524 μm from the ONH center) still appeared normal. C: A subimage from the dorsal quadrant of a treated retina showing altered F-actin distribution across the entire image. Bundle boundaries became “blurred” as they approached the ONH. D: cross sectional images of the bundles in C at 211, 323, and 435 μm from the ONH. Alteration of F-actin becomes less severe for bundles away

from the ONH. BV: blood vessels. A, B: the 3rd retina and C, D: the 4th retina in Group 2, Fig. 6.

Figure 6.

cIOP of treated eyes and extent of F-actin alteration. Each vertical bar represents the cIOP value of one eye. The 12 treated eyes are grouped into Group 1: normal appearing bundles in all measured cLSM images, Group 2: F-actin alteration only appearing near the ONH, and Group 3: F-actin alteration extending to peripheral retina. The percentage of altered F-actin is calculated for each retina in Group 3 (see text). Heavy lines are the mean cIOP $\pm$ s.d. of each group, 51 ± 20 , 85 ± 10 , and 255 ± 59 mmHg-days for Groups 1–3, respectively.

Figure 7.

F-actin alternation in dorsal retina. A: subimages around the ONH overlaid on an en face image taken with a 10× objective. Bundles outside the dorsal fan-shaped sector (dashed line) are well defined except for a focal loss of F-actin (hollow arrow). B: cross sectional images along the lines in A. Image f: the hollow arrow indicates the focal loss of F-actin. The cross sections are at a distance of about 460 μm from the ONH center. The black patch just above the ONH was caused by the retina curving out of the cLSM focal plane. BV: blood vessels. The 4th retina in Group 3, Fig. 6.

Table 1

Mean IOP of the treated and fellow control eyes

	Group 1		Group 2		Group 3	
	Control	Treated	Control	Treated	Control	Treated
IOP ₁	27.1 ± 2.0	30.0 ± 3.4	26 ± 2.2	31.5 ± 4.0	26 ± 0.8	31.8 ± 3.2
IOP ₂	25.7 ± 1.4	30.1 ± 2.2	27.2 ± 2.4	33 ± 3.2	25.8 ± 1.1	34.8 ± 3.6

IOP₁ and IOP₂ (mmHg) are the mean ± sd of IOP in the 1st and 2nd weeks, respectively.