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Tissue and urokinase plasminogen activators instigate the degeneration of retinal ganglion cells in a mouse model of glaucoma

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Abstract

Elevated intraocular pressure (IOP) promotes the degeneration of retinal ganglion cells (RGCs) during the progression of Primary Open-Angle Glaucoma (POAG). However, the molecular mechanisms underpinning IOP-mediated degeneration of RGCs remain unclear. Therefore, by employing a mouse model of POAG, this study examined whether elevated IOP promotes the degeneration of RGCs by up-regulating tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) in the retina. IOP was elevated in mouse eyes by injecting fluorescent-microbeads into the anterior chamber. Once a week, for eight weeks, IOP in mouse eyes was measured by using Tono-Pen XL. At various time periods after injecting microbeads, proteolytic activity of tPA and uPA in retinal protein extracts was determined by fibrinogen/plasminogen zymography assays. Localization of tPA and uPA, and their receptor LRP-1 (low-density receptor-related protein-1) in the retina was determined by immunohistochemistry. RGCs' degeneration was assessed by immunostaining with antibodies against Brn3a. Injection of microbeads into the anterior chamber led to a progressive elevation in IOP, increased the proteolytic activity of tPA and uPA in the retina, activated plasminogen into plasmin, and promoted a significant degeneration of RGCs. Elevated IOP up-regulated tPA and LRP-1 in RGCs, and uPA in astrocytes. At four weeks after injecting microbeads, RAP (receptor associated protein; 0.5 and 1.0 μ M) or tPA-Stop (1.0 and 4.0 μ M) was injected into the vitreous humor. Treatment of IOP-elevated eyes with RAP led to a significant decrease in proteolytic activity of both tPA and uPA, and a significant decrease in IOP-mediated degeneration of RGCs. Also, treatment of IOP-elevated eyes with tPA-Stop decreased the proteolytic activity of both tPA and uPA, and, in turn, significantly attenuated IOP-mediated degeneration of RGCs. Results presented in this study provide evidence that elevated IOP promotes the degeneration of RGCs by up-regulating the levels of proteolytically active tPA and uPA.

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Keywords

POAG; tPA; uPA; LRP-1; RAP; tPA-Stop; degeneration of RGCs

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1. Introduction

POAG is the second leading cause of preventable blindness in the United States and a major cause of blindness worldwide. Despite the fact that elevated IOP promotes the degeneration of RGCs in POAG patients (Burgoyne et al., 2005; Cedrone et al., 2008; Friedman et al., 2004; Quigley and Broman, 2006; Weinreb and Khaw, 2004), the molecular mechanisms underpinning IOP-mediated degeneration of RGCs is unclear.

Previous studies from this laboratory have reported that elevated levels of tPA and uPA promoted the degeneration of RGCs in acute mouse models of optic nerve ligation (Zhang et al., 2003) and excitotoxicity (Mali et al., 2005). However, it was unclear whether tPA and uPA play a role in the degeneration of RGCs in glaucoma, and if so, how these secreted proteases specifically promote the degeneration of RGCs. Recent studies have reported that LRP-1, a member of the LDL receptor family, functions as a cell surface receptor for tPA and uPA (Casse et al., 2012; Herz, 2003; Herz and Strickland, 2001). In addition to acting as a receptor for tPA and uPA, LRP-1 recognizes receptor-associated protein (RAP), which inhibits the binding of tPA and uPA, and plays a significant role in recycling and synthesis of these proteases (Bu, 2001; Bu et al., 1995; Bu and Schwartz, 1998; Willnow et al., 1996). However, thus far no studies have investigated the role of tPA, uPA, and their cell surface receptor LRP-1 in the degeneration of RGCs under glaucomatous conditions. Therefore, this study investigated the role of tPA and uPA in the degeneration of RGCs in a mouse model of POAG, in which the elevation in IOP and the degeneration of RGCs is chronic and progressive.

2. Materials and Methods

2.1. Materials

Plasminogen (Product# 410), fibrinogen (Product# 431), and tPA-Stop (2,7-bis-(4-amidino-benzylidene)-cycloheptan-1-one dihydrochloride; Product# 544), were obtained from American Diagnostica (Stamford, CT). Antibodies against uPA (Catalogue# MA-H77A10-1003), tPA (Catalogue# ASHTPA-102), and plasminogen (Catalogue# IMPLG) were obtained from Molecular Innovations (Southfield, MI). Antibody against LRP-1 (Catalogue# PAB-10774) was obtained from Orbigen (San Diego, CA). Antibody against actin (MAB1501) was obtained from EMD Millipore (Billerica, MA). Antibody against Tuj1 (neuronal class III beta-tubulin) was obtained from Covance (Catalogue# PRB-435P, Princeton, NJ), and antibody against brain-specific home box/POU domain protein 3a (Brn3a) was obtained from Santa Cruz Biotechnology (Catalogue# SC-31984, Santa Cruz, CA). Recombinant RAP was kindly provided by Dr. Guojun Bu (Washington University School of Medicine, St. Louis, MO). For immunohistochemical assays, appropriate secondary antibodies conjugated to AlexaFluor 568 (red) and AlexaFluor 647 (magenta) were obtained from Invitrogen (Carlsbad, CA).

2.2. IOP elevation in mouse eyes

All experiments on mice were performed under general anesthesia, according to the guidelines of Oakland University's Institutional Animal Care and Use Committee (IACUC). Adult B6.Cg-Tg (Thy1-YFPH) 2Jrs/J mice (6-8 weeks old) were anesthetized with an intra-peritoneal injection of Ketamine (50 mg/kg body weight) and Xylazine (7 mg/kg body weight). Two microliters of fifteen-micrometer polystyrene microbeads (~1000 beads) conjugated to AlexaFluor 465 were injected into the vitreous humor of right eyes in each mouse (n=18; 2 cohorts of 9). Two microliters of phosphate buffered saline (PBS) was injected into the anterior chamber of left eyes in each mouse (Sappington et al., 2010). For the results presented in figure 1A, eyes were imaged on anesthetized mice by using a Micron III camera (Phoenix Research Labs, Pleasanton, CA). All animals were maintained in a 12 h light and dark cycle. IOP measurements were made every week for a total of 8 weeks on anesthetized mice by using Tonopen XL tonometer (Reichert, Inc. Depew, NY). After applying topical anesthesia (0.5% proparacaine hydrochloride), at least 8-10 readings all within the 5% range were obtained from each eye. Statistical significance was determined by ANOVA, followed by a post hoc-Tukey's test (GB-Stat Software, Dynamic Microsystems, Silver Spring, MD). The results were expressed as the mean $\pm$ SEM. At 4 weeks after injecting microbeads, mice eyes (n=18; 2 cohorts of 9) were treated with intravitreal injections of PBS (2 μ L), RAP (2 μ L), or tPA-Stop (2 μ L) by using a NanoFil syringe equipped with a 36-gauge beveled-needle (World Precision Instruments, Sarasota, FL).

2.3. Protein Extraction

At the indicated time points, mice were euthanized with an overdose of carbon dioxide, and their eyes were enucleated (n=12; 2 cohorts of 6). Retinas were removed carefully and washed three times with PBS. Three to four retinas each were placed in Eppendorf tubes containing 40 microliters of extraction buffer (1% Nonidet-P40, 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₃VO₄, pH 7.4) without protease inhibitors, and the tissues were homogenized. Retinal tissue homogenates were centrifuged at 7840 g for 5 minutes at 4° C, and the supernatants were collected. Protein concentration in the supernatants was determined by using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

2.4. Determination of proteolytic activity

Proteolytic activity of tPA and uPA in retinal proteins extracted from PBS- or microbead-injected eyes (n=12; 2 cohorts of 6) was determined by fibrinogen/plasminogen zymography according to the general methods described previously (Ganesh and Chintala, 2011; Mali et al., 2005). Briefly, aliquots containing an equal amount of total proteins (50 μ g) extracted from PBS- or microbead-injected eyes were mixed with loading buffer and loaded onto 10% SDS polyacrylamide gels containing fibrinogen (5.5 mg/mL) and plasminogen (50 μ g/mL). After electrophoresis, gels were washed three times with 2.5% TritonX-100 (15 min each time), placed in 0.1 M glycine buffer (pH 8.0) and incubated overnight at 37°C. The gels were stained with 0.1% Coomassie Brilliant Blue-R250 and de-stained with a solution containing 25% methanol and 10% acetic acid. Relative levels of tPA and uPA were determined by scanning the zymograms on a flatbed scanner, and the relative protease levels

of tPA and uPA were quantified by using Scion image analysis software (Scion Corporation, Frederick, MD). The results were shown as mean arbitrary densitometric units $\pm$ SEM. Statistical significance was analyzed by using a nonparametric Newman-Keuls analog procedure (GB-Stat Software, Dynamic Microsystems, Silver Spring, MD).

2.5. Western Blot Analysis

Aliquots containing an equal amount of total proteins (50 μ g) extracted from the retinas of PBS- or microbead-injected eyes (n=12; 2 cohorts of 6) were mixed with gel-loading buffer, and the proteins were separated electrophoretically by using 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. After electrophoresis, the proteins were transferred onto PVDF membranes (EMD Millipore, Billerica, MA) and non-specific binding sites were blocked with 5% bovine serum albumin (BSA) prepared in Tris-buffered saline containing 0.2% Tween 20 (TBS-T). After incubating with primary antibodies against LRP-1 (1:2500 dilution), actin (1:2500 dilution), and plasminogen (1:2500 dilution) the membranes were washed with TBS-T and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). Membranes were then incubated with ECL reagent, and the signals were captured on an X-ray film. Note that the plasminogen antibody used in this study detects both a higher molecular weight plasminogen and a lower molecular weight active plasmin (Zhang et al., 2003).

2.6. Immunohistochemistry

a) Retinal cross sections—Eyes enucleated after PBS- or microbead injection (n=12; 2 cohorts of 6) were fixed in 4% paraformaldehyde (PFA) and ten micron-thick cross sections were prepared by using a cryostat. Retinal sections were washed three times with PBS, and non-specific sites were blocked for 1 hour at room temperature (RT) with 5% BSA prepared in PBS. Retinal cross sections were washed three times with PBS and permeabilized for 15 minutes by incubating them in 0.1 % TritonX-100. Retinal cross sections were then incubated at 4° C overnight with antibodies against tPA (1:100 dilution), uPA (1:100 dilution), and LRP-1 (1:100 dilution). The next day, retinal cross sections were washed three times with PBS and incubated for 2 hours at RT with appropriate secondary antibodies conjugated to AlexaFluor 568 (1:100 dilution). Retinal cross sections were washed three times with PBS and mounted on a slide by using Flouromount-G (Southern Biotech, Birmingham, AL).

b) Whole retinas—At the indicating time points, eyes enucleated from PBS- or microbead-injected (n=12; 2 cohorts of 6) mice were fixed in 4% PFA for 30 minutes at RT. Corneas and the lenses were removed, and the posterior eyecups were incubated in 4% PFA for another 30 minutes at RT. Whole retinas were removed from the eyecups and permeabilized in 0.5% TritonX-100 for 30 min at RT. Individual retinas were incubated overnight with primary antibodies against tPA (1:100 dilution), uPA (1:100 dilution), or LRP-1 (1:100 dilution) in PBS containing 5% BSA and 0.2% TritonX-100. The next day, retinas were washed three times with PBS and incubated for 2 hours at RT with appropriate secondary antibodies conjugated to AlexaFluor 568 (1:200 dilution in PBS). To determine the cells responsible for the synthesis of tPA, uPA, and LRP-1, retinas were washed and then incubated again for 2 hours with primary antibodies against GFAP (a marker for

astrocytes) and Tuj1 (a marker for RGCs). Whole retinas were washed three times with PBS and incubated for 2 hours at RT with secondary antibodies conjugated to AlexaFluor 647 (1:200 dilution). Finally, retinas were washed three times with PBS and mounted on a slide with Fluoromount-G (the vitreous side facing up).

2.7. Quantification of RGCs loss

At the indicated time points, eyes were enucleated (n=12; 2 cohorts of 6) and fixed in 4% PFA for 30 minutes at RT. Corneas and the lenses were removed, and the posterior eyecups were incubated in 4% PFA for another 30 minutes in at RT. Retinas were removed from the eyecups and permeabilized in 0.5% TritonX-100 for 30 min at RT. Retinas were incubated overnight with primary antibody against Brn3a diluted (1:100) in PBS containing 5% BSA and 2% TritonX-100. Retinas were washed three times with PBS and incubated for 2 hours at RT with secondary antibodies conjugated to AlexaFluor 568 (1:100 dilution). Finally, the retinas were washed three times with PBS and mounted on a slide with Fluoromount-G (the vitreous side facing up). The number of Brn3a-positive RGCs in the retinas was assessed by observing them under a Zeiss Imager Z.2 epifluorescence microscope as described previously (Chintala, 2015). For each retina, Brn3a-positive RGCs in six to eight areas of equal size (335 x 445 microns, 20x magnification), located at equal distance from the optic disc were photographed by using a Zeiss digital camera. Digitized images were compiled by using Adobe Photoshop Software 7.0 (Adobe Systems, Inc., San Jose, CA). The number of Brn3a-positive cells in the retinas were quantitated by using Nikon Elements AR software (Nikon Instruments, Inc., Melville, NY). Statistical significance was analyzed by using a nonparametric Newman-Keuls analog procedure (GB-Stat Software; Dynamic Microsystems, Silver Spring, MD), and the results were expressed as mean $\pm$ SEM.

3. Results

3.1. Injection of microbeads leads to IOP elevation in mice

A single injection of polystyrene microbeads into the anterior chamber of mouse eyes led to a progressive increase in IOP as measured by using a Tonopen (n=18; 2 cohorts of 9). The results presented in figure 1A indicate that at twenty-four hours after injection, a majority of the beads were lodged into the trabecular meshwork. The IOP readings presented from one cohort of 9 mice show a progressive increase in IOP over the eight-week period (figure 1B). On average, microbead-injected eyes developed elevated IOP ranging from 26-30 mm Hg when compared to PBS-injected eyes which showed IOP levels ranging from 12-14 mm Hg, consistent with the results reported by other laboratories (Chen et al., 2011; Sappington et al., 2010).

3.2. Elevated levels of IOP correlate with the degeneration of RGCs

To determine whether elevated IOP promotes the degeneration of RGCs, whole retinas were isolated at 0, two, four, six, and eight weeks after injecting microbeads, and immunostained with an antibody against Brn3a (n=12; 2 cohorts of 6). The results presented in figure 1C and D indicate that the loss of Brn3a-positive RGCs (in four to six microscope fields of identical size [335 x 445 microns], located approximately at the same distance from the optic disk) was increased by 21.00 ± 7.50 % at two weeks, 53.74 ± 5.84 % at four weeks,

73.65 $\pm$ 2.6% at six weeks, and 83.20 $\pm$ 8.9% at eight weeks after IOP elevation in the retinas isolated from microbead-injected eyes.

3.3. Elevated IOP up-regulates the proteolytic activity of tPA and uPA, and activates plasminogen into plasmin

To determine whether elevated-IOP leads to an up-regulation of the proteolytic activity of tPA and uPA, fibrinogen/plasminogen zymography assays were performed by using aliquots containing equal amounts of total proteins (50 μ g) extracted from the retinas of eyes injected with PBS or microbeads. Zymography assays (figure 2A, upper panels) and semi-quantitative analysis of protease activity (figure 2A, lower panels) indicate that a low level of tPA was expressed, constitutively, in retinal proteins extracted from PBS-injected eyes, consistent with our previous observations made in a mouse model of excitotoxicity (Mali et al., 2005). Time-course experiments indicate that tPA levels (figure 2, upper panels) were increased significantly at four weeks (by 55.8 $\pm$ 8%) and six weeks (by 62.2 $\pm$ 8%) after microbead injection. Interestingly, uPA levels, absent completely in retinal proteins extracted from PBS-injected eyes were up-regulated in retinal proteins extracted from microbead-injected eyes by 15 $\pm$ 1.5% at two weeks, 35.6 $\pm$ 3% at four weeks, 48 $\pm$ 7% at six weeks, and 48 $\pm$ 6% at eight weeks. To determine whether plasminogen activation plays a role in IOP-mediated degeneration of RGCs, aliquots containing an equal amount of retinal proteins (50 μ g) extracted from PBS- or microbead-injected eyes were subjected to western blot analysis. Results presented in figure 2B and 2C indicate that a very low level of plasminogen was present in PBS-injected eyes. In contrast, plasminogen levels were elevated by 70% in retinal proteins extracted at four weeks after microbead injection. At 6 weeks after injecting microbeads, most of the plasminogen was activated into a lower molecular weight plasmin.

3.4. Elevated IOP up-regulates tPA in RGCs

The results presented in figure 2 indicate that the levels of proteolytically active tPA and uPA were up regulated in retinal proteins extracted after IOP elevation, but the cellular source of these proteases in the retinas was unclear. Identifying the cells responsible for the synthesis of these secreted proteases is essential because they may promote the degeneration of RGCs in an autocrine or paracrine fashion. To determine the cellular source of tPA and uPA, retinal cross sections prepared from the eyes injected with PBS or microbeads were subjected to immunohistochemical analysis. Results presented in figure 3A indicate that a low level of tPA, expressed constitutively by RGCs in retinal cross-sections prepared from PBS-injected eyes was, elevated in RGCs in retinal cross-sections prepared from microbead-injected eyes. Also, at eight weeks after microbead-injection, a majority of the up-regulated tPA was localized in the ECM. To confirm the cellular origin of tPA, whole retinas isolated at six weeks after microbead injection were immunostained with an antibody against tPA and double-labeled with Tuj1, a marker for RGCs. Results presented in figure 3B indicate that the cells that showed positive immunostaining for tPA also showed positive staining for Tuj1 indicating that RGCs are responsible for the synthesis of tPA.

3.5. Elevated IOP up-regulates uPA in astrocytes

To determine the cellular source of uPA, retinal cross-sections prepared from the eyes injected with PBS or microbeads were subjected to immunohistochemical analysis. Results presented in figure 4A indicate that uPA was barely detectable in the GCL in retinal cross-sections prepared from PBS-injected eyes, consistent with the zymography results presented in figure 2. In contrast, after microbead injection, uPA protein levels were up-regulated in the GCL and correlated with increased proteolytic activity of uPA shown in figure 2. To confirm the cellular origin of uPA, whole retinas isolated at six weeks after microbead injection were immunostained with an antibody against uPA and double-labeled with GFAP, a marker for astrocytes. Results presented in figure 4B indicate that the cells that showed positive immunostaining for uPA also showed positive immunostaining for GFAP, indicating that astrocytes are responsible for the synthesis of uPA.

3.6. Elevated IOP up-regulates LRP-1 in RGCs

Although it was unclear how secreted proteases promote the degeneration of RGCs, previous studies have reported that tPA and uPA bind to their cell surface receptor, LRP-1, and by doing so, they activate intracellular signaling pathways (Bu and Rennke, 1996; Herz, 2003). However, the role of LRP-1 in glaucomatous degeneration of RGCs has not been investigated before. Therefore, to determine whether elevated IOP up-regulates the protein levels of LRP-1, western blot analysis was performed by using aliquots containing an equal amount of total proteins (50 µg) extracted from the retinas of eyes injected with PBS or microbeads. Western blot analysis (Figure 5A) and semi-quantitative analysis (figure 5B) indicate that a low level of LRP-1 was expressed constitutively in retinal proteins extracted from PBS-injected eyes. In contrast, at four and six weeks after IOP-elevation, LRP-1 protein levels were elevated significantly in retinal proteins extracted from microbead-injected eyes, but not in retinal proteins extracted from PBS-injected eyes. To determine the cellular localization of LRP-1 in the retina, cross-sections prepared from PBS- or microbead-injected eyes were subjected to immunohistochemical analysis by using an antibody against LRP-1. Results presented in figure 5C indicate that a low level of LRP-1 was expressed constitutively by RGCs in retinal sections prepared from PBS-injected eyes. In contrast, at four and six weeks after microbead injection, LRP-1 was up-regulated in RGCs and correlated with western blot results presented in figure 6A. Furthermore, to confirm the cellular origin of LRP-1, whole retinas isolated at six weeks after microbead-injection were immunostained with an antibody against LRP-1 and double-labeled with an antibody against Tuj1, a marker for RGCs. Results presented in figure 5D indicate that the cells that showed positive immunostaining for LRP-1 also showed positive immunostaining for Tuj1, indicating that RGCs are responsible for the synthesis of LRP-1.

3.7. RAP attenuates IOP-mediated degeneration of RGC

Since elevated levels of tPA and uPA (figure 2) correlated with the degeneration of RGCs (figure 3), and since their receptor LRP-1 was expressed by RGCs (figure 6), additional experiments were performed to determine whether inhibition of tPA and uPA interaction with LRP-1 attenuates the degeneration of RGCs. To investigate this possibility, at four weeks after microbead injection, PBS- or microbead-injected eyes were treated with

intravitreal injections of two different concentrations of RAP (0.5 and 1.0 μM) that inhibits the binding of tPA and uPA with LRP-1. At the end of eight weeks, retinal proteins were extracted from PBS-injected eyes, and microbead-injected eyes were ($n=12$; 2 cohorts of 6) subjected to zymography assays. The concentrations of RAP used in this study were based on one of our previous studies, in which these concentrations of RAP inhibited the proteolytic activity of tPA and uPA (Rock and Chintala, 2008). Results presented in figure 6A indicate that elevated IOP up-regulated the proteolytic activity of both tPA and uPA at eight weeks after microbead injection, consistent with the results presented in figure 2. In contrast, treatment of the eyes with RAP led to a significant reduction in the proteolytic activity of both tPA and uPA (figure 6B and 6C).

To determine whether RAP-mediated reduction in tPA and uPA proteolytic activity attenuates IOP-mediated degeneration, whole retinas isolated from the eyes injected with PBS, PBS plus RAP, microbeads, and microbeads plus RAP were immunostained with an antibody against Brn3a (figure 7A), and the number of Brn3a-positive RGCs was determined by observing whole retinas under an epifluorescence microscope. The results presented in figures 7A and 7B show the number of Brn3a-positive RGCs was decreased significantly in microbead-injected eyes by $82.75 \pm 1.27\%$, consistent with the results presented in figure 3. In contrast, 1.0 μM RAP, which down-regulated the proteolytic activity of both tPA and uPA in microbead-injected eyes, reduced the number of RGCs only by $28.56 \pm 4.07\%$ (figure 7A and 7B).

3.8. tPA-Stop attenuates IOP-mediated degeneration of RGCs

Since RAP attenuated IOP-mediated degeneration of RGCs shown in figure 8, experiments were performed further to investigate whether inhibition of the proteolytic activity of tPA and uPA by tPA-Stop also attenuates IOP-mediated degeneration of RGCs. First, to determine the effect of tPA-Stop, at four weeks after microbead injection, PBS- or microbead-injected eyes were treated with intravitreal injections of two different concentrations of tPA-Stop (1.0 and 4.0 μM). The concentrations of tPA-Stop used in this study were based on one of our previous studies, in which these concentrations of tPA-Stop inhibited the proteolytic activity of both tPA and uPA and attenuated the degeneration of RGCs in an excitotoxic mouse model of retinal degeneration (Mali et al., 2005). At the end of eight weeks, retinal proteins extracted from PBS- and microbead-injected eyes ($n=12$; 2 cohorts of 6) were subjected to zymography assays. Results presented in figure 8A indicate that tPA-Stop reduced the proteolytic activity of tPA in retinal proteins extracted from PBS-injected eyes in a concentration-dependent fashion (figure 8A, upper left panel). Also, tPA-Stop inhibited the proteolytic activity of tPA, as well as of uPA, in a concentration-dependent fashion in retinal proteins extracted from microbead-injected eyes (figure 8A, upper right panel). Semi-quantitative analysis indicates that tPA-Stop significantly inhibited the proteolytic activity of tPA and uPA in both PBS- or microbead-injected eyes (figure 8B, lower left and right panels).

Finally, to determine whether tPA-Stop attenuates IOP-mediated degeneration of RGCs, whole retinas were immunostained with an antibody against Brn3a. The number of Brn3a-positive RGCs was then determined by observing flat-mounted retinas under an

epifluorescence microscope. The results presented in figures 9A and 9B indicate that the number of Brn3a-positive RGCs was significantly in microbead-injected eyes by $81.8 \pm 2.34\%$. In contrast, 4.0 μ M tPA-Stop, which down-regulated the proteolytic activity of both tPA and uPA reduced the number of RGCs in microbead injected eyes only by $20.8 \pm 8.5\%$ (figure 9A and 9B).

4. Discussion

Irreversible degeneration of RGCs leads to blindness in POAG patients (Cedrone et al., 2008; Friedman et al., 2004; Quigley and Broman, 2006; Weinreb and Khaw, 2004). Despite the significant progress made in understanding glaucoma pathology and identifying elevated IOP as a risk factor, the mechanisms underpinning IOP-mediated degeneration of RGCs is still poorly understood. A better understanding of the mechanisms underlying IOP-mediated degeneration of RGCs is essential because it would open up avenues to rescue degenerating RGCs in glaucoma patients.

A number of hypotheses have been proposed previously, including IOP-mediated mechanical stress at the optic nerve head (ONH), insufficient retrograde transport of growth factors (Pease et al., 2000), glial cell activation (Ganesh and Chintala, 2011; Neufeld and Liu, 2003), and autonomous axonal self-destruction (Whitmore et al., 2005), but the role these events in mouse models of POAG is not corroborated.

This study utilized an established mouse model of POAG (Chen et al., 2011) in which injection of microbeads into the anterior chamber of mouse eyes leads to a progressive elevation in IOP, and then investigated whether elevated levels of tPA and uPA promote the degeneration of RGCs. Results presented in this study show that elevated IOP promotes the degeneration of RGCs by up-regulating the levels of tPA and LRP-1 in RGCs and uPA in astrocytes. The results presented in this study are important for the following reasons because they shed light on the mechanisms underlying IOP-mediated degeneration of RGCs: a) results presented in this study, for the first time, show that elevated levels of both tPA and uPA promote the degeneration of RGCs in a mouse model of POAG, in which the degeneration of RGCs is chronic and progressive. b) tPA-Stop and RAP reduced the proteolytic activity of tPA and uPA, by doing so, they attenuated IOP-mediated degeneration of RGCs indicating that these proteins can be targeted to prevent retinal damage.

However, a few important questions need to be addressed. 1). Can uPA alone promote the degeneration of RGCs? Although the mechanisms by which elevated levels of uPA alone promote the degeneration of RGCs under elevated IOP conditions are unclear at this time, a previous study reported that excitotoxicity promoted neuronal cell death in the hippocampus by astrocyte-mediated up-regulation of uPA (Cho et al., 2012), and uPA has been shown to regulate the calcium influx into the neurons (Christow et al., 1999). Thus, it is possible that elevated levels of uPA can promote the degeneration of RGCs by increasing calcium influx.

2). Can LRP-1 alone promote the degeneration of RGCs? Recent studies have reported that both tPA and uPA binds to LRP-1 (Bu and Rennke, 1996; Herz, 2003; Herz and Strickland,

2001), and LRP-1 increases the proteolytic activity of tPA and uPA at the cell surface by facilitating the clearance of uPA/PAI-1 complexes (Nykjaer et al., 1997). LRP-1 plays a role not only in endocytic clearance of various ligands in many pathophysiological conditions including neurodegenerative diseases and integrity of blood-brain-barrier (Lillis et al., 2008) but increasing evidence also indicates that LRP-1 mediates intracellular signaling pathways (Bu et al., 1995; Hussain, 2001; Martin et al., 2008), and promote the death of cerebrovascular endothelial cells (Wilhelmus et al., 2007). However, no evidence currently exists to support that LRP-1 alone plays a role in the degeneration of RGCs in the mouse model of POAG.

3). Why do RGCs synthesize abundant amounts of tPA under normal conditions, although under pathological conditions tPA released into the extracellular milieu promotes their degeneration? Recent studies indicated that tPA could potentiate N-methyl-D-aspartate (NMDA)-type glutamate receptor signaling (Martin et al., 2008). For example, a recent study reported that tPA interacts with the NR1 subunit of the NMDA receptor and proteolytically cleaves the subunit to potentiate calcium influx into neuronal cells (Nicole et al., 2001). In addition, studies from the same group of investigators reported that exogenous addition of tPA to cultured neuronal cells or injection of recombinant tPA into the striatum of mice promoted the cleavage the NR1 subunit, elevated intracellular calcium levels, and promoted neuronal degeneration (Nicole et al., 2001). Although NR1/2 subunits of the NMDA-type receptors are expressed in the retina (Fletcher et al., 2000; Pourcho et al., 2001), no concrete evidence currently exists to address how tPA might modulate glutamate receptor signaling under normal physiological conditions in the retina. Since previous studies on the CNS indicated that tPA can modulate glutamate receptor signaling under excitotoxic and ischemic conditions, and since copious amounts of tPA are expressed in RGCs under normal conditions as shown in this study and in a previous study (Mali et al., 2005), it is plausible that under normal conditions constitutive levels of tPA are needed to process glutamate receptors to aid rapid processing of the visual information received by photoreceptors. However, under glaucomatous conditions, excessive levels of tPA released into the ECM may promote their degeneration through over-activation of NMDA-type receptors.

3). Can plasmin alone promote the degeneration of RGCs by potentiating glutamate receptor signaling by cleaving the NR1 subunit of the NMDA-type receptors as described for the CNS? Although no concrete evidence currently exists to support or rule out the possible role of plasmin in NMDA-type receptor signaling in the retina, a previous study suggested that it is very unlikely that plasmin promotes the proteolytic cleavage of the NMDA-type glutamate receptors in the CNS (Nicole et al., 2001). Future studies are warranted to investigate some of these possibilities in rodent models of POAG.

5. Conclusions

Results presented in this study show that elevated IOP promotes the degeneration of RGCs by up-regulating the levels of proteolytically active tPA and uPA. Also, results presented in this study show that inhibition of the proteolytic activity of tPA and uPA, or inhibition of tPA and uPA binding to LRP-1 attenuates IOP-mediated degeneration of RGCs.

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Abbreviations

POAG	Primary Open-Angle Glaucoma
IOP	intraocular pressure
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
LRP-1	low density lipoprotein-related receptor-1
RAP	receptor associated protein
RGCs	retinal ganglion cells
CNS	central nervous system
GCL	ganglion cell layer
INL	inner nuclear layer
ONL	outer nuclear layer

References

- Bu G. The roles of receptor-associated protein (RAP) as a molecular chaperone for members of the LDL receptor family. International review of cytology. 2001; 209:79–116. [PubMed: 11580203]
- Bu G, Geuze HJ, Strous GJ, Schwartz AL. 39 kDa receptor-associated protein is an ER resident protein and molecular chaperone for LDL receptor-related protein. Embo J. 1995; 14:2269–2280. [PubMed: 7774585]
- Bu G, Rennke S. Receptor-associated protein is a folding chaperone for low density lipoprotein receptor-related protein. The Journal of biological chemistry. 1996; 271:22218–22224. [PubMed: 8703036]
- Bu G, Schwartz AL. RAP, a novel type of ER chaperone. Trends in cell biology. 1998; 8:272–276. [PubMed: 9714598]
- Burgoyne CF, Downs JC, Bellezza AJ, Suh JK, Hart RT. The optic nerve head as a biomechanical structure: a new paradigm for understanding the role of IOP-related stress and strain in the pathophysiology of glaucomatous optic nerve head damage. Progress in retinal and eye research. 2005; 24:39–73. [PubMed: 15555526]
- Casse F, Bardou I, Danglot L, Briens A, Montagne A, Parcq J, Alahari A, Galli T, Vivien D, Docagne F. Glutamate controls tPA recycling by astrocytes, which in turn influences glutamatergic signals. J Neurosci. 2012; 32:5186–5199. [PubMed: 22496564]
- Cedrone C, Mancino R, Cerulli A, Cesareo M, Nucci C. Epidemiology of primary glaucoma: prevalence, incidence, and blinding effects. Prog Brain Res. 2008; 173:3–14. [PubMed: 18929097]
- Chen H, Wei X, Cho KS, Chen G, Sappington R, Calkins DJ, Chen DF. Optic neuropathy due to microbead-induced elevated intraocular pressure in the mouse. Invest Ophthalmol Vis Sci. 2011; 52:36–44. [PubMed: 20702815]
- Chintala SK. Tissue and urokinase plasminogen activators instigate the degeneration of retinal ganglion cells in a mouse model of glaucoma. Exp Eye Res. 2015

- Choi E, Lee KJ, Seo JW, Byun CJ, Chung SJ, Suh DC, Carmeliet P, Koh JY, Kim JS, Lee JY. Neuroprotection by urokinase plasminogen activator in the hippocampus. *Neurobiology of disease*. 2012; 46:215–224. [PubMed: 22293605]
- Christow SP, Bychkov R, Schroeder C, Dietz R, Haller H, Dumler I, Gulba DC. Urokinase activates calcium-dependent potassium channels in U937 cells via calcium release from intracellular stores. *Eur J Biochem*. 1999; 265:264–272. [PubMed: 10491182]
- Fletcher EL, Hack I, Brandstatter JH, Wassle H. Synaptic localization of NMDA receptor subunits in the rat retina. *The Journal of comparative neurology*. 2000; 420:98–112. [PubMed: 10745222]
- Friedman DS, Wolfs RC, O'Colmain BJ, Klein BE, Taylor HR, West S, Leske MC, Mitchell P, Congdon N, Kempen J. Prevalence of open-angle glaucoma among adults in the United States. *Archives of ophthalmology*. 2004; 122:532–538. [PubMed: 15078671]
- Ganesh BS, Chintala SK. Inhibition of reactive gliosis attenuates excitotoxicity-mediated death of retinal ganglion cells. *PLoS One*. 2011; 6:e18305. [PubMed: 21483783]
- Herz J. LRP: a bright beacon at the blood-brain barrier. *The Journal of clinical investigation*. 2003; 112:1483–1485. [PubMed: 14617749]
- Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. *The Journal of clinical investigation*. 2001; 108:779–784. [PubMed: 11560943]
- Hussain MM. Structural, biochemical and signaling properties of the low-density lipoprotein receptor gene family. *Frontiers in bioscience : a journal and virtual library*. 2001; 6:D417–428. [PubMed: 11229872]
- Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. *Physiological reviews*. 2008; 88:887–918. [PubMed: 18626063]
- Mali RS, Cheng M, Chintala SK. Plasminogen activators promote excitotoxicity-induced retinal damage. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2005; 19:1280–1289. [PubMed: 16051695]
- Martin AM, Kuhlmann C, Trossbach S, Jaeger S, Waldron E, Roebroek A, Luhmann HJ, Laatsch A, Weggen S, Lessmann V, Pietrzik CU. The functional role of the second NPXY motif of the LRP1 beta-chain in tissue-type plasminogen activator-mediated activation of N-methyl-D-aspartate receptors. *The Journal of biological chemistry*. 2008; 283:12004–12013. [PubMed: 18321860]
- Neufeld AH, Liu B. Glaucomatous optic neuropathy: when glia misbehave. *Neuroscientist*. 2003; 9:485–495. [PubMed: 14678581]
- Nicole O, Docagne F, Ali C, Margaill I, Carmeliet P, MacKenzie ET, Vivien D, Buisson A. The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. *Nature medicine*. 2001; 7:59–64.
- Nykjaer A, Conese M, Christensen EI, Olson D, Cremona O, Gliemann J, Blasi F. Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes. *Embo J*. 1997; 16:2610–2620. [PubMed: 9184208]
- Pease ME, McKinnon SJ, Quigley HA, Kerrigan-Baumrind LA, Zack DJ. Obstructed axonal transport of BDNF and its receptor TrkB in experimental glaucoma. *Invest Ophthalmol Vis Sci*. 2000; 41:764–774. [PubMed: 10711692]
- Pourcho RG, Qin P, Goebel DJ. Cellular and subcellular distribution of NMDA receptor subunit NR2B in the retina. *The Journal of comparative neurology*. 2001; 433:75–85. [PubMed: 11283950]
- Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *The British journal of ophthalmology*. 2006; 90:262–267. [PubMed: 16488940]
- Rock N, Chintala SK. Mechanisms regulating plasminogen activators in transformed retinal ganglion cells. *Exp Eye Res*. 2008; 86:492–499. [PubMed: 18243176]
- Sappington RM, Carlson BJ, Crish SD, Calkins DJ. The microbead occlusion model: a paradigm for induced ocular hypertension in rats and mice. *Invest Ophthalmol Vis Sci*. 2010; 51:207–216. [PubMed: 19850836]
- Weinreb RN, Khaw PT. Primary open-angle glaucoma. *Lancet*. 2004; 363:1711–1720. [PubMed: 15158634]

- Whitmore AV, Libby RT, John SW. Glaucoma: thinking in new ways-a role for autonomous axonal self-destruction and other compartmentalised processes? *Progress in retinal and eye research*. 2005; 24:639–662. [PubMed: 15953750]
- Wilhelmus MM, Otte-Holler I, van Triel JJ, Veerhuis R, Maat-Schieman ML, Bu G, de Waal RM, Verbeek MM. Lipoprotein receptor-related protein-1 mediates amyloid-beta-mediated cell death of cerebrovascular cells. *The American journal of pathology*. 2007; 171:1989–1999. [PubMed: 18055545]
- Willnow TE, Rohlmann A, Horton J, Otani H, Braun JR, Hammer RE, Herz J. RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytic receptors. *Embo J*. 1996; 15:2632–2639. [PubMed: 8654360]
- Zhang X, Chaudhry A, Chintala SK. Inhibition of plasminogen activation protects against ganglion cell loss in a mouse model of retinal damage. *Mol Vis*. 2003; 9:238–248. [PubMed: 12813409]

Highlights

- The mechanisms underlying IOP-mediated degeneration of retinal ganglion cells (RGCs) is unclear.
- This study investigated whether plasminogen activators play a role in the degeneration of RGCs in a mouse model of POAG.
- IOP was elevated in mouse eyes by injecting microbeads into the anterior chambers.
- IOP elevation led to an up-regulation in tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), and their receptor LRP-1, and promoted significant degeneration of RGCs.
- Inhibition of tPA and uPA binding to LRP-1 or inhibition of their proteolytic activity attenuated IOP-mediated degeneration of RGCs.
- These results suggest that tPA and uPA play a significant role in IOP-mediated degeneration of RGCs.

Figure 1.

Injection of microbeads leads to IOP elevation in mice and promotes the degeneration of RGCs. (A) Immediately and at 24 h after injecting microbeads, mouse eyes were imaged under general anesthesia by using a Micron III camera. The red arrow indicates that at 24 h after the injections, a majority of the beads were localized in the trabecular meshwork. (B) Mean IOP readings obtained from one cohort of 9 mice indicate that IOP was elevated significantly in microbead-injected eyes (Δ) when compared to the eyes that received PBS (O). $p < 0.05$, when microbead-injected eyes were compared with PBS-injected eyes. To determine the effect of elevated IOP on the degeneration of RGCs, whole retinas isolated from PBS- or microbead-injected eyes were immunostained with Brn3a antibodies. (C) For each retina, Brn3a-positive RGCs in eight areas of equal size (335×445 microns, $20\times$ magnification), were photographed by using a Zeiss digital camera. (D) The number of Brn3a-positive RGCs in the retinas was quantified by using Nikon Elements AR software. Results presented in figure D indicate that the loss of Brn3a-positive RGCs was increased progressively in the retinas isolated from microbead-injected eyes at two, four, six, and eight weeks when compared to the retinas isolated from PBS-injected eyes. $*p < 0.05$ when Brn3a-positive RGCs in the retinas isolated from microbead-injected eyes were compared with Brn3a-positive RGCs in the retinas isolated from PBS-injected control eyes.

Figure 2.

Elevated IOP up-regulates the proteolytic activity of tPA and uPA, and converts plasminogen to plasmin. At two, four, six, and eight weeks after injecting PBS or microbeads, aliquots containing an equal amount of retinal proteins (50 μ g) extracted from PBS- or microbead-injected eyes were subjected plasminogen/fibrinogen zymography. Results presented in figure 2A (top four panels) indicate that the proteolytic activity of uPA, absent completely in retinal proteins extracted from PBS-injected eyes was up-regulated in retinal proteins extracted from microbead-injected eyes. Results presented in figure 2A (lower four panels) indicate that the levels of tPA were elevated in retinal protein extracted from microbead-injected eyes over an eight-week period. * $p < 0.05$, when tPA levels in retinal proteins extracted from microbead-injected eyes were compared with PBS-injected eyes. ** $p < 0.05$, when uPA levels in retinal proteins extracted from microbead-injected eyes were compared with PBS-injected eyes. NS, not significant. (B). Aliquots containing an equal amount of retinal proteins (50 μ g) extracted from PBS- or microbead-injected eyes were subjected to western blot analysis by using an antibody against plasminogen. Results presented in figures 2B and C indicate that a low level of plasminogen was expressed in PBS-injected eyes. In contrast, plasminogen levels were up-regulated in retinal proteins extracted at four weeks after microbead injection. In addition, at eight weeks after microbead injection, a majority of the plasminogen was converted into plasmin.

Figure 3.

Elevated IOP up-regulates tPA in RGCs. (A) Retinal cross sections prepared from PBS- or microbead-injected eyes were immunostained with antibodies against tPA. Results presented in the leftmost panel of the figure indicate that tPA is constitutively expressed in RGCs in retinal cross-sections prepared from PBS-injected eyes (arrow). Results presented in the middle panel indicate that at four weeks after microbead injection, tPA levels were increased in RGCs (arrows) and to some extent in astrocytes (arrow heads). Results presented in the rightmost panel indicate that at 8 weeks after microbead injection, a majority of the up-regulated tPA was localized in the ECM (arrow heads). (B) To confirm whether RGCs are responsible for the synthesis of tPA, whole retinas isolated at six weeks after microbead injection were immunostained with an antibody against tPA and double-labeled with an antibody against Tuj1, a marker for RGCs. Results presented in figure 3B indicate that tPA was localized in Tuj1-positive RGCs.

Figure 4.

Elevated IOP up-regulates uPA in astrocytes. (A) Retinal cross sections prepared from PBS- or microbead-injected eyes were immunostained with antibodies against uPA. Results presented in the leftmost panel indicate that a very low level of uPA was expressed in astrocytes (arrow) in retinal cross sections prepared from PBS-injected eyes. In contrast, uPA protein levels were increased progressively in astrocytes in retinal cross sections prepared from microbead-injected eyes (arrows in the middle and rightmost panels). To determine whether astrocytes are responsible for the synthesis of uPA, whole retinas isolated at six weeks after microbead injection were immunostained with an antibody against uPA, and double-labeled with an antibody against GFAP. Results presented in figure 4B indicate that uPA was localized in GFAP-positive astrocytes.

Figure 5.

Elevated IOP up-regulates LRP-1 in RGCs. (A) Aliquots containing an equal amount of proteins (50 μg) extracted from PBS- or microbead-injected eyes were subjected to western blot analysis by using an antibody against LRP-1. (B) Relative levels of LRP-1 in the western blots was determined by densitometry. Results presented in figures 5A and B indicate that the LRP-1 protein, expressed constitutively, in retinal proteins extracted from PBS-injected eyes was elevated at four and six weeks after microbead injection. (C). Immunohistochemical analysis indicate that at four and six weeks after microbead injection, LRP-1 protein levels were up-regulated in RGCs (arrow heads), but not in other cells in the ganglion cell layer. (D) To confirm whether RGCs are responsible for the synthesis of LRP-1, whole retinas isolated at six weeks after microbead injection were immunostained with an antibody against LRP-1 and double-labeled with an antibody against Tuj1. Results presented in figure 5D indicate that LRP-1 was localized in Tuj1-positive RGCs.

Figure 6.

RAP inhibits the proteolytic activity of tPA and uPA. (A) At eight weeks after the treatment, aliquots containing an equal amount of retinal proteins (50 μg) extracted from PBS-injected eyes and microbead-injected eyes treated with or without RAP were subjected to plasminogen/fibrinogen zymography. Results presented in figure 6A and B indicate that tPA and uPA levels were increased significantly in microbead-injected eyes when compared to PBS-injected eyes. In contrast, a higher concentration of RAP significantly reduced tPA and uPA levels in microbead-injected eyes (C). #p<0.05, when tPA levels in retinal proteins extracted from microbead-injected eyes were compared with retinal proteins extracted from PBS-injected eyes. *, **p<0.05, when tPA and uPA levels from microbead-injected eyes were compared with microbead-injected and RAP-treated eyes.

Figure 7.

RAP attenuates IOP-mediated degeneration of RGCs. At four weeks after injecting the eyes with PBS or microbeads into the anterior chamber, indicated concentrations of RAP or PBS were injected into the vitreous humor. At eight weeks after the treatment, whole retinas were isolated, and the remaining number of RGCs was determined by immunostaining with an antibody against Brn3a. Results presented in figure A indicate that the number of Brn3a-positive RGCs was reduced in microbead-injected eyes when compared to PBS-injected eyes. In contrast, treatment of the eyes with RAP attenuated the degeneration of Brn3a-positive cells in microbead-injected eyes. RAP treatment had no effect on the number of RGCs cells in PBS-injected eyes. (B) Quantitative analysis of Brn3a-positive RGCs indicates a significant decrease in the number of RGCs in microbead-injected eyes when compared to PBS-injected eyes. In contrast, the degeneration of Brn3a-positive RGCs was attenuated significantly in the retinas isolated from RAP-treated eyes when compared to microbead-injected eyes. * $p < 0.05$, when the number of Brn3a-positive RGCs was compared between PBS- and microbead-injected eyes. ** $p < 0.05$, when the number of Brn3a-positive RGCs was compared between microbead-injected eyes and microbead-injected and RAP-treated eyes.

Figure 8.

tPA-Stop inhibits the proteolytic activity of tPA and uPA. At four weeks after injecting PBS or microbeads, indicated concentrations of tPA-Stop or PBS were injected into the vitreous humor. (A) At eight weeks after the treatment, aliquots containing an equal amount of retinal proteins (50 μg) extracted from PBS-injected eyes treated with or without tPA-Stop, and microbead-injected eyes treated with or without tPA-Stop were subjected to plasminogen/fibrinogen zymography. Results presented in the figure 8B and C indicate that tPA and uPA levels were increased significantly in microbead-injected eyes when compared to PBS-injected eyes. In contrast, higher concentration of tPA-Stop significantly reduced tPA and uPA levels in microbead-injected eyes. # $p < 0.05$, when tPA levels in retinal proteins extracted from microbead-injected eyes were compared with retinal proteins extracted from PBS-injected eyes. *, ** $p < 0.05$, when tPA and uPA levels from microbead-injected eyes were compared with microbead-injected and tPA-Stop-treated eyes.

Figure 9.

tPA-Stop attenuates IOP-mediated degeneration of RGCs. At four weeks after injecting the eyes with PBS or microbeads into the anterior chamber, indicated concentrations of tPA-Stop or PBS were injected into the vitreous humor. At eight weeks after the treatment, whole retinas were isolated, and the remaining number of RGCs was determined by immunostaining with an antibody against Brn3a. (A) Microscopic images of Brn3a-positive RGCs indicate that at 8 weeks after microbead injection, the number of RGCs was reduced in microbead-injected eyes when compared to PBS-injected eyes. In contrast, the degeneration of RGCs was attenuated in the retinas isolated from microbead-injected and tPA-Stop-treated eyes, when compared to the retinas isolated from microbead-injected eyes alone. tPA-Stop treatment had no effect on the number of RGCs in PBS-injected eyes. (B) Quantitative analysis of Brn3a-positive RGCs indicates a significant decrease in the number of RGCs in the retinas isolated from microbead-injected eyes, when compared to PBS-injected eyes. In contrast, the degeneration of RGCs was significantly reduced in the retinas isolated from microbead-injected and tPA-Stop-treated eyes when compared to microbead-injected eyes alone. * $p < 0.05$, when the number of Brn3a-positive RGCs in the retinas from microbead-injected eyes was compared with RGCs in the retinas isolated from PBS-injected eyes. ** < 0.05 , when the number of Brn3a-positive RGCs in the retinas isolated from microbead-injected eyes was compared with RGCs in the retinas isolated from microbead-injected and tPA-Stop-treated eyes alone.