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Research article

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 μM) or tPA-Stop (1.0 and 4.0 μM) 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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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

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.

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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 Fig. 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 ± SEM. At 4 weeks after injecting microbeads, mice eyes ($n = 18$; 2 cohorts of 9) were treated with intravitreal injections of PBS (2 μL), RAP (2 μL), or tPA-Stop (2 μL) 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 μL of extraction buffer (1% Nonidet-P40, 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_3VO_4, pH 7.4) without protease inhibitors, and the tissues were homogenized. Retinal tissue homogenates were centrifuged at 7840 g for 5 min 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 μg) 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 μg/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 destained 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 ± 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 μg) 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

2.6.1. 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 h at room temperature (RT) with 5% BSA prepared in PBS. Retinal cross sections were washed three times with PBS and permeabilized for 15 min 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

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