During Glaucoma, α2-Macroglobulin Accumulates in Aqueous Humor and Binds to Nerve Growth Factor, Neutralizing Neuroprotection

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PURPOSE. Glaucoma is an optic neuropathy caused by the chronic and progressive death of retinal ganglion cells (RGCs), resulting in irreversible blindness. Ocular hypertension is a major risk factor, but RGC death often continues after ocular hypertension is normalized, and can take place with normal tension. Continuous RGC death was related in rodents and humans to the local upregulation of neurotoxic proteins, such as TNF-α. In rat models of glaucoma, ocular hypertension also upregulates the expression of α2-macroglobulin, which is neurotoxic. α2-macroglobulin upregulation in the retina is long-lived, even after high IOP is reduced with medication. α2-macroglobulin is examined as a possible biomarker in human glaucoma, and a possible neurotoxic mechanism of action is sought.

METHODS. Quantitative Western blotting of α2-macroglobulin in samples obtained from aqueous humor (human and rat) and retina (rat) was conducted. Ex vivo neuronal survival assays and nerve growth factor–α2-macroglobulin binding studies using surface plasmon resonance were used.

RESULTS. Increased soluble α2-macroglobulin protein is also present in the aqueous humor in a rat glaucoma model, as well as in the aqueous humor of human glaucoma patients but not in cataract patients. One mechanism by which α2-macroglobulin is neurotoxic is by inhibiting the neuroprotective activity of nerve growth factor via TrkA receptors.

CONCLUSIONS. This work further documents a potential novel mechanism of RGC death and a potential biomarker or therapeutic target for glaucoma. (Invest Ophthalmol Vis Sci. 2011; 52:5260–5265) DOI:10.1167/iovs.10-6691

Vision impairment caused by glaucoma affects more than 60 million people worldwide.1 The main characteristic of primary open angle glaucoma (POAG) is the visual field loss and the thinning of the retinal nerve fiber layer (RNFL) caused by the death of retinal ganglion cells (RGCs).2 Glaucoma is often concomitant with elevated IOP, but the prevalence of normal tension glaucoma (glaucoma with no increase in IOP) calls into question the traditional pathogenetic theory of high pressure.

The main treatment for POAG and for normal tension glaucoma patients is the reduction of the IOP to the “target IOP” which varies from patient to patient. While treatments are often successful at reducing or normalizing high IOP, the progressive loss of RGCs, optic nerve fibers, and visual field may continue albeit at a lower rate.3–7

The exact etiology of RGC death in glaucoma remains unknown, and it is likely multifactorial. The many proposed mechanisms of RGC death in glaucoma8–12 fail to explain the pathologic process of normal tension glaucoma, and also fail to explain why normalization of pressure does not result in the complete arrest of RGC death. There is strong evidence in experimental models,13,14 and humans,15,16 that ocular hypertension alters the expression of neurotoxic cytokines in retina, such as TNF-α, which can cause RGC death. The increases in cytokine expression are long-lived, which could in part explain the paradox of continuing RGC death with normal IOP.17

We recently reported that another gene and protein, α2-macroglobulin (α2M), was upregulated in two rat experimental glaucoma models (the episcleral vein cauterization and the hypertonic saline injection glaucoma models).18 Upregulation of α2M in retina is long-lived even after pharmacologic normalization of high IOP, which might explain continuing RGC death with normal IOP. Intravitreal injection of soluble α2M causes progressive RGC death.19 Neutralization of α2M during glaucoma is neuroprotective for RGCs.16,18,19 Moreover, α2M is produced by retinal glia, and appears to colocalize with TNF-α.20 This provides a potential link to RGC death, and a potential explanation as to why normalization of IOP does not completely arrest visual field loss.

Herein, we show that α2M protein is not only present in retina, but is also increased as a soluble factor in the aqueous humor in a rat glaucoma model. Moreover, we show that soluble α2M protein is increased in the aqueous humor of human eyes with glaucoma compared to human eyes with cataracts. One RGC death–causing mechanism for α2M protein is to bind to nerve growth factor (NGF), neutralizing the neuroprotective action of this neurotrophin. This work offers the opportunity to study molecular mechanisms underlying neuronal loss in glaucoma, and it may yield novel therapeutic approaches.

MATERIALS AND METHODS

Animals. Female Wistar rats (250–300 g; Charles River Laboratories International, Inc., Wilmington, MA) were kept in a 12-hour light/
dark cycle with food and water ad libitum. All animal procedures adhered to Institutional Animal Care and Use Committee (IACUC) recommendations and were approved by the Animal Welfare Committee.

**Induction of High IOP.** The episcleral vein cauterization (EVC) method was used to induce elevated IOP. EVC was performed in the right eye of rats under anesthesia as previously described.\textsuperscript{18,21,22} with minor modifications according to Laquis et al.\textsuperscript{23} The left eye in each animal was used as normal IOP control after sham surgery (conjunctival incisions with no cauterization). Planar ophthalmoscopy was used to confirm normal perfusion of the retina at elevated IOPs.

**IOP Measurements.** IOP was measured using a Tonopen XL tonometer under light anesthesia (a gas mixture of oxygen and 2% isofluorane mixture, at a rate of 2.5 L/min, as per IACUC recommendations), as described.\textsuperscript{24} Initially, IOP was measured immediately after anesthesia was established, and then every week after EVC until the endpoint of each experiment. Four consecutive readings were obtained from each eye with a coefficient of variation <5%. The mean normal IOP of rats under light anesthesia was ~12 mm Hg (range, 10–14 mm Hg), and in cauterized eyes it is elevated to a stable average ~21 mm Hg (range, 18–24 mm Hg).

**Biochemical Quantification of α2M**

**Sampling Retina and Aqueous Humor in Rat.** For rat retina α2M analysis, glaucomatous (OD) and the normal contralateral control (OS) retinas of each animal were detergent solubilized and then studied by Western blotting standardized to β-actin loading control. For digital quantification, membranes were scanned and analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The ratio of the OD versus OS was calculated, and results were averaged ± SEM (n = 3 to 4 animals per group). For α2M analysis in aqueous humor, samples from glaucomatous (OD) and the contralateral control eyes (OS) of each animal were studied, and were standardized to immunoglobulin-heavy chain loading control. The ratio of the glaucomatous versus normal aqueous humor was calculated, and results were averaged ± SEM (n = 3 to 4 animals per group). Aqueous humor was collected under an operating microscope through a central corneal paracentesis with a 30-gauge needle connected to a 1-ml syringe with special care taken to avoid blood contamination. Aqueous humor was immediately combined with 2× SDS loading buffer.

**Sampling Human Aqueous Humor.** Aqueous humor (0.1–0.2 ml) was rapidly and carefully collected at the beginning of the surgery through a corneal paracentesis, using a 27-gauge needle connected to a tuberculin syringe under an operating microscope with special care taken to avoid blood contamination. Aqueous humor was immediately frozen, then was combined with 2× SDS loading buffer. Patients gave informed consent to allow the collection (see Table 1 for a description of clinical data).

**Western Blot Analysis.** Fifteen micrograms of retinal proteins per lane were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The α2M protein was detected using goat polyclonal antibodies against α2M (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:3000 dilution. Pure activated rat α2M protein (Sigma Chemical, Saint Louis, MO) was loaded as control. Goat anti-rabbit antibodies conjugated with horseradish peroxidase (HRP; Sigma Chemical) were used as secondary reagents. For digital quantification, membranes were scanned and analyzed using ImageJ software.

**Retrograde RGC Labeling.** RGCs were retrogradely labeled with 4% Fluorogold (Fluorochrome, Englewood, CO) as previously described.\textsuperscript{18,21,22} Retrograde labeling was performed 7 days before rats were killed. Quantification of retrogradely labeled RGCs was performed on freshly isolated retinas from control (sham-operated) or from glaucoma eyes at the indicated days after cauterization.

**Quantification of RGC Survival.** Quantification of labeled RGCs was performed as reported previously.\textsuperscript{18,21,22,25} Seven days after retrograde labeling, both eyes were enucleated, the anterior parts were cut out, and the remaining part was fixed in 4% paraformaldehyde (PFA) for 30 to 45 minutes. Retinas were dissected and flat-mounted on glass slides with the vitreous side up. The retinas were observed under fluorescence microscopy (Carl Zeiss, Jena, Germany) with 12 pictures per slide, and counts were performed in the inner nuclear layer (INL) and outer nuclear layer (ONL). Results were averaged ± SEM (n = 3 to 4 animals per group).

**Table 1. Clinical History of the Patients Whose Samples Were Used in the Study**

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<th>Code</th>
<th>Age, y/SEX</th>
<th>Diagnosis</th>
<th>VA, C/D</th>
<th>IOP, mm Hg</th>
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A, acetazolamide; B, bimatoprost; Br, brimonidine; CACG, chronic angle closure glaucoma; C/D, cup to disc ratio (noted only in glaucoma patients); CF, count fingers; CRVO, central retina vein occlusion; D, dorzolamide; F, female; HM, hand motion; IOP, intraocular pressure (preoperative); JOAG, juvenile open angle glaucoma; L, latanoprost; M, male; NVG, neovascular glaucoma; P, pilocarpine; POAG, primary open angle glaucoma; T, timolol; VA, visual acuity; XFG, exfoliation syndrome glaucoma.

* Taken for glaucoma.
per retina at 20× magnification. For each retina, three digital images from each quadrant (superior, temporal, inferior, and nasal) at a radial distance of 1 mm, 2 mm, and 3 mm from the optic nerve were taken. Each 20× magnification field exposes an area of 0.2285 mm². In each independent retina, at least 2.742 mm² were analyzed. Microglia and macrophages that incorporated Fluorogold after phagocytosis of dying RGCs were excluded from our analysis according to their morphology, as reported.²⁶ Samples and images were coded and RGC counting was done by two experimenters blinded to the code.

**Standardization of RGC Survival.** Standardization of RGC counts and RGC loss in the test eyes were performed versus RGCs counted in contralateral normal IOP control eyes (100% RGC counts). Percent loss of RGCs were calculated using the formula \((1 - \frac{RGC_{test}}{RGC_{control}})\times100\).

**Surface Plasmon Resonance–Binding Studies.** Pure α2M was treated with methylamine to activate it, as previously described.¹⁸ For immobilization on the sensor chip, the protein was prepared at 5 μg/ml in 10 mM sodium acetate coupling buffer, pH 4.5, and immobilized to a CM4 chip using N-(3-dimethylaminopropyl)-N-ethylcarboxydimide/N-hydroxysuccinimide-coupling chemistry on a Biacore 3000 instrument (Biacore, Piscataway, NJ) as previously described.²⁷ All reagents were automatically introduced over the sensor chip in 10 mM HEPES, pH 7.4, 0.15 M NaCl, and 0.005% v/v surfactant P20 (HBS-P) at a flow rate of 30 μl/min with a blank chip subtracted as control for nonspecific surface binding. Binding isotherms were determined at 25°C. The sensor chip surface was regenerated by treating with 10 mM glycine-HCl, pH 2, at a flow rate of 10 μl/min. The sensogram data were evaluated with the BlAevaluation software (version 3.2; Biacore, Uppsala, Sweden). Increasing concentrations of wild type NGF were used as analyte. TGFs-β and α were also tested, because reports indicated that they bind to α2M.²⁸²⁹

**PC12 Survival Assay.** PC12 cells (7500 cells/well) were cultured in 96-well plates (Falcon, Lincoln Park, NJ) in protein-free hybridoma medium (PFH-M; Gibco, Grand Island, NY) supplemented with 0.2% BSA (SFM). Wells were supplemented with either serum (5% final) or with NGF (4 nM final), in the presence or absence of α2M (200 nM final). Wells containing all culture conditions but no cells were used as blanks. The growth/survival profile of the cells was quantified with 0.2% BSA (SFM). Wells were supplemented with either serum (5% final) or with NGF (4 nM final), in the presence or absence of α2M (200 nM final). Wells containing all culture conditions but no cells were used as blanks. The growth/survival profile of the cells was quantified using MTT (Sigma) 72 hours after plating. The NGF-promoted survival of PC12 cells in these conditions is known to be TrkA-mediated.³⁰⁻³¹

**Data Analysis.** Statistical analyses used Systat 10.0 (SPSS Inc., Chicago, IL). Data were subjected to ANOVA, and P values are reported. RGC loss and IOPs are reported as mean ± SEM. The fold-increase in α2M is reported as mean ± SEM. PC12 cell survival is reported as mean ± SD.

**RESULTS**

**Induction of IOP**

High IOP was induced in rat eyes by cauterizing three episcleral vessels of the right eye to reduce aqueous humor outflow, and the contralateral eyes were sham-operated and were used as controls (Fig. 1A). Cauterization causes an average increase of ~1.7-fold in IOP. The mean IOP in glaucomatous eyes was ~21 mm Hg compared with a mean IOP of ~12.3 mm Hg in normal contralateral eyes. The IOP of cauterized eyes was significantly higher than noncauterized control eyes at all days after cauterization (P ≤ 0.01), until the endpoint day 42.

**RGC Death Induced by High IOP**

Chronic high IOP causes progressive and cumulative RGC loss at a constant rate (~3% to 4% per week). From week 1 to week 6 postcauterization, there is a significant average loss of fluorogold-labeled RGCs versus normal IOP controls (P ≤ 0.01; Fig. 1B).

![Kinetics of progression of RGC loss in eyes with high IOP.](https://example.com/fig1.png)

**Localization of Soluble α2M in Retina and Aqueous Humor**

Previously, we showed that α2M is produced and upregulated in the inner plexiform layer and glia/Müller cells during glaucoma, using in situ mRNA hybridization and immunohistochemistry. Here we confirm those findings by studying α2M protein by quantitative Western blot analyses in lysates prepared from whole retina in a rat model of glaucoma, after 14 days of high IOP (Fig. 2A). In addition, because α2M is a secreted protein, we studied whether it could be present in the aqueous humor collected from the same eyes (Fig. 2B). In both the retina and the aqueous humor, there was a significant increase in α2M protein when each eye was evaluated versus a normal contralateral eye.

**DISCUSSION**

α2M protein changes during the time course of high IOP revealed that the increases in α2M protein in retina and aqueous humor take place early, with a 7 days of ocular hypertension. In this period of ocular hypertension, there is only ~4% RGC death (see Fig. 1B).

In the timeframe of these experiments, the maximal increase of α2M protein in rat retina is at day 14 (fold-increase of 5.2 ± 1.1; P < 0.01); and the maximal increase of α2M protein in rat aqueous humor is at day 28 (fold-increase of 4.1 ± 1.2; P < 0.01; Fig. 2C). These data suggest that the full increase in aqueous humor is slightly delayed with respect to retina. Similar studies using human aqueous humor (Fig. 2D) showed α2M protein to be increased in patients with either glaucoma, or glaucoma and cataracts, compared to patients with cataracts only. Quantification of the Western blots comparing glaucoma to cataracts showed a significant increase of
2M protein in human aqueous humor (fold-increase of 3.5 ± 0.77; \( P < 0.05 \); Fig. 2E).

Mechanism of Action of 2M

Among the many functions of 2M, it has been reported that it can bind to NGF, but unambiguous studies are lacking. We asked whether 2M can bind to NGF, and whether this might result in the neutralization of the neuroprotective signals that NGF mediates through the receptor TrkA.

In direct binding studies using surface plasmon resonance methods, soluble NGF binds to immobilized 2M with relatively high affinity and saturability \( (K_d \sim 172 \text{ nM}; \text{ Fig. } 3A) \). Although reports using other techniques had suggested that TNF-\( \alpha \) and -\( \beta \) bind to 2M,\(^{28,29} \) in our assays TNF-\( \alpha \) did not bind to 2M (Fig. 3A, inset). However, TGF-\( \beta \) did bind to 2M (data not shown).

In functional assays (Fig. 3B), PC12 cells cultured in serum-free conditions are stressed and die. Cells can be rescued from death by supplementing 5% serum (containing many growth factors) or by supplementing 4 nM NGF, and this protection is known to occur through TrkA activation.\(^{30,31} \) The addition of soluble 200 nM 2M antagonizes most of the protective action of NGF. These data suggest that 2M upregulated in retina during glaucoma can bind to endogenous NGF produced in the retina and prevent TrkA-NGF trophic neuroprotection.

DISCUSSION

In previous work, we showed that in two rat models of glaucoma, the 2M gene and protein were upregulated after only \( \sim 7 \) days of high IOP. The 2M expression was sustained and persisted for more than 20 days independent of continuous ocular hypertension. Induction of 2M mRNA was specific to high IOP, and it did not increase after optic nerve axotomy. Therefore, short-term ocular hypertension is sufficient to cause specific and long-lasting increases of 2M in the retina. In addition, 2M was implicated in RGC death in glaucoma: neutralization of 2M in the vitreous was protective to RGCs during glaucoma\(^{18} \), and inhibiting production of 2M in the retina during glaucoma reduced RGC death.\(^{19,20} \)
Here we expand previous literature to show higher presence of soluble a2M in the aqueous humor of rats with glaucoma, and in the aqueous humor of human eyes with glaucoma. While the biologic significance of a2M in aqueous humor remains to be determined, there may be value in using this protein as a potential biomarker of disease.

In the rat model of glaucoma, there is only a slight delay in the maximal increase of a2M protein in aqueous humor with respect to the retina. Therefore, it may be possible to use aqueous humor as a surrogate of the a2M protein levels in the retina. This may be useful because it is not possible to collect patient’s retinas, but it is possible to collect aqueous humor.

It is attractive to speculate that one source of a2M protein found in aqueous humor may be from retina. In the retina, a2M mRNA and proteins are expressed during glaucoma and are upregulated by glia. It is possible that after being secreted by retinal glia, a2M can diffuse to the vitreous and then to the aqueous humor in the anterior chamber of the eye. This is possible because the aqueous humor is actually produced in the posterior chamber and flows between the lens and iris, into the anterior chamber, where a2M may accumulate in the aqueous humor.

From the anterior chamber, the aqueous humor containing a2M and other proteins normally drains out of the eye via the trabecular meshwork, into the Schlemm’s canal via the aqueous vein or via collector channels to the episcleral veins. In the rat glaucoma models, and in human glaucoma, impaired draining may cause a “backflow” with a corresponding buildup of a2M in the aqueous humor in the anterior chamber and increased a2M in the vitreous in the posterior chamber.

However, we cannot rule out that the source of a2M that is found in aqueous humor can be also from liver or from endothelial cells. Indeed, serum a2M protein is produced primarily by the liver, from where it is secreted into circulation.

In this article, we also show a putative mechanism of action for the neurotoxicity mediated by soluble a2M. Soluble a2M can bind to NGF with high affinity and can neutralize NGF activation of the TrkA receptors. Because endogenous NGF-TrkA activity is needed for the normal maintenance of RGCs and the survival of RGCs under stress, the neutralization of NGF by a2M might be associated with RGC death. In contrast, the pharmacologic use of agents that activate TrkA but that are not subject to neutralization by a2M do protect RGCs in experimental glaucoma. These findings might explain, to some degree, why exogenous NGF does not readily protect RGCs in experimental glaucoma. Our findings might also explain the few reports that do show some efficacy by NGF. In these cases, NGF was delivered at extremely high doses and frequencies. Very high doses and frequencies of NGF might be able to overcome the neutralization of NGF related to a2M overexpression during glaucoma.

In summary, we present in vivo evidence that ocular hypertension regulates a2M as a key gene product in a rat model of glaucoma, and that this protein is present in aqueous humor of rat and human glaucoma eyes. We also show evidence that a2M can be neurotoxic by reducing the ability of NGF to activate protective signals via its prosurvival receptor TrkA. Future work will focus on studying the expression of a2M in normal tension glaucoma. This work contributes to our understanding of molecular mechanisms underlying the etiology of this disease, and may result in the identification of novel mechanisms or biomarkers of glaucoma.

Acknowledgments

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References


