

ORIGINAL ARTICLE



Variable expression of alpha-2-macroglobulin and tumor necrosis factor- α in the aqueous humor of patients with pseudoexfoliative syndrome with and without glaucoma

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**Abstract**

Purpose: The purpose of this study was to explore the involvement of two known mediators of the apoptotic processes in ganglion cells, the proteins alpha-2-macroglobulin (α_2M) and tumor necrosis factor- α (TNF- α), in the pathogenesis of primary open-angle glaucoma (POAG) and pseudoexfoliation (PXF) syndrome.

Methods: Aqueous humor samples were collected at the beginning of cataract surgery. Levels of α_2M and TNF- α were measured using western blot analysis and expressed as fold values. Commercially available pure α_2M protein and TNF- α were used as controls. For digital quantification, nitrocellulose membranes were scanned and analyzed using Image J software.

Results: Levels of α_2M level were measured in 61 samples, which included 18 with PXF, 15 with POAG, and 28 controls. Mean levels of α_2M in the aqueous humor of patients with PXF without glaucoma were comparable to control levels and lower than for patients with POAG (0.63 ± 0.1 ; 0.6 ± 0.05 ; and 0.92 ± 0.1 , respectively, $P = 0.05$). TNF- α levels were measured in 25 aqueous humor samples including eight with PXF, seven with POAG, and 10 controls. TNF- α protein fold level values were higher in patients with glaucoma, with and without PXF, and compared to control values (0.77 ± 0.1 and 0.5 ± 0.4 compared to 0.06 ± 0.01 , respectively, $P = 0.05$ post-log conversion).

Conclusion: This work further documents the role of α_2M protein in the apoptotic process in ganglion cells related to increased IOP but marks its absence in PXF material accumulation without glaucoma. Our findings regarding TNF- α levels may still suggest a role for this protein in PXF syndrome alone.

Introduction

Primary open-angle glaucoma (POAG) is a chronic progressive optic neuropathy, with progressive loss of visual field, associated with increased intraocular pressure (IOP) as a primary comorbidity or risk factor.^[1] It is still unknown why some patients have optic neuropathy despite constant normal measured IOP while others may have increased IOP and present no optic neuropathy. It is even less clear why nerve damage and visual field loss may continue to advance even in patients achieving normal IOP under optimal treatment.^[2-5]

The common pathway of the chronic glaucoma of various etiologies is apoptosis of retinal ganglion cells (RGCs), causing gradual visual field constriction, with possible blindness at the final stages.^[6] Therefore, identifying proteins participating in the apoptosis cascade are of a paramount importance, with alpha-2-macroglobulin (α_2M) and tumor necrosis factor- α (TNF- α) already shown to be part of that group.^[7-11]

The expression of these two proteins not only is upregulated in the eye after IOP elevation but also remains high long after IOP is normalized. The facts that α_2M appears to colocalize with TNF- α in Müller glia that both proteins can be neurotoxic

when elevated in their expression and that expression of both proteins remains elevated even after IOP normalization provides a potential link to RGC apoptosis, as well as a potential explanation to why IOP normalization does not always arrest visual field loss.^[12-14]

Pseudoexfoliation (PXF) syndrome, in which proteinaceous material is deposited in the trabecular meshwork, may lead to IOP elevation and further development of PXF glaucoma.^[15] This syndrome provides us with the opportunity to further investigate whether α_2 M and TNF- α are directly affected by increased IOP or confluent by additional mechanisms.

For this purpose, we measured α_2 M and TNF- α in the aqueous humor of patients with PXF, with and without glaucoma, and compared them to patients with POAG and cataract patients.

Methods

Aqueous humor sampling

Aqueous humor (20 μ L–200 μ L) was carefully collected at the beginning of cataract surgery from patients with PXF syndrome with or without glaucoma, POAG, and normal cataract patients.

Collection was performed through a corneal paracentesis, using a small cannula connected to a tuberculin syringe under an operating microscope. Special care was taken to avoid blood contamination and to collect immediately after the incision. All patients gave informed consent to allow the collection. Aqueous humor was combined with 2% sodium dodecyl sulfate (SDS) and immediately frozen to preserve the samples. The study was approved by the institutional ethics committee.

Western blot analysis

About 10 μ g of total aqueous humor proteins were resolved in SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The α_2 M protein was detected using rabbit polyclonal antibodies against α_2 M (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:2000 dilution. TNF- α protein was detected using rabbit polyclonal antibodies against TNF- α (Peprotech) at a 1:2000 dilution. Commercially available pure α_2 M protein and TNF- α were used as controls. Goat anti-rabbit antibodies conjugated with horseradish peroxidase (HRP; Sigma Chemical) were used as secondary reagents. For digital quantification, nitrocellulose membranes were scanned and analyzed using ImageJ software.

Statistical analysis

To compare between the groups, each reading was converted to its Log value, to minimize the effect of extreme values on both sides. Log data were subjected to ANOVA and Tukey's *post hoc* test, with *P* values reported. The fold increase in α_2 M and TNF- α is reported as the mean \pm standard mean of the error.

Results

α_2 M protein

Aqueous humor samples from 61 eyes of 61 patients were analyzed for the protein levels and included 18 patients diagnosed with PXF, 15 with POAG, and 28 patients with cataract served as controls. The mean level of α_2 M protein in the aqueous humor of patients with PXF without glaucoma was similar to control samples and lower than patients with POAG (0.63 ± 0.1 ; 0.6 ± 0.05 ; and 0.92 ± 0.1 , respectively) ($P = 0.05$ post-log conversion). In a small subgroup of four patients with PXF glaucoma, the mean level of α_2 M was 1.4 ± 0.5 , was highest, and comparable to the mean level in patients with POAG. Data and western blot analysis examples are presented in Figure 1.

TNF- α protein

A total of 25 aqueous humor samples were further analyzed for TNF- α , in eight eyes with PXF, seven eyes with POAG, and 10 eyes of controls. TNF- α protein fold level values were highest in patients with glaucoma, with and without PXF (0.77 ± 0.1 and 0.5 ± 0.4 , respectively), and compared to control values (0.06 ± 0.01 , $P = 0.05$ post-log conversion). Mean levels of TNF- α protein in patients with PXF without glaucoma were 0.29 ± 0.1 , suggesting an intermediate value, not reaching statistically significant change from the other study groups. Data and western blot analysis examples are presented in Figure 2.

Discussion

Lowering IOP is still the main target of glaucoma treatment. Yet, it is well recognized that none of the current medical or surgical interventions provide the anticipated prevention of further deterioration of the disease.^[16] Therefore, it is becoming clear that therapy should aim to target other mechanisms of action.^[17-19]

The concepts of neuroprotection to reduce RGC death and reduce neurotoxicity to prevent neuronal death have been pursued experimentally.^[11] The present study validates that both α_2 M and TNF- α may be etiological in retinal neuropathies, as presented by other studies in glaucoma.^[8,14,20] In our study, we also found elevated intraocular levels of both α_2 M and TNF- α in patients with glaucoma defined as optic neuropathy related to increased IOP. In addition, we also show comparable elevated levels of α_2 M in eyes with glaucoma with and without PXF, but not in eyes with PXF and no glaucoma.

TNF- α was found elevated in the aqueous humor in eyes that developed glaucoma with or without PXF. Levels in eyes with PXF and no glaucoma were slightly elevated compared to control samples, though this trend did not reach statistical significance. To the best of our knowledge, this is the first study to coinvestigate α_2 M protein and TNF- α in PXF patients.

α_2 M is a soluble acute phase protein, with both receptor mediated and intrinsic activities. It has been investigated for its

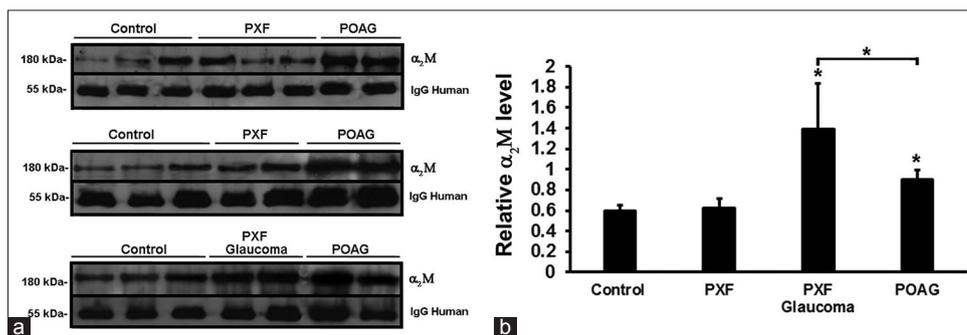


Figure 1: Comparison of alpha-2-macroglobulin (α_2M) protein levels between the four groups of patients. Western blot analysis of α_2M protein demonstrating increased expression of α_2M protein in pseudoexfoliation (PXF) glaucoma and primary open-angle glaucoma (POAG) (a). Equal amounts of protein from aqueous humor were loaded in every lane (15 μ g), and blots were probed with anti- α_2M antibody or with anti-human IgG-Fc (heavy chain) as control. Mean level of α_2M in the aqueous humor of patients with PXF glaucoma and patients with POAG was higher than the mean level in patients with PXF without glaucoma and in normal control samples (b)

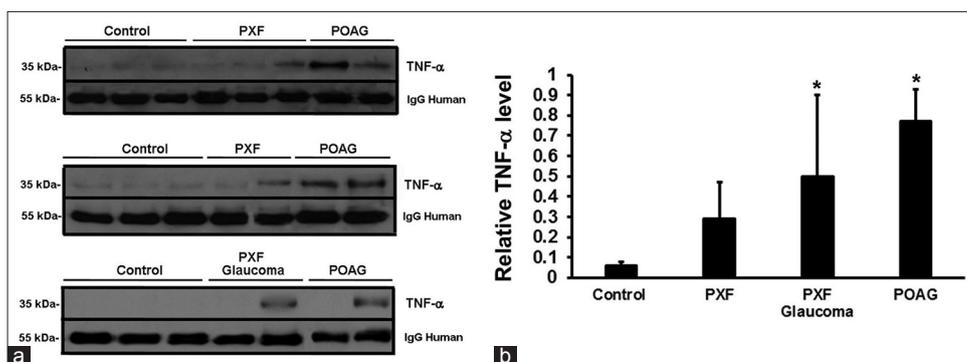


Figure 2: Comparison of tumor necrosis factor- α (TNF- α) protein levels between the four groups of patients. Western blot analysis of TNF- α protein demonstrating increased expression of the protein in PXF glaucoma and primary open-angle glaucoma (POAG) (a). Equal amounts of protein from aqueous humor were loaded in every lane (15 μ g), and blots were probed with anti-TNF- α antibody or with anti-human IgG-Fc (heavy chain) as control. Mean level of TNF- α in the aqueous humor of patients with PXF glaucoma and patients with POAG was higher than the mean level in normal control patients (b). An intermediate value was measured in patients with PXF without glaucoma

roll in other diseases including Alzheimer and Parkinson.^[21,22] Due to its protease inhibitory function, it can enhance procoagulant properties and possesses radioprotective effects.^[23] In the eye, α_2M is produced by retinal glia^[11,12] and was found elevated as a soluble factor in the aqueous humor of eyes with glaucoma.^[13] As true for other key proteins, α_2M function is multifactorial, and includes, inhibiting the neuroprotective activity of nerve growth factor (NGF) through TrkA receptors, and enhanced the neurotoxic effect of proNGF through p75NTR receptor, α_2M -LRP1 interactions, and immune bystander effects as well as glutamatergic stress.^[10-13] Shi *et al.* studied α_2M protein gene expression along with RGCs loss in a glaucoma model in rats.^[10] They found α_2M gene to be specifically regulated by ocular hypertension, not by RGCs death, as it was elevated by inducing high IOP but not by nerve axotomy. Injecting the protein to normal rat eyes induced RGC death, without increased IOP. The changes described in this study were long lasting and independent of continuous high IOP. The authors concluded that α_2M protein is a critical mediator in the cascade of RGC death. The presence of this protein in normal retinas, as

well as the delay in its effect after high IOP, suggests that it is not directly cytotoxic, but rather that it initiates a signal leading to RGC death further along the process.^[10] A significant portion of the proNGF and NGF in diseased or injured tissue is in the α_2M -bound state. This would result in potentiation of neurotoxic proNGF and neutralization of neuroprotective mature NGF in diseased tissue, thereby exacerbating the death of injured cells.^[10]

Our findings of the elevated levels of α_2M in patients with glaucoma regardless of the presence of PXF, but not in patients with PXF and no glaucoma, further support the role of α_2M in the pathogenesis of optic neuropathy triggered by increased IOP, rather than in the pathogenesis of PXF alone.

TNF- α , a well-investigated pro-inflammatory protein, targeted in the treatment of immune-mediated diseases, including uveitis, was also found to be elevated after spikes of elevated IOP.^[7] The proposed connection between TNF- α and RGCs apoptosis is also multifactorial. It acts through receptor-mediated caspase cascade, mitochondrial dysfunction, and oxidative damage to cause RGCs programmed cell death.^[7,8,10] Bai *et al.* demonstrated that TNF- α knockout mice were resistant

to glaucomatous RGC cell death. Another interesting finding in those mice was that α_2M protein levels were not elevated, in contrast to wild-type mice.^[12]

The exact process leading to the accumulation of pseudoexfoliation material in the eye is still unclear. Glaucoma occurs when this material localizes in the anterior chamber angle, leading to partial obstruction of the aqueous flow, resulting in increased IOP.^[15] Our findings, therefore, may suggest either that the pseudoexfoliation material correlates with higher levels of inflammatory mediators or that the decrease in flow and increased IOP is the cause for higher levels of these proteins. Aqueous humor of PXF and PXF glaucoma patients had been studied in the past, for the detection of various cytokines and pro-inflammatory mediators.^[24-26] Decreased concentration of ascorbic acid in the aqueous humor of patients with PXF was reported by Koliakos *et al.* and further confirmed by Sarenac Vulovic *et al.* in their patients with PXF glaucoma.^[25,26] The latter group found these low levels to be associated with increased levels of TNF- α in the aqueous humor, in both patients with PXF and PXF glaucoma alike.^[26] Another study using multiplex bead immunoassay system for cytokines' measurement did not detect differences between similar groups.^[27] Our finding further supports a possible separate role for TNF- α protein in PXF disease, not related to glaucoma, elevated IOP, or RGC death.

The limitations of our study include the small number of patients with PXF and PXF glaucoma. We found that collecting samples from patients with PXF glaucoma were particularly challenging since most patients with PXF are encouraged to undergo cataract surgery early, to reduce the risk of surgical complications. Another limitation stems from the fact that those higher factor levels could be affected by higher levels of IOP, specifically in regard to intraocular surgery.

In summary, we found α_2M protein to be elevated in the aqueous humor of patients with PXF glaucoma but not PXF alone. Its absence in eyes with PXF alone further confirms the role of this protein in the apoptosis of RGC related to increased IOP, but not PXF material accumulation. TNF- α that was found elevated not only in glaucoma patients but also to some extent in PXF without glaucoma suggests some role of this protein in PXF syndrome alone.

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