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MYOCILIN LEVELS IN PRIMARY OPEN-ANGLE GLAUCOMA AND PSEUDOEXFOLIATION GLAUCOMA HUMAN AQUEOUS HUMOR

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Abstract

Purpose—To determine the concentration of myocilin in primary open-angle and pseudoexfoliation glaucoma aqueous humor.

Methods—Aqueous humor was collected during surgery from patients with primary open-angle glaucoma (POAG), pseudoexfoliation glaucoma (PEXG), and elective cataract removal (control). Volume-equivalent aqueous samples were separated on SDS-PAGE gradient gels. Quantification of myocilin levels was performed using Western blots probed with two independent N-terminal polyclonal anti-myocilin antibodies (AB1 and AB2) followed by densitometry. Myocilin levels in aqueous humor were quantified by plotting the densitometry readings of the aqueous samples against a recombinant myocilin standard curve. Total protein concentration was determined by Bradford protein assay. Transforming growth factor beta 2 (TGFβ2) levels were assessed by ELISA.

Results—Myocilin levels are significantly elevated in human POAG aqueous humor when compared to control aqueous humor (AB1: 0.66 ± 0.53 ng/μl vs. 0.23 ± 0.20 ng/μl, $p<0.001$; AB2: 0.98 ± 0.59 ng/μl vs. 0.65 ± 0.5 ng/μl, $p<0.03$; mean ± SD). Myocilin makes up a larger percent of the total protein in POAG aqueous humor compared to control aqueous (AB1: 0.26 ± 0.20% vs. 0.10 ± 0.20%, $p<0.001$; AB2: 0.43 ± 0.32% vs. 0.28 ± 0.18%, $p<0.05$). In contrast to POAG, myocilin levels were not elevated in PEXG aqueous humor when compared to control aqueous humor. No correlation between myocilin and TGFβ2 levels was observed.

Conclusions—Myocilin is elevated in POAG, but not PEXG aqueous humor.

Keywords

Myocilin; TIGR; glaucoma; POAG; PEXG; aqueous humor; TGFβ2

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Introduction

Elevated intraocular pressure (IOP) is a risk factor for many forms of glaucoma. In human eyes, IOP is a balance between aqueous humor production and resistance to aqueous humor drainage. Aqueous humor is produced by the ciliary epithelium and flows through the pupil into the anterior chamber where it supplies nutrients and regulatory factors to nourish and maintain the avascular anterior tissues. Aqueous humor leaves the anterior chamber primarily through the trabecular meshwork and Schlemm's canal, and to a lesser extent through the uveoscleral pathway. Resistance to aqueous humor flow through these tissues is the source of intraocular pressure. In many individuals with glaucoma, the pathway of aqueous humor drainage is altered due to an increase in outflow resistance thus leading to an increase in IOP. Although the mechanism remains uncertain, the trabecular meshwork is believed to be the site of outflow resistance.

The protein component of aqueous humor may have significant effects on trabecular cell morphology and protein expression.[1] Proteins such as plasminogen activator inhibitor (PAI-1),[2] vascular endothelial growth factor (VEGF),[3, 4] soluble CD44 (sCD44),[5, 6] hepatocyte growth factor (HGF),[3] transferrin (Tf),[7] and endothelin (ET-1)[8] have all been identified as molecules that are elevated in primary open-angle glaucoma (POAG) aqueous humor. Transforming growth factor beta 2 (TGFβ2), also present in aqueous humor, has been reported to be increased in 50% of patients with POAG.[9–12]

Myocilin, a glaucoma-associated protein with unknown function, has been found elevated in some glaucomatous ocular tissues. Trabecular meshworks from POAG, low-tension glaucoma, and pseudoexfoliation glaucoma (PEXG) had increased myocilin immunoreactivity when compared to normal tissue.[13] Steroid treatment of monolayer trabecular meshwork cells or cultured human anterior segments showed a time and pressure dependent increase in myocilin protein production and secretion.[14–16] Perfusion of human anterior segment organ culture with recombinant myocilin increases outflow resistance.[17, 18] Furthermore, animal models show a correlation between increased myocilin levels and elevated IOP. Rats that spontaneously develop increased IOP had a 4-fold increase in myocilin transcription.[19]

Myocilin is present in aqueous humor where it may serve as a marker for the glaucomatous state.[20, 21] Evidence supports a role for elevated myocilin levels in canine glaucomatous aqueous humor particularly in breeds with primary glaucoma.[22] Beagles genetically susceptible to formation of glaucoma had increased levels of myocilin in their aqueous humor in a direct relation to the severity of the disease.[23] Less well understood is the relationship of myocilin levels in human glaucomatous aqueous humor, particularly in POAG and PEXG. In humans, a trend towards elevated myocilin levels in human glaucomatous aqueous humor has been reported,[24] however limited sample size prevented a glaucoma sub-type classification. In this study, we examined myocilin levels in control aqueous humor (isolated from patients undergoing cataract surgery) and compared these levels to POAG and PEXG aqueous humor to determine whether levels of myocilin are altered in these glaucoma subsets.

Materials and Methods

Aqueous Humor Collection and Classification

Aqueous humor samples were obtained at the time of surgery from 29 patients with POAG, 17 patients with PEXG and 32 patients undergoing elective cataract removal (control). Patient consent was not required since collected aqueous humor was considered waste

material. Protocol was approved by the Mayo Clinic Institutional Review Board under IRB 06-002831.

Aqueous humor was collected by making a paracentesis in the peripheral cornea. A 30-gauge cannula was inserted through the paracentesis tract. The tip of the cannula was placed in the mid anterior chamber and 50–100 μl of aqueous was slowly aspirated. Aspiration was stopped as soon as the anterior chamber began to shallow. Aqueous was transferred to a vial and placed in liquid nitrogen immediately upon removal from the anterior chamber.

The inclusion criteria for patients with POAG consisted of intraocular pressures >21 mmHg prior to treatment with glaucoma medications, optic nerve cupping and visual field loss. Patients with similar conditions and the presence of exfoliation material in the trabecular meshwork were included in the PEXG group. Control patients had no history of ocular disease other than the cataract at the time of surgery. General characteristics of the three sample groups are described in Table 1.

Total Protein Assay

The total protein concentrations of all aqueous humor samples (n = 78) were determined using the Bradford protein assay (BioRad, Hercules, CA). Briefly, 5 μl of aqueous humor was diluted to 10 μl with distilled water and combined with 990 μl of diluted dye reagent (1:5). Spectrophotometric readings were taken at an optical density of 595 nm with a UV – 1601 spectrophotometer (Shimadzu, Kyoto, Japan). All aqueous humor samples used in this study were within the reported normal range of human aqueous humor protein concentration (120–500 ng/μl).[25, 26] Aqueous samples with normal protein concentrations were used to facilitate comparisons between POAG, PEXG and control groups and reduce the possibility of elevated protein levels due to secondary aqueous contamination incurred during the retrieval process.

Recombinant Myocilin Protein Quantification

Eukaryotically expressed recombinant myocilin protein was purified from conditioned media using nickel ion affinity chromatography as previously described.[18] A Bradford protein assay (Bio-Rad) was used to quantitate recombinant myocilin levels. Quantification of myocilin concentration was verified by comparing recombinant myocilin protein amounts to known amounts of bovine serum albumin (Sigma Aldrich, St. Louis, MO) following separation on a 4–15% SDS-PAGE gradient gel (BioRad). The gel was stained with Gel Code Blue (Pierce, Rockford, IL), digitized with an EPSON Perfection 2400 Photo scanner (EPSON, Long Beach, CA), and densitometry comparison was performed using ImageJ software.[27, 28]

Determination of Myocilin Levels in Aqueous Humor

Quantification of myocilin levels in human aqueous humor was performed by Western blot analysis. Since no reliable ELISA assays are available for myocilin detection, Western blot analysis enabled comparison of multiple samples per gel, visual identification of myocilin proteins, and the use of multiple antibodies allowing for comparative studies.

To accommodate all 78 aqueous humor samples, a total of fourteen 4–15% SDS-PAGE gels were prepared with each gel containing 4–5 samples of aqueous humor (25 μl per lane) and recombinant myocilin standards (0, 5, 15, 30, and 45 ng). Each gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) in 49.6 mM Tris (pH 7.5), 384 mM glycine, and 0.01% SDS. Membranes were blocked overnight at 4°C in wash buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween) containing 2% non-fat milk. Membranes were rinsed three times with wash buffer and probed for 2-hours with an

N-terminal rabbit-polyclonal antibody (AB1) that recognizes amino acids 108–131 of myocilin.[17] Blots were incubated in wash buffer and probed with horse-radish peroxidase-linked anti-rabbit Ig (Amersham, Piscataway, NJ). Antibody:antigen complexes were detected using ECL Western blot signal detection reagent (Amersham). Kodak BioMax XAR Film was used to visualize the protein signals (Eastman Kodak, Rochester, NY). Each film was digitized with an EPSON Perfection 2400 Photo scanner.

Polyclonal antibodies developed against the same protein generally have different antigenic recognition sites and affinities. To eliminate affinity bias of a given antibody, we used a second polyclonal antibody (AB2; recognizes N-terminal amino acids 30–230 of myocilin) developed in a separate laboratory to evaluate myocilin levels in aqueous humor.[29] Following the initial analysis with AB1, the membranes were rinsed in wash buffer and incubated for 20 minutes in 7M guanidine hydrochloride to remove primary and secondary antibodies. Several blots were probed with only secondary antibody to verify that the entire primary antibody had been removed. All membranes were reprobed with AB2. Detection of antibody:antigen complexes and digitization of image was performed as described above.

The signal intensity of aqueous myocilin and recombinant myocilin standards was determined by ImageJ Software.[27, 28] For each gel, a standard curve for recombinant myocilin was determined and plotted by recombinant protein concentration on the x-axis and signal intensity readings on the y-axis. The amount of myocilin (ng/μl) was determined by using the optical density of an individual aqueous humor sample to find the crossing point within the recombinant myocilin standard curve and extrapolating the protein concentration (Figure 1). Myocilin standard curves were generated for each gel and antibody used. Myocilin standard curves were graphed using 0-intercept.

Determination of TGFβ2 levels in aqueous humor

TGFβ2 was quantified in aqueous humor using a human TGFβ2 ELISA kit (R&D Systems, Minneapolis, MN). Prior to the assay, 40 μl of each aqueous humor sample was incubated for 10 minutes with 85 μl deionized water and 25 μl 1 N HCl to activate latent TGFβ2. This reaction was neutralized with 25 μl 1.2 N NaOH/0.5 M HEPES and diluted to 800 μl with Calibrator Diluent RD51 (provided in the kit) and assayed for TGFβ2. Briefly, 100 μl of each activated sample was mixed with 100 μl of assay diluent and incubated for 2 hours in individual wells of a microtiter plate coated with TGFβ2 monoclonal antibody. Each well was washed three times with 400 μl of wash buffer and incubated with an enzyme linked polyclonal antibody. Following a 2 hour incubation, wells were washed, substrate solution was added to the bound antigen-antibody-enzyme complex, and fluorescence was calculated at 450 nm on an Infinite M200 microplate reader (Tecan Systems, Inc, San Jose, Ca) with a reference filter set at 540 nm. Amount of TGFβ2 per aqueous sample was determined using various concentrations of recombinant TGFβ2 for the generation of a standard curve.

Analysis of TGFβ2 levels was not performed on all aqueous humor samples due to limited volumes of aqueous humor collected during surgery. Therefore, only a subset of POAG, PEXG, and control aqueous humor samples that were used for myocilin determination were also assayed for TGFβ2. POAG1–4 and PEXG1–5 were from individual aqueous samples. POAG5–6 and both control samples were made by combining 3–5 aqueous humor samples since 40 μl of aqueous humor was needed to perform the ELISA.

Statistical Analysis

Myocilin levels in normal, POAG, and PEXG aqueous humor were compared by concentration (ng/μl) and as a percent of the total aqueous humor protein concentration

(within each individual sample). Statistical significance was determined by t-test comparisons.

Results

The mean age of POAG (75.9 ± 9.4 years; mean ± SD), PEXG (75.0 ± 6.2 years) and control (74.3 ± 9.2 years) aqueous humor samples was not statistically different (Table 1). Comparison of mean total protein concentration (ng/μl ± SD) between POAG and control samples was not statistically different (p=0.27). PEXG aqueous humor samples did contain elevated total protein concentrations when compared to POAG and control (PEXG vs. POAG, p<0.03; PEXG vs. normal, p<0.01). Myocilin concentrations varied from 0.00 (not detected) to 2.26 ng/μl in POAG, 0.4 to 1.27 ng/μl in PEXG, and 0.00 (not detected) to 0.68 ng/μl in control aqueous humor.

POAG vs. Control

POAG aqueous humor contained significantly elevated levels of myocilin when compared to control aqueous humor (Figure 2A). Myocilin concentrations in POAG aqueous humor increased 187% (AB1: 0.66 ± 0.53 ng/μl vs. 0.23 ± 0.20 ng/μl, p < 0.001; mean ± SD) and 51% (AB2: 0.98 ± 0.59 ng/μl vs. 0.65 ± 0.50 ng/μl, p<0.03) compared to those observed in control aqueous humor. POAG aqueous humor also contained a significantly higher percentage of myocilin per total protein than control aqueous when examined with both AB1 (0.26 ± 0.20% vs. 0.10 ± 0.20%, p<0.001) and AB2 (0.43 ± 0.32% vs. 0.28 ± 0.18%, p<0.05; Figure 2B). In POAG, the percent of myocilin per total protein concentration was increased 160% (AB1) and 54% (AB2) when compared to control aqueous humor. Approximately 70% of POAG aqueous humor samples had elevated myocilin levels when calculated either as concentrations per volume or as percent of total aqueous humor protein compared to control.

PEXG vs. Control

Myocilin levels in PEXG aqueous humor were slightly elevated, though not statistically significant, when compared to control aqueous humor (Figure 2A). Myocilin concentrations in PEXG aqueous humor increased 57% (AB1: 0.36 ± 0.40 ng/μl vs. 0.23 ± 0.20 ng/μl, p = 0.37) and 49% (AB2: 0.97 ± 0.64 ng/μl vs.0.65 ± 0.50 ng/μl p = 0.07) when compared to control aqueous humor. No difference in percent of myocilin per total aqueous humor protein concentration was observed in PEXG aqueous humor when compared to control aqueous humor (AB1: 0.11 ± 0.11% vs. 0.10 ± 0.20%, p<0.99; AB2: 0.29 ± 0.17% vs. 0.28 ± 0.18%, p<0.64; Figure 2B).

TGFβ2 in POAG, PEXG, and control aqueous humor

TGFβ2 has been found elevated in some POAG aqueous humor samples, similar to our findings with myocilin.[9–12] To determine whether myocilin and TGFβ2 levels were correlated, we compared POAG, PEXG, and control samples. POAG aqueous humor contained slightly elevated levels of TGFβ2 when compared to PEXG or control aqueous humor (Figure 3A). Only one of the six POAG samples tested, POAG3, had both elevated levels of myocilin and TGFβ2 (Figure 3B). All other POAG and PEXG aqueous humor samples had TGFβ2 levels similar to those found in control aqueous humor independent of normal or elevated levels of myocilin. This suggests that myocilin and TGFβ2 levels in POAG and PEXG aqueous humor appear independent of each other.

Discussion

Our results identified myocilin as a protein elevated in 70% of POAG aqueous humor samples in concentration per volume and as a percent of the total aqueous protein. Elevated myocilin appears unique to POAG aqueous humor, since PEXG aqueous humor did not show consistently elevated levels of myocilin, either as a concentration per aqueous volume or as a percent of total protein, when compared to control.

In addition to myocilin, other proteins such as PAI-1, VEGF, sCD44, HGF, Tf, ET-1, and TGFβ2 are also elevated in POAG aqueous humor.[2–6, 8–12] Like myocilin, elevated levels of TGFβ2 are found in some but not all POAG aqueous humor[9–12] and can increase outflow resistance in perfusion organ culture.[30, 31] Because of these similarities between myocilin and TGFβ2, we determined whether their protein levels were correlated in POAG and PEXG aqueous humor. We found that TGFβ2 levels showed a trend towards elevation in POAG aqueous humor when compared to PEXG and control aqueous humor. However, we did not see any correlation between myocilin and TGFβ2 levels, indicating their expression patterns appear to be independent of each other. Whether or not this finding is significant is unknown. However due to our limited sample size, further studies are warranted to not only compare myocilin levels to TGFβ2, but also to other glaucoma candidate marker proteins.

Whether the elevated levels of myocilin observed in POAG aqueous humor reflects a cause or effect of the disease is not addressed in this study. Not all POAG samples had elevated myocilin levels and several actually had undetectable levels. This may be explained by sensitivity limitations of the Western blot procedure used in this study or more likely due to individual variations. Furthermore, some of the undetectable or low concentrations of myocilin in POAG aqueous humor may be due to individuals with mutations or polymorphisms in the myocilin gene. While our patients were not analyzed for myocilin mutations, known incidence rates of myocilin mutations (2–8%)[32, 33] would suggest that one or two of our POAG samples (n=29) could be from patients with a myocilin mutation. The majority of proteins from mutated myocilin genes do not get secreted and form heterodimers with normal myocilin, effectively reducing the level of normal myocilin secreted into the extracellular milieu.[24, 34–37] Therefore, it is possible that in our study, if an individual had a myocilin mutation, levels of normal myocilin would be reduced, and depending on the mutation, may be undetectable in aqueous humor. These results are consistent with Jacobson et al who reported a large variation in myocilin levels in aqueous humor within their cohort of glaucomatous patients, including low concentrations of myocilin in aqueous humor in those with myocilin mutations.[24]

All POAG and PEXG samples were isolated from patients taking one or more pressure lowering medications to treat their glaucoma. Although the analysis of aqueous humor from untreated POAG and PEXG individuals would be ideal, these samples are difficult to obtain. We can not rule out the possibility that glaucoma medications may increase myocilin protein expression in some anterior segment tissues, therefore accounting for the increased levels found in many POAG aqueous humor samples. However, it is worth noting that timolol or latanoprost treatment of primary trabecular meshwork cells does not induce myocilin expression, but may slightly decrease myocilin RNA levels.[38, 39]

The effect total protein content has on glaucoma is unclear from this study. PEXG aqueous humor samples contained a significantly higher total protein concentration than either control or POAG aqueous humor. PEXG aqueous humor appears to have a higher concentration of myocilin when compared to control aqueous humor, however, when compared as a percent of total protein, no difference in myocilin levels was observed. This finding illustrates the importance of examining myocilin levels, as well as other proteins in

aqueous humor, as both a concentration per volume and as a percent of total protein. Though PEXG and control aqueous humor have a similar percent of myocilin per total aqueous protein, PEXG aqueous humor has a significantly higher protein concentration suggesting proteins other than or in addition to myocilin may have a role in the onset of glaucoma in PEX patients.

Many questions remain regarding myocilin's role in normal and glaucomatous patients. Our study does not address where the elevated myocilin levels originate nor do we know why there is elevated myocilin in POAG aqueous humor. Several studies suggest a causal relationship between myocilin and elevated IOP.[13–18] However, expression of myocilin is induced by both mechanical and oxidative stress[40, 41] and therefore, it is possible that the elevated levels of myocilin are due to a stress response, and are not the cause of POAG. Determination of myocilin's function will help to define the role myocilin has in normal physiology, and how it is altered in POAG.

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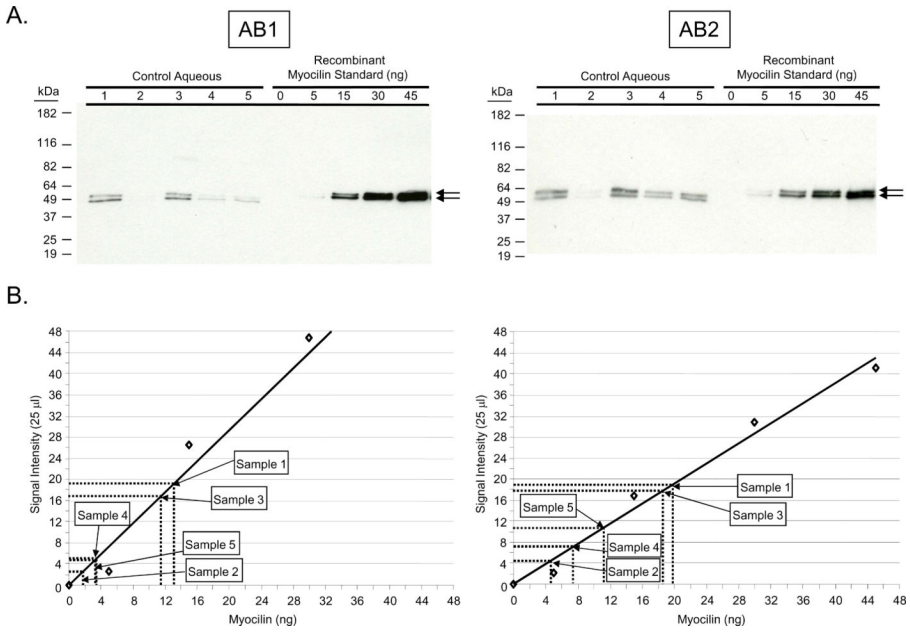

Figure 1. Western blot and densitometry analysis of myocilin levels in aqueous humor

(A) Representative Western blot of five non-glaucomatous aqueous humor samples and recombinant myocilin standards (0, 5, 15, 30, and 45 ng) with AB1 (left panel) and AB2 (right panel). (B) Standard curves of recombinant myocilin protein (diamonds) and extrapolation of myocilin amounts (dotted lines) of five non-glaucomatous aqueous humor samples. To maintain comparable y-axis between left and right panels, 45 ng recombinant myocilin standard is not shown in left panel graph.

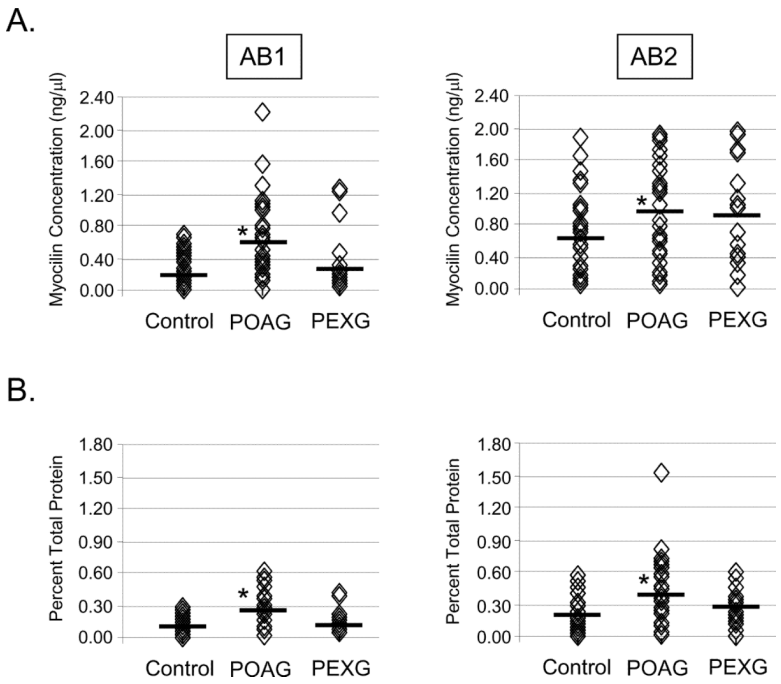

Figure 2. Myocilin levels in normal, POAG, and PEXG aqueous humor

(A) Individual (diamonds) and combined mean protein concentration (black line; mean ± SD; ng/µl) determined by AB1 (left panel) and AB2 (right panel). Statistical significance, AB1: POAG vs. control, $p<0.001$ (asterisk); AB2: POAG vs. control, $p<0.03$ (asterisk). (B) Individual (diamonds) and mean percent of myocilin per total aqueous protein determined by AB1 (left panel) and AB2 (right panel). Statistical significance, AB1: POAG vs. control, $p<0.001$ (asterisk); AB2: POAG vs. control, $p<0.05$ (asterisk).

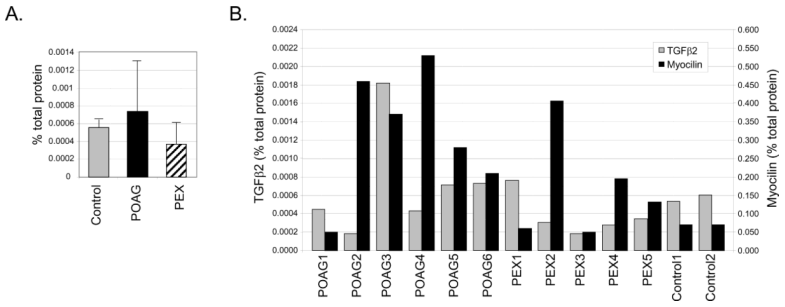

Figure 3. Comparison of TGFβ2 and myocilin levels in normal, POAG, and PEXG aqueous humor

A) TGFβ2 levels in control, POAG and PEXG aqueous humor. B) TGFβ2 and myocilin levels within individual/combined aqueous humor samples.

Table 1

	Normal	POAG	PEX
Sample Size	32	29	17
Age (years ± SD)	74.3 ± 9.2	75.9 ± 9.4	75.0 ± 6.2
Age Range (years)	52 to 89	55 to 88	62 to 85
Females	10	22	10
Males	22	7	7
Total Protein Concentration (ng/μl ± SD)	229.7 ± 85.0	254.8 ± 92.7	325.6 ± 112.6