Glaucmatous MYOC mutations activate the IL-1/NF-κB inflammatory stress response and the glaucoma marker SELE in trabecular meshwork cells

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Purpose: Activation of the IL-1/NF-κB inflammatory stress pathway and induction of SELE expression in the trabecular meshwork (TBM) is a marker for high-tension glaucomas of diverse etiology. Pathway activation stimulates aqueous outflow and protects against oxidative stress, but may be damaging in the long-term. MYOC mutations have been causally linked to high-tension forms of primary open angle glaucoma (POAG). This study investigated a possible link between MYOC mutations and activation of the IL-1/NF-κB pathway and expression of SELE.

Methods: We constructed MYOC expression vectors with mutations at sites that cause POAG. Mutations (Q368X, Y437H, A427T) were selected to represent proteins with differing POAG-causing potency (Q368X > Y437H > A427T) and intracellular retention behavior (Q368X and Y437H retained, A427T released). The constructs were made in two different kinds of vectors; one a plasmid designed for transient transfection (pCMV6), and one a doxycycline-inducible lentiviral vector (pSLIK) for stable cell transduction. The immortalized human trabecular meshwork line TM-1 was used for all expression studies. Expression of IL1A mRNA was determined by reverse transcription (RT)–PCR, as well as a set of five other genes associated with signaling pathways linked to glaucoma: IL1B and IL6 (NF-κB pathway), TGFβ2 and ACTA2 (TGF-β pathway) and FOXO1 (E2F1 apoptotic pathway). An ELISA was used to quantify IL1A protein released into culture media. To quantify intracellular NF-κB activity, we transiently transfected stably transduced cell lines with a luciferase expression vector under control of the IL8 promoter (containing an NF-κB response element).

Results: Transiently expressed wild-type MYOC was released into cell culture media, whereas mutant MYOCs Q368X and Y437H remained within cells. Both mutant MYOCs activated the IL-1/ NF-κB pathway, significantly stimulating expression of IL1A and IL1B. However Y437H, which causes a severe glaucoma phenotype, was less effective than Q368X, which causes a moderate glaucoma phenotype. In addition, the retained mutants stimulated expression of stress response genes ACTA2 and FOXO1. Unexpectedly, wild-type MYOC significantly decreased expression of IL6 and TGFβ2, to approximately half of the control levels, and expression of IL1B and ACTA2 was also slightly decreased. Induction of MYOC mutants Q368X and Y437H in stably transfected cell lines significantly stimulated the level of IL1A protein released into culture media. Once again however, the effect of the severe MYOC mutant Y437H was less than the effect of the moderate MYOC mutant Q368X. In contrast, induced expression of the intracellularly retained mutant MYOC A427T or wild-type MYOC did not change the amount of IL1A protein in culture media. Induction of Y437H MYOC plus IL1A treatment increased NF-κB activity by 25% over IL1A alone. In contrast, induction of Q368X or A427T plus IL1A treatment did not significantly affect NF-κB activity over IL1A alone. However, wild-type MYOC expression inhibited IL1A-stimulated NF-κB activity. We also observed that endogenous MYOC expression was induced by IL1A in TM-1 cells and primary TBM cell cultures. SELE was co-expressed with MYOC in the primary cell lines.

Conclusions: These results indicate that POAG-causing MYOC mutants activate the IL-1/NF-κB pathway, with activation levels correlated with intracellular retention of the protein, but not POAG-causing potency. Unexpectedly, it was also discovered that wild-type MYOC inhibits activation of the IL-1/NF-κB pathway, and that activation of the IL-1/ NF-κB pathway stimulates expression of MYOC. This is the first evidence that glaucoma-causing MYOC mutants can activate the inflammatory response and that wild-type MYOC has anti-inflammatory activity.
become blind. Additional mechanistic information is needed to identify new targets for disease intervention.

Elevated IOP, also known as ocular hypertension, results from impaired drainage of aqueous humor through the TBM and Schlemm’s canal [3]. The defect that causes primary open angle glaucoma (POAG) is at the cell and tissue level, and is influenced by genetic risk factors, the process of aging and environmental or physiologic stress [4-13]. Tissue changes include loss of TBM cells, collapse of trabecular beams, and accumulation of extracellular material [5,14,15]. Our team identified expression of the inflammatory marker endothelial leukocyte adhesion molecule-1 (ELAM-1), also known as E-selectin (SELE), as a defining feature of the diseased phenotype of the TBM in both open and closed angle forms of high-tension glaucoma of a variety of etiologies [16]. We further determined that the IL-1/NF-κB inflammatory stress response activates SELE expression, and we demonstrated the cytoprotective role of this response.

Interleukin-1 (IL-1) is a cytokine that had previously been demonstrated to lower the intraocular pressure (IOP) in rat, rabbit, and human models [17,18]. This may occur through stimulation of matrix metalloproteinase (MMP) expression [18,19], or by directly increasing paracellular permeability across Schlemm’s canal [20]. However, we proposed that the IL1/NF-kappaB stress response would lead to the pathologic hallmarks of glaucomatous trabecular meshwork if chronically activated. These findings and conclusions have been replicated and extended by other laboratories [7,8,11,21-25]. Chronic, low-grade inflammation has been suggested as an underlying mechanism linking major age-related diseases such as atherosclerosis, arthritis, osteoporosis, and cardiovascular diseases [26-28]. Inhibition of the proinflammatory mediators associated with aging and environmental stress delays the progression of several age-related pathologies [26,29]. The observed activation of a chronic inflammatory stress response in the TBM deserves special attention, as it could lead to new therapeutic approaches.

MYOC, encoding the glycoprotein called myocilin, was the first gene linked to familial forms of primary open angle glaucoma (POAG) [30]. Up to 70 different disease-causing MYOC mutations have been described, and it has been estimated that “myocilin glaucoma” accounts for 2%–4% of all cases of POAG [31-34]. MYOC has three exons and encodes a predicted 504 amino acid polypeptide containing two major homology domains: an N-terminal myosin-like domain and a C-terminal olfactomedin-like domain [34]. The majority of MYOC disease-causing variations are clustered in the olfactomedin homology domain of the third exon. Very recently, the crystal structure of the olfactomedin domain of myocilin was solved [35]. This reveals it to be a new addition to the small family of five-bladed beta-propellers, known for their ability to act as hubs for protein–protein interactions. Interacting partners identified thus far include Flotilin-1, a membrane-bound protein involved in vesicular trafficking [36], and optomedin, an olfactomedin-related extracellular protein [37]. The implications of these interactions for function remain unclear [38]. Haploinsufficiency has been excluded as the primary mechanism for POAG [39] and genetically increasing or decreasing wild-type MYOC expression also does not induce POAG [40,41]. Therefore, a gain-of-function disease mechanism has been hypothesized [42-44].

Although wild-type MYOC lacks the signal peptide sequence for routing into the classical secretion pathway, the protein is predominantly localized extracellular. One possibility is that export occurs on the surface of exosomes [45]. Most mutant MYOC forms are misfolded and aggregate in the endoplasmic reticulum, activating the unfolded protein response, a stress pathway [46,47]. Significantly, this represents a gain-of-function. It should be recognized however, that some glaucoma-causing mutations do not cause intracellular retention of MYOC protein. To account for this, an alternative mechanism has been suggested that relies on abnormal interactions of the secreted mutants with the extracellular matrix and/or cell/surface elements [48].

Recently, interest in the role of low-grade inflammation in glaucoma has been re-emerging [49]. In this study, we investigated a possible mechanistic connection between myocilin glaucoma and activation of the IL-1/NF-κB stress pathway. We hypothesized that mutant MYOC proteins would activate the IL-1/NF-κB pathway in proportion to the aggressiveness of their disease phenotype. To investigate this idea, we selected MYOC mutants that produce proteins with a range of POAG-causing potency (Q368X > Y437H > A427T) and intracellular retention behavior (Q368X and Y437H retained, A427T released).

METHODS

Cells: Throughout the text, HUGO nomenclature is used for genes and their products. Cells of the immortalized human TBM line, TM-1 [50], were grown in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS (Atlanta Biologicals, Inc., Norcross, GA), 2 mM L-glutamine, 2.5 ug/ml amphotericin B, and 25 ug/ml gentamicin. Primary human TBM cells were prepared from corneal rims discarded at the time of surgical transplantation of donor corneas, as described [51,52]. Briefly, TBM tissue was dissected from the corneal rim under a dissecting microscope and transferred to
wells of a 48 well plate containing DMEM with 10% FBS. A coverslip was placed over the tissue and the primary cells were allowed to grow out onto the bottom of the well over a period of several weeks. Once a confluent cell layer was obtained, cells were removed from the plate using trypsin and reseeded into the wells of a 6 well plate. After expansion, the identity of the cells was confirmed by morphology, by expression of the TBM cell marker AQP1, and by induction of the TBM cell marker MYOC after treatment with dexamethasone for 10 days. Primary TBM cells were used for experiments up to passage 8.

Expression constructs and transient expression: Wild-type MYOC cDNA cloned in the plasmid expression vector pCMV6 was purchased from Origene (Rockville, MD). In this vector, the inserted gene is placed under control of the strong, constitutive cytomegalovirus promoter. Mutations were created at sites that cause POAG (Q368X, A427T and Y437H) using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The oligonucleotides used for mutagenesis (Table 1) were designed according to the manufacturer’s protocol and purchased from Integrated DNA Technologies (Coralville, IA). The nucleotide changes were confirmed by sequencing (GENEWIZ, Newbury Park, CA), from both the 5’ and 3’ sides of the target DNAs.

For expression, TM-1 cells were transfected with MYOC expression constructs (1 µg) using Lipofectamine® LTX with Plus™ Reagent (Life Technologies, Carlsbad, CA), as per supplier’s instructions. Post-transfection, the cells were kept in complete medium overnight before collection of cells for further analysis.

**Lentivirus constructs and stably transduced cell lines:** Lentiviral expression vectors were created, viral particles were produced, and cell infection was performed as described [53]. Briefly, full-length wild-type MYOC and mutant MYOC cDNAs were amplified by PCR using high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA) and inserted into pre-lentiviral vector pENTT_mcs downstream of the Tetracycline Response Element (TRE). Then the target genes linked to the TRE were transferred into the lentiviral vector pSLIK-hygro by in vitro recombination (Invitrogen, Carlsbad, CA). For packaging, the lentiviral expression plasmids were co-transfected into HEK293T cells along with helper plasmids pMD.G1 and pCMVR8.91. Culture media containing viral particles were harvested after 48–72

| MYOC mutation | Direction | Oligonucleotide sequence (3′) |
|---------------|-----------|-------------------------------|
| Y437H         | Sense     | ATCATCTGTGGCACCTTGCA         |
|               | Antisense | AGGTGAGTACGTCAGGTTGCA         |
| Q368X         | Sense     | CTTCAAGCTGGCTACAGGAA         |
|               | Antisense | CCCAGATGAGACGTGCTAAG         |
| A427T         | Sense     | ATCCGTAAGCGTCAGTCAC          |
|               | Antisense | CACAGATGAGACGTGCTAAG         |

| Gene  | Direction | Oligonucleotide sequence (5′-3′) |
|-------|-----------|---------------------------------|
| IL1A  | Sense     | ATCAGTACCTCACGGCTGCT            |
|       | Antisense | TGGGTATCCCCAGCATTCC            |
| IL1B  | Sense     | CTGAAAGCCTTCCACCTCCA           |
|       | Antisense | TCATCTTTCAACACGCAGGA           |
| IL6   | Sense     | CACACAGACAGCCTCACC             |
|       | Antisense | TTCCACCGAGCAGTCCTC             |
| TGFB2 | Sense     | CGCCAAGGAGGTTTACAAA            |
|       | Antisense | TGCTGAGACGTCAAATCGAA           |
| FOXO1 | Sense     | AAGAGCGTGCCCTACACCTCA          |
|       | Antisense | CACCCCTCGGATGGAGCATC           |
| ACTA2 | Sense     | ATGCCTCTGGACGTACAACCTG         |
|       | Antisense | CGGCAGTAGTCAGAAGGAAT           |
h and used to infect immortalized TM-1 cells in the presence of 8 ug/ml Polybrene (Millipore Corp., Bedford, MA). Cell cultures were infected at a low multiplicity of infection (MOI) to ensure <30% infection frequency. Cells stably transduced with lentiviral expression vectors were selected by treating the cultures with 150 µg/ml Hygromycin (Invitrogen, Carlsbad, CA) for 3 weeks.

For induction of wild-type and mutant MYOCs, cells of stably transduced lines were seeded in 6 well plates at 200,000 cells per well. To induce the transgene, cells were then treated with doxycycline (100–1000 ug/ml) for up to 7 days. A stable cell line transduced with empty pSLIK vector was created to serve as a negative control.

Reverse transcription (RT)-PCR: Custom oligonucleotides primers (Table 1) were purchased from Integrated DNA Technologies (Coralville, IA). Total RNA was isolated from transfected TM-1 cell using TRIzol® reagent (Life Technologies). Real-time RT–PCR analysis was performed using the iScript one-step RT–PCR kit with SYBR Green (Bio-Rad, Hercules, CA) on an ABI PRISM 7900 HT sequence detection system (Life Technologies) according to the manufacturer’s instructions. The cycling parameters were set at 95 °C for 10 s (denaturation), 58 °C for 10 s (annealing) and 72 °C for 30 s (extension) and 40 cycles were performed. Relative quantification was calculated as 2\(^{-ΔΔCt}\) by the comparative Ct method.

Western blotting: Cell-conditioned culture media were harvested and clarified by centrifugation. Cells were scraped from the 6-well plate in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na\(_2\)HPO\(_4\), 1.47 mM KH\(_2\)PO\(_4\), pH 7.4) suspended in 100 µl of radioimmunoprecipitation assay buffer (RIPA buffer: Tris 50 mM, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) and protease inhibitors cocktail (Roche, Pleasanton, CA), then lysates were clarified by centrifugation. Proteins in the supernatants were separated by SDS–PAGE and transferred to PVDF membranes.

Antibodies to MYOC were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and to SELE (CD62E) and ACTB from Abcam (Cambridge, UK). The PVDF membranes were incubated with one of these primary antibodies with dilution of 1:2000 (mouse monoclonal MYOC antibody), 1:1000 (rabbit polyclonal SELE antibody) and 1:10,000 (mouse monoclonal ACTB antibody) overnight at 4 °C with gentle shaking, following the manufacturer’s instructions. The membranes were then incubated with secondary antibody–horseradish peroxidase conjugates from Santa Cruz Biotechnology (Santa Cruz, CA) with dilution of 1:10,000 for 1 h. Specific signals were developed using the ECL kit components 1 and 2 (GE Healthcare UK Limited, Buckinghamshire, UK) for 1 min. Antibody binding to proteins on the membranes was visualized by chemiluminescence exposure of photographic film (LAS-4000; Fujifilm, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA): Wild-type and mutant MYOC inducible cell lines were plated in 6-well plates at 200,000 cells per well in medium with 10% serum to promote cell proliferation. When cells in each well attained confluence, the medium was changed to serum-free and cells were left untreated or treated with doxycycline. Subsequently, media were changed every 2 days. Cell conditioned media were saved at 4 days and 6 days for quantification of cytokines by ELISA. Samples of conditioned media were concentrated by adding trichloroacetic acid to 10% of the total volume. Proteins from 1 ml of each sample were dissolved in 50 ul of PBS for ELISA (Cayman Chemical Company, Ann Arbor, MI). The protocol and calculation were performed according to manufacturer’s manual. At 6 days, cell lysates were prepared western blotting.

Luciferase assay: A luciferase expression construct driven by the IL8 promoter (pIL8/luc) was a kind gift from Dr. Ebrahim Zandi (University of Southern California). Doxycycline-inducible MYOC cell lines were plated in 6-well plates at 70% confluence and co-transfected with pIL8/luc at 2 ug/well and a control pRL-TK renilla expression construct (Promega, Madison, WI) at 1.25 ug/well using Lipofectamin LTX with Plus Reagent (Life Technologies). After transfection, cells were incubated in complete media overnight, during which time they reached confluence. Then cells were transferred to serum-free medium and treated with IL1 alpha, with or without doxycycline. After 72 h, the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to passively process (without scraping) the cells according to manufacturer’s manual, and then both renilla and luciferase activity was read using a VICTOR3V plate reader (PerkinElmer, Shelton, CT).

Statistical analysis: Results are expressed as the mean ± standard error of the mean (S.E.) The significance of differences in mean values between untreated and treated samples was determined by the Student t test after determining that the data fit a normal distribution. p≤0.05 was considered statistically significant.

RESULTS

Transient expression of MYOC mutants: We hypothesized that the misfolding of mutant myocilin would create stress and activate the NF-kappaB pathway, resulting in production of inflammatory cytokines and expression of SELE. To investigate this idea, we created wild-type and mutant MYOC expression constructs. We chose two MYOC mutations...
located in the olfactomedin-like domain of exon 3 that cause different glaucoma phenotypes [54] correlated with their differing solubility and cell retention characteristics [48]. Y437H is associated with severe juvenile POAG, exhibiting a mean maximum IOP of 43.7 mmHg, a mean age at diagnosis of 19.9 years and age of youngest diagnosis as 4 years. Q368X is associated with juvenile and adult-onset POAG and has more moderate characteristics, with a mean maximum IOP of 21.1 mmHg, a mean age at diagnosis of 37.8 years and age of youngest diagnosis as 15.1 years. The protein products of both mutants are insoluble. When expressed in cultured cells, the proteins produced by both mutants are retained within the cell.

Figure 1 shows representative results of transient transfection experiment in which cell lysates and conditioned cell culture medium were collected for analysis after 72 h in cells of the immortalized cell line TM-1 cells. Wild-type MYOC protein was found entirely in cell culture medium, while mutant proteins produced by MYOCs Q368X and Y437H were expressed and retained entirely in the cell, as revealed by western blot analysis (Figure 1A). RT–PCR analysis revealed that the level of IL1A mRNA was significantly increased in cells transfected with these two mutant constructs as compared to cells transfected with vector alone (Figure 1B). This result is consistent with our original hypothesis. However, the Y437H MYOC mutant, that causes a severe glaucoma phenotype, was less effective than the Q368X MYOC mutant that causes a moderate glaucoma phenotype. Thus IL1A was increased more than 10 fold in cells transfected with MYOC Q368X and was increased by sixfold in in cells transfected with MYOC Y437H. Interestingly, IL1A mRNA was decreased in cells transfected with wild-type MYOC, however this decrease did not attain statistical significance.
For comparison, we also examined the effects of transient transfection with the MYOC constructs on expression of a set of five other stress response genes associated with glaucoma: IL1B and IL6 (NF-κB pathway), TGFβ2 and ACTA2 (TGF-β pathway), and FOXO1 (oxidative stress; Figure 1C). Again consistent with our hypothesis, as well as with the finding for IL1A, cells transfected with mutant MYOCs significantly increased expression of all five cellular response genes. In this case, the effect of the two different MYOC mutations was not significantly different. Intriguingly, cells transfected with wild-type MYOC significantly decreased expression of IL1B, IL6, TGFβ2 and FOXO1. This finding provided an initial suggestion that wild-type MYOC overexpression might affect cellular stress response pathways, the including NF-κB pathway.

Inducible expression of MYOC mutants in stable cell lines:
Several groups have shown that transient expression of mutant MYOC forms causes cellular toxicity [47,55-57] and leads to cell death with long exposure [56]. This was also observed in our experiments (data not shown). Since this could be an independent factor in the observed changes in gene expression, we sought an approach that would reduce such toxicity by allowing us to control the level of mutant MYOC expression, and the length of time expressed. For this, we chose to create stably transduced TM-1 cell lines harboring wild-type or mutant MYOCs cloned into a lentivirus vector, placing expression under control of a doxycycline inducible promoter. We created such lentiviral vectors expressing wild-type MYOC and MYOCs Y437H and Q368X, the two mutants discussed above. For further comparison, we also created lentiviral vectors expressing MYOC A427T, a mutant that produces a mild, very late adult-onset POAG phenotype [58], and is a mild variant from a protein stability/aggregation standpoint [59]. The mean maximum IOP for this mutant is 22 mmHg, the mean age at diagnosis is 61 years, and the age of youngest diagnosis is 46 years. Importantly, the protein product is soluble and does not accumulate intracellularly.

In several different experiments, cells were incubated with various doses (100–1000 ng/ml) of doxycycline in serum-free media and cell morphology was observed. Cells treated in this way appeared completely unaffected for up to 7 days. These results are consistent with another report in which stable, inducible cell lines were employed [60].

Figure 2 shows representative results examining expression of wild-type and mutant MYOCs in the stably transduced cell lines. Expression of the transgenes was induced by doxycycline in a dose-dependent manner, and the protein products were easily detectable by western blotting. As already demonstrated in transient transfection experiments described above, mutant proteins produced by MYOCs Q368X and Y437H were found almost entirely in cell lysates, while wild-type MYOC was found entirely in the cell culture media. In addition, and as predicted, the mutant protein produced by MYOC A427T was found almost entirely in the cell culture medium.

Having established a model of mutant MYOC expression without apparent toxicity, we then re-examined the question of MYOC effects on IL1A expression. In this case, we elected to measure IL1A protein released into cell culture media by ELISA. Figure 3 shows representative results. Results were similar to those obtained in transient expression experiments and were consistent with our hypothesis. Thus, doxycycline induction (1 ug/ml) of cell lines harboring the MYOC mutants Q368X and Y437H increased the amount of IL1A protein released into culture media by ~25-fold (p=0.0007) and 10 fold (p<0.002), respectively. Once again however, the effect of the severe MYOC mutation Y437H was less than the effect of the moderate MYOC mutation Q368X. On the other hand, induction of cell lines harboring the mutant MYOC A427T or wild-type MYOC transgenes did not discernably change the amount of IL1A protein released into the culture media.

We next asked whether induction of the mutant MYOC transgenes would also increase NF-kappaB activity. A representative experiment is shown in Figure 4. The four MYOC-inducible cell lines were transfected with a luciferase reporter construct driven by the NF-kappaB-inducible transcriptional promoter for IL8 (IL8/luc) to enable quantification of NF-kappaB activity. Cells were then treated with doxycycline at 100 or 500 ng/ml to induce the MYOC transgenes, one set of cells being left untreated as a control. Parallel cultures were also treated with IL1A protein (10 ng/ml) as a positive control. After 72 h, luciferase activity was measured.

As expected, addition of IL1A stimulated NF-kappaB activity in all four lines in which transgenes were not induced with doxycycline. Induced expression (at both 100 and 500 ng/ml) of MYOC Y437H significantly increased NF-kappaB activity. Doxycycline induction of MYOC Y437H in cells co-treated with IL1A also significantly increased NF-kappaB activity by 25% over IL1A alone (p<0.05). Induced expression of MYOC Q368X caused the highest elevation of NF-kappaB activity, as was observed for IL1A expression. However the results did not attain statistical significance, likely because the error bars were large.

The mild glaucoma-causing mutant A427T did not have a discernable effect on NF-kappaB activity. Intriguingly, induction of wild-type MYOC expression inhibited IL-1alpha-stimulated NF-kappaB activity by 31%, and this was significant (p<0.04). This is consistent with the initial
suggestion provided by results of the transient transfection experiments, noted above (Figure 1).

Figure 5 shows an observation we made as part of another study using our stable cell lines harboring the MYOC expression vectors. Unexpectedly, we observed that endogenous MYOC expression is induced by IL1A in the TM-1 cell line harboring control lentivirus (Figure 5A). Co-treatment with IL1RA, a receptor antagonist, revealed that this induction occurred through the IL-1 receptor. Reprobing of the western blot revealed that SELE was co-expressed with MYOC under these conditions. At the time we made this observation it was novel, thus we followed up on its validity using two different primary TBM cell lines. We found that treatment with IL1A induced MYOC expression in both cell lines (Figure 5B). Additionally, MYOC was induced by treatment with dexamethasone, confirming an observation first made by Polansky’s laboratory [61,62]. SELE expression was also induced by IL1 in these primary cell lines, as we have previously reported [16], but was not induced by dexamethasone. This is consistent with the known anti-inflammatory effects of dexamethasone [63].

DISCUSSION

Some years ago, we reported that activation of the IL-1/NF-kappaB inflammatory stress response is a defining feature of both open and closed angle forms of high-tension glaucoma of a variety of etiologies [16]. In this study, we now provide evidence to link an additional high-tension form of open angle glaucoma to this pathway: myocilin glaucoma. Our findings support our hypothesis that IL-1/NF-kappaB inflammatory stress response represents a common mechanism operative in the pathophysiology of high-tension glaucomas.

MYOC is the first gene in which mutations were identified as causative for familial forms of glaucoma [30]. The gene is expressed in several tissues of the body, but to our knowledge, mutations cause disease only in the eye [64]. Despite intensive investigation, the biologic function of MYOC has been elusive. MYOC may play a role in cell-extracellular matrix interactions [65,66], cell migration [67] and mitochondrial function [68]. MYOC is part of the dystrophin-associated protein complex and thus may play a role as a regulator of muscle hypertrophy pathways [69]. An interesting recent study has provided evidence that MYOC may serve as an extracellular chaperone [70]. It was also recently suggested that the MYOC functions as a plasma
membrane scaffold protein involved in receptor-mediated endocytosis [71].

As mentioned in the Introduction to this paper, most mutant MYOC forms are misfolded and aggregate in the endoplasmic reticulum, activating the unfolded protein response, a stress pathway [46,47]. Specific MYOC mutations may lead to different amounts of misfolding, with varying degrees of recognition by the ubiquitin degradation pathway. Wild-type MYOC appears to contain a cryptic peroxisomal targeting signal type 1 (PTS1) and glaucoma-associated mutations were shown to activate the targeting sequence [72]. It is suggested that mutant MYOC interaction with the PTS1 receptor may mean poorer clearance from the endoplasmic reticulum and greater TBM cell dysfunction, culminating in a higher IOP phenotype [72].

In this study, we investigated a possible mechanistic connection between mutant MYOC proteins with a range of disease phenotype and myocilin glaucoma and activation of the IL-1/NF-κB stress pathway. By mapping familial glaucoma-associated lesions onto the structure of the olfactomedin domain recently resolved by crystallography [35], three regions sensitive to aggregation have been identified. The largest number of disease-causing variants is found within the core beta-sheet belt of the propeller, particularly those variants that would be most destabilized and least tolerated. The Y437H mutation associated with severe juvenile POAG is located here. Substitutions in the molecular clasp region of the core appear better tolerated. Such variants affect the tethering of the strands and/or alignment of the disulfide bond in this region. Q368X is less severe, is located here. Several additional substitutions cluster near the Ca²⁺ coordination sphere, including the mild glaucoma-causing mutation MYOC A427T. The substitution likely weakens Ca²⁺ coordination.

We found that the two intracellularly retained mutants we tested, MYOCs Y437H and Q368X, activated the IL-1/NF-κB pathway. Using both the transient transfection and stably transduced cells and two different IL1 expression

![Figure 3. Effects of MYOC Mutations on Expression of IL1A in Stably Transduced Cell Lines](image)

MYOC expression was induced in TM-1 cell lines stably transduced with wild-type or mutant MYOC lentivirus expression constructs at 1000 ng/ml doxycycline. A parallel set of cells was left uninduced for comparison. After 6 days, conditioned media and cell lysates were collected for assay. A: Lanes of an SDS-polyacrylamide gel were equally loaded with aliquots of each sample (20 ul of cell culture media or 20 ug cell lysates) and proteins were separated by electrophoresis. Western blotting was performed with anti-MYOC antibody to quantify MYOC protein. Lysate blots were re-probed with ACTB as a control for equal gel loading. The electrophoretic mobility of molecular weight size standards is indicated. B: ELISA was used to quantify IL1A protein in equal volume samples of conditioned media.
assays, we found that the moderate glaucoma-causing mutant had the greatest effect. This could be due to differences in transfection efficiencies or copy number differences, however, the western blots seemed to indicate that expression was equivalent. The reason for this observation will be followed up in future studies. Only the most severe glaucoma-causing mutant Y437H had a detectable effect on the NF-kappaB activation assay. In this case, however, error bars were large for the moderate glaucoma-causing mutant Q368X, which may have precluded the detection of true significance.

As also noted in the Introduction to this paper, some mutations that do not cause intracellular retention of MYOC protein can still cause glaucoma. To account for this, an alternative mechanism has been suggested that relies on abnormal interactions of the secreted mutants with the ECM and/or cell/surface elements [48]. In this study, we could not detect any effect of the secreted mutant A427T on activation of the IL-1/NF-kappaB pathway. However, it is important to note that this MYOC mutant has a much lower glaucoma-causing effect than the retained MYOC mutants we examined. If its ability

![Figure 4](http://www.molvis.org/molvis/v21/1071) Effects of MYOC Mutations on NF-kappaB Activity in Stably Transduced Cell Lines

TBM-1 cell lines stably transduced with wild-type or mutant MYOC lentivirus expression constructs were transfected with the IL8-luc expression construct or the renilla expression construct control. MYOC expression was then induced in at 1000 ng/ml doxycycline. A set of cultures was treated with IL1A and a parallel was left untreated for comparison. *p<0.05 n=3.

![Figure 5](http://www.molvis.org/molvis/v21/1071) Effects of IL1A on Expression of Endogenous MYOC and SELE (A) western blot of lysates from cultures of TM-1 cells stably transduced with empty lentivirus vector. Cultures were treated for 72 h with IL1A (10 ng/ml), IL1RA (100 ng/ml), or both IL1A and IL1RA. Blots were probed for MYOC and SELE. ACTB was used as a control to demonstrate equal loading of gel lanes. B: Western blot of lysates from two primary trabecular meshwork cell lines derived from separate individuals (primary line 1 and primary line 2). Cells were plated and cultures were allowed to grow to confluence. They were then treated with IL1A (10 ng/mL) or dexamethasone (100 nM) for 10 days.

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to activate the IL-1/NF-kappaB pathway is similarly low, then this effect may be beneath the level of detection of our tests.

Prior to our submission of this manuscript, a publication appeared reporting that inflammatory cytokine IL1A stimulates endogenous MYOC expression in the porcine system [70]. Independently, we found that IL1A stimulates endogenous MYOC expression in the human system. In addition, we extend this finding by our discovery that wild-type MYOC overexpression inhibits activation of NF-kappaB. Thus wild-type MYOC would participate in a negative feedback loop to inhibit activation of the IL-1/NF-kappaB inflammatory stress pathway.

We put this negative feedback loop together with the positive feedback loop activated by intracellularly retained mutant MYOC, as schematically diagramed in Figure 6. In individuals heterozygous for intracellularly retained mutant MYOC, activation of the IL-1/NFkappaB pathway by mutant MYOC should stimulate expression of both mutant and wild-type MYOC alleles; the mutant MYOC would further stimulate the pathway, but wild-type MYOC might at least partially counter this effect. However, in individuals homozygous for intracellularly retained mutant MYOC, only the positive feedback loop would be active.

MYOC was discovered in studies to understand the mechanisms of steroid-induced ocular hypertension and glaucoma identified as a protein highly induced by treatment of primary TBM cell cultures with glucocorticoids [61,62]. It has since been shown that MYOC expression can also be induced in TBM cells by TGFβ1 [73], OPTN [74], and mechanical stretch [75]. The dexamethasone-stimulated increase in MYOC expression was recently shown to be dependent on activation of NFATC1 by calcineurin [75]. NF-kappaB and NFAT pathways can crosstalk [76], suggesting this as a candidate mechanism for stimulation of MYOC expression by IL1A. MYOC modulates Wnt signaling by interacting with components of this signaling pathway [77,78]. In addition, it was recently shown that MYOC binds and activates the plasma membrane receptor ErbB2/ErbB3 in the sciatic nerve [79]. The mechanisms whereby MYOC inhibits activity of NF-kappaB remain to be determined.

Living tissues are exposed to many different stress conditions. Short-term stress typically stimulates protective responses that help maintain tissue homeostasis. However, chronic exposure can lead to changes that contribute to the progression of many age-related degenerative conditions. In our previous study, we provided evidence that activation of the IL-1/NF-kappaB inflammatory stress response in glaucomatous TBM cells is protective in the short run, but amplification of the response as a result of prolonged stress would lead to pathological tissue changes resulting in ocular hypertension [16]. Here we identify another possible mechanism for amplifying the IL-1/NF-kappaB inflammatory stress response in a familial form of glaucoma. These studies are quite timely considering recent renewed interest in the role of low-grade inflammation in glaucoma [49]. They provide additional support for the use of anti-inflammatory agents in the treatment of high-tension glaucomas.

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