Expression of Wild-Type and Truncated Myocilins in Trabecular Meshwork Cells: Their Subcellular Localizations and Cytotoxicities

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PURPOSE. To investigate the subcellular localizations and potential cytotoxicities of wild-type and truncated (Q368X) myocilin in cultured human trabecular meshwork (TM) cells.

METHODS. Full-length wild-type myocilin, truncated myocilin, and stromelysin were expressed as green fluorescence (GFP) or DsRed fusion proteins in TM cells by using adenoviral vectors, and their secretory properties and cytotoxic effects were evaluated by Western blot analysis and cell proliferation assay, respectively. To determine the subcellular localizations of myocilins, the cellular organelles of the infected TM cells were stained with organelle-specific antibodies or fluorescent indicators and examined under a confocal microscope.

RESULTS. Wild-type myocilin was expressed mainly in the perinuclear region of TM cells and was localized preferentially in endoplasmic reticulum (ER), but not in actin, microtubules, or mitochondria. Truncated myocilin was also localized in ER, and its expression was found to be potentially toxic to TM cells, leading to deformed cellular morphology and diminished cell proliferation, but it had no effect on the secretion of stromelysin. The truncated myocilin was also found to be colocalized with and appeared to aggregate with wild-type myocilin when the proteins were coexpressed.

CONCLUSIONS. TM cells participating in the turnover of trabecular extracellular matrix (ECM) components are important in regulating aqueous outflow. The truncated myocilin, colocalized and coaggregated with wild-type myocilin, is believed to cause a dysfunction of the cells, resulting in alterations in structural compartmentalization of trabecular ECM and obstruction of aqueous outflow. (Invest Ophthalmol Vis Sci. 2002;43:3680–3685)

Glaucoma, a complex ocular disease characterized by progressive optic neuropathy, is the leading cause of irreversible blindness worldwide. Primary open-angle glaucoma (POAG) is the most common form and is generally associated with elevated intraocular pressure (IOP) consequent to the abnormal resistance of aqueous outflow through trabecular meshwork (TM), a specialized tissue lining the outflow pathway of the eye.¹

Myocilin is a glycoprotein with a molecular weight of approximately 55 kDa and is composed of two major domains, an N-terminal myosin-like domain (thus named myocilin) and a C-terminal olfactomedin-like domain.²–⁴ The protein, formerly referred to as trabecular meshwork-inducible glucocorticoid protein (TIGR), is of particular interest in glaucoma genetics because of a suggested link between the protein and POAG. The evidence for the link includes (1) the induction profile of myocilin by glucocorticoid (GC), which mimics GC-induced glaucoma in dose response and time course,⁵–⁷ (2) localization of the gene encoding the protein on 1q23-q25,⁸ a GLCIA locus identified by genetic linkage analysis,⁹–¹² and (3) occurrence of various mutations associated with POAG in the gene.⁸,¹³–¹⁶

It has been assumed that myocilin is one of the aqueous outflow regulators in outflow cells, because the protein is normally present in both the trabecular cells of the uveal, corneoscleral, and juxtacanalicular regions and the endothelial cells of Schlemm’s canal.¹⁷–¹⁹ Furthermore, the perfusion of recombinant myocilin into human eye organ culture increases outflow resistance.²⁰ However, the biological activities and physiological functions of myocilin remain largely unknown.

One of the distinguishing characteristics of myocilin is that, whereas the normal form is completely Triton soluble, mutant forms are substantially insoluble.²¹ Consistently, there have been reports that normal myocilin is well secreted extracellularly, but little to no myocilin is secreted from cells that express different mutant forms of myocilin.²² Moreover, it has been recognized that the expression of truncated myocilin results in reduced secretion of normal (endogenous) myocilin.²²,²³ A recent study has revealed that the characteristics of truncated myocilin may be related to its incorrect processing in the endoplasmic reticulum (ER) and accumulation into insoluble aggregates, suggesting that the mutant myocilin acts intracellularly in the development of POAG.²⁴ To better understand the role(s) played by myocilin in glaucoma, it is necessary to determine the exact subcellular localization of myocilin in TM cells, but published results on this question have not been in agreement with each other.²⁵–²⁸

In the present study, we reexamined the subcellular localization of virally expressed normal and truncated (Q368X) myocilins in TM cells. During that work, we noted that the truncated myocilin showed a potential cytotoxic effect in the cells. The present study was therefore undertaken to investigate the subcellular localizations and cytotoxicities of both wild-type and truncated myocilins in cultured human TM cells.

MATERIALS AND METHODS

Cell Culture

All cells used in this study were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics. TM cells were a generous gift from Jon Polansky (University of California, San Francisco, CA).
Adenoviral Vector Constructions

The cDNAs for truncated and full-length wild-type myocilin were amplified separately by reverse transcription-polymerase chain reaction (RT-PCR), using total RNA extracted from TM cells treated with 250 nM dexamethasone for 7 days. The sequences of primers used were 5′-AGGAAGGCTTACCCAGGCTC (sense primer, nucleotides [nt] 13–32 in GenBank accession number U85257 for nt position numbering; GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/Genbank), 5′-ACCGGTCGCTGATGAGCTTTTCA (antisense primer for truncated myocilin, nt 1138–1119), and 5′-ACCGGTAACATCGGAGACTGTGA (antisense primer for wild-type myocilin, nt 1551–1532). The antisense primers contain four or five extra nucleotides in its 5′ end to create a flanked AgeI site and a change from C to A at nt 1550 to destroy the stop codon. The amplified cDNAs were verified by sequencing and cloned into the EcoRI and AgeI sites of the shuttle vector pACMV-GFP, resulting in the pACMV-truncated myocilin-GFP and pACMV-wild-type myocilin-GFP vectors, respectively. The green fluorescence protein (GFP) gene was originated from the pEGFP-N1 vector (Clontech, Palo Alto, CA) digested with EcoRI and NotI.

The vector expressing the myocilin with red fluorescence was generated by replacing the GFP gene in the pACMV-wild-type myocilin-GFP vector with the DsRed1 gene obtained by digestion of the pDsRed1-N1 vector (Clontech) with AgeI and NotI.

The cDNA for human stromelysin-1 was amplified by PCR, as previously described, except that the antisense primer 5′-ACCGGTATAACAAATGACAAGCTTG (nt 1477–1458 GenBank accession number X05232) was used. The primer also contains five extra nucleotides in its 5′ end to create an AgeI site and a change from C to A in nt 1476 to destroy the stop codon. The cDNA was verified by sequencing and cloned into the pACMV-GFP vector.

Replication-deficient recombinant adenoviruses were generated as described previously. Briefly, the constructed plasmids described in the prior paragraph were separately transfected into 293 cells, along with an adenovirus-5 genome-containing plasmid, pJM17, using a lipid-based transfection reagent (Lipofectamine: Gibco). Viral particles were obtained after 7 to 9 days and propagated in 293 cells. The viruses with the desired gene construction were identified by PCR, and viral titer was determined by limiting dilution on 293 cells. Contamination of the replication-competent virus was ascertained by infecting Cos-7 and HeLa cells with each viral stock. There was no sign of replication of the viruses in the cells.

Western Blot Analysis

TM cells of passages before passage 8 were seeded in 12-well plates and cultured with 0.5 mL medium to reach approximately 70% confluence. The cells were infected with the viruses at a multiplicity of infection (MOI) of 50 to 100 plaque forming units (pfu) per cell for 2 hours, washed once with culture medium, replenished with DMEM containing 2% FBS, and further cultured. After 48 hours, the culture medium was harvested and centrifuged at 17,000 rpm for 10 minutes, and the cleared supernatants were mixed with 4× sodium dodecyl sulfate (SDS) sample buffer. The plate with the adherent cells was washed twice with PBS and an equivalent amount of 1× SDS sample buffer was added (0.6 mL). The cell lysates were harvested and cleared by centrifugation.

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli. The samples were boiled for 5 minutes, and 10 μL of each sample was loaded onto 4% SDS-polyacrylamide gels and electrophoresed at 100 V for 1 hour. The resolved proteins were transferred electrophoretically to a nitrocellulose membrane. Blotted overnight with 0.5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS). The membrane was incubated for 2 hours with polyclonal rabbit anti-GFP antibodies (Clontech) diluted 1:2000 in TTBS and washed three times with TTBS for 10 minutes between washes. The membrane was probed with peroxidase-conjugated anti-rabbit antibody (Amersham, Piscataway, NJ) diluted 1:5000 in TTBS for 2 hours and developed with a chemiluminescence detection kit (Amersham, Abersham UK), according to the manufacturer’s guide.

Organelle Staining and Confocal Microscopy

TM cells were seeded onto four-well chamber slides at a confluence of 30% and infected with viruses at an MOI of 500 pfu per well for 2 hours. The cells were washed briefly, and further cultured for 48 hours. Live cell staining for Golgi apparatus, mitochondria, and lysosomes was performed with vital stains specific for each organelle (Bodipy TR ceramide D-7540, 5 μM; MitoTracker M-7512, 300 nM; and LysoTracker L-7528, 300 nM, all purchased from Molecular Probes, Eugene, OR). The staining was added to the cultures and incubated for 30 to 60 minutes according to the manufacturer’s procedure. Staining for actin microfilaments was performed by adding a lipoophilic fluorophore (250 nM; Bodipy, B-7464; Molecular Probes) to cells that had been fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. To stain microtubules and the ER, the cells were fixed and permeabilized, and incubated for 1 hour with monoclonal anti-tubulin antibody (Sigma, St. Louis, MO) diluted 1:250 in PBS containing 2% bovine serum albumin or anti-protein disulfide isomerase (PDI Stressgen, Victoria, British Columbia, Canada) diluted 1:50. After they were washed briefly with PBS, the cells were incubated for 30 minutes with tetramethylrhodamine isothiocyanate (TRITC)–conjugated secondary antibody (Zymed, South San Francisco, CA) diluted 1:50 in PBS. Images of green myocilin fluorescence were collected with a confocal laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany) using 488-nm excitation light from an argon laser and a 505- to 530-nm bandpass barrier filter. Images of red actin, tubulin, ER, Golgi, mitochondria, and lysosome fluorescence were collected using a 543-nm excitation light from the HeNe laser, a 560-nm dichroic mirror, and a 570-nm long-pass filter. Cells were illuminated only during image acquisition (3.7 seconds/frame for GFP and for TRITC) and the images were collected as a Z-series of 5 to 10 serial images at 0.5-μm intervals.

Cell Proliferation Assay

TM cells plated in a 96-well plate at a concentration of 1 × 10^4 cells per well were infected with viruses at an MOI of 15 to 1000 pfu for 2 hours. The cells were washed, replenished with 100 μL culture medium, and further cultured. After 72 hours, 10 μL of cell proliferation reagent (WST-1; Roche, Mannheim, Germany) per well was added, and absorbance at 450 nm was measured after 1 hour.

Results

Characteristics of Myocilins Tagged with GFP

Throughout this study, adenoviral vectors were used to express the desired proteins in TM cells that were robustly transfected by naked DNA. Five kinds of replication-deficient recombinant adenoviruses were generated, each carrying wild-type and truncated myocilin, stromelysin, or GFP. The first three proteins were designed expressed as GFP or DsRed fusion proteins by placing the cDNA encoding each protein to the N terminus of the fluorescent proteins to facilitate direct visualization (Fig. 1).

By Western blot analysis, it was confirmed that the tagging of proteins with a relatively large GFP did not affect the secretion property of each of the native protein. As shown in Figure 2, truncated myocilin–GFP was resolved at approximately 68 kDa and was detected only in cell lysates, whereas wild-type myocilin–GFP of approximately 83 kDa was detected in both cell lysates and culture medium when expressed solely, but only in cell lysates when coexpressed with truncated myocilin–GFP. Similarly most stromelysin–GFP was secreted into the culture medium. These results coincided well with findings previously reported.
It should be noted, however, that the secretion of stromelysin was not significantly reduced when coexpressed with truncated myocilin.

Subcellular Localization of Myocilins

In TM cells, wild-type myocilin was expressed as discrete fine vesicular structures scattered mainly in the perinuclear regions, coinciding with previous observations. However, the subcellular localization of the myocilin was not consistent with any of the previous reports. As seen in Figure 3, the protein was localized preferentially in the ER and partially in the Golgi apparatus, but gave no evidence of localization to actin filaments or mitochondria. There was also no clear colocalization of myocilin with microtubules, although sparse overlapping between GFP fluorescence and tubulin fiber staining was observed, especially in the microtubule-organizing center near the nucleus. Myocilin did not show colocalization with lysosomes.

Truncated myocilin was also localized to the ER (Fig. 4), but not to the other cytoplasmic organelles examined (data not shown). The protein, however, appeared to show more aggregation than did its wild-type form and induced PDI, an ER-resident enzyme, to aggregate. The aggregated PDI which otherwise was dispersed evenly in the ER implied that the enzyme was bound to and trapped within the myocilin aggregates.

The truncated myocilin was also found to induce wild-type myocilin (tagged with DsRed) to aggregate (Fig. 5). The wild-type and truncated myocilins may interact with each other by the formation of a dimer or oligomer. The interactions appeared to be the aggregation of truncated and wild-type myocilin and thus resulted in the colocalization of both proteins.

Cytotoxicity of Truncated Myocilin

The visualization of GFP fusion protein under a fluorescence microscope necessitates a viral infection at an MOI higher than 100 pfu. In this condition, it was found that the expression of truncated myocilin did not show dose responsiveness and seemed to be lower than expression of other proteins (data not shown). This phenomenon, also noted by other investigators, was thought to be attributable to the potential cytotoxicity of the truncated myocilin, in that the shape of cells expressing the protein was severely changed, as was clearly revealed by light microscope (Fig. 6). To confirm this possibility further, a cell proliferation assay was performed on the TM cells infected with each adenoviral vector at an MOI of up to 1000 pfu. Figure 7 clearly shows that truncated myocilin started to exert its cytotoxic effect even at an MOI of less than 100 pfu, compared with other proteins. By contrast, wild-type myocilin showed cytotoxicity comparable with GFP expression that was also slightly toxic to the cells. As expected, stromelysin, a well-known secretory protein, was the least toxic to the cells.

DISCUSSION

More than 40 different myocilin mutations have been described in the literature. The Q368X mutation occurs most commonly in patients with POAG, but little is known...
about the mechanisms by which the mutation causes POAG. In
the current study, truncated myocilin exhibited potential cy-
totoxicity in trabecular cells, giving clues to understanding
how the myocilin is involved in the pathogenesis of POAG. It
is well established that outflow resistance increases with age,
accompanied by a decreased number of TM cells and alter-
ations of the juxtacanalicular extracellular matrix (ECM).33–35
It is also well known that glaucomatous eyes exhibit fewer TM
cells and an abnormal appearance of ECM in the juxtacana-
cular region compared with age-matched control eyes.36,37
Therefore, the cytotoxicity of the truncated myocilin that in-
evitably results in diminished number and dysfunction of tra-
becular cells of the outflow tissue, may lead to decreased
outflow facility and ultimately to the development of POAG.
The reason for the cytotoxicity of truncated myocilin was
not intensely investigated in the current study, but it can be
postulated that the aggregated expression of the myocilin may
be one of the causes. It is highly probable that some mutations
in myocilin cause the protein to be folded and processed
inadequately.38,39 The subsequent accumulation of the degraded
products may support the implication that they are cytotoxic

![FIGURE 3. Subcellular localization of wild-type myocilin in TM cells. TM cells were infected for 2 hours with an adenoviral vector encoding wild-type myocilin-GFP at an MOI of 500 pfu. After 48 hours, each cytoplasmic organelle of the cells was stained and examined under a confocal microscope. Each row represents the same cell. The staining patterns of each organelle (red) and myocilin-GFP (green) are shown in the left and middle columns, respectively. The overlays of the respective pictures are shown in the right column. The colocalization generates a yellow stain. Scale bar, 10 μm.](https://doi.org/10.1167/iovs.02-0302)

![FIGURE 4. Coaggregation of PDI with truncated myocilin in TM cells. The image of PDI staining (A) and truncated myocilin-GFP (B) were merged (C). The experimental procedures were the same as in Figure 3. Scale bar, 10 μm.](https://doi.org/10.1167/iovs.02-0303)

![FIGURE 5. Colocalization and coaggregation of wild-type myocilin with truncated myocilin in TM cells. Wild-type myocilin-DsRed (red) showed punctate, fine, vesicular structures when expressed solely in TM cells (A). However, the wild-type myocilin became aggregated (B) when coexpressed with truncated myocilin-GFP (C) and showed colocalization with the truncated myocilin (D). Scale bar, 10 μm.](https://doi.org/10.1167/iovs.02-0304)

![FIGURE 6. Cytotoxicity of truncated myocilin in TM cells. TM cells were infected for 2 hours with each adenoviral vector carrying truncated myocilin-GFP (mt MYOC), wild-type myocilin-GFP (wt MYOC), stromelysin-GFP (stromelysin), or GFP at an MOI of 500 pfu, and changes of cell morphology were observed after 48 hours. Note that the cells expressing truncated myocilin became large and round.](https://doi.org/10.1167/iovs.02-0305)
altruistically misfolded proteins. It is also tempting to speculate whether E323K mutation that causes a translocational pause is part of the mechanism of POAG. This is necessary, because most patients with POAG do not carry a myocilin mutation; nonetheless, they have glaucoma. A complete appreciation of the role played by myocilin in outflow regulation will greatly contribute to the understanding of glaucoma.

References


