Non-secretion of mutant proteins of the glaucoma gene myocilin in cultured trabecular meshwork cells and in aqueous humor


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Received 30 July 2000; Revised and Accepted 20 November 2000

Until recently, very little was known about the molecular mechanisms responsible for the development of glaucoma, a leading cause of blindness worldwide. Mutations in the glaucoma gene myocilin (MYOC, GLC1A) are associated with elevated intraocular pressure and the development of autosomal dominant juvenile glaucoma and a subset of adult-onset glaucoma. MYOC is expressed in the trabecular meshwork (TM), a tissue responsible for drainage of aqueous humor from the eye, and the tissue involved in elevated intraocular pressure associated with glaucoma. To better understand the role of MYOC in glaucoma pathogenesis, we examined the expression of normal and mutant myocilin in cultured ocular (TM) and non-ocular cells as well as in the aqueous humor of patients with and without MYOC glaucoma. Normal myocilin was secreted from cultured cells, but very little to no myocilin was secreted from cells expressing five different mutant forms of MYOC. In addition, no mutant myocilin was detected in the aqueous humor of patients harboring a nonsense MYOC mutation (Q368X). Co-transfection of cultured cells with normal and mutant myocilin led to suppression of normal myocilin secretion. These studies suggest that MYOC glaucoma is due either to insufficient levels of secreted myocilin or to compromised TM cell function caused by congestion of the TM secretory pathway.

INTRODUCTION

The glaucomas are a heterogeneous group of optic neuropathies which are characterized by the progressive loss of retinal ganglion cells, excavation of the optic nerve head and characteristic visual field changes. Glaucoma is the second leading cause of irreversible blindness worldwide affecting ~66 million people (1). Approximately 2–4% of the population over the age of 40 will develop glaucoma during their lifetime, although half of the people with glaucoma are unaware that they have this ocular disease. Primary open angle glaucoma (POAG) is the most common form of glaucoma and is often associated with elevated intraocular pressure (IOP).

Three regions of the eye are involved in the pathogenesis of glaucoma. The elevated IOP associated with glaucoma is due to increased resistance to the outflow of aqueous humor from the eye. Aqueous humor is made continuously by the ciliary body to provide nutritive support for the avascular anterior tissues of the eye. Aqueous humor exits the eye through the trabecular meshwork (TM), a reticulated network of cell-lined extracellular matrix located at the junction of the cornea and iris. In most forms of glaucoma, including POAG, the efflux of aqueous humor from the eye is impaired leading to the build-up of pressure inside the eye. The loss of vision in all forms of glaucoma is due to the gradual death of ganglion cells in the neural retina. This loss of retinal ganglion cells (RGCs) is associated with distinct changes in the appearance of the optic nerve head where the 1–1.5 million RGC axons exit the eye to form the optic nerve.

A variety of risk factors are associated with the development of POAG. There is a very good correlation between the degree of ocular hypertension (i.e. elevated IOP) and the risk of developing POAG. Most forms of glaucoma are associated with advancing age, particularly after the age of 40. Some individuals develop ocular hypertension when therapeutically treated with anti-inflammatory glucocorticoids, and these patients are often referred to as ‘steroid responders’ (2,3). Steroid responders have a significantly increased risk of developing POAG in their lifetime (4,5). Race also appears to be a POAG risk factor because African-Americans have a 4-fold increased risk of developing POAG compared with the American Caucasian population (6). A family history of glaucoma is another important risk factor for the development of glaucoma.

There is clear evidence supporting the heritable nature of many subtypes of glaucoma, including POAG. The presence of a first degree relative with glaucoma is a major risk factor for
developing the disease. In addition, a variety of different loci have been mapped for POAG, congenital and developmental glaucomas, and several of these glaucoma genes have been identified (7–9). The first glaucoma locus, GLC1A, was mapped to chromosome 1q by using genetic linkage analysis (7), and the gene causing GLC1A glaucoma was identified using positional cloning methods (8). The GLC1A gene, now officially referred to as MYOC, encodes a 57 kDa protein known as myocilin. Mutations in MYOC have been shown to be responsible for the inheritance of autosomal dominant juvenile glaucoma and a subset of adult-onset POAG (8,10–12). This glaucoma gene was characterized initially as a glucocorticoid-induced gene (TIGR) in the trabecular meshwork (TM) (13,14) and as a gene expressed in retinal photoreceptors (myocilin, MYOC) (15) and thus has also been referred to as TIGR and TIGR/MYOC. In cultured TM cells, myocilin can be found intracellularly distributed in vesicles (16–18) as well as secreted into the medium as a glycoprotein (13,14). A variety of factors can induce the expression of myocilin in cultured TM cells (e.g. glucocorticoids, oxidative insult, TGF-β, stretch) (13,14,19), and it appears that myocilin expression is increased in the TM of patients with glaucoma (20). Despite considerable research effort, the function of myocilin is not known. Also unknown is how mutations in myocilin lead to the development of POAG. Expression of myocilin mRNA has been demonstrated in a variety of tissues including heart, brain, skeletal muscle, testis and various components of the eye (15,21–25).

The purpose of the present study was to examine the cellular distribution of normal and mutant myocilin in cultured ocular and non-ocular cells as well as to determine whether myocilin is present in vivo in the aqueous humor of normal, non-glaucoma patients and in glaucoma patients who have mutations in the myocilin gene.

RESULTS

Expression of normal and mutant myocilin in cultured cells

Since MYOC is expressed both in ocular and non-ocular tissues, we used ocular (TM5) and non-ocular (A549) cell lines, as well as two different transfection methods, to examine the expression of wild-type and mutant forms of the glaucoma gene coding for myocilin. All of the data are remarkably consistent regardless of the transfection technique or the cell type used.

In order to optimize the conditions for adenovirus transduction, initial experiments were conducted using the wild-type MYOC–FLAG adenovirus construct to determine the dose–response and time course of expression. A549 cells were infected with 1, 10 and 100 plaque-forming units (p.f.u.)/cell, and the presence of myocilin was evaluated in cell lysates and supernatants 24 versus 48 h after infection. FLAG-tagged myocilin was found both in intracellular and in secreted forms. The signal was much stronger at 48 h compared with 24 h post-infection. The strongest signal was seen following delivery of the 100 p.f.u. dose in both the cell lysate and the supernatant fractions (data not shown). Based on these results, we used the 100 p.f.u. dose at the 48 h time point in all subsequent experiments.

Adenovirus vectors carrying LacZ, wild-type MYOC and MYOC mutations G364V, Q368X, G364V, K423E, Y437H, I477S were used to infect A549 cells (Fig. 1) or TM5 cells (Fig. 2). Anti-MYOC antibody 129 was used to detect myocilin expression in the cell lysates and supernatants of these two cell types. There was no detectable myocilin expression in either A549 or TM5 cells infected with the LacZ adenovirus construct indicating that there is no endogenous myocilin being made at detectable levels in either of these cell lines. Myocilin was found both inside the cells and secreted into the
medium in A549 and TM5 cells transduced with the wild-type MYOC adenovirus. Intracellular myocilin was expressed as two bands of 55 and 57 kDa on SDS–polyacrylamide gels, whereas the secreted myocilin was variably seen as a single broad band of 53–55 kDa or as two bands of ∼53 and 55 kDa. Transduction with the MYOC K398R sequence variant, which is a polymorphism not associated with the development of glaucoma, gave results that were identical to the wild-type MYOC transfection (i.e. both the intracellular and secreted forms of myocilin were present).

In contrast to the results with wild-type MYOC, there was very little to no secretion of myocilin into the medium in all five of the MYOC mutations that are associated with the development of glaucoma. There was no detectable myocilin in the medium of A549 cells transduced with the G364V, Q368X, K423E, Y437H and I477S adenovirus vectors (Fig. 1B). Similarly, there was no detectable myocilin in the medium of TM5 cells transduced with the Q368X and K423E adenovirus vectors, but very small levels of myocilin were found in the medium with the G364V, I477S and Y437H mutants (Fig. 2B). Two bands of intracellular myocilin that appeared to be identical to wild-type were seen in all of the missense mutants in both cell types, and two bands of lower molecular weight (∼40 kDa) were seen for the nonsense (Q368X) mutant expressed in TM5 cells (Fig. 2A). There was no detectable intracellular expression of Q368X myocilin in the A549 cells (Fig. 1A).

An independent study evaluated the expression of MYOC mutants in TM5 cells using transient transfection with pcDNA3 expression vectors. Electroporation techniques were optimized using the pcDNA3-lacZ expression vector to give a 20% transfection frequency in the TM5 cells (data not shown). Myocilin was expressed inside the cells as well as secreted into the medium in a TM5 cell population (Fig. 3). As expected, the intracellular myocilin in the Q368X mutant was truncated. These results are very similar to those obtained with the TM5 cells transfected with the MYOC adenovirus vectors.

GLC1A (myocilin) glaucoma is inherited as an autosomal dominant disorder with high, but incomplete, penetrance. In order to determine whether the presence of a mutant myocilin affects transport of wild-type protein consistent with a dominant negative effect, co-transfection experiments were performed. Wild-type MYOC was co-transfected with either mutant MYOC (Q368X) or control vector (lacZ) into Cos-7 and TM5 cells. Q368X was chosen over the missense mutants due to its distinguishability from normal MYOC by western blot analysis. The molar ratio of wild-type to mutant or control vector was varied (1:1, 1:3.5, 1:8) for each individual co-transfection. Secretion of wild-type myocilin from Cos-7 cells was reduced when co-expressed with mutant myocilin at all plasmid ratios tested (Fig. 4). This transfection technique did not affect the overall cell viability throughout the course of this study (i.e. cell viability stayed >95% by trypan blue assessment). Increasing concentration of Q368X plasmid appeared to suppress expression of wild-type myocilin in the cell lysate as well. Co-transfection of wild-type MYOC with lacZ appeared to stimulate myocilin secretion. Secretion of wild-type myocilin from TM5 cells also was suppressed by co-transfection with Q368X MYOC (Fig. 5). Interestingly, mutant myocilin could be detected in the medium in transfections with the higher molar ratios, suggesting that at sufficiently high expression levels Q368X myocilin may overcome the secretory blockade or cause a non-secretory release of myocilin. In contrast to the results in Cos-7 cells, co-transfection of wild-type MYOC with lacZ did not appear to stimulate myocilin secretion.
Myocilin in aqueous humor

If wild-type myocilin is secreted from cells that are present in the anterior segment of the eye, such as TM cells, it is possible that myocilin would be present in the aqueous humor. We examined aqueous humor samples from 12 patients who had no history of glaucoma, 6 glaucoma patients who were genotyped as having MYOC polymorphisms and 13 glaucoma patients who did not have detectable MYOC polymorphisms. The six glaucoma patients were heterozygous for the Y437H, P361S, D208E (single patients for each polymorphism) or Q368X (three patients) MYOC mutations. Readily detectable levels of myocilin were present in the aqueous humor of all 12 normal patients (Fig. 6A). Myocilin was present as two bands of ~55–57 kDa in these reduced gels. Higher molecular weight forms of myocilin were seen in non-reducing gels (data not shown). Truncated forms of myocilin were not detectable in the aqueous humor from patients with the Q368X mutation (Fig. 6A). It appears that aqueous humor myocilin levels are quite variable among the various aqueous humor specimens. In order to determine whether there are quantitative differences in aqueous myocilin expression, all the western blots were stripped and reprobed with antibodies to transferrin, a relatively abundant protein in the aqueous humor (26). Normalization of myocilin to transferrin levels was important because there were slight differences in protein content between the various aqueous humor samples (Fig. 6A). Overall, the aqueous humor levels of myocilin tended to be modestly elevated in non-MYOC glaucoma patients and slightly reduced in MYOC glaucoma patients (Fig. 6C), but these differences were not statistically different due to the large variability and small sample sizes (Fig. 6B).

DISCUSSION

Myocilin is involved in the development of autosomal dominant juvenile glaucoma and a subset of adult-onset POAG (8,10–12). The molecular mechanisms responsible for causing this form of glaucoma are poorly defined. We have shown that normal myocilin is secreted into the medium and that there is a defect in the secretion of myocilin when myocilin mutants are transfected into cultured cells. In addition, myocilin is found in human aqueous humor. The mutant isoform of myocilin is not found in the aqueous humor of patients with Q368X MYOC glaucoma; unfortunately, we cannot currently differentiate the mutant from wild-type myocilin in the aqueous humor of glaucoma patients with missense MYOC polymorphisms.

The most common forms of glaucoma are associated with elevated IOP. One of the hallmarks of GLC1A juvenile glaucoma is the development of high IOP at a relatively early age. This finding, along with the relatively high expression of myocilin in the TM suggests that one possible function of myocilin is to regulate IOP. Increased expression of myocilin has been reported in TM cells of some glaucomatous individuals. There also appears to be a slightly higher concentration of myocilin in the aqueous humor of glaucoma patients in this study; however, this apparent increase was not statistically significant due to the relatively small sample size and the large variability. It has been proposed that secreted myocilin accumulates in the extracellular matrix leading to a blockage of aqueous humor outflow and elevated IOP (13). This hypothesis was based on two findings: (i) anti-inflammatory glucocorticoid therapy can lead to elevated IOP and open angle glaucoma in man (2,3); and (ii) myocilin expression and secretion is induced in the TM by glucocorticoids (13,14,19). Glucocorticoid-induced glaucoma is clinically similar to POAG, and there have been a number of studies suggesting the involvement of glucocorticoids in the development of POAG (3,13,18). However, there do not appear to be any promoter or coding sequence polymorphisms in myocilin that are more prevalent in ‘steroid responder’ patients compared with non-responders (27). In addition, our current study indicates that certain mutant forms of myocilin are not secreted, and thus the results of this study do not support the hypothesis that mutant forms of myocilin associate with the extracellular matrix resulting in decreased aqueous outflow.

There are several alternative explanations for the involvement of myocilin in regulating IOP. Secreted myocilin may be required for the maintenance of normal aqueous outflow. Fresh TM tissue and TM tissue from perfusion cultured eyes have relatively high levels of myocilin mRNA expression (19). In addition, myocilin is induced in TM cells by stretch (19), suggesting that it may play a role in responding to pressure changes in the TM. Our current data on the expression of myocilin in cultured cells and in aqueous humor indicate that mutant isoforms of myocilin are not secreted and may suppress the secretion of the wild-type myocilin. Myocilin forms oligomers in the absence of a reducing agent (14), and it is possible that the association of mutant with wild-type myocilin prevents secretion. Disulfide bond formation and oligomerization occur in the endoplasmic reticulum (ER) so it is likely that the normal–mutant myocilin interaction occurs in the ER. Alternatively, certain myocilin mutations may cause a centralized defect in the secretory pathway of TM cells leading to progressive...
defects in normal TM cell function. It is quite possible that certain myocilin mutations cause myocilin to accumulate in specific intracellular compartments, and we have preliminary electron microscopy data to support this supposition.

It is unclear whether myocilin haploinsufficiency alone in the absence of a dominant negative effect is enough to cause glaucoma. To date, nearly all MYOC mutations are found in the last exon and result in a protein product. One exception to this is the R46X mutation. It is not known whether this mutation results in a protein product that could affect the secretion of normal myocilin. Although we have made a construct containing this mutation, we cannot observe the protein product because the antibody used in this study does not recognize this truncated protein (data not shown). Interestingly, there are individuals with the R46X mutation who do not appear to develop glaucoma, even at advanced age (28).

A previous study also reported phenotypic differences associated with certain myocilin mutations. Zhou and Vollrath (29) prepared FLAG-tagged myocilin expression vectors for >20 MYOC variants that were reported to be associated with

Figure 6. Western immunoblot analysis of myocilin and transferrin (Tf) in human aqueous humor samples. (A) Immunostaining with anti-MYOC antibody 129 and with anti-transferrin antibody. Lanes 1–6, aqueous humor samples from normal patients (i.e., patients with no history of glaucoma); lanes 7–9, GLC1A glaucoma patients with Q368X MYOC mutations; lanes 10–12, glaucoma patients without detectable MYOC mutations. (B) Ratio of myocilin to transferrin expression in the aqueous humor of individual patients. (C) Average myocilin:Tf ratios for the normal, GLC1A glaucoma and non-GLC1A glaucoma patients (means ± SD; these groups were not statistically different from one another).
glaucoma. They transiently transfected HEK and COS-7 cells with these myocilin constructs and looked at Triton solubility of cell-associated myocilin. Under their conditions, wild-type myocilin and the K398R polymorphism were Triton soluble, whereas the Q368X, K423E, Y437H and I477S mutants were Triton insoluble. There appeared to be a very good correlation between disease-associated myocilin mutations and the Triton insolubility of myocilin in their system. Our current results are in excellent agreement with the findings of Zhou and Vollrath (29) (i.e. it appears that there is a good correlation between Triton insolubility and the lack of myocilin secretion). Interestingly, Zhou and Vollrath (29) found that myocilin containing the G364V mutation was present in both the Triton-soluble and the Triton-insoluble fractions of the cell. Our results also indicate that a small portion of G364V myocilin can be secreted. Four of the six aqueous humor samples from the GLC1A patients had mutations that were characterized in our secretion assay and the Triton solubility assay. All four appeared to have no secretion of the mutant myocilin and/or diminished aqueous humor levels of myocilin. The other two glaucoma patients have presumed myocilin mutations that will require further in vitro testing.

Evaluation of genotype–phenotype correlations in GLC1A glaucoma indicate that several of the missense MYOC mutations, including K423E, Y437H and I477S are associated with juvenile glaucoma that has an early age at onset and high IOPs (10). Myocilin encoded by these mutations fails to be or was poorly secreted from TM cells. In contrast, the G364V missense MYOC mutation is associated with a later age at onset and slightly lower IOP. In our studies, some of this mutant myocilin is secreted into the medium. There is also a good correlation between the Q368X mutation and the development of a much later-onset, milder form of glaucoma (10). Paradoxically, none of the Q368X myocilin is secreted from the transfected cells. In addition, in certain cell types (e.g. A549 cells) there is very little expression of intracellular Q368X myocilin suggesting that this form of mutant myocilin may be more susceptible to intracellular degradation. It is quite possible that the various mutant forms of myocilin are blocked at different steps of the secretory pathway, and that the severity of this blockage is correlated with the glaucoma phenotype. In fact, there is support for altered processing of the E323K MYOC mutation in an in vitro translocation processing system, in which the mutant isoform of MYOC exhibits major translational pausing (30). In addition, we have preliminary ultrastructural data to support the blockage of secretion of several of these MYOC mutants in different compartments of the secretory pathway.

Additional clinical studies of patients with GLC1A glaucoma provide interesting, but enigmatic, results. Morissette et al. (31) reported a large pedigree harboring the K423E MYOC mutation. Patients heterozygous for this mutation generally develop an early-onset autosomal dominant juvenile glaucoma. This may cause glaucoma either by haploinsufficiency or by a dominant negative effect. These investigators also identified several middle-aged patients who are homozygous for this MYOC mutation but who do not appear to have glaucoma. The authors have suggested that homoallelic complementation may be responsible for restoration of myocilin function. However, we do not see any evidence of myocilin secretion in the K423E-transfected or -transduced cells, indicating that the secretory function of this mutant is not restored by ‘homoallelic complementation’.

The source of myocilin in the aqueous humor is unclear. We and others have shown that cultured TM cells are able to secrete myocilin, and this is one possible source of aqueous humor myocilin. However, there are a variety of other anterior segment tissues that may be involved in myocilin secretion. In situ hybridization and northern blot experiments have shown that myocilin mRNA is expressed in the TM, ciliary body, iris and cornea (12,15,22–24). A comprehensive study of myocilin immunostaining in the eye also indicates that myocilin is expressed in the TM, ciliary body, iris, sclera and cornea (25), each of which could contribute to the presence of myocilin in the aqueous humor. However, to our knowledge, no one has shown that any of these tissues (other than the TM) actively secrete myocilin.

It is tempting to speculate that mutant myocilin may be misprocessed as in the case of certain mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) and the α1-antitrypsin genes. Ninety percent of patients with cystic fibrosis have a ΔF508 mutation that causes this CFTR to be retained in the ER and not be transported to the cell surface (32). α1-anti-trypsin is a glycoprotein secreted from hepatocytes and macrophages to serve as a major serum protease inhibitor. Defects in the secretion of this protein lead to α1-antitrypsin deficiency, an autosomal recessive inherited disease. Most patients with this deficiency have a mutation generating a Z form of the protein that is retained in the ER and does not progress to the Golgi apparatus for secretion (33). The misprocessing of mutations in these two genes may provide clues to understanding how mutant MYOC is handled in the TM and may lead to a better understanding of GLC1A glaucoma.

Our results are the first to show the expression of myocilin in the aqueous humor and the differential secretion of normal versus mutant myocilin. Future studies should define the precise steps in the secretory pathway at which disease-causing myocilin mutant proteins are blocked from secretion. The ER and Golgi are likely to be involved in myocilin retention because of their role in glycosylation, disulfide bond formation, proper folding and oligomer formation. It will be of interest to determine whether glaucoma results from a deficiency of normal myocilin in the aqueous humor or whether it results from toxicity to cells due to the effect of mutant myocilin on secretion. In addition, we cannot discount a direct effect of mutant myocilin on the optic nerve, since MYOC is expressed in this tissue. Ultrastructural localization and identification of myocilin-interacting proteins should help in resolving some of the questions raised by this report on altered expression of mutant myocilin in the TM and aqueous humor.

MATERIALS AND METHODS

Myocilin constructs and adenoviral particle generation

Human myocilin cDNA was cloned into the BamHI site of the mammalian expression vector pcDNA3 (Invitrogen). In some cases, a FLAG epitope sequence (5’-GACTACAAGGAC-GACGATGACAAAA-3’) was inserted at the 3’ end of the cDNA using the QuickChange Site-Directed Mutagenesis kit (Stratagene) in a three-step process according to the manufacturer’s instructions. The following mutations were introduced to both
the FLAG and non-FLAG constructs using the QuickChange kit: G364V, Q368X, K398R, K423E, Y437H and I477S. These mutations were selected because they are well-characterized and represent a spectrum of disease phenotypes (i.e. juvenile glaucoma and adult-onset glaucoma). Each mutagenesis step was followed by dideoxy-sequence analysis to confirm the entire sequence and reading frame. The sense-strand primer sequences used for mutagenesis were as follows:

G364V: 5′-CCC TGG AGC TGT CTA CCA CGG ACA GTT CCC G-3′; Q368X: 5′-GGC TAC CAC GGA TAG TTC CCAG TAT TCT TGG G-3′; K398R: 5′-CCG ATG AGG CCA GAG GTG CCA TTG TCC TCT CC-3′; K423E: 5′-GGA GAC AAA CAT CGA TGA GCA GTC AGT CGC C-3′; Y437H: 5′-GTG GCA CCT TGC ACA CCG TCA GCA GCT ACA GG-3′; I477S: 5′-AAG TAC AGC AGC ATG AGT GAC TAC AAC CCC CTG-3′.

The antisense primer sequences are the reverse and complement of the sense primer sequence for each mutation.

In order to generate adenovirus expression vectors, the mutated cDNAs were subcloned into the BamHI site of the shuttle vector pAd5R5VK-Npa (Gene Transfer Vector Core, University of Iowa College of Medicine, Iowa City, IA). The virus particles were generated using the RAPAdTM.1 procedure (34). Briefly, 15 µg of Pac1 linear shuttle plasmid was co-transfected with 4 µg of linear pcAd5 9.2-100 backbone into HEK293 cells using standard CaCl2 methods. Viral foci were evident after 4–8 days and expanded and purified by double CsCl centrifugation. Virus plaque-forming units were determined by limiting dilution on HEK293 cells. The presence of replication-competent adenovirus was tested by PCR for E1 and plaque assay on A549 cells.

Cell culture, myocilin transfection and sample preparation

The A549 cell line (human lung/airway carcinoma) (Dr Beverly Davidson, The University of Iowa Hospitals and Clinics, Iowa City, IA) was maintained in Eagle’s MEM supplemented with 10% fetal bovine serum (FBS). TM5, a stable transformed human TM cell line (35), was grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% stable transformed human TM cell line (35), was grown in Eagle’s MEM (36). Briefly, 15 µg of Pac1 linear shuttle plasmid was co-transfected with 4 µg of linear pcAd5 9.2-100 backbone into HEK293 cells using standard CaCl2 methods. Viral foci were evident after 4–8 days and expanded and purified by double CsCl centrifugation. Virus plaque-forming units were determined by limiting dilution on HEK293 cells. The presence of replication-competent adenovirus was tested by PCR for E1 and plaque assay on A549 cells.

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For adenovirus transductions, cells growing exponentially were seeded in 6-well plates at a density of 5 × 105 cells/well 18 h before use. At the time of infection, the medium was removed, cells were washed once with 1 ml of phosphate-buffered saline (PBS) + 2% FBS, and 1 ml of medium + 2% FBS was added to each well. Cells were infected with adenovirus at 100 p.f.u./cell, for 1 h at 37°C, 5% CO2. The medium was removed, and cells were washed twice with 1 ml of PBS + 1% FBS, and then cultured with medium + 1% FBS for 48 h at 37°C, 5% CO2. After the 48 h incubation, the supernatant from the adenovirus transfection cultures was transferred to 15 ml confluence tubes and centrifuged in a Beckman tabletop centrifuge at 2500 r.p.m. for 10 min. The cleared supernatants were carefully removed and placed in a new tube and stored at −80°C until used. The plates with the adherent cells were placed on ice. Each well was washed twice with 1 ml of PBS, 300 µl of triple-detergent lysis buffer (50 mM Tris–Cl pH 8.0, 150 mM NaCl, 0.02% NaN3, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin) was added to each well, and incubated for 20 min on ice. The cells were scraped off the bottom of the wells with a sterile plastic policeman and transferred to a 1.5 ml Eppendorf tube. The collected cells were lysed by rapidly passing through a 25G tuberculin syringe needle. The cell lysates were stored at −80°C until used. A BioRad DC Protein Assay, a method based on the Lowry assay (36), determined protein concentration.

For transient transfections, the long-duration electroporation procedure of Bodwell et al. (37) was followed. TM5 cells were harvested from T-175 flasks by trypsinization, pelleted by centrifugation and resuspended in ice-cold permeabilization buffer (10 mM HEPES, 137 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 2.7 mM EGTA, 1 mM ATP, pH 7.4) at a concentration of 3–10 × 105 cells/ml. Fifty microliters of DNA buffer (20 mM HEPES, 142 mM NaCl, 5.4 mM KCl, 1.3 mM Na2HPO4, 6 mM glucose, pH 7.4) containing 10 µg (−2.1 pmol) of plasmid DNA (pcDNA3.MYOC) was added to 300 µl of TM5 cells. Cells were electroporated in a 0.4 cm cuvette with a BioRad Gene Pulser II set at 170 V and a BioRad Capacitance Extender Plus set at 2500–3200 µF to achieve a time constant of 135–140 ms. Electroporated cells were resuspended in 1 ml of medium and plated into wells of a 24-well plate at a concentration of ~1 600 000 cells/well. Medium was changed after 24 h. Cells were harvested after 48 h. Serum-free medium was added 12 h prior to cell or medium collection for secretion studies. Serum-free medium was concentrated by centrifugation in a Centriforc-10 (Millipore) spin column according to the manufacturer’s recommendations to achieve an 8–10-fold concentration. Samples were stored at −20°C. The TM5 cells were rinsed with PBS, and cellular proteins were extracted in 100 µl of mammalian protein extraction buffer (Pierce) containing protease inhibitors (Complete, EDTA-free Protease Inhibitor Cocktail; Boehringer Mannheim). The cell extract was centrifuged at 12 000 g for 5 min, and the supernatant was stored at −20°C. Protein concentration was determined with the Pierce Coomassie Plus Protein Assay Reagent. Overall cell viability was assessed by trypan blue exclusion.

In an attempt to model the autosomal dominant nature of GLC1A glaucoma, wild-type MYOC was co-transfected with either mutant MYOC (Q368X) or lacZ into Cos-7 or TM5 cells. The MYOC Q368X mutation was chosen over the missense mutations due to its more common occurrence in POAG and its size-distinguishability from wild-type myocilin by western blot analysis. The amount of wild-type MYOC was fixed and the molar amount of either mutant MYOC or lacZ was varied from 1:1, 1:3.5 or 1:8 for each individual co-transfection. All transfections received equivalent total molar amounts of DNA using empty pcDNA3 vector as filler.

Aqueous humor samples

After obtaining informed consent, human aqueous humor samples were collected from patients undergoing intraocular surgery. Primary aqueous humor was taken from glaucoma patients undergoing glaucoma filtration surgery and/or cataract extraction and the aqueous humor samples from patients without a clinical history of glaucoma were taken during cataract extraction. The glaucoma patients were genotyped for mutations due to its more common occurrence in POAG and its size-distinguishability from wild-type myocilin by western blot analysis. The amount of wild-type MYOC was fixed and the molar amount of either mutant MYOC or lacZ was varied from 1:1, 1:3.5 or 1:8 for each individual co-transfection. All transfections received equivalent total molar amounts of DNA using empty pcDNA3 vector as filler.

SDS–PAGE and western blot

SDS–PAGE and western immunoblotting of transient TM5 transfections and of human aqueous humor samples were...
performed using a Novex NuPAGE (Novex) gel electrophoresis system. Five microgram samples of TM5 medium and cell extract and 5–10 μl samples of aqueous humor were electrophoresed in 10% Bis–Tris–polyacrylamide gels. Proteins were transferred to Hybond-P PVDF membranes (Amersham), blocked with gelatin and probed using an affinity-purified rabbit polyclonal anti-myocilin peptide antibody 129 (generated to myocilin peptide 156–171) and an anti-rabbit IgG secondary antibody (Amersham) with ECL Plus (Amersham) detection. After myocilin detection, aqueous humor immunoblots were stripped and re-probed using a rabbit anti-human transferrin antibody (Research Diagnostics) as an internal standard. Blots were exposed to Biomax MR film (Eastman Kodak) for imaging or were scanned on a Storm 840 Phosphorimager (Molecular Dynamics) for image quantification (ImageQuant version 5.1).

Adenovirus-transduced A549 and TM5 samples were placed in Laemmli’s sample buffer and boiled for 5 min. Between 20 and 40 μg of the cell lysates and the supernatants were electrophoresed on 10% SDS–PAGE gels (38) and electrothermally transferred to Hybond-P PVDF membranes (Amersham). The membranes were then blocked with 0.1 ml/cm² of primary blocking buffer [5% non-fat dry milk, 0.01% antifoam A (Sigma), 0.02% NaN3 and TBST (10 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20)] for 2 h at room temperature. The samples were incubated with 0.1 ml/cm² primary blocking buffer + 10 μg/ml mouse anti-FLAG monoclonal antibody M2 (Sigma) or with anti-human MYOC antibody 129 for 2 h at room temperature. The membranes were then washed three times for 10 min at room temperature with TBST at a volume of 1 ml/cm² and electrically transferred to Hybond-P PVDF membranes (Amersham). The membranes were then blocked with 0.1 ml/cm² secondary blocking buffer (5% non-fat dry milk in TBST) + rabbit anti-mouse IgG horseradish peroxidase (Amersham) diluted to 0.02 μg/ml (1:50 000) for 1 h at room temperature. The blot was washed three times in TBST for 10 min at room temperature, treated with a chemiluminescence substrate (ECL Plus; Amersham) and immediately exposed to Biomax MS film (Eastman Kodak).

ACKNOWLEDGEMENTS

This research was supported in part by the National Institutes of Health (EY 10564), the Roy J. Carver Charitable Trust, the Glaucoma Research Foundation and Alcon Research Ltd. V.C.S. is an Associate Investigator of the Howard Hughes Medical Institute.

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