Glucocorticoid Induction of the Glaucoma Gene *MYOC* in Human and Monkey Trabecular Meshwork Cells and Tissues

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Purpose. To examine the intracellular and extracellular expression of myocilin in the human and primate trabecular meshwork (TM) in the presence and absence of glucocorticoids.

Methods. Myocilin expression was examined in cultured human TM cells by Northern blot analysis and myocilin antibody–mediated immunoprecipitation. Myocilin expression was quantified using high-resolution two-dimensional polyacrylamide gel electrophoresis of radiolabeled proteins from human TM cells, TM tissue explants, and perfused human anterior segments cultured with and without dexamethasone (DEX) for 14 to 21 days, as well as TM tissue from pigtailed monkeys treated orally for 1 year with cortisone acetate. Immunofluorescence with anti-myocilin antibodies was used to localize cellular and extracellular expression of myocilin in cultured human TM cells.

Results. Glucocorticoid treatment caused a significant induction of myocilin mRNA, a tetrad of cell-associated proteins, and 8 to 20 secreted proteins (molecular mass [M_r] 56 and 59 kDa and isoelectric point [pI] 5.2 and 5.3) in some, but not all the cultured human TM cells and explanted tissues. Western immunoblot analysis using anti-myocilin peptide antibodies identified these proteins as encoded by the *MYOC* gene. There was significant induction of the myocilin proteins in three perfusion-cultured human eyes, in which DEX-induced elevated intraocular pressure developed. Monkeys treated 1 year with cortisol acetate showed steroid glaucoma-like morphologic changes in the TM that correlated with the induction of myocilin in the TM. Immunofluorescence analysis of cultured TM cells localized myocilin intracellularly in discrete perinuclear and cytoplasmic vesicular deposits as well as extracellularly on the cell surface associated with the extracellular matrix. In several DEX-treated TM cell lines, there were significant levels of myocilin secreted into the media. Enzymatic deglycosylation of proteins in the TM media converted the higher molecular weight isoforms of myocilin (~57 kDa) to the lower molecular weight isoforms (~55 kDa).

Conclusions. Although the function of myocilin is unknown, induction of these TM proteins was found in eyes in which glucocorticoid-induced ocular hypertension developed. Therefore, myocilin may play an important pathogenic role in ocular hypertension in addition to its role in certain forms of POAG. (*Invest Ophthalmol Vis Sci.* 2001;42:1769–1780)

Glaucoma is a heterogeneous group of optic neuropathies that affect 1% to 2% of the population older than 40 years.[1] It is estimated that 66 million people in the world have glaucoma, although fewer than half of those affected do not realize that they have the disease.[2] One of the major risk factors for development of the most common form of glaucoma, primary open-angle glaucoma (POAG), is elevated intraocular pressure (IOP).[3]

One important model that appears to mimic many aspects of POAG is glucocorticoid-induced glaucoma. A number of laboratories have been studying the effects of glucocorticoids on the trabecular meshwork (TM) as a model to better understand the ocular hypertension associated with POAG. The administration of glucocorticoids can cause ocular hypertension in susceptible individuals[4–6] and in a number of different animal species.[7–10] This elevated IOP is associated with morphologic and biochemical changes in the TM.[11–13] In addition, the treatment of cultured TM cells with glucocorticoids has been reported to induce changes in TM cell size,[14,15] cytoskeletal organization,[14,15] extracellular matrix deposition,[16–20] matrix metalloproteinase expression,[21,22] TM cell functions,[15,23] and TM protein expression.[23–25]

Recent findings further strengthen the link between glucocorticoids and glaucoma. The first glaucoma gene, *GLC1A*, was mapped to chromosome 1q,[26–30] and mutations in this gene are responsible for autosomal dominant juvenile glaucoma (ADJG).[31–34] Although ADJG is a relatively rare form of glaucoma, mutations in *GLC1A* also appear to account for approximately 4% to 5% randomly screened adult forms of POAG.[31,34–35] The cDNA sequence for the *GLC1A* gene is identical with a previously characterized glucocorticoid-induced gene in the TM, TM induced glucocorticoid response (*TIGR*),[23–24,36–38] a cDNA isolated from the human ciliary

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body,[39] and a gene (myocilin) isolated from a retinal cDNA library.[40] Although this gene and gene product are variably named *GLC1A*, *TIGR*, and myocilin, the currently accepted nomenclature is myocilin, with *MYOC* the gene symbol. The function of myocilin is currently unknown.

MYOC gene expression is induced by glucocorticoids in cultured TM cells.[23,24,36–38,41–43] However, there has not yet been any direct association between *MYOC* expression in the TM and the development of ocular hypertension. It has been suggested that myocilin is a stress protein,[36,37] and a recent study reports increased expression of myocilin in the TM of patients with several different forms of glaucoma.[44] Myocilin has been reported to be localized intracellularly in TM cells[41–45] and secreted into the media of glucocorticoid-treated TM cells.[23,24,36–37] In the present study, we examined the expression of myocilin in cultured human TM cells, as well as in monkey and human trabecular tissues that were treated with glucocorticoids and for which there was evidence of the development of ocular hypertension and/or glaucoma-like morphologic changes in the TM.

Materials and Methods

Culture of Human TM Cells

Human TM cells were derived from human donor eyes, as described in detail previously.[14,15,17,20] In brief, the TM was carefully dissected from the anterior segments of human donor eyes and placed in Ham's F10 culture media (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and antibiotics (Gibco BRL, Gaithersburg, MD) in a single well of a 24-well plate (Corning Costar, Cambridge, MA). TM cells migrated from the explant to form a confluent monolayer. The TM cells were serially passed using microcarrier beads (Cytodex 3; Sigma Chemical Co., St. Louis MO). In several cases, TM cell lines were initiated using the procedure of Stamer et al.[46] A total of 29 human TM cell lines were used, including lines derived from nonglaucomatous donor eyes (TM10, TM31C, TM56, TM68C, TM69C, TM70A, TM72B, TM79C, TM267, TM2341, TM2407) and glaucomatous donor eyes (GTM23, GTM59A, GTM60, GTM66, GTM63A, GTM66C, GTM74D, GTM76B, GTM81C, GTM83C, GTM86A, GTM88C, GTM90C, GTM95B, GTM426, GTM462, GTM 640, GTM1749), the latter group having documented histories of POAG. Confluent monolayers of TM cells in 25-cm^2 flasks or in 24-well culture plates were treated for 2 to 3 weeks in culture media with or without 10^{-7} M dexamethasone (DEX; Sigma) as previously described.[14,15,17,20]

Perfusion-Cultured Human Eyes

Human donor eyes were received from regional eye banks and used within 20 hours of death. Anterior segments of the eyes were set up in a constant-flow, variable-pressure perfusion organ-culture system,[47] as previously described in detail.[48] Paired eyes were perfused with media containing DEX (10^{-7} M in 0.1% ethanol) or vehicle (0.1% ethanol) for 10 days. IOP was continuously monitored using pressure transducers connected to a data recorder (DataTaker; Science Electronics, Dayton, OH). The development of steroid responsiveness and ocular hypertension was defined as pressure increases of more than 5 mm Hg from baseline during the 10 days of DEX exposure,[48] the same criterion that has been used previously to define clinical steroid responsiveness.[49,50]

Culture of Human TM Explants

Human donor eyes (median age, 82.5 years; age range, 59–93) were obtained from regional eye banks and used within 30 hours of death. The donors had no history of glaucoma, diabetes, or cancer. TM tissue was carefully dissected from anterior segments, which had iris, lens, and ciliary body removed. TM explant tissue from each eye was divided in half and placed in wells of a 24-well plate (Corning Costar) containing Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) with 0.5% fetal bovine serum (HyClone Laboratories), 100 U penicillin/ml, 100 μg streptomycin/ml, and 0.292 mg L-glutamine/ml (Gibco BRL) and incubated at 37°C in a 7% CO_2-93% air humidified incubator. One half of the TM from each eye was cultured for 3 weeks in medium containing 10^{-7} M DEX-0.1% ethanol, and the other half was cultured in medium containing 0.1% ethanol (control). Media were changed three times each week.

Long-term Treatment of Monkeys with Glucocorticoids

Pigtail macaque monkeys (*Macaca nemestrina*; mean age, 22 ± 1 years) were age-, sex-, and weight-matched into three groups that received treatment with orally administered cortisol acetate (0, 3.85, or 5.78 mg/kg body weight per day) for 12 months as part of a National Institutes of Health (NIH)– and Alzheimer's Association–sponsored study to determine glucocorticoid neurotoxicity in aged nonhuman primates.[51] IOPs were not recorded during the study, because this was not part of the original study design. At the end of the study, the monkeys were killed by barbiturate overdose, the eyes were dissected, and the anterior segments were placed in corneal preservation medium (Dexol; Chiron, Irvine, CA) for immediate shipment. Ten pairs of anterior segments (five placebo, two low-dose, and three high-dose cortisol acetate) were received. In each pair, one eye was used for protein analysis and the other eye was assessed morphologically. In one anterior segment for each pair of eyes, the TM was dissected, placed in radiolabeling medium for 24 hours, and TM proteins were analyzed as described later. In the other anterior segment, the segments were fixed and processed for ultrastructural evaluation of four quadrants per eye, as previously described.[48] Only those eyes that had acceptable morphology were used for analysis in the study. All procedures were in strict compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Northern Blot Analysis

TM cells derived from a normal donor (TM10) were used to determine the expression of *MYOC* mRNA by Northern blot analysis, using previously described techniques.[52] Normal TM cells were cultured as described earlier in the presence or absence of 10^{-7} M DEX for 14 days before harvesting total RNA. Twenty micrograms total RNA was electrophoretically separated in an agarose gel and blotted onto membranes (Gene Screen Plus; DuPont NEN, Boston MA). The Northern blot analysis was hybridized with a probe corresponding to a region of exon 3 of the human *MYOC* gene (codons 315-504). The probe was labeled with ^{32}P-(dCTP) by using DNA-labeling beads (Ready-To-Go; Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was for 16 hours at 42°C in 50% formaldehyde, 5× SSC, 1× Denhardt's solution, 20 mM phosphate buffer (pH 7.6), 1% SDS, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate. After hybridization, the blot was washed twice at room temperature in 1× SSC, rinsed twice in 1× SSC+1% SDS at 65°C, and washed once in 0.1× SSC at room temperature. Autoradiography was performed using x-ray film (XAR-5 Eastman Kodak, Rochester, NY) at −70°C with intensifying screens (Cronex Lightning Plus; DuPont, Wilmington, DE). The blot was subsequently stripped of radioactivity and rehybridized with a ^{32}P-labeled β-actin probe.

TM Protein Expression

After incubation for 2 to 3 weeks in media with and without DEX, cultured human TM cells were placed in RPMI medium (Selectamine; Gibco BRL) containing 500 μCi/ml ^{35}S-methionine (Amersham, Chicago, IL) for 1 hour before harvesting. Perfusion-cultured anterior segments from human eyes and trabecular explants from human and monkey eyes were incubated in RPMI Selectamine media containing ^{35}S-methionine at 100 μCi/ml (for 24-hour labeling) or 500 μCi/ml (for 2-hour labeling). Perfusion-cultured human eyes were perfused with radiolabeling media for 24 hours at the normal flow rate of 2 μl per

minute, whereas each of the human and monkey TM explants were placed in single wells of a 24-well culture dish (Costar) containing 0.5 ml of radiolabeling media for 2 hours and 1 ml for 24 hours, respectively. Radiolabeled proteins were extracted from the TM cells and tissues by solubilization in a solution of 9 M urea-4% Nonidet NP-40[17,20,53] using plastic pestles and microcentrifuge tubes. The homogenate was centrifuged at 15,000*g* for 15 minutes and the supernatant was stored at −80°C. Two-dimensional (2D)-PAGE of TM samples containing 0.5 to 2 × 10^6 disintegrations per minute (dpm) of ^{35}S-labeled proteins was performed with a slight modification[53] of the method of Hochstrasser et al.[54] Molecular mass (14–220 kDa) and 2D standards (Bio-Rad, Hercules, CA) were included to accurately calibrate mass and isoelectric points (pIs). The resultant 2D gels were incubated with 1 M salicylate (Sigma), vacuum dried, and exposed to x-ray film (XAR or Biomax MR; Eastman Kodak, Rochester NY) at −80°C. Autofluorograms of the gels were analyzed using a densitometer (Masterscan; Scanalytics, Billerica, MA).

Immunoprecipitation and Immunoblotting of Myocilin

A PCR product of *MYOC* encoding amino acids 316-428 was cloned into the glutathione-*S*-transferase (GST) fusion vector pGEX-2T. This plasmid was used to transform *Escherichia coli* DH5-α, and production of the myocilin fusion protein was induced with isopropyl thiogalactopyranoside (IPTG). The bacterial cells were sonicated, and the myocilin-GST fusion protein was purified from bacterial lysates by affinity chromatography on glutathione (GSH) beads. Antibodies were produced in sheep immunized with the myocilin fusion protein and subsequently boosted 8 weeks after the initial immunization. In addition, rabbit anti-myocilin antibodies were generated against myocilin peptide (amino acids 151-171) (Sigma-Genosys; The Woodlands TX).[55] Human TM cells were cultured in the presence or absence of DEX for 3 weeks, and lysates and cell culture media were immunoprecipitated and separated by SDS-PAGE or were analyzed by 2D-PAGE immunoblot analysis.[56] In addition, the media from perfusion-cultured human eyes were immunoblotted with anti-myocilin and anti-transferrin (Research Diagnostics, Inc., Flanders NJ) antibodies. For immunoprecipitation of cellular myocilin, DEX-treated GTM66 cells were lysed in Tris-buffered saline containing 1% Triton X-100, 1% hemoglobin, 1 mM iodoacetamide, and protease inhibitors (Boehringer-Mannheim, Indianapolis, IN) and centrifuged at 100,000 rpm for 60 minutes. Cell culture media and the lysate supernatant were precleared with protein beads (Protein A/G; Pierce Chemical Co., Rockford IL) before incubation with affinity-purified sheep anti-myocilin antibodies. The antigen-antibody complexes purified using protein beads (ProteinA/G; Pierce) were extensively washed and centrifuged, and the pellet was solubilized in Laemmli's sample buffer before loading onto SDS-polyacrylamide gels. Immunodetection of myocilin in SDS-PAGE and 2D-PAGE immunoblots was performed using anti-myocilin antibodies (1:250 dilution) and alkaline-phosphatase–conjugated secondary antibody (goat anti-sheep or anti-rabbit IgG) using a 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system (BioRad).

Immunofluorescent Localization of Myocilin

Human TM cells were cultured as described previously.[14,15,17,20] Twelve different TM cell lines (TM10A, TM31C, GTM63A, GTM66C, TM68C, TM70A, TM72B, TM79C, GTM831C, GTM83C, GTM86A, GTM95B) derived from donors aged 51 to 92 years were used in these studies. TM cells were grown to confluence before incubation with or without DEX (10^{-7} M) for 2 weeks. To examine intracellular myocilin, media were aspirated, and the cells were rinsed with phosphate-buffered saline (PBS) before fixation for 20 minutes in 3.7% formaldehyde. The fixed TM cells were blocked with goat antisera and exposed to anti-myocilin antibodies (1:250 dilution), rinsed in PBS, and incubated with Texas red– or Oregon green–conjugated goat anti-rabbit Ig (Molecular Probes, Eugene, OR). Actin microfilaments were visualized using Oregon green conjugated to phalloidin (Molecular Probes). Localization of microtubules, the microtubule motor protein kinesin, the Golgi apparatus, fibronectin, and type III and type IV collagen was performed using anti-tubulin (Sigma), anti-kinesin (Sigma), anti-TGN38 (Affinity Bioreagents, Deerfield, IL), anti-fibronectin (Sigma), and anti-collagen (Southern Biotechnology Associates, Inc., Birmingham, AL) mouse monoclonal antibodies followed by Texas red- or Alexa 488-conjugated goat anti-mouse IgG secondary antibodies (Calbiochem, La Jolla, CA). To detect extracellular myocilin expression, human TM cells cultured in the presence of DEX for 2 weeks were washed in PBS and incubated with anti-myocilin antibody for 60 minutes. The specificity of staining was determined by incubation with normal serum or anti-myocilin antibody plus myocilin peptide. The cells were washed again with PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Cells were washed with PBS, and secondary antibodies were added to the cells followed by a wash with PBS. The TM cells were examined by epifluorescence microscopy with a photomicroscope (Optiphot; Nikon, Tokyo, Japan) and photographs were taken (ASA 1600 film; Kodak).

Enzymatic Deglycosylation of Myocilin

Conditioned serum-free media from a DEX-treated GTM cell line (GTM1749) were concentrated 40× (Centirprep 10; Millipore, Bedford MA) and 12 μl of media were sequentially treated with O-Glycosidase DS, PGNase, and NANase II (BioRad). Samples were subjected to SDS-PAGE and immunoblotting as described earlier.

Results

Northern blot analysis showed that cultured human TM cells expressed a single 2.3-kb *MYOC* mRNA band (Fig. 1A). Exposure of cultured TM cells to 10^{-7} M DEX for 14 days led to a significant (47-fold) induction of *MYOC* gene expression. Immunoprecipitation of radiolabeled proteins from DEX-treated TM cells using sheep anti-myocilin antibodies identified two proteins on SDS-polyacrylamide gels with relative masses of 57 and 55 kDa (Fig. 1B). Immunoprecipitation of media from DEX-treated TM cells resulted in the appearance of three different proteins with approximate masses of 56, 54, and 42 kDa (Fig. 1C). Western blot analysis of these immunoprecipitated proteins using rabbit anti-myocilin antibodies showed that the upper two bands were myocilin (Fig. 1D). The identity of the 42-kDa band is currently unknown.

2D-PAGE of radiolabeled cell-associated proteins derived from one of the cultured human TM cell lines (GTM66) showed that DEX treatment altered the expression of a number of proteins, any of which may be responsible for the development of ocular hypertension. The most prominent change was a DEX-induced 9- to 20-fold increase in the expression of a tetrad of proteins with pIs of 5.2 and 5.3 and molecular masses of 57 and 55 kDa (Figs. 2A, 2B). These proteins were identified as products of the *MYOC* glaucoma gene by virtue of their immunoreactivity with an antibody generated against a myocilin peptide (Figs. 2C, 2D) that was derived from the *MYOC* gene.[52] These four proteins may represent various modified forms of the *MYOC* gene product (e.g., proteolytic processing, glycosylation, phosphorylation). The two more acidic proteins were not as immunoreactive as the two basic proteins using this anti-myocilin peptide antibody. Fifteen of 25 of the TM cell lines tested had a DEX-induction of cell-associated myocilin (Tables 1, 2). Western immunoblots of 2D gels of concentrated media from DEX-treated GTM1749 cells showed multiple (i.e., up to 20) isoforms of myocilin ranging in masses from 53 to 59 kDa and pIs from 5.0 to 5.7 (Fig. 2E). To determine whether any of these myocilin isoforms was due to glycosylation, the TM media were subjected to enzymatic deglycosylation. Western immunoblot analysis of 1D gels revealed two major protein bands (each a doublet) before deglycosylation. After deglyco-

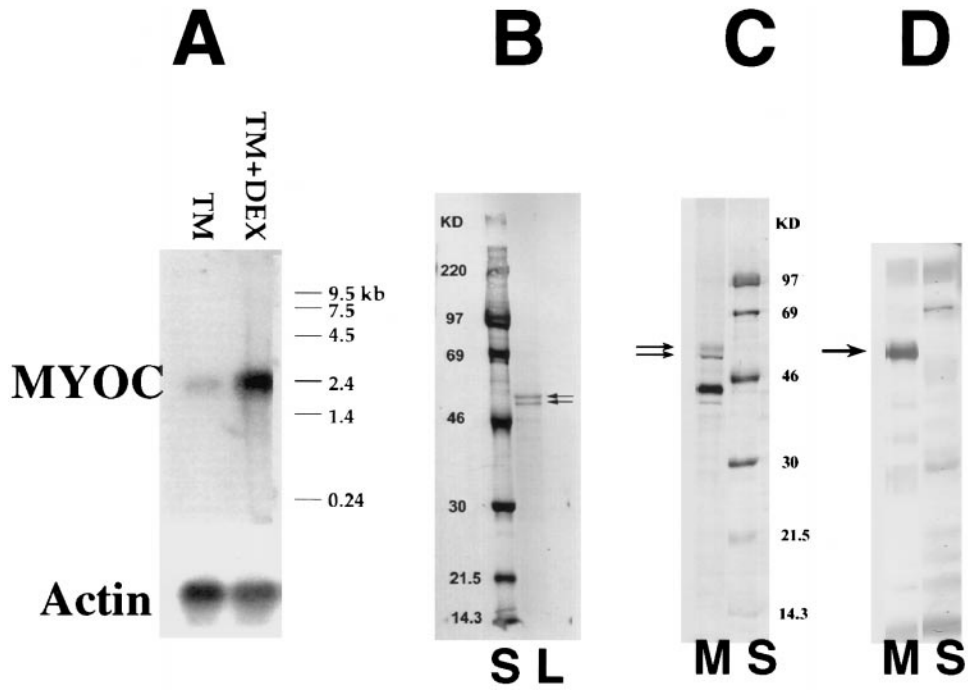

FIGURE 1. Expression of *MYOC* mRNA and proteins in cultured TM cells. (**A**) Northern blot analysis of *MYOC* mRNA expressed in TM cells cultured in the absence and presence of DEX (10^{-7} M). Expression of *MYOC* was significantly increased by treatment with DEX for 14 days. The same blot was reprobed with β-actin. (**B**) SDS-gel autofluorograph of GTM66 cell lysate (L) that was immunoprecipitated with anti-myocilin antibodies. (**C**) SDS-gel autofluorograph of GTM66 cell culture media (M) that were immunoprecipitated with anti-myocilin antibodies. (**D**) Western immunoblot of cell culture media (M) from GTM66 cells. All these TM cells were treated with DEX (10^{-7} M) for 2 weeks. (**B–D**, *arrows*) Myocilin bands. S, molecular weight standards.

sylation, only the lower molecular weight doublet remained (Fig. 2F).

Previous work in our laboratory[48] has shown that perfusion-cultured human eyes treated with DEX for 10 days can be categorized as DEX responsive (stable IOP increases, >5 mm Hg) or DEX nonresponsive (stable pressure change, <6 mm Hg). We successfully radiolabeled TM proteins in DEX-responsive eyes (average pressure increase of 9 ± 3 mm Hg after 7 to 10 days, $n = 3$) and nonresponsive ($n = 2$) and control ($n = 5$) eyes by the addition of ^{35}S-methionine to the perfusion media. There was a DEX-induced 2- to 12-fold increased expression of a quartet of proteins with molecular weights of 56 and 59 kDa and pIs of 5.2 and 5.3 in the DEX-responsive eyes compared with control eyes and DEX-nonresponsive eyes. Representative images of the 2D gels are shown in Figures 3A and 3B. These DEX-induced proteins are located in the same 2D-gel position as myocilin derived from cultured human TM cells (Fig. 2). There appeared to be a positive correlation between the amount of myocilin induced and the level of DEX-induced ocular hypertension (Fig. 4A, $r = 0.973$). Myocilin was expressed also in the media of untreated perfusion-cultured human eyes throughout the 8-day culture period with decreased expression on days 6 through 8, whereas the expression of transferrin progressively decreased throughout the culture time (Fig. 3C).

There was also increased expression of a very similar family of proteins (56 and 59.5 kDa and pI 5.2 and 5.4) in some of the human TM explants treated with DEX for 3 weeks compared with control specimens (Figs. 3D, 3E). There appeared to be a modest variation in the level of induction of these proteins among the series of 12 TM explants evaluated with several explants giving approximately a threefold induction, whereas others had only minor, barely detectable levels of these proteins after DEX exposure (Table 1).

Monkeys treated with the glucocorticoid cortisol acetate for 12 months displayed no loss in body weight, although there were significant increases in urinary and plasma cortisol and significant suppression of plasma adrenocorticotropic hormone (ACTH) in the cortisol acetate–treated groups.[51] Two pairs of eyes from each of the three groups were examined ultrastructurally and biochemically. The control placebo eyes showed relatively normal TM with abundant TM cells, intertrabecular spaces, and separate beams. Schlemm's canal was open and the endothelial lining was intact (Figs. 5A, 5B). No blebs or vacuoles were noted, because this tissue was not perfusion fixed. The TM from age-matched animals receiving cortisol acetate (Figs. 5C, 5D) appeared relatively well preserved but markedly different from control tissue. The intertrabecular space was drastically reduced and fusion of trabecular beams was common. There appeared to be fewer TM cells, and the basement membrane of those cells was thickened, with both amorphous and fibrillar material. The trabecular beams were thicker, with an apparent increase in long-spacing collagen. There also appeared to be a substantial increase of extracellular material in the juxtacanalicular tissue (JCT) region of the TM in the steroid-treated animals. Unfortunately, there is no IOP history to correlate with these morphologic findings. There appeared to be a dose-dependent induction of myocilin expression in the TM of these animals (Fig. 4B). Monkeys subjected to prolonged treatment with low or high doses of cortisol acetate had approximately a 1.5- to 3-fold increased expression of TM proteins with molecular masses of 53 and 55 kDa and pIs of approximately 5.3, compared with TM proteins from control animals (Figs. 3F–H). The position of these monkey TM pro-

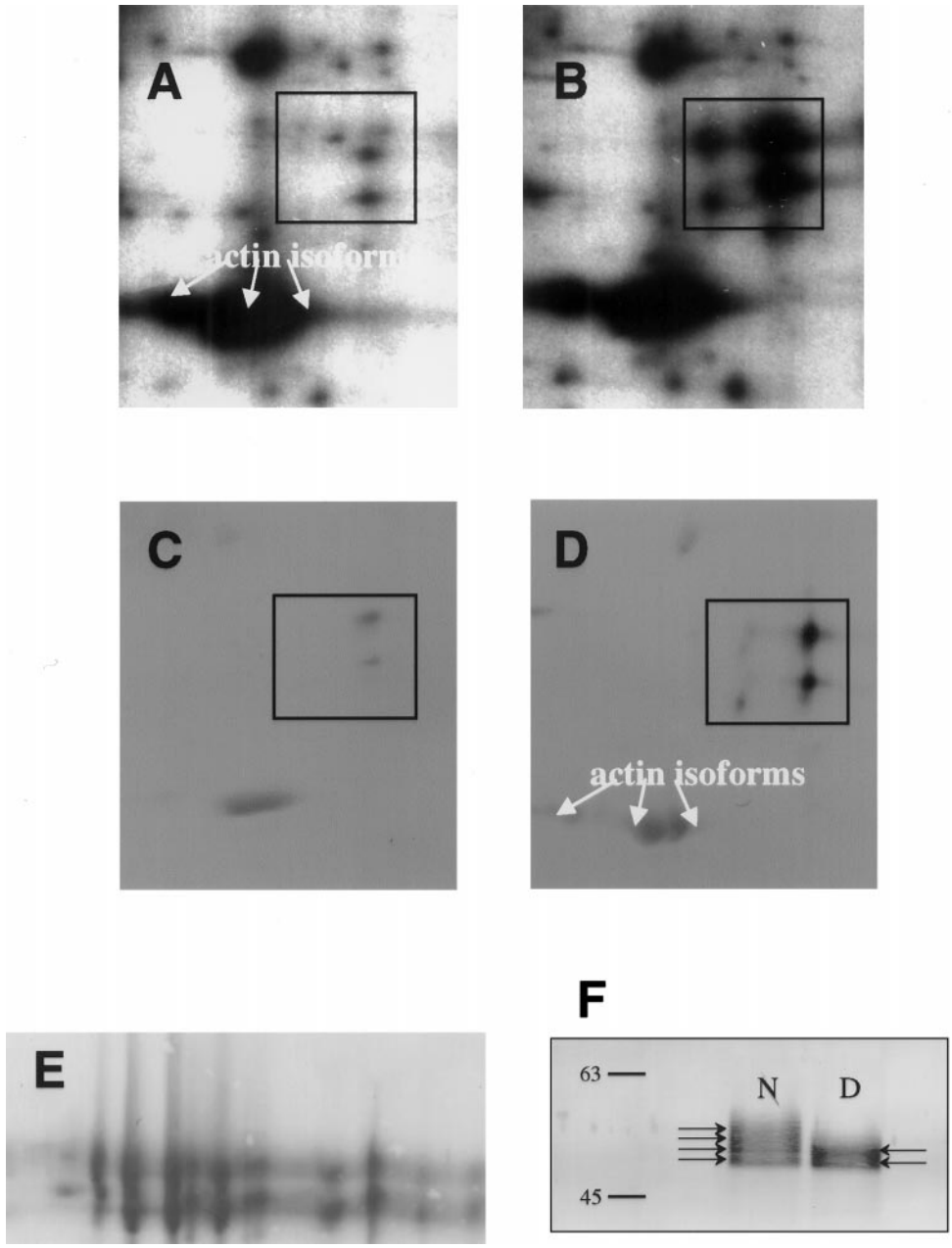

FIGURE 2. PAGE analysis of myocilin expressed in human TM cells cultured with or without DEX (10^{-7} M) for 2 weeks. Autofluorograms (**A**, **B**) and immunoblots (**C**, **D**) of cultured human TM proteins from control (**A**, **C**) and DEX-treated (**B**, **D**) GTM66 cells resolved by 2D-PAGE. The immunoblots were slightly overdeveloped to show nonspecific binding to the actin isoforms (*arrows*) for the purpose of orienting the myocilin immunoreactive spots. The expression of at least two myocilin immunoreactive proteins (within the *outlined box*) with masses of approximately 56 and 59 kDa was increased 9- and 20-fold, respectively, after exposure to DEX. (**E**) Western immunoblot of myocilin in the media of GTM1749 cells exposed to DEX. (**F**) SDS-gel immunoblot of TM cell lysate before (N) and after (D) deglycosylation.

teins in the 2D gels was similar, with slight differences, to the human DEX-responsive TM myocilin protein profiles. The molecular masses of the monkey myocilin isoforms in these 2D gels appeared to be slightly lower than those derived from the human TM.

Table 1 summarizes the glucocorticoid induction of myocilin expression in cultured human TM cells and explants, as well as in the TM of perfusion-cultured human eyes. The glucocorticoid stimulation of TM-cell–associated myocilin expression was variable. Some cultured cells, explants, and perfusion-cultured eyes did not respond at all, whereas others responded with a 2- to 20-fold induction of myocilin expression.

Indirect immunofluorescent studies of myocilin expression demonstrated intracellular and extracellular localization of myocilin (Figs. 6A–F). Not all cultured human TM cells were stained with the anti-myocilin antibody, but many TM cells showed strong staining of intracellular vesicle-like myocilin deposits surrounding the nucleus (Figs. 6A–F). In some cases, these myocilin vesicles were scattered throughout the cytoplasm. The extracellular myocilin (Fig. 6B) appeared to colo-

TABLE 1. Variability in DEX-Induced Myocilin Expression in the TM

Sample	% Responding	*n* (Responding/Total)	Range of Induction (Fold Increase)
Cultured human TM cells	33	5/15	0.9–20
Human TM explants	59	7/12	1.0–2.9
Perfusion-cultured anterior segments	67	3/5	1.0–12

Cell-associated myocilin expression was quantified by analyzing the myocilin spots on 2D-PAGE autofluorographs of radiolabeled TM proteins. The myocilin spot intensity was standardized to actin, and then the expression of myocilin spots in the DEX-treated samples were compared with the untreated control samples.

calize with components of the extracellular matrix, including fibronectin and types III and IV collagen. The normal TM cells exposed to DEX had a greater intracellular staining intensity, and a higher percentage of TM cells expressed myocilin (5.4% ± 3.8% vs. 45.2% ± 5.4%, $P < 0.001$; Table 2). The TM cells from glaucomatous eyes had a higher percentage of cells expressing myocilin than did normal TM cells (21.9% ± 4.3% vs. 5.4 ± 3.8%, $P < 0.005$) and also were DEX responsive (untreated 21.9% ± 4.3%, treated 60.7% ± 11.2%, $P < 0.002$; Table 2).

There was a reorganization of the TM microfilament cytoskeletal structure to form cross-linked actin networks (CLANs) in DEX-treated and TM cells from glaucomatous eyes (Fig. 6C), which has been seen previously.[6,14,15] The myocilin vesicles appeared to be contained within these CLANs, and myocilin was coexpressed with these CLANs in most, but not all, of the TM cells (Fig. 6C). Although the perinuclear location of myocilin was often close to the microtubule organizing center, its localization was quite distinct from the microtubules (Fig. 6D). Human TM cells also were stained with antibodies against kinesin, a microtubule motor protein. The pattern of kinesin expression in the TM cells appeared to be very similar to the myocilin pattern (i.e., kinesin and myocilin were expressed in similar vesicular-like deposits; Fig. 6E). The Golgi complex was seen at one pole of the nucleus in areas adjacent to the myocilin deposits, although the myocilin staining was much more extensive (Fig. 6F).

TABLE 2. Percentage of Responder TM Cells Expressing Myocilin in the Absence and Presence of DEX

TM Cell Line	Normal/ Glaucoma	% of TM Cells Expressing Myocilin	
		−DEX	+DEX
TM			
TM10A	N	7.4	52.1
TM68C	N	3.8	42.3
TM31C	N	2.1	38.1
TM2407	N	2.7	45.4
TM267	N	11.2	48.1
Total		5.44 ± 3.82	45.2 ± 5.36*
GTM			
GTM83C	G	17.2	52.3
GTM86C	G	27.9	65.6
GTM90C	G	23.3	77.2
GTM426	G	22.8	59.2
GTM462	G	18.2	49.0
Total		21.9 ± 4.32*	60.7 ± 11.2†

Human TM cells were cultured in the absence (−) or presence (+) of DEX (10^{-7} M) for 2 weeks, and the percentage of TM cells expressing intracellular myocilin was assessed by immunofluorescence.

* Statistically significant difference ($P < 0.005$) from untreated normal TM cells.

† Statistically significant difference ($P = 0.05$) from DEX-treated normal TM cells.

DISCUSSION

In the present study, glucocorticoid exposure of cultured human TM cells, human TM tissue explants, perfusion-cultured human eyes, and monkey TM in vivo caused an induction of myocilin in some samples. This myocilin induction was seen at the level of *MYOC* mRNA and at the protein level by gel electrophoresis and immunofluorescence. Myocilin was expressed as discrete intracellular vesicular structures in many of the cultured TM cells and appeared to colocalize with the microtubule motor protein kinesin. Myocilin also was associated with the extracellular matrix in cultured TM cells, was secreted from DEX-treated TM cells, and was present in the perfusate of perfusion-cultured human anterior segments. Not all the TM cells or TM tissues responded to glucocorticoids with an upregulation of myocilin expression. In the TM of perfusion-cultured human eyes and that of monkeys treated in vivo with glucocorticoids, there appeared to be an association with increased myocilin and the development of ocular hypertension and/or steroid glaucoma-like changes in the TM.

The discovery of genes responsible for heritable forms of glaucoma is beginning to unravel the complex molecular pathogenic mechanisms responsible for these blinding disorders.[26–34,57–59] The gene responsible for ADJG (*GLC1A*) was first mapped to chromosome 1q23-24 by Sheffield et al.,[26] and this locus has been confirmed by a number of groups.[27–30] Stone et al.[31] mapped a glucocorticoid-induced gene (*MYOC*) to the *GLC1A* locus and identified a number of mutations in this gene in families affected by juvenile open-angle glaucoma (JOAG) and in patients with adult-onset POAG.[31–35] *MYOC* has been variably named (*GLC1A*, *TIGR*, myocilin) and is expressed in the TM,[23,24,36–38,41–44,60] the ciliary body,[39,60,61] the retina,[40] and a variety of other ocular and nonocular tissues.[39,40,52,60,62]

In addition to its role in ADJG and POAG, altered *MYOC* expression in the TM may play a role in the development of glucocorticoid-mediated ocular hypertension and glaucoma. A subset of patients treated with glucocorticoids have an elevated IOP that can lead to secondary open-angle glaucoma, which is similar in many ways to POAG.[4–6] Not all TM cell strains or perfusion-cultured human eyes displayed a DEX-induced increase in myocilin expression, which correlates very well with the heterogeneity of glucocorticoid-induced ocular hypertension in the general population.[4–6] Of interest, patients with glaucoma are more likely to respond to steroid drugs,[4–6] and the TM cells from glaucomatous eyes in the current study appeared to have a higher expression of myocilin than those from nonglaucomatous eyes.

There are many similarities shared between ADJG and glucocorticoid-induced glaucoma. Both diseases are charac-

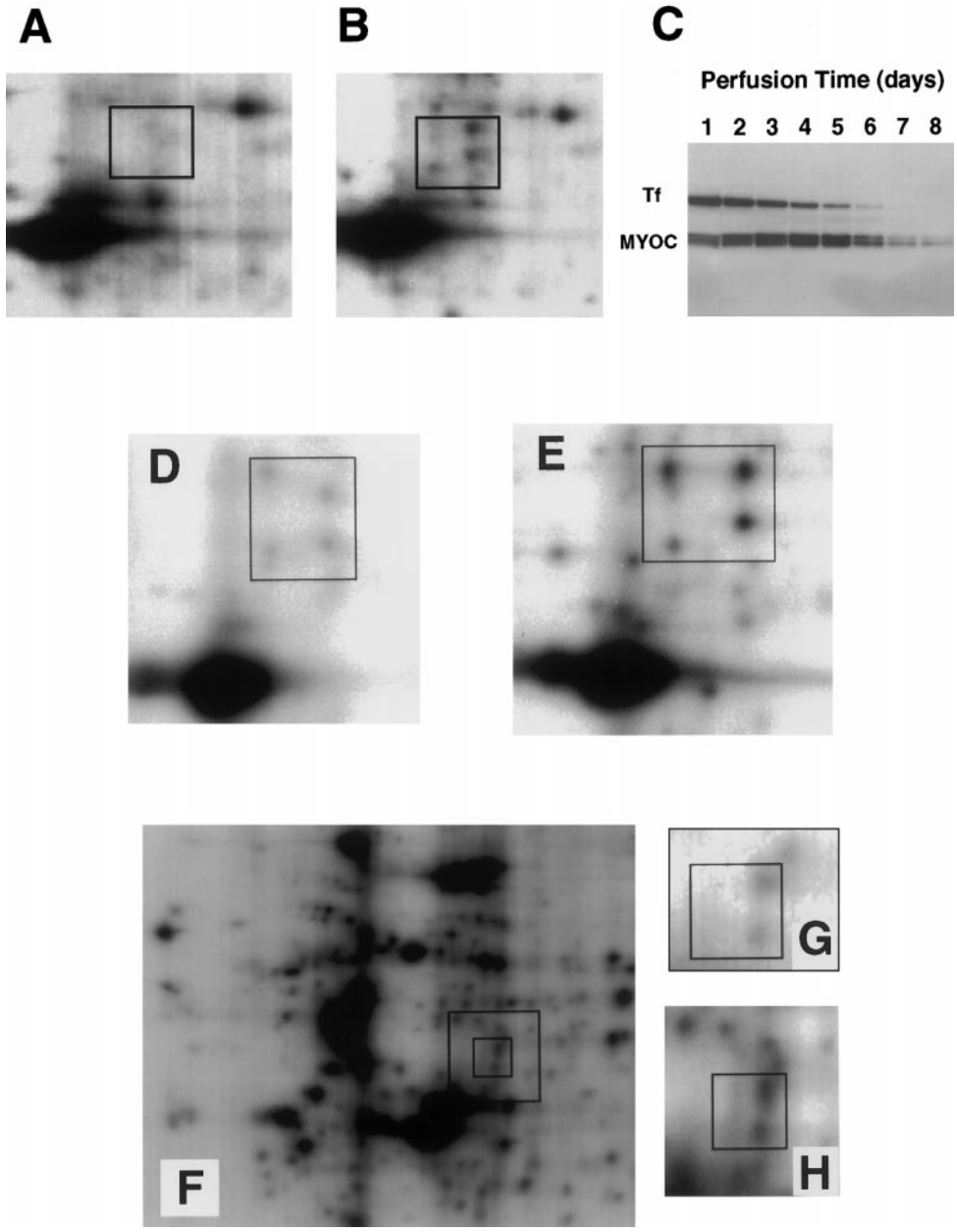

FIGURE 3. Expression of myocilin in human and monkey TM tissues. (**A**, **B**) High-resolution 2D-gel autofluorograms of TM proteins from perfusion-cultured human eyes. Protein expression profile of a pair of eyes perfused with media with (**A**) or without (**B**) 10^{-7} M DEX for 10 days. The DEX-treated eye showed a steroid-induced elevation in IOP, whereas the control eye maintained a stable baseline perfusion pressure. The expression of a group of proteins (*boxes*, with M_r 58 and 56 kDa and pI 5.2 and 5.3) corresponding to myocilin was induced by DEX treatment. (**C**) Western immunoblot of myocilin (MYOC) and transferrin (Tf) in media from perfusion-cultured human eyes. Myocilin was present in the media throughout the 8 days of culture, whereas the expression of transferrin progressively decreased. (**D**, **E**) High-resolution 2D-gel autofluorograms of TM proteins from TM tissue explants cultured in the absence (**D**) or presence (**E**) of 10^{-7} M DEX for 3 weeks. The expression of a tetrad of proteins (*box*, M_r 58 and 55 kDa and pI 5.2 and 5.4) corresponding to myocilin was induced in these DEX-treated explants. (**F–H**) High resolution 2D-gel autofluorograms of monkey TM proteins from (**F**, **H**) cortisol acetate–treated (5.78 mg/kg·d) or (**G**) control animals. The *outlined* region in (**F**) is enlarged in (**G**, **H**). There was increased expression of several TM proteins (M_r 53 and 55 kDa and pI 5.3) corresponding to myocilin in the cortisol acetate–treated animals. The glucocorticoid induction of myocilin varied from 2- to 20-fold in these TM tissues.

terized by relatively high IOPs, and the glaucomatous damage to vision appears to be due almost exclusively to pressure-induced optic neuropathy. Lowering pressure by glaucoma filtration surgery in ADJG or discontinuation of steroid therapy in corticosteroid glaucoma generally halts progression of glaucomatous visual field loss. There are also similarities in the ultrastructural morphology of the TM extracellular matrix between patients with steroid glaucoma[13] and patients with ADJG.[63] Despite these similarities, it appears that there are no mutations within the *MYOC*

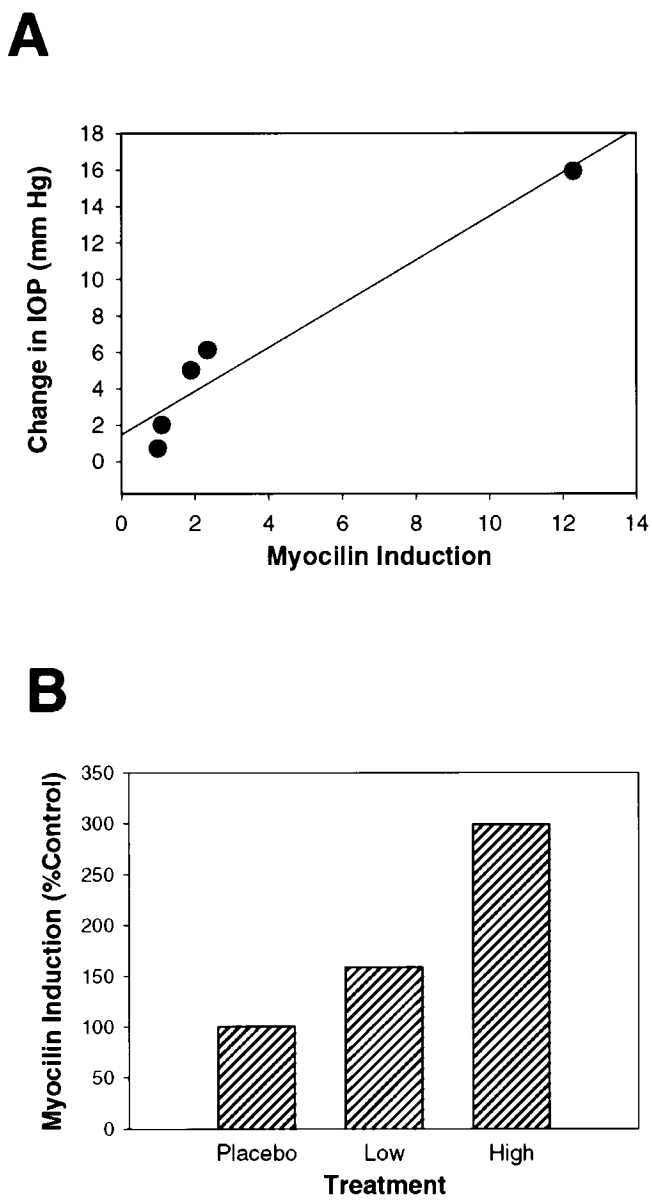

FIGURE 4. Correlation of steroid-induced TM myocilin expression with DEX-induced ocular hypertension in perfusion-cultured human eyes and with steroid dose in cortisol acetate–treated primates. (**A**) Association of myocilin induction (*x*-fold increase over control) in the TM of perfusion-cultured human eyes ($n = 5$) with DEX-induced changes in IOP ($r = 0.973$). (**B**) Dose-dependent association of myocilin induction (*x*-fold increase over placebo control) in the TM of monkeys treated with low or high doses of cortisol acetate for 1 year ($n = 2$ per group).

coding or promoter regions of steroid-responsive patients.[10,64]

Steroid effects on the TM have been investigated in attempt to identify the molecular mechanism(s) responsible for the development of ocular hypertension. Several laboratories have documented the induction of a 55-kDa glycoprotein in cultured TM cells treated with glucocorticoids. Polansky et al.[23,24,36,38] and Nguyen et al.[37] used SDS-PAGE and 2D-PAGE to report the appearance of a cell-associated 55-kDa and secreted 64- to 68-kDa glycoproteins in human TM cells treated with DEX for 10 to 14 days. The heterogeneity in molecular weight and pI of this protein are reported to be due to differences in glycosylation.[23,24,36–38] A similarly sized 56-kDa glycoprotein has been reported to be induced in porcine TM cells treated with cortisol for 3 to 4 weeks.[65]

The induction of this protein in cultured human TM cells by DEX has been reported to be unusual in several respects. First of all, the protein appears in the TM cells only after 7 to 10 days of DEX treatment, which is later than would normally be expected for a gene directly regulated by glucocorticoids. Secondly, the median effective concentration (EC_{50}) of DEX for inducing expression of this protein in the TM is approximately 10 times higher[23] than the inherent affinity of DEX for the TM glucocorticoid receptor.[66] It has been argued that these two in vitro properties of *MYOC* induction closely match the in vivo generation of glucocorticoid-induced ocular hypertension and therefore implicate the involvement of this protein in ocular hypertension.[24,25,36–38]

There have been reports of glucocorticoid response elements (GREs) upstream of *MYOC*.[36–37] In contrast, Fingert et al.[52] have indicated that there are no classic, palindromic GREs up to 1900 bp upstream of the translation start site in the *MYOC* gene from human or mouse. There appear to be up to 16 putative GR-binding sites consisting of 6-bp sequences that resemble half-sites of classic GREs. Several of these sites previously have been implicated to involve delayed induction of glucocorticoid-induced gene expression in other systems.[67] Recent studies using cultured human TM cells as well as cells transfected with a vector containing the human *MYOC* promoter fused with a reporter gene suggest that myocilin expression is indirectly regulated by glucocorticoids[68] (Shepard et al., submitted for publication), although the precise *MYOC* regulatory element(s) responsible for this induction has not been identified.

The expression of myocilin in glucocorticoid-treated cultured TM cells provides only indirect evidence of its involvement in ocular hypertension. We now present more direct evidence that glucocorticoids upregulate the expression of myocilin in the TM of ocular hypertensive human eyes and in the TM of primates treated for 1 year with glucocorticoids. There have been a variety of ultrastructural changes in the human TM associated with steroid-induced ocular hypertension as seen in patients with corticosteroid-induced glaucoma[11–13] and in perfusion-cultured DEX-responsive human eyes.[48] There is an accumulation of extracellular material in the JCT region and between trabecular beams in these steroid-treated eyes. In addition, there is a thickening of the trabecular beams and an activation of trabecular cells.[11,48] Although we do not know whether long-term administration of cortisol acetate induced ocular hypertension in the monkeys used in the present study, there were ultrastructural changes in the TM that are similar to those in humans treated with glucocorticoids. DEX-induced ocular hypertension has been reported in monkeys,[10] and it is therefore possible that the monkeys used in the present study had some degree of ocular hypertension, and elevated expression of myocilin may have been involved.

We currently do not know the function of myocilin in the TM. It has been suggested that the glucocorticoid-induced deposition of myocilin in the extracellular material of the TM leads to increased aqueous humor outflow resistance.[23,24,36–38] Several recent publications have reported opposite effects of myocilin on the outflow facility of perfusion-cultured human anterior segments. Transduction with adenovirus-*MYOC* expression vectors appears to increase outflow,[69] whereas the addition of recombinant myocilin to the perfusate appears to impede outflow.[70] Further work is required to resolve these discrepancies. In our present study, myocilin was secreted into the media of several of our DEX-treated TM cell lines and appears to be associated with the extracellular matrix in some TM cells. In addition, the data from our present study agree with findings in other studies that

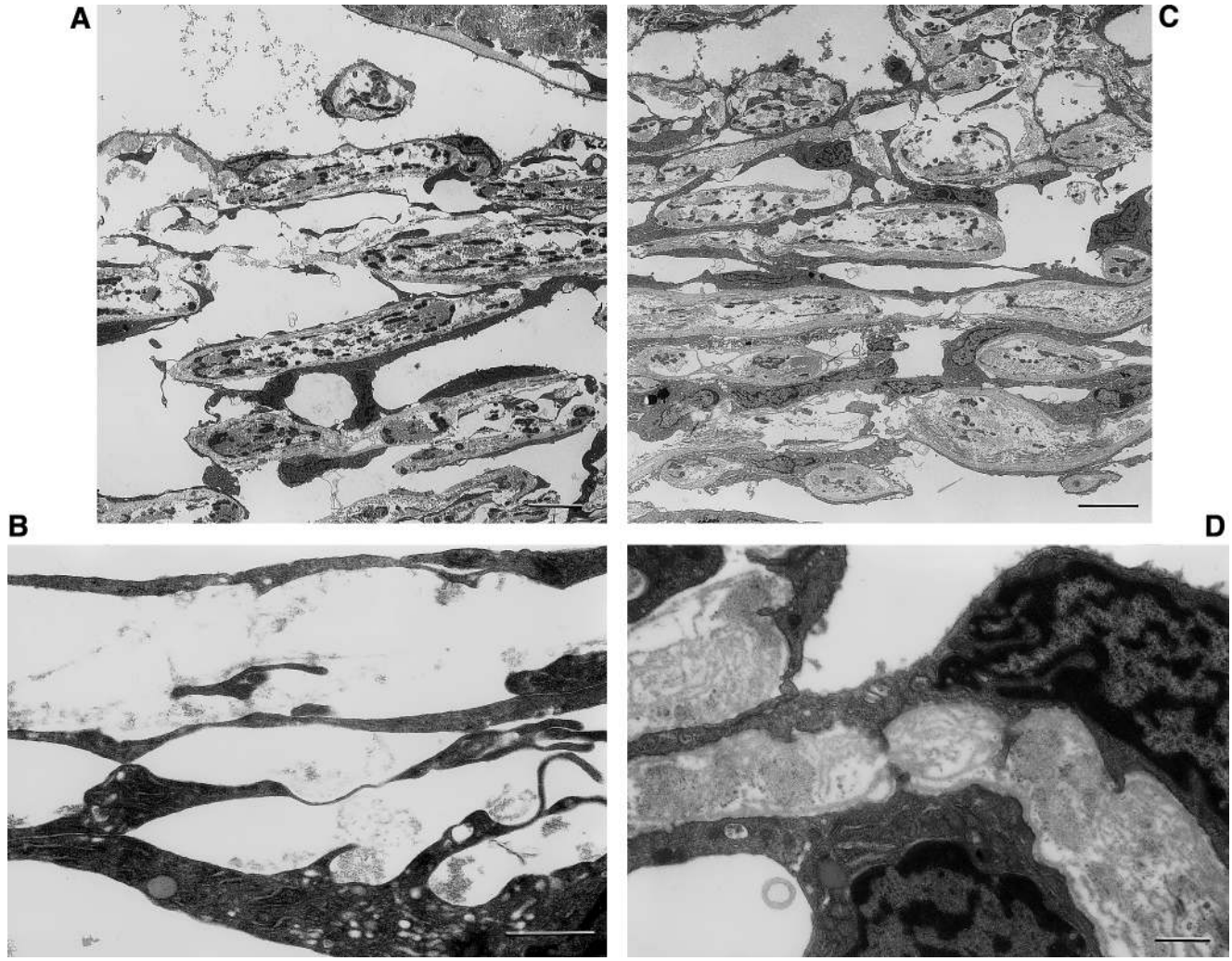

FIGURE 5. Transmission electron micrographs of the TM of primates treated for 1 year with placebo (**A**, **B**) or with 5.78 mg cortisol acetate/kg body weight per day (**C**, **D**). The control animals had normal TM morphology, whereas the TM of the cortisol acetate–treated animals showed some morphologic signs of corticosteroid-induced glaucoma, including increased deposition of extracellular matrix material and thickening of the trabecular beams. Scale bars, (**A**, **C**) 10 μm; (**B**, **D**) 1 μm.

suggest that myocilin is an intracellular protein in cultured human TM cells.[41,42,44,46] Myocilin appeared to be associated with microfilaments in some TM cells. We have previously demonstrated that glucocorticoids induce a reorganization of the cytoskeleton in cultured TM cells to form geodesic dome–like structures of CLANs.[14,15] These same CLAN structures are also found in TM cells derived from glaucomatous donor eyes.[71] CLANs have been proposed to interfere with a variety of TM cell functions, and the induction of myocilin may be involved. Results from other studies support the intracellular and cytoskeletal localization of myocilin. The amino terminal half of myocilin shares sequence homology with nonmuscle myosin, and myocilin has been found to be associated with the basal body in mammalian photoreceptor cells.[40] Immunolocalization of myocilin in TM tissue from normal and glaucomatous eyes also suggests that myocilin is located within the trabecular cells.[44]

The vesicular staining pattern that we and others have shown may indicate that myocilin is associated with other structures, such as the Golgi apparatus, the endoplasmic reticulum, and cytoskeletal motor proteins. Several previous studies have shown that glucocorticoids induce a proliferation of the Golgi apparatus, the endoplasmic reticulum, and intracellular vesicles in TM cells.[14,48] There is a reported increase in the number of intracellular vesicles in DEX-treated TM cells. These vesicles appeared to be oriented along the long axis of the cell, suggesting a cytoskeletal association (McCartney et al., submitted for publication). The apparent association of myocilin with the microtubule motor protein kinesin suggests that myocilin may be involved in vesicular transport. In a notable finding, GFP-tagged myocilin vesicles appear to move in cultured TM cells at the rate of vesicles driven by a kinesin motor.[72] The proximity and slight overlap of myocilin staining with the trans-Golgi complex also suggest that myocilin may be associated with secretory vesicles. Myocilin contains a consensus N-terminal secretory sequence, is secreted into the media, is glycosylated, and therefore would be expected to be found in secretory vesicles. Recent studies also indicate that myocilin is associated with intracellular vesicles around the nucleus in cultured TM cells.[41,42,73] However, we have not found myocilin colocalized with microtubules[74] or with mitochondria[46] as reported in previous studies.

We have presented evidence linking the expression of myocilin in trabecular tissue with steroid-induced ocular hypertension. The major challenge ahead is to determine the function of myocilin in the TM and to dissect the role of myocilin in the pathogenic process. In addition, this research may aid in the

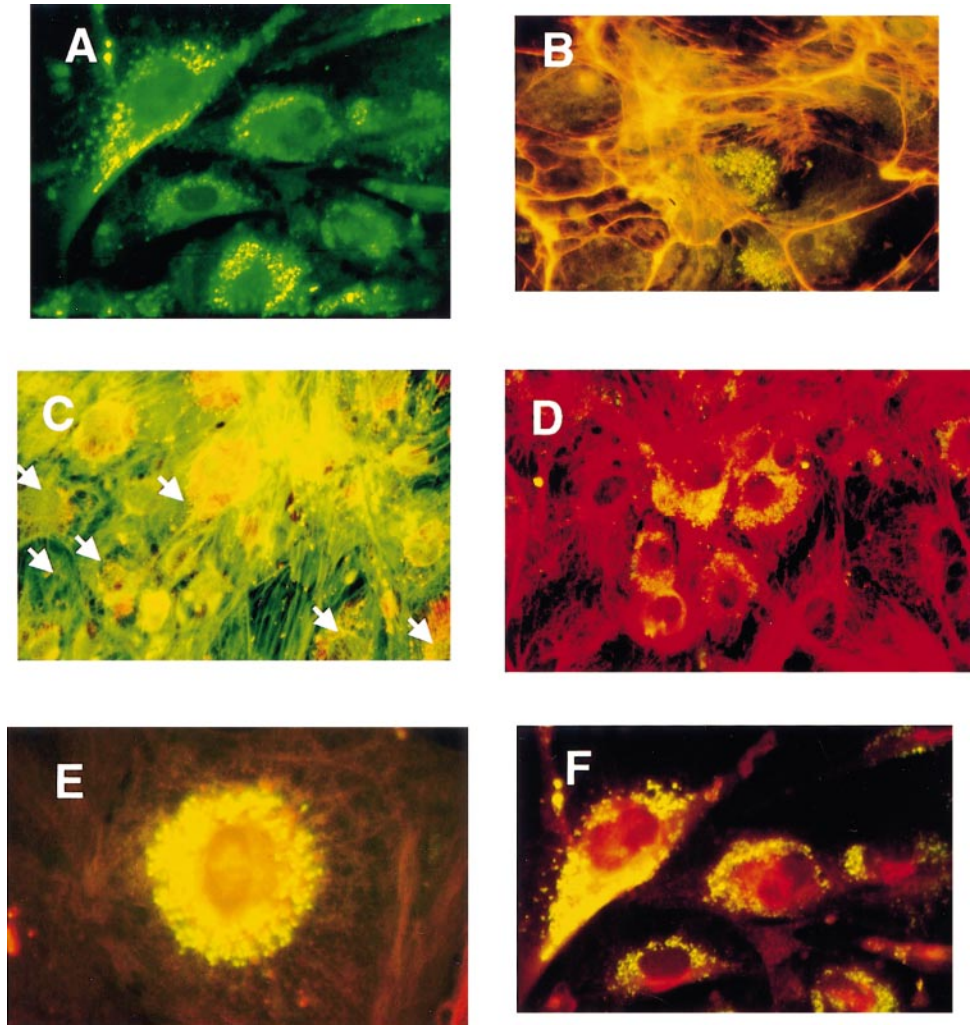

FIGURE 6. Immunofluorescent photomicrographs of myocilin, actin, tubulin, kinesin, and the Golgi complex in DEX-treated human TM cell cultures. There was intracellular, vesicular myocilin staining of many, but not all of the TM cells (**A**–**F**). (**A**) TM cells stained with anti-myocilin antibodies demonstrated punctate vesicular staining. (**B**) TM cells stained with two different anti-myocilin antibodies demonstrated both extracellular myocilin (*orange*) and intracellular myocilin (*green*). (**C**) TM cells stained for myocilin (*red-yellow*) and actin (*green*) demonstrated CLANs (*arrows*) and vesicular myocilin. (**D**) TM cells stained for tubulin (*red*) and myocilin (*yellow-green*). (**E**) TM cells stained for kinesin *(red)* and myocilin *(green)* showed an apparent colocalization of many of the vesicles. (**F**) TM cells stained with a marker for Golgi complex (*red*) and myocilin (*yellow-green*) showed a partial overlap in staining patterns.

identification of additional genes that are responsible for glaucomatous damage to the TM.

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