Characterization of Cytoskeleton-Enriched Protein Fraction of the Trabecular Meshwork and Ciliary Muscle Cells

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PURPOSE. To understand the molecular basis for the known distinct contractile characteristics of trabecular meshwork (TM) and ciliary muscle (CM) cells, the cytoskeleton-enriched protein fractions of the TM and CM cells were isolated and characterized.

METHODS. The nonionic surfactant insoluble fraction enriched for cytoskeletal proteins was isolated from human and porcine TM tissue and cells and from CM cells and was characterized by SDS-PAGE, mass spectrometry, and immunoblotting techniques.

RESULTS. The cytoskeleton-enriched protein fraction derived from both human and porcine TM cells contained Plectin 1, Filamin A, non-muscle myosin IIA, clathrin, α -actinin, vimentin, actin, caldesmon, myosin IC, and annexin A2 as major proteins and was noted to exhibit compositional similarity with the cytoskeletal protein fraction isolated from TM tissue. Importantly, the cytoskeletal protein composition of the TM cells was also found to be similar to that noted for CM and vascular endothelial cells. Although the activity of myosin II, a crucial regulator of cellular contraction and a major component of the cytoskeletal protein fraction in TM and CM cells, was regulated predominantly by Rho kinase in both cell types, myosin light chain kinase (MLCK) also appeared to control myosin II activity in CM cells.

CONCLUSIONS. These data reveal that the activity of non-muscle myosin II, a critical molecule of cellular contraction, was found to be regulated differentially in TM and CM cells by the Rho kinase and the MLCK pathways despite their compositional similarity in cytoskeletal protein profile. (*Invest Ophthalmol Vis Sci.* 2010;51:6461–6471) DOI:10.1167/iovs.10-5318

Homeostasis of aqueous humor outflow through the conventional pathway is critical for the regulation of intraocular pressure (IOP). Any abnormality or blockage of

aqueous humor outflow through the conventional pathway consisting of trabecular meshwork, juxtacanalicular tissue, and Schlemm's canal can lead to increased IOP, a major risk factor for glaucoma.¹ Glaucoma is the second leading cause of blindness in the United States, and lowering IOP by medical and surgical treatment is the primary avenue for the treatment of glaucoma.² Therefore, there is a great deal of interest in understanding the regulation of aqueous humor drainage through the TM tissue and finding novel avenues for lowering IOP in glaucoma patients.³

The conventional aqueous humor outflow pathway is pressure sensitive, and changes in IOP influence aqueous humor drainage through the TM.^{4–8} Further, the perfusion of various physiological compounds (e.g., cytokines, lipid growth factors, steroids, agonists, and peptide growth factors) is known to influence aqueous humor outflow facility.^{1,3} Importantly, changes in TM and CM contractile and relaxation properties,⁹ morphology of the outflow pathway,¹⁰ and extracellular matrix organization and synthesis¹¹ are presumed to influence the resistance of aqueous humor drainage, suggesting a role for cell adhesive interactions, cytoskeletal organization, and mechanotransduction in homeostasis of aqueous humor outflow.^{11–14} This conclusion/hypothesis is supported by several ex vivo and in vivo studies in which various cytoskeleton-interacting agents were documented to alter aqueous humor outflow facility through the conventional pathway.^{3,12,15} In addition to agents that directly disrupt cytoskeletal (both microfilament and microtubule) integrity, inhibitors of various kinases that regulate actomyosin assembly, contraction, cell shape, and cell-ECM and cell-cell interactions, including Rho kinase, protein kinase C, and myosin light chain kinase, influence aqueous humor outflow facility and IOP in animals and humans.^{12,14,16–19} Thus, multiple and consistent observations collectively support the inference that cytoskeletal proteins and their organization play a crucial role in the modulation of aqueous humor outflow resistance and eventually IOP. Despite ample evidence for the importance of cytoskeletal proteins in aqueous humor outflow pathway function, little is known about the composition of major cytoskeletal and cytoskeletal-associated proteins in the cells and tissues of the aqueous humor outflow pathway.

Additionally, although the TM and SC cells are thought to be derived from the neural crest and endothelia,^{20,21} respectively, these two cell types express smooth muscle properties and exhibit many characteristics similar to those of vascular endothelial cells. On the other hand, the CM and TM tissue, which originate from the differentiation of the neural crest,²⁰ are reported to exhibit distinct contractile and relaxation properties.⁹ Moreover, while contraction of the CM increases aqueous humor outflow facility, contraction of the TM is thought to decrease aqueous humor outflow facility.^{9,22,23} These observations indicate possible differences between the CM and TM in either the cytoskeletal protein profile or the signaling mecha-

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nisms that control contraction and relaxation between these two types of tissue.⁹ Thus, unraveling the molecular basis for the differences in contractile and relaxation properties of TM and CM might provide novel insights for targeted therapy for glaucoma.

As part of an effort to understand the contractile and relaxation properties and the mechanobiology of TM, SC, and CM tissues and the potential role of these events in modulation of aqueous humor outflow facility, in this study we identified and characterized the major cytoskeletal and cytoskeletal-associated proteins of the TM and CM cells by mass spectrometry (MS). Further, the cytoskeletal protein profiles of the TM and CM were compared with and contrasted to those of vascular endothelial cells (because SC cells are endothelial in origin) to understand the differences and similarities among these three different cell types with respect to their smooth muscle-like and mechanical properties.

MATERIALS AND METHODS

Materials

Rho kinase inhibitor (Y27632) was provided by Welfide Corporation (Tokyo, Japan). ML-7 was purchased from EMD Biochemicals (San Diego, CA). Cell culture media and fetal bovine serum were obtained from Gibco-BRL (Gaithersburg, MD). Tetrahydroamine isothiocyanate (TRITC)-conjugated phalloidin, monoclonal antibody against β -actin (catalog no. A2228), and monoclonal antibody against vinculin (catalog no. V4505) were from Sigma-Aldrich (St. Louis, MO). Monoclonal antibody against annexin II (catalog no. 610068) was purchased from BD Biosciences (Franklin Lakes, NJ). Filamin A (catalog no. MAB1678) monoclonal antibody and Millipore ultrafree centrifugal filters were purchased from Millipore Corporation (Bedford, MA). Polyclonal phospho-MLC antibody (catalog no. 3671S) was from Cell Signaling Technology (Danvers, MA). Detection reagents (ECL Plus) were from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Isolation and Culture

Human TM (HTM) tissue extracted from donor eyes (obtained from local eye banks) was minced and incubated under coverglasses in plastic dishes containing Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) until HTM cells grew from the

tissues. The HTM cells used in this study were isolated from donor eyes obtained from subjects aged 15 to 50 years of age. Human donor tissues were used in accordance with the tenets of the Declaration of Helsinki. Porcine TM (PCM) cells were cultured from TM tissue isolated from freshly obtained eyes (from a local abattoir) by collagenase digestion, as described previously by us.²⁴ Porcine ciliary muscle (CM) cells were isolated by the method described by Tamm et al.,²⁵ with minor modifications. Briefly, the lens, vitreous, and iris were removed from porcine eyes. The pars plana ciliaris was cut out, and pigmented epithelium was scraped from the ciliary body. The residual ciliary body, composed primarily of the CM, was then peeled off, and the TM was confirmed to be attached to the sclera. The isolated CM was digested using 1 mg/mL collagenase type 4 for either 2 hours (for PTM tissue) or 4 hours (for PCM tissue), in the presence of 0.3 mg/mL human serum albumin in medium-199 at 37°C with continuous agitation. After collagenase digestion, the tissue samples were centrifuged (2800 rpm for 10 minutes), suspended in cell culture medium, and plated on 2% gelatin-coated plastic dishes. TM and CM cells were cultured in DMEM containing 10% FBS, penicillin (100 U/mL) and streptomycin (100 μ g/mL). Human umbilical vascular endothelial cells (HUVECs) were procured from Clonetics (San Diego, CA) and maintained with EGM-2 medium supplemented with FBS, bFGF, IGF-1, VEGF, EGF, heparin, ascorbic acid, and hydrocortisone according to the manufacturer's recommendations (Clonetics). All cells were cultured at 37°C under 5% CO₂. HTM cells were used at passages 5 and 6, PTM and PCM cells were used at passages 3 to 5, and HUVEC were used at passage 7. For the comparative studies of TM and CM cell cytoskeletal protein profiles, both cell types were isolated from the same eyes. The human and porcine TM cells described are enriched mainly with TM, but the presence of small number of JCT and SC cells cannot be ruled out.

Isolation of Cytoskeleton-Enriched Protein Fractions

The TM and CM cells and HUVECs were cultured to confluence in plastic Petri plates. For the preparation of cytoskeleton-enriched protein fractions, nonionic surfactant (Triton X-100; Dow Chemical, Midland, MI)-insoluble fractions were isolated as described by us earlier.²⁶ Briefly, cells were homogenized in cell solubilization buffer (CSB) pH 7.1 containing 10 mM PIPES, 50 mM KCl, 10 mM EGTA, 3 mM MgCl₂, 2 M glycerol, 2 mM NaF, 1 mM Na₃VO₄, and protease inhibitors using

FIGURE 1. SDS-PAGE separation of porcine TM cell triton-insoluble protein fraction and MS identification. To identify and characterize the predominant protein components of the TM cell triton-insoluble cytoskeletal fraction, the porcine TM cell-derived protein fraction (25 μ g protein) was analyzed by SDS-PAGE using different concentrations of acrylamide (range, 5.5%–12.5% acrylamide) and blue staining. After destaining, distinctly separated major protein bands were extracted, in-gel digested with trypsin, and subjected to MALDI-TOF spectrometry-based identification. Mass spectrometry-based identity of each protein is indicated next to the corresponding protein band. (A–C) Each left lane indicates protein molecular mass markers. Only the protein bands that were visible with light box were subjected to MS analysis. These analyses were repeated using three independent primary TM cell lines derived from different eyes.

both probe (4–6 strokes; Microson Ultrasonic Disruptor; Misonix Inc., Farmingdale, NY) and bath (30 minutes; Aquasonic model 50T; VWR Scientific Products, West Chester, PA) sonicators on ice. Cell lysates were centrifuged at 800g for 15 minutes at 4°C, and the supernatants were further centrifuged at 30,000g for 30 minutes at 4°C using a Beckman tabletop ultracentrifuge. The pellets were washed with CSB and rehomogenized in CSB containing 1% Triton X-100. These detergent-treated fractions were then centrifuged at 30,000g for 30 minutes at 4°C, and the pellets were collected as triton-insoluble fractions.

For the TM tissue cytoskeletal fraction, TM tissues were isolated from 10 freshly enucleated porcine eyes and homogenized with Down's glass homogenizers and subsequently with probe sonication in CSB containing 1% nonionic surfactant (Triton X-100; Dow Chemical). The lysates were centrifuged at 800g for 15 minutes to collect supernatant. The pellets from this step were washed twice with the same buffer, and supernatants were collected from each step. The supernatants were pooled and then centrifuged at 30,000g for 30 minutes at 4°C to obtain pellets containing the triton-insoluble fraction.

The triton-insoluble fractions derived from different cell types and TM tissue were washed once again, lysed in extraction buffer containing 20 mM Tris-HCl, 300 mM NaCl, 30 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitors and were passed through a 28-gauge syringe 10 times. Protein content was determined using the Bio-Rad protein assay reagent (catalog no. 500-0006).

In-Gel Trypsin Digestion and Mass Spectrometry Analysis

The triton-insoluble protein fractions of both TM and CM cells and TM tissue were separated on SDS-PAGE gels containing 5.5%, 8%, or 12.5% acrylamide. Samples were mixed with sample loading buffer and boiled for 3 minutes before analysis (25 or 10 µg protein per cell lysate or 8 µg protein per tissue lysates loaded per well). After electrophoresis, SDS-PAGE gels were rinsed with pure water and stained overnight at 37°C, using the stain reagent (GelCode Blue, catalog no. 24590; Pierce, Rockford, IL). After staining, the gels were destained with water, and distinctly stained protein bands (viewed with a light box) were excised from the gels. Gel slices containing the protein bands were subjected to in-gel tryptic digestion using in-gel tryptic digestion kit (catalog no. 898771; Pierce) according to the manufacturer's instructions. This digestion process included the reduction and alkylation of protein samples. The peptides were dissolved in 5 µL of 50% acetonitrile, 0.1% trifluoroacetic acid containing 5 mg/mL α -4-hydroxycinnamic acid (Sigma-Aldrich) as a matrix for matrix-assisted laser desorption ionization (MALDI) MS. The peptide-matrix mixture (0.5 µL) was loaded onto a 192-spot MALDI sample plate and subjected to MALDI time-of-flight (TOF) MS and MS/MS analyses (4700 Proteomics analyzer; Applied Biosystems, Foster City, CA) and GPS software package (Applied Biosystems). Proteins were identified based on MS and MS/MS spectra using the Mascot search engine. MS spectra were submitted for protein search with a precursor mass tolerance of 0.1 Da and MS/MS spectra with fragment tolerance of 0.5 Da. Where indicated, the proteins from porcine species were identified using a mammalian database (The National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/pubmed>). The confidence interval percentage for all identified proteins was no less than 99.5%.

Myosin Light Chain Phosphorylation

To determine the relative effects of Rho kinase inhibitor and myosin light chain kinase inhibitor on myosin light chain phosphorylation in TM and CM cells, cell lysates derived from confluent cells grown in the presence of 1% FBS treated either with Rho kinase inhibitor (Y-27632) or myosin light chain kinase inhibitor (ML-7) were subjected to immunoblot analysis using anti-phospho-MLC antibody and enhanced chemiluminescence detection, as we described earlier.²⁴

Immunoblot Analysis

For determining the distribution of annexin II and Filamin A in TM cells, HTM cells were sonicated as described earlier in CBS buffer and

protein fractions derived from the total lysate (800g supernatant), cytosolic (40,000g supernatant), and membrane-enriched insoluble fractions (40,000g pellet, washed thrice) were subjected to SDS-PAGE (10% or 5.5% acrylamide), transferred to nitrocellulose membrane, and immunoblotted either with anti-Filamin A monoclonal antibody or with

FIGURE 2. (A) Comparison of PTM and HTM cell triton-insoluble cytoskeletal protein profiles. Triton-insoluble cytoskeletal protein fractions (10 µg) derived from PTM and HTM cells were separated by SDS-PAGE (8% acrylamide), and predominant protein bands were identified by MS analysis as described in Figure 1. The cytoskeletal protein profiles of PTM and HTM cells were found to be similar to each other, both by SDS-PAGE analysis and by MS identification, with exception of caldesmon and laminin receptor. *Left lane:* separation and staining patterns of protein molecular mass markers. These comparative analyses were performed twice with samples derived from two independent HTM cell lines. (B) Cytoskeletal protein profile of PTM tissue. TM tissue was isolated from freshly enucleated porcine eyes obtained from a local slaughterhouse, and triton-insoluble fractions (8 µg protein) were derived from TM tissue (pooled from 10 different eyes) and analyzed by SDS-PAGE, followed by detection using blue staining. Distinctly resolved protein bands were excised from SDS-PAGE gels, in-gel digested using trypsin, and then identified by MS analysis. The cytoskeletal protein profile of TM tissue was confirmed to be similar to that of TM cells. *Left lane:* separation of standard molecular mass protein markers. These data were based on a single analysis from pooled TM tissue derived from 10 different eyes.

annexin II monoclonal antibody in conjunction with the ECL detection system, as we described earlier.²⁶

For the detection of secretory annexin II, conditioned medium derived from the HTM cells was concentrated using the Millipore ultrafree centrifuge tubes with a 30-kDa cutoff. The concentrated medium was analyzed by SDS-PAGE, and resolved proteins were transferred to nitrocellulose membranes, which were then immunoblotted using the anti-annexin II antibody.

Immunofluorescence Staining

Immunofluorescence staining was performed as described previously.¹⁷ Briefly, PTM and PCM cells were grown to confluence on glass coverslips coated with 2% gelatin and were treated either with Y27632 or with ML-7 for 1 hour after overnight incubation with 1% FBS in DMEM. Drug-induced morphologic changes were monitored by phase-contrast microscopy (IM35; Carl Zeiss, Thornwood, NY). After treatment, cells were fixed at room temperature for 12 minutes in 3.7% formaldehyde in PBS and washed with cytoskeletal buffer (10 mM MES (2-*N*-morpholino-(ethanesulfonic ether) *N,N,N,N*-tetra acetic acid), 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM glucose, pH 6.1), followed by permeabilization for 12 minutes with 0.5% Triton X-100 in PBS and blocking with serum-containing buffer as we described earlier.¹⁷ For F-actin staining, cells were incubated with TRITC-phalloidin (1:500) for 20 minutes. For the detection of focal adhesions, cells were immunostained with monoclonal anti-vinculin (1:200) for 2 hours at room temperature. Subsequently, cells were incubated with secondary antibody conjugated with AlexaFluor488 for 30 minutes at room temperature. Coverslips were mounted, and micrographs were obtained with a fluorescence microscope (Axioplan-2; Zeiss). Similarly, HTM cells were immunostained for annexin II and Filamin A using the respective monoclonal antibodies in conjunction with AlexaFluor488 secondary antibody.

RESULTS

Cytoskeletal Protein Profile of TM Cells and Tissue

Nonionic surfactant (Triton X-100; Dow Chemical) insoluble protein fractions were prepared from confluent cultures of porcine and human TM cells grown in DMEM containing 10% FBS. The triton-insoluble protein fractions were separated by SDS-PAGE (containing 5.5%, 8%, and 12.5% acrylamide and were stained using stain reagent [GelCode Blue; Pierce]). Figures 1 and 2 illustrate the protein profile of triton-insoluble protein fraction of porcine TM cells separated on SDS-PAGE and reveal the presence of several predominant protein bands

with molecular masses ranging from 15 kDa to >250 kDa. To identify individual proteins in the triton-insoluble fraction of porcine TM cells, distinctly separated protein bands by SDS-PAGE were excised from the gels, subjected to in-gel trypsin digestion, and analyzed by MALDI-TOF MS and MS/MS. Some of the major high-molecular mass proteins (>100 kDa) identified by MS included Plectin 1, Filamin A, nonmuscle myosin IIA, clathrin, myosin IC, and α 1-actinin. Proteins in the >40 kDa molecular mass range included caldesmon, vimentin, and β - and γ -actin; annexin II/A2, with a molecular mass range below 40 kDa, was the predominant protein in the triton-insoluble fraction. In some analyses, nuclear histones and lamins were identified as some of the predominant proteins. To ensure consistency of the observed cytoskeletal protein profiles of TM cells, we repeated (three times) the protein extraction and MS analyses using TM cells derived from a different batch of eyes. Except for the histones and lamins, the data from these different experiments yielded very similar protein profiles, confirming the reproducibility of analyses.

To compare the cytoskeletal protein profiles of porcine and human TM cells, triton-insoluble fractions were isolated from human TM cells using procedures identical to those applied to porcine TM cells. As shown in Figure 2A, the cytoskeletal protein profiles of human TM cells, as assessed by SDS-PAGE analysis, was found to be similar to that of porcine TM cells. In addition, MS analysis of in-gel digested major proteins of the human TM cell triton-insoluble fraction identified Plectin 1, Filamin A, nonmuscle myosin IIA, clathrin, Actinin α 1, vimentin, actin, and annexin II as some of the major proteins. Although caldesmon was easily detectable in porcine TM cells (Fig. 2A), we did not see a distinct band corresponding to this protein in human TM cells. On the other hand, though human TM cells showed a distinct band of laminin receptor, there was no corresponding detectable protein band in PTM cells.

Because the gene expression and protein profile of cultured cells are generally expected to be altered by cell culture conditions and thus potentially differ from the profiles exhibited by the corresponding tissue of origin, we extracted the triton-insoluble protein fraction of TM tissue derived from freshly enucleated porcine eyes for a comparative analysis with the triton-insoluble fraction derived from cultured porcine TM cells. As shown in Figure 2B, SDS-PAGE in conjunction with MALDI-TOF MS analysis of the TM tissue triton-insoluble fraction identified Filamin A, myosin II, clathrin, α -actinin, vimentin, and actin as some of the major proteins, indicating close similarity in cytoskeletal protein profiles between cultured TM cells and freshly extracted TM tissue.

FIGURE 3. Comparison of cytoskeletal protein profiles between TM and CM cells. TM and CM cells isolated from the same porcine eyes were passaged identically, and the triton-insoluble fraction derived (25 μ g protein) from these cells was separated by SDS-PAGE using either 5.5% or 8% acrylamide. The predominant protein bands of the CM cell fraction were identified by MS using methods described earlier. The cytoskeletal protein profiles were found to be identical for the TM and CM cell types, based on SDS-PAGE analysis. Further, MS identified the same major proteins in both the TM and the CM cell types. (A, B) Each left lane depicts the separation of molecular mass marker proteins. This comparison was performed twice with two different cell lines.

TABLE 1. Abundant Cytoskeletal and Cytoskeleton-Associated Proteins of the Porcine TM Cells, TM Tissue, and CM Cells Identified by Mass Spectrometer

Protein	Porcine TM Cells	Porcine TM Tissue	Porcine CM Cells
Plectin	+	+	+
Filamin A	+	+	+
Nonmuscle myosin II	+	+	+
Actinin	+	+	+
Clathrin	+	+	+
Myosin I	+		+
Caldesmon	+		
Vimentin	+	+	+
Actin	+	+	+
Annexin II	+	+	+
Stomatin			+

Characterization of Ciliary Muscle Cell Cytoskeletal Protein Profile

CM and TM tissues exhibit distinct contractile characteristics despite a common developmental origin.^{9,20} To gain insight into the molecular basis for—and to determine whether differences in the cytoskeletal protein composition might be a contributory factor for—the recognized differences between these two tissue types, CM tissue was isolated and CM cells were cultured using methods similar to those used for TM cell isolation and culture. Triton-insoluble fractions were isolated from CM cells at the same passage number as the TM cells used in the current studies, separated by SDS-PAGE, and subjected to MS, as described earlier.

Surprisingly, the protein profile of triton-insoluble fractions isolated from porcine CM cells was found to be very similar to that noted for TM cells derived from the same eye (Fig. 3). Additionally, MS identification of predominant proteins from the CM cell triton-insoluble fraction confirmed similarity of the profile with TM cells, with Plectin 1, Filamin A, myosin IIA, clathrin, myosin IC, vimentin, actin, annexin II, and stomatin

representing abundant components. These data indicate a close similarity between the CM and TM cell types with respect to the makeup of the cellular cytoskeletal fraction and the presence of major protein components known to regulate contractile activity and cytoarchitecture. Table 1 lists the major cytoskeletal and cytoskeleton-associated proteins detected in the detergent-insoluble fractions of porcine TM cells, TM tissue, and CM cells.

Comparison of Human Vascular Endothelial Cell Cytoskeletal Protein Profile with Human TM Cells

TM cells are recognized to have smooth muscle-like properties similar to those of vascular endothelial cells and express α -smooth muscle actin.^{12,27} Additionally, SC cells are known to be endothelia derived, similar to vascular endothelial cells. Therefore, to obtain further insight into whether the similarity among the TM, SC, and vascular endothelial cell types extends to cell structural and functional similarities, we compared the triton-insoluble cytoskeletal protein fractions of the HUVEC line and human TM cells. Analysis of HUVECs and human TM cell triton-insoluble cytoskeletal protein fractions by SDS-PAGE (8% and 5.5% acrylamide) revealed remarkably similar protein profiles between these two types of cells (Fig. 4). Only a few proteins were found to be unique to the HUVEC phenotype. For example, Nestin, a progenitor stem cell marker and intermediate filament protein, was found only in HUVECs. On the other hand, nonmuscle myosin IIA, γ -actin and annexin II are present in both cell types. These data further confirm that the TM cells are very close to endothelial cells in the context of cellular cytoskeletal composition.

Distribution of Filamin A and Annexin II in TM Cells

Filamins are large cytoplasmic proteins that cross-link cortical actin into dynamic three-dimensional structures and transmit extracellular signals through integrin receptors into the cytoplasm. Additionally, filamins interact with a large number of other proteins with diverse functions, including cellular mole-

FIGURE 4. Comparison of cytoskeletal protein profiles for HTM cells and HUVECs. Cell cytoskeletal protein fractions were isolated from HUVECs as described for HTM cells. The protein profile of HTM cells and HUVECs (25 μ g protein) were compared by SDS-PAGE using 8% and 5.5% acrylamide. Interestingly, the protein profile of HUVECs was found to be very similar to that of HTM cells. Selected proteins sharing the same migration pattern characteristics between the two cell types were extracted from SDS-PAGE gels of HUVEC samples and identified by MS. The identity of individual proteins is indicated in the figure next to the respective protein band. One distinct protein band with a molecular mass >250 kDa, which was present only in the HUVECs, was identified as Nestin by MS. These analyses were performed twice.

FIGURE 5. Distribution of Filamin A in HTM cells. (A) HTM cells grown on glass coverslips were fixed and immunolabeled for Filamin A using a monoclonal antibody and colabeled for actin filaments with rhodamine phalloidin. Filamin A was found to be distributed throughout the cell body in a filamentous pattern colocalizing with F-actin. Images were captured using confocal microscopy. Scale bar, 20 μ m. (B) HTM cell lysate protein fractions (total, 20 μ g; cytosolic, 20 μ g; membrane-enriched, 10 μ g insoluble fractions) were separated by SDS-PAGE (5.5% acrylamide), transferred, and immunoblotted for Filamin A using monoclonal antibody. Filamin A was found to be distributed to the cytosolic and membrane fractions. These data were based on two independent analyses.

cles involved in signaling, adhesion, cellular motility, and trafficking.^{28,29} Filamin A was found to be one of the abundant proteins in the cytoskeleton-enriched protein fraction of the TM and CM cells and HUVECs (Figs. 1, 3, 4). Immunolocalization analysis conducted to determine the cellular distribution and interaction of Filamin A with actin filaments in TM cells revealed that Filamin A is distributed throughout the cell cytoplasm, exhibiting a filamentous organization colocalizing with actin filaments (based on colocalization of filamin staining with F-actin; Fig. 5). Additionally, Filamin A was found to be distributed in both soluble and membrane-rich insoluble fractions (Fig. 5B) of TM cells.

Annexin A2/or II is a member of a large family of calcium-binding proteins that also binds to phosphatidylinositol 4,5-bisphosphate and regulates actin assembly.³⁰ In this study we identified Annexin II as an easily detectable protein in the cytoskeleton-enriched fractions of the TM and CM cells and HUVECs. Annexin II is distributed throughout the cytoplasm, exhibits a fine filamentous organization (data not shown), and is present both in the cytosolic and the membrane-rich fractions based on immunoblot analysis (Fig. 6A). Interestingly, annexin II is also secreted by the TM cells (Fig. 6B). Immunoblot analysis of TM cell-conditioned media showed two distinct immunopositive bands migrating closely to each other, one migrating at 36 kDa, similar to intracellular annexin II, and the other slightly larger in molecular size. Annexin II colocalized distinctly with actin at the cell ruffles in the presence of nocodazole (data not shown).

Regulation of Contractile Characteristics of TM and CM Cells

A common feature of the TM and CM cells and HUVECs is that all three cell types contain abundant levels of non-muscle myosin IIA, Filamin A, α -actinin, and actin (Figs. 1, 3, 4). These proteins regulate actomyosin organization and actin filament cross-linking, cellular contraction, mechanical properties, and cell shape. To evaluate the similarity and differences in contractile properties between TM and CM cells, we determined the relative sensitivity of TM and CM cells to the Rho kinase inhibitor and to myosin light chain kinase inhibitor. Rho kinase and MLCK are key regulators of phosphorylation-dependent myosin II activity.^{31,32} We evaluated the effects of Y-27632 and ML-7, which are well-

characterized and specific inhibitors of Rho kinase and MLC kinase, respectively, on MLC phosphorylation in cultured porcine TM and CM cells of equivalent passage number and derived from the same eye.

Confluent cultures of porcine TM and CM cells grown in DMEM containing 10% FBS were shifted to DMEM containing 1% FBS (for 16–18 hours) before they were treated with Y-27632 and ML-7. Cells were exposed to either inhibitor for 30 minutes and at a concentration of 5 and 10 μ M Y27632 or 10 and 20 μ M ML-7 before they were assessed for changes

FIGURE 6. Distribution and secretion of Annexin II. (A) HTM cell lysate protein fractions (total, 40 μ g; soluble, 40 μ g; membrane-enriched 10 μ g insoluble fractions) were separated by SDS-PAGE (12.5% acrylamide), transferred, and immunoblotted for Annexin II using a monoclonal antibody. Annexin II immunoreactivity was positive in all three fractions. (B) To confirm the secretion of Annexin II by HTM cells, conditioned media (40 μ g protein) derived from different HTM cell lines (donors) were analyzed by SDS-PAGE, and proteins were transferred to nitrocellulose membranes, followed by immunoblotting using monoclonal anti-Annexin II antibody. HTM cells show two distinct and closely migrating immunopositive bands with molecular masses of 36 kDa and slightly higher than 36 kDa, confirming that HTM cells produce a secreted form of Annexin II. These data were based on three independent analyses.

in cell shape, actin cytoskeletal organization, focal adhesions, and MLC phosphorylation. Both TM and CM cells were found to be more sensitive to the Rho kinase inhibitor and to exhibit dramatic changes in cell shape (cell-cell detachment, cell rounding), decreased actin stress fibers, focal adhesions, and MLC phosphorylation, compared with cells treated with ML-7 (Figs. 7A, 7B, 8A, 8B). The effects of the Rho kinase inhibitor were found to be much earlier and more severe in TM cells than in CM cells. On the other hand, ML-7 was found to induce changes in cell shape and to decrease actin stress fibers, focal adhesions, and MLC phosphorylation more potently in CM cells than in the TM cells

(Figs. 7A, 7B, 8A, 8B), suggestive of subtle differences in the regulation of contractile activity of TM and CM cells despite the existence of very similar cytoskeletal protein profiles between these two cell types.

DISCUSSION

There is growing interest among the glaucoma scientific community regarding the structure and function of the cellular cytoskeletal framework in cells and tissues of the aqueous humor outflow pathway, because many agents that

FIGURE 7. Relative sensitivity of TM and CM cells to inhibitors of ROCK and MLCK. Porcine TM (A) and CM (B) cells derived from the same eye were cultured to confluence, maintained in 1% serum for 16 to 18 hours, and treated either with Rho kinase inhibitor (Y-27632, 5 and 10 μ M) or with MLCK inhibitor (ML-7; 10 and 20 μ M) for 30 minutes. After 30 minutes of drug treatment, cell morphologic changes were recorded using phase-contrast microscopy. Fixed cells were stained for actin filaments using rhodamine-phalloidin and for focal adhesions using a vinculin antibody in conjunction with AlexaFluor-488. Both TM and CM cells were sensitive to the Rho kinase inhibitor, as evidenced by changes in cell shape and decreases in actin stress fibers and focal adhesions. However, CM cells were found to be more sensitive to ML-7 than TM cells. These conclusions were based on two independent experiments performed using two different cell lines.

FIGURE 8. Relative effects of Rho kinase and MLCK inhibitors on myosin light chain phosphorylation in TM and CM cells. Porcine TM and CM cells were isolated from the same donor eye, and confluent cell cultures were maintained in 1% serum for 16 to 18 hours before treatment with either Rho kinase inhibitor (Y-27632; 5 and 10 μ M) or MLCK inhibitor

(ML-7; 10 and 20 μ M) for 30 minutes. The cell lysates (25 μ g protein) were analyzed for changes in MLC phosphorylation by immunoblot analysis using phosphospecific anti-MLC polyclonal antibody. As shown in the figure, MLC phosphorylation was dramatically reduced in both TM (A) and CM cells (B) treated with Rho kinase inhibitor compared with cells treated with ML-7. However, in contrast to TM cells, 20 μ M ML-7 was noted to markedly reduce MLC phosphorylation in CM cells, indicating subtle differences between the role of Rho kinase and MLCK in TM and CM in regulation of contractile activity of these two cell types. Actin was immunoblotted in the same samples to serve as a loading control. These results represent two independent analyses.

target the cytoskeletons influence aqueous humor outflow facility.¹² Because little is known regarding the composition of the cytoskeletons in cells and tissues of the aqueous outflow pathway,^{33–35} in this study we focused on identifying the major cytoskeletal proteins of the trabecular meshwork cells and their relative abundance with a goal to provide additional insight into the regulation of aqueous humor drainage. To our knowledge, this is the first report on the comprehensive cytoskeletal protein profile of TM and CM cells. In this study, we not only identified the major cytoskeletal proteins of the trabecular meshwork but also compared them to the CM cells and vascular endothelial cells to see how similar or different they are from each other. Interestingly, all three cell types—TM, CM, and vascular endothelial cells—revealed very similar cytoskeletal protein profiles with abundant levels of nonmuscle myosin IIA, α -actinin, Filamin A, Annexin II, actin, vimentin, clathrin, and Plectin-1 which are involved in the regulation of cellular contraction, mechanical properties, trafficking, and cell shape.

Although the regulation of aqueous humor drainage through the trabecular pathway is not understood completely, several experimental and human studies have consistently reported that agents known to affect cellular contractile and relaxation properties, cell shape, actin polymerization, cell adhesive interactions, and ECM remodeling influence aqueous humor outflow facility,^{3,9,11,12,35,36} indicating the importance of cytoskeletal proteins and their organization and regulation in aqueous humor outflow pathway. Proteomics analysis of human TM and porcine TM cell triton insoluble fractions enriched in cytoskeletal and cytoskeleton-associated proteins revealed the presence of abundant levels of non-muscle myosin IIA, Filamin A, α -actinin, Plectin-1, actin, and vimentin. The other easily detectable proteins in the cytoskeleton-enriched fraction included caldesmon, laminin receptor, clathrin, annexin II, and myosin 1C. Importantly, the protein profile of tissue-cultured TM cells was found to be very similar to that of TM tissue. Interestingly, the cytoskeletal protein profile of TM cells from human and porcine species showed only subtle differences, indicating high conservation of cytoskeletal protein composition between these two species. Given that many of the cytoskeletal proteins play a fundamental and structural role, it is expected that they maintain evolutionary conservation in their structure and function.

Non-muscle myosin II (NM II), an actin-binding protein that has actin cross-linking and contractile properties, was found to be one of the major cytoskeletal proteins in TM, CM, and HUVECs. NM II activity is regulated by the phosphorylation of its light and heavy chains.³⁷ NM II and actin together represent the major contractile proteins of smooth muscle cells that generate force and play a fundamental role

in cell adhesion, migration, cell architecture, and mechanobiology.^{37,38} Myosin II-regulated actin stress fibers provide the force for a wide array of motile and morphogenetic processes in eukaryote cells.^{38,39} In TM cells and in the outflow pathway, direct inhibitors of myosin II activity, including blebbistatin and 2,3-butanedione 2-monoxime, have been shown to induce cell shape changes, decreased actin stress fibers, focal adhesions, and morphologic changes, concomitant with increased aqueous humor outflow facility.^{15,40} NM II activity is regulated predominantly by myosin light chain kinase and Rho kinase-mediated phosphorylation,³⁷ and both Rho kinase and myosin light chain kinase inhibitor-mediated suppression of phosphorylation of the regulatory subunit of myosin II also increase aqueous humor outflow facility in association with changes in cell shape, actin stress fibers, and morphologic changes.^{19,41} These observations suggest that NM II activity plays a significant role in the regulation of aqueous humor outflow facility and homeostasis of IOP. Additionally, as we have suggested earlier,⁴⁰ NM II may serve as an important molecular target for the novel treatment of glaucoma. Moreover, because NM II activity is controlled by various extracellular stimuli and intracellular mechanisms,³¹ the regulation of NM II activity in the outflow pathway may play a vital role in both homeostasis of aqueous outflow and pathophysiology of glaucoma. Caldesmon, a known negative regulator of myosin II ATPase activity and smooth muscle contraction, was identified as one of the constituents of TM cell cytoskeletal fraction. Overexpression of caldesmon in aqueous humor outflow pathway has been demonstrated to increase outflow facility,⁴² further strengthening the importance of myosin II and its contractile activity in the regulation of outflow facility.

Interestingly, the other prominent proteins—including Plectin-1, Filamin A, and α -actinin—found in the cytoskeleton-enriched fraction of TM cells and tissue are well-characterized and versatile cytoskeletal linker proteins involved in various cellular processes in addition to being cytoarchitectural proteins. For example, Plectin-1, with a molecular mass >500 kDa, cross-links microfilaments, microtubules, and intermediate filaments and binds to cell junctional complexes. Because of its versatile cytolinker property, Plectin is known to play an important role in cytoskeletal network organization and to control the viscoelastic properties of cytoplasm and the mechanical integrity, stiffness, and resistance of cells and tissues.^{43,44} These properties of Plectin may be important for the regulation of pressure-sensitive attributes of TM tissue, architecture, and mechanotransduction.

Similarly, Filamin A is a large cytoplasmic protein that cross-links cortical actin into dynamic three-dimensional structures and transmits extracellular signals through integrin receptors into the cytoplasm.^{29,45} Based on its known interaction with the actin cytoskeleton, plasma membrane,

and signaling molecules, it is recognized to be an important scaffolding protein regulating various cellular processes, including the response of cells to their chemical and mechanical environment, by regulating changes in shape and motility.^{28,46,47} The human filamin family consists of three members, Filamins A, B, and C, that share 70% sequence homology.⁴⁵ However, Filamin A appears to be the predominant isoform expressed in TM cells and tissue. Filamin A has been shown to be induced by mechanical forces applied through $\beta 1$ integrins and has been implicated in mechanoprotection.^{46,48–50} Further, increased Filamin A production by tensile forces facilitates cell survival, in part by mechanical stabilization of cortical actin and prevention of force-induced cell depolarization.⁵¹ In TM cells, Filamin A was distributed throughout the cytoplasm, colocalizing with actin filaments and exhibiting a distribution profile spanning the cytosolic and membrane fractions. Based on these characteristics, it is reasonable to speculate that the abundant levels of Filamin A in TM cells and tissue may play a crucial role in the cytoarchitecture and mechanical properties of TM and in the homeostasis of aqueous outflow facility.

α -Actinin, which belongs to a superfamily of spectrin structural proteins, is a well-characterized actin-cross-linking protein with a critical role in actin stress fiber formation and contraction.⁵² In addition to binding to actin, α -actinin links actin to various plasma membrane proteins, including signaling molecules, and localizes to cell adhesions and cell-cell junctions.^{52–54} Based on these multiple activities, α -actinin is thought to play a central role in cellular mechanotransduction and cytoarchitecture. The many common characteristics of these different cytolinker proteins and their shared role(s) in various cellular processes suggest that they play a vital role in the regulation of mechanotransduction, cytoarchitecture, and contractile activity in pressure-sensitive TM cells and in CM cells.

Annexin II, myosin 1C, and clathrin were also noted to be present in abundant levels in the cytoskeleton-enriched fraction of TM cells and tissue. These proteins are known to regulate various aspects of membrane and protein trafficking.^{30,55} TM cells are very active in endocytosis, exocytosis, and phagocytosis.^{56–58} Interestingly, annexin II, a calcium and phospholipid binding protein, interacts with actin and regulates actin polymerization during endocytosis.^{30,59} Annexin II is also known to regulate plasminogen activation and plasmin production, both of which regulate fibrinolysis and ECM turnover.⁶⁰ In TM cells, this protein is distributed in the cytosolic and the membrane fractions and is distributed throughout the cytoplasm. Importantly, annexin II is also secreted into the cell medium by TM cells, indicating a potential intracellular and extracellular role for this cytoskeleton-binding protein in the aqueous humor outflow pathway, as has been suggested in other cell types.⁶¹ In some analysis, we noted the presence of nuclear proteins histones in the triton-insoluble fractions; these could be nonspecific contaminants because they were not found in all our samples.

Intriguingly, the cytoskeletal protein profiles of TM and CM cells were found to be very similar. In both cell types, the non-muscle myosin IIA, which regulates cellular contraction, was abundant. However, the physiological experiments carried out using the CM and TM tissue strips have been reported to exhibit distinct characteristics under the stimulation of certain agonists.⁹ Despite having similar cytoskeletal protein profiles in these two different tissues, which developmentally originated from the neural crest,²⁰ the possible explanation for the reported distinct contractile characteristics could be related to a potential difference in signal transduction at the plasma membrane, intracellular

mechanisms, or both.⁹ The cytoskeletal protein profiles of both TM and CM cells were found to be very similar to that of vascular endothelial cells. As in the case of TM and CM cells, vascular endothelial cells showed abundant levels of non-muscle myosin II, Filamin A, Plectin-1, annexin A2, and clathrin, indicating a close cell structural similarity among the TM, CM, SC, and vascular endothelial cells, especially in the context of contractile activity, cytoarchitecture, mechanotransduction, and membrane-trafficking activities. However, when comparing the cytoskeletal protein profile of these three cell types with certain other cell types (e.g., lens fiber cells), the lens fiber cell cytoskeletal protein profile included spectrin as one the abundant proteins, which was not easily identifiable (based on SDS-PAGE) in any of the three cell types discussed. Similarly, though NM II, Filamin A, and annexin II are abundant in TM and CM cells and HUVECs, they were not the major proteins in the lens fiber cell triton cytoskeletons.²⁶

Interestingly, the TM and CM cells were found to exhibit differential responses to inhibitors of Rho kinase and myosin light chain kinase in experiments designed to obtain further insight into the regulation of contractile characteristics in these two cell types. These differential responses included changes in actomyosin organization, cell shape, and myosin light chain phosphorylation, with both TM and CM cells exhibiting higher sensitivity to the Rho kinase inhibitor than the MLCK inhibitor, and indicating the importance of Rho/Rho kinase signaling in the regulation of contractile activity in both cell types. Although both Rho kinase and MLCK regulate MLC phosphorylation and contraction, MLCK-mediated MLC phosphorylation is calcium dependent, whereas Rho kinase-mediated MLC phosphorylation is calcium independent.^{31,32} However, on a relative basis, CM cells were more sensitive to the MLCK inhibitor than the TM cells, indicating subtle differences in the regulation of contractile activity between the TM and CM cells. Whether this subtle difference noted in the cell culture model system is significant in vivo in TM and CM tissues remains to be addressed and requires additional studies.

As in this study, in future studies, it would be important to compare and contrast the cytoskeletal protein profile, contractile characteristics, and expression profile of cell adhesive molecules, integrins, and cell junctional proteins between TM and SC cells to understand the distinct roles played by these two different cell types in the outflow pathway.

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