Primary Trabecular Meshwork Cells Incubated in Human Aqueous Humor Differ from Cells Incubated in Serum Supplements

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PURPOSE. To determine whether aqueous humor, the in vivo source of nutrients for trabecular meshwork cells, alters cellular and molecular characteristics in primary trabecular monolayer cell cultures when compared with standard culture conditions.

METHODS. Human primary trabecular meshwork cell cultures were grown in DMEM supplemented with 50% human aqueous humor (DMEM-AH), heat-denatured DMEM-AH, 10% fetal bovine serum (DMEM-FBS, the standard culture supplement), or heat-denatured DMEM-FBS. Confluent trabecular cells were assayed for cell proliferation and morphology for 21 days. Protein expression profiles of trabecular cell lysates were analyzed by two-dimensional polyacrylamide gel electrophoresis. Western blot analysis was used to determine the protein expression of myocilin and TIMP-1 in conditioned media collected from trabecular cells at 5, 10, 15, and 21 days. Myocilin expression was also analyzed by Western immunoblots after addition of dexamethasone (10^-7 M) or ascorbic acid (29 mg/dL).

RESULTS. Trabecular cells supplemented with DMEM-AH for 21 days showed decreased cell proliferation when compared with DMEM-FBS (11% vs. 141%). Cellular morphology was also altered: Trabecular cells incubated in DMEM-AH showed larger, broader-, and flatter-appearing cells than did the more spindle-shaped cells grown in DMEM-FBS. Protein profiles of trabecular cell lysates isolated from cells incubated in DMEM-AH differed from those incubated in DMEM-FBS. In DMEM-AH-conditioned medium, myocilin expression was increased and TIMP-1 expression was decreased at day 21. Induction of myocilin by dexamethasone was observed in conditioned medium isolated from cells treated with DMEM-FBS (442%), but only a 10% increase in myocilin was observed beyond the normal induction in DMEM-AH. Daily administration of ascorbic acid to DMEM-AH failed to increase myocilin expression beyond that obtained with DMEM-AH.

CONCLUSIONS. Addition of human aqueous humor rather than FBS to trabecular monolayer cell cultures triggers significant changes in cellular and molecular characteristics. The protein component of aqueous humor is responsible for these changes. Aqueous humor supplementation may maintain cultured trabecular cells in a more physiologic state. (Invest Ophtalmol Vis Sci. 2005;46:2848–2856) DOI:10.1167/iovs.05-0101

The trabecular meshwork accounts for most aqueous outflow resistance in the anterior chamber of the human eye.1 It is believed that the interplay between the endothelial-like cells of this tissue (trabecular cells) and the surrounding extracellular matrix are responsible for maintaining the resistance necessary for preservation of the aqueous outflow pathway. The trabecular cells are phagocytic cells, actively removing debris such as pigment granules, erythrocytes, and pseudoxfoliation material from the aqueous outflow system.2–6 They also are involved in the production and turnover of extracellular matrix.7–10 The understanding of the biochemical and molecular events that occur in these cells under normal hemostatic conditions is important for determining their functional role.

Originally described in 197911–14 and more recently modified by Stamer et al.,15 primary trabecular monolayer cell culture has been a cornerstone for investigation of the molecular and biochemical functions in this distinct cell type. Despite its widespread acceptance, monolayer trabecular cells differ from the in vivo state, particularly in the plastic substrate on which they are grown, altered extracellular matrix, lack of aqueous flow, and their nutrient source. In vitro, trabecular cells are maintained in an environment rich in nutrients (generally supplied by the addition of fetal bovine serum). The presence of the rich medium promotes cell replication that is necessary for cell expansion in culture. This differs from trabecular cells in vivo, which rarely replicate.16–21

In vivo, trabecular cells are nourished by aqueous humor, a complex mixture of electrolytes, organic solutes, growth factors, and additional proteins. Aqueous humor is produced from the ciliary body epithelium and mixes with plasma-derived proteins, originating from fenestrated capillaries of the ciliary body that flow into the anterior chamber via the iris root.22–25 Aqueous humor is not a simple diffusate of plasma, because aqueous has high concentrations of solutes and qualitative and quantitative differences in protein content from plasma.26–29 In addition, aqueous humor also contains proteins and other molecules secreted from anterior segment tissues.26,30,31

Aqueous humor also differs from fetal bovine serum in both the types and proportions of proteins and growth factors. The use of aqueous humor as a medium supplement in vitro for primary trabecular cell culture models is not a common practice. One study has reported that aqueous humor stimulates trabecular cell migration in culture.32 In corneal endothelial cells, aqueous humor reduces apoptosis and also produces immunosuppressive effects.33–35 In this study, we investigated whether incubation in human aqueous humor rather than serum supplements changes the cellular and molecular char-
acteristics of human primary trabecular cell monolayer cultures.

METHODS

Collection of Normal Human Aqueous Humor

Human donor aqueous humor was chosen as the source for aqueous humor because it was more readily available in the amounts needed to perform the experiments. Normal human donor eyes were obtained within 12 hours of death from the Minnesota Lions Eye Bank. A 27-gauge needle attached to a tuberculin syringe was inserted into the anterior chamber, and aqueous humor was slowly withdrawn. Aspiration was stopped before the anterior chamber collapsed, to minimize protein and cell debris contamination of primary aqueous humor. Approximately 100 to 200 µL of aqueous was collected per donor eye. Samples were immediately placed at ~7°C. A Bradford protein assay (Bio-Rad, Hercules, CA) was performed to determine protein concentration. Only aqueous humor obtained from donors with no known eye disease, no steroid or eye medications, and protein concentrations in the near-normal range (12–50 µg/dL) were used. Overall, 16 male and 21 female human donor aqueous humor samples were used in the study. The average age was 74 ± 11 years (mean ± SD) with a range of 47 to 95 years. Patients were taking an assortment of medications. The donor eyes were managed in accordance with the provisions of the Declaration of Helsinki for research involving human tissue.

The steroid content in two human donor aqueous humor samples was analyzed by HPLC on a triple-quadrupole mass spectrometer (model 5000; Applied Biosystems, Inc. [ABI], Foster City, CA) with electrospray ionization (ESI). One sample was an aliquot from three combined donor aqueous humor samples. The second sample was an aliquot from seven combined aqueous humor samples. Of the 14 steroids tested, only trace levels were detected. The analytes that were evaluated and the limit of detection for each were as follows: beclomethasone dipropionate (0.03 µg/dL), budesonide (0.04 µg/dL), dexamethasone (0.06 µg/dL), fludrocortisone (0.06 µg/dL), flunisolide (0.03 µg/dL), fluorometholone (0.04 µg/dL), hydrocortisone (0.04 µg/dL), megestrol acetate (0.03 µg/dL), methylprednisolone (0.06 µg/dL), fluticasone propionate (0.04 µg/dL), fluticasone (0.05 µg/dL), triamcinolone (0.3 µg/dL), and triamcinolone acetonide (0.05 µg/dL).

Trabecular Cell Monolayer Culture

The trabecular cell lines used in the study were provided by Dan Stamer, PhD, University of Arizona (Tucson, AZ): TM33 (53 years old), TM61 (66 years old), and TM88 (25 years old); and Abe Clark, PhD, Alcon Laboratories (Fort Worth, TX): TM30 (48 days old) and TM153 (58 years old). The trabecular cells were isolated according to the method of Stamer et al.13 Briefly, the trabecular meshwork was dissected from human donor eyes and subjected to enzymatic matrix digestion with collagenase A. The cells were pelleted and resuspended in DMEM (Mediatech, Inc., Herndon, VA) containing penicillin-streptomycin (100 U/mL final concentration; Invitrogen, Carlsbad, CA) and 10% FBS (DMEM-FBS). Cells were placed in a T25 flask or a 6-, 24-, or 96-well plate and grown in DMEM-FBS or DMEM-AH (DMEM containing penicillin-streptomycin [100 U/mL final concentration] and 50% human aqueous humor, but no FBS) for up to 21 days. We chose 50% aqueous humor as our supplement as the use of 100% aqueous humor was not feasible because of limited quantities from human donors. Fresh media were added on days 0, 5, 10, and 15. On days 5, 10, 15, and 21, conditioned media were collected and centrifuged at 13,000g to remove any cellular debris, and supernatant was isolated. In studies requiring multiple media changes, several aqueous humor samples were combined, mixed, and aliquoted before initiation of the experiment, so that the same aqueous humor solution would be used for a given experiment over 21 days.

Analysis of Cell Proliferation

An equal number of cells from three human trabecular cell lines were placed in multiple wells of either a 24- or 96-well plate and incubated for up to 21 days in DMEM alone, DMEM-FBS, heat-denatured DMEM-FBS, or heat-denatured DMEM-AH. For heat-denatured media, FBS and aqueous humor were heated to 80°C for 5 minutes before addition to DMEM and cells. In each experimental condition, cell media were changed on days 0, 5, 10, and 15. On days 5, 10, 15, and 21, cells from two wells incubated in DMEM alone or DMEM-FBS were collected after trypsin digestion, and cell counts were performed on each sample. In the remaining conditions, cells from two wells were isolated after trypsin digestion and counted independently on day 21.

For each of the three cell lines, the average of the two independent cell counts for each time point was calculated. The average cell count on day 0 was adjusted to 50,000 cells so that comparisons could be made between the three cell lines. The numerical ratio of the adjusted cell number (50,000) divided by the actual number of cells at day 0 was then used to multiply the average cell counts at days 5, 10, 15, and 21, to obtain the adjusted cell counts for each cell line. The mean ± SD of the adjusted cell counts at each time point was calculated. All time points contained three data points except for day-15 DMEM and day-21 DMEM-AH, which had two data points.

Analysis of Cell Morphology

Images of trabecular monolayer cells incubated in DMEM-FBS or DMEM-AH were taken on days 0, 5, 10, 15, and 21 with a digital camera (Spot Insight Color model 3.20; Diagnostic Instruments, Inc., Sterling Heights, MI). To image the same location in a well at various time points, a horizontal and vertical line were placed on the bottom of the dish so that each well was divided into four quadrants. Before a photograph was taken, an inverted light microscope (Eclipse TS100; Nikon Inc.) was focused on the area where the lines bisected, to locate the middle of the plate. The microscope was adjusted so that the bisected lines were at the bottom left corner of the viewing field (the upper right quadrant was imaged). With the camera in the same position, cells were focused and imaged.

Analysis of Trabecular Cell Lysate

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed on confluent trabecular monolayer cells after incubation in DMEM-FBS or DMEM-AH for 5 days. After incubation, cells were washed twice with PBS, trypsinized and collected by centrifugation at 1000g for 5 minutes. Cell pellets were resuspended in PBS and centrifuged again. A second resuspension in PBS followed by centrifugation was performed to remove trypsin from the cell pellet. Trabecular cell pellets were solubilized in cell lysis buffer (50 mM Tris [pH 8.0], 0.5% sodium dodecyl sulfate, 0.5% Triton, 137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4·7H2O, 1 mM KH2PO4, and protease inhibitors; Roche Diagnostics, Indianapolis, IN). Samples were vortexed and placed at 4°C for 60 minutes followed by sonication. Insoluble material was removed from lysate by centrifugation at 10,000g for 5 minutes. Supernatant was collected, and a Bradford protein assay was per-
formed to determine protein concentration (Bio-Rad). Fifty micrograms of each sample was diluted with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS [3-[3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate], 60 mM dithiothreitol, and 1% chromatofocusing medium; Pharmalyte; GE Healthcare [formerly Amersham], Piscataway, NJ), and 0.25% 3–10 NL IPG strips (GE Healthcare). After 16 hours of rehydration, the strips were focused (Protein IEF Cell; Bio-Rad) for a total of 45,000 volt-hours at 20°C. After isoelectric focusing, strips were equilibrated in SDS-PAGE running buffer (Laemmli system) in two steps: the first in the presence of 1% dithiothreitol for 10 minutes and the second in the presence of 2% iodoacetamide for 15 minutes, to reduce and alkylate the cysteines. Agarose (1% in running buffer) was used to seal the strip in place at the top of a 4% to 15% Tris-HCl gel (20 × 20 × 0.1 cm). Proteins were electrophoresed in the gels at 200 V for 6 hours at 18°C. The gels were silver stained, to visualize the proteins. Gels were scanned with a 12 bit-depth scanner (GS 800 calibrated densitometer; Bio-Rad).

**Protein Identification**

Protein spots isolated from 2D-PAGE were destained with 15 mM potassium ferricyanide and 50 mM sodium thiosulfate until clear and rinsed with DH2O several times to remove all color. The protein spots were reduced with 10 mM dithiothreitol in 25 mM Tris (pH 8.1) for 20 minutes at 55°C, followed by alkylation with 20 mM iodoacetamide in 25 mM Tris (pH 8.1) for 20 minutes at room temperature. Proteins were digested for 4 hours with 0.02 μg trypsin (Promega Corp., Madison, WI) in 20 mM Tris (pH 8.1) at 37°C. Peptide extraction was performed with 20 μL of 2% formic acid followed by 20 μL of a 70% acetonitrile-30% water-0.1% formic acid solution. The pooled extracts were concentrated to less than 5 μL on a spinning concentrator (SpeedVac; Savant Instruments, Holbrook, NY) and then brought up in 0.1% trifluoroacetic acid.

For protein identification by nanolow liquid chromatography tandem mass spectrometry (nanoLC-MS/MS), the peptide mixture was trap injected onto a 75-μm × 5-cm nanolow column (ProteoPep C18 PicoFrit; New Objectives, Woburn, MA) and eluted with a 0.1% formic acid-acetonitrile gradient (Paradigm MS4 system; Michrom BioResources Inc, Auburn, CA) coupled to an ion-trap mass spectrometer (ThermoFinnigan LTQ Linear Ion Trap, ThermoElectron, San Jose, CA). The mass spectrometer was set to scan continuously for ions in a mass range of 375 to 1600 m/z, automatically switching to MS/MS mode on the ions with intensities exceeding a preset threshold. The MS/MS data were correlated to theoretical fragmentation patterns of tryptic peptide sequences from the NCBI nr and Swissprot databases, using both Sequest50 (Sequest Technologies, Lisle, IL) and Mascot55 (Matrix Sciences, London, UK) search algorithms running on a 10-node cluster (Swissprot is provided in the public domain by the Swiss Institute of Bioinformatics, at http://www.expasy.org; NCBI nr is provided in the public domain by the National Center for Bioinformatic Information, Bethesda, MD, at http://www.ncbi.nlm.nih.gov). All searches were conducted with variable or differential modifications, allowing +16 for methionine sulfone, +57 for carboxamidomethyl-cysteines and +71 for S-propionamido cysteines. The search was restricted to trypsin-generated peptides, allowing for two missed cleavages and was left open to all species. Peptide mass tolerances were ±1.5 Da, and fragment mass tolerance was set to ±0.8 Da. Protein identifications were considered when both Mascot and Sequest gave at least two consensus peptides with individual cross correlation or probability scores exceeding a threshold dependent on the precursor charge state and ranking in the top five of all the hits for their respective MS/MS spectra.

**Western Blot Analysis of Trabecular Cell Lysate or Conditioned Media**

Trabecular cells (TM30) were incubated in DMEM-AH or DMEM-FBS for 5 days. Cells were isolated, and lysates were prepared as described earlier. In duplicate, 15 μg of each lysate was placed in Laemmli sample buffer (without β-mercaptoethanol), boiled, and separated on a 4% to 15% SDS-PAGE gel (denatured, nonreduced). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane in 1× transfer buffer (50 mM Tris, 384 mM glycine, 0.01% SDS, and 20% methanol). Membranes were probed with the following antibodies: collagen type I (Chemicon International, Temecula, CA), collagen type III (Chemicon International), and glyceraldehyde-3-phosphate dehydrogenase (Novus Biologicals, Littleton, CO). Denatured, nonreduced gels were used because the collagen antibodies would not recognize the reduced form of the protein (protocol from the manufacturer). Horseradish-peroxidase-linked anti-mouse Ig or anti-rabbit Ig (GE Healthcare) was used as secondary antibody. Antibody-antigen complexes were detected with Western blot signal detection reagent (ECL; GE Healthcare).

Western blot analyses were performed on conditioned media collected from cells at days 5, 10, 15, and 21 after incubation in DMEM-FBS, heat-denatured DMEM-FBS, DMEM-AH, or heat-denatured DMEM-AH. For the heat-denatured media, FBS or aqueous humor was heated to either 60°C for 10 minutes (n = 1) or 80°C for 5 minutes (n = 2) before addition to DMEM and cells. For the media control, DMEM-AH or DMEM-FBS was incubated at 37°C for 5 days in a well that did not contain cells. Equal volumes of conditioned media (30 μL) were placed in Laemmli sample buffer, boiled, and separated on 4% to 15% SDS-PAGE gradient gels (denatured, reduced). Proteins were transferred to PVDF membrane in 1× transfer buffer and detected by Western detection reagent (ECL; GE Healthcare) after incubation with rabbit polyclonal anti-mycoblin,56 mouse monoclonal anti-TIMP-1 antibodies (R&D Systems, Inc., Minneapolis, MN), and horse-radish-peroxidase-linked secondary antibodies. Densitometry was performed with Image J software (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Changes (x-fold) were calculated by dividing experimental sample by experimental control, and reported values are listed as the mean ± SD.

**Analysis of Ascorbic Acid Levels**

Five independent human donor aqueous humor samples were analyzed for ascorbic acid concentration by high-performance liquid chromatography (HPLC) and UV detection. Briefly, each donor sample was separated into three 1.5-mL tubes (40 μL each). To one tube from each sample, 560 μL of PBS was added, mixed, and the tube placed at −70°C. To determine degradation of ascorbic acid under culture conditions, the two remaining tubes from each sample were placed at 37°C for 24 or 48 hours. After incubation at 37°C, 360 μL of PBS was added to the appropriate tube before placement at −70°C. PBS was added to each sample to dilute the aqueous humor 10-fold because aqueous humor contains high levels of ascorbic acid.51

To determine ascorbic acid concentration, an equal aliquot from each sample was mixed with an equal volume of meta-phosphoric acid to precipitate the proteins. After centrifugation to remove precipitated proteins (2000g for 10 minutes), an aliquot of the clarified supernatant fluid was subjected to separation of ascorbic acid from the other aqueous humor components by HPLC (Licosorb C18 reversed-phase column; 250 × 4.6 mm, 5-μm particle diameter; Phenomenex, Torrance, CA). UV detection (240 nm) was used to quanitate ascorbic acid concentrations by comparison to standards of known concentration. Ascorbic acid levels were multiplied by 10 to account for the 10-fold dilution that was used in analysis.

**Treatment with Ascorbic Acid or Dexamethasone**

On day 0, TM33 cells were washed twice in PBS and then fresh DMEM-AH or DMEM-FBS was added (500 μL). To appropriate wells, 2.5 μL of 200× ascorbic acid stock was added (29 mg/dL final concentration). Cells were incubated at 37°C. Our intent was to add fresh ascorbic acid so that the final concentration was 18.6 ± 4.6 mg/dL. A 200× stock of ascorbic acid was prepared (372 mg in 10 mL of
DMEM), aliquoted, and placed at −70°C. Using HPLC and UV detection (described earlier), the prepared 18.6 mg/dL ascorbic acid solution was measured as 29 mg/dL. Therefore, the ascorbic acid concentration is reported as 29 mg/dL.

On day 1, 50 μL of media from each condition was isolated and spun at 13,000g for 3 minutes, and the supernatant was collected. To maintain ascorbic acid levels in culture, fresh ascorbic acid was added after media collection (2.25 μL on days 1 and 2, to maintain the 29-mg/dL concentration after removal of 50 μL of media on day 1). On day 3, 50 μL from each condition was isolated and prepared as described for the day-1 sample. Fresh ascorbic acid was added after media collection (2 μL on days 3 and 4 to maintain the 29-mg/dL concentration). On day 5, the remaining conditioned media were collected. For SDS-PAGE, 30 μL of each sample was separated on a 4% to 15% gradient gel, and a Western analysis was performed with an anti-myocilin antibody.40 Samples were collected for DMEM-AH and DMEM-FBS controls, which are defined as media incubated at 37°C in a well that did not contain any cells.

For dexamethasone induction, 10−7 M dexamethasone (final concentration) was added daily to trabecular monolayer cell lines TM33, TM88, and TM153. Conditioned media were collected on days 1, 3, and 5, and proteins were separated on 4% to 15% SDS-PAGE gradient gels. Western analyses were performed as described earlier.

**RESULTS**

**Analysis of Cell Proliferation**

Trabecular cells in vivo do not normally undergo cell division.16–21 However, in the presence of 10% FBS (standard culture supplement), trabecular cell monolayer cultures undergo cell division for a limited number of passages. To investigate the effects of aqueous humor on primary trabecular cell proliferation, we compared three independent confluent human trabecular cell lines for 21 days in various media. Confluent trabecular cell lines incubated in DMEM-AH showed a slight increase in cell counts at 21 days, increasing by 11% (50,000 on day 0 to 56,405 on day 21, n = 3; Fig. 1A). In contrast, cells incubated in DMEM-FBS gradually increased in number by 164% over the first 15 days (132,137 cells on day 15, n = 3; Fig. 1B). The increase was followed by a slight decrease to 120,451 cells by day 21 (141% increase compared with day 0; n = 3, Fig. 1). Cell proliferation was halted in trabecular cells after incubation in heat-denatured DMEM-AH (50,000 at day 0 compared with 45,175 at day 21) or heat-denatured DMEM-FBS (50,000 at day 0 compared with 54,632 at day 21). Trabecular cells incubated in DMEM alone (no protein component) lost 54% of the initial number of cells by day 21 (50,000 on day 0 to 22,810 on day 21). These results indicate that incubation of cells in human aqueous humor minimizes cell division in vitro, similar to expected findings with trabecular cells in vivo.

In addition to cellular proliferation, there were changes in cell morphology. Cells incubated with aqueous humor appeared larger and broader in comparison with the spindle-shaped trabecular cells grown in DMEM-FBS (Fig. 2). A flat, one-cell monolayer was formed with minimal cell overlap in DMEM-AH-incubated cells. Furthermore, after trypsin digestion, cells incubated in DMEM-AH came off as sheets, rather than as individual cells, as found with DMEM-FBS, indicating an increase in cell-to-cell contact.

**Analysis of Trabecular Cell Protein Expression**

Because cell proliferation and morphologic changes occurred in trabecular cells after incubation in DMEM-AH, we investigated whether changes in individual protein expression were also present. Protein expression rather than gene expression was studied because proteins are the final product responsible for cell maintenance and function. A preliminary comparison of protein expression between a trabecular cell line incubated in DMEM-AH or DMEM-FBS was performed with silver-stained 2D-PAGE gels (Fig. 3A). Changes in the protein expression profiles can be seen between the two conditions. For example, magnification of the same region within both gels shows protein spots that are unique to the DMEM-FBS-treated cells (Fig. 3B). Two of these protein spots were sequenced and identified as collagen α1(III) precursor and collagen α1(III) precursor. These proteins are integral components of collagen types I and III. Using antibodies directed against the nonreduced form of collagen types I and III, Western blot analysis confirmed that these proteins were present in greater quantity in the cells treated with DMEM-FBS (Fig. 3C).

To determine whether trabecular cell incubation with aqueous humor also alters protein expression of secreted proteins, we analyzed conditioned media for the expression of myocilin (glaucoma-associated protein) and TIMP-1 (an inhibitor of matrix metalloproteinase activity; Fig. 4). Western blot analysis showed that myocilin expression was approximately four times greater in day-5 conditioned media collected from trabecular cells incubated in DMEM-AH than in conditioned media collected from trabecular cells incubated in DMEM-FBS (4.7 ± 3.1 vs. 1.3 ± 0.4, n = 5 cell lines). All five TM cell lines showed myocilin induction after DMEM-AH incubation, when compared with DMEM-FBS cells. Only two of the five trabec-
ular cell lines incubated in DMEM-FBS had myocilin levels higher than fresh DMEM-FBS medium (Fig. 4, control). Myocilin maintained higher expression in DMEM-AH throughout the 21-day period (7.8 ± 3.7 vs. 2.2 ± 2.3 at day 21, n = 3 cell lines).

In contrast to the increase in secreted myocilin in DMEM-AH-incubated cells, TIMP-1 secretion was decreased over time by DMEM-AH (Fig. 4). At day 5, some cell lines showed increased levels of TIMP-1 in DMEM-AH when compared with DMEM-FBS (Fig. 4), whereas other cell lines showed a decrease. The mean calculated expression levels showed similar levels for TIMP-1 expression with a trend toward a slight decrease in cells incubated with DMEM-AH when compared with DMEM-FBS (5.5 ± 1.8 vs. 8.0 ± 4.6; n = 5). By day 21, TIMP-1 expression in conditioned medium collected from cells grown in DMEM-AH was 2.6-fold lower than in conditioned medium collected from cells grown in DMEM-FBS.

FIGURE 2. Morphologic appearance of primary trabecular cell cultures. Trabecular cells incubated in DMEM-AH or DMEM-FBS for 21 days. Cells displayed a broad, flat appearance in DMEM-AH when compared with the spindle shape of cells grown in DMEM-FBS. Magnification, ×200.

FIGURE 3. Protein expression profiles of primary monolayer trabecular cell lysates after incubation in DMEM-AH or DMEM-FBS. (A) 2D-PAGE gels of trabecular cell lysates prepared from cells incubated in DMEM-AH or DMEM-FBS for 5 days. (B) Magnified image of boxed area in (A) showing protein spot differences in cell lysates obtained from DMEM-FBS-treated cells. Two of the spots were identified as collagen α1 (I) and α1 (III), integral components of collagen types I and III, respectively. (C) Western blot analysis of collagen types I and III in DMEM-FBS and DMEM-AH cell lysates. GAPDH was used as an internal protein-loading control. Molecular masses of collagen type I and III were >180 kDa (due to the nonreduced state). GAPDH ran as a 55-kDa protein.
DMEM-AH did not increase myocilin expression when compared with DMEM-FBS cells (3.1 ± 1.1 vs. 8.1 ± 6.6, n = 5 cell lines).

Analysis of Ascorbic Acid

Ascorbic acid is present in high concentrations in humans.\textsuperscript{41} To determine whether ascorbic acid was the component in aqueous humor responsible for changes in trabecular cell protein expression, we analyzed aqueous humor ascorbic acid levels in individual donor aqueous humor (postmortem samples collected within 12 hours of death) after incubation at 37°C for 0, 24, and 48 hours (mimics culture conditions). Ascorbic acid levels at time 0 were 41 ± 12 mg/dL (mean ± SD; n = 5) in human donor aqueous humor. At 24 hours, ascorbic acid levels had decreased by 85% (6 ± 5 mg/dL; n = 5). At 48 hours, ascorbic acid levels had decreased an additional 50%, averaging only 3 ± 3 mg/dL (n = 5). This confirmed that the ascorbic acid was unstable and suggested it was not the main component in aqueous humor responsible for the cellular and molecular changes we observed. To confirm this, we incubated trabecular cells in DMEM-AH or DMEM-FBS containing fresh ascorbic acid (29 mg/dL added daily). Conditioned medium from cells incubated with DMEM-FBS and ascorbic acid did not show an induction of myocilin and hence did not reproduce the increase in myocilin that occurred when trabecular cells were incubated with DMEM-AH (Fig. 5A). Furthermore, addition of ascorbic acid to cells incubated in DMEM-AH did not increase myocilin expression when compared with cells incubated in DMEM-AH alone.

Analysis of Dexamethasone Induction of Myocilin

To verify that the increase in expression of myocilin after incubation in DMEM-AH was not the result of steroid induction due to the presence of steroids in our aqueous humor samples, we incubated trabecular cells in either DMEM-AH or DMEM-FBS and treated these cells with daily administration of 10\textsuperscript{-7} M dexamethasone over 5 days (Fig. 5B). Conditioned medium from trabecular cells incubated with DMEM-AH and treated with dexamethasone showed only a 10% increase in myocilin expression when compared with cells incubated with DMEM-AH (9.8 ± 4.7 vs. 8.9 ± 3.4, n = 3 cell lines). In contrast, myocilin levels were 442% higher in 10% FBS-conditioned medium containing dexamethasone, when compared with myocilin levels in 10% FBS (6.5 ± 3.0 vs. 1.2 ± 0.2, n = 3 cell lines).

Denaturing Aqueous Proteins

To determine whether myocilin induction results from the proteins present in aqueous humor, we incubated monolayer trabecular cells with DMEM containing heat-denatured human aqueous humor (n = 3). In the presence of heat-denatured aqueous humor, myocilin levels at day 5 increased by only 64% when compared with fresh control DMEM-AH medium (aqueous humor is known to have low levels of myocilin\textsuperscript{42,43}). In comparison, myocilin levels in an aliquot of the same aqueous humor that had not been heat-denatured increased by 261% at day 5 (Fig. 6). By day 21, myocilin expression in conditioned medium from heat-denatured DMEM-AH was reduced to DMEM-AH control levels. These results suggest that the protein component in aqueous humor is responsible for changes in myocilin expression after aqueous humor incubation.
mainly in trabecular cells incubated in DMEM-FBS. These two were visually confluent. Polansky et al.11 had noted that aqueous humor incubation, the number of trabecular cells increasing by only 11% at 21 days (Fig. 1). In contrast to also showed little proliferative activity, with the number of these changes.

Furthermore, the protein expression profile of trabecular cells incubated with DMEM-AH shows differential expression of many proteins. Our findings indicate that the proteins in aqueous humor, rather than the ascorbic acid levels, are responsible for the cell proliferation activity.

Aqueous humor has both stimulatory and inhibitory effects on growth in several tissue and cell types.44 –52 In vivo, trabecular cells displayed a broad, flat appearance in DMEM-AH when compared with the more spindle shape of cells grown in DMEM-FBS. Trabecular cells incubated in DMEM-AH also undergo little cell division, similar to in vivo trabecular cells. Furthermore, the protein expression profile of trabecular cells incubated with DMEM-AH shows differential expression of many proteins. Our findings indicate that the proteins in aqueous humor, rather than the ascorbic acid levels, are responsible for these changes.

Aqueous humor has two stimulatory and inhibitory effects on growth in several tissue and cell types.44 –52 In vivo, trabecular cells undergo little or no proliferation, actually decreasing in the number of cells throughout life.16 –21 In the present study, cultured trabecular cells incubated in aqueous humor also showed little proliferative activity, with the number of cells increasing by only 11% at 21 days (Fig. 1). In contrast to aqueous humor incubation, the number of trabecular cells increased by 141% by day 21 in DMEM-FBS, even though cells were visually confluent. Polansky et al.11 had noted that primary trabecular cells maintained in 10% FBS may lose their contact inhibition. The finding that trabecular cells proliferate differently in 10% FBS compared with 50% aqueous humor suggests that these two media supplement cause a fundamental change in trabecular cell physiology. The different supplements may also affect cytoskeletal proteins, since trabecular cells incubated in DMEM-AH became broad and flat, similar to the morphologic appearance of trabecular cells in vivo (Fig. 2). Heat denaturing the proteins in both DMEM-AH or DMEM-FBS before addition to trabecular cells verified that it was the protein component responsible for the cell proliferation activity.

In addition to cellular changes, trabecular cells also had a different protein expression profile when incubated in DMEM-AH when compared with DMEM-FBS (Fig. 3). As examples, collagen α1(I) and α1(III) precursors were expressed mainly in trabecular cells incubated in DMEM-FBS. These two proteins are integral components of collagen types I and III, respectively, and have been reported in other studies of monolayer trabecular cells.53–55 Although collagen types I and III are present in the trabecular meshwork,56 gene expression studies of human trabecular meshwork did not find collagen type I-associated transcripts and found only one clone of collagen type III,57 suggesting trabecular cells express very low levels of these proteins in vivo. We are currently performing a detailed gene and protein expression study to identify molecules that are differentially expressed between DMEM-AH- and DMEM-FBS-incubated cells.

Proteins that were secreted by trabecular cells also had different expression levels. Some had lower relative amounts in DMEM-AH-treated cells (TIMP-1; Fig. 4) while others had higher levels (myocilin, Fig. 4). The expression of myocilin from cells incubated in aqueous humor was not due to the presence of ascorbic acid, which is in high concentrations in aqueous humor, nor was it due to the presence of steroids in our donor aqueous (Fig. 5). Myocilin was expressed in aqueous humor-supplemented cells because of the types and proportion of proteins found in the aqueous humor (Fig. 6), perhaps due to either a specific protein or a combination of proteins in aqueous humor.

FBS is a rich medium containing growth factors and proteins that differ in the type and proportion from those found in aqueous humor. A 10% FBS solution contains more than six times the protein level of aqueous humor (3.2 μg/μL vs. 0.5 μg/μL). It is not surprising that cells behaved differently, with different protein expression profiles in DMEM-FBS than in DMEM-AH (Fig. 3). Changes in just a few growth factors can affect expression of genes in trabecular cells.58 Supplementation of serum-free medium with several proteins in proportions found in primary or secondary aqueous humor (TGF-β1, TGF-β2, βGF, IGF-1, and transferrin) can modulate expression of fibronectin and stromylesin-1 in trabecular monolayer cell culture. TGFβ1 or TGFβ2 can elicit significant changes in both mRNA and expressed protein such as in cytoskeletal and extracellular matrix proteins.59 Considering that single changes in protein levels can affect cell function, our results suggest that trabecular cells grown in 10% FBS medium do not have a physiologic profile that mimics trabecular cells grown in aqueous humor.

A potential limitation of our study is the use of donor aqueous humor. Although it would be ideal to use aqueous obtained during cataract surgery, it is not practical, because it would be difficult to obtain the quantity needed to perform all the experiments necessary for a study of this magnitude. Although changes in aqueous humor protein levels in postmortem aqueous have been reported,60 the use of donor aqueous humor is closer to primary aqueous than current culture methods using 10% FBS. We have analyzed all our aqueous humor samples and use only the ones with protein concentrations that are near the reported limits (12–50 mg/dL).56,59 Furthermore, aqueous is collected from eyes within 12 hours after death. This we feel reduces the chances of introducing additional proteins that may not normally be present in primary aqueous humor.

As the study of trabecular cells becomes more sophisticated with analysis of individual gene and protein expression,55,59 –61 the relevance of the results to understanding normal physiology depends on the culture condition of the cells. Maintenance of the external environment after aqueous incubation increases the chances that trabecular cells cultured in aqueous humor will maintain a more physiologic profile of expressed proteins. Because trabecular cells undergo minimal proliferation in aqueous humor, all cultures must still be initiated in 10% FBS for cell expansion. Before we performed the experiments, however, our data suggested that cells should be switched to aqueous humor.
humor, enabling them to acclimate to the aqueous environment. Further analysis will define the time needed to transform trabecular cells from a 10% FBS environment to one with aqueous humor. Our data suggest trabecular cells change their phenotype in as few as 5 days.

Several interesting questions remain to be answered. How do the protein expression changes in primary trabecular cell cultures incubated in aqueous humor compare with protein expression profiles of noncultured fresh trabecular cells? What factors in aqueous humor are important for maintaining trabecular cells in a homeostatic environment? Currently, only approximately 80 proteins have been reported to be present in aqueous humor. Delineation of the proteome within aqueous humor as well as the individual protein concentrations in aqueous humor and 10% FBS will be important for identifying candidate proteins that may be essential for trabecular cell homeostasis and for development of a defined medium that can mimic aqueous humor in vitro.

References