Na-K-Cl Cotransport in Normal and Glaucomatous Human Trabecular Meshwork Cells

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PURPOSE. Previous results from this laboratory showed that intracellular volume of trabecular meshwork (TM) cells is regulated by the Na-K-Cl cotransport system. Other studies suggest that TM cell volume, in turn, is a determinant of permeability across the TM. Given that a decrease in outflow facility across the TM is thought to be the primary cause of elevated intraocular pressure in primary open-angle glaucoma, the present study was conducted to investigate the possibility that Na-K-Cl cotransport function may be altered in glaucomatous TM cells compared with normal TM cells.

METHODS. Normal and glaucomatous human TM cells were cultured from donor eyes and trabeculectomy specimens, respectively. Trabecular meshwork cell monolayers were evaluated for Na-K-Cl cotransport activity, assessed as ouabain-insensitive, bumetanide-sensitive K influx using 86Rb as a tracer for K. Cotransporter protein expression was determined by western blot analysis, and intracellular volume was determined radioisotopically using [14C]urea and [14C]sucrose as markers of total and extracellular water space, respectively.

RESULTS. Na-K-Cl cotransport activity of glaucomatous TM cells was found to be reduced by 32% ± 2% compared with that of normal TM cells, whereas western blot analyses showed that cotransporter protein expression in glaucomatous TM cells was reduced by 64% ± 14% compared with expression in normal TM cells. Also, exposure of normal TM cells to 10 μM norepinephrine or 50 μM 8-bromo-3′,5′-cyclic adenosine monophosphate was found to diminish Na-K-Cl cotransport activity, whereas these agents were without effect on glaucomatous TM cell cotransport. Finally, resting cell volume of glaucomatous TM cells was found to be increased compared with that of normal TM cells, whereas intracellular volume of both cell types was reduced after exposure to 10 μM benzametanide or 10 μM bumetanide.

CONCLUSIONS. These findings indicate that Na-K-Cl cotransport function and regulation are altered in glaucomatous TM cells compared with that of normal TM cells. However, the observation that cell volume of glaucomatous TM cells is greater than that of normal TM cells, despite reduced Na-K-Cl cotransport activity, suggests that other volume-regulatory ion flux pathways may be involved in the reduced outflow of glaucoma. (Invest Ophthalmol Vis Sci. 1999;40:425-434)
involves increasing the extracellular space for aqueous outflow through changes in both TM cell shape and volume. In this regard, we and others have hypothesized that modulation of TM cell volume is one means by which aqueous outflow facility of the TM is regulated.\(^8\)\(^{-12}\) Previous studies from this laboratory have shown that the Na-K-Cl cotransporter of cultured TM cells plays a central role in regulating intracellular volume of the cells,\(^8\) thus, we have also hypothesized that changes in activity of this cotransport system may influence outflow facility.

The Na-K-Cl cotransporter is a plasma membrane protein that participates in vectorial transport of Na and Cl across epithelia and also regulates intracellular volume of a variety of cell types, epithelial and nonepithelial.\(^13\)\(^{-15}\) The cotransporter is an obligate symporter, requiring the presence of all three ion species, Na, K, and Cl, to operate and is specifically inhibited by "loop" diuretics, such as bumetanide and benzmetanide. Our previous studies have shown that cultured TM cells exhibit a high level of Na-K-Cl cotransporter protein expression and robust cotransport activity and that the cotransporter regulates volume of these cells in two ways: (1) it contributes to maintenance of steady state volume under basal, isotonic conditions; and (2) it mediates volume recovery after hypertonicity-induced cell shrinkage.\(^6\)\(^{,16}\) Regarding the latter, when TM cells are shrunk by exposure to hypertonic media, the Na-K-Cl cotransporter is rapidly activated to bring Na, K, and Cl into the cell. As osmotically obliged water follows the ions, intracellular volume is increased to normal levels, and cotransporter activity then decreases to normal levels as well. In this manner, the cotransporter mediates what is known as a "regulatory volume increase" in the TM cells.\(^6\)\(^,16\) Regarding maintenance of steady state cell volume, when TM cells in normal isotonic medium are exposed to bumetanide to inhibit Na-K-Cl cotransport activity, the cells shrink.\(^6\) This indicates that cotransporter activity is required to maintain TM intracellular volume, even under isotonic conditions, most likely by offsetting Na, K, and Cl efflux pathways (such as K and Cl channels\(^16\)\(^^{-17}\) and K-Cl cotransport\(^17\)\(^,18\)).

Studies from this laboratory have shown that the permeability of cultured TM cell monolayers to [\(^{14}\)C]sucrose is modulated by changes in Na-K-Cl cotransport activity and intracellular volume. That is, inhibition of cotransport activity, which decreases TM cell volume, increases movement of [\(^{14}\)C]sucrose across TM cell monolayers cultured on permeable supports. Permeation of [\(^{14}\)C]sucrose across the monolayer is also increased by hypertonicity-induced TM cell shrinkage.\(^6\) These findings led us to hypothesize that the Na-K-Cl cotransporter may contribute to regulation of TM barrier function through modulation of TM cell volume and consequent changes in the extracellular space available for bulk flow of aqueous humor through the tissue. A role for Na-K-Cl cotransport and TM cell volume in modulating outflow facility in the intact eye is supported by studies using perfused anterior chamber preparations. In these studies, using bovine\(^19\)\(^,20\) and human\(^10\)\(^,12\) anterior segments Al-Aswad et al.\(^10\) and Gual et al.\(^12\) found that perfusion of the preparations with hypotonic medium, which swells TM cells, decreased outflow facility. Conversely, perfusion with hypertonic medium (which transiently shrinks TM cells) increased outflow facility,\(^10\)\(^,12\) as did Cl-free medium or bumetanide (both of which inhibit Na-K-Cl cotransport and cause sustained shrinkage of TM cells).\(^10\)\(^,11\) In another study, Gabelt et al.\(^19\) found that bumetanide had no effect on outflow facility in the monkey eye or on intraocular pressure of organ-cultured human eyes.

If Na-K-Cl cotransport activity and TM cell volume are important determinants of TM outflow facility, it is possible that a defect in function or regulation of the Na-K-Cl cotransporter contributes to the decreased outflow facility observed in glaucoma. As an initial approach to evaluating this possibility, the present study was conducted to determine whether Na-K-Cl cotransport function is altered in glaucomatous TM cells compared with normal TM cells. Specifically, we examined Na-K-Cl cotransport activity, its responsiveness to hormonal regulation, and the level of cotransporter protein expressed among normal and glaucomatous TM cell cultures. In addition, intracellular volume of normal and glaucomatous human TM cells was assessed, as were the effects of Na-K-Cl cotransporter inhibitors on intracellular volume.

### METHODS

#### TM Cell Isolation and Culture

Human TM cells were isolated by methods based on those of Polansky et al.\(^20\) For normal human TM cultures, cells were obtained from TM explants excised from eye bank donor rims using direct microscopic visualization immediately after corneal transplantation. Postmortem donor screening by eye bank personnel excluded any donors with a known history of glaucoma; however, it is possible that some of the presumably normal donors had undiagnosed glaucoma. Glaucomatous human TM cultured cells were obtained from patients undergoing primary trabeculectomy for medically uncontrolled POAG. Patients with secondary open-angle glaucoma (i.e., pseudoxefolution, pigmented, steroid-induced) were excluded, as were patients who had undergone cataract surgery or laser trabeculoplasty. Tenets of the Declaration of Helsinki were followed, informed consent was obtained, and approval was granted for these studies by the University of California Davis Human Subjects Review Committee. Trabecular meshwork cells were grown from the primary trabeculectomy specimens excised at surgery and were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 5% bovine calf serum, essential and nonessential amino acids, penicillin/streptomycin, and l-glutamine. Cells were maintained in collagen-coated 75-cm\(^2\) tissue culture flasks, and cells from passages 2 through 6 were used.

For experiments, TM cells were removed from flasks by brief trypsinization and were subcultured onto collagen-coated 24-well plates (transport and intracellular volume assays) or onto additional collagen-coated 75-cm\(^2\) tissue culture flasks (western blot analyses). For culturing in stock flasks and 24-well plates, all cells were maintained in a 95% air/5% carbon dioxide atmosphere.

#### Transport Measurements

Na-K-Cl cotransport activity was measured as ouabain-insensitive, bumetanide-sensitive K influx, using \(^{86}\)Rb as a tracer for K. Details of this method have been published previously.\(^9\)\(^,21\) Human TM cell monolayers on 24-well plates were equilibrated in an air atmosphere for 60 minutes at 37°C in a HEPES-buffered minimal essential medium (MEM) containing (in millimoles per liter): 133 Na, 136 Cl, 5.8 K, 1.2 Ca, 4.2 HCO\(_3\), 0.4 H\(_2\)PO\(_4\), 0.4 Mg, 0.4 SO\(_4\), 5.6 glucose, and 20 HEPES (pH 7.4).
The cells were preincubated for 5 minutes with HEPES-buffered MEM containing 1 mM or 0 mM ouabain and 10 μM or 0 μM bumetanide (in some experiments, MEM contained bumetanide in concentrations ranging from 0.01 μM to 100 μM). Cotransport activity was assayed by replacing the preincubation medium with identical fresh medium containing 1 μCi/ml [32P]orthophosphate (8-Br-cAMP). The assay was terminated by aspirating and rinsing the wells with ice-cold isotonic MgCl₂ to remove extracellular radioactivity. The contents of the wells were then extracted with 1% sodium dodecyl sulfate (SDS) and the amount of radioactivity present determined by liquid scintillation (Tri-Carb model 2500 TR; Packard Instruments, Downers Grove, IL). Osmolarities of all preincubation and assay media were verified by osmometry (model 3W2; Advanced Instruments, Norwood, MA). Samples of the SDS extracts were also used to determine protein content by the biocinchoninic acid method. The K influx data are expressed as micromoles of K per gram protein per minute. K influx values are normalized to the amount of protein rather than the number of cells, because proteins can readily be determined for each radiactive sample. It is important to note, however, that in these studies, we also assessed the amount of protein per cell for the normal and glaucomatous TM cells and found no significant differences between protein-per-cell values in the cultured cells. In the present study, Na-K-Cl cotransport activity was determined as ouabain-insensitive, bumetanide-sensitive K influx: (total K flux in presence of ouabain) — (K flux in presence of 5% CO₂). Cotransport proteins of a variety of mammalian cells readily recognize Na-K-Cl cotransport protein, and the T4 antibody readily recognizes Na-K-Cl cotransport protein in this study.

**Gel Electrophoresis and Western Blot Analysis**

Human TM cell monolayers were washed with ice-cold phosphate-buffered saline (PBS)/2 mM EDTA (pH 7.4) containing the following protease inhibitors: 20 μg/ml phenylmethylsulfonyl fluoride, 88 μg/ml N-tosyl-l-phenylalanine chloromethyl ketone, 1 μg/ml pepstatin A, 2 μg/ml chymostatin, 1.25 μg/ml leupeptin, 6.3 U/ml aprotinin, and 12.5 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride. Cells then were scraped from dishes and centrifuged at 7500g for 10 minutes at 4°C. The cell pellet was resuspended in 0.2 ml to 1 ml protease inhibitor-containing PBS-EDTA, and the suspension was passed through a 1-ml, 26-gauge 3/8 syringe several times. For freshly isolated normal human TM samples, TM was excised from eye bank donor rims using direct microscopic visualization immediately after corneal transplantation and suspended in protease inhibitor-containing PBS-EDTA. Suspensions of cultured TM cells and freshly isolated TM were then sonicated for 30 seconds at 4°C (XIL2020 sonicator; Heat Systems, Farmingdale, NY) and cellular debris removed by brief centrifugation at 9500g and 4°C. Protein contents of these whole-cell lysates were determined by the BCA method before electrophoresis.

Cell lysates and prestained molecular mass markers (Bio-Rad, Hercules, CA) were denatured in SDS reducing buffer (9.2% SDS, 5% β-mercaptoethanol, 50 mM Tris/HCl [pH 7.4], 35% sucrose, and 0.012% bromophenol blue) and heated at 100°C for 3 minutes. The samples were separated by polyacrylamide gel electrophoresis using gels containing 8% polyacrylamide (Mini-Protean II; Bio-Rad). Equivalent amounts of total cell lysate proteins were added to each lane of the gel. Samples were electrophoresed at 150 V for 1 hour and the resolved proteins transferred electrophoretically to polyvinylidene fluoride (0.45 μm; Millipore, Bedford, MA) for 18 hours (30 V, 4°C). The blots were incubated in 7.5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 hour at room temperature, rinsed three times with PBS-T, and incubated for 1 hour at room temperature with primary antibody (T4 monoclonal antibody, 1:10,000 dilution), rinsed again three times with PBS-T, and incubated for 1 hour at room temperature with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG, 1:3000 dilution). After five washes in PBS-T to remove unbound secondary antibody, bound antibody was visualized by enhanced chemiluminescence assay (ECL; Amersham, Arlington Heights, IL). Quantitation of cotransporter protein present in each lane of the western blot shown in Figure 4A was done by densitometric analysis, using a digital imaging system (model IS-1000; Innotec Scientific, San Leandro, CA), where the area measured per sample was held constant.

The T4 monoclonal antibody was prepared by Chris Lytle, University of California, Riverside. The T4 antibody, generated against a highly conserved region in the carboxyl terminus of the human colonic T84 epithelial cell Na-K-Cl cotransport protein, readily recognizes Na-K-Cl cotransport proteins of a variety of mammalian cells. Details of methods using the T4 antibody for western blot analysis have been published previously by O’Donnell et al. and Sun et al.

**Intracellular Volume Measurements**

Trabecular meshwork cell volume was determined by radioisotopic evaluation of TM monolayer intracellular water space.
using $[^{14}]$Curea and $[^{14}]$Csucrose as markers of total and extracellular space, respectively. Details of this method have been published previously.$^{8,9}$ Human TM cell monolayers on 24-well plates were equilibrated in an air atmosphere for 60 minutes at 37°C in HEPES-buffered MEM. The cells were preincubated for 65 minutes at 37°C in HEPES-buffered MEM containing 10 μM or 0 μM bumetanide or for 5 minutes in HEPES-buffered MEM containing 10 μM or 0 μM benzmetanide. To assess intracellular volume, the cells were then incubated at 37°C for 10 minutes in the same media containing either $[^{14}]$Curea or $[^{14}]$Csucrose (both at 1 μCi/ml). We have found that $[^{14}]$Curea and $[^{14}]$Csucrose equilibrate by 5 minutes in normal and glaucomatous TM cells (data not shown). After the period of incubation with the radioisotopic marker, the monolayers were rinsed with isotonic ice-cold MgCl2 and 5 minutes in well plates were equilibrated in an air atmosphere for 60 minutes at 37°C for 10 minutes in the same media containing either $[^{14}]$Curea or $[^{14}]$Csucrose (both at 1 μCi/ml). We have found that $[^{14}]$Curea and $[^{14}]$Csucrose equilibrate by 5 minutes in normal and glaucomatous TM cells (data not shown). After the period of incubation with the radioisotopic marker, the monolayers were rinsed with isotonic ice-cold MgCl2 and the radioactivity of SDS extracts determined by liquid scintillation.

Specific activities (in counts per minute per milligram) of $[^{14}]$Curea and $[^{14}]$Csucrose in the assay media were determined and used to calculate intracellular water space (expressed as milliliters per milligram protein). Intracellular volume was calculated as the difference between water space determined for $[^{14}]$Curea (a marker for intracellular plus trapped extracellular space) and $[^{14}]$Csucrose (a marker for trapped extracellular space only).

To determine whether values for TM cell volume might differ in assay media containing low HCO3/5% CO2 versus high HCO3/5% CO2, we conducted a series of intracellular volume assays in media containing 26 mM HCO3 equilibrated with 5% CO2 versus 4.2 mM HCO3 equilibrated with atmospheric CO2, as described in the Methods section. We found no significant difference when normal TM cells (5-year-old donor) were assayed in the low-versus high-HCO3 media, (4.52 ± 0.18 μl/mg protein and 4.58 ± 0.17 μl/mg protein, respectively; n = 10 for each condition). We also found no significant difference in the extent to which bumetanide reduced TM cell volume under these conditions (data not shown; effects of bumetanide on cell volume are presented in the Results section and in Fig. 8).

Materials

Bumetanide was purchased from ICN Pharmaceuticals (Costa Mesa, CA). $^{86}$Rb from Du Pont–New England Nuclear (Boston, MA), norepinephrine and 8-Br-cAMP from Sigma (St. Louis, MO), Eagle's minimal essential medium from JRH Biosciences (Lenexa, KS), fetal bovine serum and calf serum from Hyclone (Logan, UT), and collagen type I from Collaborative Research (Bedford, MA). Benzmetanide was a gift from Merck (West Point, PA). Statistical analyses were performed using unpaired Student's t-tests.

RESULTS

Expression of Na-K-Cl Cotransport Protein in Normal Human Cultured TM Cells and Freshly Isolated Human TM

Studies in our laboratory have shown that the Na-K-Cl cotransporter protein is highly active and abundantly expressed in cultured TM cells.$^{8,9}$ It has been suggested that expression of Na-K-Cl cotransporter protein may be culturing-induced in certain cell types.$^{27}$ To determine whether the cotransporter is present in intact TM and not just in cultured TM cells, we evaluated Na-K-Cl cotransporter protein expression in normal human cultured TM cells and freshly isolated whole human TM. Cultured TM cells and whole TM were subjected to western blot analysis of Na-K-Cl cotransporter protein using T4 monoclonal antibody. Cellular proteins (15 μg loaded in each lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and probed with T4 monoclonal antibody, as described in the Methods section. The blot was visualized by enhanced chemiluminescence. The Na-K-Cl cotransporter protein was expressed in freshly isolated and cultured TM cells and was the same molecular size, approximately 170 kDa, in both samples. Data are from a representative experiment. Whole TM was isolated from a 66-year-old normal donor rim, as described in the Methods section, whereas the TM cell culture used in this experiment was from a 2-year-old normal donor.

Figure 1. Na-K-Cl cotransporter protein expression in normal human cultured TM cells and freshly isolated whole human TM. Cultured TM cells and whole TM were subjected to western blot analysis of Na-K-Cl cotransporter protein using T4 monoclonal antibody. Cellular proteins (15 μg loaded in each lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and probed with T4 monoclonal antibody, as described in the Methods section. The blot was visualized by enhanced chemiluminescence. The Na-K-Cl cotransporter protein was expressed in freshly isolated and cultured TM cells and was the same molecular size, approximately 170 kDa, in both samples. Data are from a representative experiment. Whole TM was isolated from a 66-year-old normal donor rim, as described in the Methods section, whereas the TM cell culture used in this experiment was from a 2-year-old normal donor.

Na-K-Cl Cotransport Activity in Normal and Glaucomatous Human TM Cells

To evaluate the possibility that TM cell Na-K-Cl cotransport function may differ in some manner between glaucomatous and normal eyes, we first examined the activity of the cotransporter in TM cells cultured from human donors with or without glaucoma (Fig. 2). When two representative age-matched TM cell cultures were evaluated (Fig. 2A), we found that Na-K-Cl cotransport activity (assessed as bumetanide-sensitive K influx) was present in normal and glaucomatous TM cells but...
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TM cells cultured from a 48-year-old donor exhibited a total K influx of 48.95 ± 1.75 micromoles K/g protein per minute, which was decreased by 10 μM bumetanide to 37.08 ± 2.03 micromoles K/g protein per minute, the difference being bumetanide-sensitive Na-K-Cl cotransport activity. Exposure of these cells to 1 mM ouabain rather than bumetanide reduced K influx to 17.47 ± 0.30 micromoles K/g protein per minute, this reduction representing ouabain-sensitive K influx or Na/K pump activity. Exposure of normal TM cells to ouabain and bumetanide reduced the K influx further to 4.20 ± 0.16 micromoles K/g protein per minute. The difference between K influx in cells treated with ouabain and ouabain plus bumetanide also represents Na-K-Cl cotransport activity, but with a Na/K pump inhibited. In the normal TM cells shown in Figure 2A, bumetanide-sensitive K influxes of 11.87 ± 1.75 micromoles K/g protein per minute and 13.27 ± 0.30 micromoles K/g protein per minute were observed in the absence and presence of ouabain, respectively. In glaucomatous TM cells, also cultured from a 48-year-old donor, total K influx was 36.6 ± 1.45 micromoles K/g protein per minute, a value significantly lower than that of the normal TM cells. Exposure of the cells to bumetanide reduced K influx to 28.22 ± 1.00 micromoles K/g protein per minute. In the presence of ouabain, bumetanide reduced K influx from 11.21 ± 0.58 micromoles K/g protein per minute to 3.45 ± 0.13 micromoles K/g protein per minute. Thus, bumetanide-sensitive K influxes of 8.38 ± 1.45 and 7.76 ± 0.58 micromoles K/g protein per minute were observed in the glaucomatous TM cells in the absence and presence of ouabain, respectively. These findings indicate that in these age-matched TM cells, cotransport activity is 30% to 40% reduced in the glaucomatous TM cells. In these experiments we also found an 8% to 12% reduction in Na/K pump activity, in the glaucomatous TM cells compared with that in the normal TM cells. Although we did not observe a significant difference in Na-K-Cl cotransport activity values measured in the presence versus absence of ouabain, to avoid the possibility that Na/K pump activity could influence the activity of the cotransporter under some conditions, in all subsequent experiments of the present study cotransport activity was assessed in the presence of ouabain (i.e., as ouabain-insensitive, bumetanide-sensitive K influx).

Mean cotransport values (ouabain-insensitive, bumetanide-sensitive K influx) for seven normal TM cell cultures (donor ages, 38–68 years) and seven glaucomatous TM cell cultures (donor ages, 41–75 years) are shown in Figure 2B. We found Na-K-Cl cotransport activities of 10.65 ± 0.15 micromoles K/g protein per minute and 7.97 ± 0.21 micromoles K/g protein per minute, respectively, in normal versus glaucomatous TM cell cultures. The total number of normal TM cell cultures available ranged in donor age from 2 to 68 years (17 normal TM cultures), however, in the studies depicted in Figure 2A we did not use normal TM cell cultures from donors younger than 38 years to age-match the cultures as closely as possible with the available glaucomatous TM cell cultures (41–75 years). To determine whether cotransport activity levels of the cultured cells vary with donor age, we examined cotransport activities of cells derived from donors of various ages. We found no significant effect of donor age on Na-K-Cl cotransport activity in either the normal or glaucomatous TM cell cultures. Further, glaucomatous TM cell cotransport activity was reduced by approximately 25%, whether compared with approximate age-matched normal samples or with the expanded normal TM cell culture donor age group.
Normal K\textsubscript{i} = 0.24 \mu M
Glaucoma K\textsubscript{i} = 0.19 \mu M

**FIGURE 3.** The effect of bumetanide concentration on K influx in TM cells was assessed as described in the Methods section. Trabecular meshwork cells were preincubated for 5 minutes in a HEPES-buffered medium containing 0.01 \mu M to 100 \mu M bumetanide in the presence of 1 mM ouabain, followed by a 5-minute incubation in media of identical composition but also containing \textsuperscript{86}Rb. Similar constants for bumetanide inhibition (K\textsubscript{i}) were determined for the normal and glaucomatous TM cell transporters. Data are mean values ± SEM \textit{n} = 4, one representative experiment. Two additional experiments were conducted on normal TM cell cultures from 3- and 68-year-old donors and produced similar results. Results were fitted by a model of inhibition at a single site, where the K influx value of 0 \mu M bumetanide was not significantly different from that of 0.01 \mu M bumetanide. Cell cultures used in these studies were from 48-year-old donors.

To test whether the observed reduction of cotransport activity in glaucomatous TM cells could be due to an altered bumetanide sensitivity, we evaluated the effects of varying bumetanide concentration on K influx in normal and glaucomatous TM cells. Bumetanide sensitivities of the Na-K-Cl cotransporter protein in normal and glaucomatous TM cells cultured from 48-year-old donors were nearly identical (Fig. 3). We determined K\textsubscript{i} values of 0.24 \mu M and 0.19 \mu M for bumetanide inhibition of K influx in the normal and glaucomatous TM cells, respectively. Assessment of bumetanide potency for K influx inhibition in two other normal cell cultures yielded K\textsubscript{i} values of 0.16 \mu M and 0.15 \mu M for TM cells cultured from 3-year-old and 68-year-old donors, respectively (data not shown). These bumetanide sensitivities are similar to those reported in a number of other cell types, including vascular endothelial cells.15-21

**FIGURE 4.** Na-K-Cl cotransporter protein expression and activity in normal and glaucomatous human TM cells. (A) Normal and glaucomatous human TM cells were subjected to western blot analysis of Na-K-Cl cotransporter protein using T4 monoclonal antibody. Cellular proteins (20 \mu g loaded in each lane) were separated by sodium dodecyt sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and probed with T4 monoclonal antibody, as described in the Methods section. The blot was visualized by enhanced chemiluminescence. Na-K-Cl cotransporter protein expression was markedly reduced in glaucomatous TM cells compared with expression in normal TM cells. Data are from a representative experiment; two other experiments produced similar results. Cell cultures used in these studies were from normal donors aged 48, 55, 60, and 68 years and from glaucoma donors aged 48, 68, 72, and 75 years. (B) Bumetanide-sensitive K influx of the same TM cell cultures used for western blot analyses shown in (A) was assessed as described in the Methods section. The blot was visualized by enhanced chemiluminescence. Na-K-Cl cotransporter protein expression was markedly reduced in glaucomatous TM cells compared with expression in normal TM cells. Data are from a representative experiment; two other experiments produced similar results. Cell cultures used in these studies were from normal donors aged 48, 55, 60, and 68 years and from glaucoma donors aged 48, 68, 72, and 75 years. The most conservative significant difference between each glaucomatous TM cell culture and any of the normal TM cell cultures: *P < 0.05 and **P < 0.001 by Student’s \textit{t}-test.

**Expression of Na-K-Cl Cotransport Protein in Normal and Glaucomatous Human TM Cells**

In these studies, we also evaluated the level of Na-K-Cl cotransporter protein present in normal and glaucomatous TM cell cultures, using western blot analysis and the T4 monoclonal antibody. A representative western blot showing the levels of cotransporter protein in four normal TM cell cultures (donor ages, 48-68 years) and four glaucomatous TM cell cultures (donor ages 48-75 years) is shown in Figure 4A. We found that cotransporter protein expression was significantly reduced in all the glaucomatous TM cell cultures when compared with expression in any of the normal TM cell cultures. It is important to note that we loaded the same amount of cell lysate protein per sample and also that we found no significant difference in the amount of protein per cell in the normal and glaucomatous TM cell cultures (as described in the Methods
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To investigate this possibility, we measured intracellular cAMP concentrations using two normal TM cell cultures (18- and 48-year-old donors) and three glaucomatous TM cell cultures (41-, 48-, and 57-year-old donors) and an enzyme immunoassay for cAMP (Cayman Chemical, Ann Arbor, MI), we found no significant difference between cAMP levels in normal versus glaucomatous TM cells; 5.29 ± 1.18 pmoles/mg protein and 6.29 ± 0.98 pmoles/mg protein in normal (n = 8) and glaucomatous (n = 11) TM cells, respectively. This finding suggests that the absence of glaucomatous TM cell cotransporter responsiveness to cAMP or norepinephrine is not caused by elevated basal cAMP levels in these cells.

Intracellular Volume of Normal and Glaucomatous Human TM Cells

In the present study, we also examined the resting intracellular volume of normal and glaucomatous TM cells, to investigate the possibility that glaucomatous TM cells rest at an elevated intracellular volume, which may contribute to the increased outflow resistance observed in POAG. Despite the reduction of Na-K-Cl cotransport activity in glaucomatous versus normal TM cells, we found that the glaucomatous TM cells exhibited a significantly greater intracellular volume than the normal TM cells (Fig. 6A). In these experiments, the resting intracellular volume of normal TM cells (i.e., the volume in the absence of any hormonal or tonicity challenges) was 4.65 ± 0.28 μl/mg protein (mean ± SEM), whereas the intracellular volume of glaucomatous TM cells was 7.10 ± 0.35 μl/mg protein. The mean values (± SEM) for water space associated with urea and sucrose in the normal TM cells were 5.41 ± 0.29 μl/mg protein and 0.76 ± 0.05 μl/mg protein, respectively, whereas the mean values (± SEM) for water space associated with urea and sucrose in the glaucomatous TM cells were 8.29 ± 0.35 μl/mg protein. The mean values (± SEM) for water space associated with urea and sucrose in the normal TM cells were 5.41 ± 0.29 μl/mg protein and 0.76 ± 0.05 μl/mg protein, respectively, whereas the mean values (± SEM) for water space associated with urea and sucrose in the glaucomatous TM cells were 8.29 ± 0.35 μl/mg protein.
The intracellular volume of TM cells was assessed radioisotopically, as described in the Methods section. Trabecular meshwork cells were assayed for 10 minutes in an isotonic HEPES-buffered medium containing \(^{14}\text{C}\)urea or \(^{14}\text{C}\)sucrose. Intracellular volume was calculated as the difference between water space determined for cells but is still sensitive to inhibition by benzmetanide or bumetanide. The cells then were assayed for 10 minutes in the same medium containing either \(^{14}\text{C}\)urea (a marker for intracellular plus trapped extracellular space) and \(^{14}\text{C}\)sucrose (a marker for trapped extracellular space). Intracellular volume of glaucomatous TM cells was significantly increased compared with that of normal TM cells. Data are mean values ± SEM in three normal cultures (donor ages, 48, 55, and 60 years; \(n = 12\) in each cell culture) and in three glaucomatous cultures (donor ages, 48, 75, and 75 years; \(n = 12\) in each cell culture). *Significant differences at \(P < 0.001\) by Student’s \(t\) test. (B) Relative changes in intracellular volume of normal and glaucomatous TM cells after exposing the cells to the Na-K-Cl cotransport inhibitors benzmetanide or bumetanide are shown in Figure 6B. We found that whether cells were exposed to 10 \(\mu\text{M}\) benzmetanide for 5 minutes or 10 \(\mu\text{M}\) bumetanide for 65 minutes, intracellular volume of normal and glaucomatous TM cells was significantly reduced. This suggests that activity of the cotransporter does indeed contribute to maintenance of intracellular volume in the glaucomatous TM cells as it does in the normal TM cells and further, that the resting intracellular volume of glaucomatous TM cells, although elevated, is still sensitive to reduction by inhibition of Na-K-Cl cotransport activity.

**DISCUSSION**

The results of the present study indicate that Na-K-Cl cotransport activity and cotransporter protein levels are reduced in cultered glaucomatous human TM cells compared with levels in cultured normal human TM cells. In addition, our findings indicate that whereas norepinephrine and cAMP inhibit Na-K-Cl cotransport activity in normal TM cells, the Na-K-Cl cotransporter of glaucomatous TM cells is insensitive to inhibition by these agents. Further, the resting cell volume of the glaucomatous TM cells is elevated compared with normal TM cells but is still sensitive to inhibition of Na-K-Cl cotransport activity. Collectively, these findings suggest that the function and regulation of the Na-K-Cl cotransporter is altered in glaucomatous TM cells.

Our studies show that the reduction in cotransporter protein of glaucomatous versus normal TM cells is much greater than the observed reduction in cotransport activity. Although the reason for this is unclear, there are some possibilities worth noting. The first possibility is that although the glaucomatous TM cells may have less total cotransporter protein, they could express a larger fraction of their cotransporter protein at the cell surface than the normal TM cells. For example, although the monoclonal antibody used in these studies can detect cotransporter protein in intracellular membrane compartments and in plasma membrane, only cell surface–residing cotransporter protein can contribute to bumetanide-sensitive K influx. A second possibility is that glaucomatous TM cells express another isoform of the cotransporter that is not recognized by the monoclonal antibody but that is nevertheless active in K flux, resulting in an altered activity-to-protein ratio in these cells. There are no obvious differences in the cotransporter proteins of normal and glaucomatous TM cells (at least in bumetanide sensitivity and size of the protein). However, this does not rule out the possibility that a variant isoform of the Na-K-Cl cotransporter, which behaves differently than the normal TM cell cotransporter, is indeed present in the glaucomatous TM cells.
The findings of the present study regarding cotransporter activity and protein expression in glaucomatous TM cells are similar to previous observations from this laboratory that dexamethasone, known to induce glaucoma in susceptible patients and to cause increased resistance to aqueous outflow across the TM, also causes a reduction in Na-K-Cl cotransporter protein expression in cultured bovine and human TM cells after 12 days' exposure. In these studies, the decrease in cotransporter protein expression was not accompanied by a decrease in cotransport activity, suggesting that spontaneous glaucoma and steroid-induced glaucoma may involve changes in expression and function of the Na-K-Cl cotransporter.

In the present study, we also found that Na-K-Cl cotransport activity of the glaucomatous TM cells exhibited a reduced responsiveness to inhibition by norepinephrine and cAMP. Our previous studies have shown that permeable cAMP analogues and norepinephrine, acting through cAMP, reduce the activity of the Na-K-Cl cotransporter in bovine TM cells and that cAMP reduces intracellular volume in bovine TM cells. This suggests the possibility that if the cotransporter does not respond to agents that normally reduce cotransport activity and TM cell volume, then changes in aqueous outflow facility may not occur as needed to regulate intraocular pressure appropriately.

Our finding that the intracellular volume of cultured glaucomatous TM cells was significantly greater than that of normal TM cells is consistent with the increased resistance to aqueous outflow observed in glaucoma. That increased TM intracellular volume may diminish outflow is supported by studies which show that perfusion of anterior chamber segments with hypotonic solutions (which swells TM cells) results in decreased outflow facility. Base on the observed reduction in glaucomatous TM cell cotransport activity, one might predict that intracellular volume of the cells would also be reduced, rather than increased. However, it is important to recognize that intracellular volume is not determined by Na-K-Cl cotransport activity alone but rather by the combined activities of ion influx and efflux pathways, with the steady state volume dictated by the sum of these pathways. The efflux pathways involved in regulating intracellular volume of TM cells have yet to be elucidated; however, in other cell types these include K-Cl cotransport and/or K and Cl channels. Studies of volume regulation in a wide variety of cells have shown that a volume-sensing mechanism, which to date is not well understood, regulates activity of the net influx and efflux pathways to maintain the cell at a volume set point. In the glaucomatous TM cells, the elevated intracellular volume could be caused by altered function of other ionic influx pathway(s), such as Na/H exchange and Cl/HCO₃ exchange or altered function of ionic efflux pathway(s), or both.

Despite the elevated intracellular volume of glaucomatous TM cells compared with that in normal TM cells, both cell types exhibited reductions in volume after exposure to bumetanide or benzametanide. This indicates that although the glaucomatous TM cotransporter is reduced in activity and protein expression and may not even be under normal regulatory influences, its inhibition by these agents nevertheless results in diminished intracellular volume, just as occurs in normal TM cells. In fact, the same relative decreases in volume occurred in the normal versus glaucomatous TM cells on exposure to bumetanide or benzametanide. Thus, inhibition of cotransport activity by bumetanide and its analogues, and subsequent TM cell shrinkage, may be of therapeutic value in mediating an increase in outflow facility and reducing intraocular pressure in POAG.

It is possible that the changes in Na-K-Cl cotransporter function do not account for the primary defect in glaucoma but rather occur secondary to another defect. For example, our studies also show that Na/K pump activity is decreased in glaucomatous TM cells, which could account for the elevated intracellular volume of these cells. This could, in turn, cause a downregulation of Na-K-Cl cotransporter activity and protein expression. Clearly, identification of the primary defect in POAG, if in fact there is a single defect, awaits further investigation. It is important to note, however, that the finding of elevated intracellular volume in glaucomatous TM cells indicates that the cells are simply not volume-regulating normally. If they were, an increase in cell volume would have altered volume-regulating ion influx and efflux pathways until cell volume was brought back to the set point. A sustained elevated volume could be caused by inappropriately high ion influx, inappropriately low ion efflux, or both.

A full understanding of the role of TM cell volume regulation in the modulation of aqueous outflow across the TM requires further investigation. Our previous studies using cultured bovine and human TM cells have documented that the Na-K-Cl cotransport system is critical for the maintenance and regulation of TM cell volume. Gual et al. and Al-Aswad et al. have shown that conditions which increase TM cell volume also decrease outflow facility in anterior chamber preparations, whereas those that decrease TM cell volume increase outflow facility. A specific role for Na-K-Cl cotransport-mediated cell volume changes in modulating aqueous outflow facility is supported by the observation that perfusion of anterior chamber preparations with either isotonic Cl-free medium or bumetanide (both of which inhibit the Na-K-Cl cotransporter and shrink TM cells) increases outflow facility. However, in another study, Gabelt et al. did not find an effect of bumetanide on outflow facility either in the monkey eye or on intraocular pressure of organ-cultured human eyes. The reason for this apparent discrepancy is unclear and awaits further investigation. Whether changes in cell shape, volume, or both do indeed provide major contributions to changes in outflow facility in health and/or disease is the subject of ongoing investigations in this and other laboratories.

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