The effect of C3 transgene expression on actin and cellular adhesions in cultured human trabecular meshwork cells and on outflow facility in organ cultured monkey eyes

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Purpose: To determine the effects of adenovirus-delivered exoenzyme C3 transferase (C3) gene expression on cultured human trabecular meshwork (HTM) cells and on outflow facility in organ cultured monkey anterior segments.

Methods: An adenoviral (Ad) vector expressing both C3 and green fluorescent protein (GFP) was used to transduce cultured HTM cells. Changes in cell morphology and the organization of actin, vinculin, and β-catenin were assessed using immunofluorescence. Cultured monkey eye anterior segments were used to test the effects of AdC3GFP on outflow facility.

Results: Treatment of HTM cells with AdC3GFP resulted in dose-dependent morphological changes 3 or 4 days post-transduction. The AdC3GFP-transduced cells were either partially retracted, rounded, or very elongated compared to non-transduced cells. Compared to AdGFP-transduced cells, AdC3GFP-transduced cells demonstrated disrupted actin cytoskeleton, reduced vinculin-positive focal adhesions, and loss of β-catenin staining. Cells transduced with AdGFP did not round up or retract. In organ culture studies, outflow facility was increased by 90±21% (n=15, p<0.001) in AdC3GFP-transduced eyes compared to baseline and corrected for AdGFP-transduced control eye washout on days 3-6 after transduction.

Conclusions: C3 transduction is effective in disrupting actin filaments, cytoskeleton, and cellular adhesions in HTM cells and in increasing outflow facility in organ cultured monkey anterior segments, suggesting that expressing the C3 gene in the trabecular meshwork may be an effective approach for glaucoma therapy.

Agents that affect the actin cytoskeleton can alter cell morphology and architecture in the trabecular meshwork (TM), and thereby change the overall geometry and decrease outflow resistance. Cytochalasins, a group of fungal metabolites that disrupt actin filaments by preventing actin polymerization from the individual globular subunits [1,2], significantly increase outflow facility in monkeys [3,4]. Other direct or indirect actin-disruptive agents such as the serine-threonine kinase inhibitor H-7 and latrunculins-A and latrunculin-B, have been extensively studied in vitro and in vivo [5-8]. H-7 and latrunculins-induced increases in outflow facility result from major morphological and architectural changes in the TM, including cell relaxation, expansion of the juxtacanalicular region, dilation of Schlemm’s canal, and lumenal protrusion of the inner wall [9,10].

Rho-mediated signaling pathways regulate the assembly and contractility of the actomyosin network. Rho guanosine triphosphatases (GTPases), which belong to the Ras superfamily of low molecular weight GTP-binding proteins, regulate many essential cellular processes including actin dynamics and cellular adhesions [11]. Previous studies have shown that Rho promotes actomyosin contractility and the resulting tension drives the formation of stress fibers and focal adhesions [12-14]. Therefore, we hypothesized that agents that inhibit Rho signaling may modulate aqueous humor hydrodynamics and facilitate outflow by changing the organization of stress fibers and focal adhesions in the TM. Rho GTPases are the preferred intracellular targets of bacterial protein toxins, such as exoenzyme C3 transferase (C3) from Clostridium botulinum, which specifically inactivates Rho by ADP-ribosylation [15]. Previous studies showed that inhibition of Rho kinase, a critical downstream effector of Rho GTPase, or expression of its dominant negative mutant, increased outflow facility in organ cultured porcine and human eyes [16-19], and in rabbit and monkey eyes in vivo [20,21].

In this study, we demonstrate that adenovirus-mediated C3 delivery alters the cytoskeleton, focal adhesions, and cell-cell adhesions of cultured human trabecular meshwork (HTM) cells, and increases outflow facility in organ cultured monkey eyes. This study supports the possible use of C3 gene delivery for the treatment of glaucoma.

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METHODS

Cell culture: The human embryonic kidney cell line HEK 293 (provided by Dr. Nader Sheibani, University of Wisconsin-Madison) was maintained in Dulbecco’s modified Eagle medium (DMEM, Mediatech, Herndon, VA) supplemented
with 10% heat-inactivated fetal bovine serum (FBS, Mediatech), 100 µg/ml streptomycin, and 100 U/ml penicillin at 37 °C in 5% CO₂. HTM cells were grown in DMEM supplemented with 15% FBS, 25 µg/ml gentamycin, and 2.5 µg/ml amphotericin B at 37 °C in an atmosphere of 8% CO₂ [8]. Once they reached confluence, they were allowed to achieve a stable morphology in the same medium but with 10% FBS. TM-1 cells [22] were grown in DMEM, supplemented with 10% FBS, 25 µg/ml gentamycin, and 2.5 µg/ml amphotericin B, in 6 well plates at 37 °C in 8% CO₂.

Construction and generation of recombinant C3 expressing adenoviral vector: The C3 cDNA was kindly provided by Dr. Larry Feig (Tufts University, Boston, MA). The AdEasy Adenoviral system (Stratagene, La Jolla, CA) was used for vector construction. The C3 cDNA was subcloned into the shuttle vector, pShuttle-CMV, with an influenza virus hemagglutinin (HA) tag fused to the carboxy terminus of C3 (Figure 1). This dual expression vector also contained the gene for GFP downstream from an internal ribosome entry site (IRES) element, so that C3 and GFP could be expressed from the same transcript. The resultant pShuttle-C3 was then used to generate adenoviral recombinants through homologous recombination with the adenoviral backbone vector, pAdEasy-1, in BJ5183 bacterial cells (Stratagene, LaJolla, CA). The adenoviral vector DNA was then transfected into HEK 293 packaging cells to prepare transducing virus. For packaging, the C3 vector DNA was linearized by digestion with PacI. The linearized DNA was then transfected into HEK293 cells using Lipofectamine Plus (Invitrogen, Carlsbad, CA). Each 35 mm well received 4 µg of linearized vector DNA mixed with 5 µl of Plus reagent in 125 µl of Optimum (Invitrogen). A separate solution containing 8 µl of lipofectamine in 125 µl of Optimum was also prepared. Both solutions were incubated at room temperature for 15 min, then mixed and incubated for an additional 30 min to allow the DNA-lipo complex formation. The mixture was then added dropwise to cells plated the day before. All cells had been rinsed once with Optimum. The cultures were then incubated at 37 °C with 5% CO₂ for 3 h at which time they were transferred to DMEM with 10% FBS. A separate adenovirus vector expressing GFP alone (AdGFP) was constructed as a control (Figure 1). Adenovirus mediated gene expression was verified in both HEK 293 and HTM cells by western blot analysis as described below.

Morphology of HTM and TM-1 cells transduced with adenovirus vectors: HTM cells were cultured to confluence for 1 week, at which time they exhibited a stable monolayer endothelial-like morphology, and were then transduced with AdGFP or AdC3GFP at multiplicities of infection (MOI) of 2.5 and 25. TM-1 cells were cultured to confluence at which point they had a stable monolayer endothelial-like morphology. They were then transduced with AdC3GFP at an MOI of 10. Images were captured using a Zeiss Axiovert 200 inverted microscope (at 20x) and an AxioCam high resolution color camera (Zeiss, Thornwood, NY), and processed using the Axiovision 4.2 software (Zeiss).

Western blotting: Immunoblotting was carried out as described previously [23]. Briefly, HEK 293 or HTM cells were transduced with adenovirus vectors and at 4 days post-transduction, lysates were prepared by scraping cells from culture dishes into 1X phosphate buffered saline (PBS). Microscopic examination indicated that >99.99% of the cells were removed with this procedure. The cells were then collected by centrifugation at 400 x g for 5 min at 10 °C. The cell pellet was resuspended in 80 µl of SDS-polyacrylamide gel electrophoresis sample buffer containing 2% β-mercaptoethanol, vortexed, and sonicated. After boiling for 5 min, equal amounts of cell extracts were electrophoresed in 10% SDS-polyacrylamide gels. The proteins were then electrophoretically transferred to nitrocellulose, blocked with 5% nonfat dry milk in 1X Genius buffer I (100 mM maleic acid, 150 mM NaCl, pH 7.5) with 0.3% v/v Tween-20, and probed with monoclonal anti-HA (sc-805; Santa Cruz Biotech, Santa Cruz, CA) or polyclonal anti-C3 antibodies (kindly provided by Dr. Christian Wilde, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Albert-Ludwigs-Universität, Freiburg, Germany). The membrane was washed and incubated with secondary rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP, sc-2031; Santa Cruz), and then developed with the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. Briefly, ECL reagents A and B were mixed (40:1) with a final volume of 0.125 ml/cm² and exposed to x-ray film.

Immunofluorescence microscopy: Immunofluorescence labeling was performed as previously described [1,4,10]. Briefly, HTM cells cultured on glass cover slips pre-coated with poly-L-lysine were transduced with AdGFP or AdC3GFP. Four days post-transduction, the cells were washed with 50 mM MES buffer, permeabilized with 0.5% Triton X-100, and then fixed with 3% paraformaldehyde in PBS. Alexa 488-conjugated phallolidin (Sigma, St. Louis, MO) was used for fluorescent labeling of actin. The primary antibodies used in this study were monoclonal anti-human vinculin (clone hVin-1; Sigma) and anti-β-catenin (clone 15B8; Sigma). Secondary antibodies were Cy3-conjugated goat anti-mouse IgG H+L (116-165-062; Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa 488 conjugated goat anti-rabbit IgG H+L (A-11070; Molecular Probes, Eugene, OR). Fluorescence was observed with a Zeiss AxioPlan 2 microscope equipped with an AxioCam HRm camera together with Axiovision 3.1 software.

To localize C3 transferase in transduced eye tissues, 6 pairs of cultured anterior segments were perfused with 4% paraformaldehyde. Wedges were cut from the anterior segments, immersed in 4% paraformaldehyde, embedded in OCT, and frozen. Sections (5 µm) were cut, mounted on glass slides, and stored at -80 °C until they were processed for immunofluorescence microscopy. Sections were warmed to room temperature and immersed for several min in PBS. They were then incubated in a 1% SDS solution for 5 min according to Brown, et al. [24], and washed in several changes of PBS prior to blocking overnight at 4 °C in PBS with 1% bovine serum albumin (BSA). Sections from eyes treated with either AdGFP or AdC3GFP were then incubated with a 1:50 dilution of rab-
bit polyclonal anti-C3 antibody for 1 h at room temperature. Rabbit antibodies were detected with Alexa 546-conjugated goat anti-rabbit IgG (A11010; Molecular Probes). Sections were labeled with Hoechst 33342 (Molecular Probes) in order to visualize nuclei. Additional wedges from the same cultured anterior segments were fixed in 4% paraformaldehyde, embedded, cut, and stained with hematoxylin and eosin.

Organ culture: A monkey anterior segment organ culture system [25] was used to test the effects of the C3 expressing adenoviral vector on outflow facility. The two-level constant pressure perfusion technique was employed [26]. Briefly, the infusion pump was stopped and the inflow ports of organ culture dish were connected to weighed reservoirs (Harvard strain gauges), which could be raised and lowered (modified

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**Figure 1.** Structure of the adenoviral vectors. **A:** AdGFP, which has an internal ribosomal entry site at the intergenic region of the genome, expresses human recombinant GFP. **B:** AdC3GFP, which has the C3 gene cloned into the multiple cloning site of the backbone and in frame with 3X HA tag, expresses C3 transferase and human recombinant GFP. The left and right inverted terminal repeat are indicated by LITR and RITR, respectively, while the multiple cloning site is indicated by MCS and the internal ribosome entry site is indicated by IRES.

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**Figure 2.** Human trabecular meshwork cell morphology after transduction with adenovirus vectors. Human trabecular meshwork cells were transduced with AdGFP or AdC3GFP for 4 days. **A:** Control, nontransduced cells. **B,C:** Control cells transduced with AdGFP at MOIs of 2.5 and 25 appeared slightly different in morphology and were arranged in a more random orientation. **D,E:** Cells transduced with AdC3GFP at MOIs of 2.5 and 25 appeared to be either partially retracted, rounded up completely (arrows), or very elongated (arrowheads) compared to control cells. No obvious cell detachment was observed in any cells. The scale bar represents 50 µm.
Harvard Precision Adjustable Screw Stand) to change the IOP. Reservoir height was changed every 4 min between two levels 10 mm Hg apart. Outflow facility for each 4 min period was calculated as the change in flow rate divided by the change in pressure. Results from successive periods were sequentially averaged. Outflow facility was usually monitored for at least 1 h. Data acquisition and screw stand control were accomplished with a National Instruments data acquisition board and custom-designed LabVIEW software (National Instruments, Austin, TX) [25]. Considering all eyes, the mean baseline low level pressure was 6.38 ± 0.37 mm Hg (mean ± SEM, n=30). Fluid infusion continued at a constant rate of 2.5 µl/min when facility was not being measured.

The two-level constant pressure method is preferred with the monkey organ culture system because detection of a change in IOP in response to a treatment is more variable when the IOP is low to start with and washout is occurring with time. IOP is lower than that typically found in the human eye organ culture system, even at the same infusion rate (2.5 µl/min); this could be due, in part, to the relatively thin sclera of the monkey eye and perhaps to removal of the highly pigmented uvea.

A total of 15 paired monkey anterior segments were studied, cynomolgus (Macaca fascicularis, 7 pairs) or rhesus (Macaca mulatta, 8 pairs). Baseline outflow facility was measured after overnight equilibration. One anterior segment was injected via the inflow port with 1.2x10^8 viral particles of AdC3GFP (80 µl), while the contralateral anterior segment received the same dose of AdGFP. The outflow facility was

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![Western blot](http://www.molvis.org/molvis/v11/a129/) Figure 3. Western blot showing dose-dependent C3 expression in AdC3GFP-transduced cells. Cells were transduced with either AdGFP or AdC3GFP at MOIs of 2.5 or 25, harvested, electrophoresed in denaturing reducing polyacrylamide gels, and blotted with antiserum specific C3 transferase. Control cells (medium) were mock transduced.

![C3-induced changes in actin cytoskeleton and focal adhesions](http://www.molvis.org/molvis/v11/a129/) Figure 4. C3-induced changes in actin cytoskeleton and focal adhesions in human trabecular meshwork cells. Human trabecular meshwork cells were transduced with AdGFP or AdC3GFP for 4 days. A-D: No virus. B,E: AdGFP-transduced. C,F: AdC3GFP-transduced. Cells were labeled with phalloidin (A-C) or anti-vinculin monoclonal antibody (D-F). Note abundant stress fibers in cells exposed to no virus (A) or transduced with AdGFP (B) which contrasts with cells transduced with AdC3GFP (C). Vinculin labeling (arrows) was associated with focal adhesions and cell-cell junctions. Note that AdC3GFP-transduced cells (F) are essentially devoid of vinculin labeling. The scale bar represents 50 µm.
measured daily thereafter for up to 4-7 days. For 4 pairs of anterior segments, AdC3GFP activity was not tested prior to organ culture experiments. For the other eyes, activity was tested either before or after the organ culture experiments.

Light microscopy of the trabecular meshwork: At the conclusion of the outflow facility studies, all eyes were perfused with 6 ml of 4% paraformaldehyde over 30 min, cut into quadrants, and then immersed in 4% paraformaldehyde. Each quadrant was either embedded in JB-4 solution (Polysciences Inc., Warrington, PA) or paraffin, 4 µm sections were cut and mounted on glass slides, and then stained with either Toluidine Blue O (Polysciences), or hematoxylin and eosin. Morphology was evaluated by light microscopy to demonstrate the quality of the organ culture. The presence of TM cells, beams, and the integrity of Schlemm’s canal (SC) were assessed.

RESULTS

Effect of C3 on HTM cell morphology: To test the efficiency of gene transfer in culture, HTM cells, which form a confluent and flat monolayer with extensive intercellular contacts, were transduced with the AdGFP or AdC3GFP at MOIs of 2.5 or 25. Cells treated with medium only and AdGFP were used as controls. As shown in Figure 2D,E, cells transduced with the AdC3GFP appeared to be either partially retracted, rounded up, or very elongated compared to controls. The number of cells showing altered morphology increased with higher MOI values, indicating a dose-dependent response. The morphological changes in AdC3GFP-transduced TM-1 cells were similar to those seen in the HTM cells (data not shown).

<p>| TABLE 1. EFFECT OF C3 ON OUTFLOW FACILITY OF ORGAN-CULTURED MONKEY ANTERIOR SEGMENTS |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Outflow facility</th>
<th>AdC3GFP</th>
<th>AdGFP</th>
<th>AdC3GFP/AdGFP</th>
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<tr>
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<td>1.90 ± 0.21*</td>
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<tr>
<td>Paired responder segments (n=11)</td>
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<tr>
<td>BL</td>
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<td>0.32 ± 0.05</td>
<td>0.97 ± 0.19</td>
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</tr>
<tr>
<td>Rx</td>
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<td>0.33 ± 0.04</td>
<td>1.63 ± 0.23**</td>
<td></td>
</tr>
<tr>
<td>Rx/BL</td>
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<td>1.16 ± 0.10</td>
<td>2.18 ± 0.23*</td>
<td></td>
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<tr>
<td>Paired segments without a response (n=4)</td>
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<tr>
<td>BL</td>
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<td>0.77 ± 0.10</td>
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<tr>
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<tr>
<td>Rx/BL</td>
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<td>1.43 ± 0.34</td>
<td>1.12 ± 0.21</td>
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</table>

The effect of C3 (1.2x10⁸ viral particles, 80 µl) on outflow facility in organ-cultured monkey anterior segments. The data are presented as the mean±SEM. Outflow facility units are calculated as µl/min/mm Hg and all ratios are unitless. The data sets are the baseline (BL) and the maximum outflow facility 3-6 days after transduction (Rx). The ratios are Rx/BL and AdC3GFP/AdGFP. Ratios were significantly different from 1.0 by the two-tailed paired t-test. The single asterisk indicates a p<0.001, the double asterisk indicates a p<0.005, the single sharp (#) indicates a p<0.05.

Figure 5. C3-induced changes in intercellular adhesions in human trabecular meshwork cells. A: β-catenin (arrows) localization around the periphery of non-transduced cells. B: AdGFP-transduced cells also localize β-catenin around their periphery. C: AdC3GFP-transduced cells demonstrated a near complete loss of intercellular junctions and prominent cell rounding (arrowheads). The scale bar represents 50 µm.
To confirm the expression of C3 transferase, cell lysates from transduced HTM cells were immunoblotted and probed with polyclonal antibodies specific for C3 transferase. As shown in Figure 3, dose-dependent expression of a 26 kDa protein corresponding to C3 was seen in AdC3GFP-transduced cells (lanes AdC3GFP, MOI of 2.5 and 25) but not in non-transduced (Medium) cells or in cells transduced with AdGFP. Lower molecular weight bands in the C3-transduced cells most likely represent degradation of the C3 protein. It should be noted that two higher molecular weight bands were also present in transduced and non-transduced samples. Since these bands are present in all samples, they most likely represent a cross reacting cellular protein.

Effect of C3 on the distribution of actin, vinculin, and β-catenin in HTM cells: As shown in Figure 4, AdC3GFP-transduced cells demonstrated a disrupted or absent actin cytoskeleton. Vinculin-positive focal adhesions were greatly reduced in number or absent in AdC3GFP transduced cells, and vinculin staining at cell-cell junctions was also reduced. Figure 5 shows a nearly complete loss of intercellular junctions, as indicated by near total loss of β-catenin staining in AdC3GFP-transduced cells. Control cells transduced with AdGFP did not show a disruption of actin fibers, nor was there a significant change in vinculin staining (Figure 4C,D). β-Catenin staining also appeared normal in AdGFP transduced cells (Figure 5B). These results confirm the functionality of the Ad-transduced C3. In other words, C3 induced changes in morphology and actin and associated cellular adhesions results from its inhibitory effects on Rho, which in turn, result in actin disruption. On the other hand, higher doses (MOI 25 or above) of AdGFP and AdC3GFP (especially the latter) ultimately led to cell death.

Effect of AdC3GFP on outflow facility of organ cultured monkey anterior segments: We next determined whether Ad mediated delivery of C3-transferase to monkey anterior segments in organ culture would alter outflow facility. The anterior segment with the lower baseline outflow facility was usually chosen to receive the AdC3GFP to minimize the confounding effect of washout. This favorably biased the detection of an increased response when compared to baseline. Comparison of the post-treatment outflow facilities in opposite anterior segments without correction for baseline, biased the result against detection of an effect. However, there were 3 pairs in which segments with the higher baseline outflow facility received AdC3GFP and 5 pairs in which baseline outflow facility was similar (difference was less than 0.02 µl/min/mm Hg). Overall, there was no significant difference in baseline outflow facility of the 15 pairs when tested by a 2-tailed paired t-test as shown in Table 1.

In all 15 pairs of anterior segments, the maximum outflow facility in AdC3GFP transduced anterior segments was increased by 116±24% (p<0.001) compared to baseline, and by 90±21% (p<0.001) compared to baseline and corrected for contralateral control anterior segment washout (23±11%). The outflow facility post-treatment was 59±21% (p<0.05) higher in the AdC3GFP-transduced, compared to contralateral control anterior segments without correction for baseline. Among the 15 pairs of anterior segments, 11 pairs responded with...
outflow facility averaging 83±23% (p<0.005) higher compared to contralateral control anterior segments without correction for baseline. The other 4 pairs did not respond to AdC3GFP.

The time course for the outflow facility response following AdC3GFP and AdGFP transduction is shown in Figure 6. The maximum outflow facility response occurred on days 3-6 after transducing virus delivery (Figure 6A). The slow response of the anterior segments to C3 is consistent with the time it took to see changes in cultured HTM and TM-1 cells. This is in contrast to the relatively rapid response (12 h) reported by Rao et al. [18] and may reflect differences in expression levels of the transduced genes used in the two studies.

**Morphology of TM:** The effect of C3 expression on the gross morphology of the anterior segment was evaluated by light microscopy at the time of the maximum response. The parameters that were examined included the degree of cellularity in the juxtacanalicular (JXT) region and along the collagen beams, the integrity of SC, and the organization of the collagen beams [27]. Of the 11 pairs of anterior segments showing changes in the outflow facility, three demonstrated no difference in morphology between AdGFP (Figure 7A) and AdC3GFP (Figure 7B) treated anterior segments. Both anterior segments contained an intact SC, organized beams, and exhibited a high degree of cellularity throughout the TM and SC. Interestingly in three pairs of anterior segments, the C3-transduced anterior segment exhibited a higher degree of cellularity and organization of the collagen beams than the control anterior segment (data not shown). Five pairs of anterior segments, however, showed a clear morphological difference between control (Figure 7C) and C3-transduced (Figure 7D) anterior segments. These differences included reduced cellularity and disorganized beams in the segments transduced with AdC3GFP. In these five samples, SC appeared intact. Morphological examination of the JXT was inconclusive and requires additional studies at the EM level.

**Immunofluorescence localization of C3 transferase in cultured anterior segments:** Attempts were made to localize C3 transferase in 6 of the 15 paired anterior segments transduced...
with AdC3GFP or AdGFP. Strong, positive staining for C3 transferase was observed in TM cells from 4 of the 6 anterior segments transduced with AdC3GFP (Figure 8A,B). The paired control segments, transduced with AdGFP, failed to demonstrate any positive staining for C3 transferase (Figure 8D,E). Two of the 6 anterior segments failed to show any positively-stained structures, due to the loss of the meshwork and ciliary body during processing. Double labeling for C3 transferase and cell nuclei (Figure 8B) confirmed that the positively stained structures were cells. We confirmed that the stained structures were trabecular meshwork tissue by comparing the frozen sections with paraffin embedded sections from the same segments stained with hematoxylin and eosin (Figure 8C,F). These results confirm that we were able to deliver the C3 transferase gene to the TM and that expression of the gene in these cells correlated with a significant increase in outflow facility.

**DISCUSSION**

In this study we evaluated the potential use of the Rho GTPase inhibitor, C3 transferase, to affect outflow facility following transduction with an adenoviral vector. Rho GTPase participates in signaling pathways that lead to formation of actin stress fibers and focal adhesions [28-30]. Rho GTPase activates Rho kinase (known also as ROCK), which phosphorylates and inhibits myosin light chain phosphatase (MLCP). When Rho GTPase is inhibited, MLCP dephosphorylates the myosin II regulatory light chain (MLC), which subsequently blocks myosin II ATPase activity. A decrease in myosin-driven contractility leads to disruption of stress fibers and focal adhesions [7,11,31]. Therefore, we hypothesized that Rho GTPase may be a potential protein target for the development of a therapeutic strategy to increase outflow facility. As expected, adenovirus-mediated delivery of the Rho GTPase inhibitor, C3 transferase [15], altered the intracellular distribution of actin, vinculin, and β-catenin in cultured HTM cells, and increased outflow facility in cultured monkey anterior segments. These results raise the possibility that delivery of the C3 transferase gene could be an effective treatment for glaucoma.
Our studies support previous work using either Y-27632, a specific ROCK inhibitor, or a dominant negative Rho [18,19] to inhibit Rho activity. Like the previous studies, inhibition of Rho activity by C3 transferase was found to cause a retraction and rounding of cultured TM cells, the disruption of actin bundles, and impaired focal adhesion formation. In all cases, inhibition of Rho activity correlated with an increase in outflow facility in both organ cultured human and porcine eyes [16,18,19] and rabbits [21] and monkeys in vivo [20]. Surprisingly, the increased outflow facility obtained with both C3 transferase and the dominant negative Rho [18,19] did not correlate with gross morphological changes within the TM and SC. Electron microscopy studies [18], however, revealed subtle changes along the inner SC wall and the collagen beams in anterior segments transduced with dominant negative Rho compared to control segments. This suggests that expression of the transduced C3 protein may result in enhanced paracellular or transcellular fluid flow across Schlemm’s canal inner wall endothelium and/or enhanced flow through the JXT, thereby increasing outflow facility.

Adenovirus vectors have previously been shown to transduce a number of anterior segment cell types, including corneal endothelium, iris pigment epithelium, and TM cells [32,33]. A number of vectors for gene therapy have been described, but adenovirus-mediated gene therapy may provide significant advantages, including high transduction efficiencies and the ability to transduce cells independently of cellular proliferation. However, adenovirus vectors have several problems that need to be addressed. The introduction of large numbers of Ad viral particles induces a variable anterior chamber inflammatory response and corneal edema in primates, the severity and duration of which can range from mild and transient, to severe and long lasting [34,35]. Repeated exposure to the viral constructs increases the likelihood of the latter response. The response can be attenuated or eliminated by lowering the dose, but this reduces the transduction efficiency. Thus methods to inhibit the inflammatory response need to be developed for the successful use of Ad vectors.

Glaucoma is a chronic condition, thus long-term expression of the transgene will be required for any gene therapy approach for this disease [36]. For the current study, we chose the CMV promoter, because it is known to be functional in several cell types and express at high levels. However, the CMV promoter does not express for long periods. In a previous study using Ad vector transduction of primate TM, expression was detected only for about a month [35], which is clearly inadequate for a chronic condition like glaucoma. The CMV promoter shut-off is most likely due to the process of an NFκB response element, since expression can be initiated with NFκB activation in some cell types [37]. Continued NFκB activation to keep the CMV promoter functional would probably provoke an inflammatory response, which would be undesirable. Thus, before our strategy of delivering the C3 transferase gene to treat glaucoma can be implemented, a more suitable promoter will need to be identified.

In summary, our study demonstrates that adenovirus-mediated C3 expression can significantly induce changes in morphology and organization of actin and cellular adhesion of HTM cells, and increase outflow facility in organ-cultured monkey anterior segments, representing a novel approach to potentially reduce IOP and treat glaucoma.

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