Corneal Endothelial Cell Migration and Proliferation Enhanced by Rho Kinase (ROCK) Inhibitors in In Vitro and In Vivo Models

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PURPOSE. To explore the role of Rho-associated kinases (ROCK) in corneal physiology and regeneration, and the effects of suppressing its activity in stimulating corneal endothelial cell proliferation and migration in vitro and in vivo.

METHODS. Immunohistochemistry was performed to detect RhoA and ROCK-1 and ROCK-2 in human corneal tissue. Adult porcine corneal endothelial cells (CECs) were isolated, grown to confluence, and further characterized. Under the treatment of ROCK inhibitors, changes in the cellular distribution profile of ZO-1 and F-actin were examined by immunofluorescence staining. Corneal endothelial cells migration was evaluated by scratch assay and analyzed with Axiovision software. Cell proliferation was quantified using Click-iT EdU HCS Assay. In vivo, the corneal endothelia of rabbits were surgically injured and H-1152 was topically applied for 10 days. Progress of wound healing was evaluated daily by monitoring corneal edema, inflammation, and thickness using slit-lamp examination, photography, and pachymetry. Rabbits were euthanized and enucleated for further evaluation.

RESULTS. H-1152 exhibited significant stimulatory effect on CEC migration and proliferation in vitro compared with both untreated and Y-27632–treated cells. Furthermore, topical administration of H-1152 led to marked reduction in corneal edema and formation of multinucleate CECs in vivo suggestive of proliferation associated with healing.

CONCLUSIONS. H-1152 exhibited a better stimulatory effect on CEC migration and proliferation in vitro than Y-27632. Our findings suggest that topical administration of H-1152 promotes healing of injured corneal endothelium in vivo. These results demonstrate the efficacy of ROCK inhibitors as a potential topical therapy for patients with corneal endothelial disease.

Keywords: corneal endothelial cells, Rho-associated kinase, ROCK inhibitor, proliferation, migration

Corneas remain the most commonly transplanted tissue in the world.[1] In 2012, 184,576 corneal transplantation surgeries were performed in 116 countries. Thirty-nine percent of these procedures were performed to correct vision loss due to Fuchs' corneal endothelial dystrophy (FECD) alone.[2] In the United States, 48% of corneal transplantation were performed on patients with some form of corneal endothelial disease (CED), establishing CED as the most common indication for keratoplasty.[3] Currently, surgical replacement of the endothelium remains the only definitive treatment option for patients with CED; however, even the most refined, targeted surgical procedures are not without risk of complications.[4,5]

To date, no pharmacologic therapy exists for CED. However, inhibition of Rho-associated kinase (ROCK) has been reported to have a stimulatory effect on corneal endothelial wound healing, a finding with significant implications for treating CED.[6] Koizumi and colleagues[6] demonstrated that inhibition of the ROCK signaling pathway with ROCK inhibitor Y-27632 resulted in inhibition of apoptosis and increased proliferation of corneal endothelial cells (CECs) isolated from the cynomolgus monkey.[7] More recently, Okumura et al.[8,9] further characterized

the effects of ROCK inhibitors on corneal endothelial wound healing by showing that topical administration of ROCK inhibitor Y-27632 augmented cell proliferation in vitro and in vivo.

ROCK is a widely studied serine/threonine kinase that functions as an effector molecule of the RhoA signaling pathway with important roles in regulating cell morphology, motility, and polarity via its rearranging effects on the actomyosin cytoskeleton. Specifically, activation of ROCK leads to formation of actin stress fibers, increased cell-cell junctions, as well as increased cell-extracellular matrix interactions.[10,11] Aberrant functioning of ROCK and the Rho GTPase pathway has been linked to the pathophysiology underlying a wide variety of disease processes, including cardiovascular disease, diabetes mellitus, asthma, and erectile dysfunction.[12–22] As a result, selective inhibition of this signaling pathway has been extensively investigated as a potential novel therapy for many of these illnesses. ROCK inhibitor agents have already been introduced into the field of ophthalmology as a viable therapeutic modality for treating glaucoma and ROCK inhibitor eye drops have been in clinical trials.[11,23–26] In fact, ripasudil

has been approved as a 0.4% ophthalmic solution for the treatment of glaucoma and ocular hypertension in Japan.[27] Interest in ROCK inhibitors within the field of ophthalmology has been further substantiated by the aforementioned investigations of ROCK inhibitors and their potential applications for corneal endothelial diseases.[7,8,28,29]

We hypothesize that, similar to previous findings, ROCK inhibitors such as Y-27632 and H-1152,[30,31] will induce proliferation and migration of CECs in vitro, and that this stimulatory effect on migration and proliferation will lead to improved corneal endothelial wound healing in vivo. We further postulate that ROCK inhibitors Y-27632 and H-1152 are a valid topical therapeutic modality for treating CED. In an attempt to confirm earlier observations and our hypotheses, we explored the potential involvement of RhoA and ROCK activities in corneal physiology and tissue regeneration, investigated the efficacy of ROCK inhibitors Y-27632 and H-1152 in stimulating proliferation and migration of CECs in vitro, and evaluated the efficacy of topical administration of H-1152 for CEC wound healing in vivo. H-1152 is a close analog of fasudil and ripasudil, which are two approved drugs in Japan. H-1152 is more specific and potent than Y-27632.[32] These experiments were performed both to assess and to compare the potential therapeutic benefits of the aforementioned agents, as well as to provide additional insight into the true mechanism of ROCK signaling pathway inhibition as it pertains to CEC behavior.

MATERIALS AND METHODS

Immunohistochemical Analysis

To determine the distribution of RhoA, ROCK-1, and ROCK-2 in the human eye, paraffin-embedded postmortem eyes from six human subjects (age, 73–90 years) with no known corneal pathology were analyzed using immunohistochemical staining, as previously performed.[33] Briefly, 5-μm sections of tissue were deparaffinized and rehydrated. Endogenous peroxidase activity was extinguished using hydrogen peroxide in methanol. Heat-induced antigen-epitope retrieval was carried out using 0.1 M citrate buffer pH 6.0 (Vector Laboratories, Burlingame, CA, USA) for 20 minutes at 100°C. Sniper Background Reducer (Biocare Medical, Concord, CA, USA) was applied after tissue was washed to prevent nonspecific staining. Tissue sections were incubated overnight at 4°C with either rabbit polyclonal anti-RhoA, ROCK-1, or ROCK-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection of bound antibody was performed using the Mach 4 Universal AP Polymer Kit (Biocare Medical) with alkaline phosphatase.

Isolation and Cell Culture

Corneal endothelial cells were isolated from freshly enucleated porcine eyes using a trypsin/EDTA digestion protocol first described by Xie and Gebhardt.[34] Briefly, after the porcine eyes were dissected and their intact corneas isolated from the globe, 30-μL 10X trypsin/EDTA solution (Sigma-Aldrich Corp., St. Louis, MO, USA) was applied directly to the endothelial surface of the cornea to disengage CECs from Descemet's membrane. Corneas were then incubated with 10X trypsin/EDTA for 5 minutes at 37°C. Corneal endothelial cells removed from Descemet's membrane were subsequently washed and collected using a 200-μL pipette tip before being directly transferred to a sterile, 60 × 15-mm Falcon petri dish coated with 10 μg/mL mouse collagen IV (Trevigen, Gaithersburg, MD, USA) and resuspended in growth media. Growth media consisted of low glucose Dulbecco's modified Eagle's medium (Gibco BRL Life Technol-

ogies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 mg/μL), gentamicin (15 μg/mL), and 75 μg/mL endothelial cell growth supplement from bovine pituitary (Sigma-Aldrich Corp.). Corneal endothelial cells were incubated at 37°C until confluence was achieved (~12 days). Growth media used in primary culture and serial passages was exchanged every 72 hours.

Immunofluorescence Analysis

Porcine CECs from primary culture were passaged and transferred to 6-well culture dishes containing coverslips coated with 10-μg/mL collagen IV (Trevigen) and grown to confluence. Corneal endothelial cells were then treated with serum-free media containing either 10-μM Y-27632 (Enzo Life Sciences, Farmingdale, NY, USA) or 2.5-μM H-1152 (Enzo) for 2 hours. Cells incubated with serum-free media served as no treatment control. Cells were fixed at 2, 24, and 48 hours following drug treatment to observe effect of ROCK inhibitor on CEC cytoskeleton. Fixation of CECs was performed at room temperature for 10 minutes in 3.7% formaldehyde (vol/vol) PBS. The CECs were then washed with cytoskeletal buffer (10-mM 2-[N-morpholino] ethane sulfonic acid [MES], containing 150-mM NaCl, 5-mM EGTA, 5-mM MgCl2, and 5-mM glucose [pH 6.1]), followed by permeabilization for 10 minutes with 0.1% Triton X-100 in PBS and blocking with serum-containing buffer (10% FBS in PBS with 0.02% sodium azide).

Immunohistochemical staining of the CEC cytoskeleton was performed as follows. F-actin staining was carried out by incubating CECs with TRITC-Phalloidin (500 ng/mL; Sigma-Aldrich Corp.) in serum-containing buffer with 0.2% saponin for 45 minutes. Corneal endothelial cell tight junctions were identified by immunostaining with monoclonal anti-ZO-1 (Invitrogen, Carlsbad, CA, USA) in serum-containing buffer with 0.2% saponin and incubated for 2 hours in a humidifier chamber. Secondary antibodies used were conjugated with FITC (Invitrogen). Coverslips were mounted onto glass slides using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, USA). Images were recorded with confocal fluorescence microscopy (Eclipse 90i; Nikon Instruments, Melville, NY, USA).

In Vitro Migration Assay

Porcine CECs from primary culture were passaged and transferred to 6-well culture dishes containing coverslips coated with gelatin (2%) and 10-μg/mL collagen IV (Trevigen). Approximately one-half million of CECs were grown to confluence (~12 days) before a 1.0-mL pipette tip was used to make a uniform, 22-mm scratch across each coverslip. The growth media was then exchanged with serum-free media containing 10-μM Y-27632 (Enzo) or 2.5-μM H-1152 (Enzo). Serum-free media lacking Y-27632 or H-1152 was used as the control. Distance between advancing cell borders on each side of the scratch was monitored at 0-, 24-, and 48-hours incubation using phase-contrast microscopy (Axio Observer.D1; Carl Zeiss, Thornwood, NY, USA) and Axiovision software (Carl Zeiss Microscopy GmbH, Jena, Germany). Immunohistochemical staining of CEC cytoskeleton was then performed, as described previously.

In Vitro Proliferation Assay

Proliferation of porcine CECs treated with Y-27632 or H-1152 was quantified using the Click-iT EdU HCS Assay (Invitrogen). Briefly, porcine CECs (~3000 cells per well) were transferred to a 96-well plate coated with 10-μg/mL collagen IV (Trevigen). Corneal endothelial cells were allowed to recover overnight in
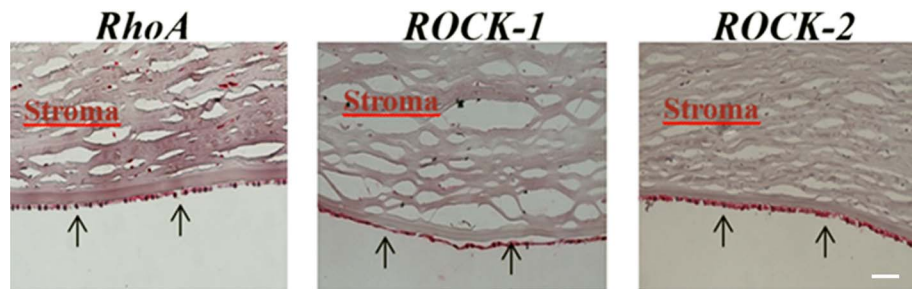
FIGURE 1. Distribution of RhoA and ROCK in the human cornea. Visualization of human cornea using light microscope and immunohistochemical staining showed positive expression of RhoA, ROCK-1, and ROCK-2 in the endothelium (*arrows*). *Scale bar*: 200 μm.

culture media containing low-glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with FBS, penicillin, streptomycin, gentamicin, and endothelial cell growth supplement, as described previously. This media was then exchanged with serum-free media containing 10-μM Y-27632 (Enzo) or 2.5-μM H-1152 (Enzo). Following 2-hours incubation at 37°C, 10-μM EdU was added to each well. EdU incubation was carried out for 24 and 48 hours before CECs were fixed and permeabilized using 3.7% formaldehyde in PBS and 0.1% Triton X-100 in PBS, respectively. The Click-iT reaction cocktail (Invitrogen) was prepared per the manufacturer's instructions and added to the permeabilized CECs for 30 minutes at room temperature for EdU detection. Fluorescence intensity of EdU signal was measured using SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

In Vivo Rabbit Study

Animals used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All studies were conducted at Duke University Eye Center (Durham, NC, USA) under a protocol approved by the Institutional Animal Care and Use Committee of Duke University and Duke University Medical Center. Six Dutch-belted rabbits were placed under general anesthesia, and the central 8 mm of each cornea was marked. Next, a transcorneal incision was made at the limbus of the eye, and the corneal endothelium was lightly scraped within the marked circle with a bent 27-G cannula. The site of incision was closed with a single suture using 10.0 nylon. The rabbit eyes were randomized to treatment versus no treatment group. The researcher making the injury was blinded to which group each rabbit would be included in. H-1152 (1 mM; Enzo) was applied topically to the experimental eye. Two sequential doses consisting of 25 μL of 1-mM H-1152, administered 15 seconds apart, were applied as eye drops four times daily for the first 3 days, then three times daily for 7 more days. Basic saline solution was applied to the contralateral eye as a control. Wound healing, characterized by corneal thinning, reduction of corneal edema, and restoration of corneal transparency, was documented once daily using slit-lamp examination, photography, and handheld ultrasound pachymeter (PachPen; Accutome, Malvern, PA, USA). The pachymeter recorded corneal thickness measurements up to 1000 μm, and each corneal thickness measurement the pachymeter recorded constituted the mean of nine individual corneal thickness measurements. Three of these average corneal thickness measurements were recorded daily and averaged to assess corneal thinning over time. After 10 days, the rabbits were euthanized and their corneas were excised from the enucleated eyes. The histologic evaluation of corneal endothelial wound healing was performed as described before.[35] Briefly, the isolated corneas were whole-mount stained with 0.25% trypan blue and 0.2% alizarin

red. After staining, the cornea was flat mounted on a glass slide and images were captured by using light microscopy (Axioplan 2; Zeiss).

Statistics

Student's *t*-test was performed to determine statistical significance between mean values in each set of two groups being compared. A *P* value less than 0.05 was considered to be statistically significant. Analyses were performed using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

RESULTS

RhoA and ROCK Expression Profile in Human Eyes

All six human eyes evaluated showed a positive staining for RhoA, ROCK-1, and ROCK-2 in the corneal epithelium (not shown) and corneal endothelium (Fig. 1). Comparing the relative immunostaining intensity of the three proteins in either human corneal epithelial or endothelial cells, there was no significant difference among these proteins.

Effect of ROCK Inhibitors Y-27632 and H-1152 on Cytoskeleton of Cultured Porcine CECs

Corneal endothelial cells were successfully cultured and passaged from the endothelial surface area of the porcine cornea using the trypsin/EDTA digestion protocol. Corneal endothelial cells formed a monolayer in culture and were positively identified by their typical polygonal morphology as well as the staining pattern of ZO-1 (Fig. 2). Monolayers of confluent CECs were treated with 10-μM Y-27632 or 2.5-μM H-1152 for 2, 24, and 48 hours in serum-free media. Confocal microscopy showed a complete collapse of actin cytoskeletal architecture with markedly reduced actin stress fibers and tight junctions within 2 hours of drug administration compared with a serum-starved control, as assessed by actin-phalloidin and ZO-1 staining (Fig. 3). Cell morphology was largely restored within 48 hours of drug treatment.

Enhanced Migration and Proliferation of Cultured Porcine CECs After Administration of ROCK Inhibitors Y-27632 and H-1152

Differences in migration of porcine CECs were found to be statistically significant with administration of 10-μM Y-27632 ($P = 0.003$ at 24 hours, $P = 0.003$ at 48 hours) and 2.5-μM H-1152 ($P = 0.016$ at 24 hours, $P = 0.004$ at 48 hours) when compared with control (Fig. 4). H-1152 demonstrated the most profound stimulatory effect on CEC migration at 24 and 48 hours, showing statistically significant enhancement of migration
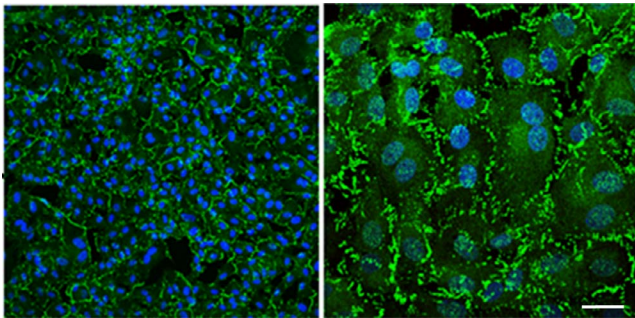
FIGURE 2. Visualization of porcine CECs isolated from freshly enucleated tissue and characterized by positive ZO-1 (*green*) staining. Using confocal microscopy images showed flattened polygonal cells and cell-cell tight junctions (*left*, 20× and *right*, 64×). Cell nuclei were counterstained with DAPI (*blue*). *Scale bar*: 20 μm.

versus control and also versus cells treated with Y-27632 (*P* = 0.01 at 24 hours, *P* = 0.02 at 48 hours). Under the treatment, cells did not migrate together as a sheet. They were dislodged from the leading edge and moved into the open space instead.

In the control group, a smaller number of cells were moved away from the edge compared with the treated group. Proliferation of porcine CECs was significantly higher with administration of 2.5-μM H-1152 compared with control (*P* = 0.0187) and also versus cells treated with Y-27632 (*P* = 0.04) at 24 hours (Fig. 5). Of note, Y-27632 treatment did not demonstrate a statistically significant improvement in proliferation of CECs versus control at 24 hours or 48 hours.

In Vivo Surgical Wound Healing With Topical Administration of H-1152

By visual observation, multiple administration of 1-mM H-1152 eye drops daily in surgically wounded rabbit eyes for 10 days improved corneal opacification associated with original injury made to the corneal endothelium (Fig. 6A). Differences in corneal thickness in eyes treated with 1-mM H-1152 showed decreased corneal swelling compared with the basic saline solution control; however, differences in corneal thickness measurements between treated eyes and control eyes approached, but did not achieve, statistical significance (*P* =
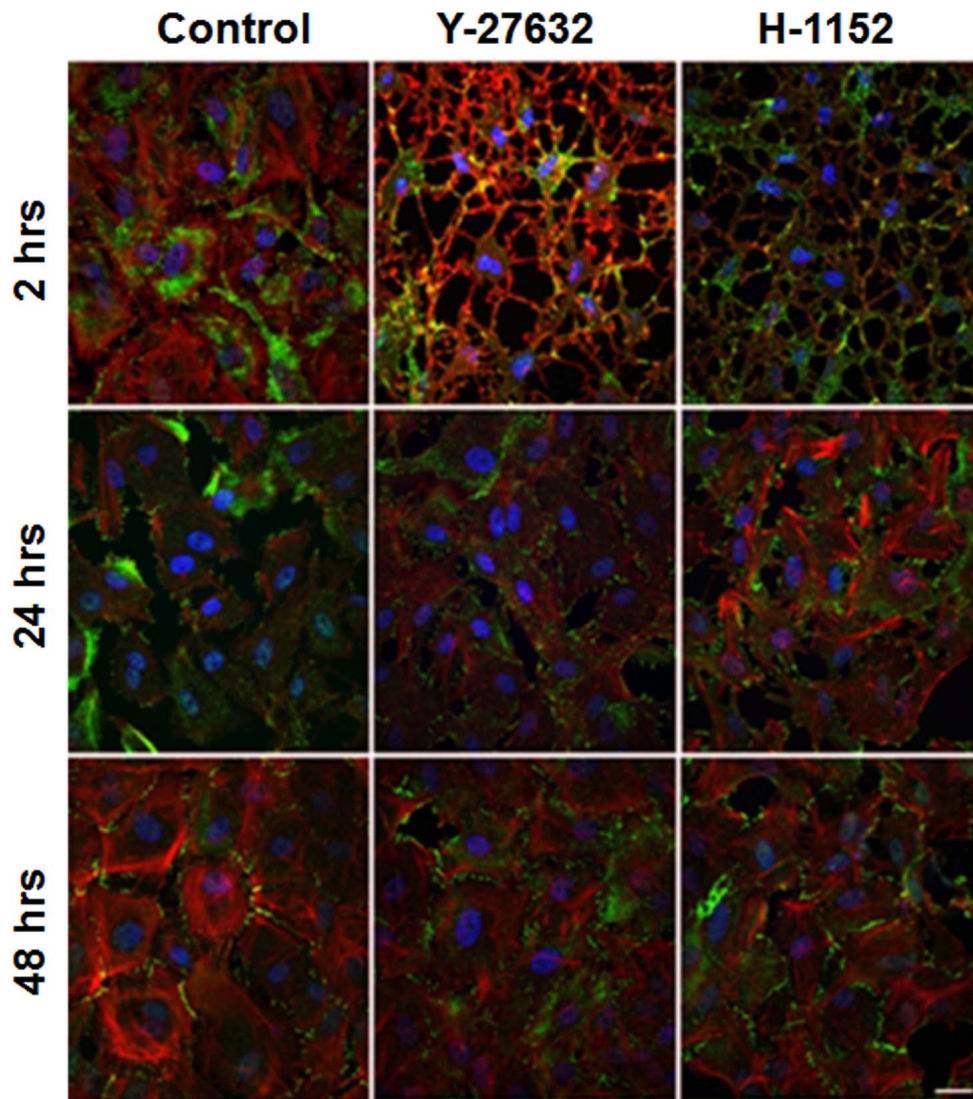

FIGURE 3. Changes in porcine CEC cytoskeleton with administration of ROCK inhibitors Y-27632 and H-1152. Porcine CEC cytoskeleton was visualized by immunofluorescence staining following administration of Y-27632 and H-1152 at 2, 24, and 48 hours. Confocal microscopy imaging showed loss of actin stress fibers and cell-cell tight junctions with positive F-actin-phalloidin (*red*) and ZO-1 (*green*) staining. Corneal endothelial cells morphology appeared largely restored by 48 hours after the chemical administration. *Scale bar*: 20 μm.
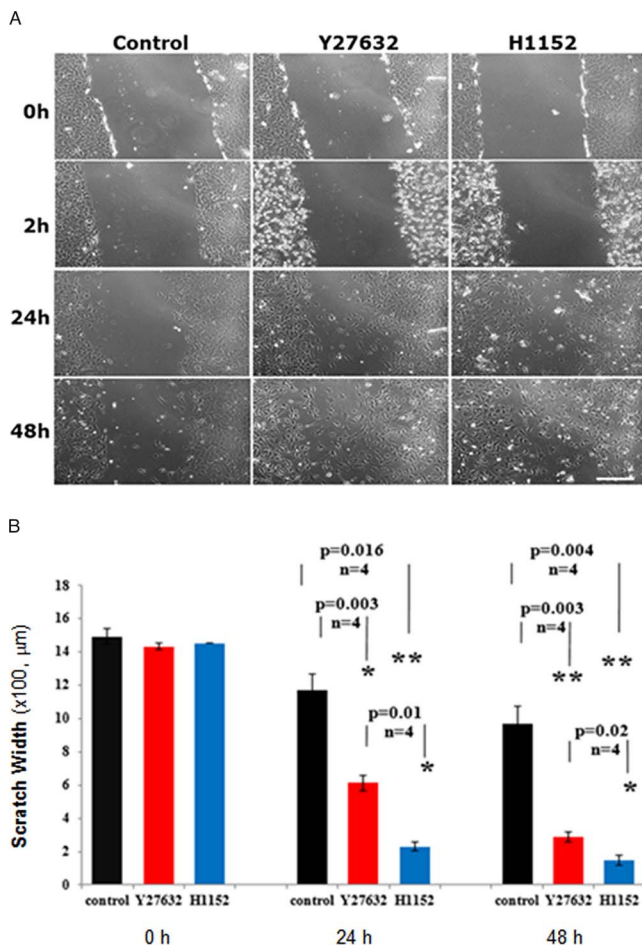
A

B

Figure 4. (A) Migration of porcine CECs following administration of ROCK inhibitors Y-27632 and H-1152. Phase contrast microscopy captured images demonstrating enhanced in vitro migration of CECs across a 48-hour time-period occurring secondary to administration of 2.5 μM H-1152 or 10 μM Y-27632. *Scale bar*: 500 μm. (B) Enhancement of porcine CEC migration by ROCK inhibitors Y-27632 and H-1152. Distance between advancing cell borders was recorded at three evenly-spaced, predetermined locations along the length of the scratch and averaged for each group of CECs treated with 2.5 μM H-1152 vs. 10 μM Y-27632 versus control at timed increments of 2, 24, and 48 hours, respectively. Results showed that both Y-27632 and H-1152 led to a statistically significant increase in wound closure (i.e., CEC migration) at 24 hours and 48 hours versus control. Additionally, compared with Y-27632, H-1152 promoted migration of CECs to a degree also found to be statistically significant at 24 and 48 hours.

0.0523) at 100 hours (Fig. 6B). To evaluate the residual wound size of corneal endothelium after the treatment by histology, the corneas of euthanized rabbits were stained with alizarin red and trypan blue. Compared with the corneas treated with balanced saline solution, the H-1152–treated ones exhibited areas of wound closure, as well as multinucleate CECs with variable morphology suggestive of enhanced migration and proliferation associated with wound healing (Fig. 7).

Discussion

The objectives of this study were to show the efficacy of ROCK inhibitors Y-27632 and H-1152 in stimulating in vitro proliferation and migration of CECs and to demonstrate that topical application of H-1152 improved corneal endothelial wound healing in an in vivo rabbit model. In an effort to further clarify the role of Rho GTPase/Rho kinase signaling and the effect of
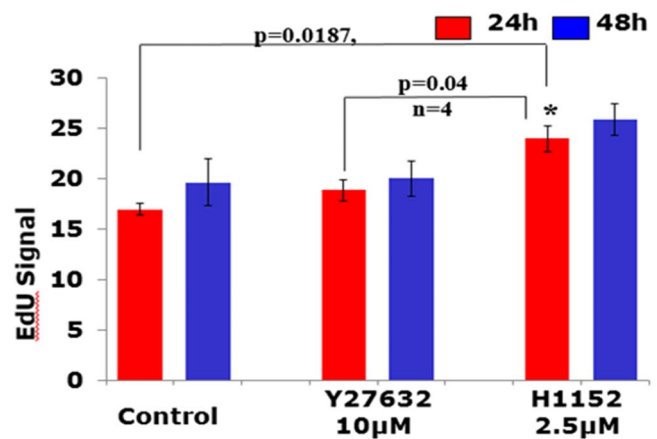

Figure 5. Enhancement of porcine CEC proliferation by ROCK inhibitors Y-27632 and H-1152. EdU was incorporated into the DNA of replicating CECs (not pictured) and signal intensity was recorded at 24 and 48 hours following incubation using spectrophotometry to illustrate effects of 2.5 μM H-1152 and 10 μM Y2763. At 24 hours incubation, H-1152 led to a statistically significant increase in CEC proliferation compared with both control and CECs treated with Y-27632.
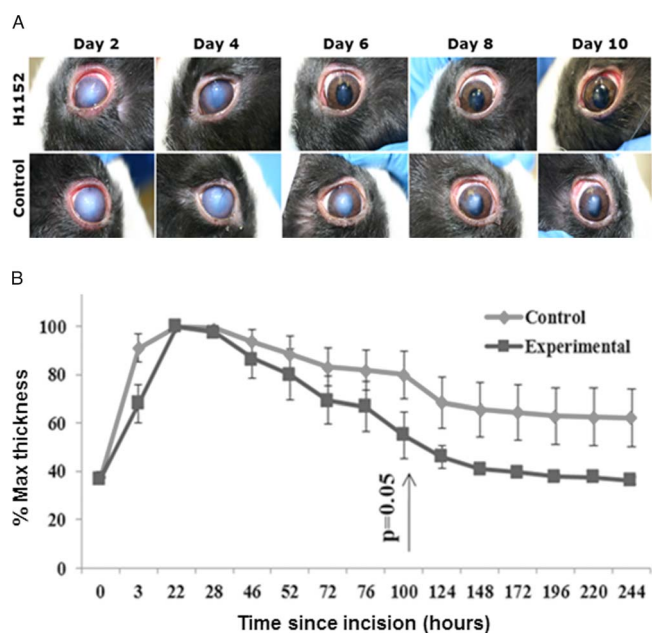

A

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Figure 6. (A) Rabbit corneal wound healing with topical administration of H-1152. Compared with control (treated with basic saline solution), topical administration of 1 mM H-1152 three to four times daily for 10 days led to faster and more complete resolution of corneal edema associated with surgical wound made to corneal endothelium of live rabbits. Rabbit eyes were examined daily using photography, slit-lamp examination, and handheld ultrasound pachymetry. (B) Effect of H-1152 on corneal thickness in vivo. Corneal thickness measurements were recorded daily for each rabbit under study, and values were averaged and categorized as either H-1152–treated experimental eyes or control eyes treated with basic saline solution. Because the handheld pachymeter was limited to measuring up to a thickness of 1000 μm, the observed changes in corneal thickness occurring over time were expressed as a percentage of maximum recorded thickness (941 nm). Graphical representation of the data collected shows that H-1152–treated eyes did undergo more corneal thinning than did control, and this was attributed to the stimulatory effect of H-1152 on CEC migration and proliferation. However, the differences in corneal thickness between experimental and control were not found to be statistically significant during first 3 days of the treatment.
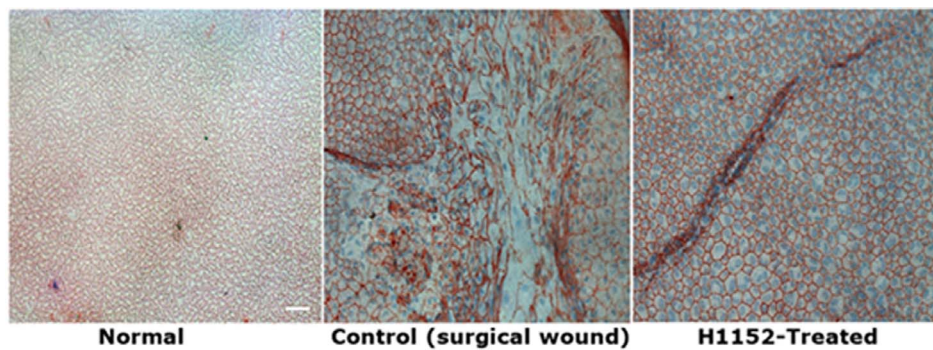
FIGURE 7. Rabbit corneal endothelium following surgery and H-1152 treatment. Rabbit eyes under study were enucleated and corneas were stained with alizarin red to evaluate wound healing. Visualization of tissue treated with H-1152 (*right*) using fluorescent microscopy displayed changes in cell morphology and multinucleate CECs suggestive of proliferation associated with healing. In comparison, eyes treated with basic saline solution (*center*) showed evidence of cell sloughing, intense alizarin red staining, and cell matrix distortion indicative of unhealed injury. *Scale bar*: 100 μm.

its inhibition on corneal tissue, we first validated the presence of RhoA GTPase, as well as its downstream effectors, ROCK-1 and ROCK-2, in the corneal epithelium and endothelium using immunohistochemical staining techniques. RhoA comprises a subfamily of Rho GTPases known to be ubiquitously expressed throughout the human body that operate by activating ROCK, which selectively phosphorylates a wide variety of substrates important for cell signaling and modulation of the cytoskeleton.[36] Specifically, RhoA/ROCK are known to promote cell contraction through formation of cell-cell tight junctions, focal adhesions, and actin stress fibers. This is accomplished primarily by increasing Ca^{2+} sensitization of smooth muscle through phosphorylation of myosin phosphatase.[11,37] Phosphorylation of this phosphatase enzyme suppresses its activity and leads to a net increase in phosphorylated myosin light chain available for actin-myosin cross-bridging, and stress fiber formation.[37,38] Other important substrates phosphorylated by RhoA activation include CPI-17 and LIM kinases 1 and 2, which indirectly stabilize actin stress fibers by inhibiting myosin phosphatase and a depolymerizing enzyme known as cofilin, respectively.[39,40] These signaling events collectively govern cell behavior by influencing cell morphology, actomyosin organization, cell motility, cell cycle progression, and apoptosis.[41–43] We observed reversible changes in porcine CEC cytoskeleton after Y-27632 or H-1152 application in vitro that consisted of loss of actin stress fibers, focal cell adhesions, and cell-cell tight junctions (i.e., the opposite net effect of Rho GTPase activation). Similar findings have been reported in primary cultures of trabecular meshwork cells treated with Y-27632 or H-1152.[22,44–47] However, further research is necessary to elucidate the precise mechanism by which selective ROCK inhibitor agents initiate these cellular changes.

We believe that these cell adhesive and cytoskeletal changes contribute to the enhanced proliferation and migration of CECs observed after application of selective ROCK inhibitors previously documented in the literature.[7–9,36] To confirm these earlier observations and to stratify efficacy of different ROCK inhibitors in achieving these target results, we administered Y-27632 and H-1152 to porcine CECs in vitro. Our findings demonstrated that H-1152 enhanced migration and proliferation of CECs to a greater degree than Y-27632, leading us to move forward with in vivo experiments using the more potent H-1152.

Our approach of topically applying H-1152 to the injured corneas of live rabbits for a period of 10 days proved to be a successful experimental treatment. Compared with control, injured eyes treated with H-1152 experienced accelerated corneal thinning and resolution of corneal edema, a finding representative of restored Na-K ATPase pump function likely due to the enhanced migration and proliferation of CECs we observed in vitro following administration of ROCK inhibitor. However, additional research is needed to determine which aspect of healing, cell migration or cell proliferation, plays a more important role in corneal endothelial wound healing. Our model of endothelial injury, in which trained ophthalmologists surgically removed the corneal endothelium from the basal surface of the cornea (Meekins L, et al. *IOVS* 2012;53:ARVO E-Abstract 6017) provided us with great control. This model also more accurately mimicked the natural end-stage of CED, in which the corneal endothelium is entirely degenerated, damaged, or removed. Similar to results of previous experiments studying effects of Y-27632 on corneal endothelial wound healing,[8,9] topical application of H-1152 in our study also improved corneal wound healing in vivo based upon changes in corneal thickness, edema, and wound size. In light of our findings, we are hopeful that ROCK inhibitors are a potentially viable topical therapy for patients with CED. As previously stated, topical ROCK inhibitor agents have already entered clinical trials for the treatment of glaucoma with minimal reported toxicologic effect to date.[23,25,42] The application of topical ROCK inhibitor agents to corneal epithelial injuries has also been explored. Topically administered Y-27632 promoted migration of corneal epithelial cells while inhibiting their proliferation,[48] a finding that implies there may be some variability in ROCK inhibitors' effects on cell migration and cell proliferation that is determined by cell type. Nonetheless, additional trials investigating topical administration of ROCK inhibitor Y-39983, an analog of Y-27632, and its effects on the corneal epithelium reported no adverse drug-induced side effects in animals under study[26]; this data demonstrates that topically administered ROCK inhibitor agents possess an attractive safety profile that support the transition of our research focus from animals to human trials.

Before moving forward with human trials, however, there are some drawbacks to this study design that should be addressed. With respect to our in vivo rabbit study, it is well documented that rabbit CECs retain a substantial proliferative potential, which allows for enhanced proliferation of CECs in vivo following endothelial injury.[49] This retained proliferative capacity may have interfered with the wound healing data we collected, and selection of an alternative source of CECs belonging to an animal species that more closely resembles the senescence of human CECs, such as the cynomolgus monkey,[6] could be used as a more effective in vivo model. Similarly, to ensure that the increased wound healing we observed in vivo was not random, routine IOP measurements[11] could have been recorded and trended over time to demonstrate appropriate ocular absorption of H-1152. Concentrations of H-1152 in the

aqueous humor could have also been obtained from enucleated rabbit eyes to show that topically administered H-1152 was passing through the cornea and making direct contact with damaged endothelial cells. Furthermore, to more clearly delineate whether migration or proliferation of CECs primarily contributed to in vivo corneal endothelial wound healing, and to more definitively show that active proliferation of CECs was taking place in the presence of H-1152, BrdU labeling of the animals' corneal endothelia prior to enucleation could have been performed. Additionally, specular microscopy provides an alternative imaging modality that could effectively capture changes in CEC density during ROCK inhibitor delivery in our in vivo model.

The major underlying assumption that we are making in these experiments is that diseased human corneal endothelium afflicted by some form of CED (i.e., FECD, posterior polymorphous dystrophy [PPMD], etc.), will respond to topical administration of ROCK inhibitors as robustly as observed here.

In conclusion, we investigated the efficacy of different ROCK inhibitors Y-27632 and H-1152 in stimulating CEC proliferation and migration in vitro and in vivo, hoping that evaluation of these parameters would help us assess the therapeutic potential of these agents. This study not only demonstrated the stimulatory effects of ROCK inhibitors Y-27632 and H-1152 on CEC proliferation and migration in vitro, but also showed that H-1152 was more effective in achieving these target results of improved wound healing. Rearrangement of porcine CEC cytoskeleton secondary to application of Y-27632 and H-1152 triggered cell adhesive changes that contributed to enhanced proliferation and migration. In vivo, topical administration of H-1152 led to marked reduction of corneal edema and furthermore, eyes treated with H-1152 displayed multinucleate CECs suggestive of proliferation and wound healing. The mode of administration (topical drops for animal models) of ROCK inhibitors being studied supports the practicality of these agents as potential noninvasive therapeutic options for patients with CED. This data not only provides greater insight into the true mechanism of ROCK signaling pathway inhibition as it pertains to CEC behavior, but also moves us one step closer to treating patients with CED more effectively, and with topical medications versus surgical intervention. These preliminary studies demonstrate the efficacy of ROCK inhibitors as potential topical therapy for patients with CED. Further investigation incorporating clinical application of this new eye drop treatment is warranted.

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