Rho kinases regulate corneal epithelial wound healing

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Yin J, Yu F-S. Rho kinases regulate corneal epithelial wound healing. Am J Physiol Cell Physiol 295: C378–C387, 2008. First published May 21, 2008; doi:10.1152/ajpcell.90624.2007.—We have previously shown that Rho small GTPase is required for modulating both cell migration and proliferation through cytoskeleton reorganization and focal adhesion formation in response to wounding. In the present study, we investigated the role of Rho kinases (ROCKs), major effectors of Rho GTPase, in mediating corneal epithelial wound healing. Both ROCK 1 and 2 were expressed and activated in THCE cells, an SV40-immortalized human corneal epithelial cell (HCEC) line, in response to wounding, lysophosphatidic acid, and heparin-binding EGF-like growth factor (HB-EGF) stimulations. The ROCK inhibitor Y-27632 efficiently antagonized ROCK activities without affecting Rho activation in wounded HCECs. Y-27632 arrested basal and HB-EGF-enhanced scratch wound healing and enhanced cell migration and adhesion to matrices, while retarded HB-EGF-induced cell proliferation. E-cadherin- and β-catenin-mediated cell-cell junction and actin cytoskeleton organization were disrupted by Y-27632. Y-27632 impaired the formation and maintenance of tight junction barriers indicated by decreased trans-epithelial resistance and disrupted occludin staining. We conclude that ROCK activities enhance cell proliferation, promote epithelial differentiation, but negatively modulate cell migration and cell adhesion and therefore play a role in regulating corneal epithelial wound healing.

corneal epithelial cells; cell migration; cell proliferation; cell-matrix adhesion; cell-cell junctions

THE CORNEAL EPITHELIUM is continuously subjected to physical, chemical, and biological insults, often resulting in a wound and the loss of barrier functions. The proper healing of corneal wounds is vital to maintaining a clear, healthy cornea and to preserving vision (24). The corneal epithelium responds rapidly to injury by migrating as a sheet to cover the defect and to reestablish its barrier function (24). Successful wound healing involves a number of processes including cell migration, proliferation, cell-matrix adhesion, and tissue remodeling, which are often driven by growth factors and other factors released coordinately into the injured area by epithelial cells (27, 65). Prominent among these epithelium-derived factors are ligands for epidermal growth factor (EGF) receptor (EGFR), such as EGF and heparin-binding EGF-like growth factor (HB-EGF; Ref. 24). In addition, other cellular components, such as ATP and lipid mediator lysophosphatidic acid (1-acyl-2-hydroxy-sn-glycero-3-phosphate, LPA), have been shown to be released from the injured cells to enhance epithelial migration and wound healing in the cornea (54, 57, 60, 64).

The Rho family of small GTPases, including Rho, Rac, and Cdc42, are small monomeric G proteins that cycle between an inactive GDP-bound form and an active GTP-bound form and regulate actin cytoskeleton, cell migration, and proliferation (10, 15). Rho regulates actin polymerization, resulting in the formation of stress fibers and the assembly of focal adhesion complex (19, 32, 38). Rho has been implicated in cell migration, actin organization, focal adhesion formation, as well as adherens and gap junction assembly in the corneal epithelium (3, 31, 40). We recently showed that wounding, HB-EGF, and LPA induced RhoA activation, and Rho activity is required for modulating both cell migration and proliferation, through cytoskeleton reorganization and focal adhesion formation in response to wounding in corneal epithelial cells (63).

Rho kinases (ROCKs), the first identified and best characterized Rho downstream effectors, are protein serine/threonine kinases with a molecular mass of ~160 kDa. Two isoforms have been identified: ROCK 1 and ROCK 2 (14, 21, 22, 28). They were initially characterized for their roles in mediating the formation of RhoA-induced stress fibers and focal adhesions, as well as actomyosin contractility, through their effects on the phosphorylation of myosin light chain (21, 39, 47). In addition, they are involved in many other cell behaviors such as cell-substrate and cell-cell adhesion, cell migration, differentiation, apoptosis, and proliferation (23, 29, 39, 44, 58).

ROCKs have been suggested to play roles in many pathological conditions such as neurological and cardiovascular disorders (23, 29). In the cornea, ROCKs have been suggested to be involved in epithelial differentiation (49), cell cycle progression (4), cell-cell adhesion (3), endothelial barrier integrity (8, 42, 43), stromal cell phenotype conversion (2, 11), cytoskeleton reorganization, contractility (17), and cell-matrix interaction (16, 20, 34). However, the precise role of ROCKs during corneal epithelial wound healing and the underlying mechanisms remain elusive.

In the present study, we demonstrated that ROCK 1 and ROCK 2 are activated in response to wounding, LPA, and HB-EGF stimulations and that inhibition of ROCK enhances wound closure by modulating cell migration, proliferation, cell-matrix adhesion, and cell-cell adhesion in HCECs.

MATERIALS AND METHODS

Materials. Defined keratinocyte serum-free medium (DK-SFM) and TRIzol Reagent were purchased from Invitrogen (Grand Island, NY). Keratinocyte basal medium (KBM) was from BioWhittaker (Walkersville, MD). Human recombinant HB-EGF was obtained from R&D Systems (Minneapolis, MN). The ROCK inhibitor Y-27632 was from Calbiochem (La Jolla, CA). Antibodies against phospho-ERK1/2 (p42/p44), ERK2 (p42 MAPK), ROCK 1, and ROCK 2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase conjugate secondary antibodies were from Bio-Rad (Hercules, CA). Antibodies against phospho-myosin phosphatase target

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subunit 1 (MYPT1, Thr-696) and MYPT1, as well as recombinant MYPT1 protein were from Upstate Signaling Technologies (Lake Placid, NY). RhoA and occludin antibodies were from Cell Signaling (Danvers, MA) and Zymed Laboratory (South San Francisco, CA), respectively. 5-Bromo-2'-deoxy-uridine (BrDU) labeling and detection kit were from Roche Applied Science (Indianapolis, IN). MTT cell proliferation assay kit was purchased from American Type Culture Collection (Manassa, VA). The 12-well Boyden chamber was from Neuroprobe (Cabin John, MD). Polycarbonate membranes (14-µm pores) were from Osmonics (Livermore, CA). Fibronectin-collagen coating mix (FNC, a 1:3 mix of fibronectin and collagen 1) was from Athena Environmental Service (Baltimore, MD). Growth factor re-coating mix (FNC, a 1:3 mix of fibronectin and collagen 1) was from BD Biosciences (San Jose, CA). Transwell Polycarbonate Transwell plates (0.4-µm pore size) were from Costar (Cambridge, MA). Mouse E-cadherin and mouse β-catenin antibodies were from BD Biosciences. Rhodamine phallidin was from Invitrogen (Carlsbad, CA). FITC-conjugated antibody against mouse or rabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and extensive wounding study. THCE cells, an SV40-immortalized human corneal epithelial cell (HCEC) line (5), were grown in DK-SFM in a humidified 5% CO2 incubator at 37°C and growth factor starved in KBM for 16 h before experiments. To create extensive wounding for biochemistry studies, cells cultured on 100-mm dishes were wounded by multiple linear scratches using a cut of 48-well shark's tooth comb for DNA sequencing gel (Bio-Rad, Hercules, CA) going from one side of the dish to the other. The dish was then rotated, and scrapes were made similarly to the original scrapes at 45, 90, and 135°. After wounding or other treatments, cells were lysed with RIPA buffer (150 mM NaCl, 100 mM Tris–HCl pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM NaF, 100 mM sodium pyrophosphate, 3.5 mM sodium orthovanadate, proteinase inhibitor cocktail, and 0.1 mM PMSF), and protein concentrations were determined using a Micro BCA protein assay kit (Pierce, Rockford, IL).

RNA isolation and semiquantitative RT-PCR. Total RNA was isolated from THCE cells using the TRIzol solution according to the manufacturer's instructions (Invitrogen), and 2 µg of total RNA were reverse transcribed with a first-strand synthesis system for RT-PCR (SuperScript, Invitrogen). cDNA was amplified by PCR using primers for human ROCK 1 (sense 5′-GAAGAAAGAAGAGCTCGAGAA-GAAGG-3′; antisense 5′-ATCTTGTAGCTCCGCATGTG-3′) and ROCK 2 (sense 5′-AATCTACGTGTGTTTCCCTGAAGATA-3′; antisense 5′-TTCTATTTCCTGTTGTAGTATGGAA-3′; Ref. 9). The PCR products were subjected to electrophoresis on 1% agarose gels containing ethidium bromide. Stained gels were captured using a digital camera.

Rho and ROCK activity assays and Western blotting. THCE cells were pretreated with 10 µg/ml Rho inhibitor Clostridium botulinum exoenzyme C3 (C3) for 24 h or 10 µM ROCK inhibitor Y-27632 for 1 h before being extensively wounded. Five hundred micrograms of proteins were immunoprecipitated with 4 µg ROCK 1 or ROCK 2 antibody overnight at 4°C. Immunocomplexes were incubated with 20 µl of protein A/G plus agarose for 3 h at 4°C, washed three times with PBS, and resuspended in 50 µl of Tris–ATP buffer (50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 10 mM magnesium acetate, and 100 µM ATP). The kinase assay was performed using the following Upstate’s protocol in the presence of 1 µg of recombinant MYPT1 at 30°C for 30 min. Phosphorylation and total levels of MYPT1 recombinant protein were detected using phospho-MYPT1 and MYPT1 antibodies, respectively. The activation status of Rho was assayed using fusion protein GST-21 as described previously (63). Briefly, 1 mg cell lysate was incubated with GST-C21 fusion protein and glutathione S-transferase beads. The beads were washed three times with lysis buffer, and bound GTP-Rho was detected by immuno blot analysis with RhoA antibody. ERK phosphorylation and total proteins of ROCK 1, ROCK 2, MYPT1, and ERK in cell lysates were detected by Western blotting using corresponding antibodies.

Scratch wounding healing studies. Growth factor-starved THCE cells were wounded with a sterile 0.1–10 µl pipette tip (TipOne, USA Scientific, Ocala, FL) to remove cells by two perpendicular linear scrapes. After suspended cells were washed away, the cells were re-fed with KBM containing 50 ng/ml HB-EGF and/or 10 µM Y-27632. Wound closure was photographed both immediately and 24 h post-wounding at the same spot with an inverted Zeiss microscope equipped with a SPOT digital camera. The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas vs. the original wound area.

Determination of cell proliferation. Cell proliferation was determined by two methods: BrDU incorporation and MTT cell proliferation assay. BrDU incorporation assay was carried out following the manufacturer's instructions. Briefly, THCE cells were grown on glass chamber slides, growth factor starved, and pretreated with 10 µM Y-27632 for 1 h. Confluent cells were wounded with a sterile 0.1–10 µl pipet tip and re-fed with fresh KBM containing 50 ng/ml HB-EGF and/or 10 µM Y-27632 and BrDU-labeling reagent for 24 h. Cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% horse serum and 1% BSA in PBS for 1 h at room temperature, and then incubated with anti-BrDU antibody (1:10) overnight at 4°C followed by incubation with anti-mouse IgG-fluorescent antibody (1:10). Cell proliferation was also measured using an MTT cell proliferation assay kit, following the manufacturer’s instructions (American Type Culture Collection). Growth factor-starved THCE cells were cultured in KBM containing 50 ng/ml HB-EGF and/or 10 µM Y-27632 for 24 h. Cells were incubated in 100 µM KBM containing 10 µM MTT reagent for 2 h. Detergent reagent (100 µl) was added to each well and cells were further incubated for 2 h. Absorbance at 595 nm was quantified on a GENios Fluorometer.

Boydren chamber analysis for cell migration. Growth factor-starved THCE cells were pretreated with 10 µM Y-27632 for 30 min. Cells were detached by 0.05% trypsin and 0.53 mM EDTA, washed with 10% donor calf serum in PBS to neutralize the trypsin, and adjusted to an equal cell number of 6 × 10^6/ml; 170 µl of cell suspension were added to top wells of the Boyden chambers, separated by a polycarbonate membrane (pore size: 14 µm) precoated with FNC (a 1:3 mix of fibronectin and collagen 1) from the lower chambers loaded with basal medium with or without 50 ng/ml HB-EGF. Cells were allowed to migrate for 3 h. After incubation, cells on the top of the filter membrane were removed by scraping. The filter membrane was then stained with a modified staining kit (Diff-Quik; Dade Behring, Dudingen, Switzerland). Cell migration was quantified as the number of migrated cells on the lower surface of the filter membrane in three random fields under ×400 magnification.

Cell-matrix adhesion assay. The adhesion of THCE cells to two matrices, FNC (1:1.3 mix of fibronectin and collagen 1) and Matrigel (BD Biosciences), were tested. The 96-well plates were coated with FNC or Matrigel (1:5) diluted in PBS for 60 min before blocked with 10% donor calf serum. BSA for 30 min to prevent nonspecific binding. Fifty microliters of basal medium containing 100 ng/ml HB-EGF, and/or 20 µM Y-27632 or 20 µg/ml C3 were added to each well. Growth factor-starved THCE cells were pretreated with 10 µM Y-27632 for 30 min or 10 µg/ml C3 for 24 h. Cells were detached by 0.05% trypsin and 0.53 mM EDTA, washed with 10% donor calf serum in PBS, and adjusted to an equal cell number of 1 × 10^6/ml. 50 µl of cell suspension was added to each well (final concentration: 50 ng/ml HB-EGF, and/or 20 µg/ml C3). Cells were allowed to adhere to the matrices for 30 min; nonadherent cells were then washed off with PBS. Cells were fixed with 5% glutaraldehyde and stained with 0.1% crystal violet solution for 60 min. Plates were extensively washed with water to remove excessive staining, and the dye was solubilized with 10% acetic acid. Absorbance at 570 nm was quantified on a GENios Fluorometer.
Immunostaining of E-cadherin, β-catenin, occludin, and actin. THCE cells cultured on 4-well glass chamber slides (E-cadherin and β-catenin), or 12-mm Transwell inserts (occludin) were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA in PBS for 1 h at room temperature, and then incubated with E-cadherin (1:250), β-catenin (1:50), occludin (1:100) antibodies, or mouse or rabbit IgG isotype controls overnight at 4°C, followed by incubation with FITC-conjugated secondary antibody (1:100) and rhodamine-phalloidin (1:50) to visualize actin filaments. The slides were then washed in PBS and mounted with Vectashield mounting medium with DAPI and examined under a Carl Zeiss fluorescence microscope Axioplan 2 equipped with an ApoTome digital camera. Negative controls (mouse or rabbit IgG isotypes) exhibited no fluorescence (data not shown).

Measurement of transepithelial electrical resistance. THCE cells were plated on 12-mm Transwell filter with 0.4-μm pore polyester membrane insert (1×10⁶ cells per insert) and cultured in DK-SFM with or without 10 μM Y-27632. Transepithelial electrical resistance (TER) was measured with STX-2 electrodes and an EVOM voltohmometer (World Precision Instruments, Sarasota, FL) daily. The background TER of blank Transwell filters was subtracted from the TER of cell monolayers.

Statistical analysis. Results are means ± SE. Statistical parameters were ascertained by software (SigmaStat) with Student’s t-test between two groups. P < 0.05 indicates a significant difference.

RESULTS

We first determined the expression of ROCK 1 and ROCK 2 in HCECs. As shown in Fig. 1, the mRNAs for both ROCK 1 and 2 were detected in THCE cells. To asess if ROCK was involved in HCEC response to epithelial injury, the kinase activities of ROCK 1 and 2 were examined at different time points postwounding. As shown in Fig. 2, ROCK activities, assessed by the phosphorylation of exogenously added ROCK substrate MYPT1, were increased as early as 10 min (the earliest time tested) and remained elevated 2 h postwounding, while the levels of total ROCK 1 and ROCK 2 proteins in cell lysates and exogenous MYPT1 protein were relatively unchanged. Since we have observed that both HB-EGF and LPA enhanced corneal wound healing and induced RhoA activation in vitro (59, 60, 63), we also assessed the effects of these factors on ROCK activation. Both LPA and HB-EGF increased ROCK kinase activities, suggesting that the RhoA/ROCK pathway may act as a downstream effector of these extracellular stimuli. Phosphorylation of ERK1/2 confirmed cell activation in response to wounding, HB-EGF and LPA.

To determine whether ROCKs function as Rho downstream effectors in HCECs, we first assessed the effects of ROCK inhibitor Y-27632 and Rho inhibitor exoenzyme C3 on Rho and ROCK activities, respectively. Wounding-enhanced kinase activities of both ROCK 1 and 2, indicated by increased MYPT1 phosphorylation, were attenuated by both C3 and Y-27632, suggesting that ROCKs act downstream to Rho in wounded HCECs and Y-27632 was effective in inhibiting ROCK activities (Fig. 3, A and B). Wounding-induced Rho activation, however, was unaffected by Y-27632, confirming...
the linear relationship between Rho and ROCK in HCECs and the specificity of Y-27632 (Fig. 3C).

To determine the role of ROCKs in epithelial wound healing, we assessed the effects of Y-27632 on epithelial wound closure of THCE monolayer (Fig. 4). The presence of Y-27632 slightly increased the basal healing of THCE cells from 25% to 35.5% 24 h postwounding. Compared with the control, exogenously added HB-EGF significantly promoted epithelial wound closure (43% healed), which was further enhanced by Y-27632 to nearly 70%, suggesting that ROCKs may negatively regulate corneal epithelial wound healing. Since we previously demonstrated that Rho inhibitor C3 delays wound closure (63), Rho and ROCKs, albeit along the same pathway, exert opposite effects on HCEC wound healing.

Since many cellular processes, such as proliferation, migration, and adhesion to extracellular matrix contribute to corneal epithelial wound closure (24), we next investigated the effects of ROCK inhibition on these processes in cultured HCECs. To elucidate the effects of Y-27632 on cell proliferation, BrdU incorporation assay was performed (Fig. 5A). While unwounded cells exhibited minimum number of BrdU-positive cells; in the wounded epithelial monolayer, there were some proliferating cells near the leading edge, indicated by positive BrdU staining. Inhibition of ROCK activities by Y-27632 had a minimal effect on the basal cell proliferation, as there was no apparent change in the number of BrdU-positive cells. HB-EGF greatly increased the number of BrdU-positive cells, indicating a mitogenic effect of the growth factor on HCECs. This HB-EGF-mediated increase in cell proliferation was diminished in the presence of Y-27632. The effects of ROCK inhibition on cell proliferation were also examined using MTT cell proliferation assay (Fig. 5B). Exogenously added HB-EGF significantly increased cell proliferation rate by 70%, compared with the control. While it exhibited no effect on basal cell proliferation, Y-27632 attenuated the HB-EGF-enhanced proliferation to a level similar to that of the control, indicating that the enhancing effect of Y-27632 on wound healing observed in Fig. 4 may result from mechanisms other than cell proliferation.

We next investigated the effects of Y-27632 on HCEC migration by Boyden chamber assay (Fig. 6). Consistent with a previous report (61), the presence of HB-EGF in the bottom chamber increased the migration of HCECs. Y-27632 significantly enhanced HCEC migration toward FNC (a 1:3 mix of fibronectin and collagen 1) and chemotaxis toward HB-EGF, suggesting that ROCKs may negatively regulate cell motility.

HCEC adhesion to different matrices, FNC and diluted Matrigel, was also investigated (Fig. 7). HB-EGF slightly increased cell adhesion to FNC and Matrigel, while Y-27632 greatly enhanced cell adhesion both in the presence and absence of HB-EGF, suggesting the accelerated wound healing by Y-27632 may result from enhanced cell-matrix adhesions. Rho inhibitor C3, on the other hand, attenuated cell adhesion, indicating that the differences between Rho and ROCKs in the regulation of cell adhesion may account for their opposing effects on wound closure.

To analyze whether ROCKs participate in the formation of adherence junctions, the effects of Y-27632 on the cellular distribution of E-cadherin were determined by immunostaining. In confluent control THCE cells, E-cadherin was localized at cell-cell borders either as typical membrane protein or in a diffuse pattern (Fig. 8A). Y-27632 treatment resulted in a decrease in E-cadherin staining intensity and loss of continuity at some cell-cell junctions (b). E-cadherin exhibited a more defused staining pattern in the presence of HB-EGF (c). Remarkably, cells treated with Y-27632 and HB-EGF simultaneously appeared to be much larger in size and displayed a more "spread and flattened" morphology with much reduced E-cadherin staining at cell-cell junctions (d).

To understand the role of ROCKs in mediating adherence junction in migratory cells, the effects of Y-27632 on actin cytoskeleton and β-catenin staining in scratch-wounded THCE cells were investigated (Fig. 8B). Migrating THCE cells displayed a strong continuous cortical actin network with apparent actin bundle staining parallel to the wounding edge (a). The presence of Y-27632 enhanced the actin bundle staining but disrupted cortical actin staining at cell-cell junctions (arrowheads in c) at the leading edge. In control migratory cells,
β-catenin staining was uninterrupted at cell-cell contact and was also seen in some areas as “line of dots” structure (b). Cells treated with Y-27632, however, displayed diminished staining of β-catenin along cell-cell junctions and strong staining at the wounding edge (arrows in f), which colocalized with thick actin bundles (h).

The role of ROCKs in the formation and maintenance of tight junction-based corneal epithelial barrier was investigated using TER measurement and occludin staining. To determine the role of ROCKs in the maintenance of barrier function, THCE cells were allowed to grow on porous Transwell membranes in growth medium and TER was measured daily. As shown in Fig. 9A, the TER reading increased gradually from ~17 to ~220 Ω within 6 days, suggesting the formation of tight junction barrier. These barrier-forming cells displayed strong occludin immunoreactivity with a well-defined tight junction like structure at cell-cell borders (Fig. 9C, a). From day 6 to day 7, the TER reading continued to increase (control 4–6) in cells cultured in growth medium, whereas cells with Y-27632 added to the culture medium had a decreased TER reading (control 1–3). The net changes in the resistance after drug treatment were significantly different from the controls (Fig. 9A, inset). Interestingly, although the TER reading was still relatively high, occludin staining was dramatically altered in Y-27632-treated cells with more diffuse intracellular staining, suggesting that the disruption of cell-cell border localization of occludin preceded the dysfunction of tight junction barrier in HCECs. To determine the role of ROCKs in barrier formation, THCE cells were cultured in the presence of Y-27632. Inhibition of ROCKs prevented the tight junction barrier from forming, as indicated by low TER readings (<30 Ω; Fig. 9B, Y1–3) and by weak and defused occludin staining (Fig. 9C, c). Withdrawal of Y-27632 from the culture medium resulted in a rapid increase in TER readings (an increase of ~100 Ω within 24 h; Fig. 9B, Y4–6) and the reformation of tight junction like structures (Fig. 9C, d) in THCE cells. Western blot analysis revealed no detectable changes of occludin protein level in the control and Y-27632-treated cells (data not shown). Taken together, these results suggest that ROCKs play a role in the tight junction barrier formation and that the inhibitory effect of Y-27632 on barrier formation was reversible.

DISCUSSION

In the present study, we examined the role of ROCKs in corneal epithelial wound healing and barrier formation. We showed that both isoforms of ROCKs are expressed in HCECs at mRNA and protein levels. Wounding, as well as LPA and HB-EGF, rapidly and robustly increased the kinase activities of ROCK 1 and ROCK 2. ROCK inhibitor Y-27632 was specific and efficient in inhibiting ROCK kinase activities without affecting Rho activity. Y-27632 accelerated basal and HB-EGF-enhanced wound healing. Y-27632 attenuated cell proliferation induced by HB-EGF, promoted cell migration, and enhanced cells adhesion to extracellular matrices; while Rho inhibitor C3 attenuated wound closure (63) and cell adhesion. ROCK activities were also required for cell-cell adhesion mediated by E-cadherin and β-catenin, as well as the formation and maintenance of barrier integrity. Our data indicate that ROCKs play important roles in corneal epithelial homeostasis and wound healing.
Small GTPases of the Rho family, including Rho, Rac, and Cdc42, are essential regulators of several cell functions, such as actin cytoskeleton organization, cell migration, and proliferation (10, 15). Rho regulates actin polymerization, resulting in the formation of stress fibers and the assembly of focal adhesion complex. Rac and Cdc42 induce the formation of filopodia and lamellipodia, respectively, which contribute to the cytoskeletal rearrangements required for cell migration (19, 32, 38). ROCKs were identified as the downstream effectors of Rho (14, 21, 22, 28) and were suggested to link Rho to stress fiber formation through enhancing myosin light chain phosphorylation (18, 46, 51). Two isoforms have been identified: ROCK 1 and ROCK 2 (14, 21, 22, 28), which share a 65% overall identity at the amino-acid sequence level and a 92% identity in their kinase domains (30). In the corneal epithelium, Rho has been implicated in cell migration, actin organization, focal adhesion formation, as well as adherens and gap junction assembly (3, 31, 40), while ROCKs have been suggested to be involved in corneal epithelial differentiation (49), cell cycle progression (4), and cell-cell adhesion (3). Our recent study revealed that Rho activity is required for cell migration, proliferation and focal adhesion formation during the healing process.

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![Figure 7](image1.png)

**Fig. 7.** Spontaneous and HB-EGF-enhanced cell adhesion were promoted by Y-27632 and attenuated by C3. Growth factor-starved THCE cells were pretreated with 10 μM Y-27632 for 30 min (A and B) or 10 μg/ml C3 for 24 h (C and D) and then allowed to attach to FNC (A and C) or 1:5 diluted Matrigel (B and D) in the presence or absence of 50 ng/ml HB-EGF for 30 min. Adherent cells were stained with crystal violet, which was dissolved in acetic acid. Cell adhesion was indicated by readings at 570 nm and are means ± SE (n = 8). *P < 0.05 and **P < 0.001 (Student’s t-test).

![Figure 8](image2.png)

**Fig. 8.** Y-27632 impairs adherence junctions and alters actin cytoskeleton. A: confluent THCE cells were cultured in the presence (c and d) or absence (a and b) of 50 ng/ml HB-EGF with (b and d) or without (a and c) 10 μM Y-27632 for 24 h. E-cadherin staining was visualized as described in MATERIALS AND METHODS (scale bar = 50 μM). Arrows and arrowheads indicate membrane and intracellular localization of E-cadherin, respectively. B: confluent THCE cells were wounded with a pipette tip and allowed to heal in the presence (e–h) or absence (a–d) of 10 μM Y-27632 for 48 h. β-Catenin and actin were visualized as described in MATERIALS AND METHODS. Arrows in f indicate concentrated β-catenin staining at cell-cell contact among cells at the wounding edge (scale bar = 20 μM). Figure represents of 2 independent experiments.
process and contributes to the regulation of wound healing in corneal epithelial cells (63). In the current study, we demonstrated that both ROCK 1 and ROCK 2 are expressed in HCECs and activated in response to wounding. Although ROCK 1 and ROCK 2 can be differentially regulated in certain circumstances, there is no evidence that ROCK 1 and ROCK 2 have different functions (39). Hence, similar expression and activation patterns of both ROCK 1 and 2 suggest they may function as a unit in the regulation of HCEC wound healing.

The biological functions of ROCKs were effectively inhibited by Y-27632, a specific antagonist against ROCK 1 and ROCK 2 with equal potency, and by Rho inhibitor exoenzyme C3, indicating that Y-27632 indeed functions downstream to Rho in wounded HCECs. Moreover, Y-27632 had a minimum effect on wound-induced Rho activation, confirming the upstream-downstream linear relation between Rho and ROCKs and suggesting that the effects of Y-27632 on cell behaviors were specific to ROCKs but not Rho. While Rho inhibition delayed wound closure (63), Y-27632 promoted healing of a scratch wound in cultured HCECs, suggesting distinct roles of Rho and ROCKs in regulating corneal epithelial wound healing. One explanation for the differential effects of Rho and ROCK inhibitions is the multiple downstream effectors for Rho (6). Other effector proteins for Rho include protein kinase N (1), rhotekin (37), citron (25, 26), and noticeably mDia (55, 56). A recent report suggested that mDia1 works concurrently with ROCKs during Rho-induced actin reorganization and that various actin fiber patterns rely on the balance between mDia and ROCK actions (55). Interestingly, both ROCK and Rho GTPase inhibitors have been shown to increase aqueous humor drainage through the trabecular meshwork, leading to a decrease in intraocular pressure.

Fig. 9. Y-27632 disrupts barrier formation. A and B: THCE cells, plated onto Transwell membranes, were cultured in growth medium without (A; C1–6) or with (B; Y1–6) 10 μM Y-27632 for 6 days. At day 6, Y-27632 was added to three samples in A (C1–3) and removed from three samples in B (Y4–6). Cells were further incubated for indicated time and TER was measured daily. Insert in A showed daily changes in TER readings from day 4 to day 5, day 5 to day 6, and day 6 to day 7. Significant difference in the changes of TER readings was observed when Y-27632 was added (a decrease in TER reading) compared with that of the control. **P < 0.001, Student’s t-test. C: THCE cells, plated onto Transwell membranes, were allowed to growth in the absence (a and b) or the presence (c and d) of 10 μM Y-27632 for 5 days. Y-27632 was then either added to (b, corresponding to C1–3; A) or removed from (d, corresponding to Y1–3; B) the culture media. Cells were further cultured for 1 or 2 days before stained for occludin (scale bar = 10 μM). 2 Transwell inserts for each condition.
pressure and to enhance ocular blood flow, retinal ganglion cell survival, and axon regeneration (36).

Using BrdU labeling and MTT cell proliferation assays, we showed that Y-27632 did not affect basal cell proliferation near a scratch wound. As Y-27632 accelerated epithelial wound closure in cultured THCE cells, the minimal effect of Y-27632 on cell proliferation would indicate that cell migration primarily accounted for the observed spontaneous (unstimulated) wound healing in these cells. The effects of Y-27632 on cell proliferation in wound epithelial cells are in sharp contrast with that of Rho inhibitor C3, which greatly attenuated cell proliferation (63). Hence, the function of ROCKs differs from its activator Rho in the regulation of cell proliferation during the healing of an epithelial wound in vitro. Intriguingly, although Y-27632 accelerated HB-EGF-enhanced epithelial wound closure, it significantly inhibited the HB-EGF-induced increase in the number of proliferating cells at the wound edge. How ROCK kinase activity might influence growth factor-induced cell proliferation remains elusive but may be related to its ability to alter the levels of cell cycle regulatory proteins via Ras and the MAPK pathway, a major mitogenic pathway of receptor tyrosine kinases such as EGFR (7).

We previously demonstrated that Rh inhibition delayed wound closure in the presence of hydroxyurea, a cell cycle blocker, suggesting that Rho also participates in cell migration during wound healing (63). In this study, we demonstrated that inhibition of ROCK activity accelerated epithelial wound closure in a cell proliferation-independent manner. Consistent with this observation, it was found that both haptotactic migration (towards matrix proteins) and chemotaxis induced by HB-EGF are enhanced by Y-27632 in Boyden chamber assay. The ability of ROCKs to negatively regulate cell migration may be related to the fact that inhibition of ROCK activities promotes cell adhesion to different matrixes as cell migration requires a dynamic interaction between the cell and its substrate (13). Rh inhibition by C3, on the other hand, led to enhanced cell adhesion, which may also account for the differential roles of Rh and ROCKs during wound healing. In line with the enhanced cell-matrix adhesion, we observed that Y-27632 in the presence of HB-EGF greatly increased the size of culture cells, resulting in a more “spread and flattened” morphology. It is likely that these “flattened” cells are strongly attached to the substratum. It has been previously reported that the Rh/ROCK signaling pathway influences the assembly of E-cadherin adherens junctions in rabbit corneal epithelium (3). It is worth noting that the third type of cell junction, gap junction, also requires Rh/ROCK signaling (3). A recent study (48) demonstrated that mice lacking the Rh-inhibitory protein, p190-B RhoGAP, are reduced in size and such reduction can be rescued by Y-27632, suggesting that ROCKs participate in the control of cell size. Similarly, migrating epithelial cells near the wound edge exhibited a large size and more apparent lamellipodia-type extension into the denuded area with strong cortical actin staining.

Disruption of epithelial barriers has been associated with an increase in microbial infection of the cornea (62). Using TER as an indication of HCEC barrier function, we observed that ROCK activities were required for the formation and maintenance of barrier properties. Staining of occludin in cells cultured on transwell inserts further confirmed that the inhibitory effects of ROCK inhibition on tight junction are reversible. Interestingly, while ROCK inhibition resulted in changes in the occludin staining pattern, the cells with disrupted occluding staining still possessed a relatively high TER reading. A similarly disturbed occluding staining was also observed in cells cultured continuously in Y-27632, and removal of the inhibitor induced redistribution of occludin to cell membrane, resulting in a rapid increase in TER reading (100 Ω/day). These results suggest that alteration of occludin localization precedes barrier breakdown or tight junction formation in cultured HCECs. Alternatively, maintaining tight junction function may not require the presence of occludin at cell-cell borders. Indeed, the barrier function of the intestinal epithelium was normal in occludin knockout mice (41), suggesting that occludin may not be directly involved in barrier function in corneal epithelial cells. Interestingly, inhibiting ROCK significantly reduced the smoke-induced lung epithelial tight junction permeability to both ions and macromolecules (33). Hence, the function of ROCK in mediating tight junction barrier formation and maintenance may be cell-type specific.

Pharmacological analyses revealed that ROCKs mediate formation of the vacuolar apical compartments and endocytosis of tight junction proteins in a myosin light chain kinase-independent manner (52, 53). Our study provides further evidence suggesting that ROCK is required for the assembly and maintenance of tight junctions, probably through its effects on the F-actin cytoskeleton organization during junctional formation.

It should be noted that while stimulating wound healing, the ROCK inhibitor Y-27632 blocked tight junction barrier formation. Thus ROCK inhibition may have beneficial as well as adverse effects on corneal epithelial cells. The contradicting roles of Y-23623 may be related to the distinct functions of ROCKs in preventing cell activation at an early stage of wound healing, including converting epithelial cells from stationary to migratory state and promoting cell differentiation, a late stage event occurring when a wound is closed. Hence, ROCK inhibitors may be useful therapeutics to treat delayed epithelial wound healing such as that observed in diabetic corneas where accelerating wound closure is important for reducing the risk of sight-threatening complications such as bacterial infection (35).

In summary, ROCKs negatively regulate corneal epithelial wound healing via modulating cell behaviors including cell migration, proliferation, cell-matrix adhesion, and cell-cell junctions. Since ROCK inhibitors such as fasudil and Y-27632 have been in clinical trials (12, 45) and side effects were reported to be minimal, understanding the role and mechanism of ROCK actions in corneal wound healing may lead to their therapeutic application in treating corneal diseases. One potential use of these inhibitors is the treatment of diabetic keratopathy, which may lead to delayed wound healing, persistent defects, and recurrent erosion of epithelium, all of which can be attributed to defects in epithelium-basement membrane interaction and adhesion (50).

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