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Role of Small GTPase Rho in Regulating Corneal Epithelial Wound Healing

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Abstract

Purpose—To determine the role of small GTPase Rho and its relation with epidermal growth factor receptor (EGFR) in mediating corneal epithelial wound healing.

Methods—Rho activity in THCE cells, an SV40-immortalized human corneal epithelial cell (HCEC) line, and primary HCECs was assessed by pull-down assay followed by Western blotting. Rho functions were inhibited with specific inhibitor exoenzyme C3 (C3) and confirmed by knockdown with small interference RNA (siRNA) transfection. Effects of Rho inhibition on wound healing were determined in porcine corneal organ culture and HCEC scratch wound models. Effects of C3 on cell proliferation and focal adhesion formation were determined by BrdU incorporation assay and immunocytochemistry, respectively.

Results—Wounding, lysophosphatidic acid, and heparin-binding EGF-like growth factor (HB-EGF) induced rapid and strong RhoA activation. HB-EGF-, but not wounding-, enhanced RhoA activity was sensitive to EGFR inhibition. In corneal organ and cell culture models, C3 attenuated spontaneous and HB-EGF-induced wound closures, confirmed by delayed wound healing in cells transfected with RhoA siRNA. C3 also retarded spontaneous wound healing in the presence of hydroxyurea, a cell cycle blocker. C3 significantly reduced the number of BrdU-positive cells near the leading edge. Treatment with C3 resulted in the disruption of the cortical actin cytoskeleton and in the disappearance of paxillin-containing focal adhesion and lamellipodia.

Conclusions—Wounding induces RhoA activation through an EGFR-independent pathway. Rho activity is required for modulating cell migration and proliferation through cytoskeleton reorganization and focal adhesion formation in response to wounding.

The corneal epithelium is continuously subjected to physical, chemical, and biological insults, often resulting in a wound and the loss of barrier function.¹ The proper healing of corneal wounds is vital to maintain a clear, healthy cornea and to preserve vision.² The corneal epithelium responds rapidly to injury by migrating as a sheet to cover the defect and to reestablish its barrier function.² 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.^{3,4} Prominent among these epithelium-derived factors are ligands for EGFR, such as epidermal growth factor (EGF) and heparin-binding EGF-like growth factor (HB-

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EGF).² In addition, other cellular components, such as adenosine triphosphate (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.^{5–8}

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. They regulate the actin cytoskeleton, cell migration, and proliferation.^{9,10} 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.^{11–13} Rho has been implicated in cell migration, actin organization, focal adhesion formation, and adherens and gap junction assembly in corneal epithelia.^{14–16} However, the mechanistic role(s) of Rho in mediating corneal epithelial wound closure remains elusive.

We previously showed that wounding induces EGFR transactivation through ectodomain shedding of HB-EGF and that this wound-induced activation of EGFR and its coreceptor, erbB2, are required for wound closure in cultured porcine corneas and human corneal epithelial cells (HCECs).^{17,18} We recently demonstrated that LPA promotes human corneal epithelial wound healing by transactivating EGFR.⁶ LPA, generated from precursors in the plasma membrane, exerts its biological activities through its interaction with LPA receptors (G-protein-coupled receptors [GPCRs]) and has the ability to activate Rac and Rho.¹⁹ In rabbit corneas, the inhibition of Rho activity attenuates LPA-stimulated corneal epithelial migration over stromal cut, suggesting Rho GTPases as downstream effectors of LPA-GPCR.¹⁴ On the other hand, the Rho family has been shown as a key signaling element downstream of the EGFR in certain cell types, especially in association with cancer progression.^{20,21} It was also reported that overexpression of RhoA-GTP induces EGFR activation in breast cancer cells.²² Therefore, the relationship between EGFR and Rho proteins seems to be cell type-, stimulus-, and event-specific. Hence, it is of great interest to elucidate whether RhoA can function as a downstream effector of EGFR signaling during corneal epithelial wound healing and how activated Rho mediates epithelial wound closure.

In the present study, we demonstrated that Rho is activated in an EGFR-independent fashion in response to wounding and that the inhibition of Rho activity attenuates corneal epithelial wound closure by modulating cell migration, proliferation, and cell-matrix adhesion.

Materials and Methods

Materials

Minimum essential medium (MEM), nonessential amino acid solution, defined keratinocyte serum-free medium (SFM), reduced serum media (Opti-MEM), transfection reagent (Lipofectamine 2000), and reagent (TRIzol) were purchased from Invitrogen (Carlsbad, CA). Agarose was obtained from MP Biomedicals (Irvine, CA). Keratinocyte basal medium (KBM) was from BioWhittaker (Walkersville, MD). Human recombinant HB-EGF was obtained from R&D System (Minneapolis, MN). LPA and EGFR inhibitor tyrphostin AG1478 were from Sigma-Aldrich (St. Louis, MO). Ca²⁺ chelator BAPTA/AM and Src kinase inhibitor PP2 were from Calbiochem (La Jolla, CA). Antibodies against RhoA were from Pierce Biotechnology (Rockford, IL) and Cell Signaling (Danvers, MA). Rabbit phospho-EGFR (Tyr1068) antibody was from Cell Signaling (Danvers, MA). HRP conjugate secondary antibodies were from Bio-Rad (Hercules, CA). Mouse anti-paxillin antibody was from Chemicon (Temecula, CA). Mouse IgG isotype control was from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated antibody against mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). Rhodamine phalloidin was from Invitrogen. LDH cytotoxicity kit and 5-

Bromo-2'-deoxyuridine (BrdU) labeling and detection kit were from Roche Applied Science (Indianapolis, IN). RhoA siRNA and nonsilencing siRNA (as a negative control) were from Santa Cruz Biotechnology and Dharmacon Inc. (Chicago, IL), respectively. All other chemicals and reagents were purchased from Sigma-Aldrich.

Cell Culture and Extensive Wounding Study

THCE cells, an SV40-immortalized human corneal epithelial cell (HCEC) line,²³ were grown in defined keratinocyte SFM in a humidified 5% CO₂ incubator at 37°C and growth-factor starved in KBM for 16 hours before experiments. Primary HCECs were isolated from human donor corneas obtained from Michigan Eye Bank. The epithelial sheet was separated from underlying stroma after overnight dispase treatment at 4°C. The dissected epithelial sheet was trypsinized, and cells were then collected by centrifugation. Primary HCECs were grown in defined keratinocyte SFM in a humidified 5% CO₂ incubator at 37°C and then used at passage 3. 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 toothcomb for DNA sequencing gel (Bio-Rad) 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% sodium dodecyl sulfate, 1% Triton X-100, 50 mM NaF, 100 mM sodium pyrophosphate, 3.5 mM sodium orthovanadate, proteinase inhibitor cocktail, and 0.1 mM phenylmethylsulfonyl fluoride) and protein concentrations, were determined using a protein assay kit (Micro BCA; Pierce).

Assay of RhoA activity

Escherichia coli BL21 cells transformed with the GST-C21 construct were kindly provided by Avraham Raz (Karmanos Cancer Institute and the Department of Pathology, Wayne State University School of Medicine). GST-C21 fusion protein contains the NH₂-terminal 90 amino acids, representing the Rho binding domain, from the Rho effector protein Rhotekin.²⁴ The activation status of RhoA was assayed using fusion protein GST-21, as described.²⁵ Briefly, THCE cell lysates from treated and control cultures were incubated with GST-C21 fusion protein and glutathione *S*-transferase. The beads were washed three times with lysis buffer, and bound GTP-Rho was detected by immunoblot analysis with RhoA antibody (1:1000) followed by HRP-conjugated secondary antibody (1:2000). Phosphorylation of EGFR at Tyr1068 was detected by immunoblot analysis with a site-specific antibody (1:500) followed by HRP conjugate secondary antibody (1:2000).

Treatment of Cells with Exoenzyme C3

Recombinant *Clostridium botulinum* exoenzyme C3 expressed in *E. coli* from the pGEX-2T vector as glutathione *S*-transferase fusion protein was kindly provided by Anne J. Ridley (Ludwig Institute for Cancer Research), and purified as described previously.²⁶ To determine incubation time required for effective Rho inhibition, THCE cells were pretreated with 10 µg/mL C3 for 3, 8, or 24 hours and then wounded. Wounding-induced RhoA activation was assayed as described, and results indicated that 24 hour-pretreatment was necessary to block Rho activation. Cytotoxicity of 24-hour exoenzyme C3 treatment was detected by measuring lactate dehydrogenase (LDH) release using a cytotoxicity kit (Roche Applied Science). Briefly, conditioned media of cells pretreated with 10 µg/mL C3 were collected. One microliter of medium was mixed with 100-µL reaction solutions and incubated at room temperature for 20 minutes before absorbance at 490 nm was quantified on a fluorometer (GENios). Conditioned media from cells lysed with 2% Triton X-100, known to produce maximum LDH release, served as the positive control (Sample number *N* = 3).

RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated from THCE cells using reagent solution (TRIzol; Invitrogen) according to the manufacturer's instructions, and 2 μ g total RNA was reverse transcribed with a first-strand synthesis system for RT-PCR (SuperScript; Invitrogen). cDNA was amplified by PCR using primers for human RhoA (5'-ATGGCTGCCATCCGGAAGAAA-3', 5'-TCACAAGACAAGGCAACCAGA-3'), RhoB (5'-GCGTGCGGCAAGACGTCTG-3', 5'-TCATAGCACCTTGCAGCAGTT-3'), RhoC (5'-ATGGCTGCAATCCGAAAGAAG-3', 5'-TCAGAGAATGGGACAGCCCCCT-3'),²⁷ and GAPDH as internal control (5'-CACCACCAACTGCTTAGCAC-3', 5'-CCCTGTTGCTGTAGCCAAAT-3').²⁸ The PCR products were subjected to electrophoresis on 1.5% agarose gels containing ethidium bromide. Stained gels were captured using a digital camera.

Porcine Corneal Organ Culture

Porcine eyes were obtained from a local abattoir, transported to the laboratory on ice in a moist chamber, and processed for culture within 24 hours. An epithelial wound was made by demarcating an area on the central cornea with a trephine 4 mm in diameter and then removing the epithelium within the circle with a surgical scalpel, leaving an intact basement membrane.²⁹ The corneas were then processed for organ culture as previously described.³⁰ Briefly, corneal-scleral rims, with the presence of approximately 4 mm of the limbal conjunctiva, were excised and cultured in MEM, leaving the epithelium exposed to the air. The corneal epithelial wounds were allowed to heal in MEM containing 50 ng/mL HB-EGF, 10 μ g/mL C3, or both, in a 5% CO₂ incubator at 37°C. Forty-eight hours after wounding, the corneas were stained with Richardson staining³¹ to mark the remaining wound area and were then photographed under a dissecting microscope (Nikon, Tokyo, Japan) and a camera (MDS290; Eastman Kodak, Rochester, NY). The extent of healing was defined as the percentage of the remaining wound areas compared with the original wound area ($N = 4$).

Scratch Wound Healing Studies

Growth factor-starved THCE cells were pretreated with 10 μ g/mL C3 for 24 hours before wounding with a sterile 0.1- to 10- μ L pipette tip (TipOne; USA Scientific, Ocala, FL) to remove cells by two perpendicular linear scrapes. After washing away suspended cells, the cells were refed with KBM containing 50 ng/mL HB-EGF, 10 μ g/mL C3, or both. Wound closure was photographed immediately and 24 hours after wounding at the same spot with an inverted microscope equipped with a digital camera (SPOT; both from Carl Zeiss, Thornwood, NY). The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area ($N = 3$). To differentiate the contributions of cell proliferation and migration to wound closure, cell cycle blocker hydroxyurea (0.5 mM) was added in the scratch wound model. Preliminary studies indicated that this concentration was sufficient to inhibit THCE cell proliferation with minimal effects on cell viability (data not shown).

RhoA siRNA Transfection

THCE cells (2×10^5) were seeded in 12-well plates and allowed to grow to 70% confluence for 24 hours. Transient transfections were performed with transfection reagent (Lipofectamine 2000; Invitrogen) according to the manufacturer's protocol. Briefly, 5 μ L RhoA siRNA (Santa Cruz Biotechnology) or nonsilencing off-target siRNA (Dharmacon) and 5 μ L transfection reagent (Lipofectamine 2000; Invitrogen) were each diluted first with 95 μ L reduced serum media (Opti-MEM; Invitrogen) and then mixed. The mixtures were allowed to incubate for 30 minutes at room temperature and then were added by drop to each culture well containing 800 μ L reduced serum media (Opti-MEM; Invitrogen; final siRNA concentration, 100 nM). The cells were cultured in the transfection media for 24 hours and then for an additional 24 hours

in defined keratinocyte SFM. Forty-eight hours after transfection, scratch wounds were made and were allowed to heal in defined keratinocyte SFM for 30 hours. The extent of healing was defined as described ($N = 3$). After photographing, cells were lysed, and the expression of RhoA protein was assayed with Western blotting. In a preliminary experiment, downregulation of RhoA expression was observed 48 to 96 hours after transfection.

BrdU Incorporation Assay

BrdU incorporation assay was carried out according to the manufacturer's instruction. Briefly, THCE cells were grown on glass chamber sides, growth factor starved, and pretreated with C3 as described. Confluent cells were wounded with a sterile 0.1- to 10- μ L pipette tip and were refed with fresh KBM containing 10 μ g/mL C3 and BrdU labeling reagent for 24 hours. Slides 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 hour at room temperature, and 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 quantified by the number of BrdU-positive cells counted under a microscope in five random fields.

Immunostaining of Actin and Paxillin

THCE cells were grown on four-well glass chamber slides at 3×10^4 /well or 1×10^5 /well, to yield subconfluent or confluent cultures, respectively, growth factor starved and pretreated with C3 as described. Confluent cells were wounded with a sterile 0.1- to 10- μ L pipette tip and further incubated for 1 hour. Slides were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA in PBS for 1 hour at room temperature, and incubated with mouse IgG1 isotype control (1:100) or paxillin antibody (1:100) overnight at 4°C. Actin filaments and paxillin were visualized using rhodamine-phalloidin (1:50) and FITC-conjugated secondary antibody (1:100), respectively.

Statistical Analysis

Results were expressed as mean $\pm$ SEM. Statistical parameters were ascertained by software (SigmaStat), with the Student's *t*-test between two groups and $P < 0.05$, indicating significant difference.

Results

To determine whether Rho is involved in the HCEC response to epithelial injury, we first assessed RhoA activity at different time points after wounding. As shown in Figure 1A, RhoA activity increased within 10 minutes (the earliest time tested) and remained elevated 2 hours after wounding, whereas the level of total RhoA protein was relatively unchanged during the course of the study. Since we observed that both HB-EGF and LPA enhanced corneal wound healing *in vitro*,^{6,17} we next assessed the effects of these factors on RhoA activity (Fig. 1B). Both LPA and HB-EGF enhanced RhoA activity, suggesting that RhoA may act as their downstream effector. Elevated RhoA activity was also observed in primary HCECs challenged with wounding, HB-EGF, or LPA (data not shown).

We next examined the regulatory mechanisms of wound-induced RhoA activation. Given that we previously demonstrated that wounding triggers EGFR activation, which is required for HCEC wound healing,¹⁷ RhoA activity was determined in the presence of AG1478, a selective EGFR inhibitor (Fig. 2A). Although HB-EGF-induced RhoA activation was attenuated by AG1478, wound-induced RhoA activation was insensitive to EGFR inhibition. Using the phosphorylation of EGFR at Tyr1068 as a readout of EGFR activation,³² AG1478 treatment was shown to block wound- and HB-EGF-induced EGFR activation. We previously observed that Src-family tyrosine kinases,³³ Ca^{2+} ,⁵ and protein kinase C (PKC; Yin J, Yu FS,

unpublished data, 2006) actively participate in wound-induced EGFR signaling. To determine the role of these intracellular signaling molecules in mediating RhoA activation during HCEC wound healing, RhoA activity was assessed in the presence of their inhibitors. As shown in Figure 2B, wound-enhanced RhoA activity was greatly reduced by Ca^{2+} blocker BAPTA/AM and attenuated to a lesser extent by Src inhibitor PP2, but not by broad-spectrum PKC inhibitor staurosporine, suggesting that Rho activity may be regulated by Ca^{2+} and Src in wounded THCE cells.

To study the role of Rho in corneal epithelial wound healing, *C. botulinum* C3 exoenzyme, an ADP-ribosyltransferase that specifically modifies and inhibits Rho GTPases and has been shown to inhibit migration of rabbit corneal epithelium in culture,¹⁶ was used to inhibit Rho activities in THCE cells. Because C3 has been reported to be poorly accessible to intact cells,^{34–36} we first assessed the time required for C3 to exhibit its inhibitory effects on THCE cells. As shown in Figure 3A, though 3- and 8-hour treatment had no effects on Rho activation, 24-hour incubation with C3 resulted in the downregulation of active Rho in THCE cells. To ensure the inhibitory effect of 24-hour C3 treatment was not due to excessive cell loss, cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release (Fig. 3B). C3 treatment induced minimal level of cytotoxicity, similar to that of the negative control and much less than that of the positive control (2% Triton X-100). RhoB expression has been reported to be upregulated by C3 treatment in endothelial cells³⁷ and fibroblasts³⁸; therefore, we next investigated whether 24-hour C3 treatment in THCE cells would alter the expression of Rho isoforms. As shown in Figure 3C, THCE cells expressed RhoA and RhoC, but not RhoB, mRNA. In C3-treated cells, the levels of RhoA and RhoC mRNA were unchanged whereas RhoB remained undetected. Taken together, our data suggest that C3 is not cytotoxic and that it exerts its effects on THCE cells by inhibiting Rho activity.

We next assessed the effects of C3 on epithelial wound closure in a porcine corneal organ culture model.³⁰ As shown in Figure 4, 4-mm diameter wounds made in the center of porcine corneas were about to heal (95.7% of the denuded area was covered) in 48 hours. The presence of C3 greatly delayed this spontaneous wound healing (only 51.9% of the wound was covered), indicating that Rho activity is required in spontaneous healing processes. Moreover, exogenously added HB-EGF promoted epithelial wound closure, and C3 treatment attenuated the HB-EGF-enhanced wound healing (wound healed 70%).

The involvement of Rho in epithelial wound healing was also tested in a scratch wound model of the THCE monolayer (Figs. 5A, 5B). Similar to what was observed in the porcine organ culture model, C3 attenuated the spontaneous and HB-EGF-enhanced wound closure by 38% and 30%, respectively ($P < 0.05$ and $P < 0.01$, Student's *t*-test). To confirm the findings in pharmacological studies, RhoA siRNA transfection was performed (Figs. 5C, 5D). Cells transfected with off-target nonsilencing siRNA displayed an extent of wound healing similar to that of untransfected control cells, whereas knockdown of RhoA by siRNA transfection significantly attenuated wound closure.

Because many cellular processes, such as adhesion to the extracellular matrix, migration, and proliferation, contribute to corneal epithelial wound closure,² we next investigated the effects of Rho inhibition on these processes in cultured HCECs. We first evaluated the effects of C3 on cell proliferation in healing HCECs using BrdU incorporation assay (Fig. 6). In a wounded epithelial monolayer, there were numerous BrdU-positive cells, especially in the area several rows of cells away from the leading edge. Inhibition of Rho activity by C3 decreased the number of BrdU-positive cells by more than 75% 24 hours after wounding, suggesting that C3 may delay corneal epithelial wound healing by negatively regulating cell proliferation.

Having demonstrated that Rho inhibition attenuated cell proliferation near the leading edge, we next sought to determine the effect of C3 on cell migration when cell proliferation was inhibited. In a preliminary study, we found that at 0.5 mM hydroxyurea, a DNA replication inhibitor, inhibits cell growth with minimal cytotoxicity assessed by the release of LDH (data not shown). As shown in Figure 7, 0.5 mM hydroxyurea attenuated the spontaneous wound healing by 37%. This migration-driven wound closure was further impaired by C3, suggesting this Rho inhibitor exerts its inhibitory effect on wound healing by modulating cell migration as well.

Given that cell-matrix adhesion and the dynamic remodeling of actin cytoskeleton are important processes of wound healing,^{39,40} we next examined the effect of C3 on actin organization and focal adhesion in THCE cells. Figure 8A shows the staining of actin cytoskeleton and paxillin of isolated THCE cells. Normal cells exhibited thick cortical actin staining and the perinuclear diffuse staining of paxillin. After C3 treatment, cells displayed a more flattened and spread-out morphology with intercellular gaps (arrows); the thick network of cortical actin fibers was disrupted, and actin appeared disorganized and diffusely distributed throughout the cytoplasm, whereas paxillin was still located in the perinuclear region.

In the wounded THCE monolayer (Fig. 8B), cells at the leading edge developed bundles of thick actin fibers parallel to the wound edge with well-defined lamellipodia extending into the denuded area. Paxillin was distributed throughout the cytoplasm and was concentrated in the cell periphery, with the leading edge forming spikes and puncta structures (arrow-heads). In the C3-treated THCE cells, the cortical actin ring was abolished, and paxillin was localized mostly in the perinuclear region. Cells at the wound edge sent out long and flat actin-positive protrusions, which were devoid of stress fibers with minimal paxillin staining, suggesting few focal adhesions were formed in those cells. Hence, the ability of C3 to impair actin cytoskeleton reorganization and the formation focal adhesion may account for its inhibition of wound healing.

Discussion

In the present study, we examined the role of Rho in corneal epithelial wound healing. We showed that wounding rapidly and robustly activated RhoA in an EGFR-independent, Ca^{2+} - and Src-dependent manner, although exogenously added HB-EGF is able to stimulate RhoA activation. In cultured THCE cells, Rho activities were effectively inhibited with the specific inhibitor exoenzyme C3 and were confirmed with siRNA transfection. In ex vivo and cell culture models, we demonstrated that Rho activity was required for epithelial wound closure. Inhibiting Rho attenuated cell proliferation and migration and disrupted the remodeling of actin cytoskeleton and the formation of focal adhesion in the lamellipodia pointing toward the denuded area. Our data indicate that Rho plays an important role in corneal epithelial wound healing.

The wounding of epithelial cells results in the coordinate release of growth factors and other mediators, such as ATP and LPA, into the injury site, resulting in the activation of an array of intracellular signaling pathways in neighboring uninjured cells.^{8,41–43} The work from our group and other groups indicates that EGFR is at the center of cell activation, in response to wounding and other pathophysiological challenges.^{17,44–46} The pivotal role of EGFR relies on its ability to converge multiple extracellular signals generated by cell injury into an array of intracellular signaling pathways, including MAP kinase, PI3K, and PLC α , that drive epithelial cells from the stationary to the migratory or proliferative state, or both. The Rho family of small GTPases is another group of intracellular signaling molecules participating in the regulation of wound healing. To date, the relationship between EGFR and Rho GTPase activation is elusive. In the present study, we, for the first time, showed that wounding elicited

Rho activation. Interestingly, we found that although EGFR activation elicited by its ligand can trigger RhoA activation, the rapid and strong activation of RhoA in response to wounding in HCECs is independent of EGFR activation because the inhibition of EGFR kinase exhibited no effects on wound-induced RhoA activation. Thus, we conclude that the RhoA signaling pathway is not an effector of EGFR in HCECs in response to wounding.

How might Rho be activated in wounded corneas? We showed that the increase of RhoA activation is abrogated by blocking Ca^{2+} influx or by the inhibition of Src nonreceptor tyrosine kinase. We previously showed that wounding-induced Ca^{2+} influx and Src activation are required for EGFR transactivation induced by wounding.^{5,33} Furthermore, we and others have shown that wounding induces the release of ATP from the injured cells and that ATP acts as an initial signal to trigger EGFR transactivation through HB-EGF ectodomain shedding, which also requires Ca^{2+} influx and Src activation.⁵ Hence, these mediators may also activate Rho GTPase parallel to the activation of the HB-EGF-EGFR-ERK (PI3K) pathway. Because Src is also a downstream effector of EGFR,³³ the HB-EGF-induced RhoA activation in HCECs may be secondary to that of Src or to that of other pathways after EGFR activation. Interestingly, in mammary MCF-7 cells, overexpression of the active form of RhoA has been shown to induce the activation of EGFR, presumably through the metalloproteinase-dependent cleavage of an EGFR ligand, resulting in the stimulation of ERK1/2 activation and urokinase production.²² Whether Rho participates in EGFR transactivation in wounded corneal epithelial cells is under investigation in our laboratory.

C. botulinum C3 exoenzyme has ADP-ribosyltransferase activity and modifies Rho GTPases at Asn41, specifically inhibiting their biological activities.³⁴ Because exoenzyme C3 lacks a cell binding and transport domain, it is poorly accessible for cells. When used as a pharmacological tool in intact cells, high concentrations (5–50 $\mu\text{g/mL}$) and long incubation times (up to 24–48 hours) are usually necessary.^{16,34–36} In the present study, we observed that 24-hour incubation with 10 $\mu\text{g/mL}$ C3 was required for effective inhibition of RhoA GTPase activity and showed that such treatment prevented wound-induced Rho activation, resulting in the retardation of epithelial wound closure.

C3 is known to inhibit all three Rho GTPases—RhoA, RhoB, and RhoC—which share extensive homology, and RhoA has been the best studied.⁴⁷ We detected the mRNA expression of RhoA and RhoC, but not RhoB, in THCE cells. The *rhoC* gene has been suggested to arise because of an incomplete duplication of the *rhoA* gene.⁴⁷ Although RhoB expression has been reported to be upregulated by C3 treatment in endothelial cells³⁷ and fibroblasts,³⁸ RhoB mRNA in HCECs remained undetected after 24-hour incubation with C3 in our study. Hence, the observed effects of C3 on corneal epithelial wound healing likely result from its inhibition of RhoA and RhoC.

In ex vivo and cell culture models, we observed that C3 attenuated spontaneous wound closure, suggesting that Rho activity is required for proper wound healing. The role of Rho in epithelial wound healing was further confirmed by siRNA transfection that downregulated RhoA expression and delayed wound closure in cultured THCE cells. Moreover, C3 also proportionally inhibited HB-EGF-enhanced wound closure, suggesting that certain functions of Rho are required for wound healing but do not overlap with that of EGFR and its downstream effectors, such as ERK and PI3K. However, because we only assessed RhoA activation, the possibility that wound-elicited EGFR may activate RhoC could not be ruled out by the present study.

Through the use of BrdU labeling, our study clearly showed the involvement of Rho in cell proliferation in the area near the leading edge. Interestingly, Rho has been suggested to facilitate cell growth in breast cancer cells by influencing the activity of cyclin-dependent kinases during

the cell cycle.^{48,49} Here we documented that Rho negatively regulates cell proliferation in corneal epithelial cells. Another major component of the healing process is cell migration, and the regulatory role of Rho proteins in cell migration has been established in various cell types.^{50,51} In corneal epithelium, exoenzyme C3 was found to inhibit migration in a dose-dependent manner in a rabbit corneal culture model, and Rho GTPase was suggested to be the effector of LPA, which is known to stimulate corneal epithelial wound healing through EGFR transactivation.^{6,14} In the present study, hydroxyurea, a cell cycle blocker, at a concentration enough to inhibit cell growth but not to be toxic to cells, was used to inhibit cell proliferation and to differentiate the distinctive roles of migration and proliferation in epithelial wound healing. The observation that in the presence of hydroxyurea C3 effectively attenuates wound healing suggests that Rho modulates corneal epithelial wound healing by affecting cell migration in HCECs as well.

Epithelial wound closure is a well-coordinated biological process, requiring directed cell migration toward the center of the wound. The actin filament cable plays a role in coordinating cell adhesion and motility.^{52,53} In our study, isolated epithelial cells (plated at low density) and cells at the leading edge of a wound (confluent and migratory) have fiberlike actin bundles, especially in cells adjacent to the wound. Thus, the actin bundles apparently define cell polarity at two levels, the bundles supporting lamellipodia, which point toward the denuded area, and the gradual decrease in the fiberlike staining away from the leading edge. The inhibition of Rho activity leads to the disappearance of stress fibers and peripheral bundles and to the formation of membrane protrusions with disorganized actin filaments. Thus, we propose that the formation of actin bundles regulated by Rho on wounding is associated with directional migration of the epithelial sheet. Among the regulatory proteins involved in actin polymerization and lamellipodia formation, paxillin has been reported to be phosphorylated by LPA in a C3-sensitive manner in HCECs, and both Rho activation and paxillin phosphorylation have been implicated in LPA-enhanced epithelial wound healing.¹⁶ We found paxillin staining at the leading lamellipodial protrusions. Consistent with the role mediating directional cell migration, our study demonstrated that the inhibition of Rho activity significantly reduced the staining of paxillin in protrusions with disorganized actin filaments. Hence, Rho appears to drive cell migration and wound healing by promoting the extension of lamellipodia at the leading edges of the cells and by promoting the formation of actin stress fiber and focal adhesion. Thus, Rho GTPase, through affecting cell proliferation and directional migration, is an important mediator for the maintenance of epithelial integrity and restitution.

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Figure 1.

RhoA is activated by wounding, LPA, and HB-EGF in THCE cells. **(A)** Growth factor-starved THCE cells were extensively wounded and incubated for the indicated time. Active RhoA protein was pulled down by incubating cell lysates with GST-fusion protein (GST-C21), separated on SDS-PAGE, and detected by Western blot analysis using mouse anti-RhoA antibody. Aliquots of respective cell lysate were also used to analyze total RhoA protein by Western blot. **(B)** Growth factor-starved THCE cells were stimulated with 1 μ M LPA, 50 ng/mL HB-EGF, or extensive wounding for 10 minutes before they were lysed. Active and total RhoA proteins were determined as in **(A)**. Results are representative of four independent experiments.

Figure 2.

Wound-induced RhoA activation requires Ca^{2+} and Src, but not EGFR, activities. **(A)** Growth factor-starved THCE cells were pre-treated with 1 μM EGFR inhibitor AG1478 for 1 hour and then stimulated with 50 ng/mL HB-EGF or extensive wounding for 10 minutes before they were lysed. Active RhoA was pulled down by incubating cell lysates with GST-fusion protein (GST-C21), separated on SDS-PAGE, and detected by Western blot analysis using rabbit anti-RhoA antibody. Cell lysates were also subjected to Western blot with phospho-EGFR (Tyr1068) antibody. **(B)** Growth factor-starved THCE cells were pretreated with 50 μM Ca^{2+} blocker BAPTA/AM (BA), 40 nM PKC inhibitor staurosporine (Stau), or 25 μM Src inhibitor PP2 (PP2) before they were extensively wounded and lysed. Active and total RhoA proteins were determined as in Figure 1. Results are representative of three independent experiments.

Figure 3.

Effects of exoenzyme C3 on RhoA activation and Rho GTPase expression. (A) Growth factor-starved THCE cells were pre-treated with 10 μ g/mL exoenzyme C3 for 3, 8, or 24 hours before they were extensively wounded for 10 minutes. RhoA activation was determined as described in Figure 1. (B) Growth factor-starved THCE cells were pretreated with 10 μ g/mL exoenzyme C3 for 24 hours, and cytotoxicity was assayed by measuring LDH release using a cytotoxicity kit. Values are expressed as mean $\pm$ SEM in fold increase compared with the negative control; 2% Triton X-100 treatment served as a positive control, as recommended by the manufacturer's instruction. ($N = 3$) (C) Growth factor-starved THCE cells were pretreated with 10 μ g/mL

exoenzyme C3 for 24 hours and mRNA expressions of RhoA, RhoB, and RhoC were determined by RT-PCR with GAPDH as the internal control.

Figure 4.

Exoenzyme C3 attenuates spontaneous and HB-EGF-enhanced wound healing in cultured porcine corneas. A 4-mm diameter epithelial wound was made and allowed to heal in MEM containing 50 ng/mL HB-EGF, with or without 10 μ g/mL exoenzyme C3, for 48 hours. Wounded corneas were stained with Richardson staining solution to show the initial (0 hr) and the remaining (48 hr after wounding [p.w.]) wounds. Micrographs (**A**) represent one of four samples performed each time (scale bar, 1 mm). (**B**) Statistical analysis of the extent of healing. Values are expressed as mean $\pm$ SEM ($N = 4$). ** $P < 0.001$ (Student's t -test).

Figure 5.

Inhibition of RhoA activity attenuates scratch wound healing in THCE cells. **(A, B)** Growth factor-starved THCE cells were pretreated with 10 $\mu\text{g}/\text{mL}$ exoenzyme C3 for 24 hours and then wounded with a 0.1- to 10- μL pipette tip. Cells were allowed to heal in KBM containing 50 ng/mL HB-EGF, with or without 10 $\mu\text{g}/\text{mL}$ C3. Wound closure was photographed immediately (0 hr) or 24 hours (24 hr) after wounding. Micrographs **(A)** represent one of three samples performed each time. Original magnification, $\times 100$. Scale bar, 200 μm . **(B)** Statistical analysis of extent of healing. **(C, D)** THCE cells were transfected with RhoA siRNA or nonsilencing siRNA. Forty-eight hours after transfection, cells were wounded and allowed to heal in defined keratinocyte SFM for 30 hours. After wound closure was photographed, cells were lysed, and RhoA protein expression was examined by Western blotting. Micrographs **(C)** represent one of three samples. Original magnification, $\times 100$. Scale bar, 200 μm . **(D)** Statistical analysis of extent of healing with RhoA expression (*inset*). Values are expressed as mean $\pm$ SEM ($N = 3$). * $P < 0.05$; ** $P < 0.001$ (Student's *t*-test).

Figure 6.

Exoenzyme C3 inhibits THCE cell proliferation. Growth factor-starved THCE cells were pre-treated with 10 $\mu\text{g}/\text{mL}$ C3 for 16 hours, then wounded with a 1- to 10- μL pipette tip and allowed to reepithelialize in KBM containing BrdU, with or without 10 $\mu\text{g}/\text{mL}$ C3, for 24 hours. BrdU incorporation was visualized using anti-BrdU antibody and an FITC-conjugated secondary antibody. Corresponding nuclear staining was performed with DAPI. Micrographs (A) represent one of five random fields visualized, and positively staining cells (*green*) are proliferating cells. Original magnification, $\times 100$. Scale bar, 200 μm . (B) Statistical analysis of the numbers of BrdU-positive cells per field. Values are expressed as mean $\pm$ SEM ($N = 5$).

** $P < 0.001$ (Student's t -test).

Figure 7.

Exoenzyme C3 attenuates scratch wound healing in the presence of hydroxyurea. Growth factor-starved THCE cells were pre-treated with 10 $\mu\text{g/mL}$ exoenzyme C3 for 16 hours and then wounded with a 0.1- to 10- μL pipette tip. Cells were allowed to heal in KBM containing 10 $\mu\text{g/mL}$ C3, with or without 0.5 mM hydroxyurea (HU). Wound closure was photographed immediately (0 hr) or 24 hours (24 hr) after wounding. Micrographs (**A**) represent one of three samples performed each time. Original magnification, $\times 100$. Scale bar, 200 μm . (**B**) Statistical analysis of the extent of healing. Values are expressed as mean $\pm$ SEM ($N = 3$). * $P < 0.05$; ** $P < 0.001$ (Student's t -test).

Figure 8.

Exoenzyme C3 disrupts actin organization and focal adhesion in THCE cells. Growth factor-starved THCE cells were pretreated with 10 $\mu\text{g}/\text{mL}$ C3 for 24 hours. Confluent cells (**B**) were wounded with a 0.1- to 10- μL pipette tip and further incubated in the presence or absence of C3 for 1 hour. Subconfluent (**A**) and wounded (**B**) cells were then fixed and incubated with mouse IgG1 isotype (negative control) or paxillin antibody, followed by visualization with FITC-conjugated secondary antibody (*green*). Actin organization was visualized with rhodamine-phalloidin (*red*). (*asterisks*) Wound edge. *Arrows*: intercellular gaps.

Arrowheads: Paxillin-positive cell protrusions. Details are displayed in two right panels in (**B**). Results are representative of two independent experiments. Original magnification $\times 400$. Scale bar, 50 μm .