Role of p38 MAP Kinase in Regulation of Cell Migration and Proliferation in Healing Corneal Epithelium

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PURPOSE. The purpose of the present study was to examine the roles of signaling pathways potentially activated by TGFβ (i.e., Smad and p38 mitogen-activated kinase [MAPK]) in regulation of cell migration and proliferation of healing mouse corneal epithelium.

METHODS. Activation of Smads or p38MAPK was evaluated by immunohistochemistry in healing mouse corneal epithelium after debridement. The role of endogenous TGFβ or p38MAPK in epithelial healing was determined in organ-cultured mouse corneas with an epithelial defect, in the presence or absence of a TGFβ-neutralizing antibody or p38MAPK inhibitors, respectively. Cell proliferation was evaluated by incorporation of bromodeoxyuridine.

RESULTS. Migrating mouse corneal epithelium had minimal cell proliferation. Smad3 and -4 were found in nuclei of normal corneal epithelium, whereas they were absent in nuclei of migrating cells in association with Smad7 upregulation on epithelial debridement. Administration of TGFβ-neutralizing antibody reduced the protein expression of Smad7 in vivo after a corneal injury. In contrast, phosphorylation and nuclear translocation of p38MAPK were markedly evident in migrating epithelium during healing, but not in uninjured epithelium. In organ culture, addition of p38MAPK inhibitors blocked cell migration more markedly than neutralizing TGFβ-antibody and enhanced cell proliferation in the injured corneal epithelium, in association with phosphorylation of Erk.

CONCLUSIONS. Endogenous TGFβ enhances migration of corneal epithelium during wound healing in mice. The p38MAPK, but not the Smad, cascade plays a major role in promoting cell migration and in suppressing cell proliferation in migrating epithelium. (Invest Ophthalmol Vis Sci. 2004;45:100–109) DOI:10.1167/iovs.03-0700

Corneal epithelial defects must be rapidly resurfaced to avoid microbial infection and further damage to the underlying stroma. Epithelial healing is achieved by migration of the epithelial sheet to cover the denuded surface and enhanced cell proliferation to reestablish the epithelial stratification quickly after resurfacing. It is of interest to note that in the early phase of healing only one of the two cellular responses, cell migration, takes place, whereas cell proliferation is suppressed.¹,² Although cell migration promotes rapid reepithelialization, the cessation of cell proliferation may impede healing if such cessation is prolonged.

Various growth factors, including transforming growth factor-β (TGFβ), are believed to orchestrate the behavior of healing corneal epithelium: for example, cell migration and proliferation, cell death, and protein synthesis.³–⁶ In mammals, three isoforms of transforming growth factor-β (β1, -2, and -3) are known. Members of TGFβ family are multifunctional cytokines involved in development, tissue repair, and other physiological or pathologic processes.⁷–⁹ It has been demonstrated that the TGFβ isoforms and their receptors are present in corneal and limbal epithelia and other supporting tissues (e.g., conjunctiva and tear fluid).¹⁰ Therefore, it has long been speculated that the TGFβ isoforms play pivotal roles in maintaining corneal homeostasis in a paracrine and autocrine fashion.¹¹–¹⁵ TGFβ is believed to inhibit corneal epithelial cell proliferation in vivo, because it reportedly inhibits cell proliferation of cultured keratinocytes and corneal epithelial cells in vitro.¹²,¹⁶–¹⁸ This notion is further supported by the observation in which the administration of anti-TGFβ-neutralizing antibodies reduces scar tissue formation in injured corneas¹⁹ and enhances epithelial cell proliferation after a penetrating injury of corneas in mice (Saika S, unpublished observation, 2002). Recently, Zieske et al.²⁰ reported that epithelial debridement causes an upregulation of TGFβ receptor expression on migrating corneal epithelial cells, suggesting that this ligand may have a pivotal role in modulation of functions of migrating corneal epithelial cells during wound healing. However, the exact mechanism by which TGFβ modulates epithelial cell behavior (cell migration and proliferation) in wound healing remains to be clarified.

In the present study, we examined the roles of signal transduction pathways that can be activated on endogenous TGFβ stimulation and may regulate epithelial cell migration and proliferation in healing corneal epithelium. To examine whether cell migration may be associated with the suppression of cell proliferation during wound healing, cell proliferation after wounding was determined by incorporation of bromodeoxyuridine (BrdU). Immunohistochemistry was used to determine whether the signaling cascades of Smads and p38MAPK are activated in migrating corneal epithelium after injury. Our data indicate that there was a cessation of cell proliferation and an absence of nuclear Smad5 and -4 in migrating epithelium. In contrast, phosphorylated p38MAPK translocated to nuclei of

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migrating epithelial cells within 1 hour after debridement. Neutralization of the endogenous TGFβ isoforms resulted in delay in epithelial resurfacing and reduction in p38MAPK activation in organ-cultured epithelium. Finally, specific p38MAPK inhibitors suppressed cell migration but induced cell proliferation, as well as MAPK-Erk activation, in healing corneal epithelium after debridement in organ culture. In our study, p38MAPK signal, but not Smad, played a major role in promoting cell migration and suppression of cell proliferation, possibly by blocking the MAPK-Erk cascade in healing epithelium after corneal epithelium debridement.

**Materials and Methods**

Animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committees of Wakayama Medical University and the University of Cincinnati Medical Center.

**Epithelial Defect in Mouse Cornea**

Adult male C57BL/6j mice (n = 42) were anesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg) or combined xylazine (13 mg/kg) and ketamine (87 mg/kg) and topical oxybuprocaine (Santen, Osaka, Japan), and a corneal epithelial debridement (2 mm in diameter) was performed in one eye, as previously reported. 21 The other eye served as the control. Our previous studies have shown that a 2-mm central corneal epithelial debridement is resurfaced in 24 hours. 22,23 In the current studies, after different periods of injury (1–22 hours), the animals were given BrDU and killed 2 hours later by CO₂ asphyxiation and cervical dislocation, as previously reported. 22 For specimens obtained immediately after epithelial debridement, central corneal epithelial debridement was performed 2 hours after intraperitoneal administration of BrDU, and the experimental animals were killed. Affected eyes from four animals at each time point were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 48 hours. Specimens were dehydrated and embedded in paraffin. 22 Affected eyes from three experimental animals were embedded in optimal cutting temperature (OCT) compound (Miles Inc., Elkhart, IN) and subjected to cryosection.

**Effect of TGFβ−Neutralizing Antibody on Expression of Smad7 In Vivo**

In adult male C57BL/6j mice (n = 34) anesthetized as described earlier, the central cornea of one eye was perforated with a 26-gauge hypodermic needle attached to a micropipette. On puncture, 3 μL of neutralizing antibodies against individual and all TGFβ isoforms in PBS was injected into an eye at the same concentration as previously reported. 21 Nonimmune host IgG at a concentration of 10 μg/mL (rabbit or goat; Cappel, ICN Pharmaceuticals, Aurora, OH) was used as the control. The animals were killed at 24 hours, and the enucleated eyes were fixed in 4% paraformaldehyde and subjected to morphologic examination. Paraffin sections from each eye were cut through the central corneal perforation and were immunostained for Smad7, as described later.

**Organ Culture of Injured Eyes in the Presence of TGFβ−Neutralizing Antibody or p38MAPK Inhibitors**

We examined the effect of a neutralizing anti-TGFβ antibody or the p38MAPK inhibitors SB202190 and SB203580 on the cell migration and proliferation of corneal epithelial cells. An organ-culture system of debrided corneas was established as previously reported, with a minor modification. 21 An epithelial defect (2 mm in diameter) was created in the center of corneas of 4-week-old C57BL/6 mice under general anesthesia by intraperitoneal injection of pentobarbital sodium. The animals were killed without reawakening, and individual enucleated eyes were then cultured in 1.0 mL of DMEM supplemented with 2.0% fetal bovine serum with either mouse monoclonal anti-TGFβ (β1, -2 and -3)–neutralizing antibody (20 μg/mL; R & D Systems, Minneapolis, MN) or p38MAPK inhibitors (SB202190 and SB203580; 10 μM in 0.5% dimethylsulfoxide; Calbiochem, San Diego, CA), respectively. The control culture contained nonimmune IgG at the same concentration as the neutralizing antibody or 0.5% dimethylsulfoxide, respectively. The closure of the epithelial defect was determined by fluorescein staining after a 2-hour labeling with BrdU after 12, 18, 24, 36, and 48 hours of culture. Four or five eyes were prepared and analyzed for each experimental condition at each time point of the TGFβ neutralization and the p38 MAPK inhibitors, respectively. Cell proliferation of enucleated eyes was determined with BrDU incubation for 2 hours after the surgery (n = 6 at each time point). Eyes were then fixed in 4% paraformaldehyde after the BrdU-labeling and embedded in paraffin. Deparaffinized sections (5 μm thick) were processed for histology and immunostaining for BrdU and phospho-activated MAPK in a mouse monoclonal IgM and IgG anti-phospho-p38MAPK antibody (1:200 or 1:100 dilution in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, and Cell Signaling Technology, Beverly, MA, respectively) in the TGFβ neutralization experiment and p38MAPK inhibitor experiments, respectively.

**Immunohistochemistry**

Deparaffinized sections 5 μm thick were processed for immunohistochemistry for Smad3, -4, and -7; phosphorylated p38MAPK, and BrDU. After blocking with 5% dry milk and 5% bovine serum in PBS, the sections were incubated with rabbit polyclonal antibody against Smad3 (1:100 dilution in phosphate-buffered saline [PBS]; Zymed, South San Francisco, CA), goat polyclonal antibody against Smad4 (1:200 dilution in PBS; Santa Cruz Biotechnology), Smad7 (1:400 dilution in PBS; Santa Cruz Biotechnology), or mouse monoclonal IgM anti-phospho-p38MAPK antibody (1:200 dilution in PBS) for 12 hours at 4°C. After washes in PBS, the specimens were treated with peroxidase-conjugated antibodies against each immunoglobulin for 4 hours at 4°C. After another wash in PBS, a peroxidase reaction was performed with 3,3′-diaminobenzidine (DAB), as previously reported. 21 After they were counterstained with methyl green, the sections were mounted in balsam and observed under light microscopy.

Phospho-p38MAPK and phospho-Erk were also detected by immunofluorescent staining. Mouse monoclonal IgG anti-phospho-p38MAPK antibody (1:100 dilution in PBS) and rabbit polyclonal anti-phospho-Erk (1:100 in PBS; Cell Signaling Technology) were allowed to react with specimens overnight at 4°C. After a wash with PBS, they were treated with FITC-conjugated antibodies against each immunoglobulin. FITC was detected under a fluorescence microscope after embedding in a medium containing 4′,6′-diamino-2-phenylindole (DAPI) nuclear staining dye (Vestashield H-1200; Vector Laboratories, Burlingame, CA). For BrDU immunostaining, paraffin sections were treated with 2N HCl for 1 hour at 57°C and then washed in PBS before the application of anti-BrdU antibody (1:1 in PBS; Roche Diagnostics, Mannheim, Germany). Cryosections 7 μm thick were processed for immunostaining with goat polyclonal anti-Smad2 antibody (1:200 dilution in PBS; Santa Cruz Biotechnology). These slides were then processed for secondary antibody reaction, DAB reaction and observed under light microscopy.

**Results**

**Cell Proliferation and Activation of the Erk-MAP Kinase Cascade in Healing Corneal Epithelium after Epithelial Debridement**

Uninjured corneal epithelium and corneal epithelium immediately after epithelial ablation contained occasional BrdU-positive basal cells (Figs. 1A–K). At 3 hours (not illustrated) and 6 hours after injury, a few BrdU-positive epithelial cells were
observed in the periphery, but none at the edge of the migrating epithelium (Figs. 1aD, aE). At 18 hours after injury, BrdU-labeled epithelial cells were primarily found in the basal layer of the limbal-peripheral (Fig. 1aF) and midperipheral (Fig. 1aG) epithelia, whereas central resurfacing and migrating monolayer epithelium (Fig. 1aH) lacked BrdU-labeled cells. Twenty-four hours after injury, several BrdU-positive cells appeared in the central regenerated epithelium (Fig. 1aK) in association with the decrease in BrdU-positive cells the limbal (aI) and midperipheral corneal epithelia (aJ). (aB, aE, aG, aJ) Corneal epithelium in the midperiphery, at the wound edge (arrowhead; edge of the remaining epithelium). (aC, aH, aK) Epithelium in the central area. (b) Number of BrdU-positive cells in each zone of the epithelium. Rapid induction of cell proliferation in peripheral epithelium was followed by induction in the midperipheral and then the central epithelium. (c) Phosphorylation of Erk in healing epithelium. Phospho-Erk was faintly detectable in the cytoplasm of basal epithelial cells of uninjured epithelium (Fig. 1cA) with occasional nuclear localization and was not detected in migrating-edge epithelial cells from 1 (cC) to 12 (not shown) hours. The cells then started to upregulate phospho-Erk in the central epithelial cell cytoplasm at 18 hours (cE). Basal cells were positive for phospho-Erk in the nuclei at 24 hours (cG). (cB, cD, cF, cH) The DAPI nuclear staining of the identical area shown in (cA, cE, cF, cG), respectively.

Subcellular Distribution of Smad Proteins during Wound Healing of Corneal Epithelium

Smad signaling has been implicated in mediating the inhibition of epithelial cell proliferation induced by TGFβ in many organs and cell types. First, to examine whether the Smad cascade is activated in migrating epithelial cells, we determined the intracellular distribution of Smad family members during corneal epithelium wound healing after an epithelial debride-
p38MAPK in Corneal Epithelial Healing

Figure 2. Immunohistochemical detection of Smads in healing corneal epithelium. (a) Intracellular localization of Smad3 protein in mouse corneal epithelium. Cells in the uninjured corneal epithelium exhibited both nuclear (yellow arrows) and cytoplasmic Smad3 immunoreactivity (aA). Nuclei of migrating epithelial cells were not labeled by anti-Smad3 antibody at 6 (aB) and 12 (not shown) hours after epithelial debridement (white arrows). At 18 hours after injury, several cell nuclei were labeled by an anti-Smad3 antibody (aC, yellow arrows). Twenty-four hours after injury, central regenerated epithelium (aD) contained cells showing nuclear immunoreactivity for Smad3 with an increase in cytoplasmic labeling. (b) Intracellular localization of Smad4 protein in mouse corneal epithelium. Localization pattern of Smad4 was similar to that of Smad3 shown in (a). In normal uninjured cornea, most of the epithelial cells exhibited a nuclear immunoreactivity for Smad4 (bA, arrowheads). Epithelial cells at the edge of the epithelial defect lacked nuclear Smad4 at 6 (BC, arrows) and 12 (BD, arrows) hours after injury, whereas cytoplasm was positive for Smad4 protein. Cells with nuclei positive (BE, arrowheads) or negative (BF, arrow) for Smad4 were both observed in the double-layered epithelium that resurfaced the central corneal epithelial defect at 18 hours after injury. At 24 hours, central regenerated stratified epithelium (BF, arrowheads) contained many nuclear Smad4-positive cells. No specific immunoreactivity was observed in negative control staining (Bb). (c) Protein expression pattern of Smad7 in the healing corneal epithelium. Uninjured corneal epithelium (cA) and that immediately after the epithelial debridement (not shown) exhibited very faint immunoreactivity for Smad7 in the cytoplasm. Epithelial cells around the defect (c) were strongly labeled by the anti-Smad7 at 6 (cB) and 12 (cC) hours after debridement. Eighteen (not shown) to 24 (cD) hours after injury, Smad7 immunoreactivity returned to the normal level of an uninjured cornea. (d) Downregulation of Smad7 expression by administration of TGFβ-neutralizing antibody. Smad7 protein expression was found to be upregulated in the entire epithelium at 24 hours after puncture injury of the cornea (dA–C). Administration of neutralizing antibodies against TGFβ1 (not illustrated), TGFβ2 (dD–F), and pan anti-TGFβ antibodies (not illustrated) reduced Smad7 protein expression in the central and midperipheral, but not in the peripheral, epithelium at the same time point. (dB, dC) High-magnification images of the left- or right-boxed areas in (dA), respectively; (dE, dF) High-magnification images of the left- or right-boxed areas in (dD), respectively.

The cytoplasm showed prominent staining for Smad3. Eighteen hours after injury, positive nuclear Smad3 staining reappeared in double-layered epithelium that had resurfaced the denuded cornea (Fig. 2aC). At 24 hours, nuclear Smad3 staining was again observed in all cells of the regenerated stratified central corneal epithelium (Fig. 2aD). Anti-Smad3 antibody labeling of nuclei of peripheral epithelium was present during healing intervals lasting from 0 to 24 hours (data not shown). Nonimmune control IgG did not label the corneas (data not shown). Subcellular localization of Smad2 (data not shown) and Smad3 in the corneal epithelium was similar to one another. In Figure 2b, the intracellular localization of Smad4 exhibited a similar pattern to Smad3 during corneal wound healing as shown in...
Sma7, an inhibitory Smad, inhibits the dimerization and nuclear translocation of Sma5 and -4 and blocks TGFβ signaling. To determine whether the disappearance of Sma3 and -4 in the nuclei of migrating cells is associated with up-regulation of Sma7, the expression pattern of Sma7 protein in healing epithelium was examined by immunohistochemistry (Fig 2c). In uninjured corneal epithelium (Fig. 2cA) and in corneal epithelium immediately after debridement (not shown) Sma7 protein was very weakly detected in the epithelial cell cytoplasm. At 6 (Fig. 2cB) and 12 (Fig. 2cC) hours after debridement, the cytoplasm of epithelial cells around the defect exhibited marked immunoreactivity to Sma7. Eighteen (not shown) to 24 hours after injury, the immunoreactivity to Sma7 returned to its basal level, as seen in uninjured corneas (Fig. 2cD). To elucidate further the mechanism of up-regulation of Sma7 on injury and its association with the loss of Sma3 and -4 nuclear localization in migrating epithelial cells, the expression pattern of Sma7 was determined in injured epithelium in the presence or absence of neutralizing anti-TGFβ antibodies (Fig. 2d). Sma7 protein expression was upregulated over the entire epithelium after a penetrating injury of cornea with control PBS injection (Figs. 2dA–C). Administration of neutralizing antibodies against TGFβ1 (not illustrated), TGFβ2 (Figs. 2dD–F), and pan anti-TGFβ antibodies (data not shown) reduced Sma7 expression in the central and mid-peripheral, but not in the peripheral, epithelium, indicating that both endogenous TGFβ1 and -2 are the factors responsible for Sma7 upregulation in injured epithelium.

Loss of nuclear localization of Sma5 and -4 in migrating corneal epithelial cells implies that Smad signaling may not be directly involved in eliciting cell movement and inhibiting epithelial cell proliferation of migrating cells. This notion prompted us to hypothesize that such cellular behavior in migrating epithelium may be mediated by signaling pathways other than Smads (e.g., p38MAPK cascade).

Phosphorylated p38MAPK in Corneal Epithelium

To examine the possibility that activation of the p38MAPK cascade may mediate the cell migration and suppression of cell proliferation during corneal epithelial wound healing, subcellular localization of phosphorylated p38MAPK was first determined by immunofluorescent staining with an anti-phospho-p38MAPK antibody. As shown in Figure 3, nuclear translocation of phospho-p38MAPK was detected in the healing corneal epithelium weakly, but positively, as early as 1 hour (Fig. 3B) and lasted until 24 hours (Fig. 3F) after injury, whereas cells of uninjured epithelium had phospho-p38MAPK mainly in the cytoplasm (Fig. 3A). Prominent accumulation of phospho-p38MAPK were identified in nuclei of migrating epithelial cells at 6 (Fig. 3D) and 12 (Fig. 3E) hours. At 18 (not shown) and 24 (Fig. 3F) hours after injury it was observed in both the nuclei and cytoplasm of regenerated epithelium. This finding promoted us to hypothesize that signaling mediated by p38MAPK has a major role in regulating cell behavior in healing epithelium.

Organ-Culture with a TGFβ-Neutralizing Antibody

To determine whether corneal epithelial healing is regulated by endogenous TGFβ through p38MAPK activation, neutralizing antibodies against TGFβ1, -2, and -3 were included in an ex vivo corneal epithelial healing model of cultured eyes (Fig. 4). The results show that the antibodies delayed the reepithelialization (Fig. 4a). BrdU-labeled cells were absent in migrating epithelium in control cultures (Figs. 4cA, cC), whereas several BrdU-labeled cells were seen in migrating epithelium of anti-TGFβ-antibody-treated corneas (Figs. 4cB, cD). Immunofluorescent staining (Fig. 4d) showed marked positive immunoreactivity for phospho-p38MAPK, mainly in basal cells of migrating epithelium in control specimens at each culture interval (Fig. 4dA), whereas staining for phospho-p38MAPK was very faint in corneal epithelium treated with TGFβ-neutralizing antibody (Fig. 4dB). These observations support the notion that endogenous TGFβ is one of the cytokines mediating p38MAPK activation, resulting in increases in cell migration and suppression of cell proliferation.

Organ Culture with p38MAPK Inhibitors

The results of organ-culture experiments revealed that endogenous TGFβ activates p38MAPK and modulates corneal epithelial wound healing. To further confirm this notion, the specific p38MAPK inhibitors SB202190 and SB203580 were added to the medium in an ex vivo wound healing model of cultured mouse eyes. The results show that SB202190 markedly delayed reepithelialization (Fig. 5). Its presence delayed resurfacing of epithelial defects (Figs. 5aF–I, b), compared with control cultures (Figs. 5aA–E, b). In the presence of SB202190, the corneal epithelial defect persisted at 48 hours in the inhibitor-treated culture (Figs. 5aI, b), whereas all five corneas were resurfaced within 36 hours (Figs. 5aD, b) in the control experiment. The addition of SB202190 produced more profound inhibitory effects on epithelial cell migration than that by the TGFβ-neutralizing antibody.

Histology of control experiments showed that the epithelial sheet started to migrate on the denuded central cornea at 12 hours and that the defect was resurfaced by a single-layered epithelium within 36 hours (Figs. 5cF, cH, cJ). In contrast, in the presence of SB202190, the edge of the epithelium remained multilayered (stratified) up to 24 hours in culture (Figs. 5cC, cE, cG) resembling that seen at the 0 time point (Fig. 5cA), and the epithelial migration was noted with a single-cell-layered epithelium at the leading edge after 48 hours in culture (Figs. 5cG, cI, cK). Figure 5d shows the lack of p38MAPK activation in healing epithelium in the presence of SB202190.

Figure 6 indicates the status of Erk-MAPK and cell proliferation activity in healing, organ-cultured corneal epithelium in the presence of SB202190. As shown in Figure 1, epithelial cell proliferation was associated with Erk activation, based on increases in its phosphorylation status, which correlated inversely with p38MAPK phosphorylation. Thus, we examined the effects of a p38MAPK inhibitor on Erk activation in epithelial cells of injured corneas. Figure 6a shows that Erk remained highly phosphorylated in the presence of SB202190 compared with the control. We therefore examined cell proliferation in these specimens by BrdU incorporation (Figs. 6b, 6c). At the 0 hour time point (incubated with BrdU 2 hours immediately after debridement) in the absence of the inhibitor, no BrdU-labeled cells were found at the edge of the debrided epithelium, whereas in inhibitor-treated specimen BrdU-labeled cells were seen at the same level found in normal uninjured cornea (data not shown). Throughout the culturing period up to 36 hours, BrdU-labeled cells were detected at the margin of the debrided epithelium in the presence of SB202190, with a peak at 18 hours (Figs. 6bB, BD, BF), while in control culture no BrdU-labeled cells were observed (Fig. 6aB, BC, BF). The cells of regenerated epithelium resurfacing the denuded cornea reentered the cell cycle at 48 hours in control culture as shown (Fig. 6bG). Figure 6c summarizes the incidence of BrdU-labeled cells at individual time points. Similar results were obtained with another p38MAPK inhibitor, SB203580 (data not shown).
cell mobility and the expression of mesenchymal cell markers (e.g., vimentin or α-smooth muscle actin) in association with the loss of epithelial markers (e.g., cadherins) as well as the loss of cell polarity and cell-cell junction. We previously demonstrated that migrating epithelial cells of injured rabbit corneas (i.e., alkali-burn transiently expressed vimentin).23 These observations imply that corneal epithelial cells may undergo phenotypic changes to gain migratory characteristics in a way similar to the EMT process mediated through activation of the p38MAPK cascade during wound healing.

In our organ culture experiment, adding p38MAPK inhibitors enhanced Erk phosphorylation and induced increases in BrdU incorporation in healing epithelium, indicating that activation of p38MAPK suppresses the MAPK-Erk cascade. The effect of p38MAPK inhibitors on cell proliferation regulation is,
corneal epithelium reenter the cell cycle at 24 hours with proliferation through expression of the cdk inhibitor p27 kip1 and lines stimulated by activin.32,33 However, p38MAPK-independent vascular endothelial cells and in some neoplastic cell hypophosphorylation of Rb tumor-suppressor protein in cultured some pancreatic carcinoma cell lines, TGF activates a serine-threonine phosphatase that suppresses the growth-factor-dependent MAPK/Erk cascade by dephosphorylating Erk2.34 Similarly, in macrophages it has been shown that activation of TGFβ-linked Smad signaling leads to downregulation of p38 by inducing MAPK phosphatase-1.35 Cells in regenerated central corneal epithelium reenter the cell cycle at 24 hours with nuclear translocation of phospho-Erk after epithelial debridement, although phospho-p38MAPK is also detected in the cell nuclei at relatively higher levels at that time point than that in uninjured epithelium. This finding suggests that MAPK-Erk cascade may overcome proliferation inhibition by phosphorylated p38MAPK in regenerated corneal epithelium at this time point.

It has been suggested that Smads may mediate TGFβ signaling resulting in inhibition of cell proliferation.16–18,36–38 On the contrary, in our results there was a lack of Smad3 and -4 localization in nuclei of migrating corneal epithelial cells after wounding in vivo, which suggests that TGFβ-Smad signaling may not be directly involved in regulation of cell proliferation and migration in mouse corneal epithelium. In addition, Smads were localized in nuclei of uninjured corneal epithelium and restratified epithelium 24 hours after debridement, comcomitant with the detection of BrdU-labeled epithelial cells, suggest-
ing that activation of other signaling pathways (i.e., MAPK-Erk pathway) may accelerate cell proliferation at this later healing phase. Alternatively, activation and nuclear translocation of Smad members may occur in migrating epithelial cells presumed to be blocked by an immediate expression of Smad7 in these cells. Upregulation of TGFβ receptors in migrating corneal epithelial cells, reported by Zieske et al., can account for our finding that Smad7 expression is upregulated in migrating epithelial cells as an immediately early gene. Indeed, this notion is further supported by our unpublished data in which the absence of Smad3 does not affect the incorporation rate of BrdU-labeled cells or the resurfacing rate in healing corneal epithelium at the time points of 6, 12, and 24 hours after debridement in Smad3-knockout mice (Saika S et al., unpublished data, 2003). Because Smad3-null mice exhibit hyperproliferation of epidermal epithelium during wound healing after an incision injury, signaling mechanisms regulating cell proliferation may differ in epidermis and corneal epithelium.

Our results in the experiment using the TGFβ-neutralizing antibody demonstrated that endogenous TGFβ is an important intrinsic factor involved in activation of p38MAPK, as well as cell migration, in injured corneal epithelium. Multiple signaling cascades are activated on TGFβ binding to its cognate receptor—that is, Smads, RhoA-related signals, MAPK-Erk-1/2, stress kinases (i.e., JNK), p38MAPK, phosphatase2A, or PI3-kinase/AKT. Thus, TGFβ can lead to great variations in cellular response, depending on which of these signaling transduction pathways are within a given cell type. For example, pathways containing Smad, Rho proteins, and PI3-kinase are involved in cell differentiation-dedifferentiation, whereas the MAPK-Erk pathway modulates cell proliferation and cell survival.
Although the present study shows that TGFβ is one of the factors regulating corneal epithelial healing through p38MAPK activation, many other growth factors and cytokines are capable of modulating corneal epithelial cell behavior. Wilson et al. demonstrated that epithelial debridement causes release of IL-1 by the injured cornea, which subsequently induces the expression of hepatocyte growth factor (HGF) and keratocyte growth factor (KGF) by keratocytes that serve as paracrine modulators of corneal epithelial cells. Moreover, it has been reported recently in cultured corneal epithelial cells that p38MAPK is activated by exogenous addition of HGF or KGF, which results in stimulation of cellular migration. In the same study, however, it was also reported that inhibition of p38MAPK activation enhanced EGF and KGF stimulation of Erk-1/2 activity and proliferation. These in vitro results are consistent with our observation that after wounding of organ-cultured corneas, p38MAPK inhibition resulted in stimulation of cell proliferation and enhanced MAPK-Erk phosphorylation. Indeed, besides TGFβ, other growth factors (e.g., HGF and KGF), stress kinases, and inflammatory cytokines are presumably involved in the cascade of p38MAPK activation in vivo. A recent study by other investigators showed that HGF augments migration of cultured corneal epithelial cells by using the MAPK-Erk pathway. In a series of our ex vivo corneal wound-healing studies with organ-cultured eyes, we have observed that the MEK inhibitors U0126 and PD98059 do not significantly alter the resurfacing rate of a corneal epithelial defect (data not shown), an observation consistent with the suggestion that the MAPK-Erk pathway does not have a significant role in epithelial cell migration in situ. Because our ex vivo wound-healing model of organ culture mimics the in vivo condition, it is postulated that the MAPK-Erk pathway may differentially regulate migration of corneal epithelial cells between in vitro and in situ/in vivo conditions.

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