EVIDENCE OF CROSS-TALK ACTIVATION BETWEEN MAP KINASE CASCADES* in Epithelial Wound Healing

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One important action of growth factors is their participation in tissue repair; however, the signaling pathways involved are poorly understood. In a model of corneal wound healing, we found that two paracrine growth factors, hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF), induced rapid and marked activation and prompt nuclear accumulation of phospho-p38 (p-p38) and -ERK1/2 (p-ERK1/2), but not of JNK (p-JNK1/2), in corneal epithelial cells. Interruption of p38 and ERK1/2 signaling pathways by pretreatment with inhibitors SB203580 and PD98059 and subsequent stimulation with HGF or KGF abolished the activation and nuclear localization. Inhibition of either one of these mitogen-activated protein kinases, p38 or ERK1/2, induced a robust cross-activation of the other. In immunofluorescence studies of wounded cornea, p-p38, unlike p-ERK1/2, was immediately detectable in epithelium after injury. Inhibition of p38 by SB203580 blocked migration of epithelial cells almost completely. In contrast, PD98059 seemed to slightly increase the migration, through concomitant activation of p38. Unlike ERK1/2, p38 did not significantly contribute to proliferation of epithelial cells. Interruption of either the ERK1/2 or p38 pathway resulted in delayed corneal epithelial wound healing. Interruption of both signaling cascades additively inhibited the wound-healing process. These findings demonstrate that both p38 and ERK1/2 coordinate the dynamics of wound healing; while growth factor-stimulated p38 induces epithelial migration, ERK1/2 activation induces proliferation. The cross-talk between these two signal cascades and the selective action of p38 in migration appear to be important to corneal wound healing, and possibly wound healing in general, and may offer novel drug targets for tissue repair.

One of the most important events in wound repair is the coverage of the defective area by migration of residual epithelial cells. Concomitant with migration, cells undergo phenotype alterations. A second important event in the repair process, cell proliferation, occurs later. Induction of signals that allow the cells to respond to these events is not well understood, although growth factors are implicated in this highly complex and interactive process. One signaling pathway linked to growth factor response is comprised of the mitogen-activated protein kinases (MAPK).1 There are three main MAPK cascades: the extracellular signal-regulated kinases (ERK1/2), the c-Jun N-terminal kinases (JNK, also known as SAPK), and p38. These serine/threonine protein kinases phosphorylate both cytoplasmic and nuclear targets. ERKs are predominantly activated by mitogenic factors, while JNK and p38 are preferentially activated by stress-inducing stimuli such as UV light, heat shock, and pro-inflammatory cytokines (1). Depending on cell type, p38 can be involved in development, differentiation, proliferation, and survival (2). Recent studies have also shown that p38 modulates cell migration. Specific inhibitors of p38 reduce platelet-derived growth factor-induced smooth muscle cell migration (3, 4), hepatic myofibroblast migration (5), and aortic endothelial cell migration (6).

Corneal epithelium can be injured by different mechanisms, including disorders such as aging or tear deficiency, or injury caused by microorganisms, chemicals, or mechanical damage, e.g. laser surgery. Injury triggers the release of growth factors and cytokines (7–10), which in turn activates several signaling pathways (11, 12). Corneal epithelial wound healing is a highly complex, dynamic process that involves migration, proliferation, and differentiation of epithelial cells. One of the experimental advantages of studying this tissue is its avascular nature, which excludes blood-borne mediators from the repair process. In this study, we employed a corneal organ culture wound model that allowed us to monitor epithelial wound closure (13). Because stromal-epithelial interactions are important in the corneal repair process, two paracrine growth factors, hepatocyte (HGF), and keratinocyte growth factors (KGF), which are synthesized in corneal stroma and act on receptors expressed in corneal epithelial cells, were used in these studies (14). Both HGF and KGF activate the ERK1/2 and phosphati-dylinositol 3-kinase/p70 S6 kinase pathways and are involved in corneal wound healing (11, 12).

In other cells, such as lung fibroblasts, ERK1/2 positively regulates cyclin D1, which could implicate this kinase in cell proliferation. In contrast, p38 may be a negative regulator of...
cyclin D1 (15), suggesting a role of this kinase in halting proliferation. Recent studies have shown that migrating corneal epithelial cells do not progress through the cell cycle, while peripheral corneal epithelial cells do (16). In the latter cells there are increases in the expression of cyclins D and E (17). In contrast, migrating, nondividing epithelium does not exhibit nuclear localization of these markers (18).

Therefore, we hypothesized that the MAPKs may have important regulatory functions in both migration and proliferation of corneal epithelial cells during wound-healing. This prompted our probes to investigate the involvement of ERK1/2, p38, and JNK in response to the actions of HGF and KGF during corneal repair.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit eyes were obtained from Pel-Freeze Biologicals (Rogers, AK). Human recombinant double-chain heparocyte growth factor (HGF) was a gift from Genentech (San Francisco, CA). Human recombinant keratinocyte growth factor (KGF) and the p38 inhibitor SB203580 were from Upstate Biotechnology (Lake Placid, NY). The MKK3 and MKK6 inhibitor PD98059 was obtained from New England Biolabs (San Diego, CA). Phosphorylated forms of ERK1/2 (p-ERK1/2), p-p38, and p-JNK (c-Jun N-terminal kinase) as well as anti-c-Jun N-terminal kinases (JNK1 and JNK2) antibodies were purchased from Sigma. Anti-ERK1 and anti-p38 were purchased from BD Transduction Laboratories (San Jose, CA). For immunofluorescence, ERK2, p-ERK1/2, p38, and p38 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated sheep anti-mouse IgG antibody and ECL Western blotting system were obtained from Amersham Biosciences. All SDS-PAGE reagents were purchased from Bio-Rad. The horseradish peroxidase protein marker detection kit was obtained from New England Biolabs (Beverly, MA). CyQuant cell proliferation assay kit was obtained from Molecular Probes (Eugene, OR).

**Cell Culture**—Rabbit (RCE) and human (HCE) corneal epithelial cells were used in the present study. Rabbit eyes were shipped on ice in Hank’s Balanced Salt Solution containing antibiotics and antimitotic (Pel-Freeze Biologicals, Rogers, AK). Eyes were kept at 4 °C and used no later than 24 h after enucleation. Corneas were collected in sterile Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM-F12, 1:1) containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin. The endothelia and Descemet’s membranes were removed with a sterile scalpel, and RCE cells were cultured essentially as described earlier (19). First passages of RCE cells were used at 80–90% confluence. Immortalized HCE cells that express the HGF and KGF receptors were obtained from Dr. R. W. Beuerlein (Department of Ophthalmology, LSUHSC), and maintained in serum-free keratinocyte growth medium (KGM, Clonetics BioWhittaker Europe) supplemented with appropriate growth factors and antibiotics. HCE cells were passaged twice per week using trypsin, which was neutralized by KGM (10% fetal bovine serum). Cells were cultured by centrifugation and resuspended in KGM and then seeded at a 1:3 split ratio. A large pool of cells was frozen at initial passage to avoid higher passage. All experiments were performed between passages 25–45 and at 60–70% cell confluence.

**Western Blotting**—RCE cells were incubated at 37 °C overnight in DMEM-F12 without serum or growth factors then stimulated with HGF or KGF (50 ng/ml) for different times (11). In some experiments, cells were preincubated for 1 h with 50 μM PD98059 (a specific inhibitor of MEK) or 20 μM SB203580 (a specific inhibitor of p38) before stimulation with growth factors. Inhibitors were dissolved in dimethyl sulfoxide (Me2SO), and similar concentrations (0.01%) were added to controls. Activation of ERK1/2, p38, and JNK was evaluated by Western blot using phosphospecific antibodies. Total protein was determined using anti-ERK1, anti-p38, and anti-JNK1/2 antibodies. Briefly, 40 μg of protein/well were subjected to SDS-PAGE (10%) gel and then transferred to a nitrocellulose membrane using a Bio-Rad Mini Trans Blot electrophoretic transfer unit. The membranes were blocked for nonspecific protein with 5% nonfat dry milk in Tris-buffered saline (TBS, 20 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20) or probed with the specific primary antibody. The membranes were then washed six times (5 min per wash) with TBS plus 0.05% Tween 20 to remove unbound antibodies and further incubated with appropriate secondary antibodies. Separated proteins were visualized by an ECL kit according to the manufacturer’s protocol. Intensities of the respective bands were examined by densitometric analysis (Bio-Rad Molecular Analyst software program).

**Corneal Organ Culture Wound-healing Model**—Corneal debridement wounds were made to central rabbit corneas using a battery-operated mechanical device (Algerbrush II, Alger Co., Inc. Lago Vista, TX). The central part of the cornea was marked with 7-mm surgical trephine and the epithelium was gently scraped off, without damaging the basement membrane. The wounds were also compared with scalpel-derived wounds (13) both by microscopy and stained with 0.5% methylene blue, and by immunohistochemistry and stained with hematoxylin-eosin. They were determined to be topographically smoother, more regular, and more consistent in shape and area of the wound as compared with scalpel-derived wounds, with the basement membrane intact (data not shown). The central part of the cornea was left 1 mm of scleral rim, rinsed three times in DMEM-F12 containing appropriate antibiotic and antimitotic solutions, and cultured in vitro as described earlier, with some modifications (13, 20) on 1% agarose mounts at 37 °C with 5:95% CO2/air. Agarose mounts were prepared on polypropylene copolymer cups, which closely match corneal curvature, using DMEM-F12 medium with antibiotics. Corneas were pretreated 4 h with SB203580 (20 μM) and/or PD98059 (50 μM), and then incubated with HGF (40 ng/ml) for 24 h. Corneas without any treatment incubated under similar conditions were used as a control. For the zero time control, they were stained immediately after injury with 1% alizarin red for 2 min and then fixed in 5% ethanol for 1 h. Photographs were made on a dissecting microscope (Shimadzu Nikon) with an attached Sony camera, recorded by Adobe Photoshop software. They were analyzed for uncovered wound areas by a computer digitizer and an image-analysis program (Image Pro-Plus, Media Cybernetics, MD) operated by a technician to whom specific specimen treatments were unknown.

**Immunohistochemistry**—RCE cells were plated in 4-well Lab-Tek chamber slides, and upon reaching 50–60% confluence, were serum-starved overnight in keratinocyte basal medium (KBM; KGM without growth factors) and then stimulated with HGF or KGF (20 ng/ml) for 15 min. In some experiments cells were pretreated for 1 h with SB203580 (20 μM) or PD98059 (50 μM) and then stimulated with HGF or KGF. After a brief wash with cold PBS, the cells were fixed and permeabilized for 6 min with methanol (prechilled at −20 °C), extensively washed with PBS, and incubated at room temperature for 15 min with 1% bovine serum albumin and 5% goat normal serum in PBS. Cells were subsequently incubated with primary antibodies against ERK2 (1:200), p38 (1:200), p-ERK1/2 (1:100), and p-p38 (1:100) at 4 °C, 18 h. After three washings with PBS, they were incubated with fluorescein isothiocyanate-conjugated sheep anti-mouse IgG antibody (1:50) at room temperature for 45 min. After extensive washing with PBS, the cells were mounted in aqueous mounting fluid (Lerner Laboratories) and observed under a fluorescence microscope (Nikon Eclipse TE200 equipped with a Nikon digital camera DXM1200). Negative controls for immunostaining were cells incubated with mouse IgG without specific primary antibody. To detect the expression of MAPK in corneal tissues, rabbit corneas were embedded in optimal cutting temperature compounds (Miles Inc.), and 6-μm serial cryostat sections were prepared, air-dried, and stored at −80 °C. Immunofluorescence staining for ERK2, p-ERK1/2, p38, and p-p38 was performed as described above.

**Cell Proliferation Assay**—RCE cells were seeded into 96-well microplates at 5000 cells/well, allowed to attach (4–6 h), and then incubated in DMEM-F12 with 1% fetal bovine serum overnight at 37 °C. Cells were supplemented with growth factor (HGF) and/or inhibitors (PD98059 and SB203580) and incubated at 37 °C for 24, 48, or 72 h. Each condition was performed in octuplicate; medium was changed at 48 h. Proliferation was measured by a CyQUANT cell proliferation assay kit (Molecular Probes, Eugene OR) as previously described (21).

**Cell Migration Assay**—RCE cell migration assays were performed with modifications to earlier reports (22–24). RCE cells were seeded into 60-mm Petri culture dishes, and a straight line was gently carved diametrically across the center, outer, bottom surface of each dish with an Alger brush. The cells were allowed to attach and spread. One side was then incubated overnight in serum-free medium, then scraped from one side of the marked line and washed two to three times with medium to remove all loose or dead cells. Dishes were carefully checked under microscope to confirm complete removal of cells from the scraped side and photographed (0 h). Cells were then stimulated with HGF or KGF or inhibitors (SB203580 and PD98059) at 37 °C for 24 h. Control dishes were similarly scraped and incubated without addition of any growth factor and/or inhibitor. Inhibitors were dissolved in Me2SO and similar concentrations of Me2SO (0.01%) were added to control dishes to determine its effect on cell migration. Cells that

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*p38 and ERK1/2 in Corneal Epithelial Wound Healing*
Fig. 1. Activation of MAPK by HGF and KGF in corneal epithelial cells. RCE cells were serum-starved overnight (16–18 h) at 80–90% confluence and stimulated with HGF or KGF (20 ng/ml) for the indicated period of time. The status of active p38, JNK, and ERK1/2 was determined by Western blotting using phosphospecific monoclonal antibodies against phospho-p38, -JNK, and -ERK1/2, as described under Experimental Procedures. Antibodies that recognized corresponding total non-phosphorylated enzymes, anti-p38, anti-JNK1/2, and anti-ERK2, were used in control cells (A and B). The experiment was repeated three times with similar results.

We used HCE cells for these studies, because they grow in a more dispersed pattern, and they stain more clearly with the antibodies that we used, than do RCE. We observed similar responses with both HCE and RCE (data not shown). HGF- and KGF-stimulated HCE cells showed p-p38 staining mainly in the nuclear area with some perinuclear staining (Fig. 3, W and X). p-ERK1/2 staining was exclusively confined to the nuclei (Fig. 3, I and J). However, in control and growth factor-stimulated HCE cells, all p38 and ERK2 was distributed throughout the cytoplasm, with none in nuclei (Fig. 3, A–C and O–Q). Nonstimulated controls for p-p38 and p-ERK1/2 were counterstained with DAPI for nuclear staining (Fig. 3, H and V).

Cells pretreated with PD98059 then stimulated with HGF or KGF were completely devoid of nuclear staining for p-ERK1/2 (cells were counterstained with DAPI for nuclear staining) (Fig. 3, K and L). Similar treatments for p-p38 produced immunofluorescence mainly in nuclear regions (Fig. 3, Y and Z); all p38 and ERK2 appeared in the cytoplasm (Fig. 3, D, E, R, and S). Cells pretreated with SB203580 followed by stimulation with HGF or KGF were completely devoid of any immunofluorescence staining for p-p38 (cells were counterstained with DAPI for nuclear staining) (Fig. 3, A1 and B1). With similar treatments, p-ERK1/2 staining was observed mainly in nuclei (Fig. 3, M and N), while all ERK2 and p38 staining was distributed in the cytoplasmic region (Fig. 3, F, G, T, and U).

Inhibition of p38 Delays Corneal Epithelial Wound Closure as Compared with Inhibition of ERK1/2—We previously demonstrated in an organ culture model that both HGF and KGF stimulate corneal epithelial wound healing (11). In order to investigate the involvement of MAPK signaling activated by growth factors, corneas were incubated with or without growth factors and MAPK inhibitors as described above. In untreated corneas, the wounded area was significantly reduced to almost 50% at 24 h after injury (Fig. 4). Treatment with PD98059 produced a small but not significant inhibition of wound healing. However, when corneas were incubated with SB203580, there was greater inhibition of wound closure compared with untreated corneas at 24 h after injury or to corneas treated with PD98059 (Fig. 4B). As reported before, HGF reduced the wound area by almost 65–70% compared with the 0-h control, and by 40% (p < 0.05) compared with 24 h without growth factor (11). When corneas were treated with PD98059 then stimulated with HGF, there was a significant (p < 0.05) inhibition in wound healing leading to an increase in the uncovered wound area by almost 1.7-fold, compared with HGF-treated corneas. However, when corneas were preincubated with SB203580, and then stimulated for 24 h with HGF, the inhibition was significantly higher compared with corneas treated with PD98059 plus HGF or corneas treated with HGF alone.
and the relative size of the uncovered wound area increased by 2-fold as compared with HGF-treated corneas at 24 h. When corneas were coincubated with both MAPK inhibitors and stimulated with HGF, inhibition of wound healing was further enhanced and the remaining uncovered wound area increased by almost 2.5-fold over HGF-stimulated corneas (Fig. 4). This inhibition was significantly higher than in corneas pretreated with PD98059 or SB203580 and stimulated with HGF, as well as with HGF alone.

**Differential Activation of ERK1/2 and p38 in Migrating and Proliferating Epithelium—Immunocytochemistry of corneal sections of migrating epithelium showed that immediately after injury there was no p-ERK1/2-positive staining, while p-p38 was detected in regions near the wound and gradually diminished in quantity with distance from the wound (Fig. 5A, B and D). ERK2 and p38 were present from the wound edge (Fig. 5A, F and N).** In corneas pretreated with PD98059 and stimulated with HGF there was inhibition of p-ERK1/2 immunofluorescence (Fig. 5A, N). SB203580 was equally inhibitory for p-p38 fluorescence (Fig. 5A, T). In migrating epithelium the p-ERK1/2 immunofluorescence was not increased by SB203580 in the presence of HGF, as compared with 24-h HGF stimulation (Fig. 5A, R and J). In contrast, the p-p38 staining was significantly enhanced by pretreatment with PD98059 plus HGF (Fig. 5A, P and I).

After corneal injury, the epithelial cells of the limbal region,
Blockade of p38 produces more delayed wound closure than does inhibition of ERK1/2. Rabbit corneal organ cultures were treated with specific inhibitors of the p38 and ERK1/2 pathways with or without HGF. SB203580 significantly delayed wound healing as compared with PD98059 in the presence of HGF. A 7-mm epithelial debridement wound was made as described under Experimental Procedures. The corneas were treated with growth factor and/or inhibitors, incubated for 24 h at 37 °C, and then stained with alizarin red. Corneas from each condition were used for immunohistochemical analysis (see Fig. 5) or stained immediately after injury for zero hour control or at 24 h (panel A). The remaining uncovered wound area was considered as a negative index for wound healing. The data in panel B represent at least 5–6 corneas in each treatment and were obtained from 3–4 separate experiments. *, p < 0.05 compared with control and PD98059 at 24 h; **, p < 0.05 compared with HGF; †, p < 0.05 compared with PD98059 + HGF; NS, non-significant compared with control at 24 h. Numbers in panel A refer to conditions specified in panel B.

distal from the wound, begin proliferating (25). We found increased p-ERK1/2 immunostaining, but not increased p-p38 immunostaining, in cells of the limbal region of corneas 24 h after injury (Fig. 5B, f and h). Pretreatment with PD98059 and stimulation with HGF inhibited p-ERK1/2 in the limbus without affecting p-p38 (Fig. 5B, j, n and l, p). However, when p38 activity was blocked by SB203580 in the presence of HGF, (Fig. 5B, t) p-ERK1/2 staining was significantly increased, compared with HGF alone (Fig. 5B, r, j). The total ERK2 and p38 immunofluorescence remained essentially the same with all treatments in migrating and limbal epithelium.

Inhibition of p38 Decreases Epithelial Migration but Does Not Affect Proliferation—In order to obtain more clear evidence of the role of these kinases in corneal epithelial wound healing, a simple cell scrape wound model assay was used, where RCE cells were scraped from one side of marked reference line (simulating a wound) and were allowed to migrate on their own or under the desired treatments. A similar approach in an earlier study induced migration without chemotaxis (22). Cells were treated with HGF and/or specific MAPK inhibitors 24 h, and cell migration was studied as described above. There were 480 ± 22 cells/area that migrated across the reference line when stimulated with HGF, compared with only 120 ± 7 cells/area in control (24 h) untreated cells (Fig. 6A, e and b; p < 0.01) (data indicate average of at least 10 different areas per dish in three independent experiments), suggesting an almost 4-fold increase in migration in HGF-stimulated cells over untreated control cells. Cell migration was completely inhibited by SB203580 alone (Fig. 6A, d), and there was a significant reduction, by 80–90%, in migration upon treatment with SB203580 in the presence of HGF: the average number of cells that migrated across the reference line was 50 ± 3 cells/area (Fig. 6A, h; p < 0.01 compared with e). In contrast, PD98059 does not decrease migration of these cells; rather, it seems to potentiate it. The average number of cells that migrated across the reference line was 210 ± 12 cells/area, which was almost 2-fold higher than in the 24-h control (Fig. 6A, f and b; p < 0.05).

Pretreatment of cells with PD98059 followed by stimulation with HGF did not inhibit migration, but slightly enhanced it, with an average of 520 ± 26 cells/area migrating across the reference line (Fig. 6A, g; p < 0.01 compared with h). A vehicle-only control with Me2SO at the same concentration as used with the inhibitors SB203580 and PD98059 (0.01%) did not show a noticeable change in migrating cells compared with the 24-h control (Fig. 6A, b and c). To determine whether cross-talk exists and whether it plays a role during cell migration, in similar experiments migrating cells were immunostained with anti-p-ERK1/2 and anti-p-p38. Cells that migrated across the reference line showed negligible staining for p-ERK1/2 (Fig. 6B, a); after HGF stimulation, cells showed a diffuse p-ERK1/2 staining localized mainly in the cytoplasm (Fig. 6B, b). Pretreatment with SB203580 followed by HGF, which significantly reduced migration, cross-activated p-ERK1/2 staining, and the few cells that did migrate showed p-ERK1/2 staining in the nucleus (Fig. 6B, c). When control cells were immunostained with anti-p-p38, more cells that migrated showed staining in the nucleus compared with control cells stained with anti-p-ERK1/2 (Fig. 6B, d and a). Cells stimulated with HGF showed a significantly higher number of cells that migrated and that had p-p38 staining in the nuclear region compared with control (Fig. 6B, e), and inhibition of ERK1/2 potentiated the nuclear localization of p-p38 immunostaining (Fig. 6B, f) compared with both HGF and control. To further corroborate these findings, we also analyzed by Western blot the activation of p38 and ERK1/2 in cells that migrated. Cells that migrated across the reference line were carefully removed and processed as described under “Experimental Procedures.” Cells stimulated with HGF and allowed to migrate for 24 h showed activation of p-p38. When the ERK1/2 pathway was blocked, there was a significant and robust up-regulation of p-p38 MAP kinase (Fig. 6C, top panel). This coincided with increased migration (Fig. 6A, g). HGF stimulation also up-regulated p-ERK1/2; however, when cells were pretreated with PD98059 and stimulated with HGF, although the cells showed robust migration, there was a significant inhibition in p-ERK1/2 compared with HGF alone (Fig. 6C, bottom panel). The total p38 and ERK1/2 remained unchanged with these treatments (Fig. 6C, lower panels).

We also investigated the role of p38 stimulated by HGF in cell proliferation. In agreement with a previous report (26), HGF stimulated proliferation by almost 20% at 48 and 72 h compared with the untreated control (Fig. 7). PD98059 treatment inhibited the proliferation of nonstimulated cells by 12 and 20% at 48 and 72 h, respectively, over the control. When cells were pretreated with PD98059 for 1 h and then stimulated with HGF, the proliferation was significantly (p < 0.05) inhibited, by almost 14 and 27% at 48 and 72 h, respectively, as compared with HGF treatment. On the other hand, when cells were pretreated with SB203580 for 1 h followed by stimulation with HGF, there was no noticeable effect on cell
FIG. 5. Immunostaining of p38 and ERK1/2 at the wound edge and at the limbal region of corneal epithelia in organ-cultured rabbit corneas. After a central 7-mm epithelial wound was made as explained under “Experimental Procedures.” Rabbit corneas were cultured for 24 h at 37 °C in a 5% CO₂ atmosphere. The corneas were embedded in O. C. T. and 6-μm sections were prepared for immunostaining. p38 and
proliferation compared with cells stimulated with HGF alone (Fig. 7).

**DISCUSSION**

The paracrine growth factors HGF or KGF have been shown to activate ERK1/2 upon stimulation of corneal epithelial cells (11, 12). In the present studies we demonstrate that another of the MAPKs, p38, was activated and translocated to nucleus. However, JNK was not stimulated by the growth factors. Our results indicate that when the ERK1/2 pathway was interrupted by deploying a specific antagonist at one level upstream, i.e. MEK (MAPK-K), this triggered a concomitant up-regulation of p38. Similarly, when the p38 pathway was chemically blocked, we observed an activation of ERK1/2. This clearly establishes that in corneal epithelium there exists a two-way cross-talk between p38 and ERK1/2 MAP kinases. There have been recent reports of one-way cross-talk between these two kinases in other cell types (27, 28) and one study suggesting an inhibitory cross-talk among MAP kinases in human umbilical vein endothelial cells (29). Both p38 and ERK1/2, when activated by growth factors, are translocated to the nucleus and may further phosphorylate specific transcription factors to regulate gene expression (30). Pretreatment of cells with an antagonist to block the ERK1/2 pathway in the presence of growth factors completely inhibited ERK1/2 activation as well as translocation to the nucleus, and at the same time, potentiated p38 activation and subsequent translocation into the nucleus. Similar inhibition of the p38 pathway accompanied by growth factor stimulation totally abrogated activation of p38, but induced a greater accumulation of p-ERK1/2 in the nucleus, especially in the presence of HGF.

Immunohistochemical studies of wounded corneas in organ culture indicated that immediately after injury, p38 is activated near the wound edge. In contrast, p-ERK1/2 levels were undetectable. The results suggest that p38 is activated in the migrating epithelium in order to help cover the wound as soon as possible. Even at 24-h post-injury, wound closure was driven by activation of p38. In these experiments growth factors were not added to the corneas; hence the response of the epithelium is a consequence of activation of endogenous components, which could include several growth factors and cytokines pro-

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ERK1/2 were immunostained in the epithelial cells and underlying stromal cells of all groups. A, immunolocalization of p38 and ERK1/2 and their phosphorylated forms in the migrating epithelium of corneas obtained immediately after injury (0 h, control) or after 24-h control, with HGF with or without 50 \( \mu \)M PD 98059 (PD) or 20 \( \mu \)M SB 203580 (SB), as described under “Experimental Procedures.” B, immunolocalization of the kinases and their active forms in limbal epithelium.

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**Fig. 6.** Blocking p38 activation affects epithelial migration. RCE cells were seeded in 60-mm dishes marked with a reference line in the center. Confluent monolayer cells were serum-starved overnight and then scraped from one side of the reference line as described under “Experimental Procedures.” Cells were incubated with the indicated treatments for 24 h. A, at 24 h after injury and indicated treatments, cells were observed under the microscope and photographed. Each individual photograph is an example of at least 8–10 randomly chosen areas from 3–4 separate experiments. B, in parallel experiments the cells that migrated at 24 h were immunostained with anti-p-ERK1/2 or anti-p-p38 as described. DAPI was used for nuclear counterstain. C, p-p38 and p-ERK1/2 expression determined by Western blot in migrating epithelial cells.
compared with control; **, p significant difference compared with HGF.

Microplates were processed for proliferation assay as described under "Experimental Procedures." The data are representative of octuplicate assays in three independent sets of experiments ± S.E. *, p < 0.05 compared with control; **, p < 0.05 compared with HGF; NS, no significant difference compared with HGF.

RCE cell proliferation. However, SB203580 had no adverse effect on proliferation. RCE cells were plated at 5000 cells/well in 96-well microplates and allowed to attach before being serum-starved overnight. The microplates were processed for proliferation assay as described under "Experimental Procedures." The data are representative of octuplicate assays in three independent sets of experiments ± S.E. *, p < 0.05 compared with control; **, p < 0.05 compared with HGF; NS, no significant difference compared with HGF.

FIG. 7. Inhibition of ERK, but not p38, affects proliferation. Blockade of the ERK1/2 pathway by PD98059 significantly inhibited RCE cell proliferation. However, SB203580 had no adverse effect on proliferation. RCE cells were plated at 5000 cells/well in 96-well microplates and allowed to attach before being serum-starved overnight. The cells were treated with growth factor and inhibitors for 48 or 72 h. The microplates were processed for proliferation assay as described under "Experimental Procedures." The data are representative of octuplicate assays in three independent sets of experiments ± S.E. *, p < 0.05 compared with control; **, p < 0.05 compared with HGF; NS, no significant difference compared with HGF.

The data are representative of octuplicate assays in three independent sets of experiments ± S.E. *, p < 0.05 compared with control; **, p < 0.05 compared with HGF; NS, no significant difference compared with HGF.

FIG. 8. Schematic representation of activation of corneal epithelial wound healing by MAPK. Injury induces the release of growth factors (HGF and KGF) that activate their receptors and induce the coordinated activation and cross-talk between ERK1/2 and p38. Blockade of either signaling cascade at the level of MEK (MAPK-K) or p38 activates the other kinase. These two pathways act together to achieve optimal healing response. Upstream regulation of these kinases has not been defined in this model.

The proliferation studies indicate that HGF-mediated ERK1/2 activation mainly governs proliferation of epithelial cells. Blockade of the p38 pathway did not affect proliferation in our assays, perhaps because the system is already maximally stimulated, and other control signals are in place to avoid hyperproliferation (e.g. MAPK phosphatases).

Our corneal organ culture studies demonstrated that inactivation of p38 induced cross-activation of ERK1/2 in the limbal epithelium. After injury, there is an increase in the expression of cyclins D and E in the cells distal from the wound (18), and ERK1/2 can activate cyclin D (15), therefore activation of this MAP kinase in limbal epithelial cells could be a mechanism to induce the proliferative state of these cells.

In conclusion, this study is the first to link activation of the MAPK signaling cascade with a tissue-repair response. We provide evidence that, in corneal epithelium, there is a two-way cross-talk activation between p38 and ERK1/2 (Fig. 8). Activation of p38 occurs rapidly after epithelial injury and is sustained at 24 h in the migrating epithelium. Moreover, p38 mediates HGF-induced epithelial migration, but not proliferation. In contrast, there is no ERK1/2 activation in the migratory epithelium, while ERK1/2 activation in the areas distal from the wound mediates HGF-stimulated proliferation. If one or both cell signals are interrupted, the wound-healing process is hampered. These signaling intersections and the cross-talk between the two kinases may be of great significance to corneal wound healing as well as in other tissues. These findings provide a better understanding of the complex wound-healing process and raise the possibility of therapeutic intervention when wounds are difficult to repair.
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REFERENCES

1. Robinson, M. J., and Cobb, M. H. (1997) Curr. Opin. Cell Biol. 9, 180–186
2. Nebreda, A. R., and Porras, A. (2000) Trends Biochem. Sci. 25, 257–260
3. Hedges, J. C., Dechert, M. A., Yamboliev, I. A., Martin, J. L., Hickey, E., Weber, L. A., and Gerthoffer, W. T. (1999) J. Biol. Chem. 274, 24211–24219
4. Iijima, K., Yoshizumi, M., Hashimoto, M., Akishita, M., Kosaki, K., Ako, J., Watanabe, T., Ohike, Y., Son, B., Yu, J., Nakahara, K., and Ouchi, Y. (2002) Circulation 105, 2404–2410
5. Tangkijvanich, P., Santiskulvong, C., Melton, A. C., Rozengurt, E., and Yee, H. F., Jr. (2002) J. Cell. Physiol. 191, 351–361
6. Matsumoto, T., Yokote, K., Tamura, K., Takekoshi, M., Ueno, H., Saite, Y., and Mori, S. (1999) J. Biol. Chem. 274, 13954–13960
7. Wilson, S., Chen, L., Mohan, R., Liang, Q., and Liu, J. (1999) Exp. Eye. Res. 68, 377–397
8. Brazzell, R. K, Stern, M. E., Aquavella, J, V., Beuerman, R. W., and Bird, L. (1991) Invest. Ophthalmol. Vis. Sci. 32, 336–340
9. Sotozono, C., Inatomi, T., Nakamura, M., and Kinoshita, S. (1995) Invest. Ophthalmol. Vis. Sci. 36, 1524–1529
10. Imanishi, J., Kamiyama, K., Iguchi, I., Kita, M., Sotozono, C., and Kinoshita, S. (2000) Prog. Retin. Eye Res. 19, 113–129
11. Chandrasekher, G., Kakazu, A. H., and Bazan, H. E. P. (2001) Exp. Eye. Res. 73, 191–202
12. Liang, Q., Mohan, R. R., Chen, L., and Wilson, S. E. (1998) Invest. Ophthalmol. Vis. Sci. 39, 1329–1338
13. Chandrasekher, G., and Bazan, H. E. P. (1999) Curr. Eye Res. 18, 168–176
14. Wilson, S. E., Walker, J. W., Chwang, E. L., and He, Y. G. (1993) Invest. Ophthalmol. Vis. Sci. 34, 2544–2561
15. Lavoie, J. N., L’Allemand, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996) J. Biol. Chem. 271, 20608–20616
16. Zieske, J. D. (2000) Prog. Retin. Eye Res. 19, 257–270
17. Joyce, N. C., Mekhr, B., Joyce, S. J., and Zieske, J. D. (1996) Invest. Ophthalmol. Vis. Sci. 37, 645–655
18. Chung, E. H., Hutcheon, A. E., Joyce, N. C., and Zieske, J. D. (1999) Invest. Ophthalmol. Vis. Sci. 40, 1952–1958
19. Hurst, J., Ma, X., and Bazan, H. E. P. (1999) Invest. Ophthalmol. Vis. Sci. 40, 790–795
20. Foreman, D. M., Panzani, S., Jarvis-Evans, J., McLeod, D., and Boulton, M. E. (1996) Exp. Eye. Res. 63, 555–565
21. Chandrasekher, G., Ma, X., Lallier, T. E., and Bazan, H. E. P. (2002) Invest. Ophthalmol. Vis. Sci. 43, 1422–1428
22. Edin, M. L., Howe, A. K., and Juliano, R. L. (2001) Exp. Cell Res. 266, 214–222
23. Rieck, P. W., Cholidis, S., and Hartmann, C. (2001) Exp. Eye Res. 73, 639–650
24. Schilling-Schon, A., Pleyer, U., Hartmann, C., and Rieck, P. W. (2000) Exp. Eye Res. 71, 583–589
25. Hanna, C. (1966) Am. J. Ophthalmol. 51, 55–63
26. Wilson, S. E., He, Y. G., Weng, J., Zieske, J. D., Jester, J. V., and Schultz, G. S. (1994) Exp. Eye. Res. 58, 665–678
27. Berra, E., Diaz-Meco, M. T., and Moscat, J. (1998) J. Biol. Chem. 273, 10792–10797
28. Xiao, Y. Q., Malcolm, K., Worthing, G. S., Gardai, S., Schiemann, W. P., Fadok, V. A., Bratton, D. L., and Henson, P. M. (2002) J. Biol. Chem. 277, 14884–14893
29. Surapisitchat, J., Hoeven, H. J., Pi, X., Yoshizumi, M., Yan, C., and Berk, B. C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6476–6481
30. Brunet, A. and Pouyssegur, J. (1997) Essays Biochem. 32, 1–16
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