Common and Distinct Pathways for Cellular Activities in FGF-2 Signaling Induced by IL-1β in Corneal Endothelial Cells

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Purpose. To determine the mechanism by which IL-1β induces FGF-2 and to elucidate the signaling pathways of IL-1β–induced FGF-2 in corneal endothelial cells (CECs).

Methods. Expression and/or activation of FGF-2, p38, ERK1/2, and Akt was analyzed by immunoblot analysis. Cell proliferation was measured by MTT assay. Pharmacologic inhibitors were used to block PI 3-kinase, p38, or ERK1/2.

Results. Brief stimulation of CECs with IL-1β activated PI 3-kinase and p38 in a biphasic fashion. The first wave of activation, triggered by IL-1β, involves the inductive activity of IL-1β on FGF-2 production; the second wave of activation, triggered by the induced FGF-2, involves the promotion of cellular activities. In both pathways, p38 acts downstream to PI 3-kinase. The inductive activity of IL-1β on FGF-2 is further evidenced by the conditioned medium, which contains a large amount of FGF-2. Stimulation of CECs with IL-1β also activated ERK1/2 in a delayed fashion. The IL-1β–induced FGF-2 exerted cellular activities using distinct pathways: the second wave of activation of PI 3-kinase and p38 was involved in cell migration, whereas cell proliferation was simultaneously stimulated by ERK1/2 and the second wave of PI 3-kinase. Likewise, the conditioned medium demonstrated cellular activities and pathways identical with those observed in cells treated with IL-1β.

Conclusions. These data suggest that CECs produce FGF-2 by IL-1β stimulation through PI 3-kinase and p38. The IL-1β–induced FGF-2 facilitates cell migration via PI 3-kinase and p38, whereas it stimulates cell proliferation using PI 3-kinase and ERK1/2 in parallel pathways. (*Invest Ophthalmol Vis Sci.* 2009; 50:2067–2076) DOI:10.1167/iovs.08-3135

The corneal endothelium is a monolayer of differentiated cells located in the posterior portion of the cornea. Human corneal endothelial cells (CECs) are known to remain arrested in the G_1 phase of the cell cycle throughout their lifespan.[1,2] This characteristic behavior of cell proliferation dictates most of the wound-healing processes that occur in the corneal endothelium: CECs cover the denuded area by migration and attenuation rather than by replacing the lost cells through cell division. Occasionally, however, during the wound-healing process, CECs are transformed into mesenchymal cells that subsequently produce fibrillar extracellular matrix (ECM) in the basement membrane environment. Thus, corneal fibrosis represents a significant pathophysiological problem—one that causes blindness by physically blocking light transmittance. One clinical example of corneal fibrosis observed in the corneal endothelium is the development of a retrocorneal fibrous membrane (RCFM) in Descemet's membrane.[3–6] In RCFM, CECs are converted to fibroblast-like cells: the contact-inhibited monolayers of CECs are lost, resulting in the development of multiple layers of fibroblast-like cells.[5,6] These morphologically altered cells simultaneously resume their proliferation ability and deposit a fibrillar ECM in Descemet's membrane. An in vitro model to elucidate the molecular mechanism of RCFM formation led us to the finding that activated polymorphonuclear leukocytes (PMNs) were able to transform the type IV collagen-synthesizing polygonal endothelial cells to type I collagen–synthesizing fibroblastic cells.[7–9] Of the several proteins released by the activated PMNs, a 17-kDa protein band that caused endothelial mesenchymal transformation (EMT) of CECs was identified as interleukin 1β (IL-1β) using protein chip array technology.[9,10]

IL-1β is a major proinflammatory cytokine that plays an important role in acute and chronic inflammatory diseases.[11–16] IL-1β also has a crucial role in the regulation of inflammation and wound healing on the ocular surface.[17–20] Numerous studies have reported that both interleukin (IL)-1α and -1β orchestrate the inflammatory process by inducing the production and release of secondary cytokines; IL-1β stimulates the expression of a variety of genes necessary for the wound repair process.[21–27] Both IL-1α and -1β markedly stimulate synthesis and release of fibroblast growth factor (FGF)-2 in a variety of cell types.[23–25] Likewise, CECs produce all isoforms of FGF-2 in response to IL-1β stimulation[10]; IL-1β activates PI 3-kinase, the enzyme activity of which was greatly stimulated after a 5-minute exposure to IL-1β. Such an early and rapid activation of PI 3-kinase greatly enhanced FGF-2 production in CECs; pretreatment with LY294002 completely blocked the induction activity of IL-1β.

FGF-2, a ubiquitous and multifunctional regulator, contributes to the regulation of many different biological processes, including cell proliferation and differentiation of a broad spectrum of mesodermal and neuroectodermal cells.[28–34] Among the five isoforms of FGF-2, the 18-kDa ECM isoform employs cell surface FGF receptors to relay signals that mediate a variety of cellular activities,[35–37] although the high-molecular-weight (HMW) isoforms of FGF-2 exert intracrine activity, the mechanism of which is yet to be defined. In our previous studies, we reported that the 18-kDa FGF-2 is the direct mediator for EMT observed in CECs.[38–45] First, FGF-2 directly regulates cell cycle progression by degrading $p27^{Kip1}$ (p27), leading to a marked stimulation of cell proliferation.[39–41] Second, FGF-2 upregulates the steady state levels of α1(I) collagen RNA by stabilizing the message and subsequently facilitates synthesis and secretion of type I collagen into the extracellular space.[42] And third, FGF-2 induces a change in cell shape from a polygonal to a fibroblastic morphology through regulation of the Rho family

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of small GTPases and subsequent reorganization of actin.[43,44] Of interest, FGF-2 uses PI 3-kinase as the major signaling pathway for these phenotypic alterations,[38–44] similar to the published report.[45]

In the present study, we attempted to link inflammatory responses to the different set of cellular responses triggered by the growth factor induced during inflammation. We had reported that FGF-2 was the direct cause of the cell shape change mediated by IL-1β. Neutralizing antibody to FGF-2 completely reversed the cell shape from an elongated to a polygonal morphology.[10] The fact that IL-1β is able to induce FGF-2, the direct mediator of EMT, led us to investigate the respective signaling pathways triggered by IL-1β and FGF-2. A brief stimulation of CECs with IL-1β activates PI 3-kinase and p38 in a biphasic fashion: The first wave of activation is triggered by IL-1β and is subsequently involved in the inductive activity of IL-1β on FGF-2 production. The IL-1β–induced FGF-2 then initiates distinct signal transduction during the course of EMT; cell migration is mediated via PI 3-kinase and p38 as a downstream effector, and cell proliferation is triggered by parallel pathways of extracellular signal–regulated kinase 1/2 (ERK1/2) and PI 3-kinase. Thus, this study indicates the sequential activation of CECs by IL-1β and FGF-2 in the context of inflammatory responses and nonphysiologic wound healing.

Materials and Methods

Anti-Akt, phospho-Akt (Thr308), phospho-Akt (Ser473), p38, phospho-p38, and ERK1/2 antibodies and anti-phospho-ERK1/2 antibody were purchased from Cell Signaling Technology (Danvers, MA); FGF-2 from Calbiochem (San Diego, CA); IL-1β, U0126, SB203580, LY294002, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), mitomycin-C, and peroxidase-conjugated secondary antibodies from Sigma-Aldrich (St. Louis, MO); anti-FGF-2 antibody and neutralizing anti-FGF-2 antibody from Upstate (Charlottesville, VA); and anti-β-actin antibody from BioVision (Mountain View, CA).

Cell Culture

Rabbit eyes were purchased from Pel Freez Biologicals (Rogers, AR). Isolation and establishment of rabbit CECs were performed as previously described.[46] Briefly, the Descemet's membrane complex was treated with 0.2% collagenase and 0.05% hyaluronidase (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37°C. Primary cultured cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) supplemented with 15% fetal bovine serum (Omega Scientific, Tarzana, CA) and 50 μg/mL of gentamicin (DMEM-15) in a 5% CO_2 incubator. For subculture, confluent primary cultures were treated with 0.05% trypsin and 5 mM EDTA in phosphate-buffered saline (PBS) for 5 minutes. First-passage CECs maintained in DMEM-15 were used for all experiments. Heparin (10 μg/mL) was added to cell cultures treated with FGF-2 (10 ng/mL), because our previous study showed that the initiation of FGF-2 activity in CECs requires the addition of supplemental heparin.[8] In some experiments, pharmacologic inhibitors were used in the presence of IL-1β or FGF-2 stimulation: SB203580 (20 μM), U0126 (10 μM), or LY294002 (20 μM). The undiluted culture media maintained for 16 hours after stimulation with IL-1β for 10 minutes was used as conditioned media in some experiments.

Protein Preparation and Immunoblot Analysis

Cells were scraped in RIPA lysis and extraction buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysates were sonicated on ice. Concentration of the resultant lysates was assessed with the Bradford protein assay system (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were separated by SDS-PAGE as described by Laemmli, by using the discontinuous Tris-glycine buffer system.[47] The proteins separated by SDS-PAGE were transferred to a 0.22-μm nitrocellulose membrane (Whatman Inc., Florham Park, NJ), and nonspecific binding sites of nitrocellulose membrane were blocked by 5% nonfat milk in TBS containing 0.1% Tween-20. The incubations were performed with primary antibodies (1:2500 dilution) and peroxidase-conjugated secondary antibody (1:5000 dilution). Membranes were treated with enhanced chemiluminescence (ECL) reagent (Amersham Biosciences Corp., Piscataway, NJ) and exposed to ECL film. The relative density of the polypeptide bands detected on ECL film was determined using commercial software (Gel-doc; Bio-Rad Laboratories, Inc.).

Purification of the Isoforms of FGF-2

Purification of the isoforms of FGF-2 was performed according to our published protocols with some modification.[48] CECs cultured in each culture condition on 100-mm tissue culture dishes were washed twice with sterile PBS. The extracellular matrix FGF-2 fraction was initially extracted with 2 M NaCl in 20 mM HEPES (pH 7.6) and then with 2 M NaCl in 20 mM sodium acetate (pH 4.5) to extract FGF-2 bound to high- and low-affinity receptors. The cells were then incubated in an enzyme-free cell dissociation solution (Chemicon, Temecula, CA) for 5 minutes at room temperature. The cells were detached by scraping, transferred to microcentrifuge tubes, and pelleted at 5000*g* for 10 minutes. Nuclear and cytoplasmic proteins were extracted with a nuclear extraction kit containing cytoplasmic lysis buffer and nuclear lysis buffer (Chemicon), according to the manufacturer's instructions. Briefly, the harvested cells were resuspended in two cell pellet volumes of cold cytoplasmic lysis buffer containing 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 0.5 mM dithiothreitol and allowed to swell on ice for 15 minutes. IGEPAL CA-630 (Sigma-Aldrich) was then added to a final concentration of 0.1%, and the swollen cells were incubated for an additional 5 minutes on ice and homogenized with a 27-gauge needle. Nuclei were pelleted at 8000*g* for 20 minutes at 4°C. The supernatant containing the cytosolic portion was transferred to a fresh tube and stored at −80°C until further use. The remaining pellet containing the nuclear portion was washed by centrifugation with cold nuclear extraction buffer containing 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 0.5 mM dithiothreitol and then resuspended in two thirds of the original cell pellet volume of cold nuclear extraction buffer. Nuclei were disrupted by drawing and ejecting 10 times, with a syringe and a 27-gauge needle. Nuclear proteins were extracted at 4°C for 60 minutes with gentle agitation, using an orbital shaker. The nuclear protein extract was clarified by centrifugation at 16,000*g* for 5 minutes at 4°C and then dialyzed (Slide-A-Lyzer MINI Dialysis Unit, 7K MWCO; Pierce Biotechnology, Rockford, IL). Nuclear and cytoplasmic protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories, Inc.) with bovine serum albumin as a standard. To verify the purity of the fractions, 15 μg of nuclear or cytoplasmic proteins were immunoblotted with lamin B and α-tubulin antibodies. These verified nuclear and cytoplasmic proteins were used for FGF-2 estimation.

FGF-2 Estimation

The amount of FGF-2 in the purified subcellular fraction was measured with a protein array system (Bio-Plex) and analyzed with the accompanying system software (Bio-Plex Manager 2.0; all from Bio-Rad Laboratories, Inc.). Since anti-human FGF-2 antibody has cross reactivity with rabbit FGF-2, we chose a human FGF-2 assay kit (Bio-Plex Human FGF-2 Singleplex; Bio-Rad Laboratories, Inc.).[10] All procedures were performed, according to the manufacturer's instructions. Briefly, 50 μL of each subcellular fraction was added to same volume of anti-FGF-2 antibody–conjugated beads in a 96-well filter plate and incubated at room temperature for 30 minutes. After a series of washes to remove the unbound proteins, 25 μL of biotinylated FGF-2 antibody (which detects a different epitope from the bead-conjugated FGF-2 antibody) was added to each well and incubated for 30 minutes, resulting in the

formation of sandwiches of antibodies with the target proteins. After another washing, 50 μL of streptavidin-PE was added to each well, followed by further washing. Thereafter, 125 μL of assay buffer (Bio-Rad Laboratories, Inc.) was added to each well, and the well contents were analyzed using the protein array system. The unknown FGF-2 concentration was determined by finding the concentration on the standard curve derived by using various concentrations of FGF-2 standards in the assay. The cytokine assay (Bio-Plex; Bio-Rad Laboratories, Inc.) allows the quantitative measurement of multiple cytokines in a small volume of subcellular fractions, which are comparable to traditional enzyme-linked immunosorbent assays (ELISA).

Scratch-Induced Directional Migration Assay

Cells were plated in six-well tissue culture dishes at a concentration of 1×10^5 cells and maintained in DMEM-15. After serum starvation, the tip of a micropipette was used to wound the cells, creating linear, cross-stripe scrapes 2 mm apart. The cells were washed with PBS to remove floating cellular debris and refed for an additional 16 hours with either serum-free medium (for use as a negative control) or experimental medium. Wound closure or cell migration was photographed when the scrape wound was introduced and at designated times after wounding, using an inverted microscope equipped with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The speed of migration was quantitated by computer-assisted image analysis (SPOT program, version 2.1.2; Diagnostic Instruments, Inc.). The individual gaps were measured in each culture condition and at each time point using this program, and the speed of migration was acquired by dividing the length of gap by the culture time. The residual gap between the migrating cells from the opposing wound edge was expressed as a percentage of the initial scraped area. All experiments were conducted in the presence of 5 μg/mL of mitomycin-C to inhibit cell proliferation.

Protein Preparation of Migratory Cells

Cells were plated in 100-mm tissue culture dishes at a concentration of 1×10^6 cells, and wounds were created as described earlier with a 10-mm separation between the linear scraped wounds. Migratory cells that moved into the wounded area were carefully scraped without contaminating the nonmigratory cells. These cells were washed with ice-cold PBS and then lysed with RIPA buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% Nonidet P-40, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin, and 1 mM PMSF on ice for 30 minutes. The lysates were centrifuged at 15,000*g* for 10 minutes at 4°C. The protein concentration of the sample was assessed with the Bradford protein assay system (Bio-Rad Laboratories, Inc.).

Cell Proliferation Assay

Cells were seeded in 96-well tissue culture plates at a concentration of 4×10^3 cells/well. On the following day, the medium was changed from DMEM-15 to DMEM for serum starvation and maintained for 30 hours. The serum-starved cells were then maintained for 24 hours in each culture condition. At the end of culture, the medium was replaced with medium containing MTT (50 μg/mL) and further maintained for 4 hours at 37°C. The MTT-containing medium was discarded and 100 μL of undiluted dimethyl sulfoxide was added to the cells. After a 1-hour incubation at room temperature, absorbance of the converted dye was measured with a spectrophotometric plate reader (Benchmark Plus Microplate Spectrophotometer; Bio-Rad Laboratories, Inc.), at a wavelength of 570 nm with background subtraction at 650 nm.

Results

FGF-2 Production Induced by IL-1β through the p38 Pathway

Our previous study[10] demonstrated that PI 3-kinase is activated in CECs within 5 minutes after activation with IL-1β. As a consequence of activated PI 3-kinase (as determined by the levels of phosphorylated Akt, the downstream effector of PI 3-kinase), CECs greatly upregulate FGF-2 expression at both the transcript and the protein levels. To further identify the intracellular signaling pathways involved in the inductive mechanism of IL-1β on FGF-2, cells were treated with IL-1β for 10 minutes. The cells were then maintained in IL-1β–free and serum-free medium (DMEM) for 1 to 48 hours. We first determined phosphorylation profiles of Akt at Ser473 and Thr308 sites. Both sites were phosphorylated in a biphasic fashion. The first peak of PI 3-kinase activation was observed in cells maintained for 1 hour after a brief stimulation with IL-1β, reaching the maximum activation at 2 hours. Activation was then markedly decreased until the second wave of PI 3-kinase activation was observed in cells maintained for 12 hours, reaching another maximum activation at 16 hours after IL-1β stimulation (Fig. 1A). We also determined whether p38 and ERK1/2 were involved in IL-1β-triggered intracellular signal transduction during FGF-2 induction. The activation profile of p38 was biphasic: phosphorylated p38 was observed in cells maintained for 1 hour, and the second peak of p38 activation was observed at 16 hours after IL-1β stimulation (Fig. 1B), similar to the activation kinetics of PI 3-kinase (Fig. 1A). On the other hand, the activation profile of ERK1/2 was very different from that of p38; the activation kinetic is not biphasic, and the earlier wave of activation of the enzyme was missing. Phosphorylation was detected in a relatively late time period (4 hours after IL-1β stimulation); phosphorylation was gradually increased to reach the maximum level 16 hours after a brief stimulation with IL-1β, and its activation state was sustained for 48 hours (Fig. 1B).

The parallel activation kinetics of p38 and PI 3-kinase led us to investigate whether p38 was also involved in the inductive activity of IL-1β on FGF-2 synthesis (Fig. 2). After a 2-hour pretreatment with SB203580, the cells were stimulated with IL-1β for 10 minutes and further maintained in DMEM for 1, 2, or 4 hours. Figure 2 shows that FGF-2 production was markedly triggered within 1 hour after brief stimulation with IL-1β and that FGF-2 synthesis was completely blocked by the p38 inhibitor. This finding clearly demonstrates that the activation of p38 is required for FGF-2 production mediated by IL-1β. Figure 2 further confirms our previous data[10] that IL-1β induced all isoforms of FGF-2 and that the 18-kDa ECM FGF-2 is the dominant isoform of FGF-2 induced by IL-1β stimulation.

We then investigated the mechanism of the biphasic activation of p38. It is likely that the first wave of activation was triggered by IL-1β, whereas the second wave of p38 activation was mediated by IL-1β, FGF-2, or both. CECs were treated with IL-1β for 10 minutes followed by treatment with the neutralizing antibody to FGF-2 for up to 16 hours to silence the activity of the FGF-2 induced by IL-1β. Phosphorylation profiles of p38 were also determined. The first wave of p38 activation (1 hour after IL-1β stimulation) was not blocked by the neutralizing antibody to FGF-2, whereas the second wave of p38 activation was completely blocked by the neutralizing antibody to FGF-2 (Fig. 3A). The respective pattern of p38 activation suggests that only the second wave of p38 activation is triggered by FGF-2, whereas the first wave of p38 activation is triggered by IL-1β. This finding was further confirmed in CECs treated with FGF-2 for 2 to 16 hours (Fig. 3B). Phosphorylation of p38 was evident after 4 hours of FGF-2 stimulation, and reached maximum level after 8 hours of continuous FGF-2 stimulation. There appears to be a 4-hour lag period in the second wave of p38 activation by IL-1β and by FGF-2. The time-difference of activation kinetics may suggest that FGF-2 production is prerequisite to the second wave activation of p38 in response to IL-1β stimulation. We also investigated whether phosphorylation of ERK1/2 was mediated by the

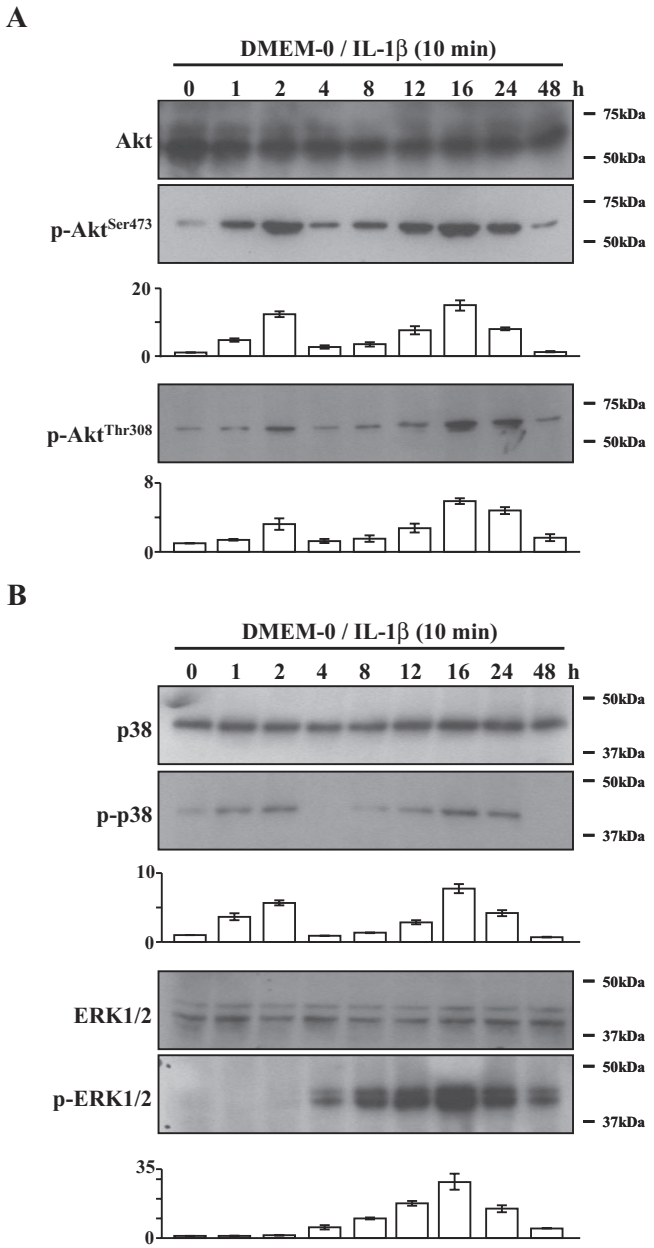

FIGURE 1. Effect of IL-1β on expression and/or activation of Akt, p38, and ERK1/2. When cells reached approximately 70% confluence, they were starved of serum for 24 hours. The serum-starved cells were treated with IL-1β (5 ng/mL) for 10 minutes and then maintained in DMEM for up to 48 hours. At the end of treatment, cells were lysed with RIPA buffer, and the cell debris was eliminated by centrifugation. After centrifugation, the supernatant was immunoblotted with the respective antibody. Relative density of immunoblotting bands was determined by using a gel documentation system. Total Akt, p38, and ERK were used to control protein concentration on immunoblot analysis. The relative differences (*x*-fold) were then compared with the number of CECs stimulated with IL-1β for 10 minutes (0 hours). The results represent data obtained in three independent experiments.

FGF-2 induced by IL-1β. Phosphorylation of ERK1/2 in response to IL-1β stimulation was completely blocked by the neutralizing antibody to FGF-2 (Fig. 3C). When FGF-2 action on ERK1/2 activation was examined in cells treated with FGF-2, phosphorylation of ERK1/2 was observed to occur in a time-dependent manner, and the activation of ERK1/2 by FGF-2 was faster than ERK1/2 activation by IL-1β (Fig. 3D). We further tested whether conditioned medium prepared with IL-1β contained FGF-2, which subsequently phosphorylates the two MAPKs: p38 and ERK1/2. Phosphorylated p38 was observed in CECs treated either with IL-1β or conditioned medium, whereas such activation was completely blocked by the neutralizing antibody to FGF-2 (Figs. 3A, 3E). Identical findings were observed with phosphorylation of ERK1/2; both IL-1β and the conditioned medium phosphorylate ERK1/2, which is blocked by the neutralizing anti-FGF-2 antibody (Figs. 3C, 3E). These findings confirmed that the active component in the conditioned medium generated with IL-1β is FGF-2, which facilitates the second wave of p38 activation and phosphorylates ERK1/2.

Finally, we measured the protein amount of FGF-2 in response to IL-1β stimulation in CECs. Cells were stimulated with IL-1β for 10 minutes and maintained in DMEM for 16 hours. The nuclear, cytoplasmic, and ECM isoforms of FGF-2 were prepared and the FGF-2 amount of each fraction was measured (Bio-Plex Protein Array System; Bio-Rad Laboratories; Fig. 4). The purities of the nuclear and cytoplasmic fractions were confirmed with lamin B and α-tubulin, respectively (data not shown). The basal levels of all isoforms of FGF-2 were very low in CECs. In the absence of serum, cells produced a barely detectable amount of FGF-2, whereas IL-1β greatly facilitated FGF-2 production in all isoforms (five- to tenfold). Of interest, almost half of the FGF-2 produced is the 18-kDa ECM isoform, which is the major isoform of FGF-2 that causes EMT of CECs.

Cell Migration Facilitated by the IL-1β–Induced FGF-2 via p38

The parallel activation kinetics of PI 3-kinase and p38 led us to investigate which of the two signaling proteins is upstream in intracellular signaling pathways triggered by IL-1β. After pretreatment of cells with LY294002 for 2 hours, the cells were briefly activated with IL-1β for 10 minutes and further maintained in DMEM for 1 to 24 hours. LY294002 completely blocked phosphorylation of Akt at both the Ser473 and Thr308 sites, and the PI 3-kinase inhibitor also completely blocked the first and second waves of p38 activation (Fig. 5A). On the other hand, SB203580, which inhibited phosphorylation of p38 during both waves of activation, did not block phosphorylation of Akt at Ser473 and Thr308 sites during the first wave of activation of PI 3-kinase (Fig. 5B), whereas the second wave of PI 3-kinase activation was blocked by the p38 inhibitor. These

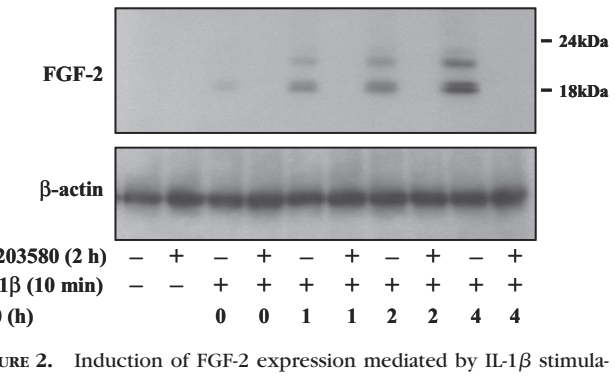

FIGURE 2. Induction of FGF-2 expression mediated by IL-1β stimulation through p38 activation. The serum-starved cells were pretreated with SB203580 for 2 hours during serum starvation before stimulation with IL-1β for 10 minutes. The cells were further maintained in DMEM for the indicated time. For positive control, the CECs were treated with only IL-1β for 10 minutes and the cells were further maintained in DMEM for the indicated time. Total cell protein preparation and immunoblot analysis were performed as described in Figure 1. β-Actin was used to control the protein concentration on immunoblot analysis. The data are representative of results in four experiments.

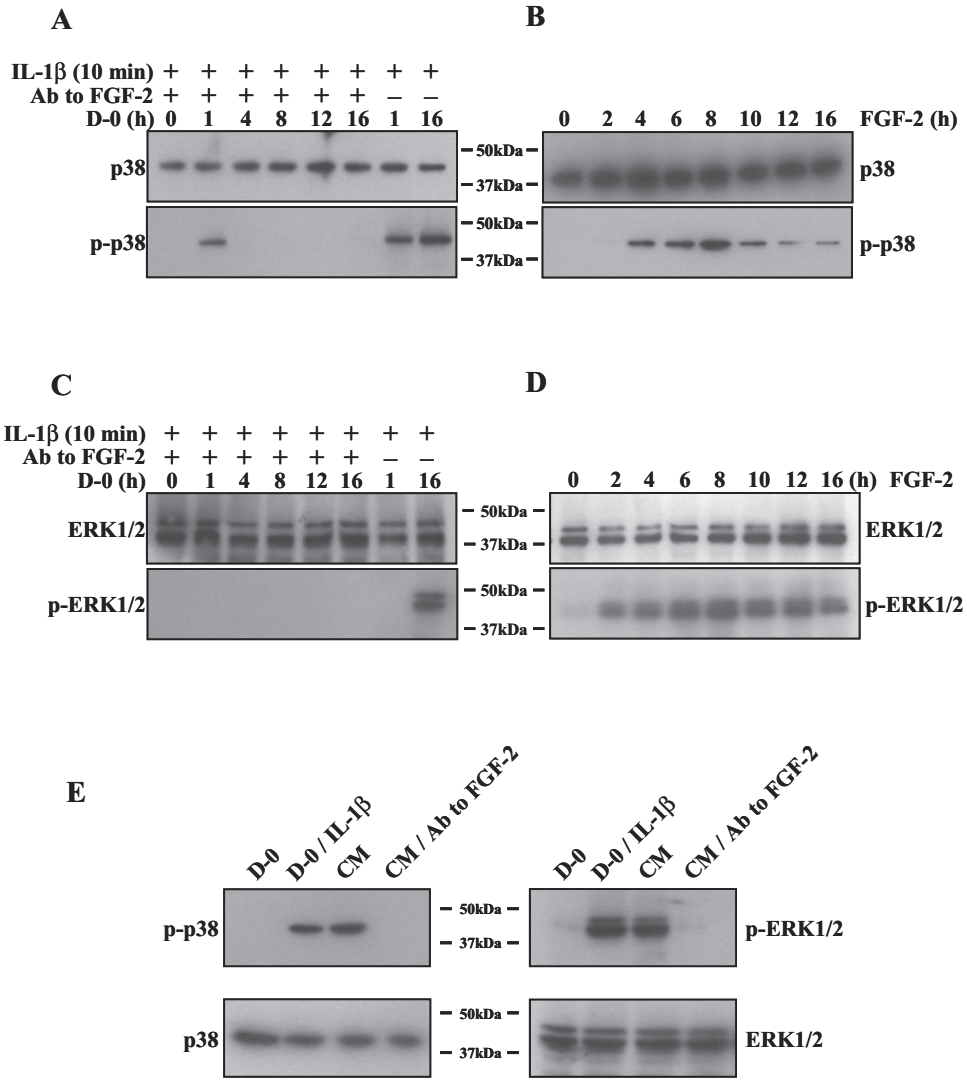

FIGURE 3. Effect of FGF-2 induced by IL-1β on activation of p38 and ERK1/2 through PI 3-kinase. The serum-starved cells were stimulated with IL-1β for 10 minutes. The cells were maintained in DMEM with neutralizing antibody to FGF-2 for the indicated times (**A**, **C**). For positive control, CECs were treated with only IL-1β for 10 minutes and the cells were further maintained in DMEM for the indicated time. The serum-starved cells were maintained in DMEM with FGF-2 for the designated times (**B**, **D**). The serum-starved cells were maintained in conditioned media with or without neutralizing antibody to FGF-2 for 16 hours (**E**). For positive and negative control, the CECs were treated for 10 minutes with or without IL-1β, respectively, and they were maintained in DMEM for an additional 16 hours. At the end of treatment, total cell proteins were prepared as described in Figure 1. Each cell lysate was then immunoblotted with the respective antibody. Total p38 and ERK1/2 were used to control protein concentration on immunoblot analysis. D-0, CM, and Ab to FGF-2 designate DMEM without serum, conditioned media acquired from IL-1β–treated cells, and neutralizing antibody to FGF-2, respectively. Data shown are representative of three independent experiments.

findings clearly demonstrated that, during the first wave of PI 3-kinase and p38 activation, PI 3-kinase signaling is unidirectional and upstream to p38. When the first wave of p38 activation is blocked by SB203580, the inhibitor blocks the inductive activity of IL-1β on FGF-2 production. Therefore, the second wave of PI 3-kinase and p38 activation that should be triggered by FGF-2 is completely abolished. We then tested which of the two PI 3-kinase and p38 is upstream in the FGF-2–mediated second wave of activation. Pretreatment of cells with LY294002 followed by stimulation with FGF-2 for 8 hours demonstrated that phosphorylation of Akt and p38 was completely blocked, whereas pretreatment of cells with SB203580 failed to block phosphorylation of Akt (Fig. 5C). These findings together suggest that p38 is downstream to PI 3-kinase in both the IL-1β–mediated FGF-2 induction pathway and FGF-2–mediated signal transduction.

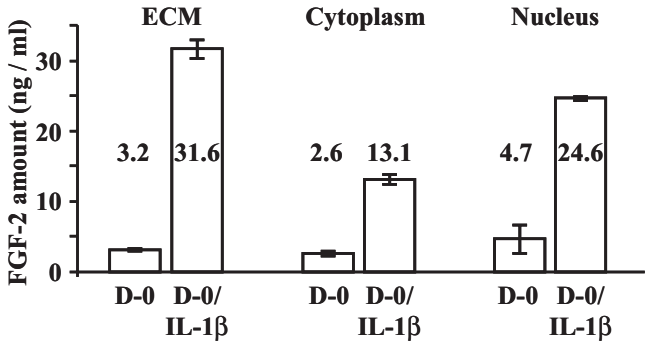

FIGURE 4. Measurement of FGF-2 production in response to IL-1β stimulation in each subcellular fraction. The serum-starved cells were treated with IL-1β for 10 minutes and then maintained in DMEM for 16 hours. Extracellular matrix and cytosolic and nuclear fractions were prepared. The amounts of FGF-2 contained in each subcellular fraction derived from cells that were either stimulated or not stimulated by IL-1β were measured by cytokine assay. The mean FGF-2 concentration in each subcellular fraction was expressed in ng/mL. The data represent results obtained in three independent experiments.

Since p38 is a major signaling molecule observed in cell migration,[49–51] we determined whether this is also true for IL-1β–facilitated cell migration in CECs. Cell migration was determined with scratch-induced directional migration assay in the presence of mitomycin-C (5 μg/mL). Migratory cells maintained in DMEM showed no detectable amount of gap closing, thus serving as the negative control, whereas those cells treated with FGF-2 that showed a marked gap closing were used as the positive control (Fig. 6A). In this scrape-wound assay over a 16-hour period, the cells stimulated with either IL-1β or maintained in conditioned medium for 16 hours recovered 44% or 47% of wound area, respectively, although cells stimulated with FGF-2 recovered almost 60% of the

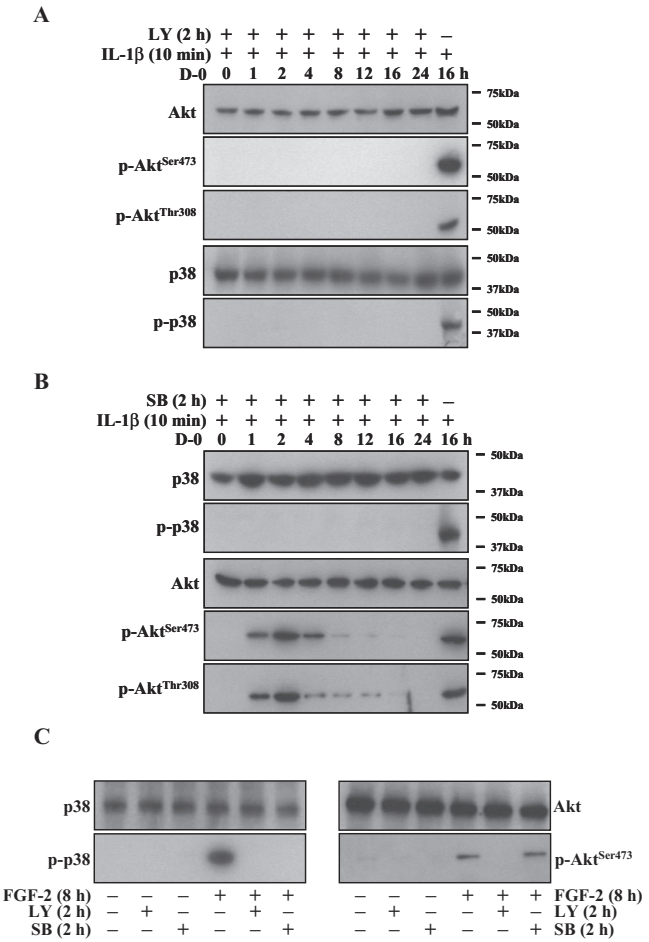

FIGURE 5. Upstream regulation of PI 3-kinase on phosphorylation of p38 in response to IL-1β and FGF-2 stimulation. The serum-starved cells were pretreated with LY294002 (**A**) or SB203580 (**B**) for 2 hours during serum starvation before stimulation with IL-1β for 10 minutes. The cells were further maintained in DMEM for the indicated time. For positive control, CECs were treated with only IL-1β for 10 minutes and cells were further maintained in DMEM for 16 hours. (**C**) The serum-starved cells were pretreated with LY294002 or SB203586 for 2 hours and then stimulated with FGF-2 for 8 hours. At the end of treatment, total cell proteins were prepared as described in Figure 1. Each cell lysate was then immunoblotted with the designated antibody. Total Akt and p38 were used to control protein concentration on immunoblot analysis. D-0, LY, and SB designate DMEM without serum, LY294002, and SB203580. The data shown are representative of results in three independent experiments.

wound area (Fig. 6A). When neutralizing antibody to FGF-2 was included in each experimental condition, the antibody greatly inhibited the cell migration in all conditions, suggesting that the active component causing cell migration in cells stimulated with IL-1β or maintained in conditioned medium is indeed FGF-2. We further tested whether p38 is involved in cell migration in response to FGF-2 induced by IL-1β. Cells between the initial wound site and the re-covered region were carefully removed and prepared for immunoblot analysis for both total and phosphorylated p38, whereas cells away from the wound site served as nonmigratory cells. The migratory cells derived from the cells that were either stimulated with IL-1β or maintained in the conditioned medium demonstrated phosphorylated p38; however, such activation was completely blocked by the neutralizing antibody to FGF-2 (Fig. 6B). These data further suggest that p38 is indeed involved in cell migration mediated by the IL-1β–induced FGF-2. On the other hand, the nonmigratory cells failed to phosphorylate p38 (Fig. 6B) in all experimental conditions. We further confirmed the involvement of p38 in cell migratory activity triggered by the IL-1β–induced FGF-2 using SB203580. Table 1 demonstrated that the migratory rate in the cells treated simultaneously with IL-1β and SB203580 was similar to the basal level observed in cells maintained in DMEM and that a similar level of inhibitory action by SB203580 was observed in the cells maintained in the conditioned medium or in the cells treated with IL-1β or FGF-2 (Table 1).

Cell Proliferation Facilitated by the IL-1β–Induced FGF-2 Via Parallel Pathways of PI 3-Kinase and ERK1/2

The findings observed in Figures 1B and 3E suggest that ERK1/2 is activated by FGF-2 induced by IL-1β. The finding that FGF-2 activates the ERK1/2 pathway further led us to determine which of the two, PI 3-kinase or ERK1/2, was upstream. Cells were pretreated with the pathway-specific inhibitors for

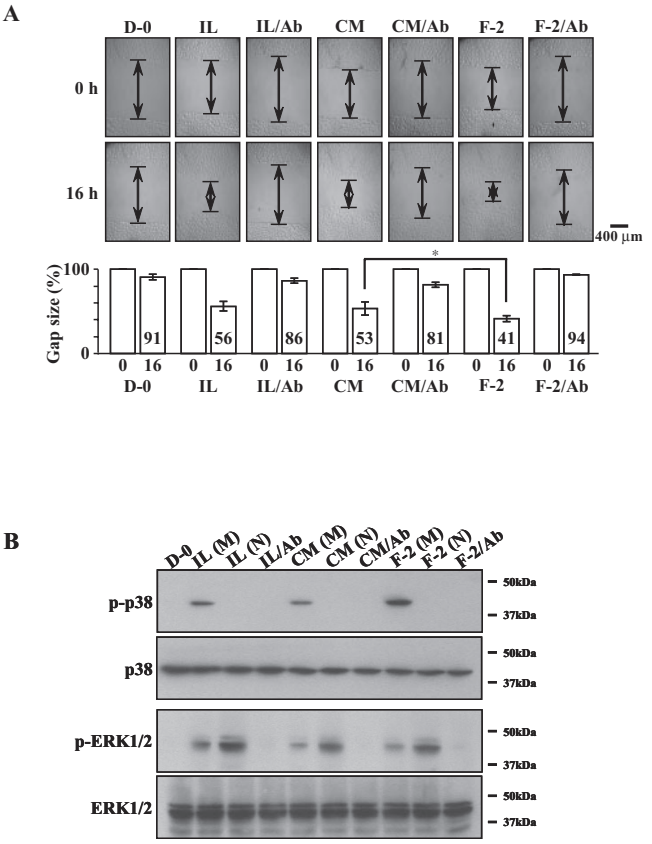

FIGURE 6. FGF-2 induced by IL-1β enhances cell migration during wound healing. First passage cells were plated in six-well tissue culture plates at a cell density of 1×10^5 in DMEM-15 for 24 hours and serum starved as described in Figure 1. The serum-starved cells were pretreated with IL-1β for 10 minutes before wound formation. Thereafter, a wound (scratch) was made at the middle of the wells, and free cells were removed by washing with PBS. Media were then changed to designated fresh media containing mitomycin-C (5 μg/mL), to block cell proliferation, and were further incubated for 16 hours. Wound closure was photographed with SPOT and quantified (**A**). Isolated migratory (M) and nonmigratory (N) cells were lysed. Each cell lysate was then immunoblotted with the designated antibody (**B**). D-0, IL, Ab, CM, and F-2 designate DMEM without serum, IL-1β, neutralizing antibody to FGF-2, conditioned media acquired from IL-1β–treated cell, and FGF-2, respectively. The data are representative of results in three experiments. $^*P > 0.1$.

TABLE 1. Migratory Rate of CECs during Wound Healing in Each Culture Condition

Culture Condition	Migratory Rate (μm/min)
D-0	0.12 ± 0.04
IL-1β	0.54 ± 0.05
IL-1β +SB	0.14 ± 0.04*
CM	0.58 ± 0.09
CM +SB	0.18 ± 0.02†
FGF-2	0.64 ± 0.07
FGF-2+SB	0.17 ± 0.05‡

The migration rate of CECs maintained in each culture condition was investigated using a scratch-induced directional migration assay. Data shown are representative of results in three experiments. D-0, DMEM without serum; SB, SB203589; CM, conditioned medium acquired from IL-1β-treated cells.

* $P < 0.01$ compared with cells treated with IL-1β.

† $P < 0.01$ compared with cells cultured in CM.

‡ $P < 0.01$ compared with cells stimulated by FGF-2.

2 hours before they were stimulated with IL-1β for 10 minutes and maintained in DMEM for 1 to 24 hours; U0126 (MEK inhibitor) failed to block phosphorylation of Akt at both Ser473 and Thr308 sites (Fig. 7A), and LY294002 failed to block phosphorylation of ERK1/2 (Fig. 7B). These findings suggest that the PI 3-kinase and ERK1/2 pathways are parallel and independent in response to stimulation with FGF-2 induced by IL-1β.

One major cellular activity triggered by PI 3-kinase and/or ERK1/2 pathways is stimulation of cell proliferation. We first determined whether cell proliferation was stimulated in cells treated either with IL-1β or conditioned medium (Fig. 8A). When compared to the positive control value derived from the cells stimulated with FGF-2, both IL-1β and conditioned media showed a strong stimulatory influence on CEC proliferation. When the neutralizing antibody to FGF-2 was simultaneously included in the culture medium, the antibody blocked cell proliferation almost to the basal level observed in cells maintained in DMEM. Use of pathway-specific inhibitors further confirmed that PI 3-kinase and ERK1/2 are the active pathways in IL-1β–stimulated cell proliferation (Fig. 8B). After pretreatment of cells with either LY294002 or U0126 for 2 hours, the cells were stimulated with IL-1β or conditioned medium for 16 hours. Cell proliferation stimulated with either IL-1β or conditioned medium was greatly inhibited by either LY294002 or U0126; similar findings were observed in the positive control cells treated with FGF-2. When both inhibitors were simultaneously added to the culture conditions, there was an additive effect of LY294002 and U0126 leading to the basal level. Of interest, the involvement of ERK1/2 in cell proliferation is different from that shown by our previous study in which PD98059 was used to block MEK; the use of PD98059 did not block cell proliferation triggered by FGF-2,[38] in contrast to the findings we obtained using U0126. It is likely either that PD98059 may not work in the CEC system or that the inhibitor is not useful for blocking MEK.

DISCUSSION

Corneal endothelium in vivo responds to diverse injury by converting to fibroblast-like cells.[3–6] We reported that these morphologically modulated cells produce fibrillar collagens, the predominant species of which is type I collagen, and that these cells deposit an abnormal fibrillar ECM.[5,6] One clinical example of such phenotypic modulation of CECs is the production of RCFM between Descemet's membrane and the endothelial monolayer, the presence of which blocks vision, thereby causing blindness.[3–6] An in vitro model that elucidates the molecular mechanism of RCFM formation led us to the finding that FGF-2 plays a key role in such EMT: first, FGF-2 directly regulates cell cycle progression by degrading p27, leading to a marked stimulation of cell proliferation[39–41]; second, FGF-2 upregulates the steady state levels of α1(I) collagen RNA by stabilizing the message and subsequently facilitates synthesis and secretion of type I collagen into the extracellular space[42]; and third, FGF-2 induces a change in cell shape from a polygonal to a fibroblastic morphology and causes loss of the contact-inhibited phenotype.[43–45] Of interest, FGF-2 uses PI

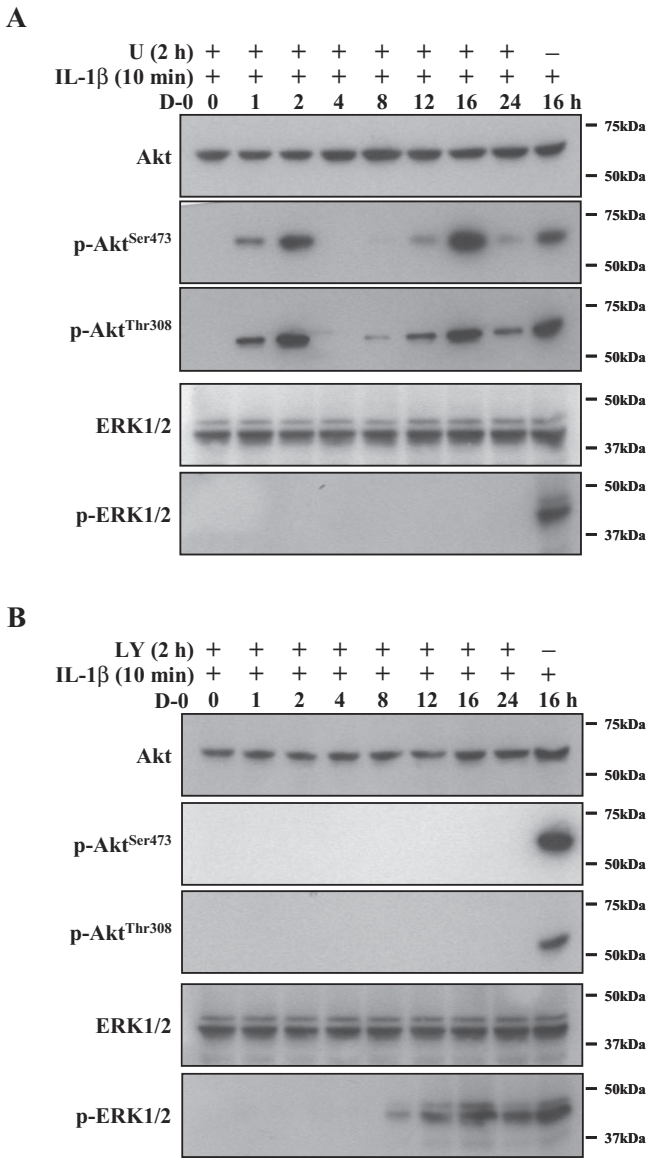

FIGURE 7. Independent activation of PI 3-kinase and ERK1/2 in response to IL-1β stimulation. When cells reached approximately 70% confluence, they were serum starved for 24 hours. The serum-starved cells were pretreated with U0126 (**A**) or LY294002 (**B**) during serum starvation before stimulation with IL-1β for 10 minutes. For a positive control, CECs were treated with only IL-1β for 10 minutes and maintained in DMEM for an additional 16 hours. At the end of treatment, total cell proteins were prepared as described in Figure 1. Each cell lysate was then immunoblotted with the designated antibody. Total Akt and ERK1/2 were used to control protein concentration on immunoblot analysis. D-0, U, and LY designate DMEM without serum, U0126 and LY294002, respectively. The data are representative of results in three independent experiments.

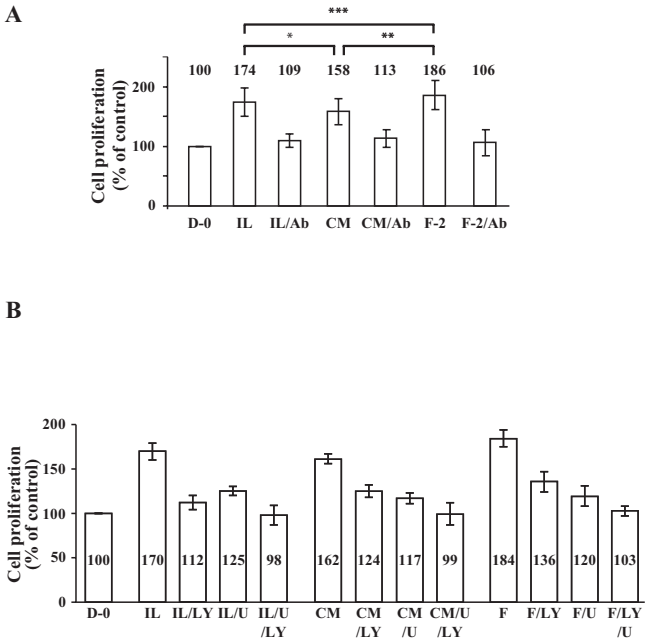

FIGURE 8. Inhibitory effect of U0126, LY294002, and neutralizing antibody to FGF-2 on cell proliferation stimulated by FGF-2 or IL-1β. Cell proliferation was determined by MTT assay. (**A**) The serum-starved cells were maintained in each culture condition, with or without neutralizing antibody to FGF-2 for 24 hours. (**B**) The serum-starved cells were pretreated with U0126, LY294002, or both for 2 hours and then maintained in DMEM with IL-1β, DMEM with FGF-2, or CM for 24 hours. At the end of incubation, MTT was added for 4 hours and then intracellular purple formazan, the MTT metabolic product by the action of dehydrogenase enzymes of metabolically active cells, was quantified with a spectrophotometric plate reader at dual wavelengths of 570 and 650 nm. Data were normalized to DMEM-treated cells. D-0, IL, Ab, CM, F-2, LY, and U designate DMEM without serum, IL-1β, neutralizing antibody to FGF-2, conditioned media acquired from IL-1β–treated cell, FGF-2, LY294002, and U0126, respectively. The data are representative of results in three independent experiments. The probabilities suggest that there are no differences in cell proliferation rate among IL-1β–, CM-, and FGF-2–treated cells: $^{*}P = 0.259$; $^{**}P = 0.145$; $^{***}P = 0.296$.

3-kinase as the major signaling pathway for all these phenotypic alterations.[38–44]

Furthermore, we discovered that IL-1β, a potent proinflammatory cytokine, is also involved in FGF-2–mediated EMT.[10] One major role of IL-1β is its stimulation of the expression of a variety of genes necessary for the wound repair process.[21–27] Both IL-1α and -1β markedly stimulate synthesis and release of FGF-2 in various cells.[23–25] Likewise, CECs produced all isoforms of FGF-2 in response to IL-1β stimulation via the PI 3-kinase pathway.[10] In the present study, we further explored the mechanism by which IL-1β induced FGF-2 and cellular activities facilitated by FGF-2, which acts as the main inducer in IL-1β–triggered signaling pathways.

A brief stimulation of CECs with IL-1β activates PI 3-kinase and p38 in a biphasic fashion, leading to the following sequential events (Fig. 9). The first wave of activation of PI 3-kinase and p38 is triggered by IL-1β and subsequently takes part in the inductive activity of IL-1β on FGF-2 production. In this pathway, p38 serves as the downstream molecule to PI 3-kinase. As a consequence of activation of both PI 3-kinase and p38 in response to IL-1β stimulation, CECs produce a large amount of FGF-2. Similar inductive activity of p38 was also reported in other systems: hypoxia activates the p38 pathway to enhance IL-8 gene transcription,[52] whereas p38 is involved in cyclooxygenase-2 production in response to FGF-2 stimulation.[53] The IL-1β–induced FGF-2 then triggers the second wave of PI 3-kinase activation, which in turn activates the second wave of p38. By now, CECs are exposed to FGF-2 in a autocrine fashion, the direct mediator of EMT. The IL-1β–induced FGF-2 then operates distinct signal transduction for its own cellular activities during the course of EMT: cell migration, cell proliferation, and phenotypic changes of ECM proteins. When the signaling pathway for cell migration was determined, the IL-1β–induced FGF-2 facilitates cell migration via PI 3-kinase and p38 pathways. On the other hand, parallel pathways of PI 3-kinase and ERK1/2 are required for the stimulation of cell proliferation mediated by the IL-1β–induced FGF-2; thus, it appears that FGF-2 utilizes PI 3-kinase and ERK1/2 as the major mitogenic pathways, whereas PLC-γ1 serves as a minor pathway.[38] Taken together, our findings indicate that the major role of IL-1β in EMT is the induction of FGF-2, whereas FGF-2 induced in such a fashion triggers the whole spectrum of the EMT process.[38–45] Of interest, p38 exerts two respective activities, according to its biphasic activation: The first wave of activation, which occurs within 1 hour after IL-1β stimulation, causes FGF-2 production; the second wave of p38, which is activated by the IL-1β–induced FGF-2 via PI 3-kinase, is involved in cell migration.

Inflammation has been recognized as a common mechanism of disease. Many diseases result from uncontrolled inflammation, but the ability to intervene in the inflammatory process is very limited because of an incomplete understanding of the many molecular pathways and interactions involved in inflammation. Of great importance, our in vitro EMT model shows that the two respective pathways (the IL-1β– and FGF-2–mediated) interact to link inflammatory responses directly to the cellular responses triggered by the growth factors induced during inflammation. Thus, it is important to dissect the inflammatory cascade and to address it in a systematic fashion. In RCFM formation, production of FGF-2 by the infiltrating inflammatory cells would be the first step in intervening in the blocking of EMT; thus, blocking the first enzyme, PI 3-kinase, may be crucial before CECs are exposed to the direct mediator

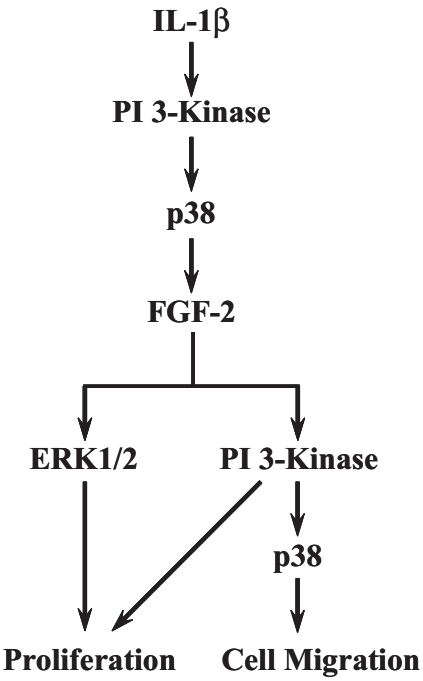

FIGURE 9. Schematic illustration of a proposed pathway induced by IL-1β. FGF-2 induced by IL-1β through PI 3-kinase and p38 increases cell migration and proliferation in CECs.

of EMT. The next stage-specific pathway in our EMT model is blocking the p38 pathway as a means of pursuing the same goal (blocking of FGF-2 production). If such an initial attempt to block the IL-1β–induced FGF-2 production fails, a pathway-specific blockade can be used depending on the stage of EMT process with respect of cell migration or cell proliferation. The ability to intervene in the stage-specific pathway may be the means to obstruct inflammatory responses, not only in RCFM, but in all fibrosis.

References

1. Joyce NC, Navon SE, Roy S, Zieske JD. Expression of cell cycle-associated proteins in human and rabbit corneal endothelium in situ. *Invest Ophthalmol Vis Sci.* 1996;37(8):1566–1575.
2. Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. *Invest Ophthalmol Vis Sci.* 2000; 41(3):660–667.
3. Michels RG, Kenyon KR, Maumence AE. Retrocorneal fibrous membrane. *Invest Ophthalmol.* 1972;11(10):822–831.
4. Waring GO III. Posterior collagenous layer of the cornea: ultrastructural classification of abnormal collagenous tissue posterior to Descemet's membrane in 30 cases. *Arch Ophthalmol.* 1982; 100(1):122–134.
5. Kay ED, Cheung C-C, Jester JV, Nimni ME, Smith RE. Type I collagen and fibronectin synthesis by retrocorneal fibrous membrane. *Invest Ophthalmol Vis Sci.* 1982;22(2):200–212.
6. Leung EW, Rife L, Smith RE, Kay EP. Extracellular matrix components in retrocorneal fibrous membrane in comparison to corneal endothelium and Descemet's membrane. *Mol Vis.* 2000;6:15–23.
7. Kay EP, Smith RE, Nimni ME. Type I collagen synthesis by corneal endothelial cells modulated by polymorphonuclear leukocytes. *J Biol Chem.* 1985;260(8):5139–5149.
8. Kay EP, Gu X, Ninomiya Y, Smith RE. Corneal endothelial modulation: a factor released by leukocytes induces basic fibroblast growth factor that modulates cell shape and collagen. *Invest Ophthalmol Vis Sci.* 1993;34(3):663–672.
9. Kay EP, Gu X, Smith RE. Corneal endothelial modulation: bFGF as direct mediator and corneal endothelium modulation factor as inducer. *Invest Ophthalmol Vis Sci.* 1994;35(5):2427–2435.
10. Lee HT, Lee JG, Na M, Kay EP. FGF-2 induced by interleukin-1β through the action of phosphatidylinositol 3-kinase mediates endothelial mesenchymal transformation in corneal endothelial cells. *J Biol Chem.* 2004;279(31):32325–32332.
11. Dinarello CA. Interleukin-1 and interleukin-1 antagonism. *Blood.* 1991;77(8):1627–1652.
12. Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood.* 1996;87(6):2095–2147.
13. Allan SM. The role of pro- and antiinflammatory cytokines in neurodegeneration. *Ann N Y Acad Sci.* 2000;917:84–93.
14. Gramantieri L, Casali A, Trerè D, et al. Imbalance of IL-1 beta and IL-1 receptor antagonist mRNA in liver tissue from hepatitis C virus (HCV)-related chronic hepatitis. *Clin Exp Immunol.* 1999;115(3): 515–520.
15. Boutin H, LeFeuvre RA, Horai R, Asano M, Iwakura Y, Rothwell NJ. Role of IL-1α and IL-1β in ischemic brain damage. *J Neurosci.* 2001;21(15):5528–5534.
16. Arend WP. The balance between IL-1 and IL-1Ra in disease. *Cytokine Growth Factor Rev.* 2002;13(4–5):323–340.
17. Cubitt CL, Lausch RN, Oakes JE. Synthesis of type II interleukin-1 receptors by human corneal epithelial cells but not by keratocytes. *Invest Ophthalmol Vis Sci.* 2001;42(3):701–704.
18. Moore JE, McMullen TCB, Campbell IL, et al. The inflammatory milieu associated with conjunctivalized cornea and its alteration with IL-1 RA gene therapy. *Invest Ophthalmol Vis Sci.* 2002;43(9): 2905–2915.
19. McDermott AM, Redfern RL, Zhang B, Pei Y, Huang L, Proske RJ. Defensin expression by the cornea: multiple signaling pathways mediate IL-1beta stimulation of hBD-2 expression by human corneal epithelial cells. *Invest Ophthalmol Vis Sci.* 2003;44(5):1859–1865.
20. Djalilan AR, Nagineni CN, Mahesh SP, Smith JA, Nussenblatt RB, Hooks JJ. Inhibition of inflammatory cytokine production in human corneal cells by dexamethasone, but not cyclosporin. *Cornea.* 2006;25(6):709–714.
21. Tamura M, Arakaki N, Tsubouchi H, Takada H, Daikuhara Y. Enhancement of human hepatocyte growth factor production by interleukin-1 alpha and -1 beta and tumor necrosis factor-alpha by fibroblasts in culture. *J Biol Chem.* 1993;268(11):8140–8145.
22. Pshenichkin SP, Szekely AM, Wise BC. Transcriptional and post-transcriptional mechanisms involved in the interleukin-1, steroid, and protein kinase C regulation of nerve growth factor in cortical astrocytes. *J Neurochem.* 1994;63(2):419–428.
23. Cronauer MV, Stadlmann S, Klocker H, et al. Basic fibroblast growth factor synthesis by human peritoneal mesothelial cells: induction by interleukin-1. *Am J Pathol.* 1999;155(6):1977–1984.
24. Sobue T, Zhang X, Florkiewicz RZ, Hurley MM. Interleukin-1 regulates FGF-2 mRNA and localization of FGF-2 protein in human osteoblasts. *Biochem Biophys Res Commun.* 2001;286(1):33–40.
25. Hayashi T, Matsuoka K, Saitoh M, Takeda S, Kimura M. Influence of α–tumor necrosis factor and β–interleukin-1 on production of angiogenetic factors and thymidine phosphorylase activity in immortalized human decidual fibroblasts in vitro. *J Obstet Gynaecol Res.* 2006;32(1):15–22.
26. Jung YD, Liu W, Reinmuth N, et al. Vascular endothelial growth factor is upregulated by interleukin-1β in human vascular smooth muscle cells via the p38 mitogen-activated protein kinase pathway. *Angiogenesis.* 2001;4(2):155–162.
27. Liu W, Reinmuth N, Stoeltzing O, et al. Cyclooxygenase-2 is up-regulated by interleukin-1 beta in human colorectal cancer cells via multiple signaling pathways. *Cancer Res.* 2003;63(13):3632–3636.
28. Ornitz DM, Itoh N. Fibroblast growth factors. *Genome Biol.* 2001; 2(3):REVIEWS3005.
29. Ornitz DM, Marie PJ. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev.* 2002;16(12):1446–1465.
30. Sørensen V, Nilsen T, Wiedłocha A. Functional diversity of FGF-2 isoforms by intracellular sorting. *Bioessays.* 2006;28(5):504–514.
31. Garcia-Maya M, Anderson AA, Kendal CE, et al. Ligand concentration is a driver of divergent signaling and pleiotropic cellular responses to FGF. *J Cell Physiol.* 2006;206(2):386–393.
32. Monfils M-H, Driscoll I, Melvin NR, Kobl B. Differential expression of basic fibroblast growth factor-2 in the developing rat brain. *Neuroscience.* 2006;141(1):213–221.
33. Yu P-J, Ferrari G, Galloway AC, Mignatti P, Pintucci G. Basic fibroblast growth factor (FGF-2): the high molecular weight forms come of age. *J Cell Biochem.* 2007;100(5):1100–1108.
34. Arnaud E, Touriol C, Boutonnet C, et al. A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. *Mol Cell Biol.* 1999;19(1):505–514.
35. Klint P, Claesson-Welsh L. Signal transduction by fibroblast growth factor receptors. *Front Biosci.* 1999;4:D165–D177.
36. Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.* 2005; 16(2):139–149.
37. Frinchi M, Bonomo A, Trovato-Salinaro A, et al. Fibroblast growth factor-2 and its receptor expression in proliferating precursor cells of the subventricular zone in the adult rat brain. *Neurosci Lett.* 2008;447(1):20–25.
38. Lee JG, Kay EP. FGF-2-mediated signal transduction during endothelial mesenchymal transformation in corneal endothelial cells. *Exp Eye Res.* 2006;83(6):1309–1316.
39. Lee HT, Kay EP. Regulatory role of PI 3-kinase on expression of Cdk 4 and p27, nuclear localization of Cdk4, and phosphorylation of p27 in corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 2003;44(4):1521–1528.
40. Lee JG, Kay EP. Two populations of p27 use differential kinetics to phosphorylate Ser-10 and Thr-187 via phosphatidylinositol 3-kinase in response to fibroblast growth factor-2 stimulation. *J Biol Chem.* 2007;282(9):6444–6454.
41. Lee JG, Kay EP. Involvement of two distinct ubiquitin E3 ligase systems for p27 degradation in corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 2008;49(1):189–196.

42. Ko MK, Kay EP. Regulatory role of FGF-2 on type I collagen expression during endothelial mesenchymal transformation. *Invest Ophthalmol Vis Sci.* 2005;46(12):4495–4503.
43. Lee JG, Kay EP. Cross-talk among Rho GTPases acting downstream of PI 3-kinase induces mesenchymal transformation of corneal endothelial cells mediated by FGF-2. *Invest Ophthalmol Vis Sci.* 2006;47(6):2358–2368.
44. Lee JG, Kay EP. FGF-2-induced wound healing in corneal endothelial cells requires Cdc42 activation and Rho inactivation through the phosphatidylinositol 3-kinase pathway. *Invest Ophthalmol Vis Sci.* 2006;47(4):1376–1386.
45. Rieck PW, Cholidis S, Hartmann C. Intracellular signaling pathway of FGF-2-modulated corneal endothelial cell migration during wound healing in vitro. *Exp Eye Res.* 2001;73(5):639–650.
46. Kay EP, Smith RE, Nimni ME. Basement membrane collagen synthesis by rabbit corneal endothelial cells in culture: evidence for an alpha chain derived from a larger biosynthetic precursor. *J Biol Chem.* 1982;257(12):7116–7121.
47. Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227(5259):680–685.
48. Gu X, Kay EP. Distribution and putative roles of fibroblast growth factor-2 isoforms in corneal endothelial modulation. *Invest Ophthalmol Vis Sci.* 1998;39(12):2252–2258.
49. Sharma G-D, He J, Bazan HE. p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing. *J Biol Chem.* 2003;278(24):21989–21997.
50. Frey MR, Dise RS, Edelblum KL, Polk DB. p38 kinase regulates epidermal growth factor receptor downregulation and cellular migration. *EMBO J.* 2006;25(24):5683–5692.
51. Shahabuddin S, Ji R, Wang P, et al. CXCR3 chemokine receptor-induced chemotaxis in human airway epithelial cells: role of p38 MAPK and PI3K signaling pathways. *Am J Physiol Cell Physiol.* 2006;291(1):C34–C39.
52. Xu L, Pathak PS, Fukumura D. Hypoxia-induced activation of p38 mitogen-activated protein kinase and phosphatidylinositol 3′-kinase signaling pathways contributes to expression of interleukin 8 in human ovarian carcinoma cells. *Clin Cancer Res.* 2004;10(2):701–707.
53. Tessner TG, Muhale F, Schloemann S, Cohn SM, Morrison A, Stenson WF. Basic fibroblast growth factor upregulates cyclooxygenase-2 in I407 cells through p38 MAP kinase. *Am J Physiol Gastrointest Liver Physiol.* 2003;284(2):G269–G279.