FGF-2 Induced by Interleukin-1β through the Action of Phosphatidylinositol 3-Kinase Mediates Endothelial Mesenchymal Transformation in Corneal Endothelial Cells*

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Our previous work demonstrated that both polymorphonuclear leukocytes (PMNs) and protein fractions released from PMNs induced de novo synthesis of fibroblast growth factor 2 (FGF-2), which in turn becomes the direct mediator of endothelial mesenchymal transformation observed in corneal endothelial cells (CECs). To identify the protein factor, we used ProteinChip Array technology. Protein fractions obtained from the conditioned medium released by PMNs were resolved by two-dimensional electrophoresis with immobilized pH gradient strips. Most of the protein spots, with molecular masses of 17 kDa, were sequentially subjected to in-gel trypsin digestion and mass spectrometry. The 17-kDa peptide band was identified as interleukin-1β (IL-1β). Biological activities of IL-1β were further determined; IL-1β altered the shape of CECs from polygonal to fibroblastic morphologies in a time- and dose-dependent manner, whereas neutralizing IL-1β antibody, neutralizing antibody to FGF-2, and LY294002 blocked the action of IL-1β. IL-1β greatly increased the levels of FGF-2 mRNA in a time- and dose-dependent manner; IL-1β stimulated expression of all isoforms of FGF-2. IL-1β initially induced nuclear accumulation of FGF-2 and facilitated translocation of FGF-2 to plasma membrane and extracellular matrix. IL-1β activated phosphatidylinositol (PI) 3-kinase, the enzyme activity of which was greatly stimulated after a 5-min exposure to IL-1β. This early and rapid activation of PI 3-kinase greatly enhanced FGF-2 production in CECs; pretreatment with LY294002 hampered the induction activity of IL-1β. These observations suggest that IL-1β takes part in endothelial to mesenchymal transformation of CECs through its inductive potential on FGF-2 via the action of PI 3-kinase.

Corneal fibrosis represents a significant pathophysiological problem that causes blindness by physically blocking light transmittance. One clinical example of corneal fibrosis observed in corneal endothelium is the development of a retrocorneal fibrous membrane (RCFM) in Descemet’s membrane (1, 2). In RCFMs, corneal endothelial cells (CECs) are converted to fibroblast-like cells; the contact-inhibited monolayers of CECs are lost, resulting in the development of multilayers of fibroblast-like cells (3, 4). These morphologically altered cells simultaneously resume their proliferation ability and deposit a fibrillar extracellular matrix (ECM) in the basement membrane environment. 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 (5–7). We also reported that the partially purified protein fractions obtained from the conditioned media of the activated PMNs demonstrated the same modulation activities as the PMNs themselves did (8, 9). Thus, the partially purified protein fraction was designated corneal endothelium modulation factor (CEMF). Among a number of proteins in the CEMF fraction, we showed that a 17-kDa protein band caused the cell shape change of CECs (9). In the present study we identified the 17-kDa protein obtained from inflammatory cells (PMNs) as interleukin 1β (IL-1β) using ProteinChip Array technology, also known as surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS). We further attempted to determine the potential novel function of IL-1β in CECs.

IL-1β is a major proinflammatory cytokine that plays an important role in acute and chronic inflammatory diseases (10–12). IL-1β also has a crucial role in the regulation of inflammation and wound healing on the ocular surface (13–18). Numerous studies report that both IL-1α and IL-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 processes (19–22). Among them, both IL-1α and IL-1β markedly stimulate synthesis and release of fibroblast growth factor 2 (FGF-2) in human peritoneal mesothelial cells (23), osteoblasts (24), and umbilical vein endothelial cells (25). We also reported that CECs treated with CEMF produced high levels of high molecular weight and ECM isoforms of FGF-2 (26). FGF-2, a ubiquitous, multifunctional growth factor, is present in Descemet’s membrane (8, 27) as well as in other tissues and cells (28–30). 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 (31, 32), polymorphonuclear leukocytes; CEMF, corneal endothelium modulation factor; IL, interleukin; SELDI-TOF MS, surface-enhanced laser desorption/ionization-time of flight mass spectrometry; FGF-2, fibroblast growth factor-2; EMT, endothelial mesenchymal transformation; PI 3-kinase, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; IEF, isoelectric focusing.

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‡ The abbreviations used are: RCFM, retrocorneal fibrous membrane; CECs, corneal endothelial cells; ECM, extracellular matrix; PMNs, polymorphonuclear leukocytes; CEMF, corneal endothelium modulation factor; IL, interleukin; SELDI-TOF MS, surface-enhanced laser desorption/ionization-time of flight mass spectrometry; FGF-2, fibroblast growth factor-2; EMT, endothelial mesenchymal transformation; PI 3-kinase, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; IEF, isoelectric focusing.

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wereas the high molecular weight isoforms of FGF-2 exert inetrane activity, the mechanism of which is yet to be defined (33).

In our previous studies (8, 9, 26) we reported that FGF-2 is the direct mediator for endothelial mesenchymal transformation (EMT) observed in CECs. Among the phenotypes altered during EMT, we reported that FGF-2 directly regulates the cell cycle progression through the action of phosphatidylinositol (PI) 3-kinase, thus leading to a marked stimulation of cell proliferation (34, 35). We also reported that FGF-2 induces a change in cell shape from a polygonal to a fibroblastic morphology and that it induces a reorganization of actin cytoskeleton via PI 3-kinase (36, 37). Similarly, PI 3-kinase is known to directly regulate cell cycle progression and morphogenetic pathways in other cell systems (38–42). Furthermore, PI 3-kinase is activated by proinflammatory cytokines, such as tumor necrosis factor or IL-1 (43–46).

We attempted to link inflammatory responses to the different set of cellular responses triggered by growth factors. To test this hypothesis, we tested whether FGF-2 was inducible by IL-1β. In the present study we demonstrated that IL-1α released by PMNs initiates EMT because it induces FGF-2, which is a direct mediator for EMT. We also showed that activation of the PI 3-kinase pathway is required to mediate the biological activities of IL-1β. Thus, this study indicates the sequential activation of CECs by IL-1β and FGF-2 in the context of inflammatory responses and non-physiological wound healing.

EXPERIMENTAL PROCEDURES

Materials—FGF-2 was from Intergen (Purchase, NY); IL-1β, PD998059, cycloheximide, LY294002, anti-β-actin, and anti-IL-1β antibodies were from Sigma-Aldrich. All SELDI-TOF MS materials were from Ciphergen (Fremont, CA). Radiochemicals were from ICN (Irvine, CA). The anti-p85 subunit of PI 3-kinase antibody was from BD Biosciences, OR, and fluorescein isothiocyanate-conjugated-secondary antibodies were from Chemicon (Temecula, CA). Mounting solution and biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA).

Cell Culture—Rabbit eyes were purchased from Pel Freeze (Rogers, AR). Isolation and establishment of rabbit CECs were performed as previously described (5). Briefly, the Descemet's membrane-corneal endothelial complex was treated with 0.2% collagenase and 0.05% hyaluronidase (Worthington Biochemical, Lakewood, NJ) for 90 min at 37 °C. Primary cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum and 50 μg/ml gentamicin in a 5% CO2 incubator. First passage CECs that were maintained in DMEM containing 15% fetal calf serum (DMEM-15) were treated with 0.05% trypsin and 5 mM EDTA in phosphate-buffered saline for 5 min. In some experiments the Descemet's membrane-corneal endothelial complex was peeled and incubated with one of the following conditions in growth medium (DMEM-15): IL-1β, IL-1β, and anti-IL-1β antibody, or IL-1β with LY294002. After 4 days of incubation, the Descemet's membrane-corneal endothelial complex was plated on slide glass and fixed with 4% paraformaldehyde in phosphate-buffered saline for 5 min. The tissue was then stained with rhodamine-phalloidin (1:100 dilution) at 37 °C for 10 min.

Preparation of Partially Purified CEMF—All animal care procedures were in accordance with the research guidelines set forth by the University of Southern California on the Use of Animals in Ophthalmic and Vision Research. Polymorphonuclear leukocytes were obtained from rabbits and isolated as described previously (9). All the methods and procedures have been presented previously (9).

Protein Preparation, Protein Assay, SDS-PAGE, RNA Isolation, Reverse Transcription, and PCR Amplification, Western Blotting Analysis, Immunofluorescent Analysis, Confocal Microscopy, and Measurement of PI 3-Kinase Enzyme Activity—All detail methods and procedures have been presented previously (35, 47, 48).

Two-dimensional Gel Electrophoresis—Our preliminary studies revealed that the optimum sample size was 500 μg of protein for the 11-cm Immobiline™ DryStrip Gel strips (Bio-Rad Laboratories). Isoelectric focusing (IEF) was performed on 11-cm, pH 5.5–6.7 Immobiline–electric focusing (IEF) was performed on 11-cm, pH 5.5–6.7 Immobiline™ DryStrip Gel strips using a Protean isoelectric focusing cell (Bio-Rad) for 35 kV-h at 20 °C. Strips were stained with Bio-Safe™ Coomassie G250.

A. Strips were equilibrated for 17 kDa. B. Strips were equilibrated for 17 kDa. C. Strips were equilibrated for 17 kDa. D. Strips were equilibrated for 17 kDa. E. Strips were equilibrated for 17 kDa. F. Strips were equilibrated for 17 kDa.

FIG. 1. Identification of CEMF as IL-1β using proteomic methodologies. Conditioned medium obtained from PMNs was concentrated, and proteins were prepared as described in "Results." A, 30 μg of partially purified CEMF was subjected to SDS-PAGE (15%) gel and stained with Coomassie Brilliant Blue (R-250). B, IEF of CEMF (500 μg) loaded on Immobiline™ DryStrip Gel strips (pH5.5–6.4) was performed for 35 kV-h at room temperature. After IEF separation, the strip was loaded onto precast 4–15% gradient SDS-electrophoresis gel. After the gel was run, it was stained with bio-safe Coomassie Blue C. Protein spots with molecular masses of 17-kDa were excised and subjected to in-gel trypsin digestion. After extraction of the digested peptides from the gel, samples were analyzed by SELDI-TOF MS. D, identification of 17-kDa CEMF was achieved with Mascot Search-Fit (Swiss-Prot) software. The underlined sequences in italics match with the sequence of rabbit IL-1β. E, the 17-kDa peptide band in the CEMF fraction was further confirmed as IL-1β by immunoblot analysis using monoclonal antibody to IL-1β. F, CECs were treated with CEMF (25 μg/ml) with or without neutralizing IL-1β antibody (5 mg/ml) (magnification, ×200).

line™ DryStrip Gel strips using a Protease isoelectric focusing cell apparatus (Bio-Rad) for 35 kV-h at 20 °C. Strips were equilibrated for 15 min in 0.375 m Tris-HCl (pH 8.8), 6.0 μm urea, 2% (w/v) SDS, 20% (v/v) glycerol, and 2% dithiothreitol, then for 10 min in the same buffer containing 2.5% iodoacetamide. Equilibrated immobilized pH gradient strips were loaded onto 4–15% precast SDS gel and overlaid with ReadyPrep-agarose in 0.375 m Tris-HCl (pH 8.8), 6.0 μm urea, 2% (w/v) SDS, and 20% (v/v) glycerol containing bromphenol blue. Gels were electrophoresed at 50 mA for 2 h at room temperature. The two-dimensional gels were stained with Bio-Safe™ Coomassie G-250.

In-gel Tryptic Digestion—Protein spots of 17-kDa range were manually excised from the gel. Spots were destained and dehydrated with 25 mM NH4HCO3, in 50% acetonitrile (v/v) and dried by vacuum centrifugation.

Sequence grade trypsin (12.5 ng/ml) with or without neutralizing IL-1β antibody (5 mg/ml) (magnification, ×200).

Mass Spectrometry Analysis—The enzymatically digested protein samples were analyzed by SELDI-TOF MS (49–51) in a Ciphergen Protein Biology System II using α-cyano-4-hydroxycinnamic acid matrix. Before SELDI-TOF MS analysis, H4 (C18 hydrophobic surface) ProteinChip surface was pre-equilibrated with 50% acetonitrile for 2 min at room temperature. One μl of the peptide samples was spotted on.
Fig. 2. IL-1β-mediated cell shape change in CECs. A, CECs were treated with IL-1β (5 ng/ml) in DMEM-15 for 30 min to 72 h. B, CECs were treated with IL-1α at concentrations ranging from 0.05 to 50 ng/ml for 24 h. C, CECs were treated with IL-1β (5 ng/ml) for 24 h, after which neutralizing IL-1β antibody (Ab) at 5 mg/ml was added for an additional 24 h. Phase contrast micrographs were taken at the end of each incubation. Data shown are representative of three experiments (magnification, ×200).

a H4 ProteinChip Array surface and allowed to dry. Because the digestion was performed using matrix-assisted laser desorption ionization (MALDI) technology, the normal washing steps for SELDI-TOF MS were omitted. 0.5 μl of α-cyano–4-hydroxycinnamic acid matrix saturated in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid was added to the H4 ProteinChip Array surface. Row data were analyzed using the computer software provided by the manufacturer. Protein identification based on peptide mass fingerprint was performed with Mascot Search-Fit (Swiss-Prot) software (www.matrixscience.com). The fact that IL-1β peptide-containing protein sample obtained from the conditioned medium of PMNs (Fig. 1A) was separated first by isoelectric focusing on immobilized pH gradient Immobiline™ DryStrip Gel strips followed by reduction and alkylation before the second-dimension separation on SDS-PAGE in an orthogonal direction (Fig. 1B). Individual protein spots with molecular masses of 17-kDa removed from the gel matrix were subjected to in-gel trypsin digestion, and peptide mass mapping was carried out by SELDI-TOF. The MS analysis of one peptide with the mass/charge (m/z) value of 2075 (Fig. 1C) was unambiguously identified by Mascot search as a peptide from IL-1β (Fig. 1D; the underlined sequences, shown in italics). The 17-kDa protein of the CEMF fraction was further confirmed by immunoblotting analysis using anti-IL-1β antibody (Fig. 1E). The fact that IL-1β is a key factor in the CEMF was further confirmed with neutralizing IL-1β antibody (Fig. 1F); a fraction of cells treated with CEMF became elongated, whereas neutralizing IL-1β antibody was able to block the action of CEMF.

One major activity that CEMF demonstrated was modulation of cell morphology from a polygonal to an elongated fibroblastic shape (6, 8, 9). Cellular activity of IL-1β in CECs was, therefore, determined using its effect on cell shape change. When cells were treated with IL-1β at 5 ng/ml for a designated time ranging from 30 min to 72 h, it appeared that the cytokine required a prolonged time to modulate cell shape; cells treated for 24 h began to acquire an elongated cell shape in some foci, whereas the majority of cells maintained a polygonal cell morphology, suggesting a mix of populations both responding to IL-1β and non-responding. When cells were treated for 48 h or longer, the fibroblastic cells outgrew the non-responding cells (Fig. 2A). These findings are exactly the same as the observations we had made with the activated PMNs in CEC cultures...
shown are representative of three experiments. Compared with the values of unstimulated cells (*, P < 0.01). Data shown are representative of three experiments.

The optimal concentration of IL-1β for causing cell shape change after 24 h of exposure to the cytokine was found to be 5 ng/ml, whereas neutralizing antibody to IL-1β blocks the modulating activity of the cytokine on cell shape (Fig. 2C).

**Up-regulation of FGF-2 Expression by IL-1β**—To explore whether IL-1β induced FGF-2 synthesis in CECs, we used real-time PCR to measure the steady-state levels of FGF-2 mRNA in cells stimulated with IL-1β. The steady-state level of the FGF-2 transcript increased in a dose-dependent manner; in response to IL-1β stimulation (5 ng/ml), the steady-state level of FGF-2 mRNA was increased more than 2-fold compared with the levels in the untreated cells (Fig. 3A). We also determined FGF-2 mRNA levels in CECs treated with IL-1β (5 ng/ml) for 1–72 h (Fig. 3B). The relative amount of FGF-2 transcript was increased in a time-dependent manner; IL-1β gradually induced FGF-2 transcription for up to 24 h, and the inductive potential of IL-1β reached a maximum at the 48-h treatment.

To determine whether expression of FGF-2 was also increased at the protein level, cells treated with IL-1β (5 ng/ml) for 30 min to 72 h were analyzed by immunoblotting analysis using anti-FGF-2 antibody (Fig. 4A). In the absence of IL-1β stimulation, CECs showed a very low level of FGF-2 (both 18-kDa and high molecular mass isoforms). Expression of FGF-2 isoforms was increased in cells treated for 8 h, after which the levels of FGF-2 were greatly increased in a time-dependent manner (12–16-fold during the 24–72 h stimulation). We further investigated whether IL-1β had an effect on protein stability; cells were treated with cycloheximide in the absence or presence of IL-1β to block protein synthesis. Cells treated with cycloheximide alone showed a half-life of FGF-2 of ~4 h, and IL-1β did not change the stability of FGF-2 (Fig. 4B).

Our previous study demonstrated that CEMP not only maintains the persistent localization of FGF-2 in the nuclei but promotes the distribution of FGF-2 in the ECM (26). To examine whether IL-1β also regulates subcellular localization of FGF-2, cells stimulated with IL-1β were stained with anti-18-kDa FGF-2 antibody. Cells maintained in DMEM-15 showed a relatively moderate nuclear staining of FGF-2 (Fig. 5A, control). Progressively differential subcellular localization of FGF-2 was observed as the exposure time to IL-1β was extended; cells treated with IL-1β for up to 8 h demonstrated a moderate nuclear staining of FGF-2, whereas a strong nuclear staining and relatively appreciable cytoplasmic staining of FGF-2 were observed in the cells treated for 24 h. A prominent membrane staining in addition to the strong nuclear staining of FGF-2 was observed in the cells treated for 36 h, whereas cells treated for 48 h demonstrated multilocations of FGF-2, including nuclei, cytoplasm, membrane, and ECM. When cells were simultaneously treated with IL-1β and PD98059, PD98059, or neutralizing antibody to IL-1β, both PD98059 and the neutralizing antibody to IL-1β were able to block accumulation of FGF-2 in locations other than the nuclei, whereas PD98059 (an inhibitor of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway) did not alter the IL-1β-induced multiple subcellular localization of FGF-2 (Fig. 5B).

**IL-1β Induces FGF-2 Expression through PI 3-Kinase Pathways in CECs**—CECs predominantly employed PI 3-kinase pathways for the mitogenic and morphogenetic pathways stimulated by FGF-2 (34, 36). Furthermore, Fig. 5B suggests that IL-1β may utilize PI 3-kinase pathways for its activity on FGF-2 expression. Therefore, we determined whether IL-1β was also able to activate PI 3-kinase activity. Cells were briefly stimulated with the cytokine (1–60 min) to determine the kinetics of PI 3-kinase activation; PI 3-kinase activity was determined using a gel documentation system. β-Actin was used for control of protein concentration on Western blot. Data shown are representative of three experiments.
tion of the enzyme was observed in cells treated with IL-1β for 5 min (6.7-fold) or 10 min (8.3-fold), after which the activation of PI 3-kinase was markedly decreased (Fig. 6A). When cells were pretreated with LY294002 for 40 min followed by stimulation with IL-1β for 10 min, activation of PI 3-kinase was greatly decreased (Fig. 6B).

To determine whether such a rapid and early activation of PI 3-kinase by IL-1β was sufficient to mediate stimulation of FGF-2 synthesis, cells were treated with the cytokine for 10 min, then maintained in growth medium without IL-1β for 1–24 h. There was a marked increase of FGF-2 production at the protein level for up to 24 h; even within the first hour after a brief stimulation with the cytokine, FGF-2 synthesis was increased by 3-fold, and 8 h after the stimulation with IL-1β, a 7-fold increase of FGF-2 production was observed (Fig. 7A). Involvement of PI 3-kinase in FGF-2 up-regulation mediated by IL-1β was further tested in cells that were pretreated with LY294002 for 2 h then stimulated with IL-1β for 10 min. Cells were then maintained in the growth medium alone for up to 24 h. CECs treated with LY294002 for 2 h then stimulated with IL-1β for 10 min. Cells were then maintained in the growth medium alone for up to 24 h. CECs treated with LY294002 for 2 h served as a control value, to which the inductive activity of IL-1β on FGF-2 synthesis was compared (Fig. 7B). CECs maintained in growth medium before treatment with both LY294002 and IL-1β showed a higher level of FGF-2 expression (2.4-fold) than did the control, whereas pretreatment of cells with LY294002 completely blocked the inductive activity of the cytokine on FGF-2 production.

To test whether FGF-2 was the direct cause of the cell shape change mediated by IL-1β, cells were simultaneously treated with IL-1β and neutralizing antibody to FGF-2. CECs treated with IL-1β for 24 h demonstrated the foci of the modulated cells (Fig. 8A), whereas the neutralizing FGF-2 antibody completely reversed the cell shape change mediated by IL-1β to the characteristic polygonal CEC morphology (Fig. 8A). Similarly, LY294002 was able to block the cell shape change mediated by IL-1β, whereas PD98059 failed to convert the altered cell morphology mediated by IL-1β to the polygonal shape (Fig. 8A). The modulation activity of IL-1β on cell shape through the PI 3-kinase pathway was further tested in ex vivo corneal endothelium by staining the tissue with rhodamine-phalloidin. A monolayer of corneal endothelium adhered on its basement membrane (Descemet’s membrane) was maintained in DMEM-15 alone or in DMEM-15 containing IL-1β, IL-1β with its neutralizing
antibody, or IL-1/β with LY294002. At the end of incubation, the tissue was stained for F-actin. The corneal endothelium maintained in DMEM-15 alone demonstrated the characteristic circumferential cortical actin ring structure in the contact-inhibited monolayer of endothelium, whereas IL-1/β caused a dramatic cell shape change from the characteristic cobblestone to an elongated morphology. The actin cytoskeleton at the cortex was partly disrupted (Fig. 8B). Both neutralizing antibody to IL-1/β and LY294002 blocked the action of IL-1/β on cell shape and actin cytoskeleton, suggesting that IL-1/β is able to regulate the morphogenetic pathway through PI 3-kinase both in vivo and in vitro.

**DISCUSSION**

The regeneration of corneal endothelium after in vivo injury appears to have two distinct pathways, 1) the regenerative pathway, by which endothelial cells do not replicate but are replaced by migration and spreading of existing endothelial cells and 2) the nonregenerative pathway (or fibrosis), by which transformed endothelial cells not only resume proliferation but alter their cell morphology and collagen phenotypes, leading in turn to the production of an abnormal ECM. One such clinical example is the formation of RCFM, the physical presence of which causes loss of vision. Any acute corneal endothelial injury is associated with a massive infiltration of PMNs in the anterior chamber and stroma with the resultant induction of inflammation and wound healing on the ocular surface (13–18). Numerous studies report that both IL-1α and IL-1/β orchestrate the inflammatory process by inducing production and release of secondary cytokines, which are necessary for wound repair processes (e.g. hepatocyte growth factor, nerve growth factor, and the other interleukin families) (19–22). Both IL-1α and IL-1/β are known to markedly stimulate synthesis and release of FGF-2 in a number of human cell systems (23–25). We also reported that CECs treated with CEMF produced high levels of FGF-2 (26). Because RCFM formation is associated with local inflammation and IL-1/β is released by the infiltrating PMNs, it is likely that IL-1/β is the major cytokine causing the corneal fibrosis observed in RCFM in vivo.

In the present study we demonstrated that IL-1/β exerts the same activities exerted by CEMF. IL-1/β alters cell shape from a polygonal to a fibroblastic morphology, it induces loss of the characteristic contact-inhibited phenotype of CECs, thus leading to multilayers of fibroblastic cells, it greatly stimulates synthesis of FGF-2 at both transcript and protein levels, and it regulates the subcellular localization of FGF-2. IL-1/β initially

**Fig. 7. Rapid induction of FGF-2 mediated by IL-1/β through PI 3-kinase activation. A, CECs were treated with IL-1/β (5 ng/ml) for 10 min, and cells were further maintained in DMEM-15 for 1 to 24 h. B, CECs were pretreated with LY294002 (20 μM) for 2 h, after which the medium was replaced with DMEM-15. Cells were further maintained for 1–24 h. Cell lysates were prepared for immunoblotting analysis for FGF-2 and β-actin expression. Relative densities of FGF-2 bands and β-actin were determined using a gel documentation system. Data were normalized to β-actin (a loading control). Relative fold differences were then compared with the values of unstimulated CECs (*, p < 0.001). Data shown are representative of three experiments.**
IL-1β-mediated cell shape change and actin cytoskeleton through FGF-2 and PI 3-kinase. A. CECs were treated with IL-1β for 24 h and were then further treated for 24 h with IL-1β-Ab or FGF-2. B. DMEM-15 was incubated for 4 days with DMEM-15, IL-1β (5 ng/ml), IL-1β with neutralizing antibody (Ab) to IL-1β, or IL-1β with LY294002 (20 μM). The tissues were fixed and stained with rhodamine-phalloidin (1:100) at 37 °C for 10 min. Data shown are representative of two experiments. Bar, 10 μm.

accumulates FGF-2 in the nuclei, but it later facilitates accumulation of FGF-2 to the cytoplasm, membrane, and ECM. We further demonstrated that IL-1β employs PI 3-kinase for its signaling pathways while up-regulating FGF-2 expression and that both neutralizing antibody to FGF-2 and PI 3-kinase inhibitor were able to block the cell shape changes mediated by IL-1β.

Of great interest is that CECs predominantly use PI 3-kinase pathways for a number of signaling functions, such as the mitogenic and morphogenetic pathways in response to FGF-2 stimulation (35, 37, 48). Likewise, CECs employ PI 3-kinase pathways in response to IL-1β stimulation. However, differential kinetics of activation of PI 3-kinase are observed depending upon the extracellular ligand; an 8-fold increase of PI 3-kinase activity was observed in CECs stimulated with IL-1β for 10 min, whereas a prolonged and continuous exposure (24 h) of cells to FGF-2 was required to produce a 3-fold activation of PI 3-kinase (35). The subsequent cellular events after PI 3-kinase activation are also different in CECs; the fast activation of PI 3-kinase by a brief stimulation with IL-1β leads to up-regulation of FGF-2 synthesis, whereas the late activation of PI 3-kinase by FGF-2 is involved in mitogenic and morphogenetic pathways, as shown in our previous studies in which FGF-2 markedly stimulated cell proliferation of CECs by directly regulating cell cycle progression (35). FGF-2 also reorganized the actin cytoskeleton of CECs in favor of de-adhesion phenotypes and simultaneously altered cell shapes (37). The differential kinetics of PI 3-kinase activation appear to be in agreement with the events that may take place after injury inflicted to the cornea in vivo: after injury, there is infiltration by inflammatory cells, mainly PMNs; the IL-1β released by these activated PMNs rapidly activated PI 3-kinase, which in turn greatly facilitated synthesis of FGF-2. After this rapid sequence of events, FGF-2 acted as a direct mediator for subsequent and attenuated events necessary for completing the wound healing process through the PI 3-kinase pathways, which are activated with much slower kinetics (Fig. 9). The regulatory subunit of PI 3-kinase interacted directly with IL-1 receptor I (IL-1RI) (44, 52, 53) or with the IL-1 receptor accessory protein (IL-1RAcP) required to form a high affinity complex with IL-1β-IL-1RI (54). Thus, PI 3-kinase plays a central role in corneal fibrosis both in the early event caused by injury-mediated inflammation, during which IL-1β induces a rapid and high level of FGF-2, and in the late stage of wound healing, during which FGF-2 takes part in cell proliferation, cell shape changes, loss of contact-inhibited phenotypes, and production of fibrillar ECM, which requires the regeneration of basement membrane phenotype. However, this nonregenerative wound healing process accounts for a minor proportion of wound healing observed in corneal endothelium, suggesting that the balance between the regenerative ability of the host and the degree of inflammation plays the key role in determining whether the tissue avoids corneal fibrosis. How the balance is maintained or impaired to cause pathophysiological phenotypes (corneal fibrosis) in corneal endothelium is yet to be determined.

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