Epidermal Growth Factor Synergism with TGF-β1 via PI-3 Kinase Activity in Corneal Keratocyte Differentiation

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PURPOSE. To investigate the action of epidermal growth factor (EGF) on corneal keratocyte differentiation and its effects in conjunction with transforming growth factor (TGF)-β1.

METHODS. Rabbit corneal keratocytes (RCKs) were treated with EGF, TGF-β1, or EGF plus TGF-β1 in the presence or absence of inhibitors of EGF-receptor (EGF-R), neutralizing concentrations of EGF antibody and of signaling kinases for 2 days to 1 week. RCK differentiation to myofibroblasts was identified with anti-aldehyde dehydrogenase (ALDH)-1 and α-smooth muscle actin (α-SMA) antibodies. Cell proliferation was evaluated with anti-Ki-67 antibody. Extracellular matrix (ECM) components were assayed by immunochrometry and Western blot. Cell migration images were captured with a camera attached to the microscope, and the area of the wound was calculated using imaging software.

RESULTS. RCKs cultured in serum-free DMEM/F12 without frequent changes of medium maintained the phenotype for more than 1 month. EGF stimulated differentiation into a prototypical fibroblast phenotype with the loss of dendritic shape and the expression of α-SMA. Treatment with TGF-β1 stimulated 12% of the cells to differentiate to defined myofibroblasts, but in the presence of EGF, TGF-β1 induced 90% of RCKs to transform into myofibroblasts. Inhibition of EGF-R activation and of the phosphatidylinositol-3 kinase (PI-3K)/Akt-1 pathway prevented the action of EGF on TGF-β1 cell differentiation. TGF-β1 in the presence of EGF also increased cell migration, which is inhibited by blocking EGF-R activation.

CONCLUSIONS. These data show that EGF contributes to the differentiation and migration of myofibroblasts induced by TGF-β1 through EGF-R activation and that it is an important modulator of wound healing and scar tissue formation. (Invest Ophthalmol Vis Sci. 2008;49:2936–2945) DOI:10.1167/iows.07-0900

The corneal stroma constitutes 90% of the corneal volume and consists of a highly organized and uniquely transparent extracellular matrix (ECM) of collagen fibrils and proteoglycans that provide both the refractive shape and the tensile strength of the tissue. Keratocytes are the principal cells of the stroma, which are responsible for the synthesis and maintenance of the ECM components. In normal adult cornea, keratocytes appear as a population of flat dendritic cells residing between the collagen lamellae and connecting to each other through a network of extensive processes.1–4 These cells are mitotically quiescent and contain few mitochondria or endoplasmic reticulum and no nucleoli. Turnover of keratocytes is low (2–3 years), and remodeling of the stromal ECM is undetectable over time.5–7 These homeostatic characteristics of stroma contribute to corneal transparency.

After injury, the quiescent keratocytes at the wound periphery become metabolically activated and transform to their repair phenotypes—corneal fibroblasts or myofibroblasts—which migrate to the damaged area, proliferate, and deposit a disorganized and fibrotic ECM to repair the wound.8–13 These cells differ from keratocytes in many aspects, including loss of dendritic shape, high proliferation rate, downregulation of keratin sulfate (KS) proteoglycans and aldehyde dehydrogenase (ALDH), and upregulation of chondroitin sulfate (CS) proteoglycans and fibronectin (FN).5–7,12,14 Corneal fibroblasts and myofibroblasts contribute to normal wound healing, but myofibroblasts play a crucial role in tissue fibrosis because they produce ECM components at a high rate and regulate contractile elements that generate the strength necessary for wound closure.6,7,13 If the remodeling of the ECM is not controlled, as in corneal disorders such as dystrophies, pterygia, pronounced wounds, or infections, it can lead to corneal opacity and loss of vision.

One of the first responses to corneal injury is inflammation, with the synthesis of several lipid mediators such as prostaglandins, lipoxigenase metabolites, platelet-activating factor,10,16 and several growth factors and cytokines.7,17 This is followed by the apoptosis of keratocytes close to the wound.18,19 The next step is a repair response with cell differentiation, proliferation of keratocytes to fibroblasts and myofibroblasts, and changes in ECM components. Keratocyte differentiation is controlled by a variety of growth factors, including transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF)-2, and insulin-like growth factor (IGF)-1.20–23 TGF-β causes keratocyte differentiation to myofibroblasts with the expression of α-SMA, along with increased CS proteoglycans and decreased KS proteoglycans.20,24 On the other hand, FGF2 and PDGF induce keratocyte differentiation to fibroblasts.22 In serum-cultured corneal fibroblasts, the addition of FGFr2 results in the downregulation of α-SMA and the upregulation of KS proteoglycans.21

Epidermal growth factor (EGF), through binding to the EGF receptor (EGF-R), stimulates its tyrosine kinase activity, leading to DNA synthesis, ECM molecule production, and cell proliferation.25,26 Receptor phosphorylation also leads to actin cytoskeletal rearrangement, which promotes cell motility. All three corneal cell types express EGF and its receptor, suggesting that EGF affects corneal cells in an autocrine, paracrine, or possibly juxtacrine manner.17,27,28 EGF stimulates the proliferation of corneal epithelial and endothelial cells and accelerates epithelial wound healing.29–32 Topical application of EGF significantly increases the tensile strength of sutured or unsutured wounds.
full-thickness corneal incisions, even under conditions in which stromal healing was impaired by corticosteroids33–39; however, little is known about the cellular and molecular mechanisms by which EGF exerts this action. Here, we have investigated the effects of EGF on the proliferation, differentiation, and expression of ECM components in isolated keratocytes, which, in turn, could maintain its phenotype for extensive periods in culture, mimicking the in vivo situation. We have also investigated the involvement of the signaling pathways activated by the EGF-R. We have demonstrated that synergisms exist between EGF and TGF-β1 that increase the differentiation and migration of myofibroblasts.

**MATERIALS AND METHODS**

**Materials**

Recombinant human EGF, TGF-β1, and goat anti-EGF antibody (AB-236-NA) were purchased from R&D Systems (Minneapolis, MN), and (4-chloroanilino)-6, 7-dimethoxyquinazoline (AG1478) was purchased from Biosource International (Camarillo, CA). 4,6-Diamidino-2-phenylindole (DAPI), mouse monoclonal α-SMA, -vimentin (clone Vim-13.2), anti-chondroitin sulfate (clone CS-56), anti-collagen type IV (clone Col-94), anti–cellular fibronectin (clone FN-32E), anti–human fibronectin (clone IST-3), anti–laminin (clone LAM-89), and anti–proliferating cell protein Ki-67 (clone pp-67) were purchased from Sigma (St. Louis, MO). Mouse anti–collagen type III (FH-7A) antibody (abcam7) and monoclonal anti–keratin sulfate antibody (clone 5-D-4) were purchased from Seikagaku Corporation (Chuo-ku, Tokyo, Japan). Mouse anti–TSP-1 was purchased from Abcam Inc. (Cambridge, MA). Mouse anti–Akt1 (2H10) and anti–phospho Akt (Ser473) monoclonal antibodies were from Cell Signaling Technology (Danvers, MA). Goat anti–EGF-R (sc-03-G) and p-EGF-R (sc-12351) were from Santa Cruz Biotechnology (Santa Cruz, CA), and the MAPK-K inhibitor PD98059 (abcam10494) and monoclonal anti–keratin sulfate antibody (clone 5-D-4) were purchased from Seikagaku Corporation (Chuo-ku, Tokyo, Japan). Mouse anti–TSP-1 was purchased from Abcam Inc. (Cambridge, MA). Mouse anti–Akt1 (2H10) and anti–phospho Akt (Ser473) monoclonal antibodies were from Cell Signaling Technology (Danvers, MA). Goat anti–EGF-R (sc-03-G) and p-EGF-R (sc-12351) were from Santa Cruz Biotechnology (Santa Cruz, CA), and the MAPK-K inhibitor PD98059 and goat anti–aldehyde dehydrogenase 1 (ALDH1) were purchased from Calbiochem (San Diego, CA). The p38 inhibitor SB203580 was from Upstate Biotechnology (Lake Placid, NY). LY294002 was from Calbiochem (La Jolla, CA). All SDS reagents were from Bio-Rad (Hercules, CA); polyvinylidene difluoride (PVDF) membranes were from Amersham Bioscience (Piscataway, NJ).

**Isolation and Culture of Rabbit Corneal Keratocytes**

Rabbit eyes (Pel-Freeze Biologicals, Rogers, AR) were shipped to the laboratory on ice in Hanks solution containing antibiotics and antimitotic and were used within 24 hours of enucleation. After marking with a 10-mm diameter trephine, the corneal epithelia and a thin layer of stroma were removed by superficial keratectomy under a dissecting microscope. The central corneal button was cut off, and the endothelium, together with the Descemet membrane, was removed with a tooth-free forceps under the microscope. Stromal buttons were cut into small pieces measuring 1 × 2 mm² and were digested with 5 mg/mL collagenase in DMEM/F12 (1:1) for 4 hours at 37°C. Isolated rabbit corneal keratocytes (RCKs) were collected by centrifugation, resuspended in DMEM/F12 with 100 µg/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin, and seeded at the concentration of 1.5 × 10⁵ cells/mL in the same medium in 12-well plates (2.25 × 10⁶ cells/well) or 100-mm dishes (1.5 × 10⁶ cells/dish). When the cells were attached, the media were changed to remove the dead cells and debris (1 mL or 10 mL, respectively). To maintain the cell phenotype, one-tenth of the original volume of new medium was added carefully every week (e.g., 100 µL/well for a 12-well plate and 1 mL for a 100-mm dish). To stimulate the cells, RCKs were incubated in the presence of EGF, TGF-β1, or a combination of TGF-β1 and EGF, added in a total volume of 100 µL DMEM/F12. Fibroblasts were obtained by incubating RCKs in DMEM/F12 containing 10% fetal bovine serum (FBS). Differentiated myofibroblasts were obtained by subculture of fibroblasts at low density and were incubated with 5% PBS for 3 days, as previously described.14-40

**Immunofluorescence Staining**

Cells were washed in PBS and fixed with 2% paraformaldehyde in 0.1 M phosphate buffer for 30 minutes at 4°C and permeabilized with 0.3% Triton X-100 solution for 5 minutes on ice. The remaining procedures were performed at room temperature. After three washes with PBS, the cells were incubated with 10% normal goat serum in PBS containing 0.1% bovine serum albumin (PBS-BSA) for 30 minutes to block nonspecific binding. Afterward, the cells were incubated for 1 hour with the corresponding primary antibodies at optimal dilutions in PBS containing 1.5% normal goat serum. After rising with PBS-BSA (3 × 5 minutes), cells were incubated with the corresponding secondary antibodies for 45 minutes. DAPI was used to counterstain the nuclei. In all assays, negative controls were prepared using normal mouse IgG (sc-2025; Santa Cruz Biotechnology) or 0.1 M PBS instead of the primary antibody to exclude nonspecific staining.

**Western Blot Analysis**

After the different treatments, cell cultures on 100-mm dishes were rinsed twice with PBS and harvested in modified RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/mL each of aprotinin, pepstatin, and leupeptin, 1 mM NaVO₃, 1 mM NaF) and were analyzed by Western blot using different antibodies, as described previously by our laboratory.14 Bound antibodies were visualized after chemiluminescence detection (ECL Plus Kit; GE Healthcare, Chalfont, St. Giles, UK) on autoradiographic film.

**Cell Proliferation and Migration**

Seven-day cultures of RCK in 12-well plates were treated with EGF, TGF-β1, or a combination of both in the presence or absence of AG1478 for 48 hours. Cultures were also treated with TGF-β1 and anti-EGF antibody. Cell proliferation was assessed by immunostaining with anti–Ki-67 antibody. DAPI was used to counterstain the nuclei. Numbers of Ki-67–positive nuclei compared with all nuclei, as shown by DAPI staining, were counted in a blind fashion in 10 different fields, clockwise at positions 3, 6, 9, 12 o’clock, and center at low magnification (10×) in two wells, and were averaged. Ki-67–positive cells were expressed as a percentage of total cells counted. To assay cell migration, the RCK cultures were wounded by a linear scratch with a sterile plastic tip in the center of the well and were incubated for 24 hours in DMEM/F12 with or without growth factors. In experiments in which AG1478 or anti–EGF antibody was used, RCK cultures were added 30 minutes before wounding. Cell migration was determined by phase-contrast images collected by a camera attached to the microscope, and the wounded area was calculated using imaging software (MetaVue; Molecular Devices, Eugene, OR). Each point represents the analysis of images collected from 10 different wounded areas in two different wells (see Fig. 7B).

**Statistical Analysis**

Data are presented as mean ± SD. Comparisons between groups at each time point during treatments were conducted by one-way ANOVA followed by Tukey test. P < 0.05 was considered statistically significant.

**Results**

**Keratocyte Culture and Phenotype Maintenance**

RCKs cultured in serum-free DMEM/F12 without frequent changes of medium retained their phenotypes for more than 1 month (Fig. 1A). These cells showed typical dendritic morphology and were connected to each other with long processes to form a network, as seen in vivo. They were strongly stained for...
vimentin, a general marker of mesenchyma-derived cells (Fig. 1B), and showed an eccentric bean-shaped nucleus. RCKs were also positively stained for ALDH1, a marker for keratocyte phenotype, but were negatively stained for \( \alpha \)-SMA (Fig. 1C). In contrast, when the cells transformed to myofibroblasts by plat-ing fibroblasts at low density,\(^\text{14,40}\) they showed the character-istic positive staining for \( \alpha \)-SMA but negative staining for ALDH1. In addition, keratocytes and myofibroblasts both ex-pressed EGF-R. These results were further confirmed by immu-noblotting (Fig. 1D), in which ALDH1 appeared as a single band of approximately 51 kDa in the RCK samples but not in the myofibroblasts, whereas \( \alpha \)-SMA was present in the trans-formed cells with stronger EGF-R expression.

**EGF Stimulation on Keratocyte Differentiation, Proliferation and ECM Expression**

One-week cultures of RCKs were treated with different concentrations of EGF for 2 days. Western blot analysis showed that EGF induced \( \alpha \)-SMA expression in a dose-dependent fash-ion, with optimal concentration at 50 ng/mL (Fig. 2A). Immuno-staining with \( \alpha \)-SMA (Fig. 2B) showed that the cells differ-entiated to a proto-myofibroblast phenotype in which the stain was prominent in the cytoplasm but was not organized into stress fibers, which are typical of well-differentiated myofibro-blasts.\(^\text{41}\) There was a loss of dendritic morphology and process connection, an upregulation of CS expression, and a down-regulation of KS expression, characteristics of altered pheno-type.\(^\text{5,7}\) EGF also stimulated the expression of several ECM components (Fig. 2C), including FN, thrombospondin (TSP)-1, laminin (LM), and collagen (Coll) types III and IV. In addition, EGF treatment induced the expression of proinflammatory cytokines such as interleukin-1\(\alpha\), interferon-\(\gamma\), and metallopro-teinase-2 (data not shown). Similar results were obtained when keratocytes were incubated with EGF for 1 week. EGF in-creased cell proliferation, as detected by Ki-67 staining (Fig. 2D). Cell proliferation was stimulated after 2 and 4 days and was decreased by day 7 (Fig. 2D).

**Activation of Akt-1 and ERK1/2 MAP Kinases by EGF Stimulation in Corneal Keratocytes**

To determine which signaling pathways are activated, RCKs were stimulated with EGF (50 ng/mL) for different times and phosphorylation of Akt-1, and the mitogen-activated protein kinases (MAPKs), ERK1/2, and p38 were determined by West-ern blot (Fig. 3A). EGF rapidly phosphorylated Akt-1 at Ser473, with a peak at 15 minutes and a decrease by 60 minutes, though the decrease was still higher than in controls at 2 hours. Growth factor also increased the phosphorylation of ERK1/2 with a peak at 15 minutes but did not have any effect on p38 activation. There were no changes in the expression of the total proteins. Immunofluorescence showed that Akt-1 in un-stimulated cells was expressed throughout the cytoplasm, and treatment with EGF for 30 minutes induced the nuclear local-ization of p-Akt (Fig. 3B).
FIGURE 2. Effects of EGF on kerato-cyte transformation, proliferation, and ECM expression. (A) Seven-day RCK cultures were treated with different concentrations of EGF for 2 days and analyzed by Western blot. (B) Immunofluorescence was performed in RCKs treated with 50 ng/mL EGF to detect the expression of α-SMA, CS, KS, and Ki-67. (C) Expression of FN, TSP-1, LM, Coll III, and Coll IV in EGF-treated cells. DAPI was used to stain the nuclei. For better contrast of images, DAPI blue was converted to red. Images of the controls in which those ECM components were not found have been omitted. Images shown are representative of three independent experiments. (C) Time course of cell proliferation induced by EGF. Data are expressed as mean ± SE of percentages of Ki-67–positive cells versus total cells counted in a blind fashion in 10 different fields of two wells. The experiment was repeated twice with similar results.

FIGURE 3. EGF induced Akt-1 and ERK phosphorylation in corneal keratocytes. (A) Seven-day RCK cultures were stimulated with EGF (50 ng/mL) for 5, 10, 15, 30, 45, 60, and 120 minutes and were analyzed by Western blot with antibodies for the phosphorylated form of Akt-1, ERK1/2, and p38. As positive control (+) for p-p38, a protein lysate of rabbit corneal epithelial cells stimulated with EGF was used. Antibodies for total Akt, ERK1/2, and p38 demonstrate similar gel loading. (B) Immunofluorescence shows the nuclear localization of p-Akt in RCKs after stimulation with EGF (50 ng/mL) for 30 minutes. DAPI stain the nuclei blue. Data shown are representative of three separate experiments.
Synergetic Effect of EGF with TGF-β1 on Keratocyte Transformation to Myofibroblasts

To test what effect EGF has on TGF-β1–induced differentiation, RCK cultures were treated with EGF (50 ng/mL), TGF-β1 (10 ng/mL), or a combination of both growth factors for 7 days, and the expression of α-SMA and FN was analyzed by immunofluorescence and Western blot. Previous work had shown that this concentration of TGF-β1 induces keratocyte differentiation. As already shown, treatment with EGF induced the transformation of 100% of the cells into a proto-myofibroblast phenotype that synthesized FN (Figs. 4A, 4B); treatment with TGF-β1 induced 88% ± 4% of RCKs to differentiate into proto-myofibroblasts and 12% ± 4% to differentiate into myofibroblasts, characterized by their large, spread-out morphology. However, treatment with TGF-β1 plus EGF induced 91% ± 4% of the cells to differentiate into definitive myofibroblasts, with significant increases in the expression of α-SMA and FN (Figs. 4A, 4C).

Abolishment of the Synergetic Effect of TGF-β1 on Keratocyte Differentiation and FN Expression by Inhibition of EGF Receptor or PI-3K Signaling

To investigate the interaction of EGF with TGF-β, RCKs were treated for 7 days with EGF (50 ng/mL), TGF-β1 (10 ng/mL), or EGF plus TGF-β with or without AG1478 (10 μM), a specific inhibitor of EGF-R activation; LY294002 (15 μM), an inhibitor of PI-3K; PD98059 (50 μM), an inhibitor of MEK-K; and SB203580 (20 μM), a p38 inhibitor. None of these inhibitors exhibited visible cell toxicity, as assayed by a viability kit (Live/Dead Baclight; Molecular Probes, Eugene, OR). Immunofluorescence (Fig. 5A) showed that AG1478 completely inhibited EGF-induced keratocyte differentiation and FN expression.

Neutralization of EGF and Inhibition of EGF-R on TGF-β1–Induced α-SMA and FN Expression

To determine whether TGF-β1 acts through the activation of EGF-R or through the induction of EGF synthesis, RCKs were treated with TGF-β1 in the presence of anti-EGF antibody at different concentrations (100 ng–100 μg/mL) or AG1478 (10 mM) for 2 days, and the expressions of α-SMA and FN were assayed by immunofluorescence and Western blot (Figs. 6A, 6B). Neutralization of EGF with anti-EGF antibody (10 μg/mL) did not prevent TGF-β1–induced α-SMA or FN expression. Increasing the concentration of antibody up to 100 μg/mL showed similar results (data not shown). On the other hand, inhibition of EGF-R by AG1478 significantly decreased α-SMA and FN expression. These results suggest that TGF-β1 may act through the transactivation of EGF-R. To further test this hypothesis, RCKs were stimulated with TGF-β1 (10 ng/mL) at different times, and the phosphorylation of EGF-R was determined by Western blot. TGF-β1 rapidly induced EGF-R activation, with a peak at 10 minutes and a decrease by 60 minutes (Fig. 6C). TGF-β1–induced EGF-R phosphorylation was com-

Figure 4. EGF and TGF-β1 promote keratocyte differentiation and fibronectin synthesis. Seven-day RCK cultures were treated with EGF (50 ng/mL), TGF-β1 (10 ng/mL), or EGF plus TGF-β1 for 1 week. (A) Immunostaining with α-SMA and FN antibodies. Nuclei were counterstained with DAPI. The color of DAPI stain in the merged images with FN was changed from blue to red for better contrast. (B) Average percentage of proto-myofibroblasts versus differentiated myofibroblasts counted in a blind fashion in 10 different fields of two-well plates. Results are representative of two separate experiments. (C) Cell total homogenates were analyzed by Western blot with antibodies for α-SMA and FN. Vimentin was used as a loading control. Data are representative of three separate experiments. Relative signal strengths of α-SMA or FN to vimentin are presented as mean ± SD.
pletely inhibited by AG1478 (Fig. 6D). These studies strongly suggest that TGF-β1 acts through the transactivation of EGF-R to promote RCK differentiation and ECM expression.

Prevention of EGF-Stimulated Cell Proliferation and Migration by Inhibition of EGF-R Activation

Proliferation and migration are two important mechanisms by which corneal stromal cells respond to injury. To determine the relationship between EGF and TGF-β1 in these two cellular responses, 1-week cultures of RCKs were treated with EGF (50 ng/mL) and EGF plus TGF-β1 (10 ng/mL) with or without AG1478 (10 μM), LY294002 (15 μM), PD98059 (50 μM), or SB203580 (20 μM) for 1 week. (A) Representative images of immunofluorescence with α-SMA and FN. DAPI stain the nuclei blue. (B) Western blot shows that inhibition of the EGF-R blocked not only the effect of EGF but also of TGF-β1 on the expression of α-SMA and FN (*P < 0.05). Relative signal strengths of α-SMA or FN to vimentin are presented as mean ± SD.

**FIGURE 5.** Effect of EGF-R and kinase inhibition on keratocyte differentiation and fibronectin expression. Seven-day RCK cultures were treated with EGF (50 ng/mL) and EGF plus TGF-β1 (10 ng/mL) with or without AG1478 (10 μM), LY294002 (15 μM), PD98059 (50 μM), or SB203580 (20 μM) for 1 week. (A) Representative images of immunofluorescence with α-SMA and FN. DAPI stain the nuclei blue. (B) Western blot shows that inhibition of the EGF-R blocked not only the effect of EGF but also of TGF-β1 on the expression of α-SMA and FN (*P < 0.05). Relative signal strengths of α-SMA or FN to vimentin are presented as mean ± SD.

AG1478 or anti-EGF antibody and were stimulated with TGF. Both EGF and TGF-β1 promoted keratocyte proliferation (Fig. 7A), but proliferation in the presence of EGF was significantly higher than with TGF-β1 alone, and there was no synergistic effect when both growth factors were combined. Inhibition of EGF-R decreased the proliferation induced by EGF and TGF-β1 to levels lower than that induced by TGF-β1 alone (*P < 0.05) and completely blocked EGF-stimulated proliferation. However, the neutralization of EGF by treatment with anti-EGF antibody did not significantly decrease the action of TGF-β1, demonstrating that the small proliferative action of TGF-β1 involved the transactivation of the EGF-R. Both EGF and TGF-β1 stimulated keratocyte migration (Fig. 7B). Migration was more rapid with TGF-β1, and further increases took place...
when EGF and TGF-β1 were combined. Inhibition of EGFR by AG1478 completely prevented EGF-induced migration and significantly decreased TGF-β1-induced cell migration (P < 0.05), whereas treatment with anti-EGF antibody in the presence of TGF-β1 had no effect on migration.

**DISCUSSION**

The cornea contains no blood vessels, and unlike epithelial and endothelial layers, which can derive nutrients directly from tear fluid and aqueous humor, keratocytes obtain their supply exclusively by diffusion. This relatively insufficient supply of nutrients may contribute to keratocyte quiescence, stromal homeostasis, and corneal transparency. In the present study, by culturing RCKs in serum-free DMEM/F12 without frequent changes of the medium, we were able to maintain the keratocyte phenotype for long times in vitro. The cells exhibited dendritic morphology, eccentric bean-shaped nuclei, and long intercellular processes that connected extensively to form a network like that seen in situ.1–4

Immunofluorescence showed that the cells were strongly stained for KS and ALDH1 but were weakly stained for CS and negatively stained for α-SMA and FN, typical of keratocytes seen in situ.5,6 Expression of EGFR was detected in keratocytes and myofibroblasts. In addition, treatment with EGF induced 100% transformation of keratocytes into a proto-myofibroblast phenotype expressing α-SMA in the cytoplasm but without the well-organized stress fibers found in myofibroblasts. Proto-myofibroblasts proliferate and also express FN, TSP-1, LM, and Coll III and IV. These ECM components were not found in the unstimulated keratocytes but had been reported in the fibroblasts of corneal repair tissue,42–45 indicating that EGF-stimulated cells have the characteristics of repair fibroblasts. EGF showed similar effects at longer times (1 week) of incubation, suggesting that growth factor–induced differentiation is not altered after long exposure to EGF. Our findings suggest that EGF is an important mediator in the initial activation of stromal keratocytes through stimulation of the EGFR and that the PI-3K/Akt-1 signaling pathway plays a key role in the EGF-induced keratocyte differentiation to proto-myofibroblasts, proliferation, and ECM production. Akt-1 regulation requires the activation of PI-3K to recruit Akt-1 to the plasma membrane, where it is first phosphorylated (activated) and then detached from the membrane to phosphorylate substrates from the cytosol and nuclei. Under our conditions, EGF induced a strong and prolonged (longer than 2 hours) Akt activation, and stimulating RCKs for 30 minutes induced the translocation of phosphorylated Akt-1 to the nuclei, suggesting that the regulation of transcription factors by Akt-1 could be an important mechanism for the differentiation of keratocytes stimulated with EGF. Studies on breast epithelial cells stimulated with IGF-1 have shown an active role of PI-3K/Akt in phosphorylation and in an epithelial-to-mesenchymal transition.46 Given that no organized α-SMA stress fibers were found in the EGF-treated cells, we conclude that EGF is not sufficient to induce keratocyte differentiation to myofibroblasts but that other mediators are required for the completion of cell differentiation. A diversity of growth factors and cytokines is present after injury in the cornea, and it is possible that more than one component is required for myofibroblast differentiation.

TGF-β1 is a potent inducer of myofibroblast transformation in a variety of cells of different tissues, including cornea.24,47–51 Under our culture conditions, TGF-β1 induced 12% of the cells to differentiate into myofibroblasts. However, EGF plus TGF stimulated 90% of cells to differentiate into myofibroblasts, with a significant upregulation of FN expression. Our results using chemical inhibition showed that the synergism between TGF-β1 and EGF involves the activation of EGF-R and PI-3K/Akt-1 signaling but not ERK1/2 or p38 activation. Blocking EGF-R activation or the PI-3K/Akt-1 pathway resulted in a significant decrease in α-SMA and FN expression when RCKs were stimulated with TGF-β1 (Fig. 5), suggesting that this specific cytokine could act through the EGF-R. In addition, our experiments with neutralizing anti-EGF antibody demonstrated that the action of TGF-β1 is not mediated by promoting the synthesis of EGF but rather by inhibiting the activation of the EGF-R (Fig. 6). Earlier studies support this possibility. For example, it has been shown that TGF-β1 induces the expression of transformed keratocytes into a proto-myofibroblast and myofibroblasts. In addition, treatment with EGF significantly decreased TGF-β1-induced cell migration (P < 0.05), whereas treatment with anti-EGF antibody in the presence of TGF-β1 had no effect on migration.

**FIGURE 6.** Effect of neutralization of EGF or inhibition of EGF-R on TGF-β1-stimulated α-SMA and FN expression. Seven-day RCK cultures were treated with TGF-β1 (10 ng/ml) with or without anti-EGF antibody (10 μg/ml) or AG1478 (10 μM) for 2 days. (A) Representative images of immunofluorescence with α-SMA and FN. DAPI stain the nuclei blue. (B) Western blot shows that the inhibition of EGF-R, but not the neutralization of EGF, decreased the effect of TGF-β1 on the expression of α-SMA and FN. Relative signal strengths of α-SMA or FN to vimentin are presented as mean ± SD. Data are representative of three separate experiments. (C) Seven-day RCK cultures were stimulated with TGF-β1 (10 ng/ml) for 5, 10, 30, and 60 minutes and were analyzed by Western blot with antibodies for the phosphorylated form of EGF-R. Antibodies for total EGF-R demonstrate similar gel loading. (D) Seven-day RCK cultures were pretreated with AG1478 (10 μM) for 30 minutes and then stimulated with TGF-β1 (10 ng/ml) for 10 minutes. Cell lysates were prepared for Western blot with antibodies for EGF-R and the phosphorylated form of EGF-R.
of high-affinity EGF-R in stromal fibroblasts. In the A431 epidermal cell line, TGF-β1 caused increased tyrosine phosphorylation of the EGF-R that was not dependent on protein synthesis. Other studies have shown that TGF-β amplifies the content of EGF-R in granulose cells from rat ovaries and increases EGF-R transcription in kidney fibroblasts. Our studies have shown that EGF-R is activated rapidly when stimulated with TGF-β1. Adding EGF to TGF-β1 did not affect proliferation; in the presence of AG1478, however, there was a small but significant decrease in proliferation stimulated by TGF-β1 (Fig. 7B), suggesting that TGF-β1 is able to induce the proliferation of keratocytes by pathways that involve EGF-R activation but not EGF synthesis. However, adding EGF to TGF-β1 promoted cell migration that was significantly impaired when the EGF-R was blocked, suggesting that EGF-R signaling is important in cell migration. These data differ from earlier findings describing that EGF increases the migration, chemotaxis, and proliferation of stromal fibroblasts differentiated by serum and that TGF-β1 decreases the proliferation and migration of the cells. However, none of these studies...
investigate the combined action of EGF and TGF-β1 in nondifferentiated keratocytes, such as those found “in situ” in the cornea.

In corneal epithelial cells stimulated with hepatocyte growth factor, p38 activation has been shown to be important for cell migration; however, we could not demonstrate the activation of p38 by EGF in RCKs in our experiments. It is possible that other signals, such as PI-3K/Akt-1, are important in migration. The role of PI-3K/Akt-1 in migration seems to be cell- and tissue-specific and dependent on the potential targets of Akt. Akt phosphorylation has been linked to the organization of the actin cytoskeleton and to the expression of genes involved in the migration and phosphorylation of proteins involved in adhesion, such as focal adhesion kinase and paxillin. The increased expression of FN contributes to the combined effect of TGF-β1 and EGF on cell migration. This glycoprotein mediates a wide variety of cellular interactions with the matrix and plays important roles in cell adhesion, migration, growth, and differentiation. The upregulated expression of FN has been found at sites of stromal wounds and is thought to provide a substrate for the attachment and to facilitate the migration of corneal fibroblasts. In vitro models of wound healing have also shown that fibronectin promotes corneal fibroblast-mediated collagen gel contraction and contributes to the maintenance of corneal shape by corneal fibroblasts during stromal wound healing. In the present study, we have shown that EGF and TGF-β1 separately induced FN expression in corneal keratocytes. However, there was a significant increase when both growth factors were added, suggesting that during stromal injury, these two growth factors may synergistically function to promote wound healing by the upregulation of fibronectin expression and to promote migration. Future studies to investigate the actions of the growth factors on message levels could preclude variables such as protein secretion to the media and protein degradation and further insight into the mechanisms of gene induction of FN and other ECM components.

In conclusion, our studies demonstrate that EGF synergizes TGF-β1 induction of keratocytes to myofibroblast differentiation, increases secretion of FN, and produces changes in ECM components through the activation of EGF-R and PI-3K/Akt-1 signaling. These results provide insight into why the use of EGF in penetrating wounds, though effective in closing the wounds, promotes scar tissue formation and compromises corneal transparency.

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References


