Effects of Epidermal Growth Factor, Fibroblast Growth Factor, and Transforming Growth Factor-β on Corneal Cell Chemotaxis

Maria B. Grant,* Peng T. Khaw,† Gregory S. Schultz,‡ Julie L. Adams,‡ and Robert W. Shimizu‡

The effects of recombinant basic fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor-beta (TGF-β) on migration of human and bovine corneal cells were determined using checkerboard analysis in Boyden chambers. EGF, FGF, and TGF-β each stimulated high levels of chemotactic migration. Each growth factor, however, induced a different dose–response pattern. Migration stimulated by FGF reached a plateau at a concentration between 100 and 200 ng/ml for endothelial, epithelial, and stromal fibroblasts. By contrast, chemotactic responses to EGF peaked between 10 and 50 ng/ml, then decreased at higher concentrations. TGF-β also stimulated a peak in migration in all three corneal cells, but the peak of migration occurred at an approximately 1000-fold lower concentration (1 pg/ml) than for EGF. Checkerboard analysis demonstrated that FGF and EGF, but not TGF-β, stimulated chemokinesis of bovine, stromal, and endothelial cells. These results demonstrate that FGF, EGF, and TGF-β induce migration in pure populations of bovine and human corneal cells and support the concept that these growth factors may play key roles in corneal wound healing by regulating migration of corneal cells. Invest Ophthalmol Vis Sci 33:3292–3301, 1992

Healing of corneal wounds is a complex biologic process not fully understood at the molecular level. Migration of corneal cells is one of the most important components of the biologic response of corneal cells to injury. Previous in vitro and in vivo studies have identified several peptide growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (FGF), and transforming growth factor beta (TGF-β), that enhance corneal wound healing. Whether these peptide growth factors affect migration of corneal cells has not been established.

EGF has been extensively studied for its effects on healing of a variety of corneal wounds. EGF accelerated reepithelialization of corneal surface injuries in rabbits and primates in vivo, and stimulated proliferation of corneal epithelial cells in vitro. EGF also was reported to increase tensile strength of corneal incisions in rabbits and primates, and to stimulate DNA synthesis of rabbit stromal keratocytes in culture. EGF stimulated mitosis of rabbit, bovine, primate, and human corneal endothelial cells in vitro and increased endothelial cell density in primates after endothelial injury in vivo. Thus, EGF has been reported to enhance wound healing in vivo and to stimulate mitosis of corneal cells in vitro.

The effects of EGF on corneal cell migration have been studied less thoroughly than its effects on corneal cell mitosis. Watanabe and colleagues reported that EGF stimulated migration of rabbit epithelial cells along the edges of corneal blocks during organ culturing but found no chemotactic activity of EGF with rabbit epithelial cells using Boyden chambers. Soong et al reported that EGF accelerated the healing of rabbit epithelial wounds by stimulating mitosis of epithelial cells rather than stimulating chemotaxis. Joyce et al demonstrated that EGF stimulates the migration of individual rabbit corneal cells from the edge of in vitro wounds during a 72 hr culture with serum. However, the effects of EGF on migration, independent of mitosis, have not been characterized. The effects of EGF on chemotactic migration of stromal endothelial or fibroblasts cells have not been previously reported using the Boyden chamber technique.

FGF has also been reported to enhance healing of corneal epithelial and endothelial wounds in vivo. As with EGF, FGF has been mainly studied for its mito-
genic effect on corneal cells. FGF stimulated proliferation of bovine corneal epithelial cells, human stromal fibroblasts, and bovine endothelial cells. FGF accelerated healing of rabbit epithelium after mild chemical injury or epithelial scrape injury and increased regeneration of corneal endothelium in cats after transcorneal freeze injury. The effects of FGF on chemotaxis or chemokinesis of corneal cells has not been reported.

TGF-β is a potent peptide growth factor that has not been evaluated in corneal wound-healing models. However, in a number of other wound-healing models, TGF-β acts as a bifunctional regulator of cell division; TGF-β generally inhibits mitosis of ectodermally derived cells and stimulates mitosis of mesodermally derived cells. TGF-β has also been reported to be a potent chemotactic factor for dermal fibroblasts and monocytes. The effects of TGF-β on corneal cell migration and corneal wound healing have not been reported.

Although a number of studies have reported an enhancement of wound healing by EGF, FGF, and TGF-β, the effects of peptide growth factors on chemotaxis and chemokinesis of corneal cells have not been fully investigated. Because in vivo models of corneal wound healing can be complicated by the presence of multiple cell types and by endogenously produced growth factors, we performed checkerboard analyses using modified Boyden chambers to determine the chemotactic and chemokinetic effects of recombinant human FGF, EGF, and TGF-β on pure cultures of bovine corneal endothelial cells and stromal fibroblasts. In addition, we studied the effects of these factors on migration of human corneal epithelial cells, stromal fibroblasts, and endothelial cells. The results demonstrate that these peptide growth factors are potent stimulators of migration of corneal cells. We postulate that the enhancement of corneal wound healing reported previously for these factors may be caused, in part, by the enhancement of chemotactic migration of corneal cells.

Materials and Methods

Endothelial Cell Culture

Primary cultures of bovine corneal endothelial cells were established using minor modifications of previously described methods. Animal tissue was obtained under procedures that conformed with the ARVO Resolution on the Use of Animals in Research. Briefly, four fresh cow eyes were obtained from a local meat-processing laboratory, extracocular tissues were removed from the globe which were then soaked for 1 hr at 4°C in minimum essential media supplemented with an antibiotic and antimitomytic solution (0.5 µg/ml amphotericin B, 100 µg/ml gentamicin, and 100 µg/ml penicillin). Three milliliters of providone iodine solution was pipetted over the globes, and in a sterile laminar flow hood, the corneas were excised with an attached 2-mm wide scleral ring. The corneas were placed endothelial side up in a Petri dish, the corneal endothelium was washed three times with Hank’s balanced salt solution (HBSS) and 0.5 ml of a trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA, 0.2%) solution was placed on the endothelium. After an approximately 1-hr exposure at room temperature, endothelial cells were gently dislodged with a rubber policeman to avoid penetration of Descemet’s membrane. The suspension of endothelial cells was added to bicarbonate buffered Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS; Gibco, Frederick, MD) and the antibiotic–antimyotic solution, and centrifuged at 900 x g for 5 min. The endothelial cell pellet containing 10^5 cells was resuspended in 10 ml of the same medium, seeded into two 25-cm² culture flasks, and incubated at 35°C in 95% air and 5% carbon dioxide. Cultures were fed three times per week, and confluence was achieved in approximately 7 days. Confluent cultures were passaged using 0.25% trypsin and 0.2% EDTA and split at a ratio of 1:4.

Human corneal endothelial cells were isolated using a similar procedure with the following modifications. Isolation was performed from a pair of donor eyes obtained from a 3-year-old child within 24 hr of death. Cells were dislodged with a lower concentration of trypsin and EDTA (0.1% and 0.08%, respectively) and were placed directly into a 25-cm² flask coated with 2% gelatin and grown in DMEM with 20% FCS, endothelial cell growth supplement (0.015 mg/ml), insulin (1 µg/ml), transferrin (5 µg/ml), selenium (2 µg/ml), and the antibiotic–antimyotic solution. Human corneal endothelial cells were passaged without trypsin and EDTA using mechanical detachment with a rubber policeman and split at a ratio of 1:3. Cell lines established from bovine eyes or human cadaveric eyes were confirmed to contain pure populations of endothelial cells by acetylated LDL incorporation. Experiments were performed with bovine or human endothelial cells in the third to sixth passage.

Stromal Fibroblast Culture

Bovine or human corneal fibroblasts were isolated using modifications of a previously described method. Briefly, extracocular tissues were removed from two fresh globes, which were then rinsed extensively with DMEM containing the antibiotic–antimyotic solution. The corneas were excised, epithelium and endothelium were removed by scraping with a
scalpel blade, and the corneas were cut into 1-mm cubes with a McIlwain (Surrey, England) tissue chopper. Tissue pieces were dissociated into single cells by incubation in 5 ml of DMEM containing 2.5 mg/ml collagenase (type III; Sigma, St. Louis, MO) for 90 min at 37°C with shaking. Dissociated cells were separated from remnants of stromal tissue by filtration through 145-μm pore nylon mesh (Small Parts, Miami, FL), and the cells were pelleted by centrifugation at 100 × g for 2 min. Stromal fibroblasts were resuspended in Medium 199 (Sigma, St. Louis, MO) buffered to pH 7.4 with 25 mmol/l HEPES and supplemented with 10% FCS and an antibiotic–antimycotic solution (penicillin 100 IU/ml, streptomycin 100 μg/ml, amphotericin B 0.25 μg/ml), seeded into flasks and grown in humidified air at 37°C. Stromal fibroblasts were passaged with trypsin and EDTA with a split ratio of 1:4. The purity of fibroblast cell cultures was assessed by spindle-shaped morphologic criteria, and experiments were performed with fibroblasts from the third to sixth passage.

Epithelial Cell Culture

Human epithelial cell cultures were established using modifications of a previously described method. 26 Briefly, human corneas with a 3-mm rim of sclera were washed three times with DMEM and streptomycin (100 μg/ml), penicillin (100 IU/ml), gentamicin (10 mg/ml), and amphotericin B (250 ng/ml). The endothelium was wiped off with a cotton-tipped swab, and the scleral rim was trimmed off. Corneas were placed in a 35-mm culture dish filled with 2 ml of a solution of Medium 199 containing 50 U/ml of Dispase II (Sigma). After 1 hr of incubation at 37°C, full-thickness sheets of corneal epithelium were removed by gentle dissection with a periosteal elevator. Sheets of epithelium were suspended in keratinocyte growth medium (KGM) supplemented with pituitary extract and EGF (Clonetic, San Diego, CA), seeded into Primaria culture flasks (Falcon, Oxnard, CA), and grown to confluence after 7–10 days of culture at 37°C in 5% CO2. The purity of epithelial cell cultures was established by their cobblestone pattern at confluence and by immunohistochemical detection of the 64-kilodalton corneal-specific cytokeratin (AE5 monoclonal antibody; ICN, Costa Mesa, CA). 27 Experiments were performed with primary cultures because of the tendency of subpassaged epithelial cells to differentiate terminally.

Chemotaxis and Chemokinesis Assays

Chemotaxis and chemokinesis of corneal cells in response to peptide growth factors were measured in two-tiered blind-well chemotaxis chambers (Neuroprobe, Bethesda, MD) using a modified checkerboard format. 28 A growth factor is considered a chemotactic agent if it induces cells to migrate from an area of lower growth factor concentration to an area of higher concentration of growth factors. This process is described as chemotaxis or the "directional migration" of cells. If the growth factor induces chemotaxis, the number of cells that migrate through the membrane will increase as the concentration of the growth factor increases in the upper well. This is illustrated in the top row of each checkerboard analysis where growth factor is present only in the top well of the chambers. Chemokinesis, by contrast, represents random non-directional cell motion and is assessed by the number of cells in the diagonal boxes of the checkerboard tables. If an agent induces chemokinesis, the number of migrating cells in the diagonal boxes increases as the concentration of growth factor increases in both the upper and lower wells.

Single-cell suspensions of corneal cells were prepared from confluent cultures by different regimens of trypsin treatment as a result of the varying sensitivities of corneal cells to trypsinization. Cultures of human and bovine corneal fibroblasts were dissociated using HBSS containing 0.25% trypsin and 0.2% EDTA at 37°C until the cells were first observed to round up, which typically occurred after approximately 3 min. DMEM containing 10% FCS was added to the culture flask to inactivate trypsin; the fibroblasts were collected by centrifugation and then resuspended in DMEM containing 1% bovine serum albumin (BSA) at 1.6 × 10^6 cells/ml.

Dissociation of cultures of human corneal epithelial cells required repeated brief exposure to lower concentrations of trypsin and EDTA to maintain chemotactic responses. One day before use in the chemotaxis assay, primary cultures of human epithelial cells were placed in KGM without growth factor supplements. Epithelial cell cultures were exposed to 0.1% trypsin and 0.08% EDTA at 22°C for 3–5 min. Dislodged cells in the trypsin solution were transferred to a conical centrifuge tube containing KGM with 10% FCS to inactivate the trypsin. Fresh trypsin solution was added to the flask of epithelial cells, and the procedure was repeated two more times, which removed a majority of cells from the flask. Epithelial cell harvests were pooled, centrifuged, and resuspended in KGM containing 1% BSA at 1.6 × 10^6 cells/ml. Suspensions of human and bovine corneal endothelial cells were prepared by brief trypsinization followed by mechanical disruption with a rubber policeman. Endothelial cell cultures were exposed to HBSS containing 0.1% trypsin and 0.08% EDTA at 22°C for 3–5 min, which caused the endothelial cells to round up but did not dislodge from the flask. Endothelial cells then
were dislodged by gentle scraping with a rubber policeman. Cells were added to DMEM containing 10% FCS, centrifuged, and the cell pellet resuspended in DMEM containing 1% BSA at 1.6 X 10^6 cells/ml.

For each migration assay, 40,000 corneal cells in 25 μl of DMEM containing 1% BSA alone or supplemented with the appropriate concentration of peptide growth factor were placed in each of the 48 lower wells. The test condition was assayed with a minimum of six replicates, each well with a degree of variation in the number of cells counted in three separate, high-power (400X) fields (HPF). When chemokinesis and chemotaxis were studied in an experiment, the DMEM contained the concentration indicated on the vertical axis of the checkerboard tables. The concentration of the growth factor which was placed in the upper well of the chamber is shown on the horizontal axis of the checkerboard tables. In experiments where only chemotaxis was measured, no growth factor additions were made to the DMEM in the well below the membrane, and only growth factors were added to the DMEM above the membrane. Wells were overlaid with a porous polyvinyl- and pyrrolidone-free polycarbonate membrane (Nuclepore, Pleasanton, CA) that had been coated with a solution of bovine dermal collagen (Ethicon, Summerville, CA). Migration experiments with endothelial cells were performed using filters with 5-μm pores because experiments using filters with 8- and 10-μm pores resulted in high background migrations that masked chemotactic responses. Using similar criteria, filters with 8-μm pores were determined to be optimal for fibroblast chemotaxis studies, and filters with 10-μm pores were determined to be optimal for epithelial cell studies.

Chemotaxis chambers were then inverted and incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C for 90 min, which allowed the cells to attach to the membrane. Chambers were then placed upright, and 50 μl of the test solutions were added to the upper well. The optimal incubation period was 3.5 hr for endothelial cells, 5 hr for fibroblasts, and 12 hr for epithelial cells. Membranes were recovered, and cells on the attachment side were scraped off, leaving only those cells that had migrated through pores of the filter. Membranes were submerged in methanol to fix the cells, which were then stained with Leukostat solution (Fisher, Springfield, NJ) and mounted onto glass slides. DMEM containing 1% BSA was used as a negative control and resulted in 13 ± 3 cells per HPF (Fig. 1, panel A). FGF was tested in a range of concentrations of 0-200 ng/ml and induced a dose-dependent increase in migration that began to reach a plateau at approximately 200 ng/ml. The maximum response to FGF was 3.2-fold greater than that stimulated by 10% FCS and 6.5-fold higher than that observed with serum-free control.

EGF was tested in the concentration range of 0-50 ng/ml (Fig. 1, panel B). EGF generated a peak migratory response at 10 ng/ml, which was 11-fold greater than the BSA control and nearly 3.1-fold more than 10% FCS. Migration decreased at concentrations of EGF higher than 10 ng/ml.

Migration of human corneal endothelial cells was very sensitive to TGF-β. This peptide was tested in the concentration range of 0-2 ng/ml and induced migration with a maximal response at 1 pg/ml (Fig. 1, panel C). At higher concentrations, there was a decline in the migratory response. The peak response to TGF-β was 5.5-fold greater than that induced by the BSA control and of similar magnitude compared with 10% FCS.

Human Stromal Fibroblasts

As shown in Figure 1, panel D, increasing concentrations of FGF induced migration that began to reach a plateau at 80 ng/ml. The maximum response to FGF was 3.7-fold higher than for BSA control and 1.6-fold higher than 10% FCS. EGF stimulated a peak of migration at 1 ng/ml, which decreased at higher
Migration of human corneal endotheial cells to FGF
Migration of human corneal fibroblasts to FGF
Migration of human corneal epithelium to FGF
Migration of human corneal endotheial cells to EGF
Migration of human corneal fibroblasts to EGF
Migration of human corneal epithelium to EGF
Migration of human corneal endotheial cells to TGF-β
Migration of human corneal fibroblasts to TGF-β
Migration of human corneal epithelium to TGF-β

Fig. 1. Migratory response of human corneal cells to FGF, EGF, and TGF-β. Migration of human corneal cells was performed using blind-well chemotaxis chambers. Background levels of migration were measured using 1% BSA, and 10% FCS was used as a positive chemotactic control. Chemotactic responses of corneal endothelial cells to FGF, EGF, and TGF-β are shown in panels (A–C), respectively. Similarly, the migratory response of corneal fibroblasts to FGF, EGF, and TGF-β are shown in panels (D–F), respectively, and the response of corneal epithelium to FGF, EGF, and TGF-β are shown in panels (G–I), respectively. Each data point represents the mean and standard error of six replicate wells.

concentrations (Fig. 1, panel E). The peak migratory response was 5.5-fold greater than the BSA control and 3.7-fold greater than 10% FCS. TGF-β generated a peak of migration at 0.5 pg/ml, which decreased at higher concentrations (Fig. 1, panel F). The peak migratory response was 3.6-fold higher than the BSA control and 1.9-fold higher than 10% FCS.

Human Epithelial Cells

Similar to human corneal endothelial cells and fibroblasts, the migratory response of human epithelial cells to FGF reached a plateau at approximately 50 ng/ml and did not significantly increase at higher concentrations (Fig. 1, panel G). The maximum FGF response was 7.4-fold higher than BSA and similar to the level of migration stimulated by 10% FCS. EGF stimulated a peak of migration at 20 ng/ml that decreased at higher concentrations (Fig. 1, panel H). The maximum response to EGF was 7.5-fold higher than the BSA control and 2-fold higher than 10% FCS. TGF-β stimulated a maximum migratory response at a concentration of 1 pg/ml (Fig. 1, panel I). The maximum response was 2.8-fold greater than the response to the BSA control and 1.5-fold higher than 10% serum.

Migratory Response of Bovine Corneal Cells

Bovine endothelial cells: The dose–response curve of bovine corneal endothelial cells migration to increasing concentrations of FGF is shown in Figure 2, panel A. Within the concentration range tested, FGF generated a sigmoidal dose–response curve that reached a plateau between 100–200 ng/ml. At the lowest concentration tested (25 ng/ml), FGF induced a 2-fold increase in migration over the BSA control whereas the highest concentration of FGF (200 ng/ml) stimulated a 14-fold increase over basal level. At a concentration of 50 ng/ml, FGF induced a 1.7-fold greater migratory response than the 10% FCS positive control.

Migration of bovine corneal endothelial cells to a gradient of FGF suggested that FGF was chemotactic for these cells. However, migration of cells in response to a gradient of FGF alone was insufficient to distinguish between chemotaxis and chemokinesis. Therefore, a checkerboard experiment was performed to determine if the migratory response stimulated by FGF represented chemotaxis or chemokinesis. The results in Table 1 show that bovine corneal endothelial cells had both chemotactic and chemokinetic responses to FGF. Chemotaxis was demonstrated by in-
Fig. 2. Migratory response of bovine corneal cells to FGF, EGF, and TGF-β. Migration of bovine corneal cells was performed using blind-well chemotaxis chambers. Background levels of migration were measured using 1% BSA, and 10% FCS was used as a positive chemotactic control. Chemotactic responses of corneal endothelial cells to FGF, EGF, and TGF-β are shown in panels (A–C), respectively. Similarly, the migratory response of corneal fibroblasts to FGF, EGF, and TGF-β are shown in panels (D–F), respectively. Each data point represents the mean and standard error of six replicate wells.

Table 1. Checkerboard analysis of FGF effect on bovine corneal endothelial cell migration

<table>
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<tr>
<th>Concentration of b-FGF (ng/ml) above the membrane</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of b-FGF (ng/ml) below the membrane</td>
<td>0</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>0</td>
<td>12 ± 4</td>
<td>24 ± 3</td>
<td>39 ± 6</td>
<td>57 ± 5</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>25</td>
<td>13 ± 6</td>
<td>22 ± 8</td>
<td>54 ± 7</td>
<td>68 ± 1</td>
<td>70 ± 6</td>
</tr>
<tr>
<td>50</td>
<td>14 ± 8</td>
<td>12 ± 9</td>
<td>37 ± 5</td>
<td>47 ± 6</td>
<td>65 ± 5</td>
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<td>29 ± 8</td>
<td>28 ± 6</td>
<td>39 ± 7</td>
<td>57 ± 4</td>
</tr>
<tr>
<td>200</td>
<td>52 ± 1</td>
<td>37 ± 8</td>
<td>41 ± 6</td>
<td>48 ± 1</td>
<td>42 ± 9</td>
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</table>

Values are the average ± SE of the number of cells per high-power field that migrated through the membrane pores.

Increasing numbers of bovine corneal endothelial cells migrating to increasing concentrations of FGF from 0–200 ng/ml (top row). Chemokinesis was demonstrated by the increased numbers of endothelial cells migrating in response to increasing concentrations of FGF above and below the membrane (diagonal boxes from top left to bottom right). The maximum chemotactic response of bovine endothelial cells to FGF was 85 ± 4 cells per HPF, whereas the corresponding chemokinetic response was 42 ± 9 cells per HPF. Thus, the chemokinetic effect of FGF was significantly less than the chemotactic effect of FGF.

EGF also was tested for its effects on bovine corneal endothelial cells migration. EGF induced a maximum migratory response at 50 ng/ml that was 9-fold higher than BSA control and 2.5-fold higher than 10% serum (Fig. 2, panel B). Higher concentrations of
transforming growth factor-β on bovine endothelial cell migration

Table 2. The effect of concentration gradients of transforming growth factor-β on bovine endothelial cell migration

Table 3. Checkerboard analysis of FGF effect on bovine corneal fibroblast cell migration

Values are the average ± SE of the number of cells per high-power field that migrated through the membrane pores.

Discussion

Migration of cells is a critical component of corneal wound healing. The effects of peptide growth factors on corneal cell migration have not been fully investigated. Our results demonstrate that EGF, FGF, and TGF-β stimulated migration of human and bovine corneal endothelium, fibroblasts, and epithelium in vitro. The ability of EGF, FGF, and TGF-β to stimulate migration of corneal cells in vitro suggests that these growth factors may enhance healing of corneal wounds in vivo, in part by stimulating migration of corneal cells to the wound site. Because peptide growth factors have been shown previously to stimulate mitosis of corneal cells and synthesis of extracellular matrix, our results indicate that peptide growth factors can stimulate all of the major components of corneal wound healing, ie, migration, mitosis, and synthesis of extracellular matrix.
An aspect of corneal wound healing that remains undetermined is whether gradients of peptide growth factors are generated at the site of a corneal injury. In the case of epithelial and stromal injuries, growth factors such as EGF, which have been detected in tears,\textsuperscript{29-31} could establish a concentration gradient by diffusion into tissues from the edge of the injury. In the case of endothelial wounds, concentration gradients could be established if peptide growth factors such as FGF are released from injured cells or Descemet's membrane after wounding.

The mechanism by which FGF accelerates healing of corneal wounds in vivo has not been fully established. FGF has been reported to stimulate mitosis of bovine corneal epithelial cells\textsuperscript{19} and endothelial cells in culture.\textsuperscript{11} The results of our experiments demonstrate that FGF also stimulates migration of corneal cells. Thus, FGF may enhance healing of corneal wounds by stimulating both mitosis and migration.

FGF has also been reported to stimulate migration of other cells in vitro. Terranova et al\textsuperscript{32} demonstrated that FGF stimulated both chemotaxis and chemokinesis of human umbilical vein endothelial cells in modified Boyden chamber checkerboard assays. The dose-response curve for FGF reached a maximum at 14 ng/ml and decreased slightly at 140 and 1400 ng/ml. In our experiments, maximum response to FGF also occurred at concentrations of FGF of 50–200 ng/ml, which were the highest concentrations tested.

The effects of TGF-\beta on corneal cells and corneal wound healing have not been extensively studied in vitro experiments have shown that TGF-\beta was a weak mitogen for bovine corneal endothelial cells in serum-free medium.\textsuperscript{11} The current results demonstrated that TGF-\beta was a potent chemotactant for corneal epithelial cells, stromal fibroblasts, and endothelial cells. The TGF-\beta dose–response curves for each of the cell types reached a peak of migration that decreased with higher TGF-\beta concentrations. The peak of migration occurred at approximately 1 pg/ml, which was approximately 10,000 times lower than the optimal concentrations of FGF or EGF. Even though the physiologic significance of a peak in migration is unknown, these results suggest that TGF-\beta may cause corneal cells to migrate to a position in a wound where the concentration of TGF-\beta is approximately 1 pg/ml (35 fmol/l) and not continue to migrate to the position in a wound with the highest TGF-\beta concentration. However, the concentrations of TGF-\beta that have been shown to stimulate synthesis of collagen in fibroblast cultures are in the range of 150–1500 pg/ml (5–50 pmol/l),\textsuperscript{33} which is significantly higher than the concentration that promotes optimal migration (1 pg/ml).

TGF-\beta has also been reported to be a potent chemotactic factor for other types of cells. This peptide stimulated chemotaxis of cultured human dermal fibroblasts, with a peak response at 10 pg/ml, but did not stimulate significant chemokinesis.\textsuperscript{35} TGF-\beta was also a potent stimulator of migration of peripheral human blood monocytes, producing a peak response at 0.5 pg/ml.\textsuperscript{24} Thus, the dose–response curve of corneal cell migration to TGF-\beta was very similar to the dose–response patterns observed with other cell types.

Studies on the effect of TGF-\beta on healing of non-corneal wounds have shown that TGF-\beta stimulated the rate of epidermal regeneration of partial-thickness injuries in pig skin\textsuperscript{34} and also increased tensile strength of skin incisions in normal and doxorubicin-treated rats.\textsuperscript{35} In vitro models of epithelial wound healing showed that TGF-\beta increased the outgrowth of epithelial keratinocytes in organ-cultured explants of pig skin.\textsuperscript{36} The ability of TGF-\beta to initiate earlier outgrowth of epithelial cells was apparently not caused by an effect on mitosis because TGF-\beta did not increase incorporation of tritiated thymidine into keratinocytes in the growing epidermal sheets. Thus, the ability of TGF-\beta to stimulate epidermal regeneration of partial-thickness skin wounds in vivo is probably a result of TGF-\beta stimulation of epithelial cell migration.

EGF has been studied more extensively than any other peptide growth factor for effects on corneal cells and corneal wound healing. EGF was reported to be a potent mitogen for all three types of corneal cells and to enhance healing of epithelial wounds, stromal incisions, and endothelial injuries.\textsuperscript{1-6,8-10,37} Our results suggest that EGF could stimulate both migration and mitosis of corneal cells during wound healing. Kitazawa et al\textsuperscript{38} reported that the acceleration of rabbit epithelial healing observed with topical EGF was associated with a twofold increase in epithelial cell division. Watanabe et al\textsuperscript{49} reported that EGF and fibronectin each stimulated the migration of rabbit corneal epithelial cells along the edges of corneal blocks in organ culture. In a subsequent study, they reported that EGF did not stimulate the chemotaxis of cultured rabbit corneal epithelial cells using a modified Boyden chamber, whereas fibronectin stimulated chemotaxis, haptotaxis, and chemokinesis.\textsuperscript{39} Soong et al\textsuperscript{48} also reported that EGF enhanced the epithelial wound closure rate of organ-cultured rat corneas but concluded that the effect of EGF was caused primarily by stimulation of proliferation rather than individual cell motility. This conclusion was based on their observations that EGF failed to stimulate the migration of rat and rabbit corneal epithelium using Boyden chambers and agarose drop studies and that addition
of colchicine, an antimitotic agent, blocked the effect of EGF on organ-cultured corneas. Recently, Nickoloff et al.\(^{40}\) reported that EGF stimulated the migration of normal human keratinocytes in a modified Boyden chamber assay, demonstrating that EGF was chemotactic for skin epithelial cells.

It is not clear why EGF induced chemotaxis of human and bovine corneal epithelial cells but did not stimulate chemotaxis of rat and rabbit corneal epithelial cells using Boyden chambers.\(^{16,39}\) It is possible that rat and rabbit epithelial cells respond differently to EGF than human and bovine epithelial cells. It is more likely that differences in methods resulted in different results. We found that several factors were crucial for demonstrating EGF stimulation of epithelial cell migration. Specifically, the duration of migration had to be extended to 12 hr for epithelial cells. Dissociation of epithelial cell cultures required repeated brief exposures to low concentrations of trypsin and EDTA. Membranes had to be coated with extracellular matrix components and had to have pores of 10 \(\mu\)m in diameter. Only primary cultures could be used successfully for migration experiments. Watanabe et al.\(^{39}\) used a 3-hr migration period with BSA coating of the membrane. In the study by Soong et al.,\(^{16}\) the ability of the rat and rabbit epithelial cells to respond to EGF or other general chemotactic factors was difficult to evaluate because a positive control demonstrating the migratory response of the rat and rabbit corneal epithelial cells was not included.

We have demonstrated that EGF, FGF, and TGF-\(\beta\) stimulate chemotactic migration of human and bovine corneal epithelium, fibroblasts, and endothelium in vitro. These studies further support the concept that peptide growth factors play important physiologic roles in modulating the cellular response of the cornea to wounding.

**Key words:** cornea, chemotaxis, EGF, FGF, TGF-\(\beta\)

**References**


