Basic Fibroblast Growth Factor Stimulates Corneal Endothelial Cell Growth and Endothelial Wound Healing of Human Corneas

Vincent P. T. Hoppenreijs,* Elisabeth Pels,* Gijs F. J. M. Vrensen,* and W. Frits Treffers†

Purpose. To determine the dose response of human recombinant basic fibroblast growth factor (bFGF) on mitogenic activity, and the supplementary role of serum in cultured bovine and human corneal endothelial cells (BCECs, HCECs). To investigate the effect of bFGF on endothelial wound healing of human corneas in vitro.

Methods. In cell culture, DNA synthesis was assessed by ³H-thymidine incorporation. Wound healing was studied using paired human corneas after mechanical damaging of the endothelium. One cornea was treated with bFGF, and the mate served as control. Wound closure was determined after staining with trypan blue. Endothelial cell density (ECD) was assessed in the closed wound area after alizarin red staining. DNA synthesis was assessed using ³H-thymidine autoradiography.

Results. In cell culture, bFGF induced a dose-dependent mitogenic response on BCECs and HCECs. Addition of serum to the culture medium shifted the dose–response curve to considerably lower bFGF concentrations. In organ culture, the time of complete wound closure shortened only marginally (0.5 day) after bFGF treatment (P < 0.01). In the closed wound center, ECD was significantly higher in 1 ng/ml bFGF-treated corneas (686 ± 134 cells/mm²) than in controls (554 ± 117 cells/mm²), an increase of +25%. Doses of 0.1 and 10 ng/ml also were effective, but less so than with 1 ng/ml (+11% and +15%, respectively), whereas a dose of 100 ng/ml even had a negative effect (−11%). DNA synthesis was marginally enhanced in bFGF-treated (1 ng/ml) corneas.


Corneal endothelial cells are essential for maintaining corneal transparency. Inadequate healing of endothelial injuries caused by trauma, surgery, or disease can lead to excessive stromal hydration and corneal clouding. The limited regenerative ability of endothelial cells of human corneas after injury or surgical intervention (eg, cataract extraction) may necessitate transplantation of a donor cornea that possesses a functional endothelium. The potential regeneration of the endothelial layer in vivo varies among species, and is a complex process involving enlargement, migration, coalescence, and mitosis.12 There is evidence that growth factors and their receptors, acting in an autocrine or paracrine way, play a key role in the regulation and stimulation of healing of the corneal endothelial layer.

There also is evidence that basic fibroblast growth factor (bFGF) plays a role in stimulating and maintaining corneal endothelial cell density and functioning. In vitro, bFGF acts on a wide variety of cells of neuroectodermal and mesodermal origins, and influences cell growth, migration, differentiation, regeneration, neovascularization, and senescence.5–8 bFGF, a 16- to
25-kD protein, belongs to the family of FGFs known as heparin-binding growth factors. Numerous studies document the ability of FGF to stimulate proliferation of corneal epithelial cells, stromal fibroblasts, and endothelial cells in vitro, in cell and organ culture systems. FGF also has been reported to enhance healing of rabbit corneal epithelium, and corneal endothelium of cats and rabbits in vivo. Because most FGF activity in the eye can be attributed to bFGF, which is 30- to 100-fold more potent than acidic FGF, bFGF was chosen for this study.

In most studies of FGF, unpurified “FGF” from natural sources, such as bovine brain or human placenta, was used, and there is some controversy over the optimal dose of the growth factor. In the current study, the dose–response effect of recombinant-derived human bFGF on the mitogenic activity of bovine (BCECs) and human corneal endothelial cells (HCECs) in cell culture was studied. The effects of serum concentration on the dose–response curve are shown.

Finally, bFGF stimulation of endothelial wound healing of human corneas preserved in organ culture medium was investigated. The effects of bFGF on wound closure, cell area, cell density, and cell shape, and on DNA synthesis in the wound area, were determined. Because heparin or heparin-like molecules, such as heparan sulfate proteoglycans (HSPG), appear to be required for bFGF high-affinity receptor binding, the role of heparin in the bFGF-induced effects on human corneas also was studied.

MATERIALS AND METHODS

Tissue Culture

BCECs were prepared and kept in culture as previously described by Yue and Baum. Briefly, eyes from young and adult cattle were obtained from the local slaughterhouse. Bovine corneal buttons were prepared within 2 to 3 hours after enucleation. Explants of the endothelium and Descemet’s membrane were placed in culture medium in 25-ml tissue-culture flasks (Nunc, Roskilde, Denmark). The culture medium consisted of Eagle’s minimum essential medium (EMEM), 10% fetal bovine serum (FBS) (EMEM and FBS; ICN Biomedicals Inc., Costa Mesa, CA), 100 U/ml penicillin (Gist-Brocades, Leiderdorp, The Netherlands), and 50 μg/ml streptomycin (Biochemie GmbH, Vienna, Austria). Cultures were maintained in a moist incubator at 37°C with 5% CO2 and 95% air. Growing between the fifth and tenth passage were used.

HCECs were collected according to Lass et al the corneal endothelium from eyes of human donors (age range, 20 to 50 years) was incubated with a solution of trypsin-EDTA at 37°C. The detached cells were allowed to adhere in culture medium enriched with 1.35% chondroitin sulfate (Sigma Chemical Company, St. Louis, MO). The culture medium consisted of L-valine EMEM (GIBCO, Grand Island, NY), 1% nonessential amino acids (ICN Biomedicals), 0.011% pyruvic acid (Sigma), 0.001% insulin, 10 ng/ml human epidermal growth factor (hEGF; Sigma), 10% FBS, and antibiotics (see above). After 1 day, the cells were settled. The culture medium was replaced three times a week. Owing to the limited availability of eyes of human donors for experimental purposes, and to the restricted number of subcultures reached (maximally four to five), HCECs were not available as continuous cell lines. Therefore, a restricted number of experiments could be carried out with these cells.

The measurement of DNA synthesis by 3H-thymidine incorporation was used to determine whether BCECs and HCECs responded to bFGF in a dose-dependent manner. Cells were removed from confluent cultures in tissue culture flasks by incubation with trypsin-EDTA solution for about 5 minutes, collected by centrifugation (200g, 5 minutes), resuspended in medium, counted, and plated out in basal medium at 1 X 10⁴ cells per well in 96-well, flat-bottomed tissue culture plates (Nunc) (day 0). Basal culture medium consisted of EMEM, 2% FBS, and antibiotics (see above). For HCECs, the basal medium was enriched with chondroitin sulfate during the first day only (day 0), to stimulate the adherence. The cells were cultured at 37°C in humidified 5% CO2 and 95% air. After 24 hours, basal or experimental medium was added (day 1). Twenty-four hours after addition of the experimental medium to the 96-well plates (day 2), cells were labeled with methyl-3H-thymidine (specific activity 15 Ci/mmol, 2.5 μCi/ml medium; New England Nuclear Research, Boston, MA) for 24 hours. After this period, the cells were detached by trypsinization and harvested with a cell harvester (day 3). Tritiated thymidine incorporation was measured by liquid scintillation counting. Using this technique, pilot experiments showed that unincorporated 3H-thymidine amounted to about 10% of the total 3H-thymidine measured (data not shown).

The dose–response effect of bFGF was studied using 10⁻⁵ to 500 ng/ml human recombinant bFGF (> 95% purity; Sigma) in basal medium without (serum free) and with 2% FBS (for details, see Figures). In a second experiment with BCECs, the effect of different concentrations of FBS (range, 0% to 40%), with or without 1 ng/ml bFGF, was determined. All assays were repeated at least four times in hexaplicate.

Basic FGF-induced enhancement of ³H-thymidine incorporation can be due to increased DNA synthesis, increased ³H-thymidine uptake, or both. To investigate whether bFGF increased ³H-thymidine incorpora-
tion and/or $^3$H-thymidine uptake, the mitotic activity of BCECs was inhibited by 5-fluorouracil (5-FU), mitomycin-C, or X-irradiation, as described previously.32

**Organ Culture: Human Corneas**

Paired human corneas, donated for transplantation to the EuroTransplant Foundation (Leiden, The Netherlands) but discarded by the Cornea Bank (Amsterdam, The Netherlands) because of scars, low endothelial cell density (ECD; $1500 < ECD < 2000$ cells/mm$^2$), or irregularity of the endothelium (polymegethism), were used. The corneas had an average ECD of $2350 \pm 280$ cells/mm$^2$ (range, 1700 to 3100). Corneas with pleomorphic, extreme polymegetic, or diseased endothelium were excluded from this study. Paired corneas were used to overcome interindividual variation in wound closure and morphometric aspects. A total of 89 pairs of corneas were used. Donor ages ranged from 51 to 95 years (mean, $75 \pm 10$ years). The interval between death and enucleation and between enucleation and corneal dissection averaged $6.9 \pm 3.4$ hours and $15.2 \pm 5.8$ hours, respectively. The onset of the experiments was always within 1 day after preparation of the corneoscleral button for the morphometric study, and within 2 days for the autoradiographic study.

**Wounding Procedure.** A central endothelial lesion was made mechanically (Fig. 1), as previously described.23 With this method, the Descemet's membrane was not injured. The wound sizes among pairs of corneas varied considerably, from $4.5$ to $7.5$ mm$^2$ (mean, $6.0 \pm 0.9$ mm$^2$). Within each pair of corneas, however, the initial wound sizes were about the same (average difference in initial wound size: $2.7\%$). The corneas with the larger wound size were randomly distributed among the untreated and treated groups.

After injury, the corneas were placed in 10 ml of sterile EMEM supplemented with 5% Dextran T500 (Pharmacia, Uppsala, Sweden), 2% FBS, and antibiotics at $31^\circ$C (see above), according to the Dutch organ culture system for preservation of human corneas.34 To the culture medium of one cornea of each pair, 0.1, 1, 10, or 100 ng/ml recombinant bFGF was added, whereas the mate, incubated in the absence of bFGF, served as control. The effects of bFGF on wound closure, morphometric parameters (ECD, cell shape), and cell division in the closed wound center were determined. The data presented are from corneas treated with 1 ng/ml of bFGF unless stated otherwise. The culture media, with or without growth factor, were replaced every day except in the autoradiographic study, in which the medium was replaced every other day.

The influence of heparin on wound closure and morphometric parameters of bFGF-treated HCECs was investigated. In this experiment, both corneas of each pair were treated with 1 ng/ml bFGF, while one cornea of this pair also was incubated with 500 ng/ml heparin (Leo Pharmaceuticals, Weesp, The Netherlands). This concentration of heparin appeared to be the optimal dose based on experiments performed in the cell culture system (data not shown). To determine this optimal dose, two proliferation assays, the DNA synthesis assay and the MTT assay, were used as described previously.32 Briefly, heparin was tested in a range of concentrations of 0 to 10,000 ng/ml. BCECs were seeded on day 0. The next day, the cell cultures were incubated with control (0 to 10,000 ng/ml heparin) or experimental medium (0 to 10,000 ng/ml heparin + 10 ng/ml bFGF; day 1). The DNA assay and MTT assay, described previously,32 were then used. It was found that 500 ng/ml heparin was the optimal dose for stimulating the bFGF effect (data not shown).

**Longitudinal Study of Wound Closure.** The endothelial side of the wounded corneas was stained daily with trypan blue, as described previously.2 A pilot study showed that trypan blue did not affect cell migration (data not shown). The wound margin was outlined directly by means of a drawing tube, and the wound area was measured using an MOP-Videoplan Image Analysis System software package, developed by Kontron.
Bildanalyse (Munich, Germany). To evaluate the effect of bFGF on wound closure, the wound area was plotted versus time for each pair of corneas. The wound closure time was defined as the day at which Descemet’s membrane no longer stained with trypan blue, and was fully covered by endothelial cells.

**Morphometric Analysis.** The morphometric analysis method has been described in detail previously. Briefly, after the wounds had closed, the endothelial cell borders and nuclei of the corneas were stained with alizarin red and trypan blue (Fig. 2). After preparing a flat, wet endothelial specimen, the endothelium in the wound center of each cornea was photographed. The photographed cells were outlined in two randomly selected fields, as described previously. The Videoplan software package was used to measure cell area, perimeter (PM), and maximal diameter (Dmx). The ECD, expressed as the number of cells per square millimeter, was calculated from the mean cell area (μm²). The coefficient of variation of area (CVarea) was calculated by dividing the standard deviation (SD) of the area by the mean cell area, and was expressed as a percentage. This index provides a quantitative parameter of variation in cell area (polymegethism). Cell shapes were described by the shape factor PM/Dmax, which describes the degree to which a cell has elongated: a shape factor below 3.0 is an indication of elongated cell shape. The coefficient of the shape factor (CVshape factor) describes the variance in cell shape (SD/mean shape factor). Shape factor and CVshape factor were used to describe the degree of cellular symmetry or regularity (a measure of pleomorphism).

Morphometric analysis was performed on paired corneas obtained at two postwounding times, at the time of wound closure (between 4 to 8 days; 60 pairs), and 15 days after infliction of the wound, corresponding to about 7 to 11 days after wound closure (10 pairs). The same corneas were used to determine the wound closure times.

**Autoradiography.** Nineteen pairs of corneas were cultured for 6 to 7 days in medium containing 3H-thymidine (New England Nuclear Research; specific activity 15 Ci/mmol, 1 μCi/ml medium; continuous labeling) with or without bFGF. After incubation, the corneas were stained with alizarin red, fixed between two glass slides in formaldehyde 3.8% for 6 hours, dried for 15 hours, and mounted on gelatinized slides. The specimens were autoradiographically processed as described previously. All corneal wounds proved to be closed. The total number of labeled nuclei in the wound area was counted for each specimen. The percent change in labeling between both groups was calculated only for corneas in which more than ten labeled nuclei were observed in the wound area.

**Statistical Analysis**

Differences in cell culture parameters were statistically tested by analysis of variance. For the organ culture study, the differences in wound closure (50% and 10% level, time of wound closure), morphometric parameters (ECD, cell shape, CVarea, CVshape factor), or labeled nuclei between the corneas of one pair were computed, ranked, and statistically tested with the Wilcoxon’s signed-rank test (two-tailed) for paired observations. The means ± SDs of the entire untreated and treated groups were not used for statistical testing; differences in parameters were determined between both corneas of one pair. Differences between groups of two different experiments were statistically tested using the Wilcoxon’s two-sample test (two-tailed). For correlations, we used the Spearman’s rank correlation coefficient (rs, two-tailed). Data given in the text are mean ± SD. In the Figures, either SD or SEM are indicated. A P-value of 0.05 was considered to be significant.
RESULTS

Tissue Culture: Dose-Response Data of bFGF and Serum Dependency

Dose-response data indicated that bFGF significantly stimulates BCEC incorporation of \(^\text{3H}\)-thymidine (Fig. 3). Using serum concentrations ranging from 0.5% to 5% FBS, bFGF was effective at levels as low as 0.1 ng/ml. The half-maximal response was reached at 0.1 ng/ml, a plateau was observed between 1 and 5 ng/ml, and at higher levels of bFGF the effect decreased. The dose-response curves obtained with 0.5%, 1%, 2%, and 5% serum were similar. Because the organ culture medium (human corneas) contains 2% serum, only the dose-response curve obtained with 2% serum is shown in Figure 3. The dose-response curves obtained with serum-free medium (0% FBS) showed that the peak concentration was reached at 100 to 250 ng/ml. In this case, doses from 0 to 10 ng/ml did not stimulate BCEC DNA synthesis. In both situations (ie, with or without serum), bFGF enhanced BCEC DNA synthesis about 2- to 2.5-fold.

Different concentrations of FBS (0% to 40% FBS) were tested in the absence or presence of 1 ng/ml bFGF for their ability to stimulate cell proliferation (Fig. 4). Without bFGF, FBS significantly increased DNA synthesis in a dose-dependent manner; a threefold to eightfold increase in DNA synthesis compared to serum-free control cultures was observed. Addition of 1 ng/ml bFGF further increased the BCEC incorporation of \(^\text{3H}\)-thymidine compared to the corresponding control samples, as long as serum was present. The maximal BCEC mitogenic response to bFGF occurred with 2.5% and 5% FBS.

Dose-response data with HCECs showed that 0.1 and 1 ng/ml bFGF significantly stimulated DNA synthesis in medium containing 2% FBS (Fig. 5). The maximal response, however, was significantly higher in BCECs than in HCECs.

To find out whether bFGF-induced enhanced \(^\text{3H}\)-thymidine labeling was due to enhanced DNA synthesis, enhanced \(^\text{3H}\)-thymidine uptake, or both, cell cultures were pretreated with mitotic inhibitors (5-fluorouracil, mitomycin-C) or were irradiated. It was found that, in mitotically inhibited cultures (MIC), the total \(^\text{3H}\)-thymidine labeling was about 10% of the total \(^\text{3H}\)-thymidine labeling seen in mitotically competent cultures. In MICs, the total \(^\text{3H}\)-thymidine labeling was not significantly different between untreated and bFGF-treated cultures (data not shown).
FIGURE 5. Stimulation of DNA synthesis of HCECs by bFGF (\(^{3}H\)-thymidine incorporation). Cells were incubated in medium containing 2% FBS and various concentrations of bFGF. Results of test conditions are expressed as the percentage of radioactivity incorporated into control cultures. Each point represents the mean ± SEM of two experiments (six wells per experiment). FGF significantly enhanced DNA synthesis at concentrations of 0.1 and 1 ng/ml.

In both subconfluent and confluent BCEC cell cultures, a difference in morphologic appearance was observed with phase-contrast microscopy between untreated and bFGF-treated cultures (Fig. 6). In control cultures, a thin cell layer with a polygonal pattern was found. In the presence of bFGF, the cells had a more bloated, more elongated, fibroblast-like aspect, with long pseudopodia, and cell-to-cell contacts were less frequent. Despite the increased mitotic activity in bFGF-treated cultures compared to control cultures, a complete confluent monolayer was reached in a later phase in the bFGF-treated cultures than in the control cultures. At confluence, a more compact monolayer was observed under phase-contrast microscopy. These results were confirmed by separate experiments; at confluence, the number of cells appeared to be higher (about 1.5-fold) in the bFGF-treated wells than in the control wells (data not shown). The change in morphologic appearance of BCECs after bFGF treatment was also observed in mitotically inhibited BCECs (data not shown).

Organ Culture: Human Corneas

Wound Closure. To evaluate the effect of bFGF treatment on wound closure, the wound area was plotted against time for each pair of corneas. Figure 7 combines the wound closure data of both groups (22 pairs), and shows a large biologic variation in wound closure, as indicated by the large SDs. Wound closure occurred between 4 and 8 days. When bFGF was present, the mean estimated wound closure time was 5.5 ± 0.8 days, compared to 6.0 ± 0.8 days in untreated controls (P < 0.01).

The mean wound area was significantly lower in the bFGF-treated group than in the control group..
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FIGURE 7. Wound closure rates of 22 paired human corneas. The mean wound area, expressed as a percentage of the initial wound area, is plotted versus time for all control and bFGF-treated corneas. Mean initial wound area is 6.0 ± 0.9 mm². Each point represents mean ± SD. For each point in time, the number of paired corneas ranged from 17 to 22. The 10% level, which is the time at which 10% of the initial wound area was left, was reached significantly earlier in the bFGF-treated corneas than in the control corneas (P < 0.01). bFGF shortened wound closure significantly, by 0.5 day (P < 0.01).

from day 3 onward, although the differences were marginal (P < 0.01). Because the shape of the wound closure curves for different corneas was not uniform, one value for inclination of the curves, and thus wound closure rate, could not be determined. Hence, for each pair we also determined the times at which 50% and 10% of the initial wound area was left (Fig. 7). In contrast to the 50% level, the 10% level was reached significantly earlier in the bFGF-treated group (mean 4.3 days) than in the control group (4.6 days) (P < 0.01). Treatment of corneas with 0.1 (12 pairs), 10 (10 pairs), or 100 ng/ml (11 pairs) did not increase wound closure.

Morphometry. Morphometric analysis was performed at the time of wound closure and 15 days after wounding (Fig. 2). The morphometric data are listed in Table 1.

At Wound Closure. In the closed wound center, the ECD was higher in all 12 corneas exposed to bFGF compared to control corneas, at a bFGF concentration of 1 ng/ml (P < 0.01). The increase in ECD in the treated corneas of each pair averaged 132 ± 69 cells/mm² (range, 32 to 263), or, given as percentage of the control corneas, 25% ± 13% (range, 5% to 45%). Treatment of corneas with 0.1 (12 pairs) and 10 ng/ml (10 pairs) bFGF resulted in a lower increase in ECD compared to 1 ng/ml (Fig. 8). A dose of 100 ng/ml significantly reduced the ECD (11 pairs) (Fig. 8). At all dosages of bFGF tested, the mean CV_area, the shape factor of the cells in the wound center, and the CV_shape factor were not significantly different between the treated and control groups. In contrast to the results found with cell-cultured BCECs, no differences in morphologic appearance of the cells were observed between control and bFGF-treated corneas.

In experiments on the effects of heparin on bFGF-treated corneas, the ECD of corneas exposed to bFGF and heparin (746 ± 168 cells/mm²) did not differ from that of their mates exposed to bFGF alone (750 ± 195 cells/mm²; n = 15 pairs). In addition, the other parameters (shape factor, CV_area and CV_shape factor) were not significantly different between both groups (data not shown).

Fifteen Days After Wounding. Fifteen days after wounding, corresponding to about 7 to 11 days after wound closure, all ten corneas cultured in the presence of bFGF had higher ECDs than their mates cul-

TABLE 1. Morphometric Analysis of Human Corneal Endothelial Cells After Wounding

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>bFGF-Treated Group</th>
<th>P Value*</th>
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<tbody>
<tr>
<td>At the time of wound closure (12 pairs)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECD (cells/mm²)</td>
<td>554 ± 117 (367-784)</td>
<td>686 ± 134 (442-890)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CV_area (%)</td>
<td>45 ± 5 (37-55)</td>
<td>47 ± 6 (36-56)</td>
<td>NS</td>
</tr>
<tr>
<td>Shape factor (PM/D_max)</td>
<td>2.57 ± 0.04 (2.50-2.64)</td>
<td>2.55 ± 0.04 (2.50-2.64)</td>
<td>NS</td>
</tr>
<tr>
<td>CV_shape-factor (%)</td>
<td>8.4 ± 0.7 (7.5-9.6)</td>
<td>8.8 ± 0.7 (7.2-9.8)</td>
<td>NS</td>
</tr>
<tr>
<td>15 days after wounding (10 pairs)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECD (cells/mm²)</td>
<td>556 ± 109 (394-783)</td>
<td>698 ± 158 (504-1052)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CV_area (%)</td>
<td>50 ± 8 (39-66)</td>
<td>54 ± 7 (44-68)</td>
<td>NS</td>
</tr>
<tr>
<td>Shape factor (PM/D_max)</td>
<td>2.67 ± 0.16 (2.53-3.10)</td>
<td>2.67 ± 0.18 (2.55-3.15)</td>
<td>NS</td>
</tr>
<tr>
<td>CV_shape-factor</td>
<td>7.5 ± 0.8 (6.8-8.7)</td>
<td>7.5 ± 1.0 (6.0-9.4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

For the calculation of the ECD, variation of the mean cell area (CV_area), shape factor, and the coefficient of the shape factor (CV_shape-factor): see Materials and Methods. Treatment: 1 ng/ml bFGF. Values are mean ± SD (range).

* Wilcoxon's signed-rank test.
FIGURE 8. The effects of bFGF on ECD in wounded human corneal endothelium. After wounding, one cornea of each pair was treated with 0.1 ng/ml (12 pairs), 1 ng/ml (n = 12), 10 ng/ml (n = 10), or 100 ng/ml (n = 11) bFGF, while the mate served as control. The increase in ECD in the treated group is expressed as a percentage of the control group. At doses of 0.1, 1, and 10 ng/ml bFGF, the mean ECD was significantly higher compared to the ECD of the control corneas (P < 0.01). The ECD in the bFGF-treated group was significantly lower at a dose of 100 ng/ml (P < 0.01). Values are mean ± SEM.

Autoradiography. Labeling of endothelial cells with ³H-thymidine was observed in all 19 paired corneas. The variation in ³H-thymidine incorporation between pairs of corneas was dramatic, however, varying from a few to hundreds of nuclei. The labeled nuclei were scattered throughout the wound area (Fig. 9). The number of labeled nuclei in both groups significantly decreased over the age ranges (51 to 91 years) studied (r = -0.553, 0.01 < P < 0.02). The number of radioactive nuclei counted in the wound center was significantly higher in the bFGF-treated group (mean, 300 ± 290) than in the untreated group (254 ± 243; P < 0.01). The average increase in labeled nuclei in the treated group amounted to 7 ± 14 nuclei/mm² (range, -8 to 53) or 19% ± 25% (range, -16% to 66%). Using the mean ECD “at the time of wound closure” (Table 1), however, the labeled cells in both groups made up

FIGURE 9. Autoradiograph of a human cornea 6 to 7 days postwounding after continuous ³H-thymidine labeling. The transition from the periphery (p), with small cells, to the wound area (w), with large, irregular cells, is shown. Labeled nuclei are restricted mainly to the wound area proper. Arrowheads indicate abnormal nuclei, possibly representing amitosis. Cell borders are stained with alizarin red.
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about 7% ± 7% of all cells in the wound area. No correlations could be found between the increase in labeling in the bFGF-treated group and the number of nuclei in the control corneas, or between the increase in labeling by bFGF and donor age.

Previously, we tested the effects of two other growth factors, hEGF and platelet-derived growth factor (PDGF, dimer BB), on the endothelial wound healing of human corneas using the same wound healing model. The results of these two growth factors were compared with those of bFGF (Table 2).

DISCUSSION

Cell Culture System

Numerous studies have reported the ability of bFGF to stimulate proliferation of corneal endothelial cells — bovine,2–11 rabbit,12,13 primate,14 and human15–19 — in vitro. Although many studies were performed with “FGF,” most of this FGF was neither of recombinant origin nor purified to homogeneity. The effects of specific growth factors of the FGF family in this case bFGF, could be investigated only after its characterization in the mid-1980s. In our study, recombinant-derived bFGF induced dose-dependent mitogenic responses in BCECs. The maximal effective concentration of bFGF for stimulating BCEC cell division turned out to depend on the presence or absence of serum in the medium. The peak concentration was reached at a lower level in serum-containing media (0.5% to 5% FBS) than in a serum-free medium (1 to 5 and 100 to 250 ng/ml, respectively). The results indicate that bFGF is more potent in serum-containing medium than in serum-free medium. This finding explains the different optimal concentrations of purified bFGF reported in other studies, about 150 to 200 ng/ml in serum-free medium,9,28 versus 0.1 to 10 ng/ml in serum-containing medium.10,13,18 The explanation for the shift in dose-response curve is not clear; the phenomenon may be caused by a number of factors. For example, it is possible that bFGF acts synergistically with serum components; that serum proteins act as carriers for bFGF, and, therefore, are involved in the regulation of bFGF bioavailability; or that serum alters the production or release of bFGF by endothelial cells or Descemet’s membrane.

Table 2. The Influence of Growth Factors on the Endothelial Wound Healing of Human Corneas

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Shortening of Wound Closure (Days)</th>
<th>Increase in ECD (Cells/mm²)</th>
<th>Increase in DNA Synthesis (Nuclei/mm²)</th>
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<tbody>
<tr>
<td>hEGF (n = 14)</td>
<td>1.1</td>
<td>257 (or 52%)</td>
<td>11 (or 32%)</td>
</tr>
<tr>
<td>PDGF-BB (n = 15)</td>
<td>0.8</td>
<td>222 (or 44%)</td>
<td>9 (or 26%)</td>
</tr>
<tr>
<td>bFGF (n = 12)</td>
<td>0.5</td>
<td>132 (or 25%)</td>
<td>7 (or 19%)</td>
</tr>
</tbody>
</table>

The effects of three growth factors, human epidermal growth factor (hEGF; 10 ng/ml), PDGF, dimer BB (10 ng/ml), and basic fibroblast growth factor (bFGF; 1 ng/ml) were tested on the endothelial wound healing of human corneas. Three parameters were studied: the time of wound closure, the ECD, and the DNA synthesis in the closed wound center. All growth factors caused a faster wound closure, an increase in ECD, and an increase in DNA synthesis (P < 0.01). The percent change in ECD and DNA synthesis between control and treated corneas is shown in parentheses. Values are the mean of the indicated number of paired corneas.

TABLE 2. The Influence of Growth Factors on the Endothelial Wound Healing of Human Corneas

Serum may contain many growth factors; that the serum used contains bFGF cannot be ruled out. Unambiguous data demonstrating the presence of any FGF in serum do not exist.3 Even if serum contains bFGF, it is not likely that a serum-induced change in the actual total bFGF concentration in the medium will cause a shift in the dose-response curve of about 10- to 100-fold. If serum increases the basal level of bFGF, a shift in the curve would be expected on a normal scale rather than on a logarithmic scale. In addition, when the dose–response curves were measured with different serum concentrations, ranging from 0.5% to 5%, the curves were about the same. Thus, although it cannot be ruled out that serum may alter the amount of bFGF, either because it contains bFGF itself or by altering the release of bFGF by cells or Descemet’s membrane, our results suggest that serum alters the bFGF action rather than the total amount of bFGF in the medium.

In our experiments, heat-inactivated serum was used. With this kind of serum, the shift in curves favors a passive role of serum in altering bFGF bioavailability rather than a process mediated by active biochemical factors in serum.

Compared to BCECs, the effect of bFGF on the growth of HCECs in tissue culture was small. In serum-containing medium, the optimal dose of bFGF for HCECs was found to be 1 ng/ml.

Another finding of this study was that the maximal bFGF-induced effect on BCEC DNA synthesis depends on the serum concentration used (Fig. 4). The maximal effect of bFGF occurred with a FBS concentration of 2.5% to 5%. These serum-dependent effects on DNA synthesis were also found with hEGF (data not shown) and PDGF.32 In contrast to PDGF, however, hEGF and bFGF were able to stimulate the proliferation of tissue-cultured BCECs in serum-free medium. FGF, as well as serum, which contains many growth factors and hormones, may stimulate proliferation of BCECs, at least in part by induction of the c-fos gene and its nuclear protein.39

Because unincorporated 3H-thymidine is scarcely measured with the technique used here, and bFGF did not increase total 3H-thymidine labeling in MICs, the
bFGF-induced increase in \(^{3}\)H-thymidine labeling was primarily due to enhanced DNA synthesis rather than enhanced \(^{3}\)H-thymidine uptake. The same results were found with another growth factor, PDGF.\(^{32}\)

**Organ Culture System**

Data demonstrate that a bFGF-like molecule normally exists in the aqueous humor of human, cat, dog, and pig eyes.\(^{25,40,41}\) It also was found that BCECs contain bFGF gene transcripts,\(^{8,42}\) that HCECs produce mRNA coding for bFGF,\(^{43,44}\) that bFGF protein is produced and secreted by corneal endothelial cells,\(^{13,42,45}\) and that FGF receptors are present on corneal endothelial cells.\(^{13,18,19,44,46}\) FGF can be extracted from the extracellular matrix deposited by rabbit and bovine corneal endothelial cells in tissue culture,\(^{13,45}\) and it is a physiological component of corneal basement membranes, including Descemet’s membrane.\(^{13,47}\) These findings, along with the mitogenic effects of bFGF on tissue-cultured cells, suggest that bFGF may influence endothelial wound healing in human corneas. The continued expression of bFGF mRNA by tissue-cultured HCECs with proliferative as well as senescent morphology\(^{48}\) also may suggest that the endothelial cells of senior donor corneas respond to exogenous bFGF. These suggestions were confirmed by our study, in which an effect of bFGF on wounded corneal endothelium of senior corneas was found. The main finding of the organ culture study was that the ECD in the closed wound area was higher in the bFGF-treated corneas than in the control corneas, studied both at the time of wound closure and 15 days after wounding. An increase in ECD also was found after post-wound injection of FGF into the anterior chamber of cats\(^{25}\) and rabbits.\(^{54,48}\) FGF, however, did not appear to increase the ECD in disseminated endothelial damage induced by NaCl in pig eyes.\(^{49}\) Except in the study by Rieck et al,\(^{24}\) the FGF preparations used in these studies were not of recombinant origin (rather, they were extracted from pituitary glands or placenta), nor were they purified to homogeneity. In our study, a dose-dependent effect of bFGF on the ECD was found. Doses of 0.1, 1, and 10 ng/ml caused significant increases in ECD of 11%, 25%, and 15%, respectively. A dose of 100 ng/ml, however, caused an unexpected decrease in ECD of 11%. This decrease in ECD in the treated group cannot be explained only by receptor downregulation, but suggests a toxic effect with high-dose bFGF. Our results demonstrate that bFGF stimulates wound repair in human corneal endothelium when the cells are maintained on their normal substrate, Descemet’s membrane. Because endogenous FGF is a physiological component of Descemet’s membrane,\(^{13,47}\) and is synthesized by corneal endothelial cells,\(^{8,42-44}\) it is possible that injury of the corneal endothelium or denudation of Descemet’s membrane may release bFGF into the culture medium. Therefore, it is possible that the observed increase in ECD is underestimated owing to the presence of endogenous FGF.

The higher ECD in closed wounds of the bFGF-treated human corneas may be the result of an increase in cell division or cell migration. Although many studies reported that replication of human and animal corneal endothelial cells in tissue culture can be stimulated by FGF,\(^{7-10}\) it was not known whether bFGF regulates the mitotic activity of HCECs in situ as well. The autoradiographic study shows that in the healed wound area, the total number of labeled nuclei was statistically higher in the bFGF-treated group than in the untreated group. In most of the bFGF-treated corneas, however, the absolute increase in ECD (mean, 132 cells/mm\(^2\)) cannot be explained by the limited stimulation of cell division indicated by the small increase in labeled nuclei per square millimeter (mean, 7 nuclei/mm\(^2\)). DNA synthesis does not always imply cell division. In addition, part of the increase in labeled nuclei in response to bFGF may be caused by the increase in ECD. In fact, when we related the number of labeled nuclei per square millimeter to the ECD of each group, it was found in both the control and treated groups that about 7% of the cells in the closed wound area were labeled. Thus, bFGF scarcely stimulates endothelial cell division in our human corneas. In the current study, however, corneas of senior donors were used, and the effect of bFGF on cell division may play a more important role in younger donors. In the animal studies that showed an FGF-induced increase in ECD after in vivo wound healing, the differential contribution of cell division and cell migration in the repair process was not determined.\(^{25,24}\) The capacity for cell division in lower mammals, such as rabbits, however, is much higher than that of humans, and thus it is likely that the bFGF-induced effects on ECD are significantly different between the human and the rabbit. During wound healing of cell-cultured cat corneal endothelial cells, bFGF enhanced the DNA synthesis.\(^{50}\)

Because in our study cell division was marginally stimulated by bFGF, cell migration induced by bFGF must play the most important role in the higher ECDs of the bFGF-treated group. bFGF enhanced wound closure in cell cultures of cat corneal endothelium.\(^{50}\) In organ-cultured bovine corneas, addition of crude FGF led to more rapid wound closure.\(^{51}\) In cats, bFGF stimulated a more completely healed endothelium 6 days after transcorneal freeze wounding in vivo.\(^{22}\) After mechanical wounding of rabbit corneal endothelium, a single intracameral injection of bFGF caused
smaller wound areas after 2 and 4 days. In human corneas, wound closure increased marginally, and only in the last phase of wound closure. Because wound closure increased only marginally, an increase in the number of migrating cells must be the most important effect of bFGF on ECD. The higher ECD in the treated corneas 7 to 11 days after wounding also favors an increased number of migrating cells. If the only factor was faster migration, a smaller or zero difference in ECD between untreated and treated groups would be expected 7 to 11 days after wound coverage. Thus, increased migration means an increased number of migrating cells, rather than a faster migration rate. Grant et al. found that bFGF can stimulate the migration of cultured corneal endothelial cells; using checkerboard analysis in modified Boyden chambers, they demonstrated that bFGF stimulated chemotaxis in HCECs and BCECs, as well as chemokinesis in BCECs.

Corneal endothelial wounding leads to polymegathism and pleomorphism, indicated by the CVarea, shape factors, and CVshape factor. After initial coverage of the wound area, cell rearrangement continues to take place in both control and bFGF-treated groups, as indicated by the increased shape factors and decreased CVshape factors of cells studied 15 days postwounding compared to cells studied at the time of wound closure. The CVarea, however, still was considerably high at 15 days postwounding. bFGF did not affect these parameters in the closed wounds. The morphologic appearance of the cells was not obviously different. In tissue-cultured BCECs, however, the morphologic appearance of cells changed after bFGF treatment; they had a more bloated and elongated aspect, and continuity of cell borders often was lost. Despite the increase in mitosis in treated cultures, a confluent monolayer was reached at a later point in time. At confluence in both groups, the saturation density was higher in the bFGF-treated group than in the control group. Polygonal morphology was not found in the treated group. This morphologic appearance of the treated group also was observed in mitotically inhibited BCECs, indicating that the bFGF-induced change in cellular shape did not depend on cell division. In rabbit corneal endothelial cells, a more elongated shape was found after treatment with bFGF in combination with heparin.

FGF interacts with specific, high-affinity cell surface receptors that possess intrinsic tyrosine kinase activity. A lower-affinity class of binding sites, the HSPGs, also has been identified (see ref. 27). The HSPGs are found on the cell surface of a wide variety of tissues, and in the extracellular matrices of cell cultures, including basement membranes. Binding of bFGF to its receptor requires prior binding either to the HSPGs on membranes or to free heparin. Although corneal endothelial cells synthesize and secrete HSPGs, it is unknown whether exogenous addition of heparin to cells can enhance the bFGF effect by functioning as a soluble form of low-affinity FGF receptor. In studies using human corneal endothelial cells, the necessity of adding heparin for bFGF activity is somewhat controversial. In addition, the heparin-enhanced bFGF effects obtained with our cell culture system (data not shown), and the low activity of bFGF observed in our endothelial wound healing model of human corneas, forced us to check the effects of exogenously added heparin on the bFGF-induced changes with respect to corneal endothelial wound healing. It was found that heparin did not enhance the effect of bFGF on ECD in the closed wound center in our human endothelial wound healing model.

After wounding, human corneal endothelium heals primarily by enlargement and migration, whereas cell division is thought to contribute relatively little to healing. Using the same endothelial wound healing model, the influence of three growth factors, namely hEGF, PDGF (dimer BB), and bFGF, have now been tested on wound closure, ECD, and DNA synthesis in the closed wound area. The results are summarized in Table 2. During the wound healing process, none of the growth factors was able to stimulate mitosis of HCECs on senior donor corneas to any important extent. Therefore, the higher ECD in the closed wounds found in growth factor-treated corneas is caused mainly by an increase in cell migration. The rank order of effectiveness of growth factors on wounded human corneal endothelium was found to be hEGF > PDGF-BB > bFGF.

The biologic importance of bFGF-induced changes with respect to corneal wound healing is not completely clear. With regard to endothelial wound healing, it was found that bFGF alone did not have very dramatic effects. Because cocktails of growth factors may act in an additive, synergistic, or even antagonistic way with respect to the mitogenic potential of a given individual growth factor, it is possible that bFGF may play a more important role in endothelial wound healing when combined with other growth factors. For example, bFGF increases the mitogenic potency of the PDGF isoforms by specific upregulation of the PDGF-α receptor in vascular smooth muscle cells. bFGF may play a more important role in maintaining cell physiology, or may have cellular functions other than stimulating wound healing.

Key Words

corneal endothelium, fibroblast growth factor, wound healing, autoradiography, morphometry
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