Effects of Platelet-Derived Growth Factor on Endothelial Wound Healing of Human Corneas

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

Purpose. To investigate the effects of platelet-derived growth factor (PDGF) on endothelial wound healing of organ-cultured human corneas.

Methods. The endothelia of paired human donor corneas (age, 71 ± 11 years; total 84 pairs) were mechanically wounded (area, 5.6 ± 0.8 mm²). Of each pair, one cornea was treated with 10 ng/ml human recombinant PDGF-BB while its mate served as control. The endothelial wound closure time was assessed by daily staining of the corneas with trypan blue. Morphometric data (endothelial cell density, shape, coefficient of variations) were obtained in the wound area after alizarin red staining. DNA synthesis was assessed using ³H-thymidine autoradiography.

Results. Although significant, the time of complete wound closure shortened only marginally on addition of PDGF to the culture medium. In the closed wound center (between 4 and 9 days), all corneas exposed to PDGF had significantly higher endothelial cell densities (737 ± 126 cells/mm²) than the control corneas (515 ± 89 cells/mm²). Fifteen days after wounding, the mean endothelial cell density averaged 526 ± 93 and 708 ± 135 cells/mm² in the control and PDGF-treated groups, respectively. PDGF did not affect the final cell shape within the closed wounds. DNA synthesis was significantly but only marginally enhanced in PDGF-treated corneas.

Conclusion. In organ-cultured human corneas, PDGF-BB promotes endothelial wound healing predominantly by cell migration, at least in corneas from senior donors. Invest Ophthalmol Vis Sci. 1994; 35:150-161

Human corneal endothelium has only a limited ability to self-regenerate after injury, intraocular surgery, or during age-related impairment. Because a proper endothelial function is essential for corneal transparency, it would be advantageous to have growth factors available that could stimulate its regeneration. Platelet-derived growth factor (PDGF) is a potent mitogen for different connective tissue cells in culture, including fibroblasts, smooth muscle cells, and glial cells. In these cells, PDGF has not only been shown to stimulate growth, but also to elicit a motility response, including cytoskeletal reorganization and cell differentiation. There is evidence that PDGF acts not only on cells of mesenchymal, but also on cells of ectodermal origin such as corneal epithelial cells. PDGF was originally purified from human platelets, but recently has been found to be produced by various other cells, for example, monocytes, megakaryocytes, vascular endothelium, smooth muscle cells, and transformed cells. The in vivo function of PDGF remains speculative, but the fact that PDGF is released by platelets and by cells involved in inflammatory reactions and that it stimulates proliferation, chemotaxis, matrix production, and gene expression in cell types considered essential for tissue repair, may indicate that it also plays a role in the wound healing process.
Effects of PDGF on Wounded Human Corneal Endothelium

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different PDGF receptor types, denoted α and β,

5,19-22 each with different ligand binding specificity.21,22

In a previous study, PDGF-BB was found to induce dose-dependent mitogenic responses in bovine corneal endothelial cells maintained in tissue culture.7

In addition, immunohistochemically detectable PDGF receptors of both α- and β-type were localized on corneal endothelium of human corneas, the β-receptor being more abundant than the α-receptor.7 However, no information is available on the influence of PDGF on human corneal endothelium in situ. The purpose of the current study was to investigate if the dimer PDGF-BB stimulates the endothelial wound healing of human corneas preserved by organ-culture. The effects of PDGF-BB on wound closure time, on morphometric parameters of endothelial cells (cell area, density, and shape), and on DNA synthesis in the wound area were determined.

METHODS

Human Corneas

Paired human corneas, donated to the Eurotransplant Foundation (Leiden, The Netherlands) for transplantation but discarded by the Cornea Bank (Amsterdam, The Netherlands) because of macula (superficial scars), low endothelial cell density (ECD) (1500<ECD<2000 cells/mm²), and/or irregularity of the endothelium (polymegethism), were used. The corneas had an average ECD of 2300 ± 320 cells/mm² (range, 1500 to 3100 cells/mm²). Polymegethism was determined by gross visualization and defined as a variation in apical cell diameter of two times or more. Corneas with pleomorphic or extreme polymegetic endothelium or corneas with diseases of the endothelium were excluded from the experiments. Paired corneas were used to overcome interindividual variation in wound closure and morphometric aspects. A total of 84 pairs of corneas were used. Donor ages ranged from 41 to 91 years (mean, 71 ± 11 years). The interval between death and enucleation and between enucleation and corneal dissection averaged 7.6 ± 4.6 hours (range, 1.5 to 20 hours) and 16.3 ± 5.2 hours (range, 4.3 to 30 hours), respectively. The onset of the experiments was always within 1 day of preparation of the cornea for the morphometric study and within 2 days for the autoradiographic study.

Wounding Procedure

A central endothelial lesion was made mechanically as previously described.23,24 Briefly, with the endothelial side up corneas were placed into a depression of a sterilized concave block of Teflon that matched the corneal curvature. A metal rod with a rounded, smoothly polished tip was placed perpendicularly on the center of the cornea and rotated a few times, thus creating a circular endothelial wound. Scanning electron microscopic inspection revealed that only the endothelium and not the Descemet's membrane was injured with this method of wounding (Fig. 1, A and B). For scanning electron microscopy the corneas were treated according to a standard procedure. Briefly, they were rinsed in a 0.08 M cacodylate-buffered solution of 1.25% glutaraldehyde/1% paraformaldehyde, pH 7.3, rinsed in the same buffer, dehydrated in a graded series of ethanol, critical point dried with CO₂, and coated with gold. Subsequently, they were inspected in a Philips 505 scanning electron microscope (Eindhoven, The Netherlands).

The wound sizes among pairs of corneas varied considerably from 4.3 to 7.5 mm², probably because of the biologic variation in curvature of the corneas or the degree of postmortem swelling of the corneas. However, within each pair of corneas, the initial wound sizes hardly varied. The difference in initial wound size between the corneas of each pair was determined and related to the largest wound size of each pair. The variation in the initial wound sizes within pairs averaged 2.1 ± 2.2%. The larger wound sizes were randomly distributed among the untreated and treated groups. The mean initial wound sizes averaged

FIGURE 1. Scanning electron microscopic images of the endothelial side of a human cornea immediately after mechanical wounding. (A) Survey picture of the central, circular wound. Arrows: remnants of endothelial cells. (B) Medium power view of the wound margin showing the intact, uncovered Descemet's membrane with the impressions of the removed cells, the sharp wound margin with damaged cells (arrowheads) and the normal endothelial cells (end) outside the wound area.
5.6 ± 0.8 mm² (range, 4.3 to 7.5 mm²) in the control group and 5.6 ± 0.8 mm² (range, 4.5 to 7.4 mm²) in the PDGF-treated group. The difference was not statistically different.

After injury, the corneas were placed in 10 ml of sterile Eagle's minimum essential medium supplemented with 5% Dextran T500 (Pharmacia, Uppsala, Sweden), 2% fetal bovine serum (ICN Biomedicals Inc., Costa Mesa, CA), and antibiotics at 31°C. To the culture medium of one cornea of each pair 10 or 75 ng/ml recombinant human PDGF-BB (Gibco, Grand Island, NY; >95% purity) was added, while the mate, incubated without PDGF-BB, served as control. The preference of the dimer PDGF-BB has been explained previously.

On account of previous observations in tissue-cultured bovine corneal endothelial cells (see Discussion), the effects of two concentrations of PDGF-BB, 10 ng/ml and 75 ng/ml, were tested on wound closure time (23 pairs), the ECD (23 pairs), and cell division (26 pairs) in the wound center at the time of wound closure. Because no significant differences were observed between both concentrations, the data presented are, unless stated otherwise, from corneas treated with 10 ng/ml of PDGF-BB (equivalent to 0.3 nM). For all experiments, the culture media, with or without growth factor, were replaced every other day unless stated otherwise.

**Longitudinal Study of Wound Closure**

The endothelial side of the wounded corneas was stained daily with trypan blue as described previously. A preliminary 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 (final magnification 36.2X). After this, the corneas were replaced in the organ culture medium. The wound area was measured using a MOP-Videooplan Image Analysis System software package developed by Kontron Bildanalyse (Munich, Germany). To evaluate the effect of PDGF-BB on the course of wound closure, the wound area was plotted versus time for each pair of corneas. The wound closure time was the day at which Descemet's membrane no longer stained with trypan blue and was fully covered by endothelial cells.

**Morphometric Analysis**

The cell borders and nuclei of the endothelium of the corneas were stained with alizarin red and trypan blue, respectively (Fig. 2). After preparing a flat, wet endothelial specimen, the endothelium in the wound center of each cornea was photographed. Photographic prints were prepared at a final magnification of 345X as calibrated by using an object micrometer.

The photographed cells were outlined in two randomly selected fields as described previously. The Videoplan software package was used to measure and calculate cell area, ECD, perimeter, maximal diameter, shape factor (see later) and coefficients of variations of area (CVarea) and shape factor (CVshape factor). The ECD, expressed as the number of cells per mm², was calculated from the mean cell area (μm²). The CVarea was calculated by dividing the standard deviation of area by the mean cell area and was expressed in percentage. The CVarea is a dimensionless index, independent of cell size and, therefore, provides a quantitative parameter of variation in cell area (polymegethism). Cell shapes can be described by the shape factor perimeter/maximal diameter (for references see reference 24). It is a dimensionless index describing to what degree a cell has elongated. This shape factor.
equals \( \pi \) (3.14) for a perfect circle and 3 for a perfect hexagon. A shape factor below 3 is an indication of elongated cell shape. The CVshape factor describes the variance in cell shapes (SD/Meanshape factor). Another generally accepted shape factor \( 4\pi \text{Area} / \text{perimeter}^2 \), describing to what extent the cell shape approximates that of a circle, yielded comparable results as perimeter/maximal diameter and therefore is not discussed further in this study. In the closed wound area of most corneas, it was not well possible to recognize the apices of the cells or to count the number of sides of each cell because of the presence of very irregular cells. In these cells, the number of neighboring cells is not indicative of the number of sides per cell. Therefore, the shape factors and their CVshape factor were used to describe the degree of symmetry or regularity of cells (a measure of pleomorphism).

Morphometric analysis was performed on endothelial cells obtained at three different times: (1) 3 days after wounding, ie, before wound closure (10 pairs); (2) at the time of wound closure (4 to 9 days; 15 pairs); and (3) 15 days after infliction of the wound, corresponding to about 6 to 11 days after wound closure (10 pairs). The corneas studied at the last two time points were also used to establish the effect of PDGF on wound closure times (25 pairs).

**Autoradiography**

The corneas were cultured for 6 to 7 days in a medium with or without PDGF-BB and supplemented with \(^3\)H-thymidine (New England Nuclear Research, specific activity 15 Ci/mmol, 1 \( \mu \)Ci/ml medium) at the onset of the experiment (continuous labeling). After this incubation period, the corneas were stained with alizarin red,\(^{26}\) fixed between two glass slides in formaldehyde 3.8% for 6 hours, dried for 15 hours, and mounted on gelatinized slides.\(^{24}\) The specimens were covered with Ilford K-2 nuclear emulsion (Ilford, England), exposed for 6 days at 4 to 5°C and developed at 20°C with Kodak D-19 developer (Rochester, NY) for 4 minutes. After photographic fixation in sodium thiosulfate 24%, they were rinsed in tap water for approximately 2 to 3 minutes, dehydrated in ethanol, cleared in carboxy-xylene, and mounted in Entellan (Merck, Darmstadt, Germany). The wounds of all corneas appeared to be closed. The total number of labeled nuclei in the initial wound area was counted for each specimen. The increase or decrease in labeled nuclei per mm\(^2\) was defined as the number of labeled nuclei/mm\(^2\) in the PDGF-treated group minus the number of labeled nuclei/mm\(^2\) in the control group. The percentage of change in labeling between both groups was only calculated for corneas in which more than ten labeled nuclei were observed in the wound area. The density of autoradiographic silver grains was large and the underlying nuclear chromatin could not be seen. Therefore, mitotic figures could not be detected.

**Statistical Analysis**

The differences in wound closure (50% and 10% level, time of wound closure), ECD, 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.

Differences among groups of two different experiments were statistically tested using the Wilcoxon's two-sample test (two-tailed). For correlations, we used the Spearman rank correlation coefficient (\( r_s \), two-tailed). Data given in the text are mean ± SD. A \( P \) value of ≤ 0.05 was considered significant.

**RESULTS**

**Wound Closure Time**

To evaluate the effect of PDGF treatment on wound closure, the wound area was plotted versus time after wounding for each pair of corneas. The wound closure data of all 25 paired corneas are combined in Figure 3.

Among pairs of corneas, a considerable variation in the closing times of the wounds was observed. Wound closure occurred between 4 and 9 days. Although significant (\( P < 0.01 \)), the mean estimated wound closure time was only marginally shorter in the PDGF-treated corneas. The differences in wound closure times were statistically tested with the Wilcoxon's signed-rank test (two-tailed) for paired observations.

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TABLE 1. Wound Closure of 25 Paired Human Corneas

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>PDGF-Treated Group</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% Level</td>
<td>2.6 ± 0.5 (1.9-3.8)</td>
<td>2.3 ± 0.4 (1.7-3.4)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>10% Level</td>
<td>4.8 ± 1.0 (3.8-7.8)</td>
<td>4.2 ± 0.9 (3.2-6.5)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Wound closure time</td>
<td>6.0 ± 1.1 (5-9)</td>
<td>5.2 ± 1.2 (4-8)</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

The times at which 50% and 10% of the initial wound area was left for each pair were determined. Wound closure time was the day at which the Descemet's membrane no longer stained with trypan blue and was fully covered by endothelial cells. Values are mean ± SD (range).

*M Wilcoxon's signed rank test.

Morphology of Wound Closure

Three days after wounding, the uncovered wound area was greatly reduced (Fig. 4A). Most cells adjacent to the wound, i.e., those immediately at the wound edge and several rows back from the edge, retained their cell-to-cell contacts (Fig. 4). The cells of this confluent monolayer were called the leading edge. Between this leading edge and the periphery, a transitional zone is located. This zone was particularly characterized by a gradual change in cell area and/or cell shape. The majority of the cells in the transitional zone did not show a normal hexagonal pattern. A pattern of clear-cut elongated cells directed at the wound center was evident in only a few corneas. As observed in scanning electron microscopy, the pattern of migrating cells did not differ between control and PDGF-treated corneas. Independently migrating cells were not frequently found in both groups (Fig. 4).

Survey photographs of the closed wounds showed that the transitional zone, formed by cells at the original wound edges, was better delineated in the group studied “at the time of wound closure” than in the group studied “15 days after wounding.”

Morphometry

The results of the morphometric data of the endothelial cells are summarized in Tables 2 and 3.

Endothelial cell density. At 3 days after wounding, the ECD in both the transitional zone and the leading edge was significantly higher in the PDGF-treated group than in the control group. The mean ECD in the leading edge was considerably lower than in the transitional zone in both groups (P < 0.01).

At the time of wound closure, all 15 corneas cultured in the presence of PDGF had a higher ECD in...
the wound center than their mates cultured in the absence of PDGF. The increase in ECD in the treated corneas of each pair averaged 222 ± 69 cells/mm² (range, 99 to 342 cells/mm²) or, given as a percentage of the control corneas, about 44%. No correlation was found between the PDGF-induced increase in ECD and donor age. Medium replacement every day (12 pairs) gave a somewhat lower increase in ECD (35%) compared to medium replacement every other day (44%) (0.05 < P < 0.1). A dose of 75 ng/ml PDGF (11 pairs) yielded morphometric results comparable to a dose of 10 ng/ml (data not shown). In contrast to the control group, the mean ECD in the closed wound center in the PDGF group studied “at the time of wound closure” was significantly higher than the mean ECD in the leading edge, studied at “3 days after wounding” (P < 0.01, Table 2).

Fifteen days after wounding, corresponding to about 6 to 11 days after wound closure, the mean ECD was considerably higher in all ten corneas exposed to PDGF compared to the control corneas. The average increase in ECD in the treated group amounted to 182 ± 88 cells/mm² (range, 53 to 318 cells/mm²) or about 35%. Again, age-related differences in the effects of PDGF on ECD were not observed. In both groups at 15 days after wounding, the mean ECDs in the center of the wound were not significantly different from the ECDs examined at the time of wound closure.

### Parameters of polyromegethism and pleomorphism
At 3 days after wounding, the mean shape factor of cells in the transitional zone averaged 2.60 in both the untreated and PDGF-treated group. In the leading edge, the mean shape factors of the PDGF-treated group were slightly smaller than those of the control group (Table 3). Both in the transitional zone and leading edge, the mean CV_area and CV_shape_factor were not significantly different between the control corneas and the treated ones.

### Table 3. Parameters Indicating Polyromegethism (CV_area) and Pleomorphism (Shape Factor, CV_shape_factor) of Human Corneal Endothelial Cells After Wounding

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>PDGF-Treated Group</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV_area (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*3 Days after wounding (n = 10)</td>
<td>40 ± 6% (32-50)</td>
<td>38 ± 8% (23-50)</td>
<td>NS</td>
</tr>
<tr>
<td>Leading edge of wound closure</td>
<td>43 ± 4% (36-48)</td>
<td>43 ± 4% (38-50)</td>
<td>NS</td>
</tr>
<tr>
<td>Transitional zone</td>
<td>45 ± 6% (32-54)</td>
<td>48 ± 8% (34-63)</td>
<td>NS</td>
</tr>
<tr>
<td>*At the time of wound closure (n = 15)</td>
<td>48 ± 7% (37-57)</td>
<td>49 ± 6% (41-58)</td>
<td>NS</td>
</tr>
<tr>
<td>*15 Days after wounding (n = 10)</td>
<td>2.59 ± 0.03 (2.53-2.66)</td>
<td>2.55 ± 0.02 (2.51-2.59)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Shape Factor (PM/Dmax)</td>
<td>2.60 ± 0.04 (2.53-2.65)</td>
<td>2.60 ± 0.03 (2.57-2.66)</td>
<td>NS</td>
</tr>
<tr>
<td>*3 Days after wounding (n = 10)</td>
<td>2.58 ± 0.04 (2.53-2.66)</td>
<td>2.57 ± 0.03 (2.51-2.65)</td>
<td>NS</td>
</tr>
<tr>
<td>Leading edge of wound closure</td>
<td>2.67 ± 0.05 (2.56-2.73)</td>
<td>2.68 ± 0.02 (2.64-2.72)</td>
<td>NS</td>
</tr>
<tr>
<td>Transitional zone</td>
<td>7.8 ± 0.7 (6.9-9.1)</td>
<td>8.4 ± 0.9 (7.4-10.2)</td>
<td>NS</td>
</tr>
<tr>
<td>*At the time of wound closure (n = 15)</td>
<td>7.4 ± 0.6 (6.5-8.6)</td>
<td>7.7 ± 0.7 (7.0-9.1)</td>
<td>NS</td>
</tr>
<tr>
<td>*15 Days after wounding (n = 10)</td>
<td>8.2 ± 0.5 (7.2-8.8)</td>
<td>8.4 ± 0.6 (7.6-9.7)</td>
<td>NS</td>
</tr>
<tr>
<td>CV_shape_factor (%)</td>
<td>6.9 ± 0.7 (5.7-8.1)</td>
<td>6.5 ± 0.4 (5.9-7.1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

For the calculation of variation of the mean cell area (CV_area), the shape factor (PM/Dmax), and the coefficient of the shape factor (CV_shape_factor), see Methods. The values are mean ± SD (range). n = number of pairs of corneas investigated.

* Wilcoxon's signed-rank test (NS = not significant).
Both at the time of wound closure and 15 days after wounding, the endothelial cells covering the wound area were irregular in shape and no longer had the typical polygonal structure. The variation in cell size was great. At both times points, the mean CV\textsubscript{area}, the mean shape factor of the cells, and the CV\textsubscript{shape-factor} in the wound center were not significantly different between the control and PDGF-treated groups. However, in both the untreated and the treated groups, the mean shape factor of cells studied 15 days after wounding was significantly larger than that of cells studied at the time of wound closure (P < 0.002). In addition, in both groups studied 15 days after wounding, the mean CV\textsubscript{shape-factor} of cells was significantly lower than that of cells studied at the time of wound closure (P < 0.002).

**Autoradiography**

Labeling of the endothelial cells with \textsuperscript{3}H-thymidine was observed in all paired corneas. However, the variation in \textsuperscript{3}H-thymidine incorporation among pairs of corneas was dramatic, ranging from a few to hundreds of nuclei.

The labeled nuclei were scattered throughout the wound area and cells outside this area were free of labeling. The pattern of labeled nuclei in the periphery, transitional zone, and wound center in a human cornea is shown in Figure 5. The number of labeled nuclei was counted in the closed wound area. The autoradiographic data of the control and the PDGF-treated corneas (10 ng/ml or 75 ng/ml) are shown in Table 4. Using the mean ECD given in Table 1 (at the time of wound closure), the labeled cells in both groups comprised about 7 ± 9% of all cells in the wound area. Although the number of radioactive nuclei per mm\textsuperscript{2} counted in the wound center was significantly higher in the PDGF-treated group than in the untreated group, the differences were small. The average increase in labeled nuclei in the treated group amounted to 9 ± 14 nuclei/mm\textsuperscript{2} (range, -3 to 55 nuclei/mm\textsuperscript{2}) or about 26%.

Because we were interested to know if the effect of PDGF on DNA synthesis was somehow related to donor age, we pooled the autoradiographic data of all the available control corneas (75 pairs). It was found that the number of labeled nuclei/mm\textsuperscript{2} in the control corneas significantly decreased over the age ranges studied (41 to 91 years; \(r_s = -0.469, P < 0.001\); Fig. 6A). For determination of correlations concerning the effect of PDGF on DNA synthesis, the autoradiographic data of both concentration groups of PDGF were combined (Table 4; 26 pairs). The increase in labeling in the PDGF-treated group was larger when more nuclei had incorporated \textsuperscript{3}H-thymidine in the

**TABLE 4. Number of Labeled Endothelial Nuclei Per mm\textsuperscript{2} in Wounded Human Corneas**

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>PDGF-Treated Group</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>*10 ng/ml Treatment  (n = 13)</td>
<td>31 ± 50 (4-195)</td>
<td>41 ± 64 (3-250)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>*75 ng/ml Treatment  (n = 13)</td>
<td>39 ± 49 (1-179)</td>
<td>48 ± 59 (2-206)</td>
<td>0.02 &lt; P &lt; 0.05</td>
</tr>
<tr>
<td>*Total (n = 26)</td>
<td>35 ± 49 (1-195)</td>
<td>44 ± 61 (2-250)</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

The effect of two concentrations of PDGF on DNA synthesis was investigated. Corneas were cultured for 6 to 7 days in medium with or without PDGF and supplemented with \textsuperscript{3}H-thymidine (continuous labeling). After the autoradiographic standard procedures, the number of labeled nuclei in the closed wound center was counted for each specimen. Values are mean ± SD (range), n = the number of pairs of corneas investigated.

* Wilcoxon's signed rank test.
DISCUSSION

To our knowledge, this is the first time that effects of PDGF on endothelial wound healing of human corneas have been found. The main finding of this study is that the ECD in the wound center is higher in the PDGF-treated corneas than in the control corneas studied at the time of wound closure and 15 days after wounding, 44% and 35%, respectively. Thus the effect of PDGF on the ECD in the wound area is still present 6 to 11 days after wound closure. No information is currently available on the effects of PDGF on ECD after wound healing in animal or human corneas or in cell culture systems. Previous studies in our laboratory, using the same human model of endothelial wound healing, demonstrated that addition of human epidermal growth factor (hEGF) to the culture medium induced a more pronounced increase in ECD than PDGF, namely 52% on average.24

The higher ECD in the closed wounds of human corneas in the PDGF-treated group may be the result of an increase in cell division or cell migration. There is evidence that the mitotic activity of wounded human corneal endothelium can be stimulated by growth factors, as, for example, EGF24>29 and possibly also PDGF. Therefore, the influence of PDGF on DNA synthesis of wounded human corneal endothelium was assessed by 3H-thymidine autoradiography. Synthesis of DNA is necessary before cell division. In agreement with previous studies,23'24'29'30 it is shown that, independent of the presence of growth factors, incorporation of 3H-thymidine occurred in all donor corneas during endothelial wound healing. However, because in the large majority of corneas, the number of labeled cells was too small to account for the coverage of the damaged area by cell division, it is postulated that injuries to the corneal endothelial layer of adult humans regenerate predominantly by enlargement and migration of uninjured cells.2>3'24>31 After wound closure, the untreated wounded corneas had a lower ECD (average,
515 cells/mm²) in the closed wound center compared to their (previous) normal, unwounded condition (average ECD before wounding: 2300 cells/mm²). The current study shows that, after injury, the DNA synthesis of human corneal endothelium was marginally stimulated by PDGF. Thus, PDGF is not a potent mitogen for endothelial cells of human senior corneas. In a previous study, using the same wound healing model, autoradiography revealed that the enhancement of the mitotic activity of endothelial cells by another growth factor, hEGF, was also limited.

In the closed wound, the mean increase in ECD in the PDGF-treated group was 222 cells/mm². The mean increase in labeled nuclei in the PDGF-treated group was 9 nuclei/mm². Therefore, in the majority of the PDGF-treated corneas, the increase in ECD cannot be explained by the limited stimulation of cell division as indicated by the small increase in labeled nuclei/mm². Besides, it is questionable whether the increased DNA synthesis always leads to nuclear or cell division.24,32,33

In human corneas, age probably has a strong negative influence on the replicative ability of human corneal endothelium.24 In agreement with our previous study, the number of labeled cells in the closed wound in the control corneas was inversely correlated with the donor age, suggesting more DNA replication in younger donors. Comparable results are also found in tissue culture systems: young donors usually give better growing cell lines.34–36 These findings suggest that a growth factor may have a more pronounced mitogenic effect on corneas that still have a potency to divide. This was confirmed for PDGF: a significant positive correlation between the increase in labeling in the PDGF group and the number of labeled cells in the control group was detected. The increase in labeling in the PDGF-treated group decreased with the age ranges studied (41 to 91 years). Such correlations were also found with hEGF.24 The effect of PDGF on cell division may play a more important role in the younger age groups.

Because cell division was marginally stimulated by PDGF, cell migration induced by PDGF must be the most important factor leading to the higher ECD in the PDGF-treated group. Endothelial wound closure mainly occurs by migration of cohorts of confluent cells rather than by migration of individual cells into the wound area.24 The PDGF-induced enhancement of the migration process may include a faster migration rate as well as an increased number of migrating cells. In the current human model of endothelial wound closure, exposure of the corneas to PDGF significantly shortened the wound closure time compared to the untreated control group. Because of the considerable variation in the wound closure curves, it was difficult to determine a wound closure rate. The shortening of wound closure time was less in corneas exposed to PDGF than in those exposed to hEGF.24 The shorter wound closure time observed in the PDGF-treated group favors a faster migration rate of cells near the wound boundary. However, the following arguments suggest that an increase in the number of migrating cells is an additional, probably more important, effect of PDGF. First, the mean ECD in the transitional zone as well as in the leading edge was higher in the PDGF-treated corneas than in the untreated control corneas 3 days after wounding. Second, the higher ECD in the treated corneas was still present 6 to 11 days after wound closure. If migration was only faster, no difference in ECD between both groups at 6 to 11 days after coverage of the wound would be expected. Third, the mean ECD in the closed wound center in the PDGF-treated group was higher than the ECD in the leading edge, studied 3 days after wounding, whereas this was not the case in the control group. The expectation that the width of the transitional zone would be higher in the PDGF-treated group than in the control group because of the increased migration process could not be confirmed (data not shown). Comparable results for the migration process were found with hEGF.24 In various cell types, including human corneal endothelial cells, PDGF can induce a rapid reorganization of actin. That injury-induced cell migration in corneal endothelium is partly based on the cooperative interactions of actin-containing microfilaments and microtubules also suggests a role of PDGF in the cell migration process.

There are several reports on the chemotactic activity (directed cell migration) of PDGF for various cell types, such as fibroblasts, vascular smooth muscle cells, as well as for osteoblastlike cells, retinal glial cells, and retinal pigment epithelial cells. PDGF is also strongly chemotactic for human monocytes and neutrophils/granulocytes, cells that do not respond mitogenically to PDGF. Although both α- and β-receptors of PDGF are able to induce a mitogenic response in various cell lines, only the β-receptor appears to be capable of mediating a chemotactic response after ligand binding. In the corneal endothelium of human corneas, the β-receptor was found to be more abundant than the α-receptor. In all responsive cell systems analyzed (see earlier), the optimal effective concentration of PDGF is in the range of about 1 to 100 ng/ml.

In a previous study, dose-response experiments have been carried out with tissue-cultured bovine corneal endothelial cells using two different proliferation assays, the DNA synthesis assay and the colorimetric MTT assay. Dose-response data of that study showed that the maximal effect on DNA synthesis were obtained at 75 ng/ml of PDGF-BB whereas the MTT assay indicated that maxi
mal cell activity was reached at approximately 10 ng/ml PDGF-BB. Despite being mindful that extrapolation of the results obtained in cell culture systems to organ culture systems may not be accurate, we have tested both concentrations of PDGF-BB (10 and 75 ng/ml) on wound closure, ECD, and DNA synthesis in our wound healing model. It was found that the PDGF-induced increase in wound closure time, ECD, and DNA synthesis was approximately identical for the two concentrations of PDGF-BB. The dosages used in our study correlate very well with dosages used in other cell lines (see earlier).

As expected, polymegethism and pleomorphism increased dramatically in the closed wound area as indicated by the CV_area, shape factor, CV_shape_factor, and the light microscopic appearance. Because the number of sides per cell with the corresponding shape factors for each cell type could not be determined, a global mean shape factor of all cells was used. PDGF did not affect the final endothelial cell shape or variation in this cell shape within the closed wounds as indicated by the identical shape factor and CV_shape_factor. During closing of the wound, however, more elongated cells were present on average in the leading edge in the PDGF group than in the control group, although the differences in shape factors between both groups were small. It is possible that this cell elongation ceases abruptly after initial coverage of the wound because of contact inhibition. This may explain that no differences in the final cell shape could be observed between both groups after wound closure. Cell elongation in tissue cultures of animal endothelial cells or organ cultures of human corneas was more obvious after treatment with hEGF (for references, see reference 24). After initial coverage of the wound area, endothelial cell rearrangement continues to take place in both control and PDGF-treated groups as indicated by the increased shape factors and decreased CV_shape_factor of cells studied 15 days after wounding compared with cells studied at the time of wound closure. However, the CV_area was still considerably high at 15 days after wounding.

Whether polymegethic endothelium responds differently to growth factors compared to normal endothelium cannot be excluded by this study because of the different reasons for discarding the corneas and the relatively small numbers of corneas in each group studied.

The experiments were performed with an organ culture medium that contained 2% fetal bovine serum. In tissue-cultured bovine corneal endothelial cells, it was found that PDGF-induced enhancement of DNA synthesis was dependent on the serum concentration used. This suggests that PDGF needs factors present in serum to become effective or to achieve an optimal response. It is not known if the effect of PDGF on the migration process also requires serum. That the serum contains factors enhancing the wound healing or the PDGF effect therefore cannot be ruled out. However, both control and PDGF-treated corneas had 2% fetal bovine serum in the culture medium, and the differences in wound closure times, ECD, and DNA synthesis must be the result of the effect of PDGF. It is worth mentioning that after injury of human corneal endothelium in vivo, serum is probably also present in the aqueous humor because of breakdown of the blood–aqueous barrier and/or hyphema.

The presence of PDGF receptors on endothelium of human corneas as well as the mitogenic effects of PDGF on cultured bovine corneal endothelial cells suggest a role for PDGF in corneal development and/or wound healing. This study showed that PDGF-BB is capable of promoting endothelial wound healing in human corneas from senior donors. The PDGF-enhanced healing process occurs predominantly by an increased cell migration, that is, by a faster migration rate and, probably more importantly, by an increased number of migrating cells. The mean donor age was high and it would be interesting to know the response of PDGF on wounds of younger donors. In addition, now that we have tested the activity of two growth factors independently, namely PDGF and EGF, it would be interesting to further study if a combination of these growth factors also have an additive, or even synergistic effect on the endothelial wound healing of human corneas. Studies have demonstrated the enhancing effects of PDGF when it is combined with other growth factors such as EGF, TGF-β, or IGF.

Recently, Tripathi et al56 could not verify the presence of PDGF in the aqueous humor. In agreement with these authors,56 we suppose that the level of PDGF will increase under conditions of breakdown of the blood–aqueous barrier and hyphema. It is also possible that PDGF is produced by cells lining the anterior chamber. Pharmacological manipulation of the corneal endothelial responses to wounding by PDGF may be advantageous in maintaining endothelial integrity and function.

Key Words

corneal endothelium, platelet-derived growth factor, wound healing, autoradiography, morphometry

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