Injured Corneal Epithelial Cells Promote Myodifferentiation of Corneal Fibroblasts

Kunibiko Nakamura, Daijiro Kurosaka, Mami Yoshino, Takeshi Oshima, and Hiroyo Kurosaka

PURPOSE. To determine whether injured corneal epithelial cells stimulate myodifferentiation in corneal fibroblasts and whether transforming growth factor (TGF)-β is involved.

METHODS. Rabbit corneal fibroblasts were cultured on collagen gel, with or without cocultured corneal epithelial cells or with partially scraped epithelial cells, on a companion plate separated by a permeable membrane. To evaluate fibroblast-induced gel contraction, gel thickness was measured daily relative to the original thickness. Total fibroblasts on the gel were counted. Myofibroblasts were counted by using immunocytochemical identification with anti-α-smooth muscle actin (α-SMA). TGF-β was assayed in the media on days 3 and 6. These procedures also were performed in the presence of anti-TGF-β antibody.

RESULTS. Gel contraction, α-SMA-positive cells, and total cell number were significantly greater on gels with injured epithelial cells than on gels without epithelial cells or with uninjured epithelial cells, as was TGF-β concentration in the media. Anti-TGF-β antibody eliminated these differences.

CONCLUSIONS. Injured epithelial cells stimulate myodifferentiation in fibroblasts through one or more soluble factors, including TGF-β. (Invest Ophtalmol Vis Sci. 2002;43:2603–2608)

After refractive surgery or corneal stromal injury, some corneal haze develops in some patients as a wound-healing response, causing visual impairment.1–2 Previous studies have shown that during stromal wound healing, fibroblasts differentiate into myofibroblasts that express smooth-muscle–specific α-actin (α-SMA). These cells are central to wound contraction and scarring.3–6 Currently, the most widely used refractive surgical technique is laser in situ keratomileusis (LASIK), which can preserve corneal epithelium and thereby reduce wound-healing problems associated with photorefractive keratectomy (PRK).7–8 This benefit suggests that the corneal epithelium is involved in corneal stromal wound healing. We have reported that α-SMA and subepithelial corneal haze does not occur with the denudation of epithelium alone or with LASIK, but does in PRK and in LASIK with denudation of epithelium.9 This indicates that intact epithelium is the key to the prevention of stromal haze after photorefractive ablation, and that myofibroblastic differentiation is not induced by stromal injury alone but by both epithelial and stromal injury. However, how the corneal epithelium is involved in corneal stromal wound healing is not fully understood. Abnormalities of the barrier function of corneal epithelium rend it permeable to cytokines and growth factors from tear fluid that then can pass into the corneal stroma, causing activation of keratocytes.10,11 Interaction between epithelial cells and keratocytes also is an important factor in corneal wound healing.12–15 Loss of contact between epithelial cells and fibroblasts may contribute to myofibroblastic differentiation.14

In the present study we set out to determine whether injured corneal epithelial cells can stimulate myodifferentiation in corneal fibroblasts through a soluble factor. We cultured rabbit corneal fibroblasts on a collagen gel in an insert dish containing a membrane permeable to soluble factors but not to cells. Cultures were incubated, with or without uninjured corneal epithelial cells or with partially scraped epithelial cells, on the companion plate on the opposite side of the membrane. We evaluated the fibroblast-induced contraction of the collagen gel, proliferation of cells on the gel, and extent of myodifferentiation.

METHODS

Corneal Epithelial Culture

Normal rabbit corneal epithelial cells (NRCE2) obtained from Kurabo (Osaka, Japan) were seeded (4000 cells/cm²) on six-well plates (Becton Dickinson, Franklin Lakes, NJ) in RCGM (a serum-free medium specific for rabbit corneal epithelial cells, containing 5 µg/mL insulin, 0.5 µg/mL hydrocortisone, 50 µg/mL gentamicin, 0.25 µg/mL amphotericin B, 0.03 mM Ca²⁺, 10 ng/mL epidermal growth factor [EGF], and 0.4% bovine pituitary extract: Kurabo). Cells were supplied every 2 days with RCGM and cultured to confluence.

Corneal Fibroblast Culture

Albino rabbits were purchased from Sankyo Laboratory Service (Tokyo, Japan). Care and treatment of the animals were in full accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The epithelium and endothelium were removed manually from an excised sclerocorneal button, and the stroma was cut into small pieces. These small pieces contained rabbit corneal fibroblasts, which then were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in tissue culture (TC)-199 medium containing 10% fetal bovine serum (FBS). Cells from the second passage were used for experiments.

Culture of Fibroblasts on Collagen Gel

Type I collagen (3 mg/mL) derived from porcine tendon (cell matrix type I-A) was obtained from Nitta Gelatin (Osaka, Japan). To make a collagen solution, we mixed the porcine collagen, 10-fold concentrated TC-199 medium, and 50 mM NaOH containing 260 mM NaHCO₃ and 200 mM HEPES at a proportion of 8:1:1 (vol/vol/vol) at 4°C. A 0.2-mL aliquot of this collagen solution was placed in the center of a 4.2-cm² insert dish (Falcon, Lincoln Park, NJ), containing a membrane with a pore size of 1.0 µm, and was incubated for 30 minutes at 37°C to polymerize the collagen. Corneal fibroblasts were suspended in TC-199 medium containing 10% FBS. A small aliquot of this medium containing corneal fibroblasts (0.085 mL containing 7.5 × 10⁴ cells) was deposited on top of the polymerized gels. After cultures were incubated overnight to permit cell adhesion, the collagen gel was

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remaining cell suspension was mixed with 1.5 mL of TC-199 medium and 1.5 mL of TC-199 medium containing 10% FBS. Dissociated cells were replated onto eight-chamber slides (Laboratory-Tex; Nunc, Naperville, IL). The slides were incubated for 12 hours to permit cell adhesion, after which the cells were rinsed three times with phosphate-buffered saline and then immersed in 95% ethanol containing 0.1% Triton-X (Wako Pure Chemical, Osaka, Japan) at 4°C for fixation. After fixation, the cells were rinsed three times with phosphate-buffered saline and were immunostained for αSMA, according to the manufacturer’s instructions. The primary antibody used was a mouse monoclonal antibody directed against human α-SMA (IgG2a, clone 1A4, code no. MB51; Dacopatts, Glostrup, Denmark). Peroxidase visualization was accomplished by adding a solution containing 3-amino-9-ethylcarbazole (AEC) and hydrogen peroxide. Finally, the cells were counterstained with hematoxylin. At least 200 cells were counted in each gel to determine the ratio of the number of positive cells to total number of cells (P/T ratio). The ratio was used to assess myofibroblastic differentiation. To determine the number of myofibroblasts per gel, we multiplied the total cell number per gel by its P/T ratio.

Coculture of Corneal Epithelial Cells and Fibroblasts

RGM was aspirated from the culture plates containing corneal epithelial cells. These plates then were rinsed three times with HEPES-buffered Ringer’s solution. Half of these plates were partially scraped with a cell scraper (Sumitomo Bakelite, Tokyo, Japan) before being rinsed with HEPES-buffered Ringer’s solution. Insert dishes of fibroblasts on collagen gels were combined in a companion plate containing uninjured epithelial cells, partially scraped epithelial cells, or no epithelial cells (Fig. 1). Other plates with uninjured epithelial cells or injured epithelial cells were not cocultured with fibroblasts. Next, 5.0 mL serum-free TC-199 medium supplemented with a serum-free defined medium supplement (0.2% TCX; Celox Laboratories, St. Paul, MN), was added to each well. The medium was changed on day 3.

Collagen Gel Contraction by Corneal Fibroblasts

Collagen gel culture has been used as a simple method to evaluate the contractile properties of fibroblasts, including corneal fibroblasts. Collagen gel contraction was estimated by a method described previously, being observed as a reduction in thickness of the gel. To determine changes in the biological behavior of corneal epithelial cells, an immunocytochemical study was conducted using vimentin, a marker for mesenchymal cells. Normal rabbit corneal epithelial cells were cocultured with fibroblasts as described earlier. On days 0, 3, and 6, the culture medium and the insert dishes of fibroblasts were removed. Epithelial cells were rinsed three times with phosphate-buffered saline and immersed in 95% ethanol containing 0.1% Triton-X (Wako Pure Chemical) at 4°C for fixation. After fixation, the cells were rinsed three times with phosphate-buffered saline and were immunostained for vimentin, according to the manufacturer’s instructions. The primary antibody was a mouse monoclonal antibody directed against human vimentin (IgG1/k, clone V9; NeoMarkers, Fremont, CA). Peroxidase visualization was accomplished by adding a solution containing AEC and hydrogen peroxide. The cells then were counterstained with hematoxylin.

Blocking TGF-β

The procedures described also were performed in the presence of 10 μg/mL of anti-panspecific TGF-β-neutralizing antibody or 10 μg/mL normal rabbit IgG (control; both from R&D Systems).

Statistical Analysis

Data are presented as the mean ± SD. One-way analysis of variance (ANOVA) was used to analyze dose dependence. Post hoc comparisons between groups were made using the Fisher protected least significant difference test. A repeated-measures ANOVA was used to analyze the time course data. P < 0.05 was accepted as indicating statistical significance.

RESULTS

Epithelial Cells Migration

The scraped area was reepithelialized by migrating epithelial cells from the surrounding area in 1 day. By day 2, epithelial cells in the reepithelialized area began to enlarge. More patches of large cells were apparent by day 3. By day 4 the patches changed in shape and showed whorled patterns (Fig. 2A). Figure 2C shows epithelial cells in the reepithelialized area in 0.4 mL fresh TC-199 medium containing 10% FBS. Dissociated cells were replated onto eight-chamber slides (Laboratory-Tex; Nunc, Naperville, IL). The slides were incubated for 12 hours to permit cell adhesion, after which the cells were rinsed three times with phosphate-buffered saline and then immersed in 95% ethanol containing 0.1% Triton-X (Wako Pure Chemical, Osaka, Japan) at 4°C for fixation. After fixation, the cells were rinsed three times with phosphate-buffered saline and were immunostained for αSMA, according to the manufacturer’s instructions. The primary antibody used was a mouse monoclonal antibody directed against human α-SMA (IgG2a, clone 1A4, code no. MB51; Dacopatts, Glostrup, Denmark). Peroxidase visualization was accomplished by adding a solution containing 3-amino-9-ethylcarbazole (AEC) and hydrogen peroxide. Finally, the cells were counterstained with hematoxylin. At least 200 cells were counted in each gel to determine the ratio of the number of positive cells to total number of cells (P/T ratio). The ratio was used to assess myofibroblastic differentiation. To determine the number of myofibroblasts per gel, we multiplied the total cell number per gel by its P/T ratio.

Expression of Vimentin by Corneal Epithelial Cells

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expressing vimentin. In the unscraped plate, epithelial cells maintained normal morphology in the central zone (Fig. 2B) and expressed vimentin only slightly (Fig. 2D).

Collagen Gel Contraction by Corneal Fibroblasts

Figure 3 presents findings for fibroblast-mediated collagen contraction. Gels containing corneal fibroblasts cultured without epithelial cells showed a slight contraction to 94.8% ± 1.2% of the original thickness after 6 days, whereas gels cocultured with injured epithelial cells showed greater contraction, to 71.1% ± 5.1% of the original thickness. Gels cocultured with uninjured epithelial cells showed a contraction to 77.6% ± 1.9% of the original thickness. Gels cocultured with injured epithelial cells contracted significantly more than the gels cultured without epithelial cells or the gels cocultured with uninjured epithelial cells.

Proliferation of Corneal Fibroblasts

Figure 4A depicts the overall cell count (Coulter), which shows the proliferation of corneal fibroblasts plated on a collagen gel (A). Total cells were counted on day 6. Significant differences are evident between groups. The ratio of the number of α-SMA-positive cells to the total number of cells (P/T ratio) was increased (B). Again, differences were significant between groups. Data are the mean ± SD results of five gels. *Significantly different from the control and noninjured cultures (P < 0.05).

Proliferation of Corneal Fibroblasts

Figure 4A depicts the overall cell count (Coulter), which shows the proliferation of corneal fibroblasts plated on the collagen gel. The number of corneal fibroblasts initially plated on the gel was $7.5 \times 10^5$ cells/gel. After a 6-day culture period, the total number of fibroblasts cultured without epithelial cells increased to $1.2 \pm 0.1 \times 10^4$ cells/gel. The total number of fibroblasts cocultured with injured epithelial cells increased to $2.3 \pm 0.4 \times 10^4$ cells/gel, whereas the total number of fibroblasts cocultured with uninjured epithelial cells increased to only $1.8 \pm 0.2 \times 10^4$ cells/gel. The total number of fibroblasts cocultured with injured epithelial cells was significantly greater than the number of fibroblasts cultured with uninjured epithelial cells or without epithelial cells.

Differentiation to Myofibroblasts

Figure 4B shows the proportion of cells showing differentiation from fibroblasts to myofibroblasts (P/T ratio): 0% of cells on gels cultured without epithelial cells, 16.5% ± 4.7% on gels
cocultured with injured epithelial cells, and 4.9% ± 1.9% on gels cocultured with uninjured epithelial cells were immunoreactive for α-SMA (Figs. 5A–C). The α-SMA positivity rate in the gels cocultured with injured epithelial cells was significantly greater than the rate in gels cultured without epithelial cells or for gels cocultured with uninjured epithelial cells.

**TGF-β in Media**

Concentrations of TGF-β in culture media are shown in Table 1. TGF-β2 was more abundant in media from fibroblasts cocultured with injured epithelial cells than in media from fibroblasts cocultured with uninjured epithelial cells or those that were not cocultured. Similarly, the concentration of TGF-β2 in media of injured epithelial cells alone was higher than in that of uninjured epithelial cells alone.

**Blockade of TGF-β**

Figure 6 shows results after culture in the presence of panspecific anti-TGF-β-neutralizing antibody (10 μg/mL). This antibody blocked the effects of coculture with injured epithelial cells on contraction of gels by fibroblasts, fibroblast proliferation, and myodifferentiation of fibroblasts.

**DISCUSSION**

In this study, we found that injured corneal epithelial cells secreted a soluble factor that crossed a membrane impermeable to cells, to stimulate collagen gel contraction by corneal fibroblasts, proliferation of corneal fibroblasts, and myodifferentiation of corneal fibroblasts. These findings suggest that interactions between injured epithelial cells and fibroblasts through the soluble factor are highly important in corneal wound healing and its possible complications.

In a recent study, corneal stromal keratocytes were found to exhibit three different states: quiescent, activated, and highly contractile, respectively corresponding to keratocyte, fibroblast, and myofibroblast phenotypes. In vivo after injury and in vitro after culture with serum, keratocytes are activated and become fibroblasts. Our finding that more cells were α-SMA-positive when gels were cocultured with injured epithelial cells than with uninjured epithelial cells support our previous findings in vivo that myofibroblast differentiation from keratocytes is not induced by purely stromal injury, but requires both epithelial and stromal injury.

In the present study, even though reepithelialization of the scraped area was completed by day 1, an effect of a soluble factor derived from injured corneal epithelial cells persisted. Daniels and Khaw reported that mature corneal epithelial cells in culture differentiate, showing enlargement and a whorled pattern. SundarRaj et al. reported that corneal epithelial cells changed shape and expressed vimentin in vivo during wound healing and that these events were linked to cell–matrix interactions in wound healing. Thus, the finding that after migration to cover a denuded area

### Table 1. The Levels of TGF-β in Media

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Uninjured Epithelial Cells Only</th>
<th>Injured Epithelia Cells Only</th>
<th>Fibroblast Only</th>
<th>Coculture with Uninjured Epithelial Cells</th>
<th>Coculture with Injured Epithelial Cells</th>
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<tr>
<td>TGFβ1</td>
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<tr>
<td>5 days</td>
<td>ND</td>
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<td>6 days</td>
<td>ND</td>
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<tr>
<td>TGFβ2</td>
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<tr>
<td>5 days</td>
<td>10.3 ± 1.6</td>
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<td>12.4 ± 1.2</td>
<td>43.5 ± 1.0</td>
<td>51.3 ± 1.5</td>
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<tr>
<td>6 days</td>
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<td>30.0 ± 1.0</td>
<td>10.2 ± 1.6</td>
<td>177.7 ± 49.2</td>
<td>438.9 ± 34.1</td>
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Data are expressed as picograms per milliliter. ND = not detected
epithelial cells gradually enlarged, showed a whorled pattern, and expressed vimentin after injury suggests that these altered epithelial cells secrete a soluble factor that stimulates myodifferentiation of corneal fibroblasts.

In the present study, such differentiation in cocultures with uninjured epithelial cells was slight, and uninjured epithelial cells showed only slight vimentin expression. These cells may cause only limited myodifferentiation of corneal fibroblasts, and this may not be evident in vivo.

Contraction of a collagen gel depends on the number of cells on the gel. An increasing number of cells is associated with an increase in gel contraction.24–25 Our data suggest that the influence of the soluble factor derived from injured corneal epithelial cells on corneal fibroblast-induced collagen gel contraction may depend on promotion of both cell proliferation and myodifferentiation of fibroblasts; Kurosaka et al.19 reported that the contractile action of myofibroblasts is much stronger than that of fibroblasts. Also, when we attempted to detect the myodifferentiation of fibroblasts by immunocytochemistry for α-SMA, we did not find myodifferentiation of fibroblasts from days 0 to 3 (data are not shown). This suggestion that myodifferentiation of fibroblasts may not contribute to collagen gel contraction between days 0 and 3 is in disagreement with the report of Kurosaka et al. However, Vaughan et al.26 observed enhanced formation of the structural elements that characterize the myofibroblast before any increase in expression of α-SMA. Thus, our chosen marker may have missed myodifferentiation of fibroblasts from days 0 to 3. Further investigations are needed to clarify the relationship between myodifferentiation and collagen gel contraction.

It has been proposed that growth factors and cytokines secreted by epithelial cells regulate functions of keratocytes and vice versa. This interaction between epithelial cells and keratocytes appears to be critical in corneal wound healing.25,27 In addition, several cytokines and growth factors stimulate collagen gel contraction by corneal fibroblasts, including EGF, platelet-derived growth factor (PDGF), TGF-β, and secreted protein, acidic and rich in cysteine (SPARC).12,13,18,19 Recent studies have shown that TGF-β plays a central role in differentiation of myofibroblasts.19,30 In the present study, TGF-β-neutralizing antibody blocked promotion by epithelial cell-derived soluble factor of contraction of gels by fibroblasts, fibroblast proliferation, and myodifferentiation. In addition, concentrations of TGF-β2 in media from injured epithelial cells were higher than in media from uninjured epithelial cells, whether cultured alone or cocultured. These findings suggest that TGF-β, especially TGF-β2, is pivotal to interactions between injured epithelial cells and fibroblasts in corneal wound healing. However, amounts of TGF-β2 in media from cocultures were higher than in media from epithelial cells alone. Thus, more complicated interactions are suspected, perhaps involving autocrine stimulation by fibroblasts, positive feedback from fibroblasts to epithelial cells, or participation of other cytokines. Further investigations are needed to reveal the precise role of TGF-β in interactions between injured epithelial cells and fibroblasts in corneal wound healing.

To our knowledge, this is the first in vitro study of the influence of injured epithelium on fibroblast phenotype and activity. Our data support the importance of the intact corneal epithelium for curbing differentiation of myofibroblasts in corneal wound healing and the wound-healing complications caused by these cells.

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References


