**Transforming Growth Factor-β1 Promotes Contraction of Collagen Gel by Bovine Corneal Fibroblasts through Differentiation of Myofibroblasts**

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**PURPOSE.** To determine whether the ability of transforming growth factor-β (TGF-β) to influence the contractile activity of corneal fibroblasts depends on their differentiation into myofibroblasts.

**METHODS.** Bovine corneal fibroblasts were cultured on collagen gel in MED 5 medium (F-12 nutrient mixture supplemented with 5% fetal bovine serum) with or without TGF-β1 (0.01–10 ng/ml). To evaluate the corneal fibroblast-derived contraction of collagen gel, the thickness of the gel was measured daily for 6 days. The total number of cells on the gel was counted with a Coulter counter. The detection of α-smooth muscle actin (α-SMA), a marker for myofibroblasts, on these cells was performed immunocytochemically by using a mouse monoclonal antibody against α-SMA. The number of myofibroblasts (α-SMA-positive cells) was determined.

**RESULTS.** The control gels containing bovine corneal fibroblasts that were cultured with the MED 5 medium alone significantly contracted to 72.3 ± 1.2% of their original thickness after 6 days. TGF-β1 increased the contraction of collagen gel mediated by bovine corneal fibroblasts in a dose-dependent manner. Approximately 0.2% of the cells on the control gels cultured with MED 5 medium alone were α-SMA positive. TGF-β1 significantly increased the expression of α-SMA in a dose-dependent manner. There was no significant correlation between the thickness of the collagen gel and the total number of cells. However, there was a significant negative correlation between the thickness of collagen gel and the number of myofibroblasts.

**CONCLUSIONS.** TGF-β1 increased the contractile activity of bovine corneal fibroblasts and their ability to differentiate into myofibroblasts. Because contractile activity was correlated with differentiation, the influence of TGF-β1 on corneal fibroblast-induced collagen gel contraction may depend on the promotion of myofibroblast differentiation. (Invest Ophthalmol Vis Sci. 1998;39:699–704)

Myofibroblasts exhibit structural and biologic properties that are intermediate between the properties of fibroblasts and smooth muscle cells.1 Myofibroblasts are found in wounds and fibrotic lesions, and they have been suggested to be involved in wound contraction and in retraction phenomena.1 Myofibroblasts also appear in healing wounds of the corneal stroma, and they are thought to be critically involved in the contraction of wounds in vivo.2–4 Collagen gel culture has been used to evaluate the contractile activity of various cell types,5–12 including corneal fibroblasts.13 Transforming growth factor-β (TGF-β) is present in the corneal epithelial cells and in tears,14–20 and it has been shown to promote wound healing in the cornea.21 Vesaluoma et al.20 reported that the release of TGF-β1 was increased after photorefractive keratectomy. Collagen gel contraction is caused by various cells and is promoted by TGF-β.10–12

It was recently reported that TGF-β induces various types of cells,11,22–25 including corneal fibroblasts,26,27 to differentiate into myofibroblasts. If myofibroblasts are involved in wound contraction, the degree of collagen gel contraction induced by TGF-β would correlate with the degree of myofibroblast differentiation.

To determine whether the ability of TGF-β to influence the contractile activity of corneal fibroblasts depends on their differentiation into myofibroblasts, we cultured corneal fibroblasts on collagen gels with TGF-β and examined the relationship between the contraction of collagen gel and the extent of myofibroblast differentiation.

**METHODS**

**Bovine Corneal Fibroblasts**

Corneas were excised from freshly obtained bovine eyes. The epithelium and endothelium were removed manually, and the stroma was cut into small pieces. These small pieces contained bovine corneal fibroblasts, which were then cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in MED...
10 medium. This medium consisted of an F-12 nutrient mixture (Life Technologies, Gaithersburg, MD), 10% fetal bovine serum (Bioserum, Victoria, Australia), 0.15% sodium bicarbonate solution (Life Technologies), 50 U/ml penicillin (Life Technologies), and 50 μg/ml streptomycin (Life Technologies). Cells from the second passage were used for these experiments. All procedures were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Assay of Collagen Contraction
To evaluate the effects of TGF-β1 on the contraction of collagen gel, collagen gels were assayed by a modification of the method of Guidry and coworkers. Type 1 collagen (3 mg/ml) derived from porcine tendon (cell matrix type 1-A) was obtained from Nitta Gelatin (Osaka, Japan). To make a collagen solution, we mixed the porcine collagen, the 10-fold concentrated F-12 medium, and 50 mM NaOH containing 260 mM NaHCO3 and 200 mM HEPES at a proportion of 8:1 (vol/vol/vol) at 4°C. This collagen solution then was mixed with MED 10 medium. The final concentration of collagen was 1.5 mg/ml, and that of fetal bovine serum was 5%. A 0.2-ml aliquot of this collagen solution was placed onto the center of a 35-mm culture dish (Falcon, Lincoln Park, NJ) and was incubated for 30 minutes at 37°C to polymerize the collagen. We also prepared MED 5 medium, which was similar to MED 10 medium except that it contained 5% fetal bovine serum. Corneal fibroblasts were suspended in MED 5 medium. A small aliquot of this medium containing corneal fibroblasts (0.085 ml containing 7.5 × 10^3 cells) was placed on top of the polymerized gels. These cultures were incubated for 90 minutes to allow cell adhesion. After this incubation, 3 ml MED 5 medium alone (control), 3 ml MED 5 medium supplemented with TGF-β1 (10, 1, 0.32, 0.1, 0.032, or 0.01 ng/ml), or a combination of TGF-β1 (1 ng/ml) and anti-TGF-β1 neutralizing antibody (10 μg/ml) was added to each dish. Gel contraction was observed as a reduction in the thickness of the gels. The gel thickness was measured on an inverted phase-contrast microscope by adjusting the plane of focus from the bottom to the top of the gel and by recording the movement of the stage. The gels were measured daily for 6 days. The medium was changed on day 3. TGF-β1, which was derived from porcine platelets, and the neutralizing antibody directed against recombinant human TGF-β1 (AB-101-NA) were obtained from R&D Systems (Minneapolis, MN).

Cell Proliferation
To determine the effect of TGF-β1 on the proliferation of the corneal fibroblasts plated on the collagen gels, we counted the total number of cells per gel by using a modification of the method of Goto et al. After the collagen gels had been cultured for 6 days, they were rinsed with phosphate-buffered saline. The gels were solubilized by the addition of a combination of 2.25 ml collagenase (1.5 mg/ml; Sigma, St. Louis, MO) and 0.25 ml trypsin (0.5%; Life Technologies). After incubation for 1 hour, the cell suspension was repeatedly pipetted to make a solution of single cells. The total number of cells per gel was determined with a Coulter Counter (Coulter Electronics, Luton, UK) by using 1 ml of this cell suspension. The remainder of this cell suspension was used for the assay of differentiation to myofibroblasts.

Assay of Differentiation to Myofibroblasts
To determine the effect of TGF-β1 on the differentiation of myofibroblasts, an immunocytochemical study was conducted using the labeled streptavidin-biotin method and a Histostain-SP kit (Zymed, San Francisco, CA) for the detection of α-smooth muscle actin (α-SMA), a marker for myofibroblasts. A volume of 1.5 ml of the remaining cell suspension was mixed with 1.5 ml MED 10. Then the cells were spun down and resuspended in 6 ml fresh MED 10. The dissociated cells were subsequently replated at low density onto eight-chamber slides (Lab-Tex; Nunc, Naperville, IL). The slides were incubated for 12 hours to allow cell adhesion, after which the cells were rinsed three times with phosphate-buffered saline and were 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 α-SMA 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. M851; Dakopatts, Glostrup, Denmark). Peroxidase visualization was accomplished by adding a solution containing 3-amino-9-ethyl carbazole 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 the total number of cells (the P/T ratio), this ratio was used to assess myofibroblast differentiation. To determine the number of myofibroblasts per gel, we multiplied the total cell number per gel by its P/T ratio.

A mouse monoclonal IgG2a antibody (clone Dak-G05, code no. X943; Dakopatts) was used as the negative control. No immunoreaction was detected in negative control sections.

Statistical Analysis
Data are presented as the mean ± SD. One-way analysis of variance (ANOVA) was used to analyze dose dependency. Post hoc comparisons between groups were made using Fisher's protected least significant difference test. A repeated measures ANOVA was used to analyze the time-course data. Pearson’s correlation coefficients and simple regression analysis were used to evaluate the relationship between collagen gel contraction and the number of cells. A probability value less than 0.05 was accepted as indicating statistical significance.

RESULTS
Control gels containing bovine corneal fibroblasts that were cultured with MED 5 medium alone showed a significant amount of contraction during the 6-day culture period (P < 0.0001; repeated measures ANOVA) causing a contraction to 72.3 ± 1.2% of the original thickness after 6 days (Figs. 1, 2). TGF-β1 increased the contraction of collagen gel mediated by bovine corneal fibroblasts in a dose-dependent manner (Fig. 2). Anti-TGF-β1 antibody (10 μg/ml) blocked the promoting effect of TGF-β1 (1 ng/ml) on the contraction by bovine corneal fibroblasts.

The number of bovine corneal fibroblasts initially plated on the collagen gel was 7.5 × 10^3 cells/gel. After a 6-day culture period, the total number of cells cultured in the MED 5 alone (control) increased to 7.9 ± 0.4 × 10^4 cells/gel. Al-
Corneal Fibroblast Contractility and Myofibroblast Differentiation

**FIGURE 1.** Time course of the increase in collagen gel contraction produced by transforming growth factor-β1 (TGF-β1). Collagen gels were cultured with MED 5 medium alone (control, solid squares) or MED 5 medium containing 0.01 ng/ml TGF-β1 (solid circles), 0.032 ng/ml TGF-β1 (solid triangles), 0.1 ng/ml TGF-β1 (open squares), 0.32 ng/ml TGF-β1 (open circles), or 1 ng/ml TGF-β1 (open triangles). Values are the mean ± SD of three gels, $P < 0.0001$ (repeated measures analysis of variance).

**FIGURE 2.** Dose-dependent increases in collagen gel contraction produced by transforming growth factor-β1 (TGF-β1; $P < 0.0001$, one-way analysis of variance). The thickness of the gel was measured on day 6. AB, anti-TGF-β1 antibody (10 μg/ml). Significant differences were detected among all groups, except between cells cultured with 0 and 0.1 ng/ml TGF-β1, between cells cultured with 0.01 and 0.032 ng/ml TGF-β1, between cells cultured with 0.32 and 1 ng/ml TGF-β1, and between cells cultured with 1 and 10 ng/ml TGF-β1 (Fisher’s protected least significant difference). Values are the mean ± SD of three gels.

**FIGURE 3.** The effect of transforming growth factor (TGF-β1) on bovine corneal fibroblast proliferation ($P < 0.0001$, one-way analysis of variance). The total cell number was measured on day 6. AB, anti-TGF-β1 antibody (10 μg/ml). Significant differences were detected between all groups, except between cells cultured with 0 and 0.1 ng/ml TGF-β1, between cells cultured with 0.01 and 0.032 ng/ml TGF-β1, between cells cultured with 0.32 and 1 ng/ml TGF-β1, and between cells cultured with 1 and 10 ng/ml TGF-β1 (Fisher’s protected least significant difference). Values are the mean ± SD of three gels.

though this proliferation was promoted by low doses of TGF-β1 (0.01 and 0.032 ng/ml), it was inhibited by high doses of TGF-β1 (0.32, 1, and 10 ng/ml) (Fig. 3). Doses of TGF-β1 greater than 0.032 ng/ml decreased the number of the cells in a dose-dependent manner. Anti-TGF-β1 antibody (10 μg/ml) partially blocked the inhibitory effect of TGF-β1 (1 ng/ml) on bovine corneal fibroblast proliferation.

Approximately 0.2% of the cells on the control gels cultured with MED 5 medium alone were α-SMA positive (Fig. 4). TGF-β1 increased the P/T ratio in a dose-dependent manner (Figs. 4, 5A, 5B). Anti-TGF-β1 antibody (10 μg/ml) blocked the promoting effect of TGF-β1 (1 ng/ml) on the P/T ratio.

Although there was no significant correlation between the thickness of the collagen gel and the total number of cells (Fig. 6), there was a significant negative correlation between the thickness of the collagen gel and the number of myofibroblasts (α-SMA-positive cells) (Fig. 7).

**DISCUSSION**

In this study, TGF-β1 promoted the contraction of collagen gel mediated by bovine corneal fibroblasts, a stimulatory effect seen with other cell types.10-12 TGF-β1 also increased the P/T ratio, an indicator of myofibroblast differentiation, of bovine corneal fibroblasts cultured on the collagen gel in a dose-dependent manner. This effect of TGF-β1 on the differentiation of bovine corneal fibroblasts into myofibroblasts was consistent with that seen for rabbit corneal fibroblasts cultured on glass coverslips.27 However, the present study also demonstrated a significant negative correlation between the thickness of the collagen gel and the number of myofibroblasts, but not between the thickness of the collagen gel and the total number of cells.
FIGURE 4. Dose-dependent increase of the ratio of the number of α-smooth muscle actin-positive cells to the total number of cells (P/T ratio) produced by transforming growth factor-β1 (TGF-β1; P < 0.0001, one-way analysis of variance). AB, anti-TGF-β1 antibody (10 μg/ml). Significant differences were detected between cells cultured with 0.032 and 0.1 ng/ml TGF-β1, between cells cultured with 0.1 and 0.32 ng/ml TGF-β1, and between cells cultured with 1 and 10 ng/ml TGF-β1 (Fisher’s protected least significant difference). Values are the mean ± SD of three gels.

of cells. These findings suggest that the ability of TGF-β1 to promote contractile activity of bovine corneal fibroblasts may depend on the myofibroblast differentiation.

The contraction of collagen gel depends on the number of cells on the gel, an increasing number of cells is associated with an increase in gel contraction. TGF-β1 influences the proliferation of fibroblasts in monolayer cultures. In the present study, TGF-β1 also influenced the proliferation of corneal fibroblasts cultured on gels. However, only a dose of 0.01 ng/ml TGF-β1 increased the contraction of the collagen gel associated with promoting corneal fibroblast proliferation. When corneal fibroblasts were cultured with doses of TGF-β1 greater than 0.032 ng/ml, the increased contraction of the collagen gel was not associated with an increase in the total number of cells. It was correlated with the number of myofibroblasts. These findings suggest that the contractility of myofibroblasts is much stronger than that of fibroblasts. In addition, the promoting effect of TGF-β1 on collagen gel contraction by corneal fibroblasts did not depend on its effect on cell proliferation.

We used α-SMA as a marker of myofibroblasts; we did not assess the activity of α-SMA in the gel contraction assay. However, the contraction of collagen gel by fibroblasts depends on actin assembly because cytochalasin D, which inhibits actin-filament polymerization, inhibits the contraction of collagen gel. α-SMA is involved in the contractile activity and in the inhibition of the migration of myofibroblasts. Arora and McCulloch reported that lines of gingival and periodontal ligament fibroblasts constitutively express α-SMA. The contraction of collagen gel mediated by these cells is dependent on the expression of α-SMA and is inhibited by electroinjecting an α-SMA antibody into them. These findings suggest that α-SMA may be involved in the contractile activity of myofibroblasts derived from corneal fibroblasts.

Not only α-SMA expression, but also cell–cell contacts differ between the corneal fibroblasts and myofibroblasts. Petridou and Masur reported that in cultured corneal fibroblasts, connexin 43, the gap junction protein, was expressed at the cell–cell contacts. However, cadherin, a cell–cell junction protein, was not detected. When the corneal fibroblasts differentiated into myofibroblasts by treatment with TGF-β1, the myofibroblasts expressed cadherin along with α-SMA. Thus, cadherin may provide a site for the insertion of actin filaments. A cadherin–actin association may support further an actin-based force generation. Changes in cell–cell contacts caused by TGF-β1 may also influence the degree of contractility between the corneal fibroblasts and myofibroblasts.

The effect of TGF-β on fibroblasts includes changes in the receptors for extracellular matrix proteins, as the integrins. The α2β1 integrin complex is a receptor for collagens. Antibodies for α2 integrin and β1 integrin inhibit the contraction of collagen gel caused by fibroblasts. TGF-β promotes the expression of α2β1 integrin complexes in the fibroblasts. The promotion of integrin expression by TGF-β may influence the contraction of collagen gel. However, no difference was found in the level of expression of the α2β1 integrin complex.

FIGURE 5. The effects of transforming growth factor-β1 (TGF-β1) on the ratio of α-smooth muscle actin (α-SMA)–positive cells to the total number of cells. (A) Immunohistochemical staining of corneal fibroblasts with a monoclonal antibody against α-SMA cultured with MED 5 medium containing TGF-β1 (1 ng/ml). (B) Negative control of A. The sections were stained with 3-amino-9-ethyl carbazole and were counterstained with hematoxylin. Original magnification, ×65.
between lines of fibroblasts that exhibit high versus low gel contraction.5

Collagen gel contraction also depends on matrix remodeling.22 TGF-β upregulates plasminogen activator inhibitor and the tissue inhibitor of metalloproteases,23 both of which inhibit extracellular matrix-degrading enzymes. TGF-β also upregulates the synthesis of collagen.33 These activities of TGF-β may influence collagen gel contraction, but the gel contraction mediated by dermal fibroblasts has been found to be independent of the activation of collagenase secretion by these cells.32

In the present study, immunocytochemical analyses were performed by using a low-density monolayer of replated cells that were removed from the gel by trypsinization. It is possible that the adhesiveness of myofibroblasts to chamber slides differs from that of the fibroblasts. This difference may influence the P/T ratio. To resolve these differences, we previously performed tests with cells on the collagen gels. Although the P/T ratios determined in these preliminary studies resembled those obtained with replated cells, we could not determine the P/T ratios with accuracy because of the high density of the cells and the difficulty in observing them on the thick collagen gel.

In conclusion, the present study showed that TGF-β1 increased the contractile activity and the differentiation in myofibroblasts of bovine corneal fibroblasts. Because contractile activity was correlated with myofibroblast differentiation, the influence of TGF-β1 on the contraction of collagen gel by bovine corneal fibroblasts may depend on the modulation of myofibroblast differentiation. Thus, TGF-β1 may be critically involved in the healing of corneal wounds.

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References


