

The Effect of Corneal Epithelial Cells on the Collagen Gel Contraction by Keratocytes

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Abstract: To understand the mechanism of the contraction of cicatricial tissues after corneal stromal wounding, we investigated the effect of corneal epithelial cells on the collagen gel contraction by keratocytes. Subcultured rabbit keratocytes were embedded in type I collagen gel and cultured. Rabbit corneal epithelial cells were cultured separately and the culture medium was collected and used as an epithelial cell-conditioned medium (ECCM). The collagen gel contraction by keratocytes was determined by measuring the diameters of the collagen disks once a day for 5 days. In the presence of fetal calf serum, the diameter of the collagen gel decreased in linear proportion to the number of keratocytes, but in inverse proportion to the concentration of collagen. The addition of the ECCM enhanced the collagen gel contraction by keratocytes in a dose-dependent manner. This stimulatory activity was found in the ECCM obtained from 3- to 7-day cultures of epithelial cells. These findings suggest that corneal epithelial cells secrete factor(s) that stimulate the collagen gel contraction by keratocytes. **Jpn J Ophthalmol 1998;42:174-179** © 1998 Japanese Ophthalmological Society

Key Words: Cicatricial contraction, collagen gel contraction, corneal epithelial cells, corneal wound healing, keratocytes.

Introduction

When the corneal stroma is injured, keratocytes in the adjacent stroma migrate to the wound, where they phagocytose denatured collagen fibrils and synthesize collagen and proteoglycans, resulting in opaque cicatricial tissues.¹ The cicatricial tissue then begins to contract.² This phenomenon is important for the restoration of normal stromal rigidity. However, this contraction of the scar tissue changes the shape of the cornea and results in a change in corneal refraction.

Recently, many ophthalmologists have advocated corneal refractive surgery, which corrects the refractive error by changing the shape of the cornea. One of the problems of this refractive surgery is the unpredictability of the results.³ Postoperative changes

in corneal curvature over a long period of time sometimes cause instability of the refraction. The contraction of the cicatricial tissue by keratocytes may be involved in this long-term change in corneal curvature. However, the mechanism of the contraction of the cicatricial tissue has not been clarified.

In corneal wound healing, the epithelial cells and keratocytes are thought to modulate cellular activities. Fibronectin, which is produced by keratocytes, reportedly facilitates epithelial migration.⁴ Both epithelial cells and keratocytes produce various kinds of growth factors^{5,6} and cytokines,⁷ many of which are thought to mediate cellular interactions.

Because keratocytes lie between collagen lamellae in vivo, it is important to study the behavior of keratocytes in a collagen matrix. We developed a model in which keratocytes were cultured in a three-dimensional collagen gel. We used this culture system to study the effects of extracellular collagen on the proliferation and phagocytic activity of keratocytes^{8,9}. Other investigators have cultured dermal fibroblasts in a collagen matrix as an in vitro model of the wound contraction of the skin.¹⁰⁻¹²

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In the present study, to understand the mechanism of corneal stromal wound healing, we investigated the collagen gel contraction activity of keratocytes cultured in a collagen matrix. We also investigated the effect of corneal epithelial cells on the contraction of the collagen gel by keratocytes.

Materials and Methods

Materials

Female albino rabbits were purchased from Hoku-setu Sangyo (Settsu, Osaka). Care and treatment of the animals were undertaken according to the National Institutes of Health Guiding Principles in the Care and Use of Animals.¹³ TC-199 culture medium, minimum essential culture medium (MEM), 0.25% trypsin solution and 0.02% ethylenediaminetetraacetic acid (EDTA) solution were purchased from the Research Foundation for Microbial Diseases of Osaka University (Suita, Osaka); fetal calf serum (FCS) was from Boehringer Mannheim GmbH, Biochemica (Mannheim, Germany); sterilized acid-soluble collagen Type I (Cell Matrix Type Ia) was from Nitta Gelatine (Yao); dispase was from Godo-Syusei (Tokyo); bacterial collagenase (clostridium histolyticum) was from Sigma Chemical (St. Louis, MO, USA); plastic culture ware (Coster 162-cm² flask, 48 wells) was from Toyobo Engineering (Tokyo).

Preparation of corneal epithelial cells and their conditioned medium. Rabbit corneal epithelial cells and keratocytes were prepared and cultured as described by Nishida et al⁸ with some modifications. Rabbits were euthanized by the intravenous injection of sodium pentobarbital. The cornea with scleral rim was excised from the enucleated eyes and washed several times with sterile phosphate-buffered saline (PBS). The epithelium on the stroma was obtained by removing the Descemet's membrane using jeweler's forceps under a dissecting microscope. Then the epithelium was incubated with dispase (2 mg/mL) in TC-199 medium at 37°C for 60 minutes. Epithelial sheets were removed from the stroma by using jeweler's forceps under a dissecting microscope and were treated with EDTA containing trypsin (0.125%) at 37°C for 15 minutes. Single-cell suspensions were obtained through repeated aspirations with a 21-gauge needle and washed in TC-199 medium containing 15% FCS.

These epithelial cells were cultured for 5 days in 162-cm² flasks with TC-199 culture medium containing 15% FCS. The medium was then changed to un-supplemented TC-199 culture medium and cultivation was continued for 3 more days. The medium

was then collected and used as the epithelial cell-conditioned medium (ECCM). After the removal of the epithelium the corneal stroma was incubated with bacterial collagenase (1 mg/mL) in MEM at 37°C for 12 hours. Keratocytes were centrifuged at $1,200 \times g$ for 5 minutes and then cultured in MEM containing 10% FCS at 37°C. Keratocytes of the third through sixth passages were used in all experiments.

Collagen gel contraction by keratocytes. Cultivation of keratocytes embedded in a collagen gel was performed as described by Nishida et al⁸ with some modifications. A Type I collagen solution (3 mg/mL) was mixed with a 10-fold concentration of MEM and neutralized with 0.2 N NaOH. Suspensions of cultured keratocytes were added and the mixture (collagen concentration; 1.9 mg/mL) was placed in a 48-multiwell plate (300 μ L/well), which had been previously coated with bovine serum albumin (BSA) (1 mg/mL). After incubation at 37°C for 1 hour to form a gel, MEM containing 12.5% FCS (300 μ L) was placed over the gel. Keratocytes were cultivated in the collagen gel for 5 days. During the cultivation the collagen gels detached spontaneously from the plastic plates and floated in the medium. The contraction of the collagen gel by keratocytes was then measured by the diameter of the gel. In some experiments we investigated the effect of different concentrations of both FCS in the medium and collagen in the gels. Various numbers of keratocytes ($0, 3 \times 10^3, 1 \times 10^4, 3 \times 10^4$ cells) were used to investigate the activity of the collagen gel contraction by keratocytes. In other experiments 3×10^4 cells were embedded in the collagen gel and various concentrations (0%, 10%, 20%, 30%, or 40% at the final concentration) of ECCM were added to the medium. In the final series of experiments we used the conditioned medium obtained after 1, 3, 5, 7, or 10 days of epithelial culture. Results were expressed as the mean of four assays (\pm SEM).

To estimate the collagen gel contractor activity of keratocytes, we calculated a contraction index (CI) based on the diameters of the gels initially and after 5 days of cultivation.

$$CI = \frac{\text{initial diameter of gel} - \text{diameter of gel after 5 days}}{\text{initial diameter of gel}}$$

Results

To investigate the collagen gel contraction by keratocytes, various numbers of cells were cultured in collagen gel with 12.5% FCS (Figure 1). The diameters of the collagen gels without keratocytes did

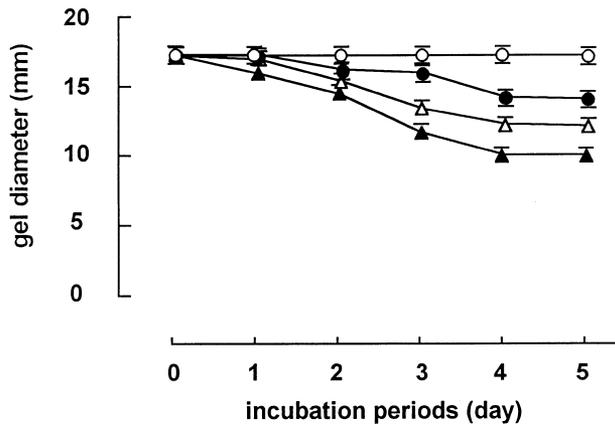


Figure 1. Collagen gel contraction by keratocytes. Various numbers of keratocytes were embedded in collagen gel (collagen concentration; 1.9 mg/mL) and cultured for 5 days in the presence of 12.5% of fetal calf serum. Gels without keratocytes did not contract. When keratocytes were embedded in gel, gel contraction depended on the initial number of cells. Each point represents mean of gel diameter in four assays (\pm SEM). \circ No keratocytes; \bullet 3×10^3 cells; \triangle 1×10^4 cells; \blacktriangle 3×10^4 cells.

not change over the 5-day incubation. However, gels with embedded keratocytes began to shrink after 1 day of cultivation and continued to decrease in diameter in proportion to the cultivation period. Furthermore, the collagen gel contraction was also found to depend on the initial number of embedded cells. When 3×10^4 cells were cultured for 5 days, the gel diameters were approximately 11 mm.

To investigate the effect of FCS on the collagen gel contraction by keratocytes, the cells (3×10^4) were cultured in gels and MEM containing various concentrations of FCS. When the cells were cultured in the absence of FCS, no collagen gel contraction was observed, as shown in Figure 2. On the other hand, the gel diameter decreased in proportion to the concentration of FCS when the cells were cultured in the presence of FCS.

The effect of collagen concentration on the gel contraction by keratocytes was also investigated. For this purpose, keratocytes (3×10^4) were embedded in gels containing various concentrations of collagen and were cultured in the presence of 12.5% FCS (Figure 3). The diameters of the gels were inversely proportional to the collagen concentration. The diameters of the gels containing collagen at the concentration of 0.24 mg/mL shrank to approximately 4 mm in diameter after 3 days of cultivation. Collagen solution thinner than 0.24 mg/mL did not form a gel.

To investigate the effect of corneal epithelial cells on the collagen gel contraction by keratocytes, we

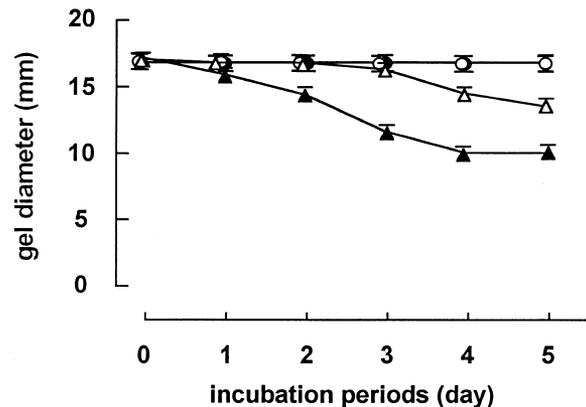


Figure 2. Effect of fetal calf serum (FCS) on collagen gel contraction by keratocytes. Keratocytes (3×10^4) in collagen gel (collagen concentration; 1.9 mg/mL) were cultured for 5 days in the presence of various concentrations of FCS. In the absence of FCS, gel diameter did not change. When FCS was added to the medium, gel diameter decreased in proportion to cultivation periods. Each point represents the mean of gel diameter in four assays (\pm SEM). \circ No FCS; \bullet 3.13%; \triangle 6.25%; \blacktriangle 12.5%.

added various concentrations of ECCM (0%, 20%, 30%, 40% at final concentration) to MEM containing 12.5% FCS. As shown in Figures 4 and 5, the diameters of the gels both with and without ECCM decreased in proportion to the cultivation periods. However, the gels cultured with ECCM shrank more rapidly than those without ECCM and this effect appeared to be dose-dependent. These findings suggested

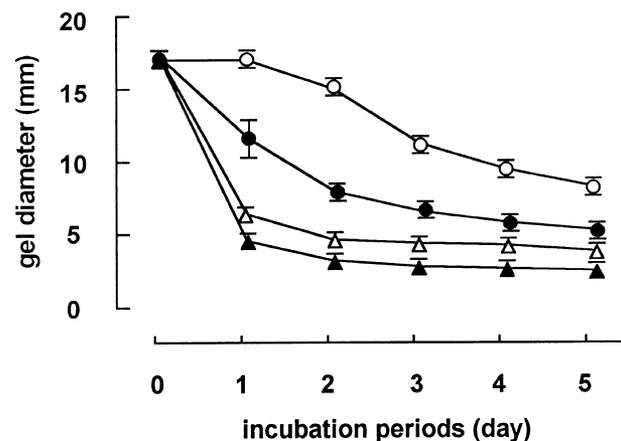


Figure 3. Effect of collagen concentration on collagen gel contraction by keratocytes. Keratocytes (3×10^4) were embedded in collagen gels containing various concentrations of collagen and cultured for 5 days in the presence of 12.5% FCS. Each point represents the mean of gel diameter in four assays (\pm SEM). \circ 1.9 mg/mL; \bullet 0.95 mg/mL; \triangle 0.48 mg/mL; \blacktriangle 0.24 mg/mL.

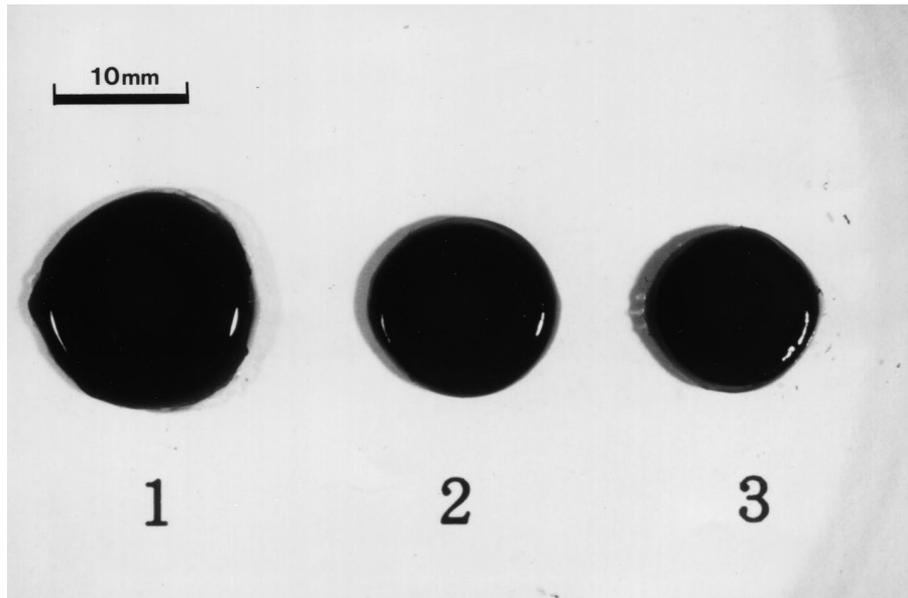


Figure 4. Collagen gel contraction by keratocytes. Keratocytes (3×10^4) were cultured in collagen gel (collagen concentration, 1.9 mg/mL) for 5 days. Then gels were fixed and stained with 1% crystal violet. (1) Gel cultured without fetal calf serum (FCS); (2) Gel cultured with FCS (12.5%); (3) Gel cultured with FCS (12.5%) and epithelial cell-conditioned medium (40%).

that ECCM contained some soluble factor(s) that accelerated the collagen gel contraction by keratocytes.

We also used the conditioned medium collected after various culture lengths (Figure 6). The medium obtained from 3- to 7-day cultures had a stimulatory effect on the collagen gel contraction by keratocytes. The medium after 5-day cultivation had the greatest stimulatory effect.

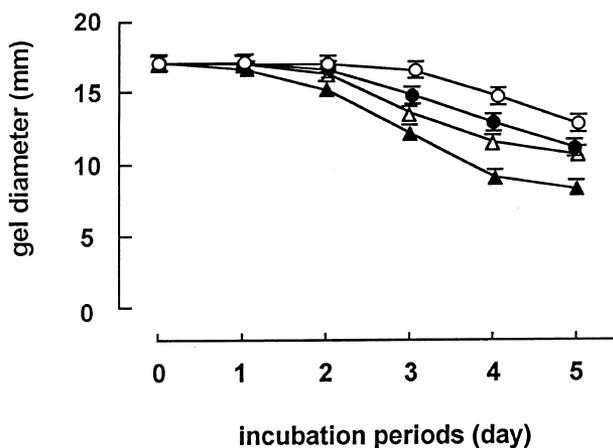


Figure 5. Effect of epithelial cell-conditioned medium (ECCM) on collagen gel contraction by keratocytes. Keratocytes (3×10^4) were embedded in collagen gel (collagen concentration; 1.9 mg/mL) and cultured for 5 days in the presence of fetal calf serum (12.5%). Various concentrations of ECCM were added. Each point represents the mean of gel diameter in four assays (\pm SEM). \circ ECCM 0%; \bullet 20%; \triangle 30%; \blacktriangle 40%.

Discussion

In the present study, keratocytes cultured in a collagen gel contracted the collagen gel in the presence of FCS; the degree of the collagen gel contraction depended on both the initial number of cells and the concentration of FCS. The contraction was also inversely proportional to the collagen concentration of the gel. ECCM accelerated the collagen gel contraction by keratocytes.

It has been well accepted that the interaction between cells and the extracellular collagen plays an important role in wound healing. Extracellular col-

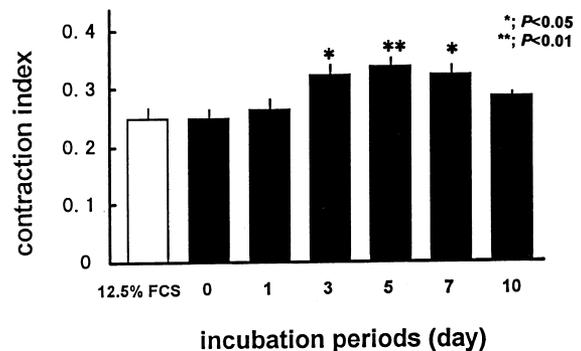


Figure 6. Effect of epithelial cell-conditioned medium (ECCM) collected after various periods of epithelial cell culture. Keratocytes were embedded in collagen gel (collagen concentration, 1.9 mg/mL) and cultured with fetal calf serum (12.5%) and ECCM (40%) for 5 days. Contraction index (CI) was calculated. Each bar represents the mean of CI in four assays (\pm SEM). *; $P < 0.05$; **; $P < 0.01$.

lagen modulates various cellular functions of keratocytes, such as proliferation.⁸ On the other hand, various types of cells (i.e., skin fibroblasts,^{10,11} melanoma cells,¹⁴ and retinal pigment epithelial cells¹⁵⁻¹⁷) have been reported to contract collagen gels. In this study, keratocytes had the same effect and our results are in agreement with those of other experiments.^{10,11,14-17} However, the mechanism of the collagen gel contraction by cells remains unclear. Guidry and Grinnel¹¹ reported that collagen degradation did not contribute to the collagen gel contraction by human skin fibroblasts. Okamoto¹⁸ also reported that keratocytes did not degrade extracellular collagen in the absence of plasmin. We confirmed that collagen degradation did not occur in this study (data not shown). These findings suggest that collagen degradation is not involved in the collagen gel contraction.

Integrins, receptors for extracellular matrix proteins on cell surfaces, are thought to be involved in the interaction between the cells and extracellular matrix proteins.^{19,20} Indeed, integrin $\alpha_2\beta_1$ was reported to be a collagen receptor^{21,22} and it has been suggested that integrin $\alpha_2\beta_1$ contributes to the collagen gel contraction because monoclonal antibodies against integrin $\alpha_2\beta_1$ inhibited the collagen gel contraction by human fibroblasts, melanoma cells and retinal pigment epithelial cells.^{11,14,23,24} The exact role of integrin $\alpha_2\beta_1$ in the collagen gel contraction by keratocytes is still unclear.

Although proliferation of keratocytes might affect the collagen gel contraction, earlier findings suggest that this is not the case. The growth of skin fibroblasts in a contracting gel was reportedly suppressed,¹² and we found that the growth of keratocytes in a collagen gel was suppressed in proportion to the concentration of extracellular collagen.⁸

The presence of FCS was necessary for the collagen gel contraction by keratocytes in our study. Other investigators likewise reported the necessity of serum for the collagen gel contraction by fibroblasts.¹¹ Various growth factors and cytokines affect the collagen gel contraction by cells; the collagen gel contraction by fibroblasts was reportedly stimulated by transforming growth factor- β (TGF- β) alone^{25,26} or with interleukin-1 (IL-1),¹⁶ platelet-derived growth factor (PDGF),^{27,28} epidermal growth factor (EGF),²⁹ or basic fibroblast growth factor (b-FGF) at low concentrations.²⁶ b-FGF at high concentrations²⁶ and interferon- γ (INF- γ)²⁸ were reported to inhibit the collagen gel contraction. It is unclear whether or not the effect of serum on the collagen gel contraction in the present study depended on the presence of these factors in the serum.

The corneal stroma is covered by the corneal epithelium. The interaction between the epithelium and the stroma is thought to play some role in wound healing. In the present study, corneal epithelial cells apparently produced soluble factors (s) that stimulated the collagen gel contraction by keratocytes. Since corneal epithelial cells reportedly synthesize cytokines, such as TGF- β ,⁶ EGF,⁵ and IL-1,⁷ these cytokines may affect various activities of keratocytes, including the growth and production of matrix metalloproteases.^{6,18,29} Therefore, this study was thought to demonstrate the actual interaction between the epithelium and the stroma in corneal wound healing. It is still unknown whether or not the contraction-stimulating factor(s) in ECCM are identical to any of these cytokines. Our future studies include isolation and identification of the contraction-stimulating factor(s) in ECCM.

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