SPARC from Corneal Epithelial Cells Modulates Collagen Contraction by Keratocytes

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PURPOSE. Contraction of the scar tissue during corneal wound healing changes the shape of the cornea and corneal refraction. In a previous study, it was found that corneal epithelial cells secrete the factor that stimulates collagen gel contraction by keratocytes in vitro. The purpose of the present study was to purify and identify the contraction-stimulating factor derived from corneal epithelial cells.

METHODS. The cultured medium of rabbit corneal epithelial cells was collected and used as an epithelial cell-conditioned medium (ECCM). Subcultured rabbit keratocytes were embedded in a collagen gel, and collagen gel contraction was investigated. The contraction-stimulating factor in the ECCM was purified through acetone precipitation, affinity chromatography (heparin Sepharose), gel filtration, and reversed-phase chromatography. The amino acid sequence of a contraction-stimulating factor was analyzed.

RESULTS. Collagen gel contraction by keratocytes was enhanced by the addition of ECCM in a dose-dependent manner. The amino acids sequence of the contraction-stimulating factor was homologous to a 32-kDa glycoprotein, a secreted protein that is acidic and rich in cysteine (SPARC). Western blot analysis confirmed that SPARC was contained in the ECCM. Collagen gel contraction by keratocytes was enhanced by the addition of purified SPARC in a dose-dependent manner. SPARC was found in the basal layer of the migrating epithelium and activated keratocytes adjacent to the wound 3 days and 1 week after perforating injury in rabbit corneas.

CONCLUSIONS. Epithelial cells secrete SPARC, which modulates the contraction of scar tissue in the corneal stroma. (Invest Ophthalmol Vis Sci. 1998;39:2547-2553)

In a recent decade, refractive corneal surgery, in which corneal stroma is incised to change the corneal curvature, has been in use worldwide. However, the mechanism of stromal wound healing of the cornea is not fully understood. Jester et al.1 have proposed that corneal wounds undergo a biphasic change in wound gape after radial keratotomy, including a precontractile and contractile phase. In the contractile phase, myofibroblasts are thought to play a central role in wound contraction.2,3 Myofibroblasts are characterized by their excessive expression of α-smooth muscle actin, N-cadherin, and integrin α5β1.4,5 Myofibroblasts have been observed in the wounded lesion of the corneal stroma, and they are thought to be transformed from keratocytes.2,5,6,7

In 1979, Bell et al.8 reported that fibroblasts incorporated in a collagen gel a progressive contraction of the gel, resulting in the formation of a dense collagen disc of markedly reduced diameter. This phenomenon has been considered to be the in vitro equivalent of the connective tissue contraction that occurs during wound healing and other biologic processes. The collagen gel contraction assay has been used as a simple method of quantitating the contractile ability of fibroblasts.9-11 Similar collagen gel contraction activity has been found in retinal pigment epithelial cells.12-14 We have also reported that keratocytes cultured in a collagen gel contract the collagen gel.15

The interaction between epithelial cells and keratocytes is another important factor in corneal wound healing. Signals transmitted from the stroma to the epithelium or vice versa constitute the basis of reciprocal epithelial-mesenchymal interactions. In theory, these epithelial-mesenchymal interactions can be mediated by such signals as extracellular matrices and cytokines. However, the signals that are actually transmitted from the epithelium to the stroma have not been clarified. In a previous study,15 we found that corneal epithelial cells secrete factors that stimulate collagen gel contraction by keratocytes. In this study, we tried to identify the contraction-stimulating factor that was derived from corneal epithelial cells.

MATERIALS AND METHODS

Materials

Female albino rabbits were purchased from Hokuseitsu Sangyo (Settsu, Osaka, Japan). Care and treatment of the animals were handled in accordance with the ARVO State-
Preparations of Cells and the Conditioned Medium of Corneal Epithelial Cells

Rabbit corneal epithelial cells and keratocytes were prepared and cultured as described previously, with some modifications. Descemet’s membrane, with the endothelium, was removed from a sclerocorneal button using a jeweler’s forceps under a dissecting microscope. Subsequently, the stromal collagen in the cornea was digested by incubation with 2 mg/ml of bacterial collagenase for 2 hours, and the keratocytes were collected by centrifugation at 1200g for 5 minutes. Single-cell suspensions were obtained by repeated pipetting. The epithelial sheets were then removed from the stroma with a spatula and treated with trypsin (0.125%) and EDTA (0.01%) at 37°C for 15 minutes. Single-cell suspensions were obtained by repeated pipetting. The epithelial cells were cultured for 5 days in 162-cm² flasks with TC-199 medium containing 15% FCS. The medium was exchanged with the un-supplemented TC-199 culture medium, and cultivation was carried out for the subsequent 3 days. The medium was then collected and used as an epithelial cell-conditioned medium (ECCM).

After the removal of the epithelium, the stromal collagen was digested by incubation with 1 mg/ml bacterial collagenase in minimum essential medium (MEM) at 37°C for 12 hours. Keratocytes were collected by centrifugation at 1200g for 5 minutes and cultured in MEM containing 10% FCS. In all experiments, keratocytes of the third through sixth passages were used.

Collagen Gel Contraction by Keratocytes

Collagen gel contraction was estimated by a method described previously. A type 1 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 300 μl/well collagen solution was placed in a 48-well plate, which had been previously coated with 1 mg/ml bovine serum albumin. After incubation at 37°C for 1 hour to form a gel, MEM containing 300 μl 10% FCS was placed over the gel. Keratocytes were cultivated in a collagen gel for 5 days. During the cultivation, the collagen gels detached spontaneously from plastic plates and floated in the medium. The collagen gel contraction by keratocytes was then estimated by measuring the diameter of the gels.

Western Blot Analysis

The acetone-precipitable fraction of the ECCM was mixed with the sample loading buffer and treated with 5% 2-mercaptoethanol at 100°C for 5 minutes. Subsequently, the samples were subjected to electrophoresis on precast 4% to 15% gradient polyacrylamide gel (Funakoshi). Electrophoresis was performed at room temperature (40 mA constant current). Proteins were visualized by silver staining. After transfer to a nitrocellulose membrane, the existence of SPARC in the conditioned medium was detected by immunoblot analysis using a mouse anti-human SPARC monoclonal antibody and rabbit peroxidase-conjugated anti-mouse IgG.

Immunohistochemical Study

After rabbits were anesthetized by intravenous injection of pentobarbital sodium, a 5-mm perforated incision was made at the center of each cornea with a razor blade. Three days and 1 week after incision, rabbits were killed by intravenous injection of an overdose of pentobarbital sodium. Corneas were dissected, fixed for more than 4 hours in 10% formaldehyde solution, and embedded in paraffin. Thin sections (4 μm) were made, deparaffinized, rehydrated, and incubated with normal goat serum diluted 10 times in 0.05 M Tris-buffered saline (pH 7.5) for 30 minutes at room temperature. Specimens were then incubated overnight at 4°C with monoclonal anti-human SPARC antibody and then incubated for 30 minutes at room temperature with biotin-conjugated anti-mouse IgG. In the control study, the anti-SPARC antibody was preabsorbed by incubation with SPARC overnight at 4°C and centrifugation. After washing three times in Tris-buffered saline, the samples were incubated with streptavidin–horseradish peroxidase avidin–biotin complex (streptABComplex/HRP, Dako) for 30 minutes at room temperature and then incubated for 30 minutes with 0.1% 3,3-diaminobenzidine tetrahydrochloride solution.

RESULTS

ECCM Stimulates Collagen Contraction by Keratocytes

First we confirmed the stimulatory effect of ECCM on collagen gel contraction by keratocytes. Keratocytes were embedded in a collagen gel and cultured for 5 days with various concentrations of ECCM in the presence of 12.5% FCS. The diameter of the collagen gel decreased in proportion to the concentration of the ECCM added (Fig. 1). These findings showed that the ECCM included soluble factors that enhanced collagen gel contraction by keratocytes. To investigate the effect of the ECCM on the proliferation of keratocytes in a contractile gel, we measured the number of the cells after 5 days’ cultivation. Collagen gels were degraded by incubation with 2 mg/ml collagenase for 2 hours, and the cells collected through centrifugation were counted using a hemocytometer. The number of the cells seemed to de-
were cultured in minimum essential medium supplemented with 12.5% serum and various concentrations of the ECCM. The diameters of gels were measured after 5 days' cultivation. After 5 days' cultivation with ECCM, the gels were degraded by incubation with 2 mg/ml collagenase, and the number of the cells dissociated from a gel was counted. Each point represents mean ± SEM; \( n = 4 \).

Purification and Identification of a Contraction-Stimulating Factor Derived from Corneal Epithelial Cells

A contraction-stimulating factor derived from corneal epithelial cells was purified and identified by the steps shown (Fig. 2A). Initially, ECCM was treated with 70% cold acetone and stored at 4°C overnight. The contraction-stimulating effect of the ECCM was maintained in the precipitable fraction of the ECCM. The precipitate was dissolved with 10 mM phosphate buffer (pH 7.0) and was applied to a column of Heparin Sepharose 4B. Contraction-stimulating activity was found in the fraction eluted with 1 M NaCl (buffer, 10 mM phosphate buffer [pH 7.0]; flow, 0.2 ml/min). After dialysis with 100 mM phosphate buffer (pH 7.0), the fraction was subjected to gel filtration using Shodex Asahipak GS 520 HQ column (buffer, 100 mM phosphate buffer [pH 7.0]; flow, 0.5 ml/min). Contraction-stimulating activity was found between the 21st and 24th fractions; their molecular masses were equivalent to approximately 40 kDa (Fig. 2B). After dialysis with distilled water, the recovered proteins were fractionated by a reversed-phase chromatography column (μRPC C2/C18 PC 3.2/3; Pharmacia Biotech) using a commercial system (SMART; Pharmacia Biotech AB, Uppsala, Sweden). The contraction-stimulating activity was observed in the fraction eluted with 51% acetonitrile (A solution 0.1% trifluoroacetic acid; B solution, 0.1% trifluoroacetic acid, 70% acetonitrile; flow, 0.2 ml/min; Fig. 2C).

Amino Acid Sequence

The amino acid sequence of the contraction-stimulating factor was analyzed using a protein-peptide sequencing system (Model 477A; Applied Biosystems, Foster City, CA). In amino acids analysis, 12 amino acids within 14 amino acids from the N-terminal end of the final preparation, APOQELP-ET-VV, were determined. This amino acid sequence was also found in rabbit, porcine, human, and bovine SPARC (Fig. 3).19-22 These findings suggest that SPARC was the contraction-stimulating factor derived from corneal epithelial cells.

Existence of SPARC in ECCM

To investigate the existence of SPARC in ECCM, the aceton-precipitable fraction of the ECCM was subjected to electrophoresis, and western blot analysis was performed using anti-human SPARC monoclonal antibody (ON1-1). Immunoreactive mass bands of SPARC in the ECCM were detected at approximately 30 kDa to 43 kDa (Fig. 4A) and were thought to be spontaneous fragmentation products, as noted by others.16,17 These findings indicated that the ECCM contained SPARC and that cultured corneal epithelial cells produced and secreted SPARC into the medium. SPARC is reported to be associated with platelet-derived growth factor (PDGF). Therefore, to rule out the possibility of the contamination of PDGF, we determined the amount of PDGF in the ECCM, the final purified preparation, and commercial human SPARC by enzyme-linked immunosorbent assay (Fig. 4B). PDGF was found in the ECCM (1 ng/ml) or in commercial human SPARC (0.1 ng/10 μg SPARC). However, PDGF in the final preparation or in MEM was lower than the sensitivity of the kit.

Effect of Purified SPARC on Collagen Gel Contraction by Keratocytes

To confirm that SPARC can stimulate collagen gel contraction by keratocytes, we added various concentrations of purified SPARC from human platelets (Hematologic Technologies) to the cultured medium. When SPARC was added, diameters of collagen gels after 5 days' cultivation decreased in a dose-dependent manner (Fig. 5). At a concentration of SPARC of more than 100 ng/ml, statistically significant differences between the treated and the control (0 ng/ml) cells were observed. These findings indicate that SPARC stimulated collagen gel contraction by keratocytes. They also suggest that SPARC played a significant role in the contraction-stimulating effect of ECCM.

Localization of SPARC in Corneal Wound Healing

The localization of SPARC in normal and wounded rabbit corneas was observed immunohistochemically (Fig. 6). In the normal cornea, immunoreactive staining was not found in the epithelium or stroma (Fig. 6A). In contrast, 3 days after perforating injury, immunoreactive products for SPARC were found in the basal layer on the migrating epithelium (Fig. 6B). SPARC was also found to be associated with keratocytes adjacent to the wound 1 week after perforating injury (Fig. 6C). These findings indicate that SPARC may play a role in corneal wound healing.
**DISCUSSION**

In this study, we found that keratocytes contracted a collagen gel, and SPARC, which was secreted from corneal epithelial cells stimulated collagen gel contraction by keratocytes. Furthermore, SPARC was found in the epithelium and the stroma of wounded cornea.

It was proposed that the interaction between corneal epithelial cells and keratocytes play an important role in corneal wound healing. This hypothesis is based on the fact that growth factors and cytokines secreted from epithelial cells regulate the cellular functions of keratocytes and vice versa. Extracellular matrices are thought to send signals from epithelial cells to keratocytes. However, there has been little evidence to prove this hypothesis. In the present study, the conditioned medium of corneal epithelial cells stimulated collagen gel contraction by keratocytes; the present results may therefore partially prove the hypothesis. Furthermore, it was shown that the contraction-stimulating factor derived from the epithelial cell was SPARC, one of the extracellular matrix proteins.

Many investigators have studied the factors that regulate collagen gel contraction by the cells. In these studies, various growth factors and cytokines (TGF-β alone, TGF-β with interleukin-1, PDGF, epithelial growth factor, basic fibroblast growth factor) stimulates collagen gel contraction by the cells. Integrins on the cell surface and matrix metalloproteinase-2 were also reported to contribute to collagen gel contraction by the cells. The present study showed that SPARC stimulated collagen gel contraction by keratocytes. The enzyme-linked immunosorbent as-

**Figure 2.** (A) Schema showing steps in purification of contraction-stimulating factor from epithelial cell-conditioned medium (ECCM). (B) The elution profile of gel filtration using Shodex Asahipak GS 520 HQ column (Funakoshi). (C) The elution profile of reversed-phase chromatography using the SMART system and the μRPC C2/C18 PC 3.2/3 column.

**Figure 3.** Comparison of the amino acid sequence of the contraction-stimulating factor. Twelve amino acids were determined within 14 amino acid sequences from the N-terminal of the purified contraction-stimulating factor. The amino acid sequence was compared with that of secreted protein that is acidic and rich in cysteine (SPARC). Amino acid sequence of SPARC (rabbit, porcine, human, and bovine) are from the SWISS-PROT protein sequence database.
say showed the contamination of commercial human SPARC with PDGF (0.01 ng/μg SPARC). However, we found that PDGF in a concentration less than 1 ng/ml did not affect the collagen gel contraction by keratocytes (data not shown). Recently, Iruela-Arispe et al. 35 also found that the addition of exogenous SPARC enhanced collagen gel contraction by type 1 collagen-deficient mouse fibroblasts. These findings may indicate that SPARC enhances the interaction between cells and fibrillar collagen. It was also suggested that SPARC plays a significant role in collagen gel contraction by keratocytes.

SPARC, which is also called BM 40, 43 K protein, or osteonectin, is a secreted glycoprotein widely distributed in human and murine tissues undergoing developmental regulation. 36 However, the specific role of SPARC in vivo remains elusive. It inhibits the spread of newly plated cells and promotes rounding of attached cells, diminishes focal adhesions and promotes cytoskeletal (actin) rearrangement, increases levels of matrix metalloproteinase and plasminogen activator inhibitor-1, decreases extracellular matrix production, and modulates the cell cycle. 36 The expression of SPARC has been associated with cell-matrix interactions during remodeling, morphogenesis, migration, and the proliferative process. 37 SPARC has been found to interact with extracellular matrix components, growth factors, and cytokines and to regulate matrix metalloproteinase expression. 36–38 Such diverse functions indicate that SPARC may profoundly influence cell-matrix interactions. In the present study, we found that after perforating injury, SPARC appeared in the epithelium and around keratocytes adjacent to the wound. Latvala et al. 39 reported similar findings in which SPARC appeared transiently at the basal epithelial cells after photorefractive keratotomy. These findings suggest that SPARC plays a certain role in the wound healing of the cornea. They also reported that SPARC was not detected in the stroma of normal and wounded corneas. The difference between our results and those in previous reports may arise from the extent of damage of the stroma.

### Table 1

<table>
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<th>PDGF concentration</th>
<th>10% FCS in MEM</th>
<th>N.D.</th>
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<tbody>
<tr>
<td>ECCM</td>
<td>1.1 ng/ml</td>
<td></td>
</tr>
<tr>
<td>Commercial SPARC</td>
<td>0.01 ng/μg SPARC</td>
<td>N.D.</td>
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<tr>
<td>Final preparation</td>
<td>N.D.</td>
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**FIGURE 4.** (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis. Lane 1: Molecular weight markers; lane 2, the acetone-precipitable fraction of the epithelial cell-conditioned medium (ECCM); lane 3, immunoblot of lane 2 with anti-human secreted protein that is acidic and rich in cysteine (SPARC) mouse monoclonal antibody and peroxidase-conjugated anti-mouse IgG. (B) Amount of PDGF in the epithelial cell-conditioned medium.
The present results suggest that SPARC from corneal epithelial cells contributes to the wound contraction of the cornea. Further investigation examining the precise role of SPARC in corneal wound healing are needed.

References


25. Wilson SE, Walker JW, Chwang EL, He YG. Hepatocyte growth factor, keratinocyte growth factor, their receptors, fibroblasts


