Serum-Free Spheroid Culture of Mouse Corneal Keratocytes

Satoru Yoshida,1,2 Shigeto Shimmura,1,3 Jun Shimazaki,3 Naoshi Sbinozaki,1 and Kazuo Tsubota1,5,4

PURPOSE. To develop a serum-free mass culture system for mouse keratocytes.

METHODS. Corneas of C57BL/6/J mice were enzyme digested after the epithelium and endothelium were removed. Stromal cells were cultured in serum-free DMEM/F12 (1:1) containing epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and B27 supplement. Primary spheres were dissociated by trypsin and subcultured as suspended secondary cells. Cells from postnatal day (P)6 to P10 spheres were subcultured onto plastic dishes or type I collagen gels for phenotype analysis. The expression of the keratocyte markers keratocan, aldehyde dehydrogenase (Aldh), and CD34, were analyzed by RT-PCR, and vimentin and α-smooth muscle actin (α-SMA) were examined by immunocytochemistry.

RESULTS. Primary keratocytes formed spheres, which were cultured for over 12 passages. Suspended sphere cells expressed vimentin, keratocan, CD34, and lumican, but were negative for cytokeratin K12 (K12) and Pax6. Sphere cells subcultured on plastic exhibited a dendritic morphology characteristic of keratocytes, and maintained keratocan, Aldh, and CD34 expression in serum-free medium. Sphere cells subcultured with 10% serum became fibroblastic, and expressed α-SMA when stimulated by transforming growth factor (TGF)-β. α-SMA-positive cells demonstrated contractile properties on collagen gels, compatible with the myofibroblast phenotype.

CONCLUSIONS. The phenotype of mouse keratocytes can be maintained in vitro for more than 12 passages by the serum-free sphere culturing technique. (Invest Ophthalmol Vis Sci. 2005;46:1653–1658) DOI:10.1167/iovs.04-1405

The corneal stroma is characterized by a well-organized extracellular matrix consisting of a dense network of collagen fibrils and proteoglycans that are produced by keratocytes, the principal stromal mesenchymal cell of cranial neural crest origin.1,2 In adult tissue, keratocytes are mitotically quiescent cells with a flat, dendritic morphology. Keratocytes form a three-dimensional network of cells through their extensive dendritic processes, linked via gap junctions.3–10 and secrete collagens and keratan sulfate proteoglycans such as lumican, mimecan, and keratan.11–15 The corneal stroma is rich in total keratan sulfate proteoglycan content,16 but contain relatively small amounts of dermatan sulfate proteoglycans.17

During corneal wound healing, the quiescent keratocytes are activated and transform into fibroblasts and/or myofibroblasts, losing their characteristic dendritic morphology. Keratan sulfate proteoglycans are downregulated,11,12 whereas keratocytes proliferate and migrate to the site of injury, causing scar formation.19–22 The conversion to myofibroblasts, characterized by intense expression of the contractile protein α-smooth muscle actin (α-SMA),23,24 is induced by endogenous and exogenous transforming growth factor (TGF)-β.25–27

Ex vivo expansion of keratocytes is often performed to investigate keratocytes in vitro, and various culture techniques have been reported involving the use of plastic substrates. However, when cultured in serum-containing medium, collagenase-isolated keratocytes from bovine28 and rabbit29,30 corneas readily lose their in vivo quiescent phenotype and acquire a fibroblastic phenotype with altered physiological properties.28,30,31 In the presence of 2% to 10% serum, keratan sulfate proteoglycan production is greatly reduced or absent in keratocyte-derived fibroblasts.28,32,33 whereas production of dermal sulfate proteoglycans is upregulated. Furthermore, TGF-β stimulation or culture at low densities30 causes corneal fibroblasts to differentiate further into myofibroblasts with a more spread-out morphology.26,29,32,34 Serum-free cultures have been reported to be effective in the maintenance of the dendritic morphology of keratocytes and the production of keratan sulfate proteoglycans.27,28,30–33,35,36 However, the cultivation of a large quantity of cells by subculturing has been difficult.

In this report, we introduce our method for subculturing mouse corneal keratocytes in large quantities, using a modified version of a suspension culture method originally described for neural stem cells.37–39 In our study, the sphere culture of keratocytes did not require serum, and the dendritic keratocyte phenotype was restored when subcultured on plastic substrate in serum-free medium.

MATERIALS AND METHODS

Cell Culture

All animals were handled in full accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Stromal cells were dissociated from adult C57BL/6/J mice (7–8 weeks old) and then cultured as described previously40 with modifications. In brief, cornea tissue was excised in Hanks balanced salt solution (HBSS) supplemented with 10% fetal bovine serum (FBS) by circular incision outside the limbus. The iris, ciliary body, and Descemet’s membrane including the endothelium were bluntly dissected from the cornea. The remaining stroma with epithelium was incubated in 5 mg/mL of Dispase II (Roche Diagnostics, Indianapolis, IN) at 4°C overnight. Loose epithelial sheets were then removed, and corneal stromal discs were cut into small segments and digested in 0.05% trypsin (Sigma-Aldrich, St. Louis, MO) for 50 minutes at 37°C, followed by 78 U/mL...
collagenase (Sigma-Aldrich) and 38 U/mL hyaluronidase (Sigma-Aldrich) for 30 minutes at 37°C.

Stromal cells were mechanically dissociated into single cells, and cultured in DMEM/F12 (1:1) supplemented with 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 10 ng/mL of fibroblast growth factor 2 (FGF2, Sigma-Aldrich), B27 supplement (Invitrogen, Carlsbad, CA), and 100 U/mL leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA) at a density of 5 × 10⁵ cells/ml in a 37°C 5% CO₂ incubator. Initial culture was performed in 24-well plates or 35-mm dishes and then subcultured to 25-cm² culture flasks. The spheres were then further subcultured in 75 cm² culture flasks after 7 to 14 days, which was repeated every 7 to 14 days. Medium was changed every 5 to 7 days. All dishes and flasks used for sphere culture were polystyrene, noncoated vessels obtained from Asahi Techno Glass (Tokyo, Japan). Stromal sphere cells were examined by immunocytochemistry and RT-PCR. To allow cells to differentiate, cells dissociated from cornal spheres were cultured in serum-free or DMEM/F12 medium (10% FBS) supplemented with or without 2 ng/mL TGF-β (Sigma-Aldrich) for 4 days. Subcultured cells were stained by calcein-AM (Dojindo Laboratories, Tokyo, Japan), as described, to visualize cell morphology. Primary stromal discs of mouse cornea were mechanically dissociated into single cells, and immediately frozen in liquid N2. cDNAs were synthesized with a cDNA synthesis kit (Life Sciences, Inc., St. Petersburg, FL) from total RNA also prepared with a kit (RNeasy; Qiagen, Hilden, Germany). Gene-specific primers used for cytokeratin K12 (K12), Pax6, vimentin, keratocan, lumican, CD44, aldehyde dehydrogenase (Aldh), and Gapd are shown in Table 1. PCR was then performed (GeneAmp 9700; Applied Biosystems, Foster City, CA). The PCR products were analyzed by agarose gel electrophoresis.

**Collagen Gel Contraction Assay**

Collagen gel contraction assay was performed as described previously, with some modifications. Collagen gels were prepared according to instructions provided by the manufacturer (Cellmatrix Type I; Nitta Gelatin, Osaka, Japan). In brief, collagen was mixed with 106-fold concentrated DMEM/F12 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. Then a 0.2 mL aliquot of the solution was placed in the center of each well of a six-well cell culture cluster (Corning Inc., Corning, NY) and allowed to polymerize at 37°C for 30 minutes in a cloning ring 10 mm in diameter (Asahi Techno Glass). Cells cultured in medium containing 10% FBS were harvested and suspended at 2 × 10⁶ cells/mL. Eighty-five micrometers of the cell suspension was applied to a polymerized collagen gel and incubated overnight in a 37°C 5% CO₂ incubator. On day 1, the cloning ring was removed, and 2.5 mL of 10% FBS-containing medium was added to each well to submerge the cells. To examine TGF-β-dependent collagen gel contraction, TGF-β was added at a 0.1- or 1-ng/mL final concentration. As an inhibitor, an anti-TGF-β antibody (0.1 ng/mL) was also added in the medium for selected dishes. FBS-containing media with or without TGF-β and/or TGF-β antibody were changed on day 3. Gel thickness was measured on day 5 with an inverted phase-contrast microscope, by adjusting the plane of focus from the bottom to the top of the gel and recording the distance that the stage had been moved. Data are expressed as the mean ± SD. Post hoc comparisons between groups was performed with the Tukey procedure. Differences were considered significant at P < 0.01.

**RESULTS**

**Sphere Formation from Stromal Cells**

More than five mice were used to prepare corneal stromal cells in each experiment. From 10 corneas, 1.32 ± 0.16 × 10⁷ cells (n = 3) were isolated, and subcultured cells proliferated into spheres, to yield an average of 7.97 ± 0.35 × 10⁷ cells per 75 cm² flask (n = 6) after four passages (P4). Sphere cells were propagated for >12 passages through 5 months without loss of viability. To avoid contamination of epithelial and endothelial cells, stromal discs were carefully prepared as described in the Materials and Methods sections. Dissociated cells from mouse stromal discs formed spheres when cultured in serum-free

**Table 1. PCR Primers**

<table>
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<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>Product Size (bp)</th>
<th>GenBank Accession ID</th>
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<td>Gapd</td>
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<td>453</td>
<td>NM_008084</td>
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</table>

**Materials and Methods**

- Collagen gel contraction assay was performed as described previously, (*3-5*) with some modifications. Collagen gels were prepared according to instructions provided by the manufacturer (Cellmatrix Type I; Nitta Gelatin, Osaka, Japan). In brief, collagen was mixed with 106-fold concentrated DMEM/F12 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. Then a 0.2 mL aliquot of the solution was placed in the center of each well of a six-well cell culture cluster (Corning Inc., Corning, NY) and allowed to polymerize at 37°C for 30 minutes in a cloning ring 10 mm in diameter (Asahi Techno Glass). Cells cultured in medium containing 10% FBS were harvested and suspended at 2 × 10⁶ cells/mL. Eighty-five micrometers of the cell suspension was applied to a polymerized collagen gel and incubated overnight in a 37°C 5% CO₂ incubator. On day 1, the cloning ring was removed, and 2.5 mL of 10% FBS-containing medium was added to each well to submerge the cells. To examine TGF-β-dependent collagen gel contraction, TGF-β was added at a 0.1- or 1-ng/mL final concentration. As an inhibitor, an anti-TGF-β antibody (0.1 ng/mL) was also added in the medium for selected dishes. FBS-containing media with or without TGF-β and/or TGF-β antibody were changed on day 3. Gel thickness was measured on day 5 with an inverted phase-contrast microscope, by adjusting the plane of focus from the bottom to the top of the gel and recording the distance that the stage had been moved. Data are expressed as the mean ± SD. Post hoc comparisons between groups was performed with the Tukey procedure. Differences were considered significant at P < 0.01.

**Reverse Transcription–Polymerase Chain Reaction**

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medium containing EGF and FGF2 (Fig. 1A, left). To exclude the possibility that spheres may have originated from contaminating epithelial cells, we first performed primary cultures of mouse corneal discs, with or without dispase treatment, followed by epithelium separation. KSFM with low Ca\(^{2+}\) was used to examine epithelial expansion.\(^{48,49}\) When untreated discs were cultured, migration of epithelial and stromal cells was observed in K-SFM and in DMEM/F12 containing 10% FBS, respectively (Fig. 1B, left). There were no epithelial cells migrating from dispase-treated discs in both media, whereas fibroblasts migrated from the discs in DMEM/F12 with serum (Fig. 1B, right). We further cultured dissociated epithelial cells under conditions that allowed stromal spheres to form by 14 days. As a result, no spheres were observed in the epithelial culture (Fig. 1A, right). To demonstrate whether the spheres were hollow or solid, confocal microscopy of 4',6'-diamino-2-phenylindole (DAPI)-stained spheres was performed. Imaging in different focal planes showed that the inside of spheres was filled with cells, not hollow (Fig. 1C).

We then examined the expression of epithelial and stromal markers in primary and subcultured spheres (P10) by RT-PCR. Stromal markers examined were the proteoglycans, keratocan, and lumican,\(^{13,14,50,51}\) as well as CD34, which was recently reported to be expressed in keratocytes.\(^{52–54}\) As shown in Figures 2A and 2B, in addition to the mesenchymal intermediate filament vimentin, the expression of the genes described earlier were detected in the stromal spheres. On the contrary, K12 and Pax6 were not detected in the spheres (A). In contrast, the keratocyte markers keratocan, lumican, and CD34 were detected (B). Immunocytochemical analysis showed expression of the mesenchymal marker, vimentin, in spheres (C). Blue: nuclei of cells counterstained with DAPI. Scale bar, 50 \(\mu m\).

Characteristics of Sphere Cells

Sphere cells plated on collagen I-coated dishes in serum-free medium exhibited a dendritic morphology consistent with keratocytes (Fig. 3A).\(^{3–10}\) RT-PCR showed that expression of keratocan and Aldh were retained under these conditions (Fig. 4). In contrast, the morphology of sphere cells subcultured in 10% serum were fibroblastic, and the expression of these genes was not detected (Fig. 4). Corneal sphere cells further differentiated to express \(\alpha\)-SMA after exposure to TGF-\(\beta\), which is consistent with the myofibroblast phenotype (Figs. 3C, 3D). Furthermore, when cells were subcultured on collagen gels in the presence of TGF-\(\beta\), fibroblast-mediated gel contraction was observed (Fig. 5). Without TGF-\(\beta\), contraction to 68.5% \(\pm\) 1.75% of the original gel thickness was observed, whereas contraction was enhanced to 50.2% \(\pm\) 3.96% or 29.4% \(\pm\) 1.96% of the original thickness in the presence of 0.1 ng/mL or 1 ng of TGF-\(\beta\), respectively \((P < 0.01)\). TGF-\(\beta\)-dependent contraction was reduced to control levels when anti-TGF-\(\beta\) antibody was added to the medium.

**Discussion**

We successfully isolated and subcultured sphere-forming cells from the mouse corneal stroma, yielding a multifold increase in available cells for further experiments. Zhao et al.\(^{40}\) have re-
ported that cells present in limbus-derived spheres are derived from the limbal epithelium but not the stroma. However, the corneal sphere cells that we isolated did not express the epithelial markers K12 or Pax6 throughout the study, and furthermore, exhibited properties of corneal keratocytes when subcultured in serum-free medium. The morphology of the subcultured cells as shown in Figure 3 was similar to that of keratocytes in situ, and together with the expression of keratocan, lumican, Aldh, and CD34 in the subcultured cells, the collective evidence shows that these cells were of keratocyte origin. Although most genes were expressed during sphere cultures and maintained after adhesion to plastic dishes, keratocan, and Aldh were exclusively expressed in the keratocyte phenotype in serum-free medium (Fig. 4). Although the biological role of Aldh is not known, abundant expression of the water-soluble enzyme was shown to be expressed in the keratocyte phenotype, but not by the fibroblasts or myofibroblasts.32,59

Plated sphere cells can further be induced to differentiate into the fibroblast and myofibroblast phenotypes. Sphere cells seeded onto plastic in the presence of 10% serum exhibited the morphology and properties of stromal fibroblasts.28 The transition to α-SMA-positive myofibroblasts by exposure to TGF-β, causing collagen gel contraction (Fig. 5), is also a functional property of stromal fibroblast primary cultures.29,43,60 Therefore, subcultured sphere cells can be conditioned to express all three known phenotypes of keratocytes after expansion by sphere culture. Berryhill et al.36 reported that the fibroblast phenotype can be partially restored to the keratocyte phenotype in terms of extracellular matrix production and morphology. However, biological functions, including Aldh activity, were not restored, suggesting that reversal to keratocyte phenotype after mass culture in serum-containing medium is not practical. Espana et al.41 have reported the use of amniotic membrane (AM) as a substrate for keratocyte cultures in the presence of serum. They have shown that even with the use of serum, primary keratocytes maintained dendritic morphology on AM. The expression of keratocan and lumican was also present for up to five passages, which is a significant improvement over previous reports using artificial substrates. Still, the scarcity of keratocytes in tissue usually necessitates the use of human tissue or cells from larger animals, such as cows.28,36 rabbits.30 and rhesus monkeys.61 Biochemical and molecular analysis of such cells are difficult due to the lack of available antibodies and genomic information.

During subcultures of spheres, cells that failed to form spheres were found attached to the dish as nondividing, fibroblast-like cells (data not shown). Although these cells may have had low viability, there may be a selection process that allows only cells with high growth potential to propagate as spheres. Once secondary spheres are successfully initiated, subsequent passages continue to produce spheres for at least 12 passages, the longest that we observed. To our surprise, cells from later passages continued to show the keratocyte phenotype when subcultured on plastic, suggesting the possible presence of...
committed progenitor cells during the sphere culture stage. Many aspects of the keratocyte are still not understood, and the availability of cells from the mouse cornea should be a powerful tool in studying the biology of these cells.

Acknowledgments

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References


