Sphere Formation and Expression of Neural Proteins by Human Corneal Stromal Cells In Vitro

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PURPOSE. To demonstrate the presence of corneal stromal precursors that express neural markers in vitro.

METHODS. To isolate sphere-forming cells, human corneal stromal cells were subjected to a reaggregation-free neurosphere assay in medium containing methylcellulose gel matrix. To promote differentiation, the isolated sphere colonies were plated in wells with medium containing fetal bovine serum. Expression of nestin, vimentin, keratocan, α-smooth muscle actin (αSMA), βIII tubulin, neurofilament M (NFM), and glial fibrillary acidic protein (GFAP) was examined in the sphere colonies and their progeny (i.e., cells migrated from sphere colonies), by immunocytochemistry and/or reverse transcription–polymerase chain reaction (RT-PCR).

RESULTS. Human corneal stromal cells formed sphere colonies that had no self-renewal capability. The frequency of sphere-forming cells was 1.5% ± 0.1% (range, 1.3%–1.6%). Most of the cells within these colonies expressed nestin and vimentin, whereas some expressed βIII tubulin, NFM, GFAP, and αSMA by immunocytochemistry. Ninety-one percent and 89% of the progeny expressed vimentin and αSMA, respectively, whereas nestin was undetectable. βIII tubulin, NFM, and GFAP-positive cells were detected in the progeny at the frequency of 7.2%, 0.9%, and 0.5%, respectively. Semiquantitative RT-PCR showed that nestin, NFM, GFAP, and keratocan gene expression was higher in the sphere colonies, whereas vimentin and αSMA expression increased in the progeny.


A recent study revealed that cells with neural potential can be isolated from the limbal explant tissue of the cornea in rodents and expanded in vitro.1 Cells isolated from the corneal limbus proliferated to form spheres when suspension culture was performed in the presence of epidermal growth factor and basic fibroblast growth factor, and withdrawal of these growth factors caused differentiation into neural cells. In contrast to the abundance of data on cells from the corneal epithelium, cells from the corneal stroma have been poorly investigated.

In this study, we isolated cells from the human corneal stroma, by using sphere-forming culture, and examined whether the isolated cells would undergo differentiation.

MATERIALS AND METHODS

Isolation of Human Corneal Stromal Cells

This study was conducted in accordance with the Declaration of Helsinki. Corneas (n = 27) were obtained from the Central Florida Lions Eye Tissue Bank and Rocky Mountain Lions Eye Bank at 7 to 8 days postmortem. The age of the donors was 41 to 78 years.

The basal medium used for culture was DMEM/F12 (1:1; Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen), 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO), and 40 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich). Corneal epithelium was carefully removed from the corneal stroma by scraping the outer surface of the cornea. The corneal endothelium and Descemet’s membrane were peeled away in a sheet from the periphery to the center of the inner surface of the cornea with fine forceps, according to a procedure described previously.2 Next, the peripheral cornea (including the limbal region) was dissected from the corneal stroma, to avoid possible contamination by corneal limbal epithelial cells. The stroma was cut into small pieces approximately 10 mm in diameter, which were incubated overnight at 37°C in basal medium containing 0.02% collagenase (Sigma-Aldrich). Subsequently, the tissue pieces were washed with phosphate-buffered saline (PBS), incubated in PBS containing 0.2% EDTA for 5 minutes at 37°C, and dissociated into single cells by trituration with a fire-polished Pasteur pipette. After centrifugation at 800g for 5 minutes, the cells were resuspended in the basal medium. The isolated stromal cells were counted with a hemocytometer. The viability of the cells was >80%, as assessed by trypan blue staining (Wako Pure Chemical Industries, Osaka, Japan). To investigate whether isolated stromal cells were contaminated with corneal epithelial cells, expression of corneal epithelial markers such as keratins K3 and K12 was assessed by reverse transcription–polymerase chain reaction (RT-PCR) before culturing.

Isolation of Sphere Colonies from Corneal Stromal Cells

The primary cell culturing technique was the neurosphere assay. Sphere-forming culturing is a widely used method for the isolation of stem, or progenitor, cells. With this method, isolated cells are allowed to proliferate in suspension culture to form clusters (sphere colonies). Previous investigators have demonstrated that multipotential progenitor cells can be isolated from various organs and tissues, including the brain,3,6 skin,7,8 inner ear,9 and corneal limbal explant tissue1 by clonal (neuro)sphere-forming culture. Basal medium containing methylcellulose gel matrix (1.5%; Wako Pure Chemical) was used to prevent reaggregation of the cells, as described previously.8,10 Cells were plated at a density of 10 viable cells/μL (10,000 cells per well, i.e., 5,000 cells/cm2) in uncoated 24-well culture plates (BD Labware, Bedford, MA). Under these conditions, reaggregation did not occur, and most (or all) of the sphere colonies were derived from single cells.8,10 Culturing took place in a humidified incubator with an atmosphere of 5% CO2.

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Submitted for publication March 14, 2004; revised August 11 and December 16, 2004; accepted January 14, 2005.

Disclosure: S. Uchida, None; S. Yokoo, None; Y. Yanagi, None; T. Usui, None; C. Yokota, None; T. Mimura, None; M. Araie, None; S. Yamagami, None; S. Amano, None

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After 7 days, cells formed clusters (i.e., sphere colonies). In some experiments, the spheres were incubated with 10 μg/mL bromodeoxyuridine (BrDU; Sigma-Aldrich) overnight before fixation. Expression of nestin, vimentin, β-III tubulin, neurofilament M (NF-M), glial fibrillary acidic protein (GFAP), α-smooth muscle actin (αSMA), keratocan, and BrDU in the sphere colonies was determined by immunocytochemistry and/or semiquantitative RT-PCR.

The number of primary spheres was counted after 7 days of culturing. To distinguish growing spheres from dying clusters, only the clusters with a diameter of >50 μm were counted. The mean diameter of spheres (20 spheres from 1 cornea) was calculated, and the average from four corneas was examined. For passaging, primary spheres (day 7) were treated with 0.5% EDTA, dissociated into single cells, and plated in 24-well culture plates at a density of 10 cells/μL. Culturing proceeded for a further 7 days in basal medium containing methylcellulose gel matrix.

**Adherent Culture of Sphere Colonies**

To assess the potentiality of isolated sphere colonies, we transferred individual primary spheres (day 7) onto 13-mm glass coverslips coated with 50 μg/mL poly-1-lysine (PLL; Sigma-Aldrich) and 10 μg/mL fibronectin (BD Biosciences, Billerica, MA) in separate wells, as described previously. To promote differentiation, 1% fetal bovine serum (FBS) was added to the basal medium to form differentiation medium, and culturing continued for another 7 days. In some experiments, the progeny were treated with 10 μM/mL BrDU on days 2, 4, and 6 of culture before fixation. The cells were then subjected to immunocytochemical analysis to examine the expression of nestin, vimentin, β-III tubulin, NFM, GFAP, αSMA, and BrDU.

Furthermore, mRNA was isolated from the progeny after 7 days of culturing, and semiquantitative RT-PCR was conducted to investigate the expression of nestin, vimentin, β-III tubulin, NFM, GFAP, αSMA, and keratocan.

**Immunocytochemistry**

Immunocytochemical analysis was performed on 7-day spheres and on their progeny in cultures adhering to glass coverslips after 7 days. Cells were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries) in PBS for 10 minutes. After they were washed in PBS, the cells were incubated for 30 minutes with 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS containing 0.3% Triton X-100 (BSA/PBST) to block nonspecific binding. The cells were incubated for 2 hours at room temperature (RT) with specific primary antibodies diluted in BSA/PBST. The following antibodies were used: mouse monoclonal anti-vimentin antibody (1:300; Dako, Glostrup, Denmark), mouse monoclonal anti-nestin antibody (1:200; BD Biosciences, Mountain View, CA), rabbit polyclonal anti-p75 NTR antibody (1:200; Promega Corp., Tokyo, Japan), mouse monoclonal anti-neurofilament 145 kDa (NF-M, 1:400; Chemicon, Temecula, CA), rabbit polyclonal anti-β-III tubulin antibody (1:2000; Covance Research Products, Inc., Denver, CO), rabbit polyclonal anti-GFAP antibody (1:400; Dako), mouse monoclonal anti-αSMA antibody (1:400; Sigma-Aldrich), mouse monoclonal anti-tubulin (1:10; Chemicon), rabbit polyclonal anti-peripherin antibody (1:100; Chemicon), mouse monoclonal anti-αSMA antibody (1:200; Sigma-Aldrich), and mouse monoclonal anti-BrDU/fluorescein antibody (1:100; Roche Diagnostics, Basel, Switzerland). As a control, IgG was used instead of the primary antibody. After washing in PBS, the cells were reacted for 1 hour at RT with the appropriate secondary antibodies diluted in BSA/PBST. The following secondary antibodies were used: labeled goat anti-mouse IgG antibody (Alexa Fluor 488, 1:200; Molecular Probes, Eugene, OR), labeled goat anti-rabbit IgG antibody (Alexa Fluor 594, 1:400; Molecular Probes). Nuclei were counterstained with Hoechst 33342 (1:2000; Molecular Probes). After cells were washed with PBS, examination was performed with a laser scanning confocal microscope (Fluoview; Olympus, Tokyo, Japan). When anti-αSMA or p75NTR antibody was used, the cell permeabilization step was omitted. For double-labeled immunocytochemistry with BrDU and the neural marker β-III tubulin, cells were first stained for β-III tubulin, which was visualized with red fluorescent dye (Alexa 594; Molecular Probes). After treatment with 2 M HCl for 60 minutes, the cells were stained with FITC-conjugated anti-BrDU antibody. The number of marker-positive cells was counted and calculated as a percentage of Hoechst-positive cells (at ×200 magnification in five randomly selected areas of adherent cells). For double-labeled immunocytochemistry with GFAP and NFM or vimentin and NFM, the cells were first stained with GFAP or vimentin antibody, which was visualized with green fluorescent dye, and subsequently the cells were stained with NFM antibody and visualized by red fluorescent dye. Approximately 1000 cells were counted per well and two sphere colonies were analyzed per experiment (n = 4).

**Preparation of RNA and RT-PCR**

Total RNA was isolated with a kit (Isogen; Nippon Gene, Tokyo, Japan), according to the manufacturer’s instructions, from primary sphere colonies, adherent progeny of sphere colonies, human corneal stromal cells before culture, and cultured human corneal epithelial cells. First-strand cDNA was synthesized with a reverse transcription system (Promega). cDNA was constructed from 1 μg of total RNA in a 20-μL reaction mixture volume. The PCR was performed with Taq polymerase (AmpliTaq Gold; ABI, Foster City, CA), in a 50-μL reaction mixture volume. After incubation at 95°C for 9 minutes, amplification was performed at 94°C for 30 seconds and then at 60°C for 30 seconds and 72°C for 30 seconds, in a thermal cycler (I-Cycler; Bio-Rad Laboratories, Hercules, CA). Products were separated on a 2% agarose gel and then visualized by staining with ethidium bromide.

For semiquantitative RT-PCR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to equalize the quantity of cDNA loaded and the amplification of PCR products from each sample was examined at three-cycle intervals. Within the linear range of amplification, six sets of PCR products were prepared under appropriate cycling conditions, and the band densities of each gene were compared. Preliminary experiments established that the amount of each transcript was semiquantitatively assessable under these conditions. When reverse transcriptase was not added, no products were obtained, suggesting no contamination by genomic DNA. The primer pairs and the number of PCR cycles used are shown in Table 1.

**RESULTS**

**Isolation of Sphere Colonies from Corneal Stroma**

To isolate precursor cells from corneal stroma, we used a sphere-forming assay, which is a technique that was initially devised to enrich neural stem cells and has been applied to isolate multipotent stem cells from the skin, inner ear, and corneal limbal explant tissue. Corneal stroma was dissociated into single cells and plated in uncoated wells at a density of 10 viable cells/μL (5000 cells/cm²). Culture was done in basal medium containing methylcellullose gel matrix to prevent reaggregation. The stromal cells were isolated without contamination by corneal epithelial cells, as demonstrated by RT-PCR analysis of the K3 and K12 genes (Fig. 1A). Almost complete dissociation into single cells was confirmed by counting the percentage of single cells, double cells, and triple cells, which demonstrated that >99% of the cells were single (Fig. 1B). After 3 days of culturing, small floating spheres were formed. After 7 days, the spheres grew larger, whereas nonproliferating cells died and were eliminated (Figs. 1C, 2A). Under identical culture conditions, we and others have demonstrated that most of the sphere colonies are derived from proliferation and not by reaggregation of the dissociated cells. To confirm that the increase of colony size was actually due to proliferation, we added BrDU (a thymidine analogue), at 24 hours before fixation and found that some of the cells within the each colony were
TABLE 1. Primers Used for Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size (bp)</th>
<th>Cycles (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
<td>5′-CACCTGGCTGGACGCTTTCTCTAA-3′</td>
<td>5′-CCACCGAATTTCCATCCCTTA-3′</td>
<td>361</td>
<td>29</td>
</tr>
<tr>
<td>Vimentin</td>
<td>5′-GGCTCAGATTGGAAGACAGC-3′</td>
<td>5′-GCTTCAACGCAAATTTTCCT-3′</td>
<td>327</td>
<td>23</td>
</tr>
<tr>
<td>Tubulin</td>
<td>5′-TCTGCAAGCCTGACCTGTC-3′</td>
<td>5′-TGGAGGCAAGTGCCTGAC-3′</td>
<td>292</td>
<td>35</td>
</tr>
<tr>
<td>NFM</td>
<td>5′-GGAGAAAGAAGATCTCCGAGA-3′</td>
<td>5′-CGGATTGCGCTTCTTCAT-3′</td>
<td>339</td>
<td>35</td>
</tr>
<tr>
<td>GFAP</td>
<td>5′-CTGGGCTCAACGAGCATTACC-3′</td>
<td>5′-AATGCCTGCTTCCTCCATC-3′</td>
<td>429</td>
<td>35</td>
</tr>
<tr>
<td>αSMA</td>
<td>5′-TGCTCAGCAGCTCCTGCT-3′</td>
<td>5′-GCCTGGGAAAGATGACAGAG-3′</td>
<td>280</td>
<td>29</td>
</tr>
<tr>
<td>Kerotan</td>
<td>5′-GGCTCAGAAAGATCAGGACAGCA-3′</td>
<td>5′-ACGGAGGTAGCGAAGATGAGGT-3′</td>
<td>358</td>
<td>35</td>
</tr>
<tr>
<td>CK3</td>
<td>5′-CATATTGTGGAATGGTGGTG-3′</td>
<td>5′-TCTCGGAGTGGTCGTGGGG-3′</td>
<td>291</td>
<td>35</td>
</tr>
<tr>
<td>CK12</td>
<td>5′-TTGTGAGATCTCAGATCAATCA-3′</td>
<td>5′-TACTCCAGGTGCGGACAGAAG-3′</td>
<td>398</td>
<td>35</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GGTGAATGCGGTGGTGAACGGA-3′</td>
<td>5′-TGGATTGGGTTCGCTCTGC-3′</td>
<td>223</td>
<td>23</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

labeled by BrdU on day 7 (Fig. 1C), indicating that the colonies contained dividing cells. Taken together, these results suggested that the sphere colonies could be produced from single isolated corneal stromal cells.

The number of digested stromal cells from individual corneas was 1.1 × 10^6 (mean range, 0.8 × 10^5 to 1.3 × 10^5, n = 4). The number of spheres grown per well was 147 ± 23 (mean ± SD, n = 4), and the number of spheres grown from one cornea was 1566 ± 211. The frequency of sphere-forming cells was 1.5% ± 0.1% (range, 1.3%–1.6%). These spheres were 87 ± 20 μm in diameter (mean ± SD, n = 4).

The replating efficiency between the primary sphere colonies and the secondary colonies decreased dramatically. When the primary sphere colonies were dissociated into single cells and were cultured in the presence of methylcellulose gel matrix, no secondary colonies were generated. This indicated that the capacity for self-renewal of sphere colonies derived from corneal stroma was limited.

**Immunocytochemistry of Sphere Colonies**

Spheres were immunostained for nestin,^1^ because nestin is expressed by immature cells within multipotent sphere colonies derived from the brain^5,6, skin,^7^ inner ear,^7^ and retina.^12,13^ Because the corneal stromal cells are derived from neural crest mesenchymal fibroblasts, the expression of vimentin as a marker of mesenchymal cells, αSMA as a marker of fibroblasts, and the p75 neurotrophin receptor (p75 NTR) as a marker of neural crest stem cells^14^ was investigated by immunocytochemistry. Most of the cells in the spheres were immunoreactive for nestin (Fig. 2B) or vimentin (Fig. 2C), and some cells in the spheres were stained by anti-αSMA antibody (Fig. 2D), but were not stained by anti-p75 antibody (not shown). When >50 sphere colonies were subjected to immunocytochemical analysis for nestin and vimentin, the expression of both markers was detected in all the colonies.

We next examined whether the sphere colonies could give rise to cells expressing neuronal lineage markers. Immunocytochemistry of sphere colonies showed that some cells expressed βIII tubulin, a marker of immature neurons (Fig. 2E). When the sphere colonies were stained for NFM, a minor population of the cells was immunoreactive (Fig. 2F). Moreover, some cells in the sphere colonies were positive for GFAP, a glial cell marker (Fig. 2G).

**Immunocytochemistry of the Progeny of Individual Sphere Colonies**

To test whether the progeny of the spheres possess the characteristics of mesenchymal cells, single day-7 spheres were transferred onto PLL/fibronectin-coated glass coverslips in 24-well plates and cultured in differentiation medium containing 1% FBS. After 7 days, many cells migrated from the spheres and were not contaminated with corneal epithelial cells. (A) RT-PCR showed that K5 and K12 were not expressed by human corneal stromal cells before culture, suggesting that the isolated cells were not contaminated with corneal epithelial cells. (B) Human corneal stromal cells before culture; ep, cultured human corneal epithelial cells. (B) Bright-field image of cells immediately after plating showing that the corneal stromal cells had dissociated into single cells. (C) Bright-field image of sphere colonies after culture of isolated dissociated corneal stromal cells for 7 days. Some cells in the primary spheres were immunoreactive for BrdU (green; inset). Scale bars: (B) 200 μm; (C) 100 μm.

**Figure 1.** Isolation of sphere colonies from human corneal stroma. (A) Bright-field image of a typical sphere colony. The colonies are immunostained for nestin (B), a marker of immature neural progenitor cells; vimentin (C), a marker of mesenchymal cells; αSMA (D), a marker of fibroblasts; βIII tubulin (E); NFM (F); and GFAP (G). As a control, IgG was used instead of the primary antibody (H). Sphere colonies were not immunostained for p75 NTR (not shown). Scale bars, 100 μm.

**Figure 2.** Immunostaining of sphere colonies. (A) Bright-field image of a typical sphere colony. The colonies are immunostained for nestin (B), a marker of immature neural progenitor cells; vimentin (C), a marker of mesenchymal cells; αSMA (D), a marker of fibroblasts; βIII tubulin (E); NFM (F); and GFAP (G). As a control, IgG was used instead of the primary antibody (H). Sphere colonies were not immunostained for p75 NTR (not shown). Scale bars, 100 μm.
neuronal lineage markers. Immunocytochemistry of the progeny grown in adherent culture (referred to as “adherent progeny”) in the presence of FBS showed that 7.2% ± 1.1% of the cells were positive for β-III tubulin, a marker of immature neurons (Fig. 3D). When the adherent progeny were immunostained with NFM, a marker of mature neurons and peripherin, a marker of peripheral nerve neurons, 0.9% ± 0.1% of the progeny were immunoreactive for NFM (Fig. 3E), and none of the cells were positive for peripherin (not shown). In addition, 0.5% ± 0.2% of the adherent progeny with a flattened morphology were immunostained for GFAP, a glial cell marker (Fig. 3F). None of the adherent progeny were immunoreactive for O4, a marker of oligodendrocytes (not shown). By analyzing six sphere colonies, we found that all the colonies gave rise to cells that were positive for vimentin, β-III tubulin, NFM, and GFAP, and the percentage of cells positive for each marker was similar.

Many of the adherent progeny were immunopositive for BrdU (Fig. 4A). Double immunocytochemistry showed that BrdU-positive cells were also immunoreactive for β-III tubulin (Figs. 4B, 4C). In addition, NFM-positive cells were not stained with GFAP antibody nor vimentin antibody (Fig. 5), suggesting that the cells were representatives of unique developmental lineages.

**Semiquantitative RT-PCR**

Semiquantitative RT-PCR demonstrated that nestin mRNA expression was high in the spheres and reduced in the adherent progeny, whereas vimentin and αSMA gene expression was upregulated in the progeny, confirming the results of immunocytochemistry. Semiquantitative RT-PCR demonstrated a similar level of β-III tubulin gene expression in the colonies and their adherent progeny, whereas NFM and GFAP gene expression was higher in the colonies than in the progeny. In addition, expression of keratocan, a marker for keratocytes, was detected in the sphere colonies but was reduced in the progeny. However, the genes for K3 and K12 molecules, which are expressed in the corneal epithelium, were not detected by RT-PCR (Fig. 6).

**Expression of Neural Markers by Monolayer Culture**

We found that no cells expressed neural markers when the dissociated corneal stromal cells were directly plated and cultured in the medium containing 10% FBS (data not shown). To examine whether the corneal stromal cells also express neural cell markers in monolayer culture, we attempted to investigate...
the cells cultured under the identical culture condition for sphere formation (i.e., cells cultured in serum-free culture medium containing bFGF and EGF and then exposed to 1% FBS). However, we found that the cells from nonfractionated primary stromal cells or fractionated primary stromal cells did not firmly attach to the culture wells under this condition, precluding subsequent immunocytochemical analysis. Thus, in an attempt to minimize the effect of serum and maintain the cells in the immature state, we cultured nonfractionated or fractionated cells in a reduced serum concentration (1% FBS) supplemented with bFGF and EGF. Although nonfractionated cells did not firmly attach to the culture wells under this condition, many fractionated cells attached to the wells. We found that 0.8% ± 0.1% and 7.0% ± 0.8% of the cells expressed neuronal markers NFM and βIII tubulin, respectively.

**DISCUSSION**

The results of this study suggest that the corneal stroma contains cells that can generate sphere colonies that give rise to cells expressing markers for mesenchymal fibroblasts and neurons. Because each of the sphere colonies or their progeny gave rise to cells expressing markers for fibroblasts as well as neurons, it seems that the individual precursor cells gave rise to cells of two lineages. It should be emphasized that the sphere colonies were grown under reaggregation-free conditions in this study, to exclude the possibility that the colonies may appear to be multipotent because they were formed by a mixture of dissociated unipotent cells committed to different lineages. Our results also demonstrated that human corneal stromal cells can generate neural cell-marker-positive cells when monolayer culture is used, indicating that at least some keratocytes can respond in the same manner as the sphere-forming cells do by monolayer culture. This is the first study to demonstrate that corneal stroma, which was considered to be composed of fibroblast-like cells, contains precursor cells with neural potential.

Our results provide two indicators that the cells in sphere colonies derived from human corneal stroma differentiate spontaneously during suspension culture. First, the individual sphere colonies grown from the corneal stroma were heterogeneous, with most of the cells expressing nestin and vimentin, while some expressed neuronal and glial cell markers. Second, cells that dissociated from the sphere colonies generated few secondary colonies, indicating that these precursor cells did not show self-renewal capability. Results of most of the studies performed in rodents suggest that sphere colonies obtained from various tissues contain immature cells expressing nestin, whereas expression of mature neuronal and glial markers is not detected by immunocytochemistry. However, sphere colonies are not thought to be composed solely of undifferentiated cells but are believed to contain heterogeneous cell populations, including immature cells, lineage-restricted progenitor cells, and postmitotic cells. Indeed, a recent study demonstrated by RT-PCR analysis that sphere colonies isolated from the adult mouse forebrain express GFAP, a mature astroglial marker. Because immature cells within sphere colonies can give rise to daughter colonies, the fact that cells dissociating from the colonies generated few secondary colonies suggests that sphere colonies derived from human corneal stroma contain relatively few undifferentiated cells. In addition, it should be emphasized that we used human cells in this study. Although several other investigators have used human cells, results in previous studies have suggested that it is difficult to maintain undifferentiated human cells in culture. It has been demonstrated that sphere colonies isolated from the human brain contain heterogenous cells including mature neuronal cells, demonstrated by immunocytochemistry, suggesting that sphere colonies derived from human tissues tend to differentiate during culture. However, with modification of the passaging method, cultures of human brain-derived progenitor cells can be maintained for a long period (several months to years). Thus, optimization of culture conditions may promote growth of undifferentiated cells from specimens of human corneal stroma.

In our study, corneal stromal precursor cells showed a high propensity to differentiate into mesenchymal cells in the culture conditions that we used. Because the progeny of the sphere colonies showed higher vimentin and αSMA expression and lower expression of NFM, GFAP, and keratocan than did cells in the sphere colonies, it seems likely that selective death of cells expressing neural markers as well as cells expressing keratocan (i.e., presumable keratocytes), occurred during culture and/or αSMA- and vimentin-positive mesenchymal fibroblasts grew faster than did neural cells and keratocytes in adherent culture. It should also be noted that cell fate change may well be affected by the culturing conditions used and not simply by cellular proliferation. Further studies are necessary to address this point.

It has been recognized that bone-marrow–derived progenitor cells can circulate and reside in many peripheral tissues. To determine whether precursor cells from the corneal stroma may have been derived from bone marrow, we separated collagenase-digested stromal cells into CD45 (pan-leukocyte marker)-positive and negative cells by using a magnetic bead cell-separation system, which does not affect the labeled or unlabeled cell fractions. Sphere colonies can be formed from CD45-negative, but not CD45-positive cells (Yamagami S, unpublished observation, 2004), suggesting that obtained spheres are derived from resident stromal cells and CD45-positive leukocytes in corneal stroma have no proliferative capacity.

In summary, we demonstrated that cells from the corneal stroma can propagate in vitro to give rise to both mesenchymal fibroblasts and neural cells. Further investigation, in vitro and in vivo, of the physiological function of neuronal- and glial-marker-positive cells is needed.

**References**