Comparison of Rabbit Corneal Endothelial Cell Precursors in the Central and Peripheral Cornea

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PURPOSE. To compare the distribution and self-renewal capacity of rabbit corneal endothelial cell precursors in the central and peripheral regions of the cornea.

METHODS. The corneal endothelium (CE) and Descemet’s membrane of New Zealand White rabbit corneas were divided into a peripheral region (6.0–10.0 mm in diameter) and a central region (6.0 mm in diameter). Then a sphere-forming assay was performed to isolate precursors from the CE of each region. Numbers of primary and secondary sphere colonies and sizes of primary spheres were compared between the central and peripheral regions.

RESULTS. Primary spheres were isolated from the peripheral and the central regions of the CE. The rate of primary sphere formation in the peripheral region (34.4 ± 10.4/10,000 cells) was significantly higher than in the central cornea (26.8 ± 6.6/10,000 cells; P = 0.0042), but there was no significant difference in the size of primary spheres between the two regions. Self-renewal capacity was higher in the peripheral region than in the central region, as evidenced by a significantly higher secondary sphere formation rate for cells from the periphery (39.0 ± 8.8/10,000 cells) compared with that for cells from the central region (25.4 ± 4.2/10,000 cells; P = 0.00028).

CONCLUSIONS. These findings demonstrate that peripheral and central rabbit corneal epithelia contain a significant number of precursors but that the peripheral endothelium contains more precursors and has a stronger self-renewal capacity than the central region. (Invest Ophthalmol Vis Sci. 2005;46: 3645–3648) DOI:10.1167/iovs.05-0630

The corneal endothelium (CE) is derived from the neural crest1,2 and forms a single layer of hexagonal cells. In the human CE, wound healing occurs predominantly by cell migration and enlargement without discernible cell proliferation.3,4 In various tissues that show vigorous proliferative activity, the existence of adult stem cells or precursors is predictable. However, the human CE shows weak to no proliferative capacity in vivo, unlike the results for cultured cells.5–7 Hence, little attention has been directed to the investigation of adult stem cells or precursors of CE.

The sphere-forming assay has been widely used to isolate adult stem cells or precursors.8–20 If dissociated single cells from each tissue form cell clusters (spheres) under serum-free conditions and the spheres and their progenies show respective self-renewal and multipotential capacity, the spheres should contain adult stem cells or precursors. Using this method, the existence of adult stem cells or precursors has been reported in various tissues, including the central nervous system,21 bone marrow,22,23 skin,24,25 inner ear,13,15 retina,14,15 pancreas,16 corneal limbal explants,17 and corneal stroma.18 We isolated precursors from human and rabbit CE by the sphere-forming assay and showed that these CE-derived precursors could have a propensity to differentiate into corneal endothelial cell-like cells that produced neuronal and mesenchymal proteins.19,20 The proliferative activity and density of cells at the peripheral and central regions of the human cornea are controversial. In vitro, peripheral CE has a higher mitotic activity than CE from the central region.21 The cornea has a higher density of endothelial cells in the peripheral region of the CE than in the central region.22,23 p53, involved in the negative regulation of cell division, is more highly expressed in the central CE.24 Moreover, BrdU incorporation in unwounded CE and telomerase activity were detected in CE limbal area only (Whikehart DR, et al. IOVS 2002;43:ARVO E-Abstract 1627; Whikehart DR, et al. IOVS 2004;45:ARVO E-Abstract 3765). On the other hand, Konomi and Joyce (Konomi K, et al. IOVS 2005;46:ARVO E-Abstract 4710) reported that there was no significant difference in the proliferative capacity of human CE between the central and the peripheral regions. The density of peripheral CE was reported to be lower than that of the central CE in humans.25 Therefore, clarification of the distribution of CE precursors may provide some clues about the proliferative capacity and density of the CE at the center and the periphery.

In this study, we investigated the distribution and proliferative capacity of rabbit CE precursors from the central and peripheral regions by a sphere-forming assay. We used rabbit corneas because the distribution of the CE can be examined at a younger age after birth.

MATERIALS AND METHODS

Primary Sphere-Forming Assay and Sizes of Spheres from Peripheral and Central CE

Rabbits were treated in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. CE and Descemet’s membrane were separated from the periphery (6.0–10.0 mm in diameter) and from the central area (6.0 mm in diameter) in New Zealand White rabbits (15–18 weeks old; Saitama Experimental Animals Inc., Saitama, Japan) using forceps and were incubated for 3 hours at 37°C in basal medium containing 0.02% collagenase (Sigma, St Louis, MO). Next, they were incubated in 0.2% EDTA at 37°C for 5 minutes and then were dissociated into single cells by trituration with a fire-polished Pasteur pipette. Viability of the isolated CE cells was greater than 90%, as shown by trypan blue staining (Wako Pure Chemical Industries, Osaka, Japan). The sphere-forming assay was used for primary cell culture.6 Basal medium containing a methylcellulose gel matrix (0.8%; Wako Pure Chemical Industries) was used to prevent cell re-
The number of viable cells/L (50,000 cells/well; 2500 cells/cm²) in the uncoated wells of 60-mm culture dishes. The basal medium was Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium supplemented with B27, epidermal growth factor (EGF; 20 ng/mL), and basic fibroblast growth factor (bFGF; 40 ng/mL). The number of spheres per 10,000 cells was calculated for each well. To measure sphere colony diameter, cultures were observed under an inverted phase-contrast microscope. These experiments were performed twice; representative results are shown (n = 10) after 7 days of culture (unpaired t test). The experiment was repeated twice from different sets of animals, and representative data are shown. *P = 0.0042. Scale bar, 50 μm.

Secondary Sphere Formation from Primary Spheres Derived from Peripheral and Central CE

For passaging, primary spheres (day 7) were treated with 0.5% EDTA and were dissociated into single cells plated into 60-mm culture dishes at a density of 10 cells/μL. Culture was continued for 7 days in basal medium containing methylcellulose gel matrix to prevent reaggregation. These experiments were performed twice; representative results are shown (n = 10) (see Fig. 3).

Statistical Analysis

The unpaired t test was used to compare mean values. Significance was set at P < 0.05, and all analyses were performed using a statistical software package (StatView Version 5; Abacus Concepts, Berkeley, CA).

RESULTS

Primary Sphere Formation and Sphere Size

The CE was disaggregated into single cells cultured for 7 days. During that period, the spheres grew larger, whereas nonproliferating cells were eliminated. To compare the densities of precursors between peripheral and central regions, primary spheres were isolated from the peripheral and the central CE. Photographs of representative spheres from the periphery and the center are shown in Figure 1A. When the number of sphere colonies obtained was counted, a significantly higher number of spheres (34.2 ± 10.4/10,000 cells, mean ± SD) was obtained from the peripheral region of the CE compared with the central region (26.8 ± 6.6) per 10,000 cells (Fig. 1B). No statistically significant difference was noted with respect to the size of primary spheres from the two regions after 3, 5, and 7 days, indicative of no difference of proliferative capacity in spheres derived from each region (Fig. 2).

Secondary Sphere Formation

To evaluate the self-renewal capacity of the CE, primary spheres were passaged under the same culture conditions as those for the growth of primary spheres. Secondary spheres were generated from dissociated primary spheres derived from the peripheral or the central CE. The number of secondary spheres per 10,000 cells was significantly higher with spheres derived from the peripheral region than from the central region (39.0 ± 8.8 vs. 25.4 ± 4.2, respectively; P = 0.00,028; unpaired t test; Fig. 3A). Photographs of representative secondary spheres are shown in Figure 3B.

DISCUSSION

We have previously shown that spheres derived from rabbit CE have a high proliferative capacity and that their progeny express a mesenchymal marker (α-smooth muscle actin) and neuronal lineage markers (neuron-specific enolase and microtubule-associated protein-2).20 This study further demonstrated that peripheral and central regions of the rabbit CE contain precursors and that the number of precursors is significantly higher in the peripheral CE than in the central region. The rate of secondary sphere formation was significantly higher for cells from the peripheral region than from the central region. Moreover, the progeny of rabbit CE spheres from the peripheral and central regions did not show any differences in mesenchymal and neuronal marker expression (TM, unpublished observation, 2004). These findings imply that rabbit CE precursors reside preferentially in the peripheral region of the CE and have potent self-renewal capacity compared with the central region.
CE while demonstrating similar multipotentiality of precursors from both regions.

Neural crest cells, from which the CE originates,\(^1,2\) migrate and differentiate during corneal development in two waves.\(^{28,29}\) In the first step, the corneal epithelium forms and synthesizes the primary stroma—that is, pericellular mesenchymal cells of neural crest origin—after which neural crest cells migrate to the margin of the optic cup and migrate between the lens and the corneal epithelium to contribute to development of the CE and trabecular meshwork. During the second wave of migration, neural crest cells invade the primary stroma and differentiate into corneal keratocytes. This embryological process may be compatible with our finding that more precursors with a high proliferative potential reside at the edge to the CE and supply differentiated cells centripetally to the central cornea during early development.

Rabbit CE precursors that are highly proliferative in vitro should proliferate in the anterior chamber of the eye because rabbit CE can proliferate at sites of CE defects in vivo. Under physiological conditions, CE are arrested in the G\(_1\)-phase of the cell cycle,\(^{10,31}\) and TGF-\(\beta\) in the aqueous humor suppresses entry into the S-phase.\(^{32}\) Cell-cell contact inhibition may also be an important mechanism for inducing cell cycle arrest to maintain the mature endothelial monolayer in a nonproliferative state.\(^{32}\) Although various transplantation techniques have been proposed for CE defects,\(^{20,33-35}\) the existence of CE precursors suggests that in vivo stimulation of precursors in the human CE by growth factors or other unknown chemical mediators may activate the proliferative potential of these precursors, leading to a novel treatment strategy for bullous keratopathy.

In rodents, the CE can repopulate after injury, but migration and enlargement without proliferation are the main mechanisms of wound healing for the human CE. Indeed, data based on assessment of the rabbit CE with proliferative capacity in vivo may not be relevant to the adult human CE from donor corneas. Further investigation is required to elucidate the distribution and proliferative capacity of precursors in the adult human CE. Our study, however, may contribute to the understanding of CE embryology, CE changes with aging, and the CE in the infant shortly after birth.

In summary, we compared the distribution and self-renewal capacity of rabbit CE precursors from the central and peripheral regions of the cornea by the sphere-forming assay. Our findings demonstrated that the peripheral and central CE contain a significant number of precursors and that the peripheral region of the rabbit CE has a higher density of precursors with stronger self-renewal capacity than the central region.

**References**


