Replication Competence and Senescence in Central and Peripheral Human Corneal Endothelium

Tatsuya Mimura and Nancy C. Joyce

Purpose. To compare replication competence and senescence in human corneal endothelial cells (HCECs) between the central and peripheral areas and between younger and older donors.

Methods. Human corneas were obtained from the eye bank and separated into two groups: young (younger than 30 years) and old (older than 50 years). Corneas were cut in quarters and a 2-mm scrape wound was created in the endothelium from the periphery to the center. Unwounded endothelium acted as a negative control. Corneal pieces were incubated for 24, 36, 48, 60, 72, 84, and 96 hours in medium containing 8% fetal bovine serum (FBS) plus additional growth factors. Tissue was fixed, immunostained for minichromosome maintenance (MCM)-2, a marker of replication competence, and mounted in medium containing propidium iodide (PI) to visualize all nuclei. Fluorescence microscope images were used to count PI-stained and MCM2-positive HCECs in three 100-μm^2 areas within the central and peripheral wound area. Results are expressed as mean number of cells/100 μm^2. Senescent HCECs in ex vivo corneas were identified by staining for senescence-associated β-galactosidase activity (SA-β-Gal). Whole corneas were cut in quarters and incubated in staining solution containing SA-β-Gal at pH 6.0. The number of cells stained for SA-β-Gal and the grade of SA-β-Gal intensity in three 100-μm^2 areas were averaged for the central and peripheral areas from each donor. For all studies, results were compared between central and peripheral cornea and between younger and older donors.

Results. In both age groups ($n = 4$/group), cells repopulated the wound area in a time-dependent manner. In corneas from older donors, significantly fewer HCECs migrated into the wound bed in the central cornea than in the periphery. At each time point, the density of cells in the central wound area was lower in corneas from older donors than from younger donors. In both age groups, the mean percentage of MCM2-positive cells increased with time until wound healing. In both age groups, more MCM2-positive cells were present in the wounded area of the peripheral than of the central cornea. At 36, 48, 60, and 72 hours after wounding, the percentage of MCM2-positive cells in the central or peripheral area of older corneas was significantly less than in the corresponding region in younger corneas. No MCM2-positive staining was observed in unwounded areas at any time point. HCECs in corneas from younger donors ($n = 4$) showed little to no SA-β-Gal activity in either the central or peripheral area. SA-β-Gal activity was easily detectable in corneas from older donors ($n = 4$) and a significantly higher percentage of central HCECs showed strong SA-β-Gal activity compared with HCECs in the periphery.

Conclusions. In ex vivo corneas, HCECs from the peripheral area retain higher replication competence, regardless of donor age. HCECs in the central area of corneas from older donors retain replicative competence, but the relative percentage of cells that are competent to replicate is significantly lower than in the periphery or in the central area of corneas from younger donors. This reduction in replicative competence negatively correlates with the observed increase in the population of central HCECs exhibiting senescence-like characteristics. (*Invest Ophthalmol Vis Sci.* 2006;47:1387–1396) DOI:10.1167/iovs.05-1199

The corneal endothelium is derived from the neural crest[1,2] and forms a single layer of hexagonal cells that is located between the corneal stroma and aqueous humor. Corneal endothelial wound healing occurs predominantly by cell migration and enlargement rather than by cell division to replace dead cells.[3–5] This type of healing, as well as the fact that endothelial cell density decreases with age, strongly suggests that corneal endothelial cells do not normally proliferate in vivo. Studies from this[6–9] and other laboratories[10,11] indicate, however, that human corneal endothelial cells (HCECs) retain the ability to proliferate, but are strongly inhibited from dividing in vivo. Our laboratory has also demonstrated an intrinsic age-related difference in the relative growth capacity of HCECs, using both an ex vivo corneal wound model[6] and cultured HCECs.[7,8,12] Morphologic studies have demonstrated that the density of HCECs is greater in the periphery than in the central cornea[13,14] and that, with donor age, the density decreases in both areas. The reason for this density difference is currently not known.

We recently compared regional differences in proliferative capacity by culturing HCECs isolated from the central and peripheral areas of corneas from young (younger than 30 years) and older (older than 50 years) donors.[12] The relative proliferative capacity was determined by comparing population-doubling time and by immunostaining for minichromosome maintenance protein (MCM)-2 as a marker for replication competence.[15–17] The results of these studies showed no significant difference in population-doubling time or in replication competence between central and peripheral HCECs within either age group, although there was a tendency for central cells, particularly from older donors, to exhibit longer population-doubling times. These studies indicate that HCECs cultured from both central and peripheral cornea retain proliferative capacity.

The present study directly addresses whether regional differences in proliferative capacity exist within the endothelial population in vivo. An ex vivo wound model was used to compare the replication competence of HCECs in the central and peripheral area of corneas from young and older donors using MCM-2 protein expression as a marker. In addition, the relative distribution of senescent cells within the endothelial

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Supported by Bausch & Lomb (TM) and National Eye Institute Grant R01 EY12700 (NCJ)

Submitted for publication September 8, 2005; revised December 6, 2005; accepted February 10, 2006.

Disclosure: **T. Mimura**, Bausch & Lomb (F); **N.C. Joyce**, None

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TABLE 1. Donor Information

Donor Age (y)	Cause of Death	Death to Preservation	Preservation to Culture
Young			
3	Head trauma	8	63
12	Bronchitis	12	65
14	Drowning	7	63
16	Motor vehicle accident	9	281
16	COPD	10.5	75
18	Motor vehicle accident	13	147
19	Motor vehicle accident	8	104
21	Heroin overdose	2.5	31
Average		8.8 ± 3.1	103.6 ± 74.4
Old			
53	COPD	10.5	87.5
54	Cardiac arrest	3	75
59	Adenocarcinoma	4.5	50.5
59	Polio	4.5	90.5
62	Motor vehicle accident	11	88.5
68	Lung cancer	9.5	41
68	Cardiovascular accident	7	63
75	Intracranial bleed	5	52
Average		6.9 ± 2.9	68.5 ± 18.3
Young vs. old (unpaired t-test)		$P = 0.2585$	$P = 0.2601$

COPD, Chronic obstructive pulmonary disease.
Data are expressed in hours.

population was determined by immunohistochemical staining for senescence-associated β-galactosidase (SA-β-Gal).[18]

MATERIALS AND METHODS

Human Corneal Tissue

All donor human corneas were obtained from National Disease Research Interchange (NDRI, Philadelphia, PA), shipped in wet ice, and preserved at 4°C for 1 week or less (Optisol-GS preservative; Chiron Vision, Irvine, CA) before use. All corneas were considered to be unsuitable for transplantation. Additional exclusion criteria were similar to those previously described[9] and include: longer than 24 hours between time of death and time of preservation; low endothelial cell density (<1500 cells/mm^2); history of diabetes or glaucoma, sepsis, or ocular infection; and and extended period of chemotherapy or high doses of chemotherapeutic agents. Handling of donor information by the source eye bank, NDRI, and this laboratory adhered to the tenets of the Declaration of Helsinki of 1975 and its 1983 revision in protect-

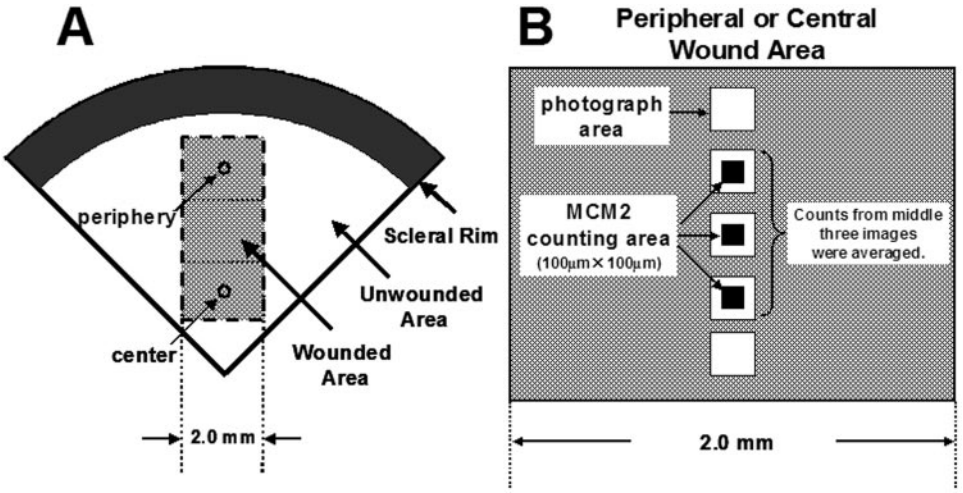

FIGURE 1. Schematic illustration of endothelial wound and microscopic fields. Whole corneas were cut in quarters and a 2-mm-wide scrape wound was made in the endothelium from the periphery to the center (**A**). The density of HCECs in the central and peripheral cornea was compared by separating the linear wound into central, middle, and peripheral areas. Five 20× magnification micrographs were taken of the wound center in both the central and peripheral areas (**B**). Only the middle three micrographs of the five were selected for analysis, to ensure positional accuracy. Cells were counted with a micrometer grid in the central 100-μm^2 portion of each micrograph. Diagram in (**A**) modified, with permission, from Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. *Invest Ophthalmol Vis Sci.* 2000;41:660–667. © ARVO.

ing donor confidentiality. Information about donor corneas and ex vivo culture conditions is presented in Table 1.

Ex Vivo Wound Model

Donor corneas were separated into two groups: younger donors (30 years of age or less) and older donors (50 years of age or more). Whole corneas were cut into quarters. The endothelium of each quarter was gently scraped twice with a silicon-coated needle measuring 1.0 mm in diameter (Alcon, Fort Worth, TX), forming a linear wound 2-mm wide based on needle diameter, as shown in Figure 1A. The width of the wound was confirmed with a millimeter-scale rule under a stereoscopic microscope (AO 580; American Optical, Buffalo, NY) and an objective micrometer (1 mm in width, and minimum resolution 0.01 mm; Olympus, Tokyo, Japan) under a phase-contrast microscope (Eclipse TS 100; Nikon, Avon, MA). HCECs in the unwounded area of each quarter served as a negative control. Corneal pieces were then placed endothelial-side-up in individual wells of a 24-well tissue culture plate (Falcon; BD Biosciences, Lincoln Park, NJ). Pieces were incubated in culture medium containing OptiMEM-1 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 8% fetal bovine serum (Hyclone, Logan, UT), 5 ng/mL epidermal growth factor (EGF; Upstate Biotechnologies, Lake Placid, NY), 20 ng/mL nerve growth factor (NGF; Biomedical Technologies, Stoughton, MA), 100 μg/mL bovine pituitary extract (Biomedical Technologies), 20 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO), 200 μg/mL calcium chloride (Sigma-Aldrich), 0.08% chondroitin sulfate (Sigma-Aldrich), 50 μg/mL gentamicin (Invitrogen Life Technologies), and antibiotic–antimycotic solution diluted 1:100 (Invitrogen Life Technologies). Medium was changed daily. Cultures were maintained for various periods at 37°C in a 5% carbon dioxide, humidified atmosphere.

Immunolocalization of MCM2

Corneas were obtained from young (12, 14, 16, and 18 years) and older donors (53, 54, 59, and 59 years). At 24, 36, 48, 60, 72, 84, or 96 hours after wounding, corneal pieces were fixed for 10 minutes in 1 mL of cold (−20°C) methanol and rinsed three times in phosphate-buffered saline (PBS). They were then permeabilized in 1.0% Triton X-100 (Sigma-Aldrich) in PBS for 10 minutes at room temperature, followed by incubation with 4% bovine serum albumin (Fisher Scientific Co., Fairlawn, NJ) in PBS for 10 minutes at room temperature to block nonspecific binding. Tissues were incubated for 2 hours at room temperature with mouse monoclonal anti-human MCM2 IgG (BD-PharMingen, San Diego, CA) diluted 1:200 in blocking buffer. Each piece was rinsed in PBS three times over 30 minutes, followed by incubation with blocking buffer for 10 minutes at room temperature. Fluorescein-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:200 in blocking buffer, was applied to the corneal pieces for 2 hours at room temperature. The negative control consisted of using nonspecific mouse IgG (1:100, BD-PharMingen) as primary antibody. The corneal pieces were then washed three times in PBS over 30 minutes at room temperature. Excess sclera was removed to flatten the pieces. Each corneal quarter was placed on a glass slide with forceps. The mean central corneal thickness in humans has been reported to be approximately 530 μm.[19] Hence, 4 drops of glue (Elmer's Glue-All; Borden, Inc., Columbus, OH) were applied to each slide around the corneal piece to accommodate the thickness of the sample. Mounting medium containing propidium iodide (PI; Vector Laboratories, Burlingame, CA), diluted 1:1 in PBS, was added, to permit visualization of the nuclei. A glass coverslip was then placed on the tissue, and a plastic dish was set on top of the slide for 30 minutes to fix the slide, the corneal pieces, and coverslip in a horizontal position. Images were captured by a fluorescence microscope (Eclipse e800; Nikon Corp.), equipped with a digital camera (Spot; Diagnostic Instruments, Sterling Heights, MI). Images of corneal quarters from four different donors were saved to a personal computer and analyzed for each time point ($n = 4$ per age group).

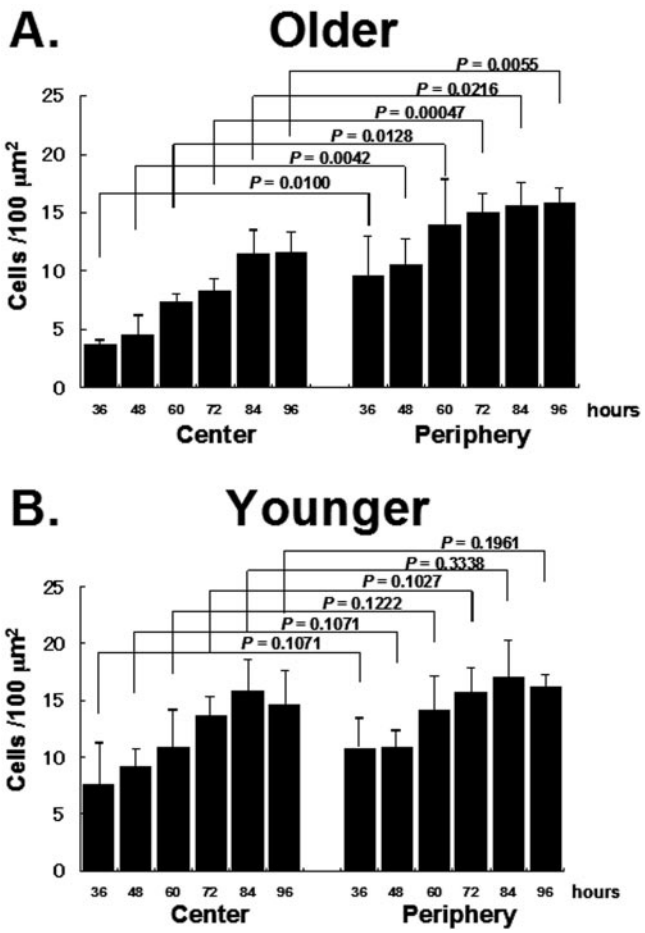

FIGURE 2. Comparison of the mean density of PI-positive HCECs in the wound area of central and peripheral cornea from older (**A**) and younger (**B**) donors. A scrape-wound was made in the endothelium of each corneal quarter. Tissue was then incubated in culture medium. The four corneal quarters were fixed at each time point and stained for PI. Three representative micrographs of the wound area in the central and peripheral cornea were used to calculate the density of PI-positive cells in a 100-μm^2 area. The number of cells from four different donors were averaged for each age group at each time point ($n = 4$). Data are presented as the mean ± SD and are considered statistically significant at $P < 0.05$.

Evaluation of Replication-Competent Cells

Fluorescence immunocytochemistry for MCM2 was used to evaluate replication competence. To compare the relative percentage of MCM2-positive HCECs in central and peripheral cornea, we divided the total wound area (approximately 2 × 4 mm) equally into central, middle, and peripheral regions (Fig. 1A). Five successive 20× magnification micrographs were taken in the center of the wound bed in both the central and peripheral regions of the corneal quarter (Fig. 1B). Only the middle three micrographs of the five were evaluated to ensure proper identification of the wound area. As a negative control, three micrographs were taken in the unwounded area. Images were analyzed on computer (Photoshop ver. 6.0, Japanese version; Adobe Systems Inc., San Jose, CA). A micrometer grid was superimposed on the images. Total PI-stained cells were counted within a 100-μm^2 area to determine the total number of cells present in the center of the wound bed at each time point. MCM2-positive cells were counted within the same microscopic field. All counts were expressed as the number of positive cells/100 μm^2. Counts from the three images were averaged, and the percentage of MCM2-positive cells was calculated by the following

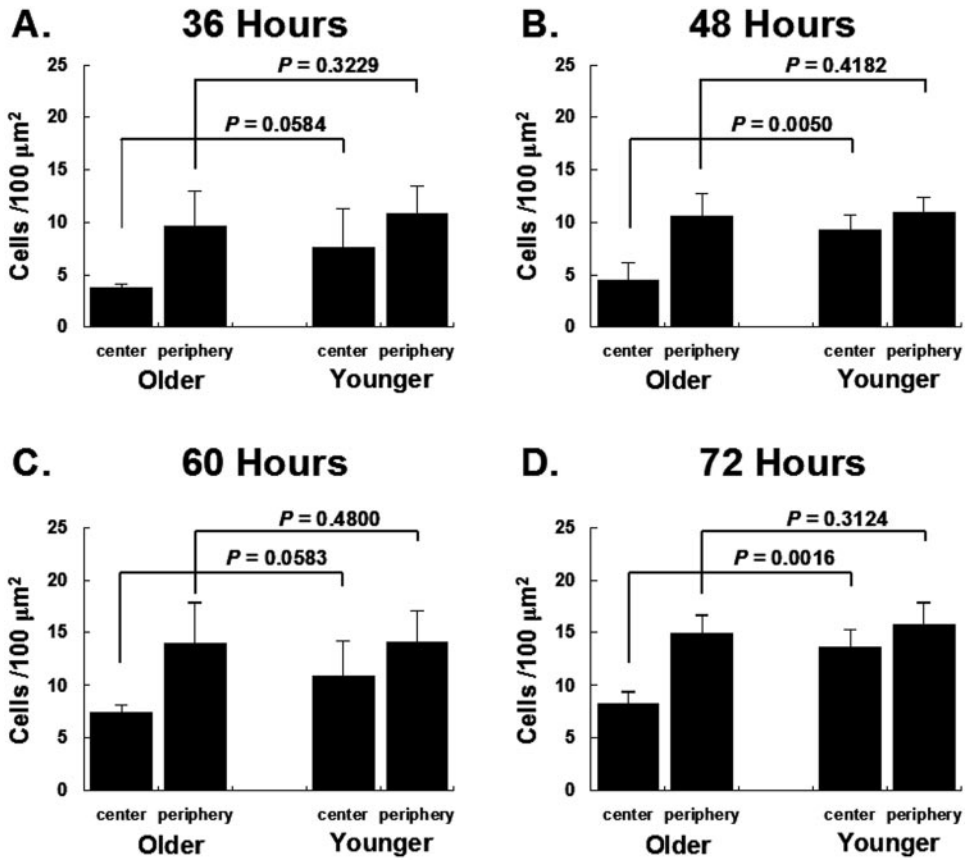

FIGURE 3. Comparison of the mean density of PI-positive HCECs between young and older donors at 36 (**A**), 48 (**B**), 60 (**C**), and 72 (**D**) hours after wounding. Data were obtained from the same study as in Figure 2, but are presented to compare HCEC density between age groups. Density within a 100-μm^2 area was averaged from four donors per age group and time point ($n = 4$). Data are presented as the mean ± SD and are considered statistically significant at $P < 0.05$.

formula: 100 × (total number of MCM2-positive cells/total number of PI-stained cells).

Evaluation of SA-β-Gal Activity

Assays to detect SA-β-Gal activity were performed with a commercial Senescence Detection Kit (BioVision Research Products, Mountain View, CA). Corneas were obtained from four young (3, 16, 19, and 21 years) and four older (62, 68, 68, and 75 years) donors. Whole corneas were cut in quarters and then washed with PBS. Each corneal quarter was fixed for 15 minutes at room temperature and then incubated at 37°C overnight in staining solution containing 5-bromo-4-chloro-3-indoyl-β-galactopyranoside at pH 6.0 according to the kit protocol. The negative control for this assay consisted of incubating corneal quarters in staining solution adjusted to pH 4.0 to detect normal lysosomal β-galactosidase.[18] The following day, tissues were washed with PBS over 15 minutes and mounted in medium containing PI as described

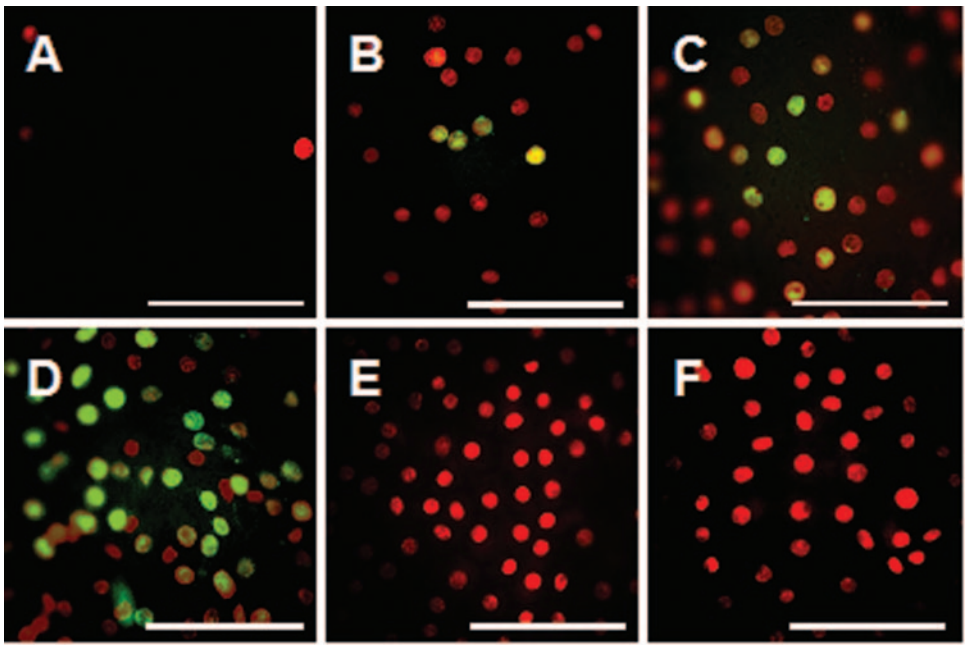

FIGURE 4. Representative images of MCM2 staining in HCECs from a 59-year-old donor. Corneas were cut in quarters, wounded, incubated, and stained for MCM2. Micrographs were taken in the midregion of the linear wound, between the central and peripheral areas at 24 (**A**), 36 (**B**), 48 (**C**), and 72 (**D**) hours after wounding. The relative number of MCM2-positive nuclei increased with time. No MCM2-positive cells were present in an unwounded area examined 72 hours after wounding (**E**). No staining was observed in the negative control incubated with mouse IgG instead of the primary antibody in wounded endothelium 72 hours after wounding (**F**). *Green*: MCM2; *red*: propidium iodide. Scale bars, 100 μm.

earlier. Development of a blue color indicated the presence of SA-β-Gal activity. Staining was visualized at 20× magnification, and both fluorescence (PI staining) and bright-field micrographs (SA-β-Gal) were taken within the central and peripheral areas of each corneal quarter, as described earlier. Images were evaluated on computer (Photoshop ver. 6.0; Adobe Systems). SA-β-Gal intensity was graded in each cell as follows: 0, no staining; 1, focal weak staining; 2, multifocal moderate staining; and 3, multifocal intense staining (see Fig. 8). The graded cells were counted with Image-J software (http://rsb.info.nih.gov/ij/ developed by Wayne Rasband and provided in the public domain by National Institutes of Health, Bethesda, MD). Individual cells were identified for counting by observation of cell–cell borders by bright-field microscopy or PI-stained nuclei. The number of cells stained for SA-β-Gal and the grade of SA-β-Gal intensity in three 100-μm^2 areas were averaged for the central and peripheral areas from each donor (Fig. 1B). Cells with unclear images were eliminated from the data. Cells from older donors produced more obscure images than those from younger donors. As a result, approximately 510 cells were counted per area in younger corneas, and an average of 312 cells were counted in older corneas. The percentage of cells stained for SA-β-Gal was calculated by the following formula: 100 × (number of cells stained with SA-β-Gal/total number of cells).

Statistics

The unpaired Student's *t*-test was used to compare mean values, and the level of significance was set at $P < 0.05$. All analyses were performed on computer (Stat View statistical software package ver. 5.0; Abacus Concepts, Berkeley, CA).

Results

Comparison of HCEC Response to Wounding in the Central and Peripheral Corneas of Young and Older Donors

HCECs exhibited morphologic changes and migrated into the wound area in a manner similar to that previously reported in ex vivo endothelial wounds.[6] By 24 hours after wounding, cells at the wound edge had begun to migrate into the wound and continued to migrate and/or proliferate until confluence was reached between 72 and 96 hours after injury. PI-positive cells were counted to compare the density of HCECs within the central and peripheral wound area over time, thereby determining the relative ability of HCECs to respond to wounding. In corneas from older donors (Fig. 2A), the mean density of HCECs increased in a time-dependent manner. Cell density in the central area plateaued by 84 hours after wounding, whereas density in the peripheral area plateaued by 72 hours, indicating formation of a confluent monolayer and healing of the wound. At each time point from 36 to 96 hours, significantly more PI-positive cells were observed in the peripheral than in the central area. This suggests that, in corneas from older donors, fewer HCECs in the central cornea respond to wounding than in peripheral cornea. In corneas from younger donors (Fig. 2B), the density of PI-positive HCECs also increased with time, and the number of cells in both areas tended to plateau by approximately 72 hours after wounding. Unlike in corneas from older donors, there was no significant difference in the mean density of cells at the center versus the periphery at any time point examined, indicating a similar ability of HCECs to respond to wounding, regardless of position within the endothelial monolayer.

Mean cell density was also compared in the wound areas of young and older donors (Fig. 3). No significant difference in the density of HCECs in the peripheral corneas of young or older donors was observed at any time point. In contrast, the density of HCECs in central cornea was more variable. Although no significant difference in mean density was observed at the 36- or 60-hour time points ($P = 0.058$ and $P = 0.058$, respectively), statistically significant differences were observed in central corneal wounds at 48 and 72 hours ($P = 0.0050$ and $P = 0.0016$, respectively). Together, these results indicate that the relative ability of HCECs to respond to wounding is similar in the peripheral cornea, regardless of donor age. Of note, there was a consistent age-related difference in the number of HCECs in the wound area of central cornea. This difference suggests that fewer cells in the central region of corneas from older donors were able to respond to wounding. It should be noted that PI-staining alone could not distinguish whether cells were present within the wound area as the result of migration or proliferation.

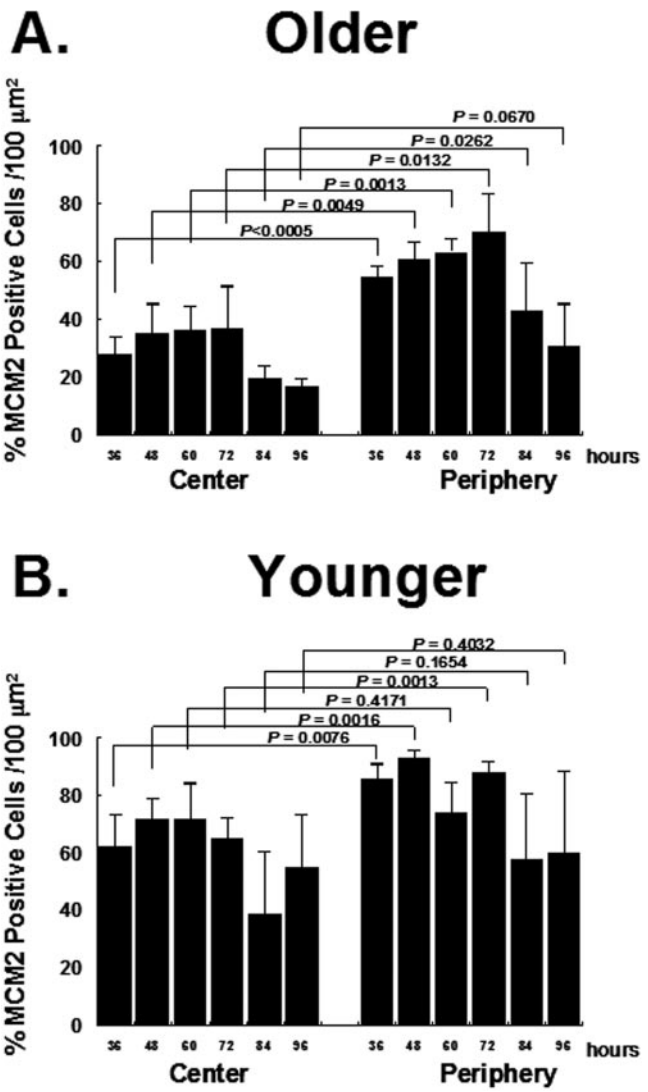

Figure 5. Comparison of the average percentage of MCM2-positive HCECs in the wound area of the central and peripheral cornea from older (**A**) and young (**B**) donors. Three representative micrographs of the central and peripheral wound areas were used to calculate the percentage of MCM2-positive cells in a 100-μm^2 area. The number of cells from four different donors was averaged at each age group and time point ($n = 4$). Data are the mean ± SD and are considered statistically significant at $P < 0.05$.

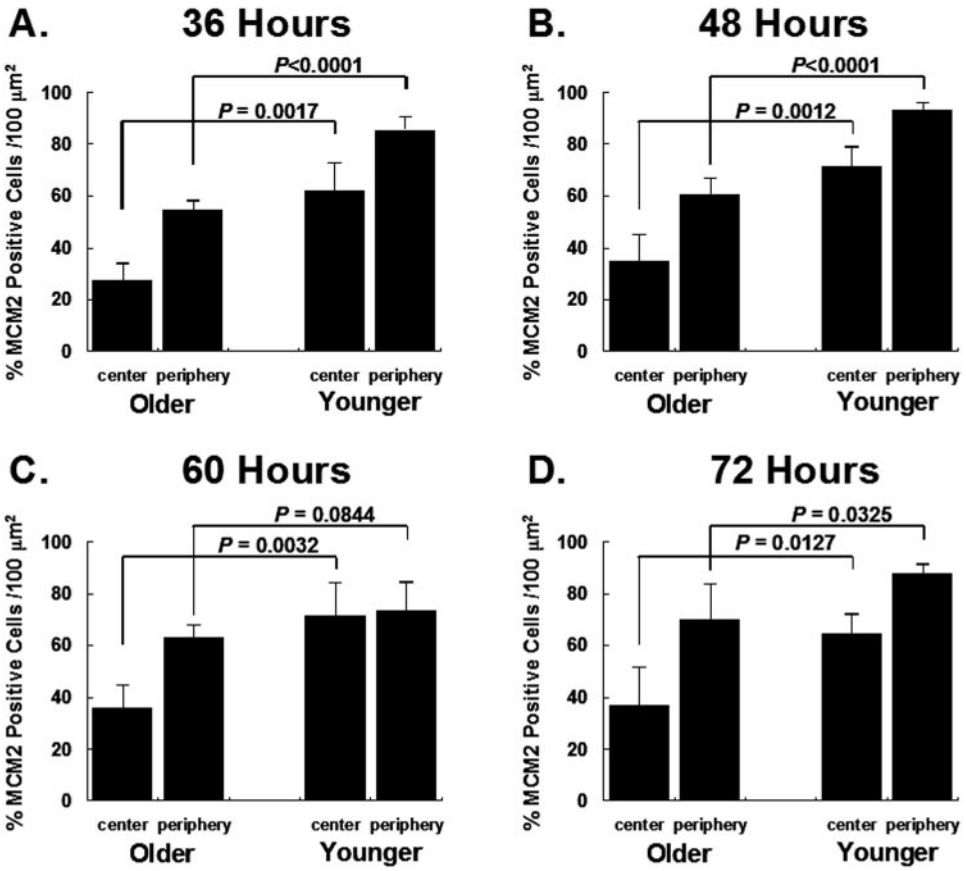

FIGURE 6. Comparison of the average percentage of MCM2-positive HCECs between older and younger donors at 36 (**A**), 48 (**B**), 60 (**C**), and 72 (**D**) hours after wounding. Density within a 100-μm^2 area was averaged from four different donors per age group and time point ($n = 4$). Data are presented as the mean ± SD and are considered statistically significant at $P < 0.05$.

Comparison of Replication Competence in Central and Peripheral Areas of Endothelium from Young and Older Donors

Formation of origin-recognition complexes on DNA is a requirement for S-phase entry. These origins contain a specific complex of proteins that associate with DNA during the G_1-phase and are present at sites on chromatin at or near future sites of initiation, making chromatin competent (licensed) for replication.[15,20] MCM2 is a member of this origin-recognition complex. It is synthesized during the G_1-phase and dissociates from the complex during the S-phase.[20] Expression of MCM2 protein is considered to be a reliable marker to identify replication-competent cells and is used to detect the replication-competent fraction of cells in malignant and normal tissues.[16,17] For this reason, MCM2 staining was used to detect replication-competent HCECs in an ex vivo wound model. In the wound model, cells at the wound edge lost contact inhibition, entered the G_1-phase of the cell cycle, and began synthesis of MCM2. Figure 4 presents representative images of MCM2 staining at various times after wounding. At 24 hours after wounding (Fig. 4A), relatively few cells had migrated into the wound bed, and no cells were stained for MCM2. With time, more cells migrated into the wound area and cells became competent to replicate as indicated by positive MCM2 nuclear staining (Fig. 4B–D). HCECs in unwounded areas of the same corneal piece showed no staining for MCM2 (Fig. 4E). No staining was seen in the negative control, in which wounded corneal quarters were incubated with mouse IgG instead of the primary antibody (Fig. 4F).

The percentage of MCM2-positive HCECs within the central and peripheral wound areas was calculated at 12-hour intervals from 36 to 96 hours after wounding. It should be noted that the same corneal pieces used to count PI-positive cells, as described earlier, were used to determine the percentage of MCM2-positive cells. Results were compared between central and peripheral HCECs within and between age groups. In corneas from older donors (Fig. 5A), the relative percentage of MCM2-positive cells in both the central and peripheral areas gradually increased between 36 and 72 hours after wounding, after which the number decreased. The timing of this decrease in MCM2-positive staining correlates well with the time at which the density of PI-positive cells plateaued, because it is known that MCM2 protein levels decrease rapidly as cells stop dividing and become quiescent.[15,20,21] The percentage of MCM2-positive HCECs was significantly higher in the periphery than in the central cornea at all time points from 36 to 84 hours after wounding. In corneas from younger donors (Fig. 5B), MCM2-positive cells were observed in both the central and peripheral areas, although the time dependence of the increase was less obvious than in corneas from older donors. A decline in MCM2-positive cells occurred in both areas by 72 to 84 hours after wounding, as was seen in corneas from older donors. The percentage of MCM2-positive cells in the peripheral area was significantly higher than in the center at 36, 48, and 72 hours after wounding. As shown in Figure 6, the percentage of MCM2-positive cells in both the peripheral and central areas was consistently higher in corneas from younger donors. Together, these results demonstrate that the replicative potential of HCECs is higher in the peripheral than in the central cornea, regardless of donor age, and that replication competence decreases with increasing donor age, particularly in the central cornea.

In the ex vivo wound model described herein, in which corneal quarters were used, the peripheral area consisted of a

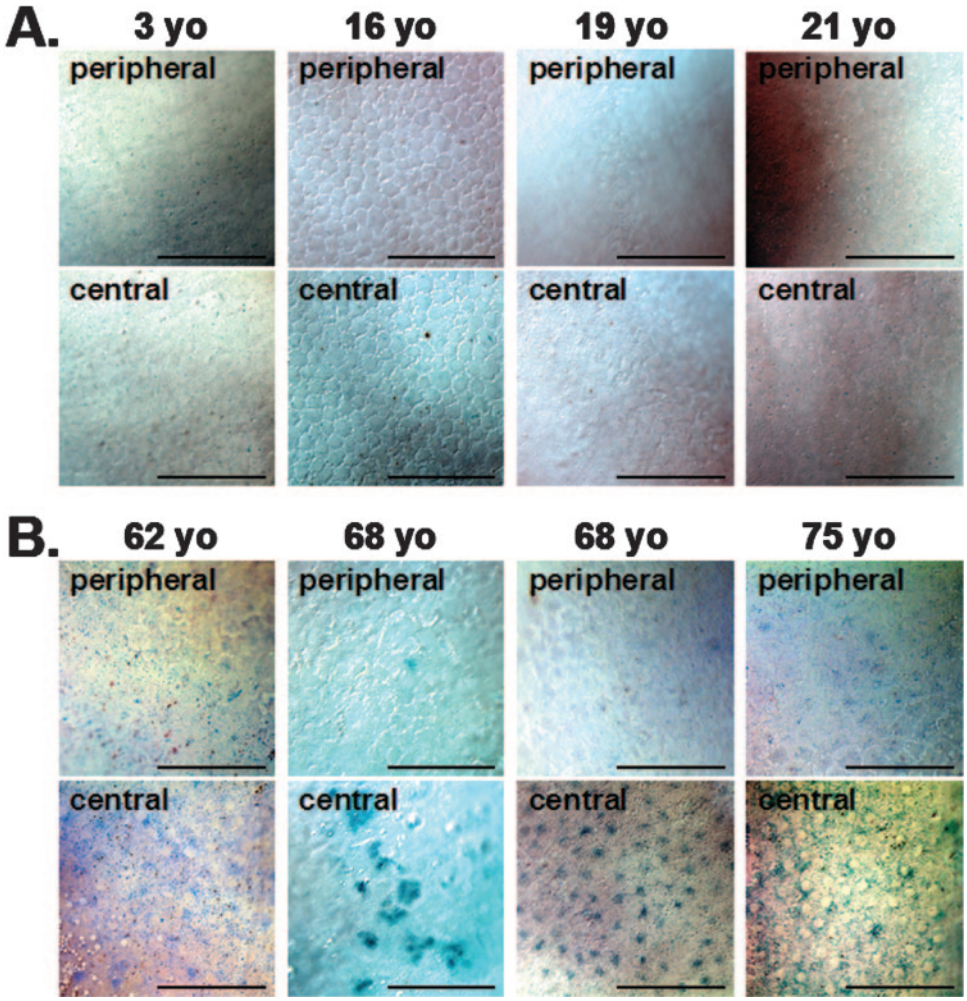

FIGURE 7. Histochemical analysis of senescence-associated β-galactosidase (SA-β-Gal) activity in HCECs in ex vivo corneas. (**A**) In younger donors, there is no or occasional faint staining for SA-β-Gal. (**B**) By contrast, older donors demonstrated a generally low level of positive staining in the peripheral area and greatly increased staining in the central area. Scale bars, 100 μm.

larger total number of cells than the central area as shown in Figure 1A. An additional study (Konomi K, Joyce NC, personal observation, 2005) was conducted to compare the effect of the reservoir of cells within the unwounded area on the relative percentage of MCM2-positive cells that had migrated into the wound area in the peripheral and central regions. In this study, a linear wound was made across the entire diameter of the cornea from periphery to periphery, and the relative percentage of MCM2-positive cells was calculated. Results from a limited number of corneas showed that the average percentage of MCM2-positive cells was higher in the periphery than in the center in corneas from both young and older donors (central versus peripheral area, 15% vs. 34% in corneas from 3-and 15-year-old donors, and 12% vs. 30.5% in corneas from 59- and 68-year-old donors). As observed previously, there were no MCM2-positive cells in the unwounded area. These results were similar to those of the wound model using corneal quarters. Therefore, our findings show that the difference in total cells within the central and peripheral areas did not appreciably affect the number of MCM2-positive cells in the wound area.

SA-β-Gal Activity in Human Corneal Endothelium

β-Galactosidase activity (pH 6.0) has been correlated with senescent cells both in culture and in vivo.[18,22] HCECs in corneas from young and older donors were stained for senescence-associated β-galactosidase (SA-β-Gal), to determine the relative percentage of senescent cells in the endothelial population. As shown in Figure 7A, little-to-no staining for SA-β-Gal activity was detected in the endothelium of corneas from young donors. In contrast, strong positive staining for SA-β-Gal was consistently observed in the endothelium of older donors and was particularly evident in the central area (Fig. 7B). SA-β-Gal staining was graded as a means of measuring relative staining intensity. Micrographs in Figure 8A illustrate the different grades of SA-β-Gal staining observed in the endothelium of older donors. The graph in Figure 8B shows that no positive SA-β-Gal staining was observed in either the central or peripheral area of the endothelium in corneas from younger donors. In corneas from older donors, the percentage of HCECs staining positive for SA-β-Gal was higher in the central than in the peripheral area (77.7% ± 22.3% vs. 51.9% ± 24.7%). The relative percentage of central HCECs positive for SA-β-Gal was 22.3% ± 25.4% at the intensity grade of 0; 36.9% ± 17.1% at 1; 29.6 ± 19.0% at 2; and 11.1 ± 3.5% at 3. The relative percentage of positive peripheral HCECs was 48.1% ± 24.7% at a score of 0; 50.3% ± 24.9% at 1; 1.4% ± 0.5% at 2; and 0.2% ± 0.3% at 3. These results indicate that there were few to no senescent cells in either the central or peripheral area of the endothelium in corneas of younger donors. In contrast, HCECs from older donors show an increased number of cells that exhibited characteristics of senescence, with central endothelium containing the highest percentage of senescent cells. The positive control showed normal β-galactosidase staining at pH 4.0 in all cells, regardless of relative position or donor age (data not shown).

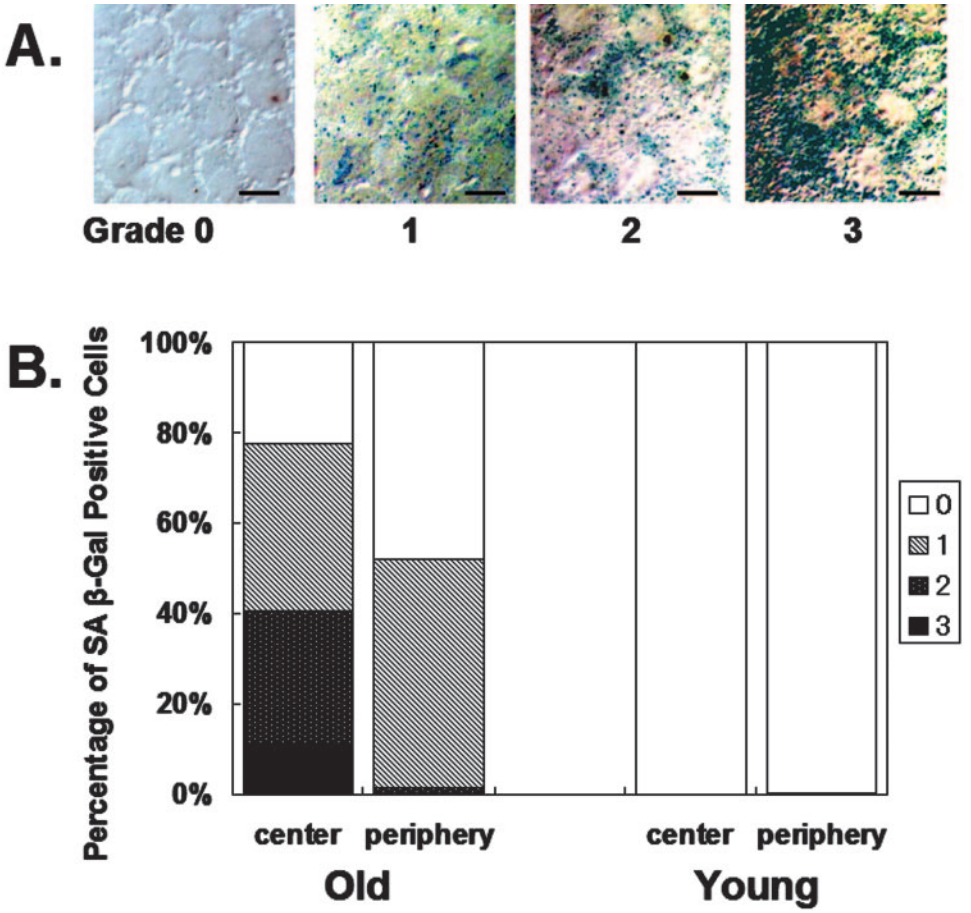

FIGURE 8. Scoring of SA-β-Gal activity in HCECs in ex vivo corneas. (**A**) SA-β-Gal intensity was graded as 0 (no staining), 1 (focal weak staining), 2 (multifocal moderate staining), or 3 (multifocal intense staining). (**B**) Average percentage of SA-β-Gal-positive cells at each grade. Corneas from four different donors were examined for each age group ($n = 4$).

DISCUSSION

The results of these studies in ex vivo corneas reveal that the corneal endothelial cell population becomes increasingly heterogeneous with donor age. Calculation of the density of PI-positive HCECs provided information regarding the relative ability of cells to respond to wounding. No significant difference was observed in HCEC density between the central and peripheral areas in corneas from younger donors, indicating that cells throughout the endothelium had a similar ability to respond to wounding. However, in corneas from older donors, the HCEC density after wounding was significantly higher in the peripheral than in the central area. These findings suggest that either fewer total cells in the central area are capable of migrating into the wound bed or the rate of response to wounding is significantly slower.

As in an earlier tissue culture study,[12] we chose to evaluate the relative proliferative capacity of HCECs by immunostaining for MCM2, rather than for Ki67, as was used in a prior study.[6] Studies comparing MCM2 and Ki67 as prognostic indicators in malignant tissue have found that the total replication-competent fraction indicated by MCM2 is consistently larger than that indicated by Ki67.[16] This is most likely because the relative timing of MCM2 synthesis is earlier in the G_1-phase, and MCM2 staining is easier to visualize because its nuclear staining pattern does not change with cell cycle status, as occurs with Ki67. MCM2 is considered to be a better marker to detect cells with the potential to replicate, because it is directly involved in making chromatin competent for duplication.[15,21] In contrast, Ki67 is believed to detect the actual growth fraction of cells. Therefore, MCM2 is considered to be a more sensitive marker to detect replication-competent cells than is Ki67.[16,17] The percentage of HCECs positively stained for MCM2 tended to be higher in the peripheral than in the central area of corneas of younger donors, although statistically significant differences were not noted at all time points. More striking were the differences found in corneas of older donors, where significantly fewer cells in the central region stained for MCM2 than did cells from the periphery. These results strongly suggest that, of the cells present in the wound bed, far fewer cells in the central endothelium of older donors are capable of replication than in the periphery. Taken together, the results suggest that, regardless of donor age, peripheral HCECs have a greater capacity for self-renewal than cells in the central cornea. It can be inferred from these data that, in corneas of older donors, the higher density of HCECs in the peripheral wound area results from proliferation as well as migration, whereas the density of HCECs in central wounds results mainly from cell migration.

Senescent cells are characterized by an increase in cell size and lysosomal mass, accumulation of lipofuscin, an increase in SA-β-Gal activity, inability to replicate, and overexpression of the cyclin-dependent kinase inhibitor p21CIP1/WAF1/Sdi1.[18] SA-β-Gal does not appear to detect a different form of β-galactosidase, but staining for SA-β-Gal appears to reflect an age-related increase in the cellular content of classic lysosomal β-galactosidase, which is detectable at pH 6.0 rather than the usual pH 4.0.[18,23] In the current studies, staining for SA-β-Gal activity was used to detect HCECs exhibiting characteristics of senescence. The lack of detectable SA-β-Gal activity in either the central or peripheral region of the endothelium in corneas from

younger donors strongly suggests that there are few if any senescent cells in younger tissue, regardless of location. In HCECs from older donors, SA-β-Gal activity was clearly detectable and the highest percentage of cells graded 2 or 3 was detected in central endothelium, indicating the presence of a greater population of senescent cells in this area. These results negatively correlate with the MCM2 data and indicate that, in corneas from older donors, the endothelial population contains a higher percentage of cells exhibiting growth-arrested, senescence characteristics than in corneas from younger donors. In corneas from older donors, the endothelium also exhibits regional differences evidenced by the highest population of senescent cells within the central region. We hypothesize that increased central cell senescence contributes to age-related endothelial disease.

Several groups have studied HCEC replication and measured cell density in the peripheral and central areas. Tissue culture studies by Bednarz et al.[24] indicate that HCECs from the peripheral area are able to replicate, but that cells from the center exhibit little to no mitotic activity. In contrast, our laboratory recently reported that there was no significant difference in replicative capacity between HCECs cultured from the central and peripheral areas.[12] It is possible that the differences in these results are due to the specific methods and media used for culturing and/or to the relative viability of the donor corneal tissue. In the current ex vivo cornea study, the central endothelium of older donors contained the greatest population of senescent-appearing cells; however, even in this region there also remained a population of replication-competent cells. Comparison of results from our tissue culture and ex vivo cornea studies strongly suggests that, when central endothelial cells are cultured from older donor corneas, there is a selection of healthy, nonsenescent cells and that it is this selected subset of the population that is capable of proliferation.

Schimmelpfennig[13] and Amann et al.[14] reported that the density of HCECs in the peripheral area is higher than that of the central area. It is also well known that the density of HCECs gradually decreases after birth. Our results provide a novel mechanism to explain these observations. Perhaps, with age, HCECs in the central cornea become more stressed than cells in the peripheral area, which leads to loss of proliferative capacity and the development of a more senescent phenotype. Our data suggest that greater loss of cells from central endothelium leads to gradual cell rearrangement and the centripetal movement of cells from the denser periphery toward the central region. This movement replicates embryonic development of the cornea in which neural crest cells migrate centripetally and differentiate into mature corneal endothelium.[1,25,26] Further study is needed to test this hypothesis. Careful investigation is also needed to demonstrate whether a corneal endothelial stem cell population exists and to determine its role in maintaining endothelial cell density. It is clear that the mechanisms underlying maintenance of the endothelial monolayer are less efficient that those that mediate cellular replacement in the corneal epithelium.

In conclusion, studies of corneal endothelium in ex vivo corneas indicate that the percentage of replication-competent HCECs is higher in the peripheral than in the central cornea. Significantly fewer central HCECs in corneas from older donors retained the competence to replicate compared with the central area of younger donors, and HCECs, particularly in the central cornea, undergo senescence-like changes with advancing donor age.

Acknowledgments

The authors thank Kenji Konomi, MD, for providing the data regarding MCM2 staining in whole corneas; Kenji Konomi and Cheng Zhu, MD, for advice on handling donor corneas; and Kikuko Enomoto, MD, and Deshea L. Harris, MS, for advice regarding how to conduct the immunocytochemical studies.

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