

Cell Cycle Kinetics in Corneal Endothelium from Old and Young Donors

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PURPOSE. To compare cell cycle kinetics in corneal endothelial cells from young and old donors.

METHODS. Human corneas were obtained from the eye bank and separated into two groups: young (19 corneas, <30 years of age) and old (40 corneas, >50 years of age). Corneas were cut in quarters, and the endothelium was released from contact inhibition by producing a 2-mm scrape wound. Unwounded endothelium acted as a negative control. Corneal pieces were exposed for 24, 36, 48, 60, 72, and 84 hours to medium containing 10% fetal bovine serum, 20 ng/ml fibroblast growth factor, and 50 mg/ml gentamicin or the same medium supplemented with 10 ng/ml epidermal growth factor (EGF). Tissue was fixed, immunostained for Ki67 (a marker for the late G1-through M-phase) or for 5-bromo-2'-deoxyuridine (BrdU; a marker for the S-phase), and mounted in medium containing propidium iodide (PI) to visualize all nuclei. Confocal images were evaluated using an image analysis program to count Ki67-positive and PI-stained cells and to evaluate cell cycle position. Cells were counted in $15 \times 100 \mu\text{m}^2$ areas randomly selected from each wound, and the mean was used for subsequent calculations.

RESULTS. Human corneal endothelial cells could be reliably scored for their position within the cell cycle using Ki67 staining patterns. In both age groups, cells repopulating the wound area stained positively for Ki67, whereas no Ki67 staining was observed in unwounded areas under any condition tested. Cells from old donors treated with fetal bovine serum and FGF stained positively for Ki67, indicating that these cells were actively cycling. Compared with cells from young donors, old cells entered the cell cycle more slowly (48 versus 36 hours), the peak of Ki67 staining occurred later (72 versus 60 hours), and fewer cells proliferated (23% versus 47%) or exhibited mitotic figures (4% versus 7%). Addition of EGF to the culture medium increased Ki67 staining in both groups, but the effect on old cells was more dramatic. More cells from old donors entered the cell cycle by 36 hours after wounding, the number of proliferating cells increased 1.6-fold, and the relative number of mitotic figures increased 2.5-fold over cells treated in the absence of EGF.

CONCLUSIONS. Regardless of donor age, corneal endothelial cells can enter and complete the cell cycle. In the presence of fetal bovine serum and FGF, cells from old donors can proliferate but respond more slowly and to a lesser extent than cells from young donors. EGF added to the medium stimulates cells from old donors to enter the cell cycle faster, increases the relative number of actively cycling cells, and increases the number of cells exhibiting mitotic figures. The resultant hypothesis is that it is possible to stimulate a significant proliferative response in corneal endothelial cells from old individuals. Administration of an optimal combination of stimulatory growth factors is required under conditions in which cells have been transiently released from contact inhibition. (*Invest Ophthalmol Vis Sci.* 2000;41:660-667)

The endothelium is the single layer of cells located at the posterior of the cornea between the stromal layer and the aqueous humor. The major function of this tissue is to maintain corneal transparency. The density of human corneal endothelial cells decreases with age,^{1,2} disease,^{3,4} and intraocular surgery^{5,6} or laser procedures.⁷ Maintenance of corneal clarity

requires an intact endothelial layer, and transparency can be lost when endothelial cell density is reduced below a critical level. The age-related decrease in endothelial cell density suggests that the rate of cell division does not keep pace with the rate of cell loss. Although human corneal endothelial cells do not normally exhibit proliferative activity in vivo^{8,9} and are generally difficult to establish in long-term culture,^{10,11} they retain proliferative capacity. Joyce et al.^{12,13} have demonstrated that, unlike suprabasal cells of the epithelium, corneal endothelial cells in vivo do not exit the cell cycle but are arrested in G1-phase. When G1-phase arrest is overcome in cultured human corneal endothelial cells by transfection with viral oncoproteins, such as simian virus (SV) 40 large T antigen^{14,15} or human papilloma virus E6/E7,¹⁵ the cells proliferate for several generations before the onset of replicative senescence.

Ki67 is detected in the nucleus of proliferating cells in all active phases of the cell cycle from the late G1-phase through

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the M-phase but is absent in nonproliferating and early G1-phase cells and in cells undergoing DNA repair.¹⁶⁻¹⁸ Ki67 antibodies have been used widely for the estimation of the growth fraction of clinical samples of human neoplasm^{19,20} and of normal cells,^{21,22} including corneal cells.^{12,13,23-25} Immunolocalization studies have reported that Ki67 antibody staining produces characteristic patterns in cells depending on their position within the cell cycle.²⁶⁻²⁸ For example, in mouse fibroblasts, Ki67 staining during the late G1-phase is detected as granular foci in the nucleoplasm. During the S- and G2-phases, it is detected as a large dotlike pattern associated with the nucleolus. During mitosis, Ki67 staining is distributed in a reticulate structure surrounding the condensed chromosomes.

The long-term goal of our studies is to increase corneal endothelial cell density in old individuals by stimulating division in a regulated manner. Studies by a number of investigators have demonstrated proliferative activity in human corneal endothelial cells,^{8,29-42} but the relative ability of cells from young and old donors to enter and complete the cell cycle has not been rigorously compared. The purpose of the current studies was to compare the ability of corneal endothelial cells from young (<30 years old) and old donors (>50 years old) to enter and complete the cell cycle. These studies used an ex vivo wound model to permit observation of age-related differences in an environment in which exposure to mitogenic stimulation could be controlled and cells could respond to wounding while associated with normal Descemet's membrane. Ki67 antibody staining was used to quantify cell cycle entry and completion in the two groups.

MATERIALS AND METHODS

Human Corneal Tissue

Donor human corneas were obtained from National Disease Research Interchange (Philadelphia, PA). All corneas were preserved at 4°C for 1 week or less in Optisol (Chiron Vision, Irvine, CA) before use. Endothelial cell counts were more than 2000 cells/mm². Criteria for exclusion of corneas from these studies included history of endothelial dystrophy, presence of central guttata, low endothelial cell density, and presence of ocular inflammation or disease.

Ex Vivo Wound Model

Human corneas were separated into two groups: young donors 30 years of age or less, and old donors 50 years of age or more. Nineteen corneas were obtained from young donors (mean age: 19.3 ± 6.4; range: 4 months to 29 years), and 40 corneas were obtained from old donors (mean age: 64.9 ± 8.8; range: 50-81 years). Whole corneas were cut in quarters, and the endothelium was released from contact inhibition by producing a 2-mm scrape wound (Fig. 1) with a silicon-coated needle (Alcon, Fort Worth, TX). Unwounded endothelium acted as a negative control. Corneal pieces were placed endothelial side up in individual wells of a 24-well tissue culture plate (Falcon, Lincoln Park, NJ). Pieces were exposed to Medium-199 (GIBCO, Grand Island, NY), containing 10% fetal bovine serum, 20 ng/ml fibroblast growth factor (FGF; Biomedical Technologies, Stoughton, MA), and 50 mg/ml gentamicin, or the same medium supplemented with 10 ng/ml epidermal growth factor (EGF; Upstate Biotechnologies, Lake Placid, NY). Cultures

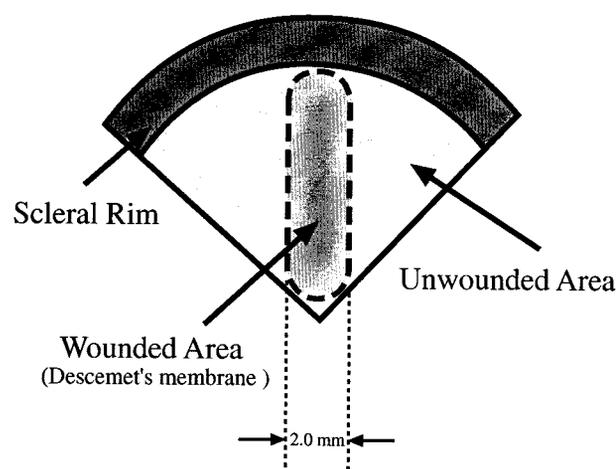


FIGURE 1. Whole corneas were cut in quarters, and the endothelium was removed by scraping with a silicon-coated needle. No damage to Descemet's membrane was observed.

were maintained for various periods at 37°C in a 5% carbon dioxide, humidified atmosphere. Time points of examinations after wounding were 24, 36, 48, 60, 72, and 84 hours.

Immunolocalization of Ki67

At the desired time point, each corneal piece was fixed for 10 minutes in ice-cold methanol and rinsed three times in phosphate-buffered saline (PBS). Pieces were incubated for 10 minutes in 1% Triton X-100 in PBS to permeabilize the cells, followed by incubation for 10 minutes with 4% bovine serum albumin in PBS to block nonspecific binding. Corneal pieces were then incubated for 2 hours with mouse monoclonal anti-human Ki67 IgG (clone MIB-1; Zymed Laboratories, San Francisco, CA) at room temperature in a humidified chamber. This antibody was prediluted by the supplier and was applied directly to the corneal pieces. Each piece was then washed three times with PBS. Fluorescein-conjugated anti-mouse IgG (Jackson Laboratories, West Grove, PA.), diluted 1:200 in blocking buffer, was applied to the corneal pieces for 2 hours. Pieces were then washed with PBS and mounted in medium containing propidium iodide (PI; Vector, Burlingame, CA) for visualization of nuclei. Slides were viewed using a confocal microscope (model TCS 4D; Leica, Deerfield, IL) equipped with a laser (model DMRBE; Leitz Lasertechnik, Heidelberg, Germany) and software (SCANware ver. 4.2; Leica). Images were collected using a ×16, ×40, or ×100 oil-immersion lens. Laser power and gain controls were adjusted to achieve an optimal range of output signal intensity for each channel. Confocal images were collected, and micrographs were printed (Photoshop ver. 4.0; Adobe Systems, San Jose, CA). For some micrographs, the printing contrast was adjusted to provide a clearer image.

Immunolocalization of BrdU

Staining for 5-bromo-2'-deoxyuridine (BrdU) was used to identify DNA-synthesizing cells in the ex vivo wound model. Corneal pieces were placed in medium containing BrdU (1:1000 dilution) for the same periods indicated earlier. Each piece was then rinsed three times in PBS to remove unbound BrdU, fixed for 10 minutes in ice-cold methanol, and then permeabilized

and incubated in blocking buffer as described earlier. Incorporated BrdU was detected by incubating the corneal pieces for 2 hours in mouse monoclonal anti-BrdU IgG containing nuclease (10 IU/ml; Amersham, Amersham, UK). This antibody was prediluted by the supplier and was applied directly to the corneal pieces. Pieces were incubated for 2 hours in fluorescein-conjugated anti-mouse IgG, diluted 1:200 in blocking buffer, washed, and mounted on slides as described earlier.

Evaluation and Quantification of Cells in Specific Cell Cycle Phases

Fluorescence confocal immunocytochemistry for Ki67 and BrdU was used to evaluate corneal endothelial cells for their ability to enter and complete the cell cycle. All nuclei were stained with PI. Positive BrdU staining identified DNA-synthesizing cells, and Ki67-staining patterns acted as markers for specific phases of the cell cycle identified according to the criteria of Kill²⁶ and Starborg et al.²⁷ Completion of the cell cycle was determined by observation of mitotic figures stained with Ki67. Three representative confocal micrographs were taken per wound using a $\times 40$ objective lens. A semiquantitative analysis of cell cycle response was made by counting total PI-stained nuclei, total Ki67- or BrdU-positive cells, and total G1-, S/G2-, and M-phase cells using a software program (NIH Image ver. 1.62; NIH, Bethesda, MD). Cells were counted in five $100\text{-}\mu\text{m}^2$ areas of each micrograph (15 areas counted per wound). Counts were averaged and the percentage of actively cycling cells (total Ki67-positive cells/total PI-stained cells) was calculated. The percentage of cells in each phase of the cell cycle was determined in a similar manner. Differences between the Ki67 and BrdU results were tested statistically using Wilcoxon's rank test. Statistical comparison of kinetic results from young and old donor corneas was made using a paired Student's *t*-test. Both analyses were conducted using a software program (StatView ver. 4.11; Abacus Concepts, Berkeley, CA).

RESULTS

Ki67 Staining Patterns in Human Corneal Endothelial Cells

Ki67 antibody produces characteristic staining patterns in cells depending on their position within the cell cycle.^{26,27} These staining patterns were used to classify cells in the late G1-phase through the M-phase. Representative micrographs of typical Ki67 staining patterns in human corneal endothelium are shown in Figure 2. Diffuse granular staining within the nucleus was considered to represent the G1-phase (Fig. 2A) and a more distinct, large, dotlike pattern was representative of the S/G2-phase (Fig. 2B). The M-phase was determined by observation of mitotic figures stained with Ki67 (Figs. 2B, 2C, 2D).

Comparison of Positive Staining for Ki67 and BrdU

Petroll et al.²⁵ have used Ki67 staining patterns to identify specific cell cycle phases in cat corneal endothelial cells; however, this is the first known study in which Ki67 has been used to identify cell cycle phases in human corneal endothelial cells. BrdU is incorporated during DNA synthesis and, under the incubation conditions used in this study, remained associated with the newly synthesized DNA throughout the remaining phases of the cell cycle. To confirm our identification of the

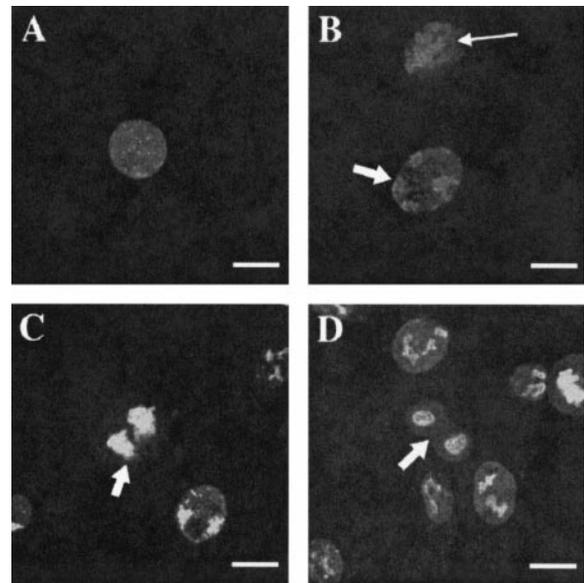


FIGURE 2. Representative micrographs of Ki67 staining patterns in human corneal endothelial cells. During the late G1-phase, Ki67 is detected as numerous small foci in the nucleoplasm (A). During the S- and G2-phases, Ki67 appears in a large dotlike pattern associated with the nucleolus (B, large arrow). During prometaphase, Ki67 was distributed in reticulate structures surrounding the condensed chromosomes (B, fine arrow). Ki67 remained associated with chromosomes in anaphase (arrow in C) and telophase (arrow in D). Bar, 20 μm .

S/G2-phase using Ki67, BrdU staining was correlated with Ki67 staining patterns. Studies were conducted at each time point to compare the percentage of Ki67- and BrdU-stained cells in the endothelium of the same donor (Fig. 3). As expected, total Ki67 staining (representing late G1- through M-phase) was higher at each time point than total BrdU staining. When the percentage of G1-phase cells was subtracted from total Ki67 staining, the resultant percentage was very similar to that of BrdU-stained cells. These results indicate that Ki67 staining correctly identified the S/G2-phase of the cycle.

Changes in Ki67 Pattern with Time

Micrographs of the wound area in Figure 4 are representative examples illustrating Ki67 pattern changes with time. Endothelial cells at the wound edge began to extend pseudopodia onto Descemet's membrane within 8 to 12 hours after wounding (data not shown). By 24 hours after wounding, cells began to migrate into the wound area, but were not stained for Ki67 (Fig. 4B). Positively stained cells were visible within 36 to 48 hours after wounding (Fig. 4C). Observation of the endothelium under low magnification (Fig. 4A) indicates that the majority of Ki67-positive cells were located within the wound bed and in cells immediately adjacent to the wound edge. The relative number of Ki67-positive cells increased and Ki67 staining patterns changed over time (compare Figs. 4C, 4D, 4E). Mitotic figures appeared 48 to 60 hours after wounding. No positive staining was observed in unwounded areas of the same corneal piece (Fig. 4F).

Cell Cycle Progression in Young and Old Corneal Endothelium

The first series of studies compared the response of cells from young and old donors to culture medium containing 10%

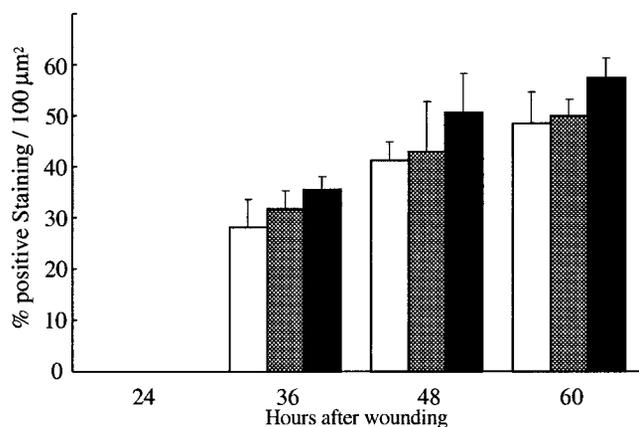


FIGURE 3. Comparison between positive staining for Ki67 and BrdU in human corneal endothelial cells from a 22-year-old donor. Corneas were incubated in 10% serum plus 10 ng/ml EGF (*open bars*, percentage of cells stained positively for BrdU; *gray bars*, percentage of total cells stained positively for Ki67 minus percentage exhibiting a G1-phase staining pattern; and *black bars*, percentage total cells stained positively for Ki67). Each column represents the mean \pm SEM for 15 \times 100 μm^2 areas randomly selected from each wound.

serum and 20 ng/ml FGF, a medium formulation that supports proliferation of rabbit corneal endothelial cells in culture.⁴³ When a very modest proliferative response was observed in old cells using this medium (see below), 10 ng/ml EGF was added to the medium to determine whether the response could be further stimulated, as occurs with rabbit cells.

In endothelial cells of young donors incubated in 10% serum, 20 ng/ml FGF, Ki67-positive staining was visible by 36 hours after wounding (Fig. 5A). The total number of positive cells increased with time, reaching a peak of 46.7% by 60 hours, after which cell counts appeared to plateau. A maximum of approximately 7% of total cells exhibited mitotic figures at 60 hours after wounding. This represents approximately 15% of all Ki67-positive cells at the 60-hour time point. Addition of 10 ng/ml EGF to the culture medium (Fig. 5B) did not appear to alter the kinetics of cell cycle entry in cells from young donors. EGF treatment increased the total number of Ki67-positive cells to approximately 60% and the number of cells in the M-phase to approximately 13.3% at 60 hours after wounding. This represents approximately 22% of all Ki67-positive cells at this time point. Proliferative activity appeared to peak at the 60-hour time point and declined thereafter. Cells exhibiting the late G1-phase staining pattern were present at all time points tested, regardless of the presence or absence of EGF. This finding indicates that cells within the healing wound continued to enter the cycle throughout the observation period under both growth conditions.

When treated with 10% serum, 20 ng/ml FGF, endothelial cells from old donor corneas took longer to enter the cycle than did cells from young donors (Fig. 5C)—that is, very few cells were positively stained at 36 hours after wounding. The total number of Ki67-positive cells reached a peak of approximately 23% at 72 hours and, as with the young group, appeared to plateau after that time. Under these treatment conditions, the percentage of mitotic figures was quite low, reaching a peak of approximately 4% by 72 hours after wounding; however, this represents approximately 17% of all Ki67-positive cells at that time point—a percentage similar to that of

cells from young donors. As observed with young donors, cells continued to enter the cycle throughout the study period. More cells entered the cycle by 36 hours when EGF was added to the culture medium (Fig. 5D). The total number of Ki67-positive cells increased with time, with a peak of approximately 37% reached by 72 hours. This represents approximately a 1.6-fold increase in actively cycling cells after EGF addition. By the 84-hr time point, the relative number of Ki67-positive cells began to decrease. EGF treatment significantly increased the percentage of old endothelial cells exhibiting mitotic figures. By 72 hours after wounding, approximately 10% of cells were in the M-phase of the cell cycle. This represents 27% of all Ki67-positive cells at that time point and a 2.5-fold increase compared with cells maintained in the absence of EGF.

DISCUSSION

Previous studies of human corneal endothelial cell proliferation, although of much value, have not directly compared the kinetics of cell cycle entry in young and old donors. In addition, there are few studies that have determined the relative ability of young and old cells to enter and complete the cell cycle in response to different mitogenic stimulation. Some studies have had small sample sizes.^{8,29,30} Other excellent studies, such as those conducted by Treffers³¹ and Couch et al.,³² provided valuable kinetic information, but they combined data over a wide range of ages rather than directly comparing age-related differences in proliferative response. In many studies, methods have been used that investigate only one portion of the cell cycle. For example, methods, such as scanning electron microscopy,³³ specular microscopy,⁸ inverted phase-contrast microscopy,³⁰ and light microscopy of stained cells³² identify only mitotic figures and do not provide information regarding other phases of the division cycle. [³H]thymidine incorporation studies have great value in that they can identify cells in the S- through M-phases (depending on the incubation conditions) and permit quantification of kinetic data. Unfortunately, the majority of studies using this method have not specifically compared the proliferative response of cells from young and old donors.^{29,31} A number of studies have also been conducted using cultured human corneal endothelial cells. These studies indicate that cells obtained from young (<20 years old) donors grow better and can be passaged longer than cells from old donors,^{10,34–40} but cell cycle kinetics were not directly examined. Those studies that have tested the effect of growth factors on human corneal endothelial cell proliferation have not compared age-related responses.^{10,35,39–42}

In the current ex vivo studies, the relative ability of corneal endothelial cells from young and old donors to enter and complete the cell cycle was directly examined. A relatively large sample size was studied, and Ki67 staining patterns were used to follow cell cycle progression from late G1-phase through mitosis. Comparison between Ki67 and BrdU staining indicated that Ki67 patterns were able to accurately categorize cells in specific phases of the cell cycle. The study design permitted observation of cell cycle kinetics within single donor samples and among grouped samples.

Several important observations were made in this study. Results clearly indicated that human corneal endothelial cells are capable of both entering and completing the cell cycle. The

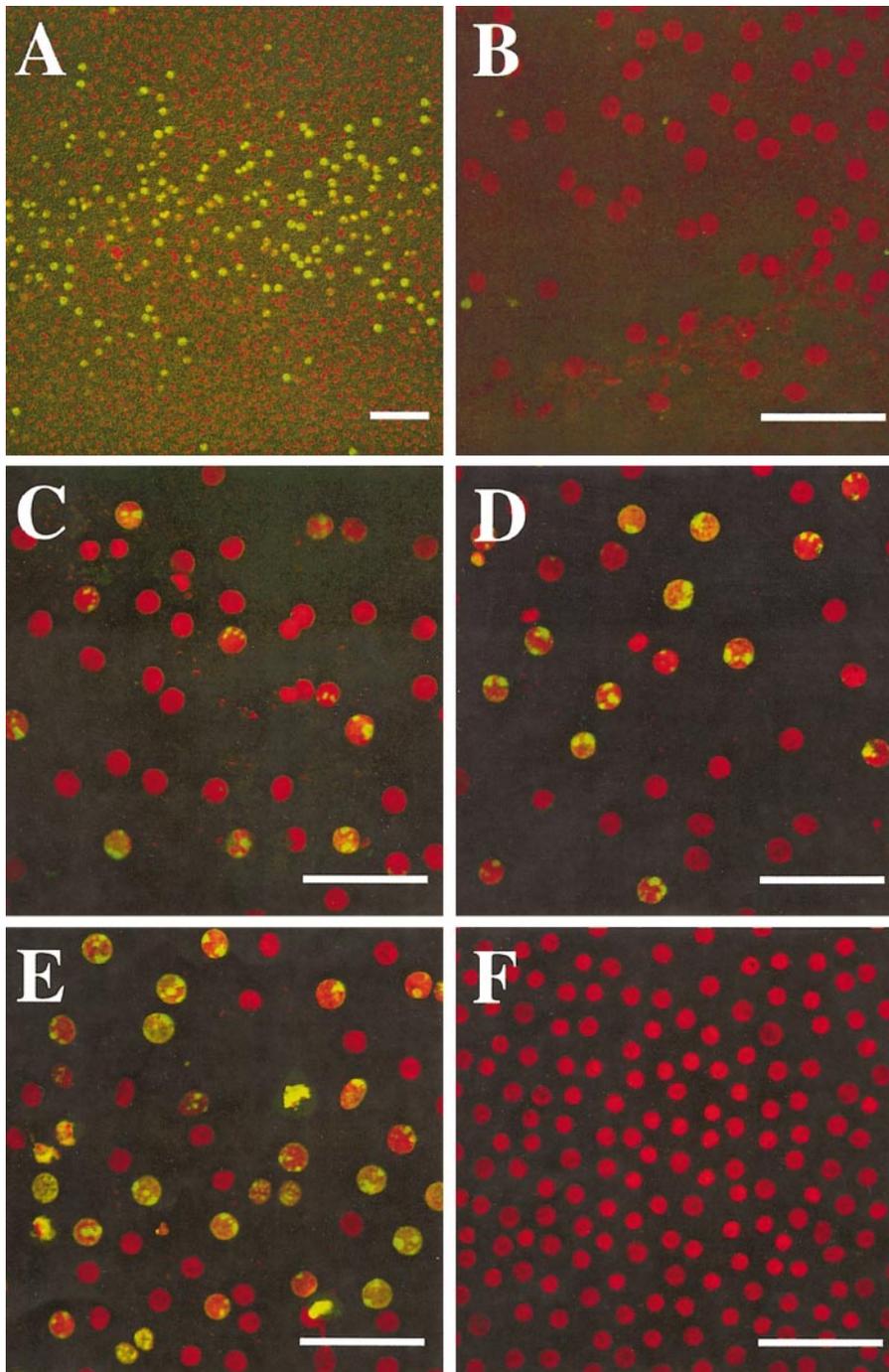


FIGURE 4. Changes in Ki67 staining with time in wounded corneal endothelium from a 22-year-old donor. Corneal pieces were incubated in 10% serum, 20 ng/ml FGF, and 10 ng/ml EGF. Low-magnification micrograph in (A) reveals Ki67-positive cells within healing wound 48 hours after wounding. The relative number of Ki67 stained cells and Ki67 staining patterns changed over 24 (B), 36 (C), 48 (D), and 60 hours (E). No Ki67-positive cells were present in an unwounded area 60 hours after wounding (F). *Green*, Ki67 staining; *red*, propidium iodide staining. Bar, 100 μ m.

major differences observed in these studies were in the kinetics and extent of the proliferative response of the two age groups and the more significant response of old cells to EGF. The majority of cells from old donors entered the cell cycle more slowly (48 versus 36 hours) and reached a peak of proliferative activity later than cells from young donors. These results are similar to those of Zagorski and Naumann,⁴⁴ who also used an *ex vivo* corneal wound model to study endothelial proliferation. They observed the presence of mitotic figures in endothelial cells from 30 to 72 hours after injury and found that the time interval between injury and the appearance of mitotic figures was longer in cells from old donors. The finding that significantly fewer cells from old donors entered the cell cycle

compared with cells from young donors is not novel. What is important is that these cells also appear to have completed the cycle, indicated by the presence of mitotic figures. Many late telophase cells were observed in the endothelium from old donors. The nuclei of these cells were in proximity but separated from each other, and both nuclei were smaller than those of neighboring cells. These morphologic characteristics are compatible with the formation of daughter cells, but it was not possible to confirm this fact by Ki67 staining.

The significantly greater response of old cells to EGF was surprising. Addition of EGF to the incubation medium generally shortened the response time of old cells from 48 to 36 hours. The total number of proliferating cells was consistently

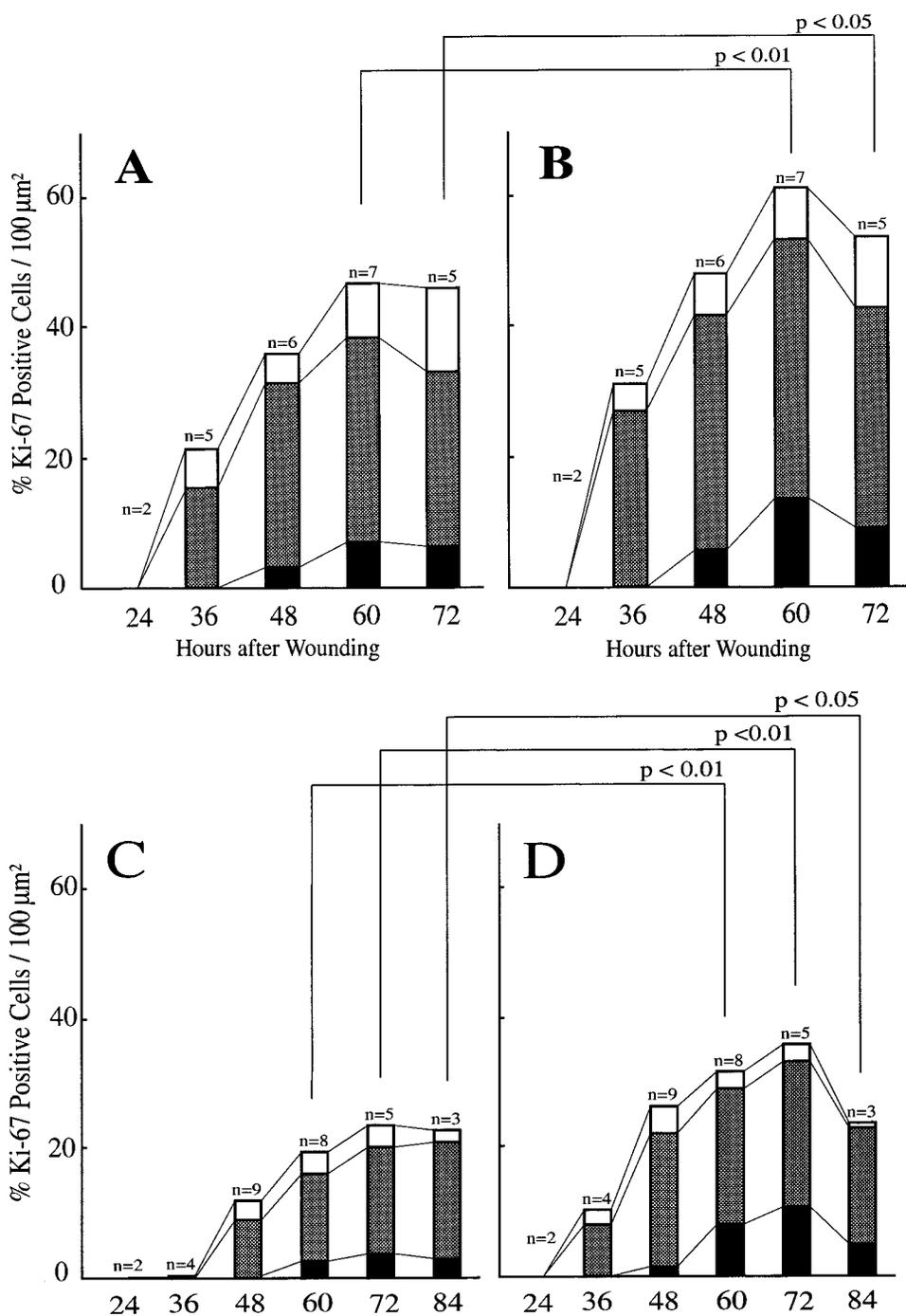


FIGURE 5. Cell cycle progression in corneal endothelial cells from young (A, B) and old donors (C, D). Corneas were incubated in 10% serum, 20 ng/ml FGF alone (A, C), or the same medium supplemented with 10 ng/ml EGF (B, D). Open bars, percentage of Ki67-positive cells exhibiting a G1-phase pattern; gray bars, percentage of Ki67-positive cells with an S/G2-phase pattern; and black bars, percentage of Ki67-positive cells with M-phase patterns.

lower in cells from old donors than in cells from young donors, regardless of treatment; however, EGF significantly increased the number of proliferating cells in the old group. In fact, this number increased to nearly that observed in young cells incubated without EGF. It is noteworthy that the percentage of total Ki67-positive cells exhibiting mitotic figures was similar in both age groups. In cultures exposed to serum plus FGF, the maximum percentage of actively cycling cells that exhibited mitotic figures in the young group was 15% compared with 17% in the old group. EGF increased the percentage of M-phase cells to a maximum of 22% in young cells and 27% in old ones. An interesting set of cell culture studies by Blake et al.⁴⁰ showed that human corneal endothelial cells from both young

and old donors could be successfully grown on bovine corneal endothelial matrix, and an age-related difference in culture characteristics was noted. Cells from old donors grew in primary culture and generally could be passaged one time, after which they exhibited morphologic changes characteristic of senescence. In contrast, cells from young donors could be passaged at least 3 to 4 times without exhibiting these characteristics. Studies were then conducted to determine the effect of growth factors, including EGF, on endothelial cell proliferation using this novel model system. Unfortunately, cells from newborns and from a 37-year-old donor were evaluated, but the effect of these factors on proliferation of cells from old donors was not tested.

The specific mechanism by which EGF increased the proliferative response of cells from old donors is unclear. Also unclear is why this growth factor did not have a more significant effect on young cells. It is well known that the signaling pathways stimulated by serum, FGF, and EGF are different. Perhaps serum, FGF, and EGF produce a strong additive or synergistic effect that is required to induce aging cells to enter the cell cycle, but is not necessary in young cells to produce the same effect.

In the presence of EGF, cells from both age groups exhibited a clear peak of proliferative activity, whereas proliferation in cells treated without EGF appeared to plateau. One reason for this differential response may be the ability of EGF to stimulate endothelial cell migration, as well as proliferation. Wound healing studies^{45,46} indicate that EGF increases migration of cultured corneal endothelial cells in response to wounding. Wound closure would then be faster in endothelium treated with EGF, because it enhances both proliferation and migration, leading to a decrease in the number of actively cycling cells.

No proliferating cells were observed within unwounded areas of the corneal quarters; however, confocal microscopy revealed Ki67-positive cells along the cut edge (data not shown) as well as in the wound area. Treffers³¹ had a similar finding, in that no [³H]thymidine-labeled cells were found in the central area of unwounded corneas, but positive cells were present in the corneal periphery. The findings in both these studies differ from those of Couch et al.,³² who observed mitotic figures in unwounded areas, as well as at the cut edge of the corneal tissue. It is possible that handling of the corneal tissue in their studies caused local damage to central areas of the endothelial monolayer sufficient to induce a proliferative response. In fact, Couch et al. discussed the possibility that endothelial cells in "isolated points of injury to the human cornea" were capable of proliferating in response to mitogenic stimulation.

In summary, an ex vivo corneal wound model was used to examine the relative ability of corneal endothelial cells from young and old donor populations to enter and complete the cell cycle. Ki67 staining patterns followed cell cycle progression from the late G1-phase through the completion of the M-phase, providing more information than would have been possible using BrdU or [³H]thymidine incorporation. Old cells were capable of both entering and completing the cell cycle, but significant differences in their response were observed compared with young cells. Old cells entered the cell cycle more slowly, and significantly fewer actively cycling cells were observed than in young cells. EGF had relatively little effect on the overall response of young cells. In contrast, it significantly increased the rate of cell cycle entry in cells from old donors and increased the total number of old cells that entered and completed the cell cycle.

References

- Laing RA, Sandstrom MM, Berrospi AR, Leibowitz HM. Changes in the corneal endothelium as a function of age. *Exp Eye Res.* 1976; 22:587-594.
- Murphy C, Alvarado J, Juster R, Maglio M. Prenatal and postnatal cellularity of the human corneal endothelium: a quantitative histologic study. *Invest Ophthalmol Vis Sci.* 1984;25:312-322.
- Schultz RO, Matsuda M, Yee RW, Edelhauer HF. Corneal endothelial changes in type I and type II diabetes mellitus. *Am J Ophthalmol.* 1984;98:401-410.
- Gagnon MM, Boisjoly HM, Brunette I, Charest M, Amyot M. Corneal endothelial cell density in glaucoma. *Cornea.* 1997;16:314-318.
- Rao GN, Shaw EL, Arthur E, Aquavella JV. Morphologic appearance of the healing corneal endothelium. *Arch Ophthalmol.* 1978;96: 2027-2030.
- Bergmann L, Hartmann C, Renard G, Saragoussi JJ, Pouliquen Y. Damage to the corneal endothelium caused by radial keratotomy. *Fortschr Ophthalmol.* 1991;88:368-373.
- Brooks AM, Gillies WE. Effect of angle closure glaucoma and surgical intervention on the corneal endothelium. *Cornea.* 1991; 10:489-497.
- Laing RA, Neubauer L, Oak S, Kayne HL, Leibowitz HM. Evidence for mitosis in adult corneal endothelium. *Ophthalmology.* 1984; 91:1129-1134.
- Svedbergh B, Bill A. Scanning electron microscopic studies of the corneal endothelium in man and monkeys. *Acta Ophthalmol.* 1972;50:321-326.
- Yue BYJT, Sugar J, Gilboy JE, Elvart JL. Growth of human corneal endothelial cells in culture. *Invest Ophthalmol Vis Sci.* 1989;30: 248-253.
- Hyldahl L. Primary cell cultures from human embryonic corneas. *J Cell Sci.* 1984;66:343-351.
- Joyce NC, Mekler B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. *Invest Ophthalmol Vis Sci.* 1996;37:645-755.
- Joyce NC, Navon SE, Roy S, Zieske JD. Expression of cell cycle-associated proteins in human and rabbit corneal endothelium in situ. *Invest Ophthalmol Vis Sci.* 1996;37:1566-1575.
- Wilson SE, Lloyd SA, He YG, McCash CS. Extended life of human corneal endothelial cells transfected with the SV40 large T antigen. *Invest Ophthalmol Vis Sci.* 1993;34:2112-2123.
- Wilson SE, Weng J, Blair S, He YG, Lloyd S. Expression of E6/E7 or SV40 large T antigen-coding oncogenes in human corneal endothelial cells indicates regulated high-proliferative capacity. *Invest Ophthalmol Vis Sci.* 1995;36:32-40.
- Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer.* 1983;31:13-20.
- Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol.* 1984;133:1710-1715.
- Key G, Kubbutat MH, Gerdes J. Assessment of cell proliferation by means of an enzyme-linked immunosorbent assay based on the detection of the Ki-67 protein. *J Immunol Methods.* 1994;177: 113-117.
- Brown DC, Gatter KC. Monoclonal antibody Ki-67: its use in histopathology. *Histopathology.* 1990;17:489-503.
- Gerdes J. Ki-67 and other proliferation markers useful for immunohistological diagnostic and prognostic evaluations in human malignancies. *Semin Cancer Biol.* 1990;3:199-206.
- Kill IR, Faragher RG, Lawrence K, Shall S. The expression of proliferation-dependent antigens during the lifespan of normal and progeroid human fibroblasts in culture. *J Cell Sci.* 1994;107:571-579.
- Braun N, Papadopoulos T, Muller-Hermelink HK. Cell cycle dependent distribution of the proliferation associated antigen in human embryonic lung cells. *Virchows Arch B Cell Pathol Mol Pathol.* 1988;56:25-33.
- Joyce NC, Harris DL, Zieske JD. Mitotic inhibition of corneal endothelium in neonatal rats. *Invest Ophthalmol Vis Sci.* 1998;39: 2572-2583.
- Nishida K, Yamanishi K, Yamada K, et al. Epithelial hyperproliferation and transglutaminase 1 gene expression in Stevens-Johnson syndrome conjunctiva. *Am J Pathol.* 1999;154:331-336.
- Petroll WM, Jester JV, Bean J, Cavanagh HD. Labeling of cycling corneal endothelial cells during healing with a monoclonal antibody to the Ki67 antigen (MIB-1). *Cornea.* 1999;18:98-108.
- Kill IR. Localisation of the Ki-67 antigen within the nucleolus. Evidence for a fibrillar-deficient region of the dense fibrillar component. *J Cell Sci.* 1996;109:1253-1265.

27. Starborg M, Gell K, Brundell E, Hoog C. The murine Ki-67 cell proliferation antigen accumulates in the nucleolar and heterochromatic regions of interphase cells and at the periphery of the mitotic chromosomes in a process essential for cell cycle progression. *J Cell Sci.* 1996;109:143-153.
28. Verheijen R, Kuijpers HJH, Driel R, et al. Ki-67 detects a nuclear matrix-associated proliferation-related antigen: localization in mitotic cells and association with chromosomes. *J Cell Sci.* 1989;92:531-540.
29. Simonsen AH, Sorensen KE, Sperling S. Thymidine incorporation by human corneal endothelium during organ culture. *Acta Ophthalmol (Copenh).* 1981;59:110-118.
30. Singh G. Mitosis and cell division in human corneal endothelium. *Ann Ophthalmol.* 1986;18:88-94.
31. Treffers WF. Human corneal endothelial wound repair: In vitro and in vivo. *Ophthalmology.* 1982;89:605-613.
32. Couch JM, Cullen P, Casey TA, Fabre JW. Mitotic activity of corneal endothelial cells in organ culture with recombinant human epidermal growth factor. *Ophthalmology.* 1987;94:1-6.
33. Olsen EG, Davanger M. The healing of human corneal endothelium: an in vitro study. *Acta Ophthalmol (Copenh).* 1984;62:885-892.
34. Baum, JL, Niedra R, Davis C, Yue BYJT. Mass culture of human corneal endothelial cells. *Arch Ophthalmol.* 1979;97:1136-1140.
35. Nayak SK, Binder PS. The growth of endothelium from human corneal rims in tissue culture. *Invest Ophthalmol Vis Sci.* 1984;25:1213-1216.
36. Pistsov MY, Sadovnikova EY, Danilov SM. Human corneal endothelial cells: isolation, characterization and long-term cultivation. *Exp Eye Res.* 1988;47:403-414.
37. Engelmann K, Bohnke M, Friedl P. Isolation and long-term cultivation of human corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 1988;29:1656-1662.
38. Insler MS, Lopez JG. Microcarrier cell culture of neonatal human corneal endothelium. *Curr Eye Res.* 1990;9:23-30.
39. Samples JR, Binder PS, Nayak SK. Propagation of human corneal endothelium: in vitro effect of growth factors. *Exp Eye Res.* 1991;52:121-128.
40. Blake DA, Yu H, Young DL, Caldwell DR. Matrix stimulates the proliferation of human corneal endothelial cells in culture. *Invest Ophthalmol Vis Sci.* 1997;38:1119-1129.
41. Schultz G, Cipolla L, Whitehouse A, Eiferman R, Woost P, Jumblatt M. Growth factors and corneal endothelial cells, III: stimulation of adult human corneal endothelial cell mitosis in vitro by defined mitogenic agents. *Cornea.* 1992;11:20-27.
42. Hoppenreijns VPK, Pels E, Vrensen GF, Oosting J, Treffers WF. Effects of human epidermal growth factor on endothelial wound healing of human corneas. *Invest Ophthalmol Vis Sci.* 1992;33:1946-1957.
43. Raymond GF, Jumblatt MM, Bartels SP, Neufeld AH. Rabbit corneal endothelial cells in vitro: effects of EGF. *Invest Ophthalmol Vis Sci.* 1986;27:474-479.
44. Zagorski Z, Naumann GOH. Factors of corneal endothelial proliferation. *Bull Soc Belge Ophthalmol.* 1987;224:15-21.
45. Joyce NC, Matkin ED, Neufeld AH. Corneal endothelial wound closure in vitro: effects of EGF and/or indomethacin. *Invest Ophthalmol Vis Sci.* 1989;30:1548-1559.
46. Joyce NC, Meklir B, Neufeld AH. In vitro pharmacologic separation of corneal endothelial migration and spreading responses. *Invest Ophthalmol Vis Sci.* 1990;31:1816-1826.