

Age Differences in Cyclin-Dependent Kinase Inhibitor Expression and Rb Hyperphosphorylation in Human Corneal Endothelial Cells

Kikuko Enomoto, Tatsuya Mimura, Deshea L. Harris, and Nancy C. Joyce

PURPOSE. Human corneal endothelial cells (HCECs) are considered to be nonreplicative *in vivo*; however, isolated HCECs can be cultured and grown successfully, indicating that they retain proliferative capacity. This capacity to replicate tends to decrease with donor age. Cyclin-dependent kinase inhibitors (CKIs) are important negative regulators of the cell cycle. Of those CKIs, p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1} are expressed in corneal endothelium. To help reveal the mechanism of this age-related difference, the relative expression of those CKIs and the kinetics of hyperphosphorylation of the retinoblastoma protein, Rb, were analyzed in HCECs from various aged donors.

METHODS. Fresh-frozen sections of corneas from an 18-year-old and a 74-year-old donor were immunostained to reveal the expression and localization of the three CKIs in corneal endothelium *in situ*. HCECs from eight donors of various ages were isolated and cultured until they reached passage 4. After the cells reached confluence, total protein was extracted, and the relative expression of p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1} was determined by Western blot analysis. A parallel analysis was performed with primary cultures of HCECs obtained from eight different donors. Subconfluent passage 2 HCECs from eight donors were serum starved and, at different times after growth factor stimulation, protein was extracted, and Western blot analysis was used to compare the overall expression of Rb protein and the kinetics of Rb hyperphosphorylation.

RESULTS. Immunocytochemistry confirmed the expression and nuclear localization of p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1} in HCECs *in situ*. Western blot studies revealed an age-related increase in p16^{INK4a} and p21^{WAF1/Cip1} protein expression in cultured HCECs. Expression of p27^{Kip1} tended to decrease with the donor's age in passage-4 cells; however, there was no significant difference in p27^{Kip1} expression level between young and older donors in primary cultured HCECs. No age-related difference in total Rb protein was observed in the Western blots; however, the rate of Rb hyperphosphorylation was significantly slower in HCECs from older donors.

CONCLUSIONS. p16^{INK4a}, p21^{WAF1/Cip1}, p27^{Kip1}, and Rb were all expressed in HCECs, regardless of donor age. Age-related differences in the relative expression of p16^{INK4a} and

p21^{WAF1/Cip1} and in the kinetics of Rb hyperphosphorylation led to the conclusion that, in addition to the normal inhibitory activity of p27^{Kip1}, there is an age-dependent increase in negative regulation of the cell cycle by p16^{INK4a} and p21^{WAF1/Cip1}. This additional molecular mechanism may be responsible, at least in part, for the reduced proliferative response observed in HCECs from older donors. (*Invest Ophthalmol Vis Sci.* 2006; 47:4330–4340) DOI:10.1167/iovs.05-1581

Corneal endothelium is the single layer of cells at the most posterior part of the cornea, bordering the anterior chamber. The endothelium plays an important role in maintaining corneal clarity by regulating corneal hydration through its barrier and Na⁺,K⁺-ATPase and bicarbonate pump functions.^{1,2} Human corneal endothelial cells (HCECs) are known to be nonproliferative *in vivo*, and therefore loss of HCECs as the result of surgery, disease, or aging is usually compensated by cell migration and enlargement from the surrounding intact area.^{3–6} Previous studies showed that HCECs can be successfully cultured by the stimulation of growth factors.^{7–12} This means that HCECs do not lose their proliferative capacity, but retain the potential to proliferate. Previous studies in our laboratory revealed that there are age-related differences in the proliferative capacity of HCECs.^{13,14} Significantly fewer HCECs from older donors respond to mitogens and those that respond require stronger mitogenic stimulation than do their younger counterparts. The mechanism underlying this age-related difference in proliferative capacity has yet to be investigated.

HCECs *in vivo* are arrested in the G₁-phase of the cell cycle.^{15,16} Cell cycle progression is controlled by the activity of several cyclin-dependent kinases (CDKs). These CDKs must bind appropriate cyclins to be activated, and the expression level of cyclins varies, depending on the stage of the cell cycle. Cyclin-dependent kinase inhibitors (CKIs) inhibit cell-cycle progression and prevent activation of the kinase activity of cyclin-CDK complexes. CKIs play important roles in maintaining G₁-phase arrest, in part by preventing cyclin/CDK-induced hyperphosphorylation of the retinoblastoma protein Rb. There are two families of CKI proteins. The CIP/KIP family (p21^{WAF1/Cip1}, p27^{Kip1}, and p57^{Kip2}) is inhibitory against all G₁-phase cyclin-CDK complexes.^{17–19} The INK4 family (p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}) interferes with cyclin D binding to CDK4/6 kinases and decreases their activity.^{20–24} Previous studies have shown that p16^{INK4a}, p21^{WAF1/Cip1}, p27^{Kip1}, and Rb are expressed in corneal endothelial cells from several species.^{16,25–28}

p16^{INK4a} is a member of the INK4 family. When p16^{INK4a} is bound to CDK4, interaction of G₁-specific cyclin D and CDK4 is inhibited, resulting in suppression of the hyperphosphorylation of Rb by these kinases. In its hypophosphorylated state, Rb protein tightly binds and inactivates the E2F transcription factor, whose activity is essential for entry into the S-phase of the cell cycle.²⁹ An age-related increase in p16^{INK4a} protein expression has been reported in some cell types.^{30,31} p16^{INK4a} is also known to act as a tumor suppressor. Genetic alterations of this

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TABLE 1. Antibodies

Primary Antibodies	Dilutions	Secondary Antibodies	Dilutions
Immunocytochemistry			
p16 (N-20)*	1:50	FITC-conjugated donkey anti-rabbit IgG†	1:200
p21 (C-19)*	1:50	FITC-conjugated donkey anti-rabbit IgG†	1:200
p27 (P2092)‡	1:50	FITC-conjugated donkey anti-mouse IgG†	1:100
Western Blot			
p16 (N-20)*	1:400	Peroxidase-conjugated donkey anti-rabbit IgG†	1:10,000
p21 Waf1/Cip1 (DCS60)§	1:2,000	Peroxidase-conjugated donkey anti-mouse IgG†	1:2,000
p27 (N-20)*	1:200	Peroxidase-conjugated donkey anti-rabbit IgG†	1:10,000
Rb (Ser 807/811)*	1:400	Peroxidase-conjugated donkey anti-rabbit IgG†	1:10,000
Rb (IF8)*	1:500	Peroxidase-conjugated donkey anti-mouse IgG†	1:10,000
β-Actin (A1978)‡	1:10,000	Peroxidase-conjugated donkey anti-mouse IgG†	1:20,000

* Santa Cruz Biotechnology, Santa Cruz, CA.

† Jackson ImmunoResearch, West Grove, PA.

‡ Sigma-Aldrich, St. Louis, MO.

§ Cell Signaling Technology, Beverly, MA.

gene, including deletions or mutations, have been detected in many kinds of cancers.^{32,33}

p21^{WAF1/Cip1} is a member of the CIP/KIP family and helps regulate the G₁/S-phase transition. Protein expression of p21^{WAF1/Cip1} increases when cells are in an unfavorable environment for proliferation, such as in oxidative stress,³⁴ senescence,³⁵ or differentiation.^{36,37} Accumulation of p21^{WAF1/Cip1} protein results in growth arrest in the G₁-phase.³⁸ p21^{WAF1/Cip1} expression can be induced in a p53-dependent³⁹ or p53-independent manner, by several types of transcription factors⁴⁰ and during terminal differentiation.³⁷

p27^{Kip1} is another member of the CIP/KIP family. The level of p27^{Kip1} protein expression is high in the G₁-phase and decreases in the S-phase. Interaction of p27^{Kip1} with CDK2 in the G₁-phase leads to inactivation of CDK2 kinase activity and results in G₁-phase arrest. p27^{Kip1} helps mediate cell cycle arrest induced by cell-to-cell contact and TGF-β.⁴¹⁻⁴³ Different from p16^{INK4a} and p21^{WAF1/Cip1}, p27^{Kip1} protein expression is not solely dependent on its mRNA level, but is also regulated posttranslationally.⁴⁴ Some studies have revealed accumulated p27^{Kip1} protein in aged cells in several types of tissue,^{45,46} but there are also reports that p27^{Kip1} protein expression is not age dependent.⁴⁷

There is a possibility that the expression of CKIs increases in an age-dependent manner in HCECs and causes the age-related decrease in proliferative capacity that is observed in these cells. To begin testing this hypothesis, we compared the protein expression of p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1}, and the kinetics of hyperphosphorylation of Rb protein in HCECs cultured from young and older donors.

MATERIALS AND METHODS

Donor Human Corneas

Donor human corneas were obtained through National Disease Research Interchange (NDRI, Philadelphia, PA). Handling of donor information by the source eye bank, NDRI, and this laboratory adhered to the tenets of the Declaration of Helsinki 1983 revision in protecting donor confidentiality. All corneas were preserved (Optisol-GS; Bausch & Lomb, Rochester, NY) at 4°C. Exclusion criteria were applied as previously indicated.⁴⁸ Corneas were divided into two age groups: young (<30 years old) and old (>50 years old).

Immunocytochemistry

Fresh-frozen transverse sections of corneas from an 18-year-old and a 74-year-old donor were fixed for 10 minutes in methanol at -20°C. All

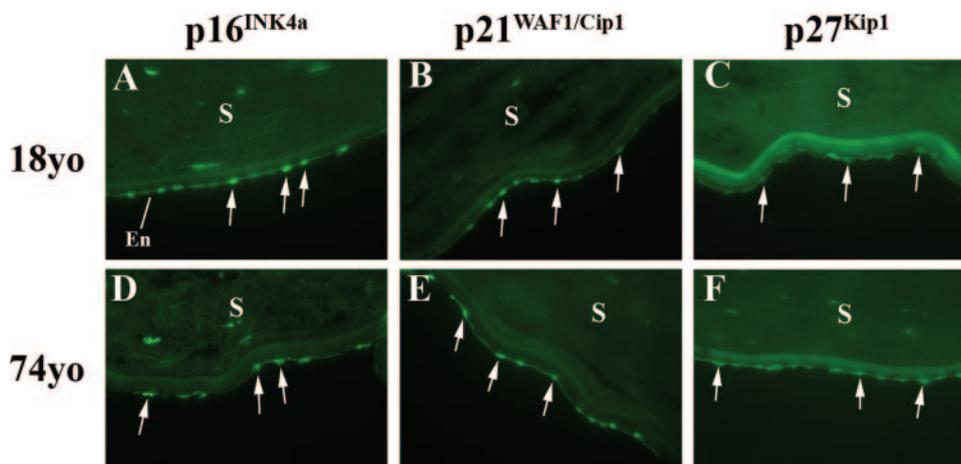


FIGURE 1. Fresh-frozen sections of human cornea from an 18-year-old (A-C) and a 74-year-old donor (D-F) were stained with antibodies for p16^{INK4a} (A, D), p21^{WAF1/Cip1} (B, E), or p27^{Kip1} (C, F). Positive staining for all three CKIs was clearly visible in endothelial nuclei from both donors. Arrows: positive staining in corneal endothelial cells. Anterior Descemet's membrane (DM) showed relatively strong autofluorescence in some sections. S, stroma; En, endothelium. Original magnification, ×60.

Donor Information for 4th Passage Cultures of Corneal Endothelial Cells

Donor Age	Cause of Death	Density (cells/mm ²) (OS/OD)	Days	
			Death to culture	Dissection to Harvest
12	Multi-system organ failure (Goldenher syndrome)	<2000	5	43
15	Motor vehicle accident	3194 (OD)	5	47
18	Overdose of acetaminophen	3106 (OS)	4	20
24	Trauma	3178 (OS)	5	49
Average ± SD (young)			4.75 ± 0.5*	39.8 ± 13.4
54	Esophageal cancer	2861/2890	3	101
65	Cardiac arrest	3086/3205	3	118
66	Small cell carcinoma	3076/3058	3	35
69	Lung cancer	3448/3496	3	90
Average ± SD (old)			3.0*	86.0 ± 35.9

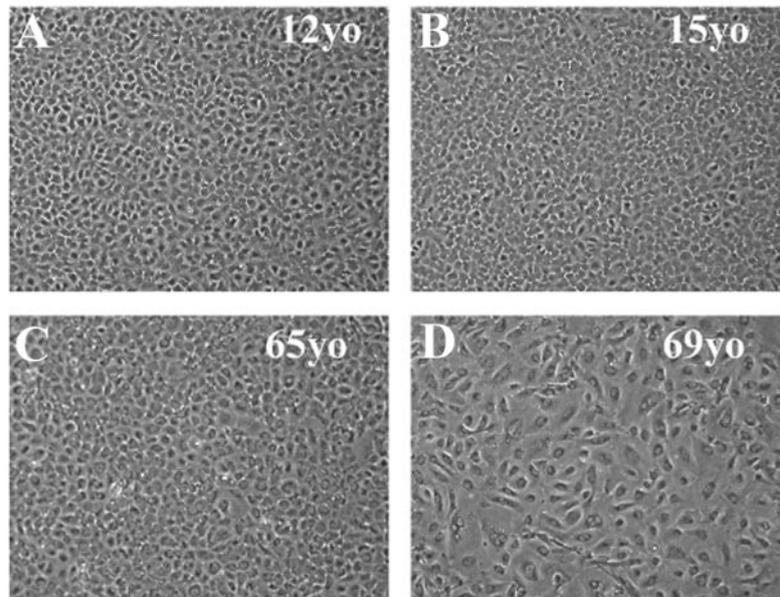
**p* < 0.01

FIGURE 2. The table provides donor and culture information for Western blot studies conducted in passage 4 HCECs. Representative phase-contrast images are shown in (A) through (D). HCECs at passage 4 were grown to confluence and then maintained an additional 3 to 7 days, to ensure formation of a contact-inhibited monolayer. The size of the cells from younger donors (A, B) was consistently smaller than that from older donors (C, D). An increase in polymorphism was clearly noted with increasing donor age. Original magnification, $\times 40$.

further incubations were at room temperature. Before they were stained with antibodies, the sections were rinsed with phosphate-buffered saline (PBS; Invitrogen-Gibco, Grand Island, NY) and incubated in blocking buffer (PBS containing 2% bovine serum albumin). After a 2-hour incubation in diluted primary antibodies, the slides were rinsed with PBS and reincubated in blocking buffer. Antibodies and their dilutions are shown in Table 1. Negative controls consisted of primary antibody preabsorbed with its antigen or incubation of tissue in secondary antibody alone. Subsequently, the slides were incubated with fluorescein-conjugated secondary antibody for 1 hour and then rinsed with PBS. Coverslips were applied, and staining was visualized (Eclipse E800 Microscope; Nikon, Melville, NY) with an epifluorescence attachment (VFM; Nikon Inc., Melville, NY) equipped with a digital camera (Spot camera with ver. 4.0.5 Software; Diagnostic Instruments, Sterling Heights, MI).

Culture of Human Corneal Endothelial Cells

Corneal endothelial cells were isolated from donor corneas and cultured according to a previously described method.^{12,14} Briefly, De-

scemet's membrane with endothelium was dissected in small strips and then incubated overnight in culture medium (OptiMEM-I; Invitrogen-Gibco) supplemented with 8% fetal bovine serum (FBS; 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 μ g/mL bovine pituitary extract (Biomedical Technologies), 20 μ g/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO), 200 mg/mL calcium chloride, 0.08% chondroitin sulfate (Sigma-Aldrich), 50 μ g/mL gentamicin (Invitrogen-Gibco), and antibiotic-antimycotic solution (Sigma-Aldrich) diluted 1:100. After centrifugation, the strips were incubated in 0.02% EDTA solution (Sigma-Aldrich) at 37°C for 1 hour to separate cells. After cells were pipetted and resuspended cells in the culture medium described earlier, cells and pieces of Descemet's membrane were pelleted by centrifugation, then resuspended in medium, and plated in precoated six-well tissue culture plates (FNC Coating Mix; Biological Research Faculty & Facility, Inc., Ijamsville, MD). Once cells reached confluence, they were cultured in medium without EGF, NGF, or pituitary extract for several days, to stabilize the monolayer and optimally reflect its in

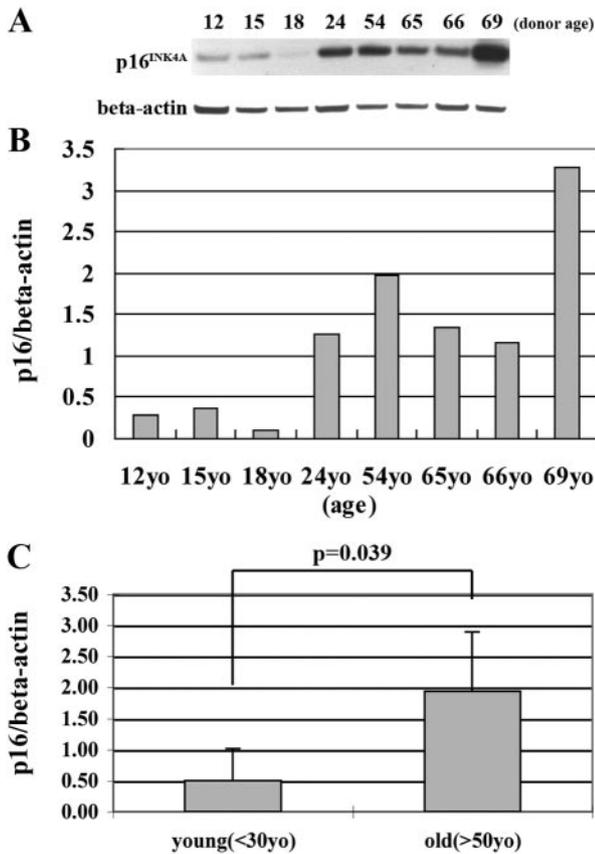


FIGURE 3. Western blot and densitometric analysis of p16^{INK4a} protein expression in passage 4 HCECs from young and older donors. Protein samples from all eight donors were electrophoresed, and Western blots were prepared to determine the relative expression of p16^{INK4a} in each sample (A). Densitometric results with β -actin used for normalization are shown in (B). The average level of p16^{INK4a} expressed in HCECs from young and older donors was compared in (C). Bars, SD. Results indicate a significant increase ($P = 0.039$) in p16^{INK4a} expression in HCECs from older donors.

vivo morphology. For some experiments, cells were passaged by sub-culturing at a 1:2 ratio.

Protein Extraction

Cultures of HCECs used to detect relative CKI expression were removed from the culture plate 3 to 7 days after reaching confluence. Subconfluent cultures were used in studies to detect Rb phosphorylation (described later). Protein was extracted by incubating cells for 30 minutes at 4°C in lysis buffer containing 1% Triton X-100, 250 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl (pH 7.4), 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (all from Sigma-Aldrich), followed by homogenization and centrifugation. Supernatants were stored at -80°C until use in SDS-PAGE and Western blot analyses.

Western Blot Analysis of CKI Expression

Soluble protein was loaded on 10% Bis-Tris gels for SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Nonspecific binding was blocked by incubation of the membrane for 1 hour at room temperature in 5% milk diluted in PBS containing 1% Triton X-100. Membranes were incubated with diluted primary CKI antibodies overnight at 4°C. Membranes were incubated with primary antibody for β -actin for only 1 hour at room temperature. All antibodies were diluted in 5%

milk in PBS containing 1% Triton X-100. Antibody dilutions are indicated in Table 1. Blots were then rinsed three times for 10 minutes each with PBS containing 1% Triton X-100 and incubated 1 hour at room temperature with secondary antibody. Membranes were washed three times with PBS containing 1% Triton X-100 for 10 minutes, and antibody binding was visualized using a chemiluminescent substrate (SuperSignal West Pico; Pierce, Rockford, IL). Immunoblots used to detect p21^{WAF1/Cip1} expression were stripped by incubation in buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8) and 100 mM 2-mercaptoethanol (all from Sigma-Aldrich) for 15 minutes at room temperature and then re-probed with p27^{Kip1} antibody. All membranes were stripped and re-probed with β -actin antibody as the loading control. Duplicate gels and immunoblots were run for all samples.

Analysis of Phosphorylated Rb

Passage 1 HCECs were grown to confluence and then trypsinized with 0.05%:0.02% trypsin-EDTA solution (Sigma-Aldrich). Cells were seeded at a density of 100,000 cells per well into six-well dishes. These subconfluent cells were incubated for 24 hours in basal medium without FBS, EGF, NGF, or pituitary extract, to induce mitotic quiescence. Complete culture medium, containing EGF, NGF, pituitary extract, and FBS, was then added to stimulate entry into the cell cycle. At 0, 24, 48, and 72 hours after growth factor was added, protein was extracted for Western blot analysis of total and hyperphosphorylated

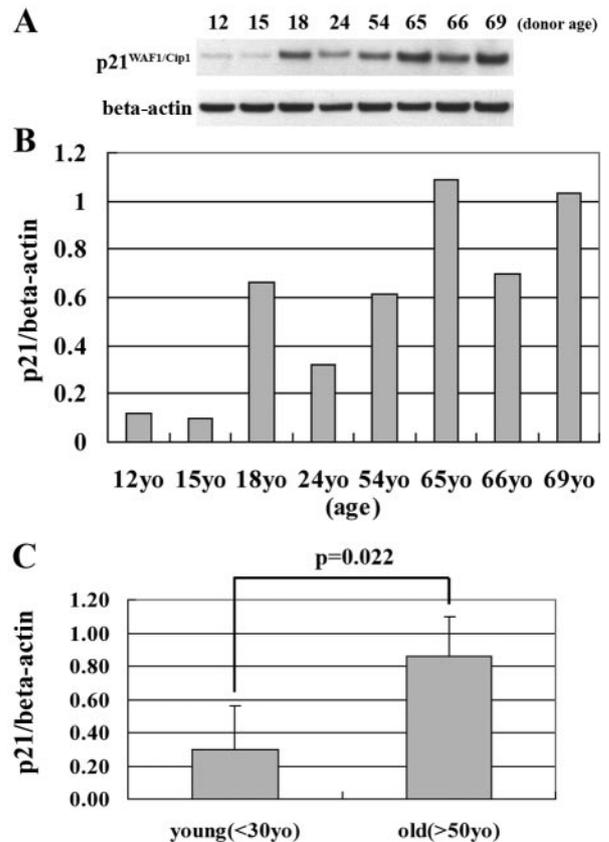


FIGURE 4. Western blot and densitometric analyses of p21^{WAF1/Cip1} protein expression. Western blots were prepared with protein samples from the same eight donors as in Figure 3, to determine the relative expression of p21^{WAF1/Cip1} (A). Densitometric results with β -actin used for normalization are shown in (B). The average level of p21^{WAF1/Cip1} expressed in HCECs from young and older donors was compared (C). Bars, SD. Results indicate a significant increase ($P = 0.022$) in p21^{WAF1/Cip1} expression in HCECs from older donors.

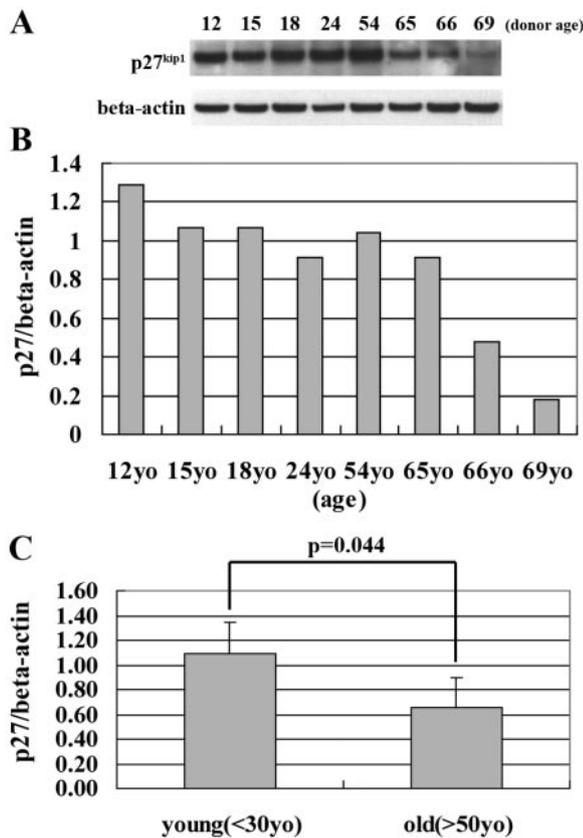


FIGURE 5. Western blot and densitometric analyses of p27^{Kip1} protein expression. The same blot used for analysis of p21^{WAF1/Cip1} protein expression in Figure 4 was chemically stripped, to remove all bound antibodies and was reprobed to analyze p27^{Kip1} protein expression (A). Densitometric results with β -actin used for normalization are shown in (B). The average level of p27^{Kip1} expressed in HCECs from young and older donors was compared (C). Bars, SD. Results indicate a significant decrease ($P = 0.044$) in p27^{Kip1} expression in HCECs from older donors.

Rb. Gel electrophoresis, protein transfer, and immunoblot analysis were performed as described earlier. Primary and secondary antibody dilutions are indicated in Table 1. To test the specificity of anti-Rb Ser 807/811, blocking peptide (5:1 peptide-to-antibody ratio; Santa Cruz Biotechnology) was used as a negative control. For this negative control, blocking peptide was preincubated with primary antibody before incubation with the PVDF membrane. Membranes were washed, and antibody binding was visualized using a chemiluminescent substrate as described earlier. Duplicate gels and immunoblots were run for all samples.

Densitometric Analysis

Quantification of protein bands was performed by densitometry using NIH Image software (NIH Image 1.34; National Institutes of Health, Bethesda, MD; available in the public domain at <http://rsb.info.nih.gov/ij/>). Protein expression was represented as a value relative to β -actin expression. Statistical analyses were performed using unpaired Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Immunocytochemical Localization of p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1}

Expression of p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1} has been demonstrated in corneal endothelium from several different

species.^{16,25–28} Immunofluorescence studies were initially performed to confirm expression of these three CKIs in human corneal endothelium *in situ*. Figure 1 presents representative micrographs of cross-sections of posterior human cornea from an 18- and a 74-year-old donor. Results confirm that all three CKIs are expressed in HCECs. Positive staining for p16^{INK4a} (Figs. 1A, 1D), p21^{WAF1/Cip1} (Figs. 1B, 1E), and p27^{Kip1} (Figs. 1C, 1F) show similar nuclear localization in endothelial cells from both donors. Positive staining for all three CKIs was confirmed either by preabsorbing primary antibody with its specific antigen or by incubating tissue in secondary antibody alone (data not shown).

p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1} Protein Expression in Passage-4 HCECs

To perform semiquantitative analysis of the protein expression of p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1}, we obtained corneas from eight donors and passaged isolated HCECs four times. The corneas were divided into two age groups. The young group consisted of four donors: 12, 15, 18, and 24 years old. The older group also consisted of four donors: 54, 65, 66, and 69 years old. Information about corneas from these donors is shown in the table in Figure 2. Although there was a statistically significant difference in days from death to culture between the two groups, all corneas were preserved less than 1 week (Optisol-GS; Bausch & Lomb). As described in previous studies,¹⁴ it tended to take longer to culture HCECs from older donors, but the difference in days from HCECs isolation to protein harvest between the two age groups was not statistically significant ($P = 0.052$). Representative phase-contrast images of confluent HCECs at passage 4 from donors of different ages are also presented in Figure 2. Cells formed a normal monolayer and retained the characteristic morphology of HCECs—that is, the hexagonal pattern of organization was still apparent, especially in cultures from younger donors. As was demonstrated previously,^{14,48} an increase in polymorphism and cell size was clearly noted with increasing donor age.

Proteins extracted from passage 4 HCECs from the same eight donors were used to perform semiquantitative Western blot analyses for p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1}. Figure 3 shows that expression of p16^{INK4a} in cells from donors in their teens was extremely low compared with that of older donors. Comparison of the results between the two age-groups revealed a statistically significant difference ($P = 0.039$) in p16^{INK4a} expression. Data for p21^{WAF1/Cip1} are shown in Figure 4. The relative expression of this CKI was similar to that observed for p16^{INK4a}. p21^{WAF1/Cip1} protein expression was very low in cells from the 12- and 15-year-old donors and gradually increased with donor age. There was a statistically significant difference in p21^{WAF1/Cip1} expression ($P = 0.022$) between the young and older groups. In contrast to the results for p16^{INK4a} and p21^{WAF1/Cip1}, expression of p27^{Kip1} appeared to decrease in an age-related manner in HCECs at passage 4 (Fig. 5). After the Western blot for p21^{WAF1/Cip1} was completed, the same membrane was chemically stripped and used for the Western blot of p27^{Kip1}. This made the background of the p27^{Kip1} blot a little higher, but the opposite tendency in protein expression of p21^{WAF1/Cip1} and p27^{Kip1} was obvious. The difference in p27^{Kip1} protein expression between the two age groups was statistically significant ($P = 0.044$). Similar results were obtained from duplicate blots. Together, Western blot analysis using passage-4 cells revealed that HCECs from older donors expressed significantly higher levels of p16^{INK4a} and p21^{WAF1/Cip1} than cells from younger donors. In contrast, expression of p27^{Kip1} tended to decrease with age.

**Donor Information for Primary Cultures of
Corneal Endothelial Cells Used for CKI Studies**

Donor Age	Cause of Death	Density (cells/mm ²) (OS/OD)	Days	
			Death to culture	Dissection to Harvest
2	Motor vehicle accident	N/A	2	7
19	Overdose of aspirin	3515 (OS)	3	13
20	Gunshot	2907/2495	3	20
20	Motor vehicle accident	2481 (OS)	2	17
Average (young)			2.5 ± 0.6	14.3 ± 5.6
51	Cardiac arrest	2755/2610	2	20
59	Myocardial infarction	2519/2582	4	33
60	Gunshot	2577/2358	5	20
76	Cardiomyopathy	3012 (OS)	2	24
Average (old)			3.25 ± 1.5	24.3 ± 6.1

N/A: Cell counts not available

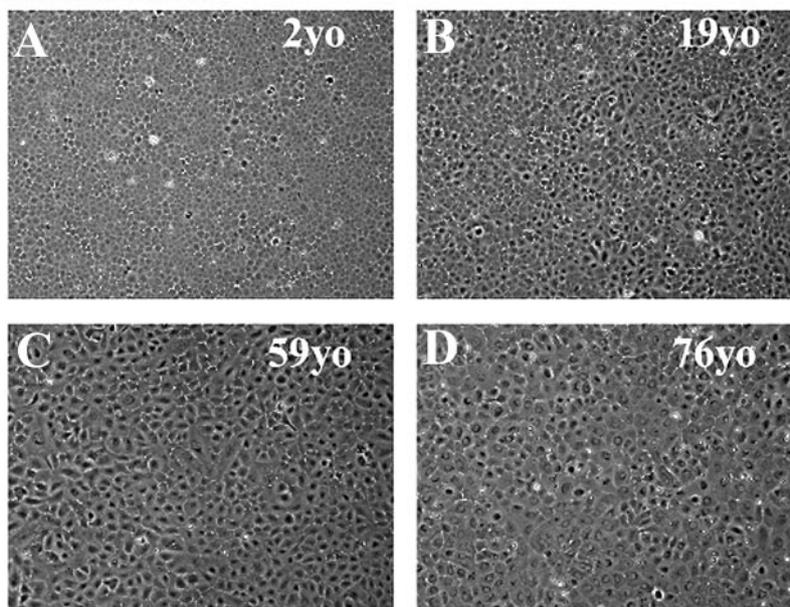


FIGURE 6. Table provides donor and culture information for studies conducted in primary cultures of HCECs for the CKI studies. Representative phase-contrast images are shown in (A) through (D). Primary cultures of HCECs were grown to confluence and then maintained an additional 3 to 7 days to ensure formation of a contact-inhibited monolayer. A similar age-related difference in cell shape was observed in primary cultures as seen in passage-4 cells (Fig. 2). Cells from younger donors (A, B) tended to be more homogeneous and smaller than those from older donors (C, D). Original magnification, $\times 40$.

p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1} Protein Expression in Primary Cultures of HCECs

As shown in the table in Figure 2, it took a long time for the passage-4 cells to reach confluence. This long culture time and the fact that cells were passaged four times may have affected CKI protein expression, and the culture may not reflect relative CKI expression of HCECs in vivo. To minimize possible artifactual modifications in CKI expression, parallel analyses were performed with primary cultures of HCECs from young and older donors. The table in Figure 6 shows information about corneas used for these studies. As in the prior experiments, corneas were divided into two age groups. The young group consisted of four donors: two aged 2 and 19 years, and two aged 20 years. The older group also consisted of four donors aged 51, 59, 60, and 76 years. Overall, the morphology of the

HCECs after primary culture was more homogeneous than in passage-4 cells (Fig. 6); however, age-related changes in morphology, such as increased cell size and heterogeneity, were also observed after primary culture.

Results of the Western blot analysis of p16^{INK4a} in primary cultures of HCECs are shown in Figure 7. Those for p21^{WAF1/Cip1} and p27^{Kip1} are in Figures 8 and 9, respectively. Expression patterns for two CKIs were similar to those of passage-4 cells. That is, HCECs from the older donors expressed p16^{INK4a} and p21^{WAF1/Cip1} at significantly higher levels ($P = 0.026$ and $P = 0.022$, respectively) than did cells from younger donors. Of interest was the fact that there was no statistically significant difference ($P = 0.885$) in the relative expression of p27^{Kip1} in primary cultures of HCECs from the young and older donors, unlike that observed in passage-4 cells. Similar results were obtained from duplicate blots.

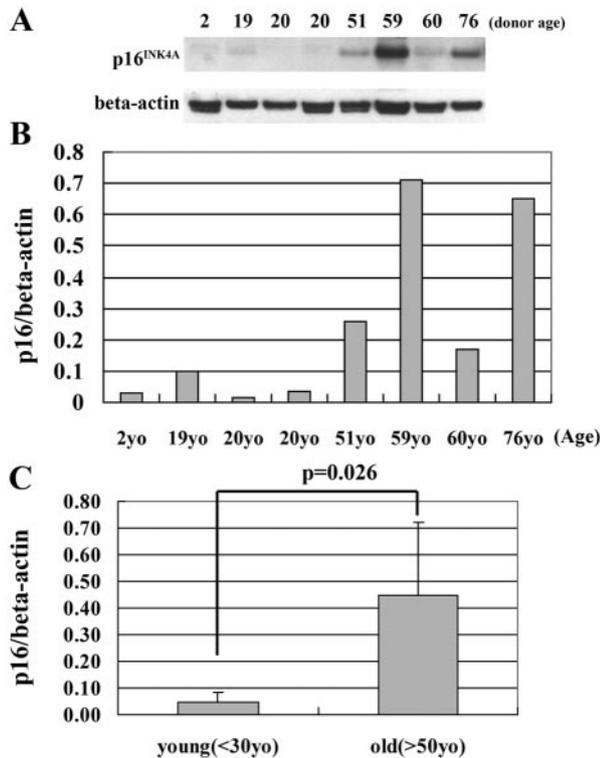


FIGURE 7. Western blot and densitometric analyses of p16^{INK4a} protein expression in primary cultures of HCECs from young and older donors. Protein samples from eight donors were electrophoresed and Western blots were prepared, to determine the relative expression of p16^{INK4a} in each sample (A). Densitometric results with β -actin used for normalization are shown in (B). The average level of p16^{INK4a} expressed in HCECs from young and older donors was compared (C). Bars, SD. Results indicate a significant increase ($P = 0.026$) in p16^{INK4a} expression in HCECs from older donors.

Kinetics of Rb Hyperphosphorylation

One of the important events leading to movement of cells from G₁- to S-phase of the cell cycle on mitogenic stimulation is hyperphosphorylation of the retinoblastoma protein, Rb, by active cyclin-kinase complexes. This hyperphosphorylation event results in inactivation of the inhibitory activity of Rb, subsequent activation of the E2F transcription factor, and entry into the S-phase. CKIs inhibit cyclin-kinase activity, preventing hyperphosphorylation of Rb and inhibiting cell cycle progression. Previous studies from this laboratory^{13,48} have demonstrated that HCECs from older donors enter the cell cycle more slowly than cells from younger donors. The finding that the expression of p16^{INK4a} and p21^{WAF1/Cip1} increases with donor age suggests that these CKIs may suppress cyclin-kinase-dependent hyperphosphorylation of Rb. We therefore conducted studies to determine whether there is any age-related difference in the kinetics of Rb hyperphosphorylation in growth factor-stimulated HCECs cultured from four young and four older donors.

The table in Figure 10 presents information regarding corneas used as a source of HCECs for these studies. This figure also presents representative phase-contrast images of confluent cultures of passage 1 HCECs. For this study, equal numbers of passage 1 cells were plated at subconfluent density and incubated for 24 hours in culture medium minus growth factors to induce mitotic quiescence. Growth factors were then added as

indicated in the Materials and Methods section to stimulate cell cycle entry, and samples were removed 0, 24, 48, and 72 hours after addition of growth factor. Western blot analyses were prepared with an antibody that recognizes total Rb protein (IF8) or an antibody that recognizes Rb specifically phosphorylated on Ser807/811 to detect the hyperphosphorylated fraction of Rb. Densitometry was performed using β -actin for normalization. Figure 11 demonstrates that there was very similar expression of total Rb protein in HCECs, regardless of donor age or time after growth factor was added. The same samples probed for hyperphosphorylated Rb (Ser 807/811) revealed an age-related difference in the kinetics of Rb hyperphosphorylation (Fig. 12). Preincubation of this antibody with a specific blocking peptide eliminated all antibody binding within the blot (data not shown). In HCECs from younger donors, Rb hyperphosphorylation increased to maximum levels within 24 hours, and the level remained high during the entire 72-hour test period. Of note, HCECs from older donors demonstrated a lower basal level of Rb hyperphosphorylation, and cells did not reach maximum levels until 48 hours after the addition of growth factors. Similar results were obtained from duplicate blots. Together, results indicate that there is no significant age-related difference in the relative expression of total Rb protein in HCECs; however, the kinetics of Rb hyperphosphorylation differed in an age-dependent manner, indicating a slower response to growth factor stimulation in HCECs from older donors.

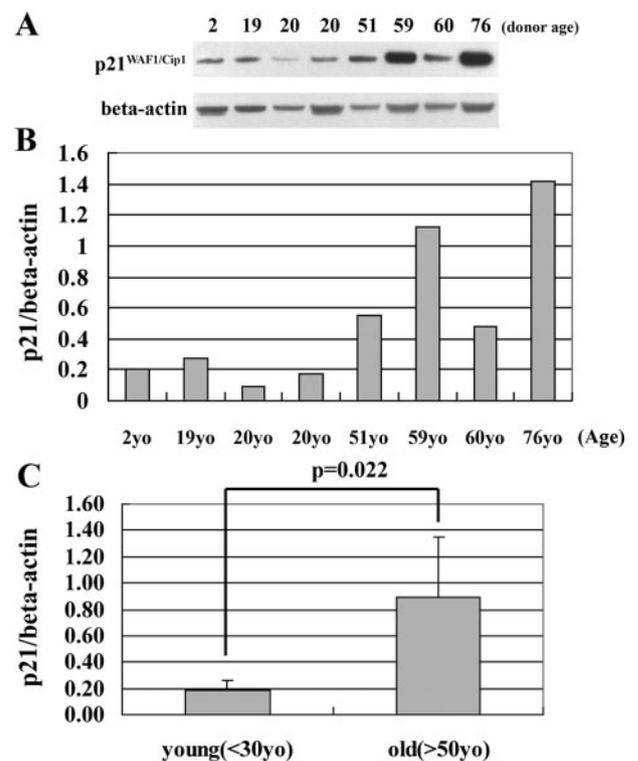


FIGURE 8. Western blot and densitometric analyses of p21^{WAF1/Cip1} protein expression in primary cultures of HCECs from young and older donors. Protein samples from the same eight donors as in Figure 7 were electrophoresed, and Western blots were prepared, to determine the relative expression of p21^{WAF1/Cip1} in each sample (A). Densitometric results with β -actin used for normalization are shown in (B). The average level of p21^{WAF1/Cip1} expressed in HCECs from young and older donors was compared (C). Bars, SD. Results indicate a significant increase ($P = 0.022$) in p21^{WAF1/Cip1} expression in HCECs from older donors.

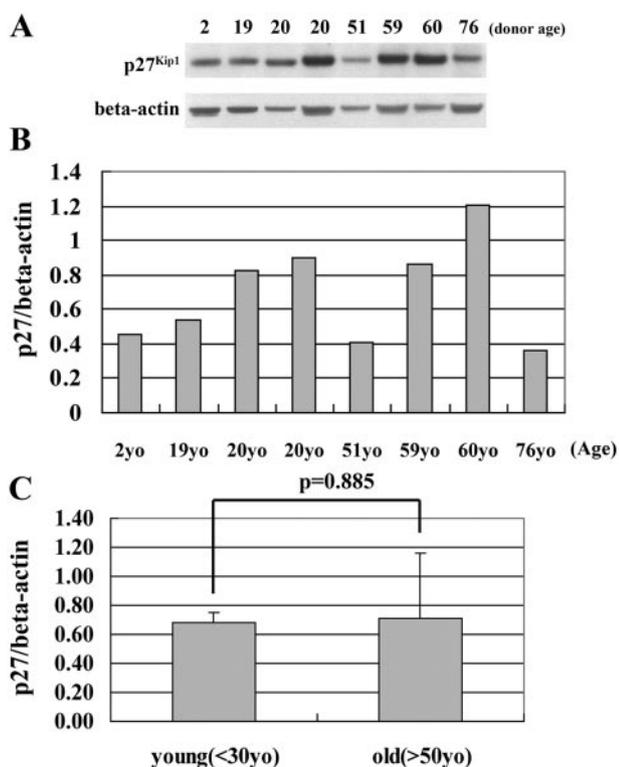


FIGURE 9. Western blot and densitometric analyses of p27^{Kip1} protein expression in primary cultures of HCECs from young and older donors. The same blot used for analysis of p21^{WAF1/Cip1} protein expression in Figure 8 was chemically stripped to remove all bound antibodies and reprobed to analyze p27^{Kip1} protein expression (A). Densitometric results with β -actin used for normalization are shown in (B). The average level of p27^{Kip1} expressed in HCECs from young and older donors was compared (C). Bars, SD. In primary cultures of HCEC, there was no significant difference ($P = 0.885$) in the relative expression of p27^{Kip1} between young and older donors.

DISCUSSION

Today the only treatment for the dysfunction of corneal endothelium due to low cell density is either penetrating keratoplasty (PK) or deep lamellar endothelial keratoplasty (DLEK). Both methods require donor corneas and involve a surgical procedure. Most corneal donors are older (i.e., >50 years old). Corneal endothelial cell density (ECD) is often not high enough to use corneas from older donors for transplantation. This decrease in ECD can be due to aging, history of surgeries, or trauma. It may be possible to induce HCECs to proliferate transiently in situ as demonstrated in a previous study.⁴⁹ For example, in donor corneas, the ability to induce transient proliferation and increase cell density would contribute to better outcomes of corneal transplantation and expansion of the number of donor corneas acceptable for transplantation. A final goal could be treatment or prevention of poor visual acuity because of low ECD by increasing ECD in vivo, that is, directly in patients' eyes, without the need for transplant surgery. This would be epoch-making progress in the treatment of HCEC dysfunction caused by low ECD. Careful analysis of the proliferative mechanism in HCECs is essential for the establishment of this new treatment. Previous studies have revealed age-related differences in proliferative capacity in HCECs.^{13,14,48,50} Because of the reasons mentioned, the future target of the

transient proliferation of HCECs will be older donors or patients. As such, it is very important to reveal the underlying causes of the observed age-related differences in proliferative capacity and to explore methods to enhance this in HCECs from older donors.

This is the first known study to compare relative CKI expression in human corneal endothelium from young and older donors. Results from both the immunocytochemistry and Western blot studies confirm the expression of p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1} in human corneal endothelium. Overall, results from primary cultures of HCECs indicate that p16^{INK4a} and p21^{WAF1/Cip1} protein levels increase as a function of donor age, whereas p27^{Kip1} levels remain relatively steady. The finding of increased expression of p16^{INK4a} and p21^{WAF1/Cip1} is consistent with results of age-related studies in other cell types.^{30,31} An age-related increase in p16^{INK4a} and p21^{WAF1/Cip1} expression was observed not only in primary cultures, but also in passage-4 cells. The consistency of the levels of these proteins under both culture conditions reflects their known mRNA and protein stability and suggests that, as in other cell types,³² these two CKIs may be involved in long-term cell cycle arrest rather than in controlling short-term cellular responses. In a recent review article,⁵¹ we presented results of preliminary studies of the relative expression of p16^{INK4a}, p21^{WAF1/Cip1}, and p27^{Kip1} from a 24- and a 65-year-old donor. With this small sample, it appeared that only p21^{WAF1/Cip1} increased with donor age. Data from these two samples was included among the larger number of samples used for the current studies; however, with the larger sample, it became clear that p16^{INK4a}, as well as p21^{WAF1/Cip1}, increased in an age-dependent fashion.

In primary cultures of HCECs, p27^{Kip1} levels did not appear to differ with donor age; however, in passage-4 cells, p27^{Kip1} levels were significantly lower in HCECs from older donors. This finding may reflect a difference in cellular response to multiple passaging rather than to an intrinsic age-related difference in p27^{Kip1} expression. p27^{Kip1} protein is regulated at both the level of translation and turnover,⁴⁴ permitting more short-term regulation of the cell cycle in response to various environmental changes, including exposure to TGF- β and formation of mature cell-cell contacts.⁴¹⁻⁴³ From results of the primary cultures, it can be concluded that, in addition to the normal inhibitory activity of p27^{Kip1}, there is an age-dependent increase in negative regulation of the cell cycle by p16^{INK4a} and p21^{WAF1/Cip1} and that this additional molecular mechanism is responsible, at least in part, for the replicative senescence-like, reduced proliferative response observed in HCECs from older donors. Further study is needed to determine why increased passaging results in apparently lower p27^{Kip1} protein levels.

Results of the current studies should be considered in light of previous reports from our laboratory^{25,26} and from Yoshida et al.²⁸ regarding the role of p27^{Kip1} in regulating corneal endothelial proliferation. Together, those studies indicate that, during postnatal corneal endothelial development in neonatal rats^{25,26} and mice,²⁸ increased p27^{Kip1} expression correlates with the decreased proliferation that occurs on formation of a mature, contact-inhibited monolayer, strongly suggesting that p27^{Kip1} helps negatively regulate proliferation in developing endothelium. Results from our current studies comparing relative CKI expression in endothelium from young and older donors suggest that different molecular mechanisms are responsible for the replicative senescence-like reduction in proliferative capacity observed in the endothelium of older donors and for the decrease in proliferation that occurs on maturation of the developing endothelial monolayer.

Donor Information for Corneal Endothelial Cells Used for Rb Studies

Donor Age	Cause of Death	Density (cells/mm ²) (OS/OD)	Days	
			Death to culture	Dissection to Harvest
14	Glioblastoma	3300/3328	4	21
18	Collapse during football	3300/3328	5	31
22	Methadone overdose	3200/3200	2	21
25	Motor vehicle accident	3311/2900	2	28
Average (young)			3.25 ± 1.30	25.25 ± 4.38
56	Myocardial infarction	2810 (OS)	3	30
59	Gunshot	2570/2262	6	22
60	Cardiac arrest	2672/2262	3	27
65	Cardiac arrest	2500/2183	3	26
Average (old)			3.75 ± 1.37	26.25 ± 2.86

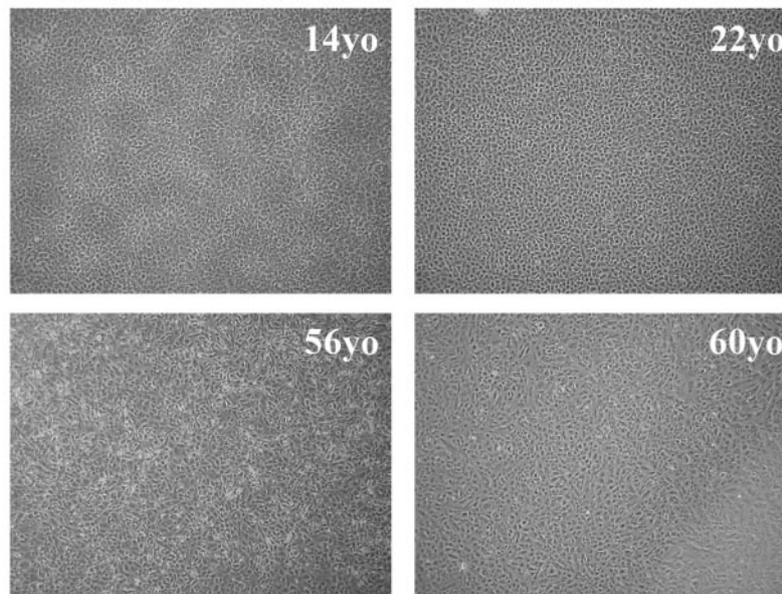


FIGURE 10. Table provides donor and culture information for studies conducted to compare the kinetics of Rb hyperphosphorylation in HCECs cultured from young and older donors. Representative phase-contrast images of confluent passage-1 HCECs are also shown. Original magnification, $\times 10$.

In several previous studies from this laboratory, a rat model was used to study cell cycle regulation in corneal endothelium. Certain observations using this model may appear to conflict with more recent findings in human corneal endothelial cells; however, these results do not conflict, if relative donor age is considered. As with humans, the density of corneal endothelial cells in both rats and mice decreases in an age-dependent manner. Fitch et al.⁵² showed a progressive decline in the number of cells and an increase in pleomorphism in rat corneal endothelium from age 6 to 30 months, closely paralleling changes reported in human endothelium in individuals from 20 to 70 years old. Similar results were obtained by Meyer et al.⁵³ Studies of mouse corneal endothelium⁵⁴ demonstrated an age-dependent increase in cell area and decrease in hexagonal cells corresponding to an age-dependent decrease in the relative number of endothelial cells over a period of 1 through 27 months. These results suggest that there is a similar age-related decrease in the proliferative capacity of corneal endothelium in rodents and in humans. In all studies of rat tissue, we used young rats, approximately 6 weeks old.

Thus, we believe the data we have obtained from our rat studies most closely reflect the phenotype and cell cycle behavior of HCECs obtained from young donors.

This age-related difference is important in interpreting studies from this laboratory that were designed to determine the effect of reducing p27^{Kip1} levels on corneal endothelial cell proliferation. Kikuchi et al.⁵⁵ reported that p27^{Kip1} antisense treatment promoted proliferation in fully confluent cultures of rat corneal endothelial cells, indicating that this CKI is a negative regulator of proliferation in corneal endothelium. Similar studies were conducted in confluent cultures of HCECs from young and older donors (Kikuchi M, et al., manuscript submitted). Results of those studies indicate that p27^{Kip1} siRNA treatment promoted proliferation in confluent cultures of HCECs from young donors (<30 years old), but not in cultures from old donors (>50 years old). Taken together, it can be seen that reduction of p27^{Kip1} protein levels in corneal endothelial cells from young rats or young human donors was sufficient to promote proliferation. However, the lack of proliferation in p27^{Kip1} siRNA-treated HCECs from older donors strongly suggests that the

age-related decrease in proliferative capacity observed in HCECs from older donors is regulated by other molecular mechanisms in addition to that of p27^{Kip1}.

Although expression of Rb protein has been reported in rabbit and human corneal endothelium,¹⁶ this is the first time that the kinetics of Rb hyperphosphorylation has been determined in HCECs. Results of the Western blot analyses indicated that there was no age-related difference in the overall protein expression of Rb in HCECs, as indicated by use of the IF8 antibody; however, hyperphosphorylation of Ser807/811 of Rb did differ in an age-dependent manner. Of interest is the fact that Ser807/811 is one of four motifs on Rb whose phosphorylation is regulated by cyclin-dependent kinase activity.⁵⁶ The demonstrated age-related difference in the kinetics of deactivation of Rb provides suggestive evidence that regulation of this important process differs with donor age. Because the expression of both p16^{INK4a} and p21^{WAF1/Cip1} is increased in HCECs from older donors, and these CKIs are known to inhibit the activity of the cyclin/kinase complexes that are responsible for hyperphosphorylating Rb, it is reasonable to hypothesize that the increased expression of p16^{INK4a} and p21^{WAF1/Cip1} contributes to the reduced proliferative response observed in HCECs from older donors. Although the data obtained in the current studies provide only evidence correlating increased p16^{INK4a} and p21^{WAF1/Cip1} expression with decreased Rb phosphorylation, it does not prove a direct relationship. Additional functional studies must be conducted to determine specifically whether increased expression of p16^{INK4a} and p21^{WAF1/Cip1} is directly responsible for decreased Rb hyperphosphorylation,

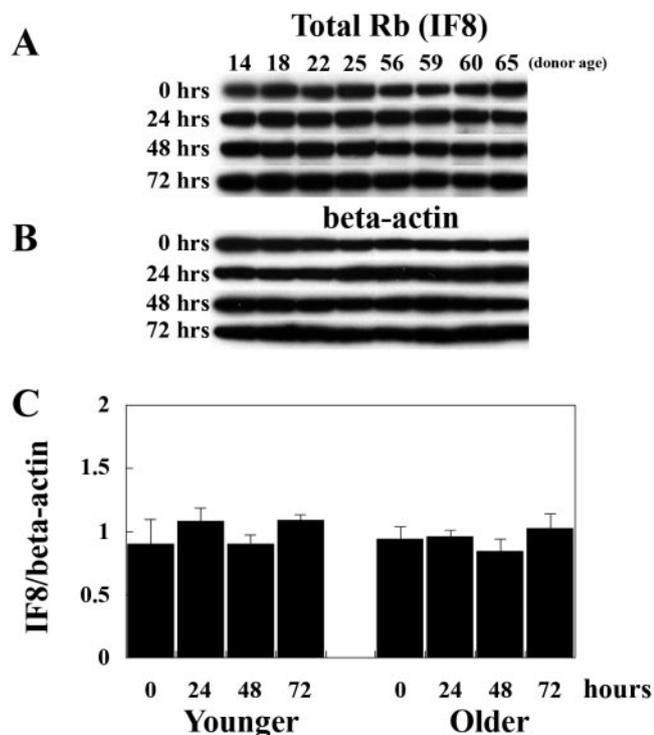


FIGURE 11. Western blot analysis using mouse anti-human Rb (IF8) to demonstrate total Rb protein expression in subconfluent, passage-2 HCECs at 0, 24, 48, and 72 hours after growth factor addition (A). Blot showing a β -actin band from all samples (B). Densitometric analysis using β -actin for normalization (C) demonstrated a similar level of total Rb protein expression in all HCECs, regardless of donor age or time after growth factor addition. Bars, SD.

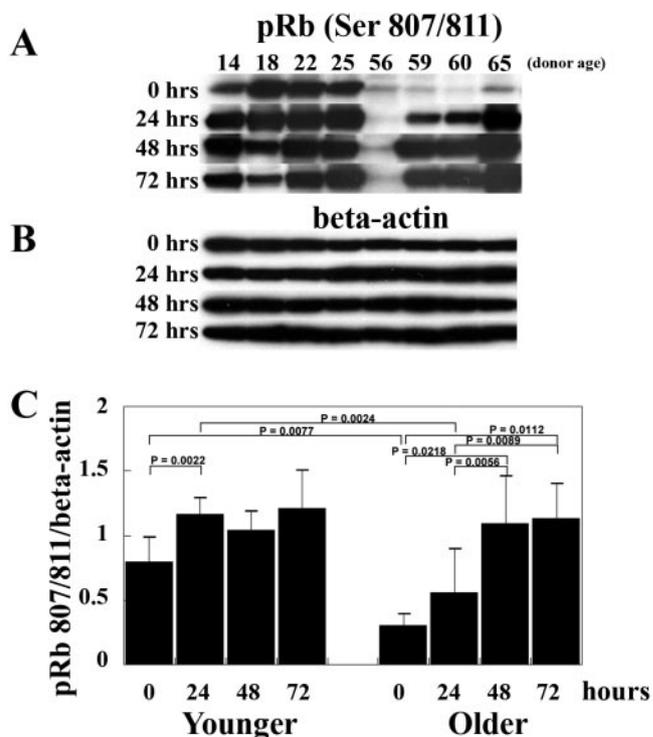


FIGURE 12. Western blot analysis using rabbit anti-human Rb (Ser 807/811) demonstrate the kinetics of Rb hyperphosphorylation in the same samples of subconfluent HCECs as used in Figure 11. Samples were taken at 0, 24, 48, and 72 hours after growth factor addition (A) Blot showing β -actin band from all samples (B). Densitometric analysis with β -actin used for normalization (C) demonstrated a maximum increase in Rb hyperphosphorylation in HCECs from younger donors within 24 hours of growth factor addition, whereas maximum hyperphosphorylation of Rb did not occur in HCECs from older donors until 48 hours after growth factor stimulation. Bars, SD. Probabilities demonstrate statistically significant differences.

leading to the age-related decrease in proliferative capacity of HCECs.

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