Relationship among Oxidative Stress, DNA Damage, and Proliferative Capacity in Human Corneal Endothelium

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Purpose. To determine whether human corneal endothelial cells (HCECs) exhibit signs of oxidative DNA damage and to test whether oxidative stress affects the proliferative capacity of HCECs.

Methods. Donor human corneas were divided into two age groups: young (<30 years) and older (>50 years). An 8-hydroxy-2′-deoxyguanosine (8-OHdG) ELISA assay was used to quantify oxidative DNA damage in HCECs freshly isolated from ex vivo corneas. 8-OHdG immunostaining localized the sites of oxidative DNA damage in corneal wholemounts and cultured HCECs. To test whether oxidative stress induces oxidative DNA damage, HCECs cultured from young donors were treated with increasing concentrations of hydrogen peroxide (H_2O_2) and immunostained for 8-OHdG. To test the effect of oxidative stress on proliferative capacity, HCECs cultured from young donors were treated with H_2O_2 and cell numbers determined by WST-8 assay.

Results. 8-OHdG levels were significantly higher ($P = 0.0031$) in the central endothelium of older donors than of young donors. Intense nuclear staining for 8-OHdG was observed in central endothelium of older, but not young, donors. The relative intensity of 8-OHdG in the nuclei of cultured HCECs was similar to that observed in ex vivo corneas. Treatment of cultured HCECs from young donors with increasing concentrations of H_2O_2 resulted in a dose-dependent increase in nuclear 8-OHdG staining and a decrease in proliferative capacity similar to that observed in untreated HCECs from older donors.

Conclusions. Age-dependent and topographical decreases in proliferative capacity observed in HCECs resulted, at least in part, from nuclear oxidative DNA damage. (*Invest Ophthalmol Vis Sci.* 2009;50:2116–2122) DOI:10.1167/iovs.08-3007

Human corneal endothelial cells (HCECs) do not normally divide in vivo[1,2]; however, they retain the ability to proliferate under appropriate conditions.[3,4] Studies conducted in this laboratory indicate that although HCECs retain the ability to divide, they exhibit differences in relative proliferative capacity based on donor age. These differences have been observed in an ex vivo corneal wound model[3] and in culture.[4] Western blot analysis[5] indicates that HCECs of older donors (>50 years) express significantly higher levels of the G1-phase inhibitors p21Cip1 and p16INK4a than do cells of young donors (<30 years), suggesting an age-dependent increase in the negative control of the corneal endothelial cell cycle. Immunostaining of HCECs for minichromosome maintenance protein (Mcm)-2, a marker of replication competence,[6] supports these findings and indicates that HCECs exhibit not only age-related differences in proliferative capacity but also topographical differences.[7] In both age groups, the relative number of Mcm-2 positive HCECs in central endothelium (6-mm diameter) was significantly lower than in peripheral endothelium (6- to 9.5-mm rim), with the central area of corneas from older donors having the lowest number of positively stained cells. Together, these data indicate that HCECs in central cornea, particularly corneas of older donors, exhibit reduced proliferative capacity.

The cell cycle characteristics exhibited by HCECs of older donors are similar to those observed in senescent cells.[8–11] "Senescence" has been defined as a viable state of growth arrest distinct from mitotic quiescence, and it acts as an important tumor-suppressive mechanism.[12] Senescent cells typically exhibit decreased saturation density and slower cell cycle kinetics, become stably arrested with a G1-phase DNA content, and express higher protein levels of p21Cip1 and p16INK4a. Researchers in the field of aging distinguish two forms of cellular senescence: replicative senescence and stress-induced premature senescence. Replicative senescence results from the successive shortening of telomeres that occurs during DNA replication.[13] Once telomeres have eroded to a critically short length, the senescence program is activated, and cells become irreversibly inhibited from dividing. Stress-induced premature senescence (SIPS) is caused by exposure of cells to certain environmental stresses.[14,15] Among these stresses is sublethal oxidative stress, which occurs when the concentration of reactive oxygen species (ROS) exceeds antioxidant defenses.[16–18] SIPS is considered to be premature because cells lose their ability to proliferate before telomere exhaustion. Thus, in SIPS, cells retain proliferative potential based on telomere length but stop dividing because of inhibitory mechanisms activated by stress-induced damage. Ectopic expression of telomerase (hTERT) can prevent the entrance of cells into replicative senescence by preventing the formation of critically short telomeres; however, SIPS cannot be overcome by ectopic hTERT expression.[14,19] Studies indicate that there is no statistical difference in relative telomere length between HCECs of young and older donors or between HCECs located in central and peripheral endothelium,[20,21] providing strong evidence that the age-related decrease in proliferative capacity exhibited by HCECs cannot be attributed to replicative senescence. Together, these results strongly suggest that HCECs retain the potential to divide based on telomere length and that the observed age-dependent and topographical decreases in proliferative capacity result from a form of SIPS.

Because sublethal oxidative stress can induce SIPS and HCECs are highly metabolically active and lie directly in the light path, making them susceptible to high levels of ROS, it is reasonable to hypothesize that the age-dependent and topographical decreases in proliferative capacity observed in HCECs result, at least in part, from oxidative DNA damage. The present study was conducted to help test this hypothesis by determining whether HCECs exhibit oxidative DNA damage

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and examining whether oxidative stress affects the proliferative capacity of HCECs.

Materials and Methods

Human Corneal Tissue and Culture of HCECs

Human donor corneas were obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA). Donor confidentiality was maintained by the original eye bank, NDRI, and this laboratory in accordance with the tenets of the Declaration of Helsinki. Donor information is presented in Table 1. Corneas were accepted for study based on published exclusion criteria.[22] Corneas were accepted only if the donor history and condition of the corneas indicated no damage to the health of the endothelium. Endothelial cell counts for all accepted corneas were at least 2000 cells/mm^2. Corneas were divided into two groups: those from young (<30 years) donors and those from older (>50 years) donors. In some cases, one cornea of a pair was used for immunocytochemical localization and the other cornea was used for isolation and culture of HCECs. HCECs were cultured to passage 2 according to previously described protocols.[4,23] Culture medium consisted of medium (OptiMEM-1; Invitrogen-Life Technologies, Carlsbad, CA) supplemented with 8% fetal bovine serum (FBS; Hyclone, Logan, UT), 5 ng/mL epidermal growth factor (EGF from mouse submaxillary gland; Upstate Biotechnologies, Lake Placid, NY), 20 ng/mL nerve growth factor (from mouse submaxillary gland; Biomedical Technologies, Stoughton, MA), 100 μg/mL pituitary extract (Biomedical Technologies), 20 μg/mL ascorbic acid, 200 μg/mL calcium chloride, 0.08% chondroitin sulfate, antibiotic/antimycotic solution diluted 1/100 (all from Sigma-Aldrich, St. Louis, MO), and 50 μg/mL gentamicin (Invitrogen-Life Technologies).

Rabbit Corneal Tissue

Young pigmented rabbits were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fresh eyes were obtained from four rabbits under an Institutional Animal Care and Use Committee–approved protocol. Corneas were dissected and cut into quarters. Some quarters were immediately fixed for 10 minutes at −20°C in 100% methanol, then washed 3 times in phosphate-buffered saline (PBS) and stored at 4°C. Other quarters were immediately placed in corneal storage medium (Optisol; Chiron Ophthalmics, Irvine, CA) and stored at 4°C for 5 days. A positive control for oxidative DNA damage was prepared based on a published protocol.[24] Briefly, the tissue was incubated for 4 hours in 0 μM or 100 μM hydrogen peroxide (H_2O_2) prepared in culture medium (Medium-199; (Gibco/BRL, Life Technologies, Grand Island, NY) supplemented with 50 μg/mL gentamicin (Gibco/BRL, Life Technologies) and 10% FBS. No ascorbic acid (an antioxidant) was present in the culture medium. After this incubation, tissue was postincubated for 24 hours in the same culture medium without H_2O_2. Before immunostaining, corneas stored at 4°C in corneal storage medium (Optisol; Chiron Ophthalmics) were incubated for 2 hours at 37°C to bring them to the same temperature as the positive controls. All tissue was then immunostained for 8-OHdG, as described, to visualize oxidative DNA damage. Duplicates were prepared for all conditions.

Table 1. Donor Information

Donor Age (y)	Time*	Days†	Cause of Death
2	06:40	5	Porencephalic cyst
11	02:30	6	Traumatic brain injury
18	12:00	2	Undetermined
19	02:00	7	Pulmonary embolism
20	09:00	2	Gunshot wound
21	06:00	8	Pulmonary embolism
22	05:05	5	Suicide
26	17:00	2	Cardiac arrest
27	09:32	2	Pulmonary embolism/ATV accident
27	02:00	2	Herniated brain tumor
28	14:00	2	Internal bleeding/MVA
55	03:17	10	Brain cancer
60	08:40	2	COPD
65	07:00	2	Heart disease/probable MI
69	12:50	2	CVA
69	03:00	3	Cardiac arrest/heart disease
71	12:00	3	Rhabdomyelysis
73	03:42	6	Gastrointestinal bleed
74	02:30	2	Cardiac arrest
78	04:30	3	Heart disease
85	14:00	3	Stroke

* Time in hours:minutes between death and corneal preservation.
† Number of days of preservation in corneal storage medium at 4°C.

Quantification of 8-OHdG

Whole human corneas were washed with PBS and divided into central (6-mm diameter) and peripheral (6- to 9.5-mm diameter) areas using a 9.5-mm corneal vacuum punch (Barron; Katena Products, Denville, NJ) and a 6-mm punch (Acu-Punch; Acuderm, Fort Lauderdale, FL). Descemet's membrane with attached endothelium was dissected from each trephined corneal sample and placed in buffer (Buffer ATL from a QIAgen DNeasy Blood and Tissue Kit; QIAgen, Valencia, CA). DNA was extracted according to the kit protocol. DNA was quantified with a spectrophotometer (Nanodrop ND-1000; Nanodrop Technologies, Wilmington, DE) and then digested with 200 mM sodium acetate and 6 U nuclease P1 (both from Sigma-Aldrich) at 37°C for 30 minutes, followed by the addition of 1 M Tris-HCl buffer (Sigma) plus 2 U alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany) and incubation at 37°C for 30 minutes. Enzymes and other macromolecules were removed by filtering through a centrifugal filter unit (Micron YM-10; Millipore, Billerica, MA) at 14,000 rpm for 10 minutes. Competitive 8-OHdG ELISA assays were performed with a high-sensitivity kit from Northwest Life Science Specialties (Vancouver, WA). Briefly, 8-OHdG antibody plus sample DNA were added to a 96-well plate precoated with 8-OHdG and incubated overnight at 4°C. After the plate was washed, horseradish peroxidase–conjugated secondary antibody was added for 1 hour at room temperature. After washing, 3,3′,5,5′-tetramethylbenzidine was added and incubated for 15 minutes at room temperature in the dark. The reaction was terminated by the addition of phosphoric acid, and absorbance was measured at 450 nm. All assays were performed in duplicate. Negative controls and 8-OHdG standards (0.125–10 ng/mL) were included in the assay. The average concentration of 8-OHdG, normalized per nanograms of total DNA, was calculated for each sample based on the standard curve. Results from the central and peripheral areas of corneas from young and older donors were then averaged and compared using an unpaired Student's *t*-test. Data were considered statistically significant at $P < 0.05$.

Immunostaining for 8-OHdG

Whole corneas and subconfluent passage 2 cultures of HCECs were immunostained for 8-OHdG according to established protocols.[3] Whole corneas from young and older donors were incubated in corneal storage medium (Optisol; Chiron Ophthalmics) for 2 hours in a 37°C water bath. Corneas were then washed in prewarmed phosphate-buffered saline (PBS), fixed for 10 minutes in 100% methanol at −20°C, washed in PBS, and cut into central and peripheral areas as described. Tissue was incubated for 10 minutes in blocking buffer containing 5% donkey serum diluted in PBS, incubated for 2 hours in a 1/200 dilution of goat anti–8-OHdG (Millipore), washed, and incubated for 1 hour with a 1/200 dilution of donkey anti–goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Both antibodies were diluted in blocking buffer. Corneal tissues incubated only in secondary antibody acted as negative controls. Corneal pieces were then washed and placed endothelial-side up in mounting medium containing propidium iodide (PI) to stain all nuclei. Fluorescence confocal microscopy was

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used to visualize positive staining. Laser images (0.5 μm) were digitally recorded. Cultured HCECs were stained for 8-OHdG using the same protocol. Staining of cultured cells was observed under a fluorescence microscope (Eclipse E-800; Nikon, Tokyo, Japan) equipped with a digital camera (Spot; Diagnostic Instruments, Sterling Heights, MI). All samples were prepared in duplicate. All staining was performed using corneas from at least three donors per age group.

Effect of H_2O_2 on Oxidative DNA Damage in HCECs

HCECs were cultured from young donors, as described, and were treated with H_2O_2 based on a model used to study the effect of oxidative stress in human diploid fibroblasts.[24] Passage 2 HCECs were treated for 4 hours at 37°C with 0, 25, 50, 100, 200, or 500 μM H_2O_2 and were postincubated for 24 hours in medium containing 8% FBS without ascorbic acid or H_2O_2 to permit recovery of the cells. Cells were then fixed and prepared for immunostaining of 8-OHdG, as described, to evaluate oxidative DNA damage. To test for the effect of H_2O_2 treatment on viability, cells were stained using an assay kit (Live/Dead; Invitrogen/Molecular Probes, Eugene, OR).

Effect of Oxidative Stress on Proliferation of HCECs

The effect of oxidative stress on the proliferation of HCECs was tested with the use of a protocol published by Chen et al.[25] to study the effect of oxidative stress on the proliferative activity of human diploid fibroblasts. Passage 2 HCECs from three young donors were plated at low density, and cell numbers were counted with a spectrophotometric assay kit (WST-8; Dojindo Molecular Technologies, Gaithersburg, MD). The numbers obtained were considered day 0 counts. Cells were then treated for 1 hour at 37°C with 0, 25, 50, or 100 μM H_2O_2 in the culture medium, as described, containing 8% FBS (minus ascorbic acid and growth factors). After treatment, cells were washed and incubated for 4 days in the same medium (minus ascorbic acid, growth factors, and H_2O_2) to permit recovery from the H_2O_2. On day 4, cells were counted, retreated with H_2O_2 as earlier, washed, and incubated in medium without H_2O_2. Cell numbers were then determined on days 6, 8, and 11. All samples were prepared in duplicate.

Results

Quantification of Oxidative DNA Damage in HCECs Isolated from Ex Vivo Corneas

ELISA for 8-OHdG, a recognized marker of oxidative DNA damage,[26] was used to quantify oxidative DNA damage in HCECs isolated from the central (6-mm diameter) and peripheral (6- to 9.5-mm rim) areas of ex vivo corneas. Individual results from four young and four older donors are shown in Figure 1A; Figure 1B shows the average concentration of 8-OHdG detected in the central and peripheral endothelium from each age group. Results clearly indicate that the level of oxidative DNA damage was higher in HCECs from older donors, primarily because of a statistically significant increase ($P = 0.0031$) in oxidative DNA damage within the central endothelium.

Localization of Oxidative DNA Damage in HCECs In Situ

Results of the ELISA assays clearly indicate that the level of oxidative DNA damage was highest in the central endothelium of older donors; however, the results do not indicate where this damage was located because both mitochondrial and nuclear DNA would have contributed to the ELISA assay results. To determine the subcellular location of this damage, whole-mounts of ex vivo corneas were immunostained for 8-OHdG and were examined by fluorescence confocal microscopy. Figures 2A and 2B present representative images of 8-OHdG staining in the central and peripheral endothelia of corneas of a young and an older donor. Specificity of 8-OHdG staining was demonstrated by the negative controls in which corneas were incubated in secondary antibody alone. Interestingly, differences in subcellular localization of 8-OHdG were noted, as were age-related and topographically related differences. In most peripheral cells, cytoplasmic staining for 8-OHdG appeared to be equal to or more intense than nuclear staining. The punctate pattern of 8-OHdG staining in the cytoplasm was more clearly seen in the magnified image presented in Figure 2C. This type of staining pattern was consistently observed in peripheral cells, providing evidence of mitochondrial oxidative DNA damage. Overall, in peripheral endothelium, the relative intensity of 8-OHdG staining and the specific staining patterns did not appear to differ with donor age. In central endothelium of both age groups, staining patterns were different from those in the periphery. In central endothelium of young donors, a low level of punctate 8-OHdG staining was observed within the cytoplasm, suggesting mild oxidative DNA damage in mitochondria. Central cells in these corneas showed a low level of positive staining in nuclei. Central endothelium of older donors showed an increase in the relative intensity of cytoplasmic staining compared with cells from young donors. Most striking,

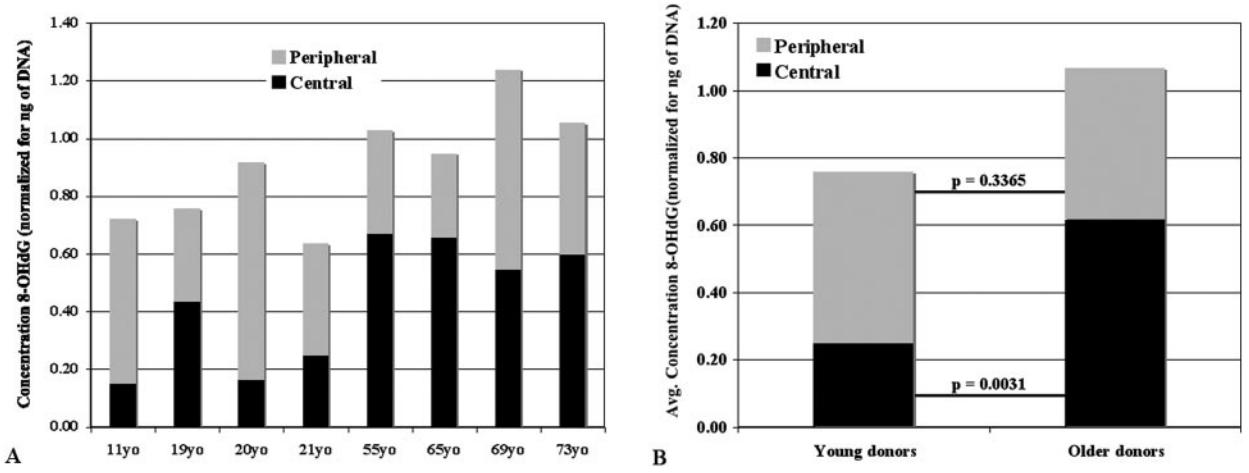

Figure 1. Quantification of 8-OHdG in the central and peripheral endothelium isolated from ex vivo corneas of four young and four older donors. (**A**) Results from donors. (**B**) Average concentrations of 8-OHdG in the central and peripheral endothelia of young and older donors. Note the statistically significant increase ($P = 0.0031$) in the amount of 8-OHdG detected in central endothelial cells from older donors.

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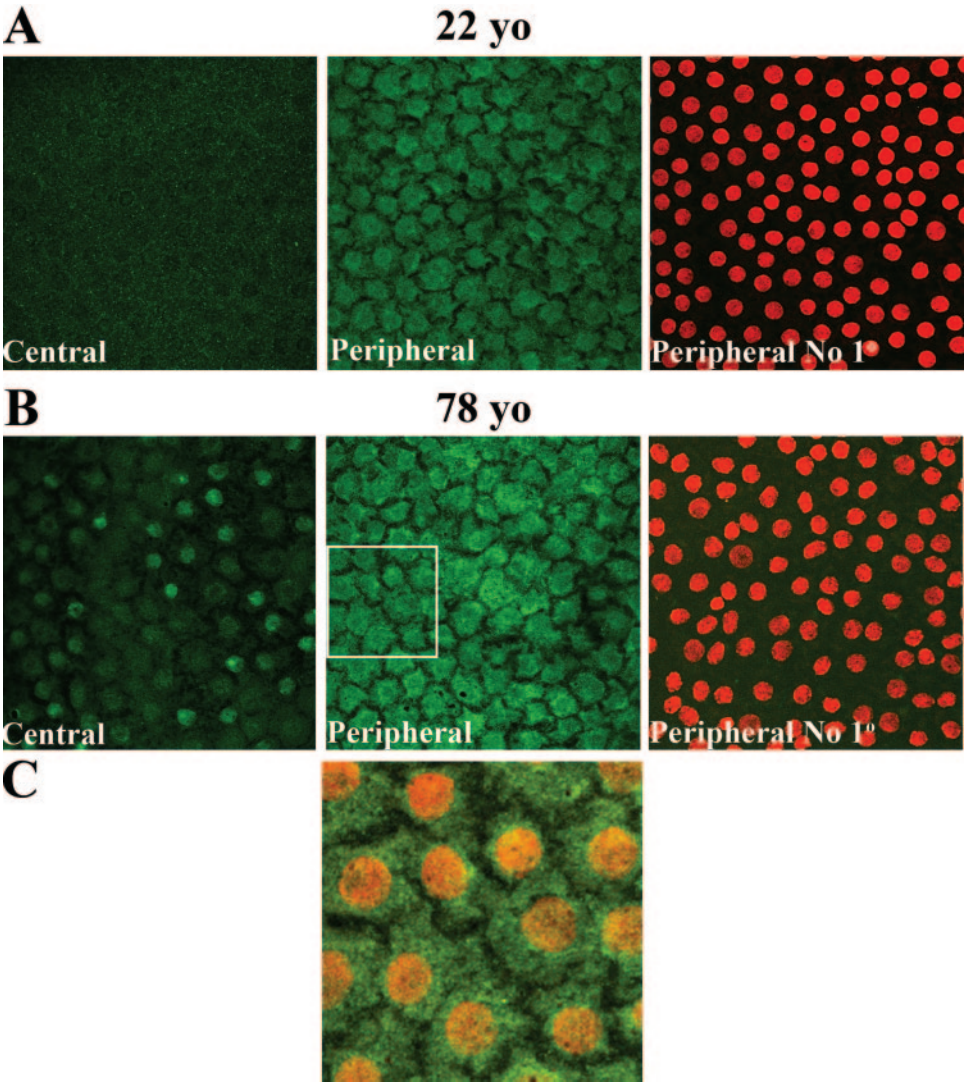

FIGURE 2. Staining for 8-OHdG in corneal endothelium of ex vivo corneas from a (**A**) 22-year-old and a (**B**) 78-year old donor. FITC (*green*) images of 0.5-μm laser sections show differences in relative 8-OHdG staining patterns and age-related and topographical differences in relative staining intensity for 8-OHdG. (**A**, **B**, *right*) Stacked images from the FITC channel and the rhodamine channel, identifying nuclei by PI staining. These images were taken in the peripheral region of corneal tissue that had been incubated in secondary antibody alone and demonstrate the specificity of primary antibody staining. The image in (**C**) is part of the same peripheral region as shown for the 78-year-old donor in (**B**). Note the area within the *white square*. Images of this region taken in both the FITC and rhodamine channels have been overlaid to show nuclei and more clearly demonstrate the punctate staining for 8-OHdG in the cytoplasm. Original magnification: (**A**, **B**) 40×, zoom 2; (**C**) 40×, zoom 5.7.

however, was the intense nuclear staining of many, but not all, cells. Overall, results strongly suggest a topographical and age-related difference in the extent of nuclear oxidative DNA damage, with HCECs in the central endothelium of older donors exhibiting the highest level of damage.

Test to Determine Whether 8-OHdG Staining in Ex Vivo Corneas Is a Postmortem Storage Artifact

It is possible that the 8-OHdG staining observed in ex vivo human corneas is a postmortem artifact generated by storage of the corneas in corneal storage medium (Optisol-GS; Chiron Ophthalmics) at 4°C before study. To explore this possibility, fresh corneas were obtained from young rabbits, cut into quarters, and incubated under various conditions before immunostaining for 8-OHdG. Representative images of the results are presented in Figure 3. Endothelial cells in corneal quarters that were fixed immediately after dissection (Fig. 3A) show a low level of positive staining in the cytoplasm and little to no staining in nuclei. Similar staining patterns were observed in endothelial cells of corneal quarters stored in corneal storage medium (Optisol; Chiron Ophthalmics) at 4°C for 5 days (Fig. 3B). The positive control in which quarters were treated with 100 μM H_2O_2 (Fig. 3C) shows intense nuclear staining in some cells, indicating an increase in oxidative DNA damage. Negative controls incubated in secondary antibody alone were not

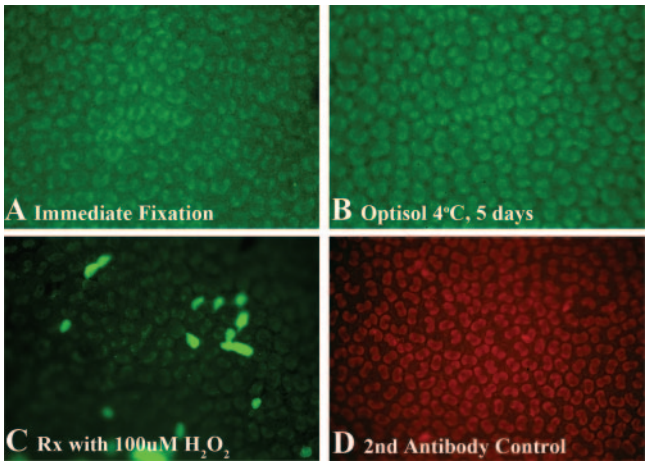

FIGURE 3. 8-OHdG staining in endothelium of ex vivo rabbit corneas (**A**) fixed immediately after dissection, (**B**) stored in corneal storage medium at 4°C for 5 days, or (**C**) treated with 100 μM H_2O_2. (**D**) Cornea incubated in secondary antibody only is an overlay from both the FITC and rhodamine channels. *Green*: 8-OHdG; *red*: PI. Original magnification, 40×.

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stained (Fig. 3D). Overall, no difference in the 8-OHdG staining pattern or relative staining intensity was observed in the endothelia of rabbit corneas fixed immediately after surgical removal (and, therefore, closely representing the in vivo status of the cells) or stored for 5 days in corneal storage medium (Optisol; Chiron Ophthalmics) at 4°C, a period longer than the average storage time for human corneas used in the present study. Together, the results strongly suggest that the HCEC staining patterns for 8-OHdG observed in ex vivo corneas were not the result of a storage artifact.

Cultured HCECs Retain Age-Related Differences in Oxidative DNA Damage

Studies were then conducted to determine whether cultured HCECs retained the same relative patterns of oxidative DNA damage observed in ex vivo corneas. For these studies, HCECs were isolated from the entire endothelium of young and older donors rather than from the central or peripheral areas, grown to passage 2, and immunostained for 8-OHdG. Figure 4A presents representative images of HCECs cultured from a young and an older donor. In cells from young donors, a low level of positive staining for 8-OHdG was observed in the cytoplasm and nucleus. This staining appeared to reflect a basal level of oxidative DNA damage contributed from the in vivo state or from time in culture. Nuclei in HCECs cultured from older donors were consistently more intensely stained than nuclei from young donors. No staining was observed in cells incubated in secondary antibody only. Results demonstrated that although all cultured HCECs exhibited low levels of oxidative DNA damage, the greatest nuclear DNA damage was observed in the cells of older donors. Importantly, results from the cultured cells were similar to those of HCECs in ex vivo corneas, providing evidence that oxidative DNA damage is retained in cultured cells and validating the use of cultured HCECs as a model to study the relationship between oxidative DNA damage and proliferative capacity.

H_2O_2 Treatment of HCECs from Young Donors

Studies were conducted to determine whether H_2O_2-induced oxidative stress in HCECs cultured from young donors induces oxidative DNA damage similar to that observed in untreated HCECs of older donors. The model of oxidative stress used was based on a method described by Gurjala et al.[24] to study the effect of oxidative stress in human diploid fibroblasts. Briefly, passage 2 HCECs cultured from young donors were treated with increasing concentrations of H_2O_2, postincubated in medium containing 8% FBS without H_2O_2 to permit recovery of the cells, fixed, and immunostained for 8-OHdG to evaluate oxidative DNA damage. Similar results were obtained in all cultures tested. Figure 4B presents representative images of 8-OHdG staining in cells from an 18-year-old donor. Exposure to increasing concentrations of H_2O_2 increased 8-OHdG staining in the cytoplasm and the nucleus, reflecting increased oxidative damage to mitochondrial and nuclear DNA. The 8-OHdG staining pattern for HCECs cultured from the 18-year-old donor and maintained under control conditions (0 μM H_2O_2) or after exposure to 25 μM H_2O_2 was similar to that seen in untreated HCECs from the 27-year-old donor in Figure 4A. The staining pattern after exposure of HCECs from the 18-year-old donor (to 50–200 μM H_2O_2) was similar to that observed in cells from the 69-year-old donor in Figure 4A. Treatment with 500 μM H_2O_2 caused significant cell loss, and remaining cells stained intensely for 8-OHdG, indicating significant oxidative DNA damage. Staining of H_2O_2-treated cells with an assay kit (Live/Dead; Invitrogen/Molecular Probes) confirmed a significant increase in cell death when cells were exposed to 500 μM H_2O_2 (data not shown). Results indicate that the treatment of HCECs cultured of young donors with increasing concentrations of H_2O_2 caused nuclear oxidative DNA damage that closely resembled the damage observed in untreated HCECs cultured from older donors. Thus, treatment of young HCECs with increasing concentrations of H_2O_2 can be used as a relevant model of age-related oxidative stress.

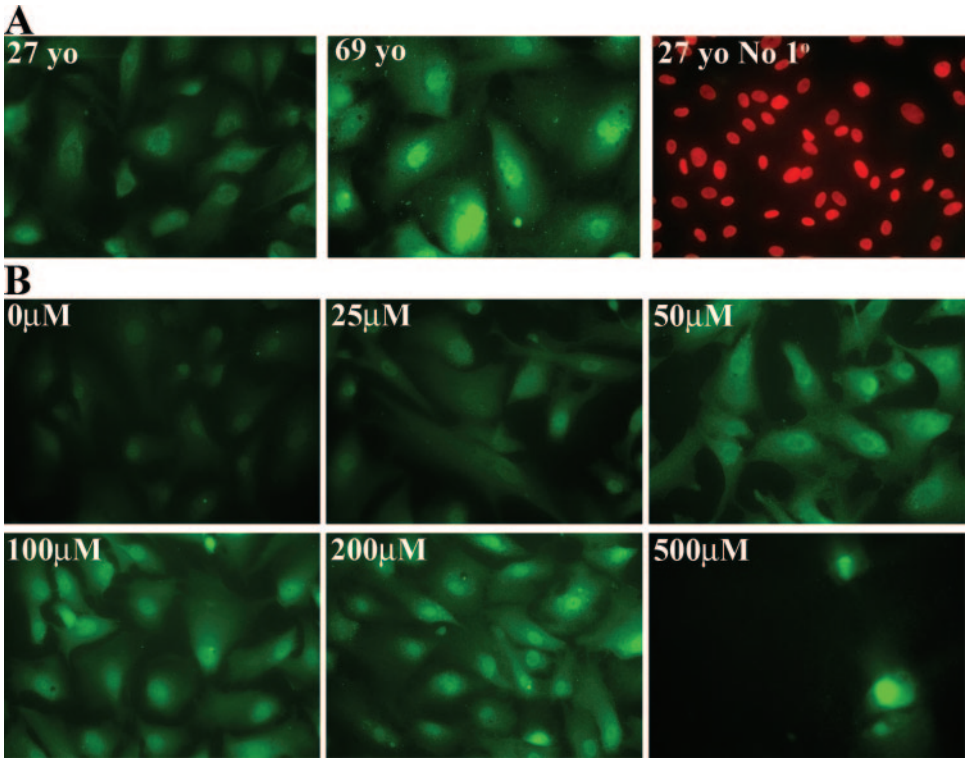

FIGURE 4. (**A**) Representative images of 8-OHdG immunostaining in subconfluent passage 2 HCECs cultured from a 27-year-old and a 69-year-old donor. *Right*: cells incubated in secondary antibody alone; this is an overlay of both the FITC and rhodamine channels. *Green*: 8-OHdG; *red*: PI. (**B**) Dose-dependent effect of H_2O_2 on induction of oxidative DNA damage in HCECs cultured from an 18-year-old donor. Subconfluent passage 2 HCECs were treated with increasing concentrations of H_2O_2 and immunostained for 8-OHdG. Negative control was stained with secondary antibody alone and is an overlay of both the FITC and DAPI channels. *Green*: 8-OHdG. Original magnification (**A**, **B**) 40×.

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Oxidative Stress–Reduced Proliferative Capacity in HCECs from Young Donors

Studies were conducted using passage 2 HCECs cultured from three young donors (2, 26, 27 years) to test whether oxidative stress affects the proliferative capacity of HCECs. To model oxidative stress, subconfluent cells were incubated in culture medium containing 8% FBS plus 0, 25, 50, or 100 μM H_2O_2 according to a protocol used by Chen et al.[25] to study the effect of oxidative stress on proliferation of human diploid fibroblasts. Cell counts were determined by a WST-8 spectrophotometric assay over 11 days. Similar results were obtained from the three donors, and a graph presenting representative results from the 26-year-old donor is shown in Figure 5. Incubation of subconfluent cells in culture medium without H_2O_2 resulted in a steady increase in cell numbers from day 0 through day 4, after which the cells became confluent, resulting in no net increase in cell numbers. A relatively similar growth curve was obtained when cells were incubated with 25 μM H_2O_2, indicating little effect on growth at this low concentration of oxidizing agent. In contrast, cells incubated in 50 μM or 100 μM H_2O_2 showed a reduced rate of growth. Cells grew more slowly from day 0 to day 4 and reached saturation density by day 6. This density was consistently lower than that observed in untreated cultures or in cultures treated with 25 μM H_2O_2.

Discussion

The relative proliferative capacity of HCECs decreases in an age-dependent and topographical manner and appears to be caused by a form of stress-induced premature senescence rather than by critically short telomeres. High metabolic activity and exposure to light, particularly in the ultraviolet wavelengths, are potential sources of oxidative stress because they both generate high levels of ROS.[26–29] Given that HCECs are metabolically active and lie directly in the light path, it is reasonable to hypothesize that the decreased proliferative capacity observed in HCECs is, at least in part, the result of oxidative DNA damage. The present study was designed to begin to test this hypothesis by determining whether HCECs exhibit evidence of oxidative DNA damage and whether this damage affects their ability to proliferate.

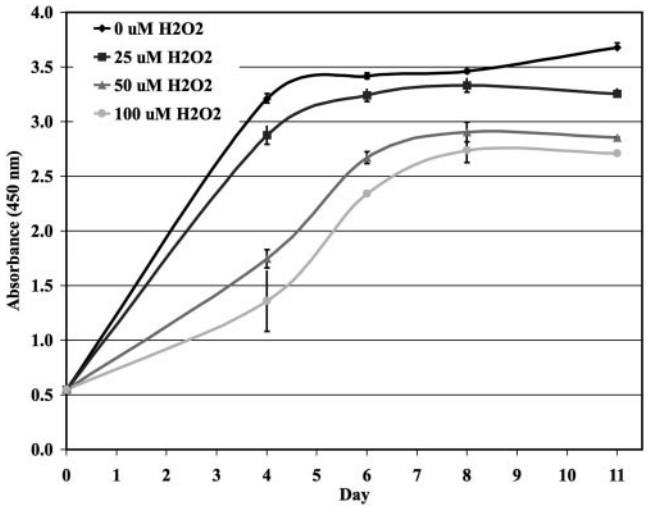

Figure 5. Representative example of the effect of mild oxidative stress on the proliferative response of HCECs. Subconfluent HCECs from a 26-year-old donor were exposed to 0, 25, 50, or 100 μM H_2O_2. Cell numbers were determined over 11 days using a WST-8 spectrophotometric assay. This graph presents cell numbers as a function of absorbance at 450 nm. Note the decrease in growth rate and saturation density on exposure of young HCECs to 50 or 100 μM H_2O_2.

Oxidation of guanine to form 8-OHdG acts as a marker of oxidative DNA damage.[30] In the present study, results of the 8-OHdG ELISA assay clearly demonstrated the presence of oxidative DNA damage in the central and peripheral areas of the endothelium, regardless of donor age. Importantly, the assay revealed a statistically significant increase in oxidative DNA damage in central endothelial cells from older donors. The punctate 8-OHdG staining pattern in peripheral endothelium was relatively similar in both age groups and suggested that, in peripheral endothelium, oxidative DNA damage is greater in mtDNA than in nuclear DNA. Nuclear staining for 8-OHdG was most intense in the central endothelium of older donors, suggesting that nuclear oxidative DNA damage is greatest in that area. Interestingly, the relative staining intensity appeared to vary from cell to cell within this area, suggesting that the extent of DNA damage differs in individual cells. Together, both studies provide strong evidence that oxidative DNA damage increases in an age-dependent and topographical manner in HCECs and that nuclear oxidative DNA damage is greatest in the central endothelium of older donors. Further investigation is needed to explain the difference in relative 8-OHdG staining intensity in the mitochondria between the central and the peripheral endothelium. The apparently lower level of mtDNA staining observed in central endothelium of both age groups may reflect differences in overall metabolic activity, antioxidant activity, and DNA repair capability.

In the present study, a conscious effort was made to minimize oxidation artifacts. For the 8-OHdG ELISA, the endothelium was dissected from the corneas, and DNA was extracted immediately after arrival in the laboratory to minimize artifactually induced oxidative DNA damage. In addition, studies were conducted with rabbit corneas to determine whether the 8-OHdG staining patterns obtained with the human corneas might have resulted from an artifact produced by storage in corneal storage medium (Optisol; Chiron Ophthalmics). The 8-OHdG staining patterns in rabbit endothelium did not differ between corneas fixed immediately on removal and corneas stored for 5 days in corneal storage medium (Optisol; Chiron Ophthalmics) at 4°C, a period longer than the average storage time for the human corneas used in the present study. Positive controls in which corneas were specifically incubated in H_2O_2 showed bright nuclear staining patterns, providing evidence that the intense nuclear 8-OHdG staining observed in the central endothelium of older donors resulted from oxidative stress.

When DNA is damaged, cells initiate a response that is appropriate for the extent of the damage. This response can include DNA repair, cell cycle delay, entry into senescence, or induction of apoptosis.[31] Stress-induced DNA damage inhibits proliferation and induces cellular senescence through the activation of specific checkpoint pathways.[16,32] The DNA damage response appears to be actively maintained in senescent cells, suggesting that DNA damage signals persist and that cell cycle inhibition is maintained as long as DNA has not been appropriately repaired.[33,34] Results of the present study reveal an excellent correlation between the finding of a statistically significant increase in 8-OHdG levels in the central endothelium of older donors and the intense nuclear localization of 8-OHdG observed in this area. These findings correlate with previous data demonstrating a reduced proliferative capacity exhibited by central endothelial cells from older donors using an ex vivo cornea wound model[3] and cultured cells,[35] and they provide strong evidence that nuclear oxidative DNA damage is responsible, at least in part, for the observed reduction in proliferative capacity.

The similarity in the relative intensity and pattern of 8-OHdG staining of HCECs in ex vivo corneas and in culture

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indicates that the relative level of oxidative DNA damage was not substantially changed on culturing and supports the use of cultured HCECs as a relevant model to study the effect of oxidative stress on proliferative capacity. Treatment of HCECs cultured from young donors with increasing concentrations of H_2O_2 resulted in a dose-dependent change in the relative intensity of nuclear 8-OHdG staining that closely resembled patterns observed in untreated HCECs cultured from older donors. Mild H_2O_2 treatment was sufficient to affect cellular function by decreasing the proliferative capacity of HCECs from young donors. Importantly, the resultant growth curves in H_2O_2-treated cells from young donors closely resemble growth curves previously obtained from HCECs from older donors in an ex vivo corneal wound model[3] and in culture.[4]

Results obtained in the present study strongly suggest that long-term, mild oxidative stress—probably induced by exposure to ultraviolet light or high metabolic activity—may be responsible for the age-related and topographically related reductions in proliferative capacity observed in HCECs. Further study will be needed to verify a correlation between oxidative stress and the activation of the DNA damage checkpoint pathway in HCECs, leading to increased levels of cell cycle inhibitors. Further study is also needed to determine whether there are age-related and topographically related decreases in antioxidant enzyme expression in HCECs. It will also be important to determine whether there is an age-related decrease in the relative expression of DNA repair enzymes, as has recently been found in the retinal pigment epithelium and the choroid of aged rats.[36]

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