Increased Proliferation and Replicative Lifespan of Isolated Human Corneal Endothelial Cells with L-Ascorbic acid 2-phosphate

Nobuyuki Shima,[*,1] *Miwa Kimoto,*[1] *Masahiro Yamaguchi,*[1,2] *and Satoru Yamagami*[*,1,3]

Purpose. To explore an alternative culture method for human corneal endothelial cells (HCECs) and to examine the effect of L-ascorbic acid 2-phosphate (Asc-2P) on the growth of these cells.

Methods. The influence of various mitogens, extracellular matrices (ECMs), and Asc-2P on growth of cultured HCECs was examined. HCECs were obtained from donors ranging in age from 12 to 74 years, and primary cultures and subcultures were performed with or without Asc-2P. Expanded HCECs were characterized with immunostaining and reverse transcription polymerase chain reaction (RT-PCR) and evaluated for generation of 8-hydroxy-2-deoxyguanosine (8-OHdG) with immunostaining and an enzyme-linked immunosorbent assay (ELISA).

Results. Culture with Asc-2P and bFGF on atelocollagen promoted the proliferation of HCECs in both primary cultures and subcultures as efficiently as conventional culture using ECM derived from bovine corneal endothelial cells. Zonula occludens-1, N-cadherin, connexin 43, and Na^+/K^+-ATPase were localized at plasma membranes of cultured HCECs. mRNAs of the voltage-dependent anion channels (*VDAC2* and *VDAC3*), sodium bicarbonate cotransporter member 4 (*SLC4A4*), and chloride channel proteins (*CLCN2* and *CLCN3*) were detected by RT-PCR. During multiple passages, cultures without Asc-2P showed a decrease in growth and irregular cell morphology, whereas cultures with Asc-2P sustained cell growth and maintained the characteristic polygonal morphology. ELISA for 8-OHdG showed that the levels in mitochondrial DNA significantly decreased when HCECs were subcultured with Asc-2P.

Conclusions. Combination of Asc-2P and bFGF on atelocollagen allows successful culture for HCECs. Asc-2P extends the lifespan of cultured HCECs, partly due to protection against oxidative DNA damage. (*Invest Ophthalmol Vis Sci.* 2011;52: 8711–8717) DOI:10.1167/iovs.11-7592

Human corneal endothelial cells (HCECs) play a crucial role in maintaining corneal transparency by regulating corneal hydration, but have weak proliferative capacity in vivo.[1] Thus, the only way to restore lost HCECs is by transplantation of a full or partial donor cornea. On the other hand, HCECs have been found to proliferate in vitro[2,3] raising the possibility of developing HCEC grafts by tissue engineering,[4–10] although successful culture requires complicated combination of mitogens, the appropriate extracellular matrix (ECM), or a conditioned medium from embryonic stem cells.[11–16] In our experience, ECM from bovine corneal endothelial cells (BCECM) is essential for the successful primary culture of HCECs.[17] When expanding cultured HCECs for clinical use, the risk of contamination by pathogens should be minimized, so an alternative to BCECM is needed because bovine eyes are listed among the specified materials with a potential risk of bovine spongiform encephalopathy (BSE). Another technical difficulty is that HCECs from adult donors have a shorter proliferative longevity than those from young donors.[17] Because most donors are older people, improvement of the replicative capacity of HCECs from such donors is an important challenge to overcome before starting clinical trials of tissue-engineered HCECs.

L-Ascorbic acid (Asc) has been used as a supplement for culture of various cell types including HCECs,[11,13] but its use is limited by its rapid oxidation.[18] L-Ascorbic acid 2-phosphate (Asc-2P) is an oxidation-resistant derivative of ascorbic acid that is known to be more stable and stimulates the growth of various cells more effectively than Asc.[19–22] Asc-2P has also been shown to extend the replicative lifespan of human vascular endothelial cells[23] and keratinocytes.[24]

In the present study, we investigated the effects of various mitogens, ECMs, and Asc-2P on HCECs growth. We found that a combination of Asc-2P and bFGF with atelocollagen as the ECM was successful for primary culture and subculture of HCECs.

Materials and Methods

Corneas were obtained from Sight Life and the Rocky Mountain Lions' Eye Bank. Low-glucose Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), human insulin, anti–zonula occludens-1 (ZO-1) antibody, and Alexa-Fluor488-labeled anti-mouse, anti-goat, and anti-rabbit antibodies were obtained from Invitrogen (Eugene, OR); collagenase A, chondroitin sulfate, mouse or rabbit normal IgG, bovine serum albumin (BSA), nuclease P1, and trypsin/EDTA were from Sigma-Aldrich (St. Louis, MO); human basic fibroblast growth factor (bFGF), Asc, Asc-2P, proteinase K, and paraformaldehyde were from Wako (Osaka, Japan); human epidermal growth factor (EGF) was from BD Biosciences (Bedford, MA); donkey serum (ab7475), antibodies to N-cadherin (ab18203), and connexin 43 (ab11370) were from Abcam (Tokyo, Japan); and antibodies to Na^+/K^+-ATPase and anti-8-OHdG were from Millipore (Temecula, CA).

From the [1]Corneal Regeneration Research Team, Foundation for Biomedical Research and Innovation, Kobe, Japan; the [2]Department of Ophthalmology, Juntendo University School of Medicine, Tokyo, Japan; and the [3]Corneal Transplantation Section, University of Tokyo Graduate School of Medicine, Tokyo, Japan.

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*Each of the following is a corresponding author: Nobuyuki Shima, Corneal Regeneration Research Team, Foundation for Biomedical Research and Innovation, TRI307,1-5-4, Minatojima-Minamimachi, Kobe, 650-0047, Japan; n-shima@fbri.org.

Satoru Yamagami, Corneal Transplantation Section, University of Tokyo Graduate School of Medicine, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655; syamagami-tky@umin.ac.jp.

Optimization of HCEC Culture Conditions

Primary culture of HCECs using BCECM was performed, as described elsewhere.[17] Studies were conducted in accordance with the Declaration of Helsinki. HCECs from passages 3 to 4 were seeded at 500 cells/cm^2 in 12-well plates coated with bovine atelocollagen (50 μg/mL; Koken, Tokyo, Japan) in a basal medium (DMEM supplemented with 15% FCS and antibiotics), with or without the following additives: Asc-2P (0.1–1.5 mM), Asc (0.1–1.5 mM), bFGF (2 ng/mL), EGF (5 ng/mL), and insulin (20 μg/mL). The cells were cultured in a 5% CO_2 incubator at 37°C for 1 to 2 weeks, with a medium change every other day. After digestion with 0.05% trypsin/EDTA, the cells were counted (Coulter counter; Beckman-Coulter, Hialeah, FL). All experiments were performed in triplicate using cells from at least three different donors.

Alternative Method for Primary Culture of HCECs

HCECs from 18 donors ranging in age from 12 to 74 years (Table 1) were subjected to primary culture as follows. Cells (together with Descemet's membrane) were stripped off with fine forceps and cut into small pieces, as described elsewhere[17] and then were digested at 37°C for 3 hours in basal medium with 2 mg/mL collagenase A. Next, the cells were washed by centrifugation, incubated with 0.05% trypsin/EDTA for 5 minutes at 37°C, washed, and cultured on atelocollagen-coated dishes in basal medium with bFGF in the presence or absence of Asc-2P (0.3 mM). Primary culture of HCECs was also performed after seeding on BCECM in basal medium containing bFGF. Cells were cultured for 2 to 3 weeks with medium exchange every 2 to 3 days. The primary cultures were trypsinized, and the cells were reseeded at 500 cells/cm^2 in atelocollagen-coated six-well plates with the above-mentioned medium and were cultured for 2 weeks. Passaging was repeated several times. The population-doubling level (PDL) was calculated by using the following equation: PDL = log($\times$1/$\times$0)/log 2, where $\times$0 is the initial cell number and $\times$1 is the achieved cell number.

Immunohistochemistry

For ZO-1, N-cadherin, connexin 43, and Na^+/K^+-ATPase staining, HCECs from passages 3 to 4 were seeded at 4000 cells/mm^2 on atelocollagen inserts (Koken) in basal medium containing Asc-2P and bFGF and were cultured for 1 week at confluent culture condition. For 8-OHdG staining, HCECs were subcultured five times with or without Asc-2P and were seeded at 4000 cells/cm^2 in atelocollagen-coated six-well plates and cultured with or without Asc-2P. Then the cells were fixed in phosphate-buffered saline (PBS) with 4% paraformaldehyde for 10 minutes at room temperature for ZO-1, N-cadherin, and connexin 43 staining. For Na^+/K^+-ATPase and 8-OHdG staining, the cells were fixed in cold methanol for 10 minutes. For 8-OHdG staining, the cells were incubated for 30 minutes with 10 μg/mL proteinase K in PBS at room temperature, washed with PBS, blocked for 10 minutes in blocking buffer containing 5% donkey serum in PBS, incubated for 2 hours with goat anti-8-OHdG (1:200), washed, incubated for 2 hours with anti-goat IgG (1:200), washed, and mounted in antifade medium (Vectashield; Vector Laboratories Inc, Burlingame, CA). For immunostaining of the other targets, the cells were washed with PBS containing 0.15% Triton X-100 (Rohm & Haas, Philadelphia, PA) and then blocked for 30 minutes in blocking buffer containing 3% BSA and 0.3% Triton X-100 in PBS. Primary and secondary antibodies were diluted with the blocking buffer. Then, the primary antibody (anti-ZO-1 1:50, anti-N-cadherin 1:120, anti-connexin 43 1:40, anti- Na^+/K^+-ATPase 1:200) was incubated with the cells for 1 hour. After the cells were washed, the secondary antibody (1:200) was incubated with them for 2 hours, after which the cells were washed and mounted. Negative controls were prepared by using nonimmune IgG of the same species, subtype, and concentration. The cells were observed under an inverted fluorescence microscope equipped with an epifluorescence attachment (Eclipse TS100; Nikon, Tokyo, Japan). All staining was done in triplicate and was performed on cells of at least three different donors.

Table 1. Donor Information

Age (y)	Preservation (*n* Days)	HCECs (*n* OS/OD)
12	7	3333/3389
14	8	3378/3322
44	6	1838/2506
46	8	2202
48	6	2159
49	9	2544/2493
53	6	N/A
55	9	N/A
60	9	2900
61	6	956/2688
62	7	2136
62	7	3225/2976
66	7	N/A
67	6	N/A
68	8	2100
69	5	1545/1364
69	8	2132/2645
74	6	N/A

N/A, not available; OS, left eye; OD, right eye.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

HCECs from passages 3 to 4 were seeded at 4000 cells/mm^2 on atelocollagen inserts in basal medium containing Asc-2P and bFGF. Total RNA was extracted from 1-week cultured HCECs and donor corneal endothelium, together with Descemet's membrane (RNeasy Mini Kit; Qiagen, Hilden, Germany). First-strand cDNA was synthesized with 50 ng of total RNA and RT-PCR reaction mixture (Clontech, Palo Alto, CA). The cDNA samples were subjected to PCR with specific primers for functional genes of HCECs (Table 2). After an initial denaturation step of 2 minutes at 94°C, amplification was performed for 35 cycles at 94°C, 59°C, and 72°C for 30 seconds each. Amplified products were transferred on 3% agarose gels for electrophoresis followed by ethidium bromide staining.

Measurement of 8-OHdG Level

HCECs were subcultured six times with or without Asc-2P and were seeded at 10,000 cells/cm^2 in atelocollagen-coated 10-cm dishes and cultured with or without Asc-2P for a week. Mitochondrial DNA was isolated (mtDNA Extractor CT kit; Wako, Osaka, Japan), as described by the manufacturer. DNA samples were digested with 200 mM sodium acetate and 6 U nuclease P1 at 37°C for 30 minutes, followed by the addition of 1 M Tris-HCl buffer containing 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 (Vivaspin 500; Sartorius Stedim, Japan, Tokyo, Japan) at 15,000*g* for 20 minutes. A competitive ELISA for 8-OHdG was performed according to the manufacturer's protocol (Nikken Seil Co. Shizuoka, Japan). Briefly, 8-OHdG antibody and sample DNA were added to a 96-well plate precoated with 8-OHdG antibody and incubated overnight at 4°C. After the plates were washed, the secondary antibody was added and incubated for 1 hour at room temperature. After another wash, 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 at 450 nm was measured. Assays were performed in triplicate. The average concentration of 8-OHdG, normalized per microgram of mitochondrial DNA, was calculated for each sample based on the standard curve ranged from 0.125 to 10 ng/mL.

TABLE 2. Oligonucleotide Sequences Used in RT-PCR Study

Gene	Accession No.	Primer Sequence (5′-3′)	Size (bp)
SLC4A4	NM_003759	F: CCAAGAAATCCAACCTTCGG R: GTCATTCAGACTGGAGGAAG	134
AQP1	BC022486.1	F: GTCCAGGACAACGTGAAGGT R: GAGGAGGTGATGCCTGAGAG	218
VDAC2	NM_003375	F: CAGTGCCAAATCAAAGCTGA R: TTGCAGAAATGGAAGCAGTG	233
VDAC3	NM_005662	F: CAGACCCTTCGACCAGGAGT R: TTCGCAACCCCTAGACTTCAG	268
CLCN2	NM_004366	F: TCCTCACCCTGGTCATCTTC R: GCAGGTAGGGCAGTTTCTTG	351
CLCN3	NM_173872	F: GCCTTAGTGCGTTGTGGTA R: CAGCTGATAGCACCTCCCTT	468
GAPDH	NM_002046	F: GCACCGTCAAGGCTGAGAAC R: TGGTGAAGACGCCAGTGGA	138

SLC4A4, sodium bicarbonate cotransporter member 4; *AQP1*, aquaporin 1; *VDAC2* and *VDAC3*, voltage-dependent anion channels; *CLCN2* and *CLCN3*, chloride channel proteins; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase

Statistical Analysis

Data were expressed as the mean ± SD. Statistical comparisons of two groups were performed with the unpaired Student's *t*-test. Multiple comparisons among groups were made with the Tukey-Kramer test.

RESULTS

Optimization of HCEC Culture Condition and Effect of Asc-2P

To determine the optimum conditions for subculture of HCECs, we used cells from passages 3 and 4 after conventional primary culture with BCECM as the matrix. As a result of these experiments, we selected DME with 15% FCS as the basal medium, because our attempts to use low-serum or serum-free medium were unsuccessful (data not shown). We selected atelocollagen (nonimmunogenic telopeptide-free collagen) as the coating matrix, because cultures with BCECMs, a fibronectin-collagen coating mix (FNC Coating Mix; Athena ES, Baltimore, MD), and atelocollagen all achieved comparable maximum growth, which was better than in cultures with type I or type IV collagen, a solubilized basement membrane matrix (Matrigel; BD Biosciences, San Diego, CA), and a chondroitin/laminin mixture (data not shown), and because of the potential risk of pathogenic contamination associated with use of BCECM and FNC coating mix.

Addition of Asc-2P to the basal medium significantly stimulated cell growth, whereas addition of Asc achieved only weak growth stimulation (Figs. 1A, 1B). The most effective dose of Asc-2P was slightly different for cells from each donor and ranged from 0.06 to 0.3 mM, but its growth-promoting effect was sustained until at least 1.5 mM (data not shown).

We selected bFGF as a growth supplement for the basal medium of HCECs, as described previously,[5,7,11,13,17] because bFGF showed the most potent growth-promoting effect in comparison with other growth factors such as hepatocyte growth factor (HGF), EGF, platelet-derived growth factor (PDGF), and insulin (data not shown). A combination of Asc-2P and bFGF led to significantly increased cell growth, but supplementation with other growth factors such as EGF and/or insulin did not have any additional effect (Fig. 1C). Accordingly, we selected the combination of Asc-2P and bFGF with atelocollagen as the matrix for optimum subculture of HCECs.

Effect of Asc-2P on Primary Cultures of HCECs

Next, we tested whether our novel method was appropriate for primary culture of HCECs. Cells from 18 donors (Table 1) were cultured in DMEM containing FCS and bFGF on atelocollagen-coated plates, with or without Asc-2P. Cultures without Asc-2P usually showed poor growth or no proliferation, and only 2 of 18 cultures reached confluence, whereas addition of Asc-2P strongly promoted the proliferation of HCECs in all primary cultures, and confluence was usually reached within 2 to 3 weeks (Fig. 2A). As shown in Figure 2B, cells from a 12-year-old donor grew rapidly in both the presence and absence of Asc-2P, reaching confluence after 10 days. On the other hand, cells from a 53-year-old donor grew well only in the presence of Asc-2P, and no proliferation was observed in its

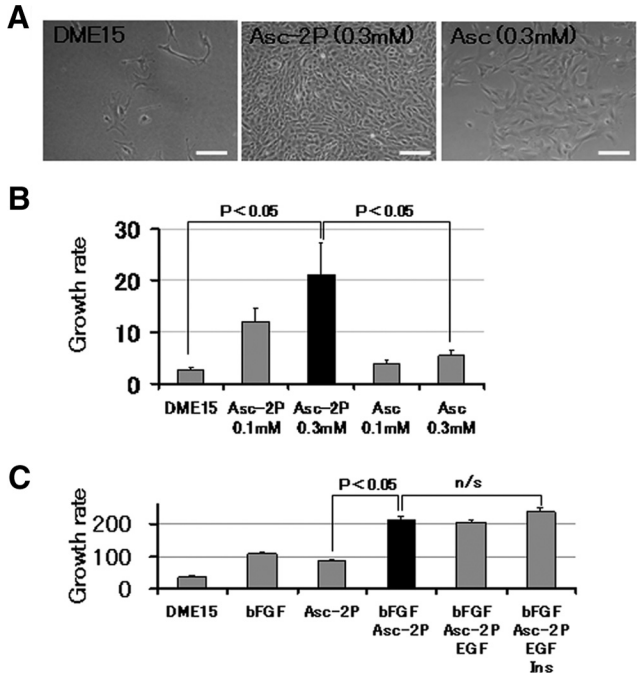

FIGURE 1. Effect of Asc-2P on growth of HCECs and optimization of growth culture conditions. (**A**) HCECs cultured in DMEM with 15% FCS (DME15) and DME15 with Asc-2P (0.3 mM) or ascorbic acid (Asc, 0.3 mM). Scale bar, 100 μm. (**B**) Effect of Asc-2P on the growth of HCECs. Asc-2P stimulated cell growth more effectively than Asc. (**C**) Combined effect of several growth factors on HCECs. A combination of Asc-2P and bFGF significantly increased cell growth but addition of insulin (Ins) and epidermal growth factor (EGF) did not further augment growth. Data are represented as mean ± SD of triplicate determinations.

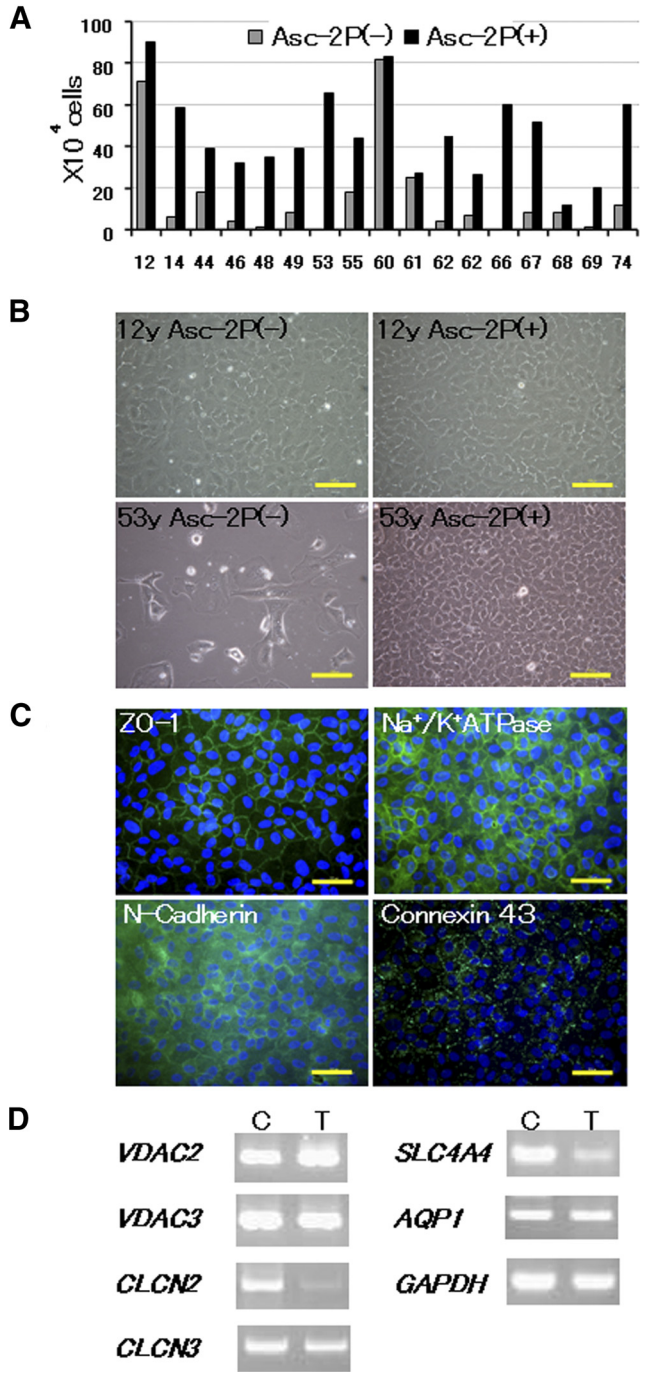

Figure 2. Effect of Asc-2P on primary cultured HCECs. (**A**) Expanded number of cells in primary culture. HCECs from 18 donors were cultured with or without Asc-2P. Cells in cultures without Asc-2P tended to grow poorly, whereas addition of Asc-2P markedly increased the proliferation of HCECs in all the primary cultures. (**B**) Representative phase-contrast images of primary cultures from donors of different ages. Cells from a 12-year-old donor grew in both the presence and absence of Asc-2P, but cells from a 53-year-old donor grew well only in cultures with Asc-2P. Confluent culture with Asc-2P from the 53-year-old donor shows a similar cell size and homogeneity of morphology to the culture from the 12-year-old donor. (**C**) Representative images of ZO-1, Na^+/K^+-ATPase, N-cadherin, and connexin 43 immunostaining of a confluent monolayer of third-passage HCECs from a 62- year-old donor. Proteins are clearly localized at plasma membranes of cells outlined with a hexagonal shape. (**D**) Expression analysis of voltage-dependent anion channels (*VDAC2* and *VDAC3*), chloride channel proteins (*CLCN2* and *CLCN3*), sodium bicarbonate cotransporter member 4 (*SLC4A4*), and aquaporin 1 (*AQP1*) by RT-PCR. Cultured HCECs (third passage from a 62-year-old donor) expressed all critical mRNAs for HCEC function. C, cultured HCECs; T, tissue HCECs; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. Scale bar: (**B**) 100 μm, (**C**) 50 μm.

absence. When the passage 3 cells were cultured on atelocollagen sheets for 1 week, Na^+/K^+-ATPase and intercellular junction proteins, such as tight junction (ZO-1), adherent junction (N-cadherin), and gap junction (connexin 43), were localized at plasma membranes of cells with a hexagonal shape, which was very similar to in vivo HCECs (Fig. 2C). Connexin 43 showed a discontinuous ring, consistent with the previous findings.[14] The mRNA of voltage-dependent anion channels (*VDAC2* and *VDAC3*), chloride channel proteins (*CLCN2* and *CLCN3*), sodium bicarbonate cotransporter member 4 (*SLC4A4*), and aquaporin 1 (*AQP1*) were expressed in both the cultured HCECs (passage 3) and tissue HCECs (Fig. 2D). These results imply that cultured HCECs derived from our culture method can produce essential proteins to maintain intact barrier and ionic pump functions.

Effect of Asc-2P on the Lifespan of HCECs

To test whether primary cultures of HCECs with Asc-2P (Fig. 3A, left) could be successfully passaged to proliferate efficiently in subsequent subculture, we serially subcultured primary HCECs, with or without Asc-2P. During multiple passages, the cultures without Asc-2P showed a decline in growth rate with every passage (Fig. 3A, right). The cells became irregular in shape and eventually were found to be large and flat (typical of senescent cells) after six passages (Fig. 3B). In contrast, cultures with Asc-2P maintained a stable cell growth rate after six passages (Fig. 3A, right), and the proliferating cells kept their characteristic polygonal shape (Fig. 3B). Cells cultured without Asc-2P underwent senescence at PDL 27.3, whereas cells cultured with Asc-2P did not show senescence over PDL 40 (Fig. 3C). An increase in the lifespan of cells cultured with Asc-2P was observed in all subcultures obtained from 15 different donors (data not shown).

Primary cultures without Asc-2P were serially subcultured in the absence of Asc-2P, and passage 2 HCECs were obtained under these conditions. Then, we evaluated the cell growth rate from passages 3 to 7, when cells were cultured with or without Asc-2P. As can be seen in Figure 3D, cells cultured without Asc-2P showed a decrease in growth after each passage, whereas culture with Asc-2P stimulated the cell growth for several passages. These findings excluded the possibility of selective expansion of rapidly growing or long-lived cells during primary culture with Asc-2P and suggested that Asc-2P prolongs the replicative lifespan of cultured HCECs.

To examine whether the combination of Asc-2P and atelocollagen provided an alternative to the previous method of primary culture using BCECM, we compared the replicative lifespan of cells grown by the two methods. Cell growth in the primary cultures was higher in BCECM, but serial subcultures showed that the cells cultured in Asc-2P plus atelocollagen grew better than those cultured in BCECM (Fig. 3E). Similar findings were obtained with cells from two other donors (data not shown).

Effect of Asc-2P on 8-OHdG Production

To investigate the effect of Asc-2P on oxidative stress, we compared 8-OHdG levels, when HCECs were cultured with or without Asc-2P. HCECs passaged five times without Asc-2P had an irregular morphology and prominent 8-OHdG staining

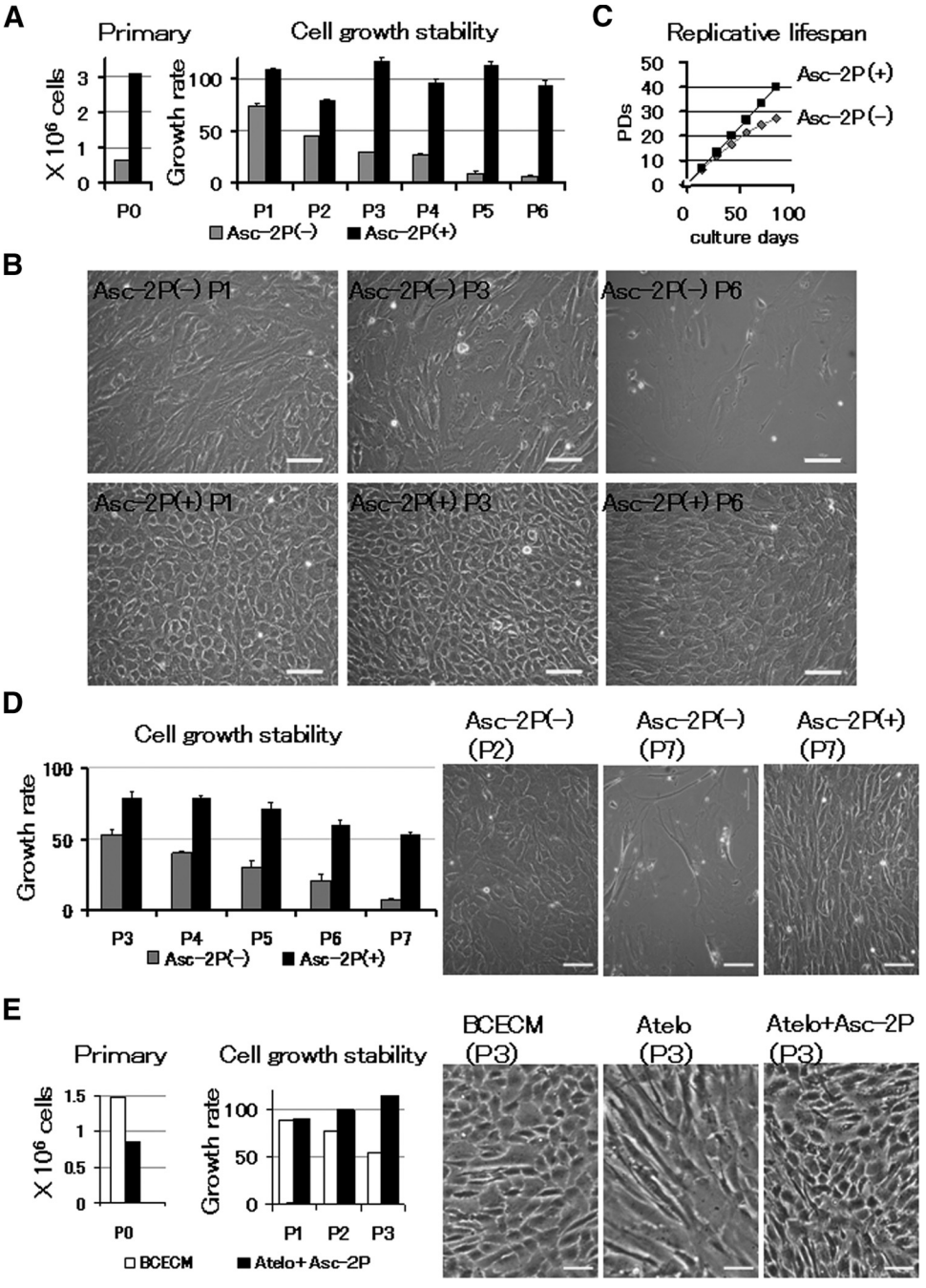

FIGURE 3. Effect of Asc-2P on the growth stability and lifespan of HCECs. (**A**) Effects of Asc-2P on growth. HCECs from a 67-year-old donor were subjected to primary culture and serial subculture, with or without Asc-2P. In cultures without Asc-2P, the growth rate decreased and cells had irregular shapes. Asc-2P supported cell growth at a higher rate, and the cells maintained their characteristic polygonal morphology. (**B**) Representative phase-contrast images of subcultured cells. In cultures without Asc-2P, senescent-like large and flat cells are seen after 6 passages, while cells cultured with Asc-2P maintain their characteristic polygonal morphology. (**C**) Effect of Asc-2P on the replicative lifespan. Cultures without Asc-2P show senescence at PDL 27.3, but cultures with Asc-2P do not show senescence over PDL 40. (**D**) Effect of Asc-2P on subcultured cells. Cells from a 14-year-old donor were cultured without Asc-2P and then subcultured with or without Asc-2P. In cultures without Asc-2P, cell growth decline and the cells showed irregular shapes. Asc-2P supported growth, and cells maintained their characteristic morphology. (**E**) Comparison of HCEC lifespan between the conventional method using ECM from BCECM and our new method. HCECs from a 60-year-old donor were cultured (primary and subculture) with BCECM and on atelocollagen with Asc-2P (Atelo+Asc-2P). In serial subcultures, the combination of Asc-2P and atelocollagen achieved better growth than BCECM. Scale bar, 100 μm. P, passage.

(Fig. 4A). In contrast, HCECs passaged five times with Asc-2P maintained a polygonal shape and showed weak 8-OHdG staining. Similar findings were obtained with cells from four other donors (data not shown). When 8-OHdG levels in mitochondrial DNA were measured by an ELISA, the levels were significantly higher in HCECs cultured without Asc-2P than those in cells cultured with Asc-2P (Fig. 4B). These results indicate that Asc-2P attenuated the production of 8-OHdG during culture in the ambient oxygen environment (21% O_2).

DISCUSSION

Some laboratories have developed successful primary culture techniques for HCECs from elderly donors, even though culturing HCECs is more difficult when the donor is older.[13,17] However, these techniques require various growth-promoting agents and/or ECMs.[11–17,25] In contrast to these techniques, we developed a simple culture method using Asc-2P. We found that the addition of Asc-2P to primary culture markedly stimulated the proliferation of HCECs from donors over a wide range of ages, including elderly donors (74 years old), whereas most attempts at primary culture from elderly donors were unsuccessful in the absence of Asc-2P (Fig. 2). The decreased proliferation of HCECs from elderly donors is considered to be due to poor responsiveness of their cells to growth-promoting agents,[13] so the addition of various mitogen cocktails is thought to be effective for culturing HCECs from such donors. In contrast to such mitogens, the growth-promoting effect of Asc-2P was independent of donor age.

At present, we cannot explain the exact mechanisms by which Asc-2P promotes cell growth, but the cell growth seems to be mediated through the scavenging of reactive oxygen

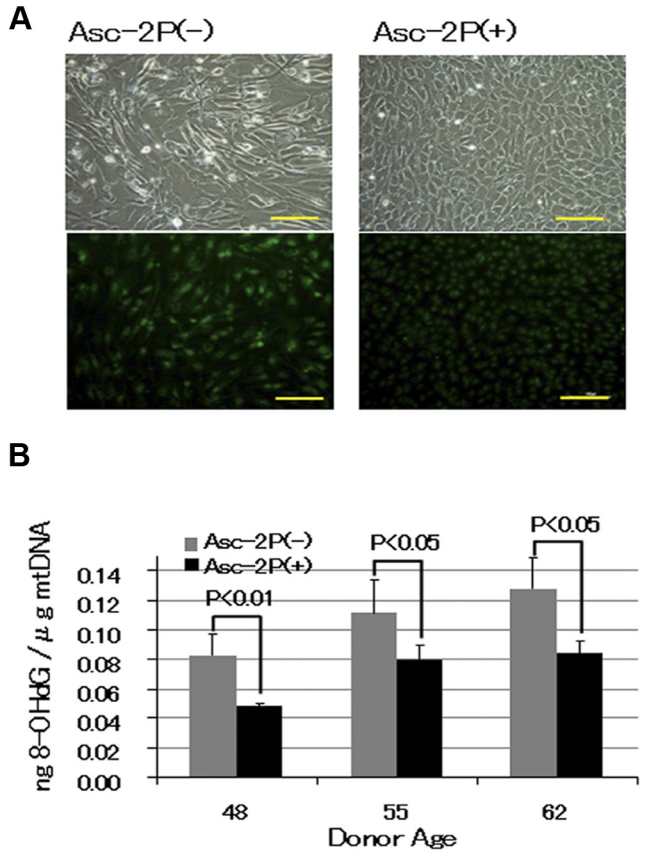

FIGURE 4. Effect of Asc-2P on 8-OHdG production. (**A**) Representative images of 8-OHdG staining. HCECs from a 62-year-old donor were serially subcultured five times, with or without Asc-2P and then immunostained for 8-OHdG. Cells cultured without Asc-2P had irregular shapes and prominent 8-OHdG staining, but cells cultured with Asc-2P had a polygonal morphology and weak 8-OHdG staining. Scale bar, 100 μm. (**B**) Quantification of mitochondrial 8-OHdG. HCECs from 48-, 55-, and 62-year-old donors were serially subcultured six times, with or without Asc-2P, and the level of 8-OHdG in mitochondrial DNA was quantified by an ELISA. The levels showed a significant decrease when cells were subcultured with Asc-2P. Data are represented as the mean ± SD of triplicate determinations. mtDNA; mitochondrial DNA.

species (ROS) and regulation of the synthesis of proteins related to cell growth. Exposure of cells to subcytotoxic levels of H_2O_2 has been shown to push these cells into a nonproliferative and quiescent stage, a phenomenon known as stress-induced premature senescence (SIPS).[26,27] Treatment of cultured HCECs with H_2O_2 also causes a dose-dependent decrease in cell proliferation.[28] Conversely, hypoxia stimulates the growth of various cells,[29,30] along with a decrease of intracellular ROS,[31] and decreased expression of a negative cell cycle regulator, p21 Cip1.[32] Taken together with our data that Asc-2P potently diminished intracellular ROS generation (Shima N, unpublished observation, 2009), Asc-2P may promote HCEC growth by reducing intracellular oxidative stress. Because ROS can act as subcellular messengers for signal transduction pathways,[33] it is possible to hypothesize that antioxidants upregulate the production of proteins related to cell growth. Asc-2P is a co-factor for collagen synthesis and is known to promote both growth and collagen synthesis by various mesenchymal cells, such as fibroblasts,[19] osteoblasts,[20,21] and mesenchymal stem cells.[22] It has been demonstrated that Asc-2P promotes cell growth through stimulation of collagen synthesis, because its growth-promoting effect is abolished by treating cells with collagen synthesis inhibitors[19] or SiRNAs for collagen types I and III.[21] Considering that a collagen coating stimulates HCECs growth,[25] these findings suggest involvement of collagen synthesis in the growth-promoting effect of Asc-2P. Further experiments are in progress to determine the mechanisms of the proliferative effect of Asc-2P on HCECs.

We showed that Asc-2P significantly induced the growth of HCECs, whereas Asc did not. Engelmann and Friedl[11] reported that Asc enhances the growth of HCECs in a dose-dependent manner up to a concentration of 25 μg/mL (75 μM), whereas a further increase of the concentration inhibits cell growth. We also confirmed that a high concentration of Asc (1 mM) had a strong cytotoxic effect (Shima N, unpublished observation, 2009). Millimolar concentrations of Asc are known to induce the apoptotic death of various types of cells.[34] Unlike the situation in vivo, Asc is rapidly oxidized in cultures with ambient oxygen (21% O_2) and produces various ROS, mainly H_2O_2. Therefore, addition of a high concentration of Asc to HCEC culture is likely to cause the production of cytotoxic levels of H_2O_2. In contrast, it has been shown that Asc-2P is resistant to auto-oxidation in culture medium, does not produce ascorbyl radicals, and does not induce apoptosis of a human cell line.[34] Asc-2P is thought to be dephosphorylated to form Asc by alkaline phosphatase on the cell membrane.[20] Taken together, the strong growth-promoting effect of Asc-2P at high concentrations (0.3–1.5 mM) could be explained by effective internalization of a high dose of Asc by cells without the production of cytotoxic ascorbyl radicals. Interestingly, the normal concentration of Asc in the human anterior chamber is approximately 500 μM,[35] which is much higher than the plasma concentrations (9–27 μM),[36] suggesting that a high concentration of Asc may have an important influence on HCECs not only in vitro but also in vivo.

We found that addition of Asc-2P not only stimulated the growth of HCECs, but also extended their replicative lifespan. HCECs cultured with Asc-2P showed a normal polygonal morphology and were not senescent-like, suggesting that Asc-2P delays the onset of senescence, leading to an increase of PDL. The 8-OHdG level was higher in senescent-like HCECs cultured without Asc-2P than in cells cultured with Asc-2P, implying that the senescence of HCECs is associated with an increase in oxidative DNA damage. When DNA is damaged, cells attempt to repair it by arresting the cell growth cycles at a specific checkpoints.[37] These repair systems, however, tend to fail with age, and accumulation of DNA damage, especially mitochondrial DNA mutations, is thought to be a major contributor to ageing.[38] Significantly higher levels of 8-OHdG are found in both ex vivo and in vitro expanded HCECs from elderly donors compared with cells from young donors.[32] Taken together, the extension of HCEC longevity by Asc-2P may be explained in part by a protective effect against oxidative DNA damage.

The combination of Asc-2P and bFGF with an atelocollagen coating was found to be a successful culture method for HCECs that was superior to the conventional method using BCECM.[17] BCECM is derived from bovine eyes and regulatory agencies prohibit its use for pharmaceutical manufacturing because of a potential risk of bovine spongiform encephalopathy (BSE). In contrast, bovine atelocollagen is derived from the dermis and is not prohibited for use in pharmaceutical manufacturing. In fact, it has been extensively used for pharmaceutical and medical purposes. We tried to develop an FCS-free culture method, but were unsuccessful. Thus, establishment of an FCS-free culture method is an important challenge from the safety perspective, and the use of autologous human serum may be an alternative, although clinical grade FCS is commercially available from companies such as Invitrogen. Further experiments are needed to establish a safe and effective culture procedure for future clinical application.

In summary, Asc-2P increased the proliferation and replicative lifespan of HCECs from donors of a wide range of ages. The 8-OHdG level in mitochondrial DNA showed a significant decrease when cells were subcultured with Asc-2P. Our finding suggests that Asc-2P extends the lifespan of cultured HCECs, partly by protecting the cells from oxidative DNA damage.

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