hTERT Extends Proliferative Lifespan and Prevents Oxidative Stress-Induced Apoptosis in Human Lens Epithelial Cells

Xiao-Qin Huang,1,2 Juan Wang,2,5,4 Jin-Ping Liu,2,5 Hao Feng,1 Wen-Bin Liu,1 Qin Yan,1 Yan Liu,1 Shu-Ming Sun,1 Mi Deng,1 Lili Gong,1 Yun Liu,1 and David Wan-Cheng Li1,3,5

PURPOSE. Telomerase is a specialized polymerase that catalyzes synthesis of telomeres in most eukaryotes. When introduced into somatic cells, it extends the proliferative lifespan and prevents replicative senescence. Whether it has similar functions in lens epithelial cells, especially in human lens epithelial cells (HLECs) remains to be determined. In this study, the human telomerase reverse transcriptase (hTERT) catalytic subunit was introduced into HLECs. A stable cell line expressing hTERT was established and the functions of hTERT were studied.

METHODS. The telomeric repeat amplification protocol (TRAP) assay was used to analyze the telomerase activity. Western blot analysis was used to examine hTERT expression. Southern blot analysis was used to detect telomere length. HLECs isolated from intact lenses were cultured in DMEM and transfected with hTERT cDNA. The expression of the exogenous hTERT was examined with RT-PCR, Western blot analysis, and TRAP assay. The functions of hTERT were examined with various techniques.

RESULTS. Among the human, bovine, and rabbit lenses examined, only the central epithelium from the 6-month rabbit lens displayed telomerase activity. In both transparent and cataractous human lenses, hTERT activity and expression were not detected. However, the template RNA was present in both types of human lenses. The telomeres in transparent lenses were approximately 1 kb longer than those in cataractous lenses. The primary cultures and later passages of HLECs also displayed no detectable telomerase activity. Introduction of hTERT cDNA into HLECs followed by G418 selection yielded a stable line of HLECs expressing hTERT. In this line, hTERT has supported normal growth after 48 population doublings (PDs) to date and also enhanced antiapoptotic activity against oxidative stress.

CONCLUSIONS. Telomere lengths may be associated with cataractogenesis. hTERT introduced into HLECs prevents replicative senescence through telomere synthesis. Furthermore, hTERT displays functions beyond telomere synthesis in normal HLECs. (Invest Ophthal mol Vis Sci. 2005;46:2503–2513) DOI:10.1167/ iovs.05-0154

Telomeres are specialized structures present at the ends of eukaryotic chromosomes, consisting of tandem arrays of highly conserved hexameric (TTAGGG)n repeats in vertebrates.1,2 They have been implicated in stabilizing linear chromosomes from exonuclease degradation and chromosome-to-chromosome fusions. Moreover, they are actively involved in preventing other forms of aberrant recombination and the attachment of chromosomes to the nuclear matrix. They also act as a “mitotic clock” in determining the maximum replicative capacity of human somatic cells.1,2 Different from normal DNA replication, telomere replication is mainly executed by a ribonucleoprotein enzyme complex named telomerase that contains a catalytic protein subunit and a polymerase template RNA.3–5 In the presence of the cognate template RNA, telomerase adds telomeric sequences onto chromosome ends, providing a mechanism to balance the loss of repeats from chromosome ends during cell division.1,2 In the absence of telomere synthesis, telomeres shorten with each cell division.6 In humans, the catalytic subunit of telomerase (hTERT) is a 127-kDa protein.7–10 The encoding sequence for this catalytic subunit has been cloned in humans and other species.11 Apparently, telomerase from these different sources shares both sequence and functional similarity with the reverse transcriptase.11 Recently, the gene encoding the telomerase RNA has been cloned in 32 different species, and the topological structures of these different telomerase RNAs are well conserved.12,13 To study its functions, hTERT has been overexpressed in a variety of human cell lines, including human retinal epithelial cells (RPE-340) and foreskin fibroblasts,14 human Werner syndrome fibroblasts,15 the immortal cell lines A431 and 29,16 fibroblasts,17 and others. The expression of hTERT in these cell lines displays distinct functions. For example, introduction of hTERT into telomerase-negative normal human RPE cells and foreskin fibroblasts enables these cells to obtain elongated telomeres and extended their lifespan.14 Whether hTERT has similar functions in normal lens epithelial cells, especially in normal human lens epithelial cells (HLECs) remains to be studied.

To explore the functions of hTERT in lens epithelial cells, we have previously introduced the hTERT cDNA into two immortalized lens epithelial cell lines: the naturally immortalized rabbit lens epithelial cells, N/N1003A18 and the viral gene, SV-40 large-T transfected HLECs.19 Our studies with these hTERT-transfected lens epithelial cells have led to two impor-
TABLE 1. Human Eye Lenses Used for Primary Cultures, Analysis of Telomerase Activity, hTERT and hTR Expression, and Telomere Length

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* These materials isolated for primary cultures were used for establishment of the hTERT-HLECs line.
† These materials were harvested for extraction of total proteins (25%), total RNAs (25%) and genomic DNAs (50%) at the same time. Each lens capsular epithelium was separated into the central and periphery regions.
‡ These lenses had clear yellow pigment accumulation but no cataract record.
§ These lenses not only showed clear yellow pigment accumulation but also lens opacification.

Table 2. Human Eye Lenses Used for Primary Cultures, Analysis of Telomerase Activity, hTERT and hTR Expression, and Telomere Length

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tant discoveries. First, hTERT is functionally compatible with non-human telomerase template RNA.18 Second, hTERT displays functions that are beyond telomere synthesis. For example, in N/N1003A cells, hTERT modulates the expression of the apoptosis-regulatory genes, p53 and bcl-xS, to attenuate apoptosis induced by camptothecin, the topoisomerase I inhibition but also lens opacification.

cataract record.

RNA Preparation and Reverse Transcription-Linked Polymerase Chain Reaction
Total RNAs were extracted from human lens epithelium or cultured cells (Trizol reagent; Invitrogen, Inc., Carlsbad, CA) as described.16–20 and 2 μg of total RNA was used for reverse transcription in a 20 μL reaction (Superscript II; Invitrogen, Inc.), as described before.16–20 For hTERT mRNA analysis, 2 μL of the reverse transcripts were used for semiquantitative RT-PCR, with β-actin as the internal control in each reaction. The primers used in this study were 5’-GTGGGGCGGCCTGCGAG- GCACCA-3’ (forward) and 5’-CTCTTAAATGCTACGACAGTTT-3’ (reverse) for the β-actin gene; 5’-GCTGATGAGTGTGTACGTCG-3’ (forward) and 5’-CCTCTTCTGCGAAGCTG-3’ (reverse) for the hTERT gene. PCR was run four cycles with hTERT primers before β-actin primers were added for another 30 cycles. For analysis of the human telomerase template RNA, the primers used were 5’-CCACGCTGCTAT- TCTAGAAGCAA-3’ (forward) and 5’-CCGAGAGACCGCGGCTGCA-3’ (reverse). The same PCR procedure was used for human telomerase template RNA (htRT) analysis, except that β-actin primers were not included.

Cell Extract Preparation and Telomerase Activity Assay
Cellular extracts from the lens epithelial and fiber cells of human, bovine, and rabbit lenses; the primary and secondary cultures of HLECs; or the transfected HLECs pCH1HLEC and pCH1H1TERT-HLEC were prepared in 1× CHAPS (3-[3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate) lysis buffer and cleared by centrifugation for 20 minutes at 4°C (12,000g). These extracts were assayed for telomerase activity with a PCR-based telomeric repeat amplification protocol (TRAP)21 using a kit (Intergen, Purchase, NY). Briefly, telomeric repeats were synthesized onto the oligonucleotide, TS (5’-AATTGCGTGAAGCAGGGTTT-5’) in an extension reaction containing each cell extract sample and run at 30°C for 30 minutes. After extension, the products were amplified by PCR with γ[32P]ATP end-labeled TS primer and a downstream TRAP primer mixture (Intergen) for 30 cycles (94°C, 30 seconds, and 59°C, 30 seconds). A 36-base pair internal standard was included for each reaction. At the end of each reaction, 25 μL of the reaction products was analyzed in a 10% polyacrylamide gel.

Southern Blot Analysis for Telomere Length
To determine the length of telomeres, we extracted genomic DNA samples from the pooled lens epithelial and cortical fiber cells of transparent and cataractous human lenses and also from the transfected cell lines pCH1HLEC (after selection) and pCH1H1TERT-HLEC (after 25 population doublings [PDs]). Ten volumes of extraction buffer (10 mM Tris-HCl [pH 8.0], 0.1 M EDTA [pH 8.0], 0.5% SDS, and 20 μg/mL pancreatic RNase) was added to the cell samples, which were either homogenized on ice with an Eppendorf tube micropestle (Brinkman Instruments, Inc., Westbury, NY), for capsular epithelium and cortical fiber cells, or resuspended by gentle pipetting, for parental or transfectected cell lines pCI-HLEC (after selection) and pCI-hTERT-HLEC (after 20 population doublings [PDs]). After digestion with the restriction enzymes Hinfl and RsaI (Promega, sterilized twice in 75% ethanol for 5 minutes and then rinsed in sterile phosphate-buffered saline (PBS). At the limbus, a stab incision was made, and the cornea and the iris were removed. As a result of these procedures, the anterior lens surface was exposed. The anterior lens capsule with adherent lens epithelium was removed by continuous curvilinear capsulorrhexis. The anterior lens capsule was cultured in 60-mm Petri dishes with Dulbecco’s modified Eagle’s minimum essential medium (DMEM) and 20% fetal bovine serum (FBS) in the presence of 50 U/mL penicillin and 20 μg/mL streptomycin.

MATERIALS AND METHODS
Sources of Human, Bovine, and Rabbit Lenses
Human eyeballs with transparent or cataractous lenses were provided by the National Disease Research Interchange (NDRI, Philadelphia, PA) and the Idaho and Oregon eye banks. The basic information on the donors is listed in Table 1. The eyes were obtained and managed in accordance with the guidelines of the Declaration of Helsinki for research involving human tissue. Bovine eyes, 24 months old, were obtained from Bringhurst Meats (Berlin, NJ). The rabbits used in this study were handled in compliance with the Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Six-month and 2-year-old New Zealand White rabbits were purchased from Harlan Sprague-Dawley (Indianapolis, IN).

Culture of HLECs
To establish the primary cultures of HLECs, we obtained eyeballs from donors between 34 and 51 years of age from NDRI. The eyeballs were harvested between 34 and 51 years of age from NDRI. The eyeballs were harvested between 34 and 51 years of age from NDRI.
Madison, WI) at 37°C 4 hours and then precipitated with 2.5 volumes of 100% ethanol in the presence of 20 µg Escherichia coli tRNA as the carrier. The DNA fragments were resolved by 0.8% agarose gel electrophoresis at 100 V for 4 hours. After depurination by soaking in 0.1 M sodium citrate (pH 3.0) for 30 minutes and denaturation with 1.5 M NaCl and 0.5 M NaOH for 20 minutes twice, the DNA fragments were neutralized with 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.0) and then transferred onto a nitrocellulose membrane in 10× SSC (1.5 M NaCl and 0.15M sodium citrate). The membrane was hybridized with a (γ-32P)-ATP-labeled oligonucleotide (TTAGGG)₇ probe at 40°C for 30 hours and washed in moderate-stringency conditions three times. DNA fragments were visualized by autoradiography.

Protein Preparation and Western Blot Analysis

Western blot analysis was conducted as we have described elsewhere.22,25 Total proteins were extracted from capsular epithelium of transparent and cataractous lenses or cultures cells (HLECs, pCHLLECs, and pCHtH-TERT-HLECs), as previously described.22,23 Fifty micrograms of total protein from each sample were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Invitrogen-Gibco, Grand Island, NY). The protein blots were blocked with 5% milk in TBS (10 mM Tris-HCl (pH 8.0) and 150 mM NaCl) overnight at 4°C; and then incubated for 1 hour with anti-hTERT antibody (Calbiochem, La Jolla, CA) and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected with an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) according to the company’s instructions.

Karyotype Analysis

The karyotype analysis was conducted according to Hsieh.24 The pCHtH-TERT-HLECs after 12 PDs were grown to 60% confluence, and then treated with 0.05 µg/mL colcemide (cat. no. 15210-040; Invitrogen) for 60 minutes and harvested for chromosome analysis. GTW Banding was used to characterize chromosomes as described by Hsieh.

Cell Growth Assay

To determine the cell growth rate, we seeded an equal number of cells from different cell lines (specified in the figure legends) in six-well plates at day 0. The media were replaced every 2 days, and the cells in each well were collected by trypsinization and counted in triplicate with a hemocytometer, at an interval of 24 hours for 5 days. At the same time, trypan blue staining was used to monitor cell death in the parental HLECs, and the vector- and hTERT-transfected cells.

Senescence-Associated β-Gal Assay

The senescence-associated β-galactosidase (SA-β-Gal) assay27 was used to determine cellular senescence. Senescent cells express a higher level of lysozymal β-Gal activity, which is detectable in the presence of X-Gal at pH 6.0 (on cell cleavage, a local blue precipitate formed).25 Briefly, 3 × 10⁶ cells of pCHLLECs and pCHtH-TERT-HLECs were plated in slide culture chambers and allowed a total of 48 hours’ growth. The media were then removed, and the cells in each chamber were washed with PBS twice, followed by fixation with 3% formaldehyde for 5 minutes. After fixation, the cells in each chamber were washed with PBS twice and then incubated for 12 hours with fresh SA-β-Gal staining solution (1 mg/mL X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, in PBS).25 For quantitative analysis of the number and flat size of the senescent cells, six fields in three slide chambers were observed. The recorded results are shown in Table 2.3

Analysis of H₂O₂ Degradation

Parental HLECs at 12 PDs, pCHLLECs after selection, and pCHtH-TERT-HLECs at 25 PDs were grown in DMEM containing 20% fetal calf serum without (for parental HLE) or with (for transfected cells) 400 µg/mL G418, and 3 × 10⁵ cells were plated into a 60-mm culture dish. After 12 hours of cell growth, the media in the different cultures were replaced with 10 mL serum-free MEM containing 150 µM H₂O₂ (the concentration at the starting point). The treatment was continued for 0 to 24 hours, and the H₂O₂ level in the medium of different cultures was determined at 0, 30, 60, and 90 minutes by using a chemical method as previously described.26,27 The results were the average (±SD) of three independent experiments.

Analysis of Induced Apoptosis

Parental HLECs after 12 PDs, pCHLLECs after selection, and pCH-tH-TERT-HLECs at 25 PDs were grown in DMEM containing 20% fetal calf serum without (for parental HLECs) or with (for transfected cells) 400 µg/mL G418, and 3 × 10⁵ cells were plated into a 60-mm culture dish. After the cells grew for 12 hours, the media in the different cultures were replaced with 10 mL serum-free MEM containing 150 µM H₂O₂ (the concentration at the starting point) for 0 to 24 hours. The percentage of apoptotic cells and the apoptotic nature of cell death were determined as previously described.29–31 The quantitative results of apoptosis are the average (±SD) of three independent experiments.

Assay of Caspase-3 Activity

The caspase-3 activity in various cell lines after treatment by H₂O₂ was assayed as previously described.29–31 The final results are the average (±SD) from three independent experiments.

RESULTS

Telomerase Activity Is Present Only in the Central Epithelium of the 6-Month-Old Rabbit Lens

To determine telomerase activity in the vertebrate lenses, we examined eyes of the bovine and rabbit. The lens epithelium and the cortical fiber cells were dissected from the lenses. The lens epithelia were further divided into the central epithelium (the inner circle, a half diameter in size, containing the epithelial cells in the quiescent G₀ stage) and the peripheral epithelium (the outer circle, also a half diameter in size, consisting of the germinal zone and the equatorial region). The telomerase...
activities in these different samples were examined with the PCR-based TRAP assay. As shown in Figure 1A, no telomerase activity was found in the central (CE) or peripheral (PE) epithelium or in the cortical fiber cells (F) of the 24-month-old bovine lenses. When similar compartments of tissue samples from rabbit lenses of 6- and 24-month-old animals were examined for telomerase activity, only the central epithelium from the 6-month-old rabbit lens displayed telomerase activity (Fig. 1B). No telomerase activity was found in the remaining lens tissues (Fig. 1B).

Telomerase Expression and Activity in Transparent and Cataractous Human Lenses

To determine the telomerase activity in both transparent and cataractous human lenses, we examined the lens epithelium in the central and peripheral regions and also the cortical fiber cells from these lenses. Examination of telomerase activity at each compartment in both types of human lenses with matched ages revealed no difference (Fig. 2A). They were all negative in telomerase activity. Next, we analyzed the protein level of the telomerase catalytic subunit in the epithelial cells from the two types of lenses. Western blot analysis revealed that hTERT was not detectable in both types of lenses (Fig. 2B). Because the holoenzyme of telomerase consists of hTERT and the template RNA (hTR), we next examined whether the hTR was expressed in the two types of lenses. RT-PCR revealed the presence of an expected 186-bp DNA band amplified from the mRNA samples isolated from both types of lens epithelial cells (Fig. 2C). Thus, the absence of telomerase activity in human lenses is the result of the absence of hTERT expression. To compare the telomere lengths in human transparent and cataractous lenses, genomic DNAs were extracted from the pooled capsular epithelium and cortical fiber cells of both transparent and cataractous human lenses and analyzed by Southern blot, as described before. The maximum telomere length in transparent lenses was approximately 12 kb, which was ~1 kb longer than that observed in cataractous human lenses (approximately 11 kb; Fig. 2D).

Establishment of Primary Cultures of Human Lens Epithelial Cells

After human lens capsular epithelia containing epithelial cells were isolated, the capsular epithelia were cultured in 60-mm Petri dishes with DMEM containing 20% FBS in the presence of 50 U/mL penicillin and 50 μg/mL streptomycin. In 2 to 3 weeks, the cultures became 100% confluent (data not shown). The 100% confluent lens epithelial cells were passaged into gelatin-coated 60-mm dishes at a ratio of 1 to
and grew in the same DMEM plus 20% FBS. After another passage, the cells were transfected with hTERT expression construct (kindly provided by Robert A. Weinberg, Department of Biology, Massachusetts Institute of Technology) or the corresponding vector (pCI-neo; Promega). Thirty-six hours after transfection, G418 at a concentration of 400 μg/mL was added to each culture dish. After a 6-week selection, a stable line of the hTERT-transfected HLECs was established (Fig. 3A).

Expression of the Exogenous hTERT in the Stable Clone of the hTERT-Transfected HLECs

After establishment of a stable clone of the exogenous hTERT-transfected HLECs (Fig. 3A), we next determined whether the exogenous hTERT is expressed in the HLECs. To do so, RT-PCR was conducted. The specific DNA band was only detected in the reaction with the total RNAs from pCI-hTERT-HLECs but not with the RNAs from the vector-transfected cells (pCI-HLECs; Fig. 3B) or the parental HLECs. To confirm the expression of the exogenous hTERT, Western blot analysis was conducted. hTERT protein was expressed only in hTERT-transfected cells (Fig. 3C). To confirm further the expression of the exogenous hTERT in these HLECs, we next performed a TRAP assay. Telomerase activity was detected only in the extracts derived from pCI-hTERT-HLECs, not from the vector-transfected or parental HLECs (Fig. 3D). Thus, the established pCI-hTERT-HLECs display telomerase activity. To further characterize the established pCI-hTERT-HLECs cell line, karyotype analysis was conducted with these cells after 12 PDs with the methodology described by Hsieh. G-banding revealed that the established pCI-hTERT-HLECs have a normal karyotype with 46 chromosomes (data not shown).

Effect of hTERT on Growth of the Transfected HLECs

During our establishment of the HLEC line, we noticed that the vector-transfected HLECs hardly grew after the selection by G418, but the hTERT-transfected stable line grew at a faster rate. A more quantitative analysis of the growth rate of these transfected cells revealed that the hTERT-transfected cells revealed that the hTERT-transfected cells (pCI-hTERT-HLECs) at 25 PDs after establishment grew (from $2.5 \times 10^4$ to $2.98 \times 10^5$ within 5 days) more than three times faster than the vector-transfected cells (from $2.5 \times 10^4$ to $8.28 \times 10^4$ within 5 days), after a 6-week selection under the same culture conditions (Fig. 4). The parental cells after 12 PDs displayed a growth rate (from $2.5 \times 10^4$ to $8.45 \times 10^4$ within 5 days)
of the vector-transfected cells were strongly β-Gal positive (Fig. 5, dark blue) and 36% of the vector-transfected cells were β-Gal positive (light blue). In contrast, no strong β-Gal-positive cells were found in the culture of hTERT-transfected HLECs (Fig. 5B) and less than 10% of light β-Gal-positive cells were noted. These results show that hTERT can prevent cellular senescence in HLECs.

Prevention of Telomere Shortening by hTERT in pCI-hTERT-HLECs

To explore the possible mechanism by which hTERT prevents cellular senescence, we examined telomere length in the vector-transfected cells pCI-HLECs right after G418 selection and in the hTERT-transfected pCI-hTERT-HLECs at 25 PDs. The genomic DNA samples were isolated and digested with HindIII and RsaI and resolved in 0.8% agarose gel and further analyzed. The vector-transfected HLECs had a maximum telomere length of approximately 10 kb (Fig. 6). In contrast, the hTERT-transfected HLECs have a telomere length of approximately 13 kb. These results suggest that hTERT prevents cellular senescence through telomere synthesis.

Resistance of pCI-hTERT-HLECs on H₂O₂-Induced Caspase-3 Activation and Apoptosis

Our previous studies have demonstrated that hTERT attenuates the transfected rabbit lens epithelial cells from camptothecin-induced apoptosis and that this attenuation occurs through regulation of p53 and Bcl-XS. To examine further the anti-apoptotic activity of hTERT, we treated both the vector- and the hTERT-transfected HLECs with the oxidative stressor H₂O₂, which may play a role in cataractogenesis and induces apoptosis in lens epithelial cells. A single dose of 150 μM H₂O₂ was used to treat the three types of lens epithelial cells. The decomposition of H₂O₂ was monitored within the first 90 minutes (Fig. 7A) using a chemical assay, as previously described. Under this condition, approximately 60% apoptosis of the parental HLE and vector-transfected cells were detected at the end of a 12-hour treatment and by 24 hours, all the treated parental HLECs and vector-transfected cells underwent apoptosis (Fig. 7B). In contrast, less than 25% of the hTERT-transfected cells underwent apoptosis after a 12-hour insult, and approximately 40% of the hTERT-transfected cells survived after a 24-hour treatment under the same conditions. A longer culture might reveal more apoptosis. The apoptotic nature of the cell death was verified by DNA fragmentation (Fig. 7C). To examine the possible mechanism, we measured the caspase-3 activity in the two types of cells 12 and 24 hours after H₂O₂ treatment. Twelve hours after H₂O₂ treatment, the caspase-3 activity in both parental and vector-transfected cells, H₂O₂ induced a 3.6- to 3.8-fold upregulation of the caspase-3 activity (Fig. 7D). In contrast, in hTERT-transfected cells, only a 1.4-fold upregulation of caspase-3 activity was observed. Twenty-four hours after H₂O₂ treatment, however, the caspase-3 activity in hTERT-transfected cells was also increased to 2.5-fold, consistent with the increased apoptosis at this stage of H₂O₂ treatment. By this time, the caspase-3 activity in vector-transfected cells became slightly decreased because of the death of the cells. Thus, in hTERT-transfected cells, activation of caspase-3 and apoptosis were substantially delayed and also attenuated to some degree.

Discussion

In the present study, we demonstrated the following. First, using the TRAP assay, we showed that among the central epithelium, peripheral epithelium, and cortical fiber cells isolated from human, bovine, and rabbit lenses, only the central
epithelium from the 6-month-old rabbit lens displayed telomerase activity. Third, we introduced the cDNA encoding the telomerase reverse transcriptase catalytic subunit (hTERT) into the early passages of the HLECs derived from primary cultures and established a stable line that expresses the exogenous hTERT and displays normal karyotype after 12 PDs. Finally, we have shown that hTERT, when expressed in telomerase negative HLECs, is capable of supporting growth of normal lens epithelial cells and suppressing cellular senescence through telomere synthesis. A similar observation has been reported in which hTERT was inducted into the human lens epithelial cell line FHL12.4 and was shown to overcome replicative senescence (Reddan JR, et al. *IOVS* 2004;45:ARVO E-Abstract 2626). hTERT also enhances the transfected cells’ ability to detoxify hydrogen peroxide and delays and partially attenuates H2O2-induced apoptosis. Our establishment of the hTERT-transfected human lens epithelial cell line provides an additional system for the study of lens biology and disease, besides the previously established various cell lines including N/N1003A, FHL12.4, αTN4-1, HLECs, HLE-B3, and animal models.57

**Prevention of Cellular Senescence through Maintenance of Telomere Length**

Cellular senescence, also called replicative senescence, or the Hayflick limit, is a process of terminal growth cessation and morphologic change displayed by normal cells after they have undergone a finite number of PDs in vitro.58 Cells undergoing replicative senescence remain adherent to the growth surface and metabolically active for an extended period after cessation of proliferation.59 The proliferative potential of a given cell population in culture correlates directly with the number of prior cell doublings in vivo.60 Several theories have been proposed regarding the basis of senescence: error-genetic damage and programmed control.61,62 In the error-genetic damage model, it is suggested that senescence occurs as a result of the accumulation of mutations in DNA or deleterious changes in RNA or proteins due to age-related errors or macromolecular damage caused by free radicals, metabolic processes, or other mechanisms. Recent studies63,64 have suggested that the progressive accumulation of oxidative damage may act as an important mechanism for organism aging. Several studies have attempted to relate oxidative damage to replicative aging of cultured fibroblasts.65– 67 In the programmed control theory, it is thought that a genetic program becomes activated or manifests at the end of the proliferative lifespan of a normal cell, causing the characteristic morphologic changes and growth arrest.61,62 Telomere length and telomerase activity can be part of the genetic program. In the absence of telomere elongation, telomeres shorten with each cell division.6,68,69 In each round of chromosome replication, telomeres typically lose ~150 bp of nucleotide sequence at the 5’ end of a DNA molecule. Thus, an increasing number of cell divisions is usually accompanied by declining telomere length. Such DNA replication-dependent loss of telomere length seems to be a mitotic clock for counting the number of cell divisions and signaling cellular senescence.6 Indeed, in our present study, we have demonstrated that the telomere length becomes shortened during division of HLECs. If new synthesis does not compensate for the decreased telomere length, as in the case of the vector-transfected HLECs, the cells then become senescent (Fig. 5). It is well documented that telomerase activity is present in germ cells and in most tumor cells but is absent in most somatic cells.21 The presence of telomerase activity in the central epithelium of the 6-month-old rabbit lenses suggests that the central epithelial cells may be the stem cells of the ocular lenses. We are currently analyzing the expression of other markers for stem cells to test this possibility. Like most
Somatic cells, the lens epithelial cells isolated from adult human lenses lack detectable telomerase activity (Figs. 2A, 3D). These cells when cultured under in vitro conditions can only be passaged for a limited number of PDs. Introduction of the hTERT into these lens epithelial cells confers telomerase activity on the transfected cells (Figs. 3B–D), which provides the basis for new telomere synthesis. The hTERT-transfected HLECs have been passaged for 48 PDs at this writing and are still growing normally. However, the vector-transfected cells displayed an extremely slow growth rate right after selection with G418 as was true of the parental cells after 12 PDs (Fig. 4). Senescence-associated β-Gal assays of both the vector- (after G418 selection) and the hTERT- (after 25 PDs) transfected HLECs revealed that most vector-transfected HLECs have entered cellular senescence (Fig. 5, Table 2).

Thus, hTERT prevents cellular senescence in HLECs with telomerase activity. To explore the possible mechanism of this protection, genomic Southern blot analysis was performed, and the obtained results demonstrated that the maximum telomere length in the hTERT-expressing cells was approximately 3 kb longer than that in the vector-transfected HLECs. Our results are consistent with numerous earlier studies in which hTERT was overexpressed in a variety of human cell lines.14–17,70–74 In all cases, the hTERT-transfected cells have extended telomere length and lifespan to various degrees. Such cells display all the identifiable characteristics of healthy young cells without genetic instability (Reddan JR, et al. IOVS 2004;45:ARVO E-Abstract 2626)14–17,71,72 or the hallmarks of malignant transformation.70–73

Two Independent Mechanisms That Maintain Telomere Length in Lens Epithelial Cells

A comparison of our studies of hTERT expression in rabbit LECs and HLECs reveals the presence of two independent mechanisms by which telomere length is maintained. In rabbit lens epithelial cells, the telomere length is approximately 12 kb, and expression of hTERT does not change telomere length.18 Moreover, rabbit lens epithelial cells derived from the intact lenses of either 6-month- or 2-year-old rabbits can be passaged continuously without signs of senescence. (We have cultured rabbit lens epithelial cells for >120 PDs and these cells show no difference in growth rate compared with the initial primary cultures.) Telomere length does not shorten as cell PDs increase with or without hTERT expression (Ref. 18 and data not shown.) These results suggest that rabbit lens epithelial cells use a telomerase-independent mechanism for the maintenance of their telomere length, as is true in several other systems. For example, in Drosophila melanogaster, telomeric DNA can be elongated by transposition of specific retrotransposons to chromosome ends.75 In yeast, telomere extension can occur by homologous recombination of nonre-
ciprocal telomeric DNA between telomeres of homologous or heterologous chromosomes. In HLECs, however, the situation is different. The hTERT-transfected cells with normal growth have a maximum telomere length of approximately 13 kb. Shortening of this telomere length to a certain degree signals cellular senescence, as observed in vector-transfected HLECs, which have a maximum telomere length of approximately 10 kb. Thus, HLECs use a telomerase-dependent mechanism to maintain their telomere stability.

hTERT Functions beyond Telomere Synthesis

Although the major function of telomerase is to synthesize telomeres in most eukaryotes, recent studies from several laboratories including ours have clearly demonstrated that telomerase has functions beyond telomere synthesis.87 One of such functions is to regulate cell proliferation. We have previously shown that hTERT, when introduced into an SV40 large-T antigen-immortalized human lens epithelial cell line, accelerates cell growth through regulation of the RB/E2F pathway.19 After our report, similar results were observed in non-lens cells. Gronthos et al.87 have recently demonstrated that ectopic expression of hTERT accelerates cell-cycle progression of human bone marrow stromal stem cells from the G1 to the S phase through modulation of expression of cyclin D3, cyclin E1, E2F-4, and DP2. Smith et al.79 have also reported that hTERT enhances cell proliferation of human mammary epithelial cells. Another important function of telomerase is the regulation of apoptosis. In non-lens systems, the antiapoptotic ability of telomerase has been demonstrated in different cell lines.80–84 In the lens system, we have recently shown that in telomerase-negative immortalized rabbit lens epithelial cells (N/N1003A cells), introduction of hTERT into these cells can enhance their protection against apoptosis induced by the topoisomerase I inhibitor camptothecin.18 This attenuation occurs through regulation of expression of the apoptosis-related genes, including p53 and bcl-X. In the present study, we have shown that hTERT when transfected into normal HLECs enhances the transfected cells the ability to detoxify H2O2 (Fig. 7A) and also substantially delays H2O2-induced activation of caspase-3 and apoptosis. Similar results have been reported in hTERT-transfected telomerase-negative human embryonic lung fibroblasts and human T-cells.85,86 Our result that hTERT substantially delays and partially attenuates activation of caspase-3 is also consistent with early observations in hTERT-transfected human embryonic lung fibroblasts.85 How could hTERT enhance the p53-H2O2/HCT-116 cells and delay as well as attenuate H2O2-induced activation of caspase-3 and apoptosis? First, recent studies have shown that H2O2 can trigger nuclear export of the telomerase catalytic subunit.88 We speculate that hTERT, once it has entered the cytoplasm, can antagonize the antiapoptotic regulators of the intrinsic pathway temporally, as the mutated p53 does,88 thus delaying caspase-3 activation and apoptosis (Fig. 7). Alternatively, hTERT may, to some degree, positively regulate the expression of the antioxidative stress enzymes to detoxify H2O2, which explains why H2O2 is decomposed faster in hTERT-transfected cells than in vector-transfected or parental HLECs and why activation of the apoptotic program by H2O2 in hTERT-transfected cells is substantially delayed. Regardless of what mechanisms may be involved, hTERT displays functions beyond telomere synthesis in both immortalized lens epithelial cells (N/N1003A and SV40 large-T-transfected HLECs) and normal human lens epithelial cells.

References


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