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Influence of Telomerase Expression on Cell Cycle Proteins, Type I Collagen and Interstitial Collagenase in Human Dermal Fibroblasts

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Telomere shortening has been implicated as an important mechanism that drives somatic cells into termination of cellular division and finally into cellular senescence. However, in germ line cells and tumor cells, which show distinct characteristics of ceaseless celluelar division without entering cellular senescence, the presence of telomerase prevents the telomere shortening. The expression of the catalytic subunit of human telomerase in normal human fibroblasts allows them to escape replicative senescence. In this study, we expressed hTERT in human neonatal foreskin fibroblasts (NFB-hTERT) and investigated the influence of hTERT on the cell cycle and metabolism of type I collagen. With increased passage, the NFB-hTERT cells showed down-regulation of p16 and cyclin D1 levels while the expression of CDK4 did not change. The basal level of type I collagen decreased with increased passage in NFB but the level did not change in NFB-hTERT. Basal level of matrix metalloproteinase-1 was reduced in aging NFB and NFB-hTERT and treatment of TNF-α resulted in less induction in NFB-hTERT. These results suggest the mechanism of inhibition of senescence by hTERT in human dermal fibroblasts.

Key words: Telomerase, Cellular senescence, Human fibroblast, hTERT Immortalization

INTRODUCTION

Increasing lifespan and delaying aging are the research challenges of the new century. Many theories have been proposed to explain the aging process but damage to deoxyribonucleic acid (DNA) is essential to this phenomenon. Recently, telomere shortening and the loss of telomerase in normal somatic cells has been implicated as a potential molecular clock that triggers cellular senescence[1], loss of proliferative capacity, and age-related pathologies[2,3]. Human telomeres consist of repeats of the sequence 5'-TTAGGG-3' at chromosome ends and these repeats are synthesized by the ribonucleoprotein enzyme telomerase[4,5]. Overexpression of the human telomerase catalytic subunit, human telomerase reverse transcriptase (hTERT), in primary cells showed that the overexpression was responsible for the restoration of telomere length and delays cellular senescence in these cells[6].

Tumor necrosis factor-α (TNF-α) has been implicated in a number of pathological conditions related to chronic inflammation, cancer, and aging. Transforming growth factor-β (TGF-β) is a profibrotic cytokine synthesized in a wide variety of tissues that modulates neocollagenesis, angiogenesis, cellular migration and proliferation, and the degradation of matrix proteins. Studies have shown that TNF-α and TGF-β modulate the expression of collagen and matrix

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metalloproteinase-1 (MMP-1) genes in fibroblasts[7-10,11]. Not only the expression of MMP-1 gene is modified by the cytokine but also by senescent cells, which have been shown to accumulate within aged human skin. The senescent cells display altered pattern of MMP expression as compared to young, replication-competent cells[12,13]. In particular, interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) production are known to be significantly enhanced in late passage skin fibroblasts[14-16].

The objective of this study was to generate human skin fibroblast cell lines with an extended lifespan by ectopic expression of hTERT and to study their molecular profile as well as their ability to respond to skin aging and/or cytokines. We have used retrovirus to infect neonatal foreskin fibroblasts with hTERT (NFB-hTERT) to investigate its effect on MMP-1 and type I collagen production. In addition, essential cell cycle proteins, all of which take significant parts in the proliferation and senescence of cells, were measured to analyze the effect of the presence of hTERT.

MATERIALS AND METHODS

Culture of human neonatal foreskin fibroblasts

Primary human dermal fibroblast cultures, established by explanting tissue specimens obtained from neonatal foreskin. The cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies Inc., Stafford, TX, U.S.A.) supplemented with 20% fetal calf serum (FCS; Life Technologies Inc.) and 2 mM glutamine in a humidified atmosphere of 5% CO2 for 7-10 days. Cells were subcultured using DMEM supplemented with 10% FCS. Transforming growth factor-beta (TGF-β; Pepro Tech Inc., Rocky Hill, NJ, U.S.A.), 5 ng/ml, and tumor necrosis factor-alpha (TNF-α; Boerhinger Mannheim, Germany), 10 ng/ml, were used for this experiment.

Retroviral infection and colony isolation

pBABE-hTERT is a puromycin-resistant amphotropic retrovirus, based on a pBABE series vector backbone, that expresses the catalytic subunit of human telomerase (hTERT), and has been previously described[17]. For retroviral infections, normal and progeric fibroblasts were seeded at a density of 8.4×10^4 cells in 6 cm diameter standard tissue culture dishes (Life Technologies). This was done approximately 24 h prior to infection such that the cultures were still substantially sub-confluent at the time of infection. One hour prior to infection the cells were re-fed with fresh medium containing 8 mg/ml of polybrene (Sigma, St. Louis, MO, USA). Two milliliters of supernatant from psiCRIP producer cell lines containing either pBABE-hTERT or control (pBABE -puro) retrovirus was then added to each 6 cm dish keeping the polybrene concentration constant at 8 mg/ml. The cultures were then incubated for 2 h in a tissue culture incubator at 37°C, after which time the retroviral supernatant was diluted with fresh medium. After 24 h the supernatant was replaced with fresh medium and cells were incubated for 24 h more. Subsequently, each culture was passaged into a series of 10 cm tissue culture dishes in a split ratio range of 1 : 2 - 1 : 32 in order to produce cultures at a suitable density for subsequent clonal isolation. Twenty four hours after passage, the cultures were re-fed with selective medium (MEM containing the supplements given above together with 1.5 mg/ml puromycin). The concentration of puromycin required to provide effective selection had been determined by cytotoxicity studies undertaken in advance of the experiment for each cell strain (data not shown). Colonies were isolated by standard techniques using cloning rings (Sigma). and were transferred without either counting or discarding any cells until each clone had expanded sufficiently to fill a 25 cm^2 tissue culture flask. At this passage total cell numbers were estimated and the population doublings the clone had undergone were estimated assuming minimal cell loss and a single cell founder for the colony. Clones typically underwent approximately 20 population doublings during the isolation procedure.

TRAP assay

Cell lysates were prepared using the CHAPS detergent lysis method as previously described[18]. Briefly, the cells were resuspended in ice-cold lysis buffer (0.5% CHAPS, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM EGTA, 5 mM β- mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) and incubated on ice for 30 min. After

centrifuge at 14,000 × g for 30 min at 4°C, the supernatant was frozen and stored at -80°C. The protein concentration of cell lysates was measured using Bradford (BioRad, Mississauga, ON, Canada) protein assay kit. The TRAP assay was performed as follows: the TS primer (5'-AATCCGTCGAG CAGAGTT-3') was allowed to be extended by telomerase for 15 min at 37°C during incubation with the cell lysates in reaction mixture containing 10 × TRAP buffer (200 mM Tris-HCl, pH 8.3, 15 mM MgCl2, 630 mM KCl, 0.05% Tween 20, 10 mM EGTA, and 1 mg/ml BSA). The CX primer (5'-CC CTTACCCTTACCCTTACCCTAA-3'), *Taq* polymerase, and [α-P^{32}]dCTP were then added. The reaction solutions were heated at 94°C for 90 s to inactivate telomerase and then subjected to 31 cycles of PCR including denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 45 s. The PCR products were analyzed by acrylamide gel. The gel was then dried, exposed with an image plate, and analyzed with a Phosphoimager (Fuji). An internal telomerase assay standard (ITAS), consisting of a 213 bp DNA sequence with primer sites for TS and CX at the ends was included to confirm that the PCR amplification was not inhibited by the cell lysate[19].

Cell viability assays

Cell viability was assessed by counting trypan blue-negatively stained cells. In addition, reduction of the formazan salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Boehringer, Mannheim, Germany) (12) was used as an index of cellular respiration. Briefly, MTT (0.5 mg/ml) was incubated with the cells during the last 4 h of each assay. At the end of this period, cells were washed and reduced MTT was dissolved in 200 (l DMSO (dimethyl sulfoxide) (Sigma) and absorbance was measured at 570 nm. NFB p 14, NFB-hTERT p 15, NFB p 26 and NFB-hTERT p 27 cells were plated at a density of 2×10^4 in a 96-well plate and incubated at 37°C overnight. MTT (Sigma) stock (5 mg/ml MTT in PBS) in DMEM and was added at a dilution of 1:10, then the cells were re-incubated for 4 h. After removing media, 200 μl of DMSO was added to each well and incubated at 37ºC for 5 minutes. The result was quantified by measuring absorbance at 570 nm using a scanning multiwell spectrophotometer (Dynatech, VA, USA).

Cell cycle analysis

To obtain aged NFB cells, the NFB and NFB-hTERT cells were passaged in culture until enlarged morphology was evident (figure not shown). Subconfluent cultures were harvested, fixed in 70% ethanol, and stained with 5 mg/ml propidium iodide (Sigma). Cell cycle analysis was performed using a FACScan (Becton Dickinson, San Jose, CA, USA) and data were analyzed using Modfit software (Verity Software House Inc., Topsham, ME, USA).

RT-PCR

Total RNA was purified from cultured cells using the RNeasy mini kit (Qiagen, MD, USA) and quantified spectrophotometrically at 260/280 nm. To obtain first strand cDNA, 1.0 μg of total RNA was reverse transcribed using AccuPower® RT PreMix (Bioneer, Daejeon, Korea) in a total volume of 20 $\mu\ell$. The cDNA obtained (1 $\mu\ell$) was used in a final reaction volume of 20 $\mu\ell$ for PCR amplification with the primers: p16-S: 5'-AGC ATG GAG CCT TCG GCT GAC T-3' and p16-AS: 5'-CTG TAG GAC CTT CGG TGA CTG AT-3', cyclin D1-S: 5'-CTG GCC ATG AAC TAC CTG GA-3' and cyclin D1-AS: 5'-GTC ACA CTT GAT CAC TCT GG-3', CDK 4-S: 5'-CCA AAG TCA GCC AGC TTG ACT GTT-3' and CDK 4-AS: 5'-CAT GTA GAC CAG GAC CTA AGG ACA-3'. Reactions were performed using 2.5 units of *Taq* polymerase (Qiagen). Parameters for DNA amplification were 94°C for 30 s, annealing temperature (58, 52, 58°C respectively) for 30 s, and 72°C for 30 s. Oligonucleotide primers used for DNA amplification were purchased from Bioneer. Mutiplex amplification of β-actin was performed to confirm the quality of the cDNA.

Protein Expression and Immunoblotting

Cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.005% bromophenol blue). Proteins (30-50 μg) from the whole cell lysate were separated by SDS-PAGE in 7.5 or 15% gels. Proteins were detected immunologically following electro-transfer onto PVDF membranes (BioRad). Protein molecular weight markers (BioRad, Mississauga, ON, Canada) were used and

membranes were revealed by Ponceau S. Membranes were blocked in PBS containing 5% powdered milk and 0.05% Tween-20 for 2 h at 4°C. Membranes were then incubated overnight at 4°C with primary antibodies in blocking solution, then with horseradish peroxidase-conjugated goat anti-mouse or anti- rabbit (1:4000) IgG for 1 h. Blots were visualized using the ECL solution (SantaCruz, Delaware, CA, USA). Protein concentrations were measured using the Bradford method with bovine serum albumin as the standard.

Data Presentation and Statistical Analysis

Assays were performed in either duplicate or triplicate. All results were analyzed by the Mann-Whitney test and were considered statistically significant at $p < 0.05$. Typical RT- PCR and Western blots shown are the representative of 3 independent experiments.

RESULTS

Establishment of the hTERT-expressing neonatal foreskin fibroblasts

As shown in Fig. 1, telomerase activity was only detected in the clone expressing hTERT. Thus, hTERT-expressing stable line (NFB-hTERT p10) was established.

Cellular growth and cell cycle in early and late passage of NFB and NFB-hTERT cells

Senescence was confirmed by measuring growth and cell cycle analysis as *in vitro* aging of NFB cells is accompanied by the loss of proliferative activity and by differential expression of certain genes and proteins. As the passage of cell increased, growth of NFB was gradually decreased from 1.41 to 1.2 fold on the day 3 and 1.79 to 1.6 fold on day 4, but the growth of NFB-hTERT did not change with increased passage (Fig. 2). Cell cycle analysis showed that the proportion of S phase was 27.14% in p17 NFB but it was higher at 40.03% in p38 NFB-hTERT. The proportion of S phase decreased to 15.43% in NFB as the passage increased to p29 but it was still higher at 26.10% in NFB-hTERT although the passage was increased to p50.

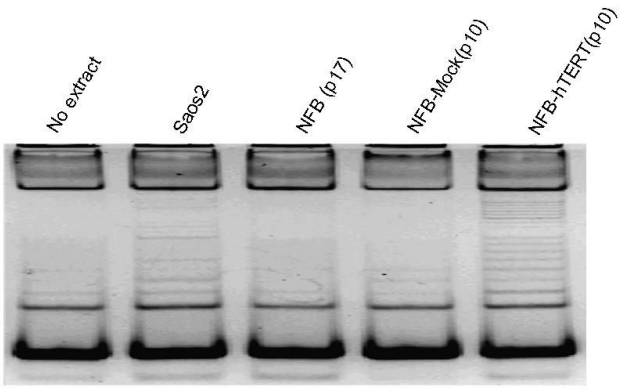


Fig. 1. TRAP assay of control cells and cells transfected with retrovirus containing hTERT or mock vector. Each assay was performed with 1,000 cell equivalent of total cell lysate (Saos2-virus only / NFB-neonatal foreskin fibroblast / NFB-Mock-virus with mock vector).

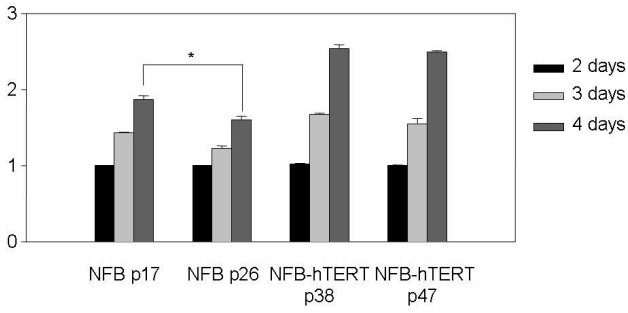
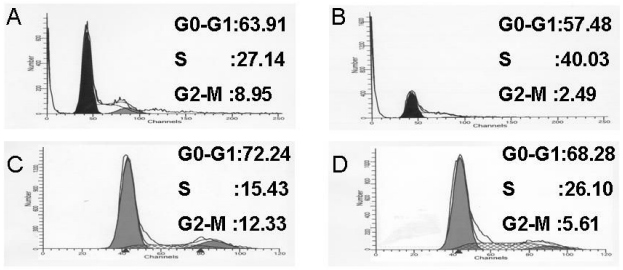


Fig. 2. MTT assay shows that the growth of NFB was significantly reduced by day 4 with increased passage. However, the growth of NFB-hTERT was not affected by increased passage (* $p<0.05$).

The proportion of cells in G1 phase was also lower in NFB-hTERT cells (Fig. 3).

Loss of p16 expression

To investigate if the pRb-p16 pathway showed any change in the hTERT immortalized cells, we performed PCR and western blot analysis on the NFB and NFB-hTERT cells. Analysis of the PCR results indicated that p16 expression increased with passage in NFB but it was significantly lower in the NFB-hTERT (Fig. 4A). Western blot analysis also revealed that, despite the increase in p16 expression in NFB, its expression in NFB-hTERT was significantly lower (Fig. 4B). CDK4 and cyclin D1 expressions showed no significant changes.



A. NFB p17 B. NFB-hTERT p38
C. NFB p29 D. NFB-hTERT p50

Fig. 3. Cell cycle analysis using FACS shows that S phase is increased and G0-G1 phase is decreased in NFB-hTERT compared to NFB cells.

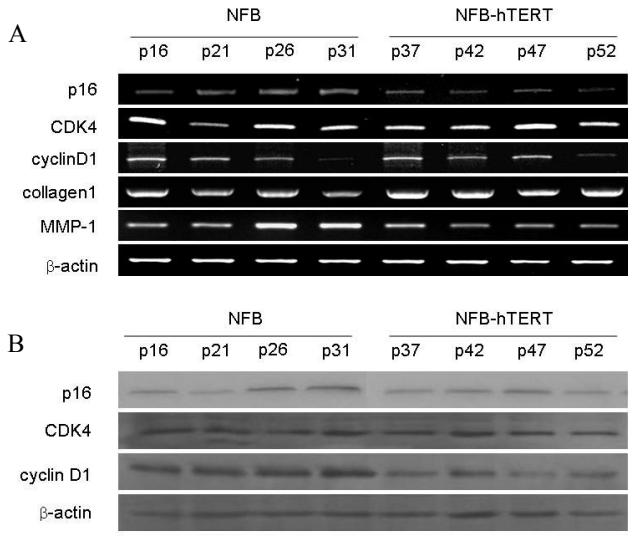


Fig. 4. Senescence-related mRNA (A) and protein (B) expressions of p16, CDK4, cyclinD1, type I collagen and MMP-1 in different passages of NFB and NFB-hTERT. β-actin was loaded as a control.

Influence of hTERT on type I collagen and MMP-1 expression

To investigate the effect of hTERT on type I collagen and MMP-1 production, in NFB and NFB-hTERT, we checked their basal levels with increasing passage and after treating them with TGF-β and TNF-α. The basal expression of type I collagen decreased with passage in NFB but it was significantly higher in NFB-hTERT and showed no change in expression with passage. However, MMP-1 basal expression showed significant increase with passage in NFB but the expression was lower in NFB-hTERT and showed no change in the expression with passage (Fig. 4A). Stimulating

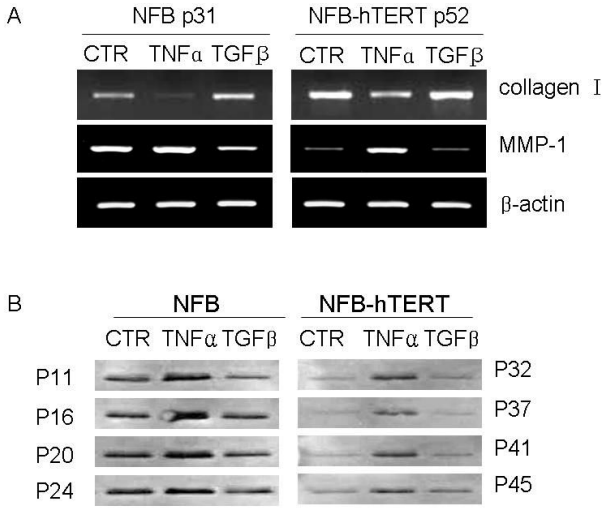


Fig. 5. mRNA (A) of type I collagen and MMP-1, and protein expression (B) of MMP-1 after the NFB and NFB-hTERT cells were treated with TGF-β and TNF-α for 24 h. β-actin was loaded as a control.

the cells with TGF-β resulted in elevated collagen expression but the basal level and growth factor-activated change was much more pronounced in the NFB-hTERT.

The basal expression of MMP-1 was much lower in NFB-hTERT and TNF-α stimulation of the cells showed that the cytokine-activated change was less pronounced in NFB-hTERT also (Fig. 5A). The basal and TNF-α-activated expressions of MMP-1 increased with passage in NFB the levels were constantly lower in NFB-hTERT at a protein level (Fig. 5B)

DISCUSSION

Telomeres are located at the ends of chromosomes and consist of nucleoprotein complexes. They are made up of tandem repeats of double-stranded TTAGGG, a 3' single strand overhang and telomere binding proteins[20,21]. It has been known that the telomeres play a critical role in the maintenance of chromosomal integrity. They shorten slightly with each cell division and contribute to the replicative senescence in adult somatic cells, which lack the mechanism to replenish the shortening telomere. However, in germ cells and tissue stem cells, a unique RNA-protein complex called telomerase operates to synthesize and maintain the telomere repeats and thus to escape the inevitable consequence. The

telomerase consists of a telomerase RNA component (TERC), a template that serves to add telomere repeats, and a telomerase reverse transcriptase (TERT), which is a protein component[22]. While TERC is widely expressed in embryonic as well as somatic tissue, hTERT is not detected in most somatic cells[23]. Therefore, expressing only hTERT in human somatic cells enables the cells to attain telomerase activity and thereby achieve telomere elongation and extended replicative life span[6,24]. When human foreskin fibroblasts were immortalized with hTERT, these cells retained their normal phenotype for the first few 100 population doublings (PDs) while the normal fibroblasts senesced at PD 40-45[25]. In our experiment, expressing hTERT in human foreskin fibroblasts led to telomerase activity, as shown by the TRAP assay, and increased growth compared to earlier passage NFB cells. Cell cycle analysis also showed the later passage NFB-hTERT cells to be in more proliferative phase than NFB.

As cells divide and reach senescence, telomere shortening is known to lead to loss of structural integrity of the telomere nucleoprotein, resulting in activation of the p53-p21 and / or pRB-p16 tumor suppressor pathways[26]. Replicative senescent cells arrest in G1 phase of the cell cycle and express high levels of p16 and p21[27,28]. However, the two proteins has been shown to contribute differentially to cellular senescence. In normal human fibroblasts, when growth arrest is induced as the initial stage of senescence, p21 was found to accumulate and decline at a later stage. In contrast, when the late stage of senescence is reached, as manifested by the appearance of SA-β gal and senescence-specific morphology, p16 was found to be increased[29,30]. In our experiment, the mRNA expression of p16 did not elevate as the passage of NFB increased. However, the mRNA expressions of p16 and cyclin D1 in NFB-hTERT were significantly lower than those of earlier passage NFB. When this phenomenon was checked at the protein level, p16 and cyclin D1 expressions were noticeably elevated with increased passage in NFB and their expressions were significantly lower in NFB-hTERT. This discrepancy between the mRNA and protein expressions in NFB needs to be further explored using later passage cells.

Type I collagen is the main component of human dermis and its homeostasis is regulated by the presence of MMP-1. TGF-β has been reported to stimulate fibroblast proliferation31, upregulates collagen synthesis[7-9] and prevents MMP-I expression[10]. TNF-α is an inflammatory cytokine and induce MMP-1 expression via NF-κB pathway[11]. Their composition is changed in the chronologically aged as well as photo-aged skin. In sunprotected skin of an aged individual, both the number of fibroblasts and their ability to synthesize type I collagen are reduced when compared with the skin of a young[32]. In addition, ultraviolet (UV) irradiation activates protein kinase-mediated signaling pathway and results in the increased expression of metalloproteinase (MMP-1)[33,34]. MMPs cause connective tissue damage to contribute to the aging and wrinking of aged skin and MMP-1, interstitial collagenase, initiates the degradation of type I and III collagens[35]. Our data showed that the presence of hTERT in human dermal fibroblast increases the basal expression of type I collagen and decreases that of MMP-1. Presence of hTERT also enhanced the cytokine responsiveness of the cells.

In conclusion, expressing hTERT in human dermal fibroblasts could be used as an anti-aging tool to stabilize and regenerate the connective tissue component of the dermis and further investigation is needed to search the in-depth mechanism of hTERT-induced anti-aging in human skin.

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