Evidence That High Telomerase Activity May Induce a Senescent-like Growth Arrest in Human Fibroblasts

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Expression of the catalytic subunit of human telomerase (hTERT), in normal human fibroblasts allows them to escape replicative senescence. However, we have observed that populations of hTERT-immortalized human fibroblasts contain 3–20% cells with a senescent morphology. To determine what causes the appearance of these senescent-like cells, we used flow cytometry to select them from the population and analyzed them for various senescence markers, telomere length, and telomerase activity. This subpopulation of cells had elevated levels of p21 and hypophosphorylated Rb, but telomere length was similar to that of the immortal cells in the culture that was sorted. Surprisingly, telomerase activity in the senescent-like cells was significantly elevated compared with immortal cells from the same population, suggesting that high telomerase activity may induce the senescent phenotype. Furthermore, transfection of normal fibroblasts with a hTERT-expressing plasmid that confers high telomerase activity led to the induction of p21, a higher percentage of SA-β-galactosidase-positive cells, and a greater number of cells entering growth arrest compared with controls. These results suggest that excessive telomerase activity may act as a hyperproliferative signal in cells and induce a senescent phenotype in a manner similar to that seen following overexpression of oncogenic Ras, Raf, and E2F1. Thus, there must be a critical threshold of telomerase activity that permits cell proliferation.

Normal human somatic cells undergo a limited number of divisions before entering an irreversible growth-arrest state defined as senescence (1). Replicative senescence is thought to provide a barrier against the unlimited proliferation and formation of cancer. The best described counting mechanism for replicative senescence involves the telomere-shortening hypothesis. Telomerase is not expressed in most human tissues; therefore, telomeres shorten with every cell division. When telomeres reach a critical length, irreversible growth arrest is activated.

It has been demonstrated that ectopic expression of the catalytic subunit of human telomerase (hTERT) can immortalize fibroblasts (2, 3), retinal pigment cells (2), and endothelial cells (4). Whether reconstitution of telomerase activity alone is sufficient for immortalization of cell types other than fibroblasts has been a subject of debate. Kiyono et al. (5) have shown that inactivation of Rb/p16INK4A is required in addition to telomerase for immortalization of epithelial cells. O'Hare et al. (6) have found that telomerase was not sufficient to immortalize adult mammary fibroblasts and endothelial cells. However, subsequently, Ramirez et al. (7) demonstrated that telomerase activity alone could immortalize epithelial cells if they were grown on feeder layers, and they proposed that the results of the earlier report (5) were because of inadequate growth conditions (7).

Although immortalization of normal human fibroblasts by ectopic expression of hTERT has been convincingly demonstrated, it is becoming apparent that immortality is not the universal outcome of hTERT expression. It has been observed that cultures of hTERT-transduced fibroblasts may undergo a crisis-like stage or acquire a senescent phenotype (8, 9). It was found that senescence was not related to the loss of telomerase activity, because there was no correlation between telomere length and immortalization, and among the clones that entered growth arrest, some had long and others short telomeres. The mechanism(s) that induces senescence, despite the presence of telomerase activity, remains unclear. One possibility is that in some cells senescence is triggered by the activation of a stress response pathway. Another explanation for the apparent lack of immortalization in some fibroblasts is that immortalization is achieved only at a certain level of telomerase activity, which may not be always reproduced by ectopic expression of hTERT.

We have observed that in three different lines of human fibroblasts, 3–20% of the cells in the population immortalized by expression of hTERT and grown for >70 PDs had a senescent-like morphology. In this study, we aimed to examine the mechanism(s), that caused the senescent-like growth arrest in telomerase-expressing cells. To this end, we developed a flow cytometry-based method to isolate these cells from the population of actively growing immortalized cells. We then compared them to the actively growing cells from the same population. The isolated cells had features typical of senescent cells. Unexpectedly, we found that telomerase activity in the senescent-like cells was significantly elevated compared with immortal cells from the same population. Furthermore, the overexpression of hTERT from a CMV promoter from a plasmid induced a senescent-like phenotype in a number of the newly transfected young cells. These results suggest that excessive telomerase activity may induce the senescent-like phenotype. We discuss

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¶ The abbreviations used are: hTERT, human telomerase catalytic subunit; PD, population doubling; CMV, cytomegalovirus; FACS, fluorescence-activated cell sorter; LTR, long terminal repeat; GFP, green fluorescent protein; TRAP, telomeric repeat amplification protocol.

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the potential mechanism of telomerase-induced senescence and its similarities to premature senescence induced by oncogenes or proliferative signals.

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions—**IMR-90 and LF1 are normal human lung fibroblasts. WI-38 fibroblasts and IMR-90 cells were from the Coriell Institute for Medical Research, and LF1 fibroblasts were a kind gift from J. Sedivy (10). HCA2 human foreskin fibroblasts were isolated in our laboratory. IMR-90-hTERT and HCA2-hTERT were kindly provided by J. Campisi. LF1-hTERT was kindly provided by J. Sedivy (11, 12). Cells were grown in Hank’s minimal essential medium or Earle’s minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, and sodium pyruvate.

**Isolation of Senescent Cells by Flow Cytometry—**In a typical experiment, $10^7$ to $10^8$ cells were collected by trypsinization, resuspended in 2 ml of fresh EMEM medium containing 10% fetal calf serum, placed on ice, and immediately used for sorting. Cells were sorted by a Beckman-Coulter EPICS Altra using Expo 32 MultiComp software (Applied Cytometry Systems).

The sorting parameters for selection of “large-senescent” and “small-young” cells were determined for each cell line using the corresponding young and senescent cultures without hTERT as standards (Fig. 1). The following two areas were selected: area A contained the majority of young cells (>70%); and area B covered the large-senescent cells and contained <1% of the dividing cells. Small and large cells were sorted simultaneously using two-way casting. Following sorting, cells were aliquoted and frozen for subsequent experiments, and a small aliquot (10^5 cells) was plated into a 35-mm tissue culture dish for SA-β-galactosidase staining.

SA-β-Galactosidase Staining—Cells were fixed and stained for SA-β-galactosidase as described previously (13). The percent of SA-β-galactosidase-positive cells was calculated by counting at least 500 cells/sample.

**Comparison of Telomere Length by Flow Cytometry—**Cells were hybridized in situ with a fluorescent telomere-specific peptide nucleic acid probe as described previously (14–17). Frozen cells (10^5) were thawed, washed in phosphate-buffered saline, and resuspended to 10^5 cells/100 μl of a hybridization mixture containing 70% dimethylformamide, 20 mM Tris, pH 7.0, 1% bovine serum albumin, and 0.3 μg/μl telomere-specific (CCCTAA)₃ fluorescein isothiocyanate-conjugated peptide nucleic acid probe (Applied Biosystems). After 10 min at 82°C, samples were incubated overnight at room temperature in the dark. Following hybridization, cells were spun down, washed twice with phosphate-buffered saline at 40°C for 10 min, and finally resuspended in phos-
phate-buffered saline containing 0.1% bovine serum albumin, 10 μg/ml RNase A, and 0.1 μg/ml propidium iodide. After 4 h at room temperature in the dark, cells were analyzed by FACS on Beckman-Coulter EPICS XL-MCL using System II version 3.0 software. Events were gated according to propidium iodide fluorescence to restrict the analysis to cells with diploid DNA content.

Western Blot Analysis—Adherent fibroblasts were harvested and lysed in protein sample buffer and boiled for 10 min, and equal numbers of cells were loaded on SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose membrane using a semidry transfer cell (Bio-Rad). Membranes were hybridized with the following antibodies: anti-p21(WAF1(Ab1) (Oncogene) and anti-Rb (Rb Ab1(1F8) (LabVision). Equivalent loading of lanes was verified by hybridization with anti-actin antibodies (Calbiochem).

Colony Size Distribution—Cells were transfected with pcDNA3.1-hTERT or pcDNA3.1 plasmids plated at a density of 200 cells/100-mm dish, and they were incubated undisturbed in the presence of 1 mg/ml G418 for 2 weeks. Dishes were then fixed and stained with 1% crystal violet. The number of cells in individual colonies was determined by microscopy.

RESULTS

Experimental System—The introduction of telomerase activity into normal human fibroblasts allows them to escape replicative senescence (2, 3). However, we have observed that immortal cultures of human fibroblasts contain some cells with a senescent morphology. In order to examine whether this was associated with other senescent cell markers and related to changes in telomere length or telomerase activity, we analyzed three lines of human fibroblasts that have been frequently used to study senescence: fetal lung fibroblasts IMR-90 and LF1 and foreskin fibroblasts HCA2. The fibroblasts were immortalized by infection with a retroviral vector containing an hTERT expression cassette. IMR-90+hTERT and HCA2+hTERT cells were propagated as mass cultures following infection, whereas LF1+hTERT was a clonal isolate. Cells were grown for at least 70 PDs after infection to confirm the immortalized phenotype. Although the cultures were actively proliferating, we observed some enlarged flat cells with a senescent morphology in the population. To quantify the percentage of senescent cells, we used SA-β-galactosidase staining and determined that the immortal cultures contained 3–20% SA-β-galactosidase-positive cells (Table I).

Isolation of Senescent-like Cells from the Populations of hTERT-expressing Cultures and Analysis of Senescence Markers—The biochemical analysis of the senescent cells that arise within populations of proliferating cells has been limited.

![Fig. 2. Morphology and SA-β-galactosidase activity of large-senescent (area A) and small-young (area B) cell fractions from cell sorting shown in Fig. 1. Following sorting, an aliquot of cells was plated and stained for SA-β-galactosidase. All of the panels are shown at equal magnification (×100). A, LF1+hTERT small. B, LF1+hTERT large. C, IMR-90+hTERT small. D, IMR-90+hTERT large. E, HCA2+hTERT small. F, HCA2+hTERT large.](image-url)
by the inability to isolate a sufficient number of living senescent cells from the growing mass culture. To overcome this limitation, we developed a method to purify senescent cells by flow cytometry using a FACS cell sorter. Senescent cells are characterized by increased cell volume; therefore, we have sorted cells according to size (Fig. 1). To allow for subsequent biochemical analysis and to avoid fluctuations in cell volume that might result from the use of fixatives, we sorted live unfixed cells. To determine the cut-off parameters for selection of large-senescent and small-young cells for each cell line, we first analyzed the size distribution of young (PD 18–24) and senescent (PD 55–75) cultures of normal IMR-90, HCA2, and LF1 fibroblasts that were not expressing hTERT. Two areas were selected: area A contained the majority of young cells (>70%); and area B contained the large-senescent cells with <1% of young cells (Fig. 1). We observed that in normal senescent cultures, a larger percentage of cells was in area B when compared with young cultures. In addition, the percent of SA-β-galactosidase-positive cells corresponded to the percent of large cells in the population.

Following sorting, aliquots of cells were plated and stained for SA-β-galactosidase activity (Table I) (Fig. 2). The large cells contained 60–95% SA-β-galactosidase-positive cells and had a typical senescent morphology, whereas the small cells were SA-β-galactosidase-negative and had a young morphology indicating that the sorting procedure that we used was capable of selectively isolating senescent cells.

To test whether isolated cells had other senescent features, we analyzed the level of p21 and Rb status in the sorted cells. Large-senescent cells had elevated levels of p21 and hypophosphorylated Rb compared with the small-young cells from the same culture (Fig. 3), similar to what has been observed in normal replicative senescent cells.

To directly test whether excessive telomerase activity can induce a senescent-like phenotype, we transfected young HCA2 fibroblasts with a hTERT expression vector and examined the newly transfected cells. hTERT cDNA was cloned into pcDNA3.1(+) vector (Promega) under the control of CMV promoter to enable strong expression. The resulting plasmid pcDNA3.1-hTERT or pcDNA3.1 vector as control was transfected into HCA2 normal human fibroblasts at PD 21. Transfection was done with either FuGENE 6 reagent or Amaxa electroporator using the maximum allowed DNA concentration in order to introduce a high number of plasmid copies per cell. Telomerase activity was analyzed by TRAP assay 3 days after transfection and compared with telomerase activity in the hTERT-immortalized HCA2 cells. Telomerase activity in the newly transfected cells was significantly stronger than in the immortalized cell line (Fig. 6A). We then allowed the cells to divide for 10 days and compared the number of senescent cells in the pcDNA3.1-hTERT and control transfections using SA-β-galactosidase staining. pcDNA3.1-hTERT-transfected cell population contained 10 times more SA-β-

**Fig. 3. Status of Rb and p21 in senescent cells isolated from hTERT-immortalized cell cultures.** Following sorting, aliquots of 10⁵ cells were used for Western blot analysis with the antibodies against Rb (A) and p21 (B). The membranes were then hybridized with anti-actin antibodies (C) to demonstrate equal loading of samples.
galactosidase-positive cells than the control cells transfected with the pcDNA3.1 vector (Fig. 6B). Western blot analysis also showed the induction of p21 in the pcDNA3.1-hTERT-transfected cells compared with control cells (Fig. 6C). We also examined the division potential of pcDNA3.1-hTERT-transfected cells by colony size distribution. pcDNA3.1-hTERT-transfected cells formed smaller clones than the control cells, and the majority of the clones stopped proliferation after 1–4 divisions (Fig. 6D). These results demonstrate that excessive telomerase activity can induce a senescent-like phenotype in normal human fibroblasts.

**DISCUSSION**

Many years ago, Martinez et al. (19) and Pereira-Smith and Smith (20) described the fact that in cultures of tumor-derived and SV40-transformed immortal human cells, there was a sub-population of cells with very limited division potential. These studies involved a clonal analysis of the proliferative capacity of the cells. The percentage of these cells varied from 1 to 30% depending on the cell line studied, and we have observed this in many other immortalized cell lines.² The plausible explanation at the time was that this was a consequence of the genomic instability of the cells, causing some cells to drop out of the cell cycle. However, in this study, we have analyzed the similar phenomenon in hTERT-immortalized cells that are karyotypically normal.

To examine the factors that trigger senescence in immortalized cell populations, we have developed a method to purify living senescent cells from the populations of human fibroblasts immortalized by hTERT. This allowed us to perform analysis of telomeres and telomerase activity in these cells. We have found that telomere length in the senescent cells was not different from the immortal cells of the same culture. This result corroborates an earlier observation (7) that did not find a direct correlation between telomere length and immortalization status. The majority of fibroblast cultures expressing hTERT have longer than "physiological" telomeres; however, such long telomeres have not been associated with growth arrest or apoptosis (8, 21). Surprisingly, we found that telomerase activity was significantly elevated in senescent cells compared with immortal proliferating cells from the same culture. Similar results were obtained with three different cell lines of immortalized human fibroblasts. hTERT cDNA was expressed from a constitutive LTR promoter, which is not known to be up-regulated in senescent cells. We also did not observe any activation of the LTR promoter in senescent cells in our control experiments with an LTR-GFP construct. When high levels of telomerase activity were induced in the young cells by overexpression of hTERT from a plasmid under a CMV promoter, some of the newly transfected cells acquired senescent-like phenotype and entered growth arrest. Therefore, these results suggest that excessive telomerase activity may induce the senescent-like phenotype.

The idea that high telomerase activity may induce senescence seems reasonable if telomerase is viewed as a proliferative signal. A growing body of data suggests that hyperproliferative signals may induce a senescent-like state in normal human cells. Thus, a loss of proliferative potential is induced by

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² O. M. Pereira-Smith, unpublished observations.
FIG. 6. Effect of hTERT overexpression in HCA2 normal human fibroblasts. HCA2 cells at PD21 were transfected with the plasmid that contains hTERT cDNA under the CMV promoter. A, typical telomerase assay gel is shown. Sorted cells were lysed, and protein concentrations were measured. Serial dilutions of the protein extract were prepared, and TRAP assay was performed with 1,000, 100, and 10 ng of protein. TRAP assay was performed using TRAPEze kit (Interogene) according to manufacturer’s instructions. Telomerase-positive cells provided with the TRAP assay kit were used as a positive control, and 500 ng of extract was used. B, quantitation was done using Molecular Dynamics densitometer and ImageQuant software. The intensity of the positive control lane was taken as 100%. The experiment was repeated three times, and error bars represent mean ± S.D.

FIG. 5. Telomerase activity in senescent and proliferating fibroblasts from immortalized cultures determined by TRAP assay. A, typical telomerase assay gel is shown. Sorted cells were lysed, and protein concentrations were measured. Serial dilutions of the protein extract were prepared, and TRAP assay was performed with 1,000, 100, and 10 ng of protein. TRAP assay was performed using TRAPEze kit (Interogene) according to manufacturer’s instructions. Telomerase-positive cells provided with the TRAP assay kit were used as a positive control, and 500 ng of extract was used. B, quantitation was done using Molecular Dynamics densitometer and ImageQuant software. The intensity of the positive control lane was taken as 100%. The experiment was repeated three times, and error bars represent mean ± S.D.
the expression of activated RAS (22) or RAF (23) or overexpression of E2F1 (24). This type of premature senescence is thought to be a fail-safe mechanism that prevents normal cells that experience potentially oncogenic insults from proliferating.

Telomerase activity is reconstituted by overexpression of hTERT from a strong promoter, which provides much higher levels of expression than is normally achieved from the endogenous hTERT promoter. In normal tissues, telomerase activity is tightly regulated and the hTERT promoter is turned on in specific cell types only for limited time periods, for example, during clonal expansion of lymphocytes (25) or in endometrial specific cell types only for limited time periods, for example, during clonal expansion of lymphocytes (25) or in endometrial cells during the proliferative phase of the menstrual cycle (26–29). Therefore, it is reasonable to assume that there are some feedback mechanisms to down-regulate the hTERT promoter when telomerase activity is no longer needed. When hTERT is expressed ectopically from a constitutive viral promoter, the cell is unable to down-regulate its expression and may respond by arresting growth.

The level of hTERT expression is not the only determinant of the level of telomerase activity, because it is also regulated by phosphorylation (30–32) and nuclear targeting (32). Furthermore, telomerase is not a classical oncogene, and hTERT-immortalized cells do not display the characteristics of cancer cells (33). This may explain why telomerase-induced senescence is not as robust a process as oncogene-induced senescence and is only induced in a fraction of cells in which the telomerase activity becomes too high. In summary, we present the first evidence that high telomerase activity could induce a senescence-like phenotype. Understanding the mechanisms of this process will be of great importance in developing applications of hTERT in cell therapy and tissue engineering.

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