Immortalization of human small airway epithelial cells by ectopic expression of telomerase

Chang Q.Piao1,*, Li Liu1,†, Yong L.Zhao1,†, Adayabalam S.Balajee1, Masao Suzuki3 and Tom K.Hei1,2

1Center for Radiological Research, College of Physicians and Surgeons and 2Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY 10032, USA and 3International Space Radiation Laboratory, National Institute of Radiological Science, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan

*To whom correspondence should be addressed at: Center for Radiological Research, Columbia University, 630 West 168th Street, New York, NY 10032, USA. Tel: +1 212 305 0846; Fax: +1 212 305 3229; Email: cp16@columbia.edu

Two immortalized human airway epithelial cell lines were established by the ectopic expression of human telomerase reverse transcriptase (hTERT). These cell lines have been continuously cultured for >200 population doublings (PDs). They are characterized by an overexpression of hTERT mRNA, elongated telomere length and higher telomerase activity. Early passage of these cells (20 PDs) expressed the p16 protein at a level comparable to their parental cells. In later passages (>150 PDs), p16 protein was decreased but recovered to the early passage level upon treatment with a methylation inhibitor, 5-Aza-CdR. Chromosome analysis showed a near-diploid karyotype albeit with gain or loss of certain chromosomes and a few stable translocations in both cell lines. No p53 gene alterations were found in these cell lines. They remained anchorage dependent in growth and were non-tumorigenic in nude mice. These two cell lines are the first reported immortalized human airway epithelial cell lines by hTERT expression without incorporation of virus or other genes, which may serve as a useful model system for studies on bronchial carcinogenesis.

Materials and methods

Cell culture

Normal human small airway epithelial cells (SAEC) from two non-smoker donors at passage 2 were purchased from Bio-Whittaker/Clonetics (Walkersville, MD). One of the donors was a 54-year-old female (SAEC-F) and the other one was a 25-year-old male (SAEC-M). The cells were cultured in a serum-free SAGM medium supplemented with various growth factors supplied by the manufacturer and maintained at 37°C in a humidified 5% CO2 incubator. These primary cells could only be cultured for about 10 passages in our laboratory before entering crisis and senescing.

Transfection of hTERT

The construct pBabe2t, in which the cDNA encoding hTERT was subcloned into the retroviral vector pBabe under the control of the promoter present in the Molony murine leukemia virus LTR, was kindly provided by

Abbreviations: hTERT, human telomerase reverse transcriptase; PD, population doubling; SAEC, small airway epithelial cells; SAGM, small airway epithelial growth medium.

These authors contributed equally to this work.
Dr Homayoun Vaziri (7). The retroviral constructs were packaged using the highly efficient and helper-free cell line Phoenix A (ATCC). Phoenix A cells were plated in 10-cm diameter dishes and transfected when reaching 80% confluence, with 4 μg/ml of retroviral plasmid DNA in 10 ml of medium using lipofectAMINE plus reagent (Gibco-BRL, Rockville, MD), according to the manufacturer’s instructions. The viral titer was assayed at 48 h post-transfection using NIH 3T3 cells and titers of >3×10^5 transducing units/ml were obtained. The SAEC-F and SAEC-M cells at passage 5 were infected with the viral supernatant in the presence of 4 μg/ml of polybrene. After a 24-h incubation period, the culture medium was replaced with complete SAGM medium. Colonies resistant to G418 were formed after 3–4 weeks of continuous culture. These colonies were then trypsinized and propagated as AE-F-hTERT and AE-M-hTERT cell lines. The two cell lines generated were, therefore, derived from mixed colonies. It should be noted that there was no colony formation in comparably treated parental cells infected with the viral vector (pBabe) alone, all of which senesced and died.

Cytokeratin staining

Cells were grown in chamber slides for 3 days and then fixed with acetone for 20 min at −20°C. To detect cytokeratin expression, a monoclonal antibody against human cytokeratin 18 (Santa Cruz) coupled with Vectastain ABC kit and DBA substrate kit (Vector Laboratories, Burlington, CA) was used, according to the manufacturer’s instructions (18).

Detection of hTERT by RT-PCR

Total RNA was isolated from cells by the use of TRIZol Reagent (Gibco-BRL). The primers 5'-CTCAGCTTTGGAAGCTCTCA-3' and 5'-ATAAGAATGCGGCCGCTCAGTGGGGAAGAAGTGGAGA-3' were used. The hTERT mRNAs were amplified using Titan One Tube RT-PCR kit (Roche, Summerville, CA), according to the manufacturer’s instructions.

Determination of telomerase activity and telomere length

Telomerase activity was analyzed using the TRAPeze kit (Intergen, Purchase, NY), and telomere length was determined by the Telo TAGGG TeloMer Length Assay kit (Roche, Summerville, CA), according to the instructions of the manufacturer.

Detection of p16 gene by PCR

PCR analysis was performed for each of the three exons of the p16 gene. The primers used were exon 1: 5'-GGAGGAAGAAGGAGGGGAG-3', 5'-ACTTCTGTCTCCAGATCGT-3', and exon 3: 5'-TGTTGACGCTGCAAACTCAG-3'. DNA was isolated from cells, and amplified using Ready To Go PCR Beads (Amersham Pharmacia Biotech), according to the manufacturer’s instructions. The PCR products were analyzed by electrophoresis in 2% agarose gel.

Analysis of p16 protein by western blot

Protein was extracted from the cells by lysis in extraction buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate and 1 mM phenylmethylsulfonyl fluoride). The protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equivalent amounts of protein (100 μg) were fractionated by electrophoresis in SDS-polyacrylamide gel. The protein was subsequently transferred to Immobilon-P nitrocellulose membrane under semi-dry conditions. The rabbit polyclonal antibody p16-H156 (Santa Cruz) was used as a primary antibody. Expression of the p16 protein bands was detected using goat anti-rabbit IgG-Ap (Santa Cruz) as a secondary antibody and coupled with ProtoBlot II AP system (Promega, Madison, WI) as a substrate.

Demethylation experiments

5-Aza-2'-CdR (Sigma, St Louis, MO) was dissolved in 50% acetic acid as a 10 mM stock solution and frozen at −80°C. A working solution was prepared in SAGM medium. Exponentially growing cells were treated with 5-Aza-CdR at a dose of 5 μg for 72 h. Cells were harvested at 96 h after the initial treatment to ensure a complete recovery from the immediate toxic effect of 5-Aza-CdR. Expression of the p16 protein was analyzed by western blot. Untreated cells under similar conditions were used as a control.

Sequencing of p53 coding regions

Total RNA was isolated from the two hTERT-immortalized cells at their 150 population doublings (PDs) by TRIZol Reagent (Gibco-BRL). First-strand cDNA was synthesized using 5 μg of total RNA with superscript II reverse transcriptase and oligo (dT) primer (Gibco-BRL). The entire open reading frame of p53 cDNA (1.2 kb) was then PCR-amplified with one-tenth of the synthesized cDNA by high-fidelity MasterAmpTM DNA polymerase (Epicerin, Madison). Primers that contain HindIII and NotI restriction enzyme recognition sites, 5'-ACCAAGCTGTACGAACCCTCTCGAGATC-3', 5'-ATAAGAATGCGGGCGTCTAGTGGAAGAAGTAGGAGA-3', were used. PCR amplification was performed for 30 cycles at 94°C (30 s), 56°C (1 min) and 68°C (2 min) followed by 10 min of extension at 68°C. After digestion with HindIII and NotI, the PCR products were cloned into the HindIII–NotI site of pcR/CMV2 vector (Invitrogen, Carlsbad, CA). The plasmid DNA prepared from positive clones was sequenced by p53-specific primers.

Cytogenetic analysis

M-FISH analysis was performed with multicolor fluorescence in situ hybridization (19). For metaphase chromosome preparation, cells in the exponential growth phase were treated with 0.5 μg/ml of colchicine for 3 h. Cells were trypsinized and treated with a hypotonic solution (0.56% KCl) for 25 min at 37°C. Cells were then fixed in acetic acid/methanol (1:3) and the metaphase chromosome spreads were prepared by air-dry technique. The slides were aged for a week at 4°C for M-FISH analysis. M-FISH and post-hybridization washings were performed according to the manufacturer’s specifications (MetaSystems, Belmont, MA). A cocktail of DNA probes specific for all the human chromosomes (24XcYte, Metasystems) was used for M-FISH. After pepsin (0.005% in 0.1 N HCl) treatment for 2 min at 37°C and post-fixation of the slides for 10 min in formaldehyde (3%), the slides were heat denatured together with the DNA probes (12 μl/slide) at 75°C for 5 min and incubated for 2–4 days at 37°C in a moist chamber. The slides were briefly washed in 1× SSC at 75°C to remove the non-specific binding of the probe. Images were captured using a Nikon Axioplan fluorescence microscope and the metaphases were analyzed using Metasystems software. At least 10 metaphase spreads were analyzed for each cell line to characterize their chromosome constitution.

Anchorage-independent assay

To test for soft-agar colony growth capacity, hTERT-immortalized cells were plated at a density of 1×10^3 cells in 3 ml of 0.7% agar base in a 60-mm diameter culture dish. Cultures were fed every 3 days and colonies with >50 cells were scored after 4 weeks in cultures under a dissecting microscope.

Tumorigenicity assay

Male Nu/Nu mice from Harlan Sprague-Dawley (Indianapolis, IN) were used. Each animal was anesthetized lightly with isoflurane (Anaquest, Madison, WI) and injected subcutaneously at two different sites with 5×10^5 cells in 0.2 ml of saline. Five animals were injected for each cell line. Animals were maintained under sterile conditions for 4 months and palpated for tumor appearance once a week.

Results

Both the parental SAEC-F and SAEC-M cells ceased dividing after ~10 passages in culture. The SAEC-F and SAEC-M cells transfected with the empty pBabe vector senesced soon after transfection as well. In contrast, SAEC-F and SAEC-M cells transfected with pBabest2 vector containing the cDNA encoding hTERT, formed colonies and were resistant to the G418 treatment. Two isolated, mixed clonal lines have been continuously cultured for >20 months, with >200 PDs since the original G418 selection began. The cell lines were designated as AE-F-hTERT and AE-M-hTERT, respectively. Both the cell lines have a cobblestone epithelial morphology (Figure 1) and showed a strong positive staining for cytokeratin, indicative of their epithelial origin (Figure 2). In contrast, human fibroblasts, which were used as a negative control, did not show any immunocytochemical staining for the epithelial marker. At ~150 PDs, the population doubling time was 24 h for AE-F-hTERT and 20 h for AE-M-hTERT compared with 27 h for the parental SAEC cells at passage 5 (Figure 3). The plating efficiency of SAEC cells varied between 12 and 15%, whereas the AE-F-hTERT and AE-M-hTERT ranged from 20 to 25%.

Expression of hTERT mRNA, telomerase activity and telomere length were analyzed at >100 PDs in both AE-F-hTERT and AE-M-hTERT cells and the results were compared with the parental SAEC cells. While hTERT mRNA and telomerase activity were overexpressed in AE-F-hTERT and
Fig. 1. Morphology of the hTERT-immortalized human airway epithelial cells at 150 PDs. (A) AE-F-hTERT cells and (B) AE-M-hTERT cells (×100).

Fig. 2. Immunocytochemical staining of cytokeratin. (A) AE-M-hTERT cells and (B) AE-F-hTERT cells with positive cytokeratin stain. (C) Control fibroblast cells with negative cytokeratin stain.

Fig. 3. Growth curves of AE-F-hTERT and AE-M-hTERT cells at 150 PDs, SAEC cells at passage 5.

Fig. 4. hTERT mRNA expression by RT-PCR. 1, Marker; 2, SAEC-F cells; 3, AE-F-hTERT cells; 4, AE-M-hTERT cells; 5, SAEC-M cells. hTERT mRNA was expressed in AE-F-hTERT and AE-M-hTERT cells and not detectable in SAEC-F and SAEC-M parental cells.

Fig. 5. Expression of telomerase activity. 1, SAEC-F; 2, SAEC-M; 3, AE-F-hTERT; 4, AE-M-hTERT at 100 PDs; 5, negative control. AE-M-hTERT cells were heated in 85°C for 10 min. Telomerase activity was inactivated. 6, Positive control cells from kit. Telomerase activity was overexpressed in AE-F-hTERT and AE-M-hTERT cells, but non-detectable in their parental control SAEC-F and SAEC-M cells.
AE-M-hTERT cells, they were not detectable in the parental SAEF cells (Figures 4 and 5). Furthermore, the telomere length was also significantly elongated, compared with the parental SAEF cells (Figure 6).

Since p16 inactivation has been suggested to be a prerequisite for the immortalization of human mammary epithelial cells by hTERT, the status of p16 protein in AE-F-hTERT and AE-M-hTERT cells was ascertained by several different approaches. Analysis of the p16 gene by PCR amplification demonstrated no deletion in any of the three exons examined in AE-F-hTERT and AE-M-hTERT cells (data not shown). Furthermore, western blot analysis of the p16 protein expression in early passages of hTERT-immortalized cells (≤50 PDs) showed a similar level of expression when compared with their parental counterparts. However, the level of p16 expression was decreased by >5-fold after 150 PDs (Figure 7A). The decrease in p16 expression in AE-F-hTERT and AE-M-hTERT cells at 150 PDs could be recovered to control levels by treatment with the methylation inhibitor, 5-aza-cytidine (5 mM for 72 h, Figure 7B). The results suggested that the p16 gene could be partially methylated during a prolonged growth in culture after hTERT transfection.

To determine the status of the p53 gene in hTERT-immortalized AE-F-hTERT and AE-M-hTERT cells, the coding sequences of exon 2 through part of exon 11 of the p53 gene at 150 PDs were analyzed. Results showed no genetic alterations, and the sequences were found to be identical to those reported in the GeneBank (Accession No. NM_000546).

Karyotypes of the hTERT-immortalized cell lines were analyzed by M-FISH and the representative pictures are given in Figure 8. Results from the AE-F-hTERT cells at 200 PDs showed that the diploid number of chromosomes varied from 44 to 53 with a gain (>2) or loss (<2) of different chromosomes. M-FISH analyses revealed extra copies of chromosomes 2, 15 and 20 in >50% of the cells and chromosome 5 in >90% of the metaphases analyzed. Loss of chromosomes randomly involved 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 18 and 21. In addition, reciprocal and non-reciprocal translocations were observed involving chromosomes 5/9, (4.3%) 2/9 (8.7%), 21/5 (4.3%) and 15/5 (4.3%). The AE-F-hTERT was relatively more stable, both at 20 and 200 PDs with a diploid number of 44–47 and >75% of the cells showed a normal diploid number of 46 chromosomes. M-FISH revealed the gain of chromosomes 5 and 15 and the loss of chromosomes 8, 9 and 22. In 80–90% of the metaphases examined, translocations involving chromosomes 5, 4 and 12 were observed.

Finally, the two immortalized cell lines showed no colony growth in soft agar and were non-tumorigenic in nude mice when examined at >150 PDs (data not shown).

Discussion

It has been suggested that the replicative senescence represents a barrier that a cell must overcome in order to become immortal, and immortalization is an essential step during the malignant transformation of normal cells (20, 21). Progressive telomere shortening has been proposed to be one critical determinant of senescence. Telomeres provide a protective function, preventing end-to-end chromosomal fusions by capping the end of mammalian chromosomes. With each round of DNA replication and cell division, the telomeric DNA sequence is under-replicated, leading to the progressive shortening of the telomeres. Eventually, the shortened telomeres may no longer be able to protect the end of the chromosome, and the unprotected chromosomal DNA end may release a senescence-inducing signal causing the cells to cease division (22, 23). Recent reports demonstrated that the ectopic expression of hTERT, the catalytic subunit of telomerase, restores telomere length in normal human fibroblasts and several other cell types and allows them to circumvent senescence and become immortalized (6–8).

Available evidence suggests that in certain human cell types, such as human keratinocytes or mammary epithelial cells, in which telomerase expression alone is insufficient to bypass senescence, the additional inactivation of p16INK4a by genetic or epigenetic mechanisms is required to render the cells immortal (10–12). However, more recent data indicated that the use of a fibroblast feeder system can bypass the need for p16INK4a inactivation in the immortalization of either a keratinocyte or a mammary epithelial cell line (15, 16). In the present study, two immortalized human airway epithelial cell lines were established by the ectopic expression of hTERT. We found that expression of the p16 protein in early passages of hTERT-transfected cells was similar to parental SAEC cells and no deletions in the p16 gene were detected. While expression of the p16 protein level obviously decreased in
later passages of the immortalized cells, p16 level could be recovered by treatment with the methylation inhibitor (5-Aza-CdR). 5-Aza-CdR appears to incorporate into the cellular DNA, where it inhibits DNA methyltransferase that methylates cytosine residues in eukaryotic DNA, and acts as a non-competitive inhibitor of the normal methylation maintenance process. 5-Aza-CdR has been widely used as an experimental tool for demethylation, especially in studies of the p16 gene (24-26). The results suggest that the p16 gene may, at least, be partially methylated and thus silenced in the later passages during the immortalization process of human epithelial cells by hTERT transfection.

There is evidence that the cell type and genetic background of the donors may also influence the pathways of immortalization or the development of neoplastic transformation. Milyavsky et al. (27) showed that the prolonged culture of telomerase-immortalized human fibroblasts led to an expression of premalignant phenotype. In contrast, Akagi et al. (28) provided the evidence that normal human diploid fibroblasts are refractory to oncogene-mediated transformation. Wise et al. (29) also demonstrated that the life span extension of primary human bronchial fibroblasts by telomerase expression did not affect Cr(VI)-induced cytotoxicity or genotoxicity. In this study, the two hTERT-immortalized cell lines derived from different genetic background displayed varying degrees of chromosomal alterations. The AE-F-hTERT cells, derived from a 54-year-old female, showed a near-diploid karyotype while the AE-M-hTERT cells, derived from a 25-year-old male showed a hyper-diploid karyotype. This observation, along with the relatively stable genotype between the very early passage (20 PDs) and late passage (200 PDs) AE-F-hTERT cells, suggested that the cytogenetic alterations in the two immortalized cell lines are probably due to factors other than the hTERT expression. Based on the negative results of colony growth in soft agar and the lack of tumorigenicity in nude mice, our data clearly indicated that the two immortalized cell lines did not exhibit any neoplastic transformation phenotype, at least up to 150 PDs.

The most common method to generate immortalized cells is the use of oncogenic viruses, such as Simian virus 40 or human papilloma virus, in which both the p53 and Rb tumor suppressing pathways are frequently inactivated (30,31). Immortalization of primary human airway epithelial cells by a combination of SV40 and hTERT transfection has been reported previously (32). Although inactivation of both the p53 and the pRb pathway is essential for the immortalization of human cells, the process is not sufficient in preventing the terminal proliferation arrest, which must occur before a cell could become immortal (33,34). SV40-transformed human cells continue to lose telomeric DNA during their extended life span and...
telomeres continue to shorten until the cell can no longer divide, resulting in crisis. In contrast to normal somatic cells, immortal cells do not exhibit telomere shortening during continuous in vitro growth (5,35,36). Furthermore, the ectopic expression of hTERT is sufficient for pre-crisis SV40-transformed cells to escape crisis and become immortal (37–39). These data suggest that human cells must activate the telomere maintenance mechanisms in order to overcome the terminal proliferation arrest of crisis. The telomere model of senescence extends to explain the escape from crisis and immortalization of human cells. Our study summarizes the first successful immortalization of human airway epithelial cells by the ectopic expression of hTERT with non-tumorigenic phenotypes.

There is evidence that the senescence mechanism in keratinocytes has a p53-dependent component, which is independent of the telomere status (32). Furthermore, Opitz et al. showed that primary oral keratinocytes could be immortalized by cyclin D1 overexpression and p35 inactivation in a telomerase-independent mechanism (40). We did not find p53 gene alterations in our two immortalized SAEC cell lines. Consistent with other published findings (6,7), our results demonstrated that the exogenous hTERT expression could directly confer an unlimited replicative potential without disrupting the p53 pathway.

The ability of hTERT expression to bypass senescence, however, is not universal. In several independent studies, exogenous hTERT expression, despite conferring high levels of telomerase activity, fails to induce a life span extension in some human cells. Disruption of the pRb/p16INK4 pathway in addition to telomere expression has been found to be required for true immortalization (10,37). The immortalized human cells, by ectopically expressing hTERT, do not progress to tumorigenicity. Even after the introduction of activated oncogenes, the telomerase-immortalized cells may differ from cells that have p53 and pRb gene defects in their tumorigenic potential (41,42). Thus, although some cells expressing hTERT could be immortalized, hTERT itself is not an oncogene. The hTERT-immortalized SAEC cell model developed by us in our laboratory should be of great value in studying the mechanisms of malignant transformation induced by environmental carcinogens.

Acknowledgements

The authors thank Ms Sarah Baker for critical reading of the manuscript. This study was supported by National Institute of Health grants ES 05786, ES 11804, CA 49062, Superfund grant P42 ES 10349 and Environmental Center grant ES 09189.

References


Received July 20, 2004; revised December 23, 2004; accepted January 8, 2005

Immortalization of human SAEC