Senescence Delay of Human Diploid Fibroblast Induced by Anti-sense p16INK4a Expression*

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p16INK4a, a tumor suppressor gene that inhibits cyclin-dependent kinase 4 and cyclin-dependent kinase 6, is also implicated in the mechanisms underlying replicative senescence, because its RNA and protein accumulate as cells approach their finite number of population doublings in tissue culture. To further explore the involvement of p16INK4a in replicative senescence, we constructed a retroviral vector containing antisense p16INK4a, pDOR-ASp16, and introduced it into early passages of human diploid fibroblasts. The introduction of this construct significantly suppressed the expression of wild-type p16INK4a. It also imposed a finite increase in proliferative life span and significant delay of several other cell senescent features, such as cell flattening, cell cycle arrest, and senescence-associated β-galactosidase positivity. Moreover, telomere shortening and decline in DNA repair capacity, which normally accompany cell senescence, are also postponed by the ASp16 transfection. The life span of fibroblasts was significantly extended, but the onset of replicative senescence could not be totally prevented. Telomerase could not be activated even though telomere shortening was slowed. These observations suggest that the telomere pathway of senescence cannot be bypassed by ASp16 expression. These data not only strongly support a role for p16INK4a in replicative senescence but also raise the possibility of using the antisense p16INK4a therapeutically.

Primary mammalian cells exhibit finite proliferative potential in vitro, the so-called Hayflick limit (1). They undergo a limited number of population doublings (PD) before entering a state of permanent growth arrest, referred to as senescence, in which they remain alive and metabolically active but are completely refractory to mitogenic stimuli (1). Although various physical and chemical factors (2–4) are able to induce a senescence-like state (pre-mature senescence) at a PD level below the Hayflick limit, cell senescence is considered as genetically programmed (5–7). Some tumor suppressors and cell cycle inhibitors are indicated to be involved in the cell senescence process. p16INK4a has been known as a tumor suppressor gene, because it was identified as a cell cycle inhibitor that negatively regulates the cell cycle kinases CDK4 and CDK6 (8). Its loss or inactivation has been found in a broad range of tumors (9, 10). Meanwhile, a substantial body of evidence suggests a role for p16INK4a in senescence of cultured cells. p16INK4a accumulation occurs in diploid fibroblasts, T-lymphocytes, keratinocytes, mammary epithelial cells, uroepithelial cells, and prostate cells as cells enter senescence. p16INK4a also can induce some features of cell senescence (6, 11–14). Expression of p16INK4a in glioma cells, diploid fibroblasts, and squamous carcinoma cells is associated with cell flattening and expression of the senescence-associated marker, pH 6.0 optimum β-galactosidase (SA-β-gal) (15). Sustained p16INK4a expression can cause a prolonged growth inhibition in several tumor cell lines and can cooperate with p53 to induce apoptosis (16). However, the actual mechanisms by which p16INK4a imposes cell senescence remain to be elucidated. A key unresolved issue is whether p16INK4a accumulation is the cause or result of cell senescence. Our studies try to address this question by sustained overexpression or inhibition of p16INK4a in human diploid fibroblasts, and further, we explore the possible regulation pathways of p16INK4a in cell senescence.

MATERIALS AND METHODS

Cell Culture and Medium—Human embryonic lung diploid fibroblast 2BS cells (obtained from the National Institute of Biological Products, Beijing, China) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% heat-inactivated fetal bovine serum. All cells were grown in medium containing 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO₂. (17, 18).

Construction of Retroviral Vectors and Transfection of Human Diploid Fibroblasts—p16INK4a cDNA in pBluescript was a kind gift from Dr. David Beach (Howard Hughes Medical Institute, Cold Spring Harbor, NY). The full-length p16INK4a cDNA (800 bp) was placed into the retroviral vector pDOR-neo (19) in both orientations. The p16 cDNA is driven by the 5′-long terminal repeat promoter, and the neoR cassette is driven by the SV40 early promoter. These two constructs were termed pDOR-p16 and pDOR-ASp16. They were transfected by Lipofectin reagent (Life Technologies, Inc.) into 4×10⁵ early passage 2BS cells (PD20) (60–70% confluence). pDOR-neo vector was also transfected as a control. The transformants, after sustained selection by G418 (400 μg/ml Life Technologies, Inc.), were termed as 2BS/p16, 2BS/ASp16, and 2BS.neo, respectively.

Southern, Northern, and Western Blot—10 μg of genomic DNA of each cell line, which had been digested with HindIII and PstI, was used for Southern blotting. DNA was electrophoresed on a 0.8% agarose gel, blotted onto nitrocellulose membrane (Bio-Rad), and hybridized to either a p16 cDNA or a neo probe labeled with [α-32P]dCTP by the Prime-a-Gen labeling system (Promega). The membrane was exposed to a Kodak x-ray film at ~80 °C (19). Total RNA was isolated from cells by guanidinium isothiocyanate (20). 15-μg samples of total cellular RNA were electrophoresed on a denaturing agarose gel, blotted onto nylon membrane (Hybond-N; Am-
ersham Biosciences), and probed with p16INK4a DNA. β-Actin was used as an internal control for normalization (19, 20).

For Western blotting, 100 μg of cell lysates were run on 12% SDS-polyacrylamide gel, transferred to nitrocellulose membrane (Bio-Rad), and probed with anti-p16INK4a monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The signals were detected with alkaline phosphatase staining.

**Measurement of PD**—Clones of transfected cells were obtained with G418 selected cultures. The PD number of a clone grown to about 10⁶ cells (70–80% confluence of a 100-mm culture bottle) is about 20. Then we started to count the doubling number of transfected cells, the 2BS/ASp16 was counted as A0, the 2BS/p16 as P0, and the 2BS/neo as N0. However, the actual PD number of the transformants should be increased by 10 PD when they are compared with the untransfected cells.

**Cell Cycle Analysis**—When cells reached 70–80% confluence, they were washed with PBS, detached with 0.25% trypsin, and fixed with 75% ethanol overnight. Samples were resuspended in 0.5 ml PBS and stained with propidium iodide in the dark for 30 min, and the DNA contents were measured by fluorescence-activated cell sorting on a Becton-Dickinson FACScan flow cytometry system. The data were analyzed using CellFIT software.

**SA-β-Gal Staining**—Cells were washed twice in PBS, fixed for 3–5 min at room temperature in 3% formaldehyde, and washed with PBS again. Then cells were incubated overnight at 37 °C (without CO₂) with freshly prepared SA-β-gal stain solution (1 mg/ml X-gal, 40 μM citric acid/sodium phosphate, pH 6.0, 5 mg potassium ferrocyanide, 5 mg potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂). (21).

**Telomere Length Assay**—30 μg of genomic DNA per sample was digested by restriction enzyme EcoRI, electrophoresed on a 0.8% agarose gel, and transferred to nylon membrane. The membrane was baked at 80 °C and hybridized with a 5'-end 32P-labeled telomeric (TTAGGG)n repeat probe. Hybridization and washing were carried out as described. The membrane was autoradiographed on Kodak XAR-5 x-ray film for 12–24 h at room temperature. The image was analyzed using NIH Image software. The mean telomere restriction fragment output, and L₀ is the length of the DNA at position i (22).

**Telomerase Assay**—The telomerase repeat amplification protocol assay was performed with several modifications to the original protocols (23, 24) to decrease false positives and effects of inhibitors of PCR amplification. Cells were lysed in CHAPS lysis buffer. The protein concentration of lysates was measured using the Bradford method. 6 μg of each cell extract were incubated initially in the presence of [γ-32P]CTP-labeled TS (5'-AACTCCGTCTGAGCAGAGGATT-3') oligonucleotide (0.1 μg), Taq DNA polymerase (5 units), and dNTPs (50 μM/m)litter. After 2 h at 37 °C, the telomerase was inactivated at 90 °C. C' (25) (TGCGTTTGGTTTCCTTAA83') oligonucleotide and dCTP, dGTP, and dATP were then added to the reaction for 30 PCR cycles. The 6-bp ladder patterns of PCR products were analyzed on 12% polyacrylamide nondenaturing gels and exposed to x-rays film indicate the telomerase activity.

**Undigested DNA Synthesis (UDS) Assays**—10⁶ cells were inoculated into each culture dishes (35 mm; Costar). Monolayer cells were washed once with serum-free medium and incubated in the medium containing 0.5% serum for 3 days to reach quiescence. Cells were washed once with serum-free medium and incubated in the media containing 5 mM hydroxyurea (HU; Sigma) and incubated for 1 h. Then they were exposed to H₂O₂ by fresh prepared SA-Galactosidase (SA-Gal) staining (26). This state is characterized by irreversible growth arrest and morphologically enlarged, flattened cells with prominent lipofuscin granules. To determine the effects of the p16INK4a expression level on cell senescence, the clones of transfected cells were obtained by sustained selection with G418 after transfection of pDOR-p16 and pDOR-ASp16. They were analyzed for the relative senescence markers compared with the untransfected 2BS cell control and pDOR-neo vector-transfected cell control, all of which came from the same batch of early passage cells.

**Telomerase Activity**—To observe the proliferative changes after transfection, the growth of the transformants and the control cells were compared (Fig. 2). The growth of 2BS/ASp16 (A5, PD45) cells are like young (PD24) cells, whereas the 2BS/p16 (P1, PD41) showed nearly complete growth inhibition like senescent cells. As the control, transfection of pDOR-neo vector had no significant effect on the cell growth rate or the growth potential compared with the same batch of normal cells without transfection.

**Asp16 Postpones G1 Cell Cycle Arrest—p16INK4a can inhibit the activity of CDKs, thereby blocking the entry of proliferating cells from G₀ to S phase. To determine the mechanisms of the growth rate inhibition described above, the cell cycle profile analysis was performed by flow cytometry (Fig. 3). In contrast to the irreversible G₁ arrest imposed by pDOR-p16 transfection, pDOR-ASp16 transfection postponed the G₁ irreversible arrest. Control, 2BS-neo cells had a similar percentage of cells in G₁ phase as normal 2BS cells. Thus, Asp16 expression effectively delayed cell senescence by postponing the G₁ arrest caused by p16INK4a accumulation.

**Cell Morphologic Changes and the SA-β-Gal Senescence-associated Marker**—We monitored morphological changes after transfection. The 2BS/p16 cells showed increasing gross enlargement, flattening, and accumulation of granular cytoplasmic inclusions. However, no significant morphologic changes were observed in 2BS/ASp16 cells (A5, PD45), which retained a refractive cytoplasm with thin and long projections like young control cells (PD25) (Fig. 4). The specific senescence-associated marker, pH 6.0 optimum β-galactosidase (SA-β-gal), was assayed by X-gal staining (Fig. 4). Only sporadic SA-β-gal-positive cells were seen in 2BS/ASp16 (A5, PD45) and young (PD29) cells, whereas almost all of 2BS/p16 (P3, PD43) cells were strongly stained, as were the senescent 2BS control cells (PD58).

**RESULTS**

Integration of pDOR-p16 and pDOR-ASp16 and Their Expression in 2BS Cells

One of the advantages of a retroviral vector as a gene transfer vector is that it is capable of integrating into the genome of the host cells. In our study, the full length of p16INK4a cDNA was inserted in both orientations into the retroviral vector. The constructs, pDOR-ASp16 and pDOR-p16, were transfected into early passage 2BS cells, respectively, and G418-resistant cell clones were obtained by sustained drug selection. Southern blot (Fig. 1A) showed neo bands in 2BS/neo (3.4 kb), 2BS/ASp16 (4.2 kb), and 2BS/p16 (4.2 kb) cells and exogenous p16INK4a bands (4.2 kb) only in 2BS/ASp16 and 2BS/p16 cells. These results suggest that the exogenous p16INK4a cDNA had integrated into the genome of 2BS cells. The mRNA expression of p16INK4a in 2BS/ASp16 (A5, PD45), 2BS/neo (N5, PD45), and middle-aged (PD40) cells are similar but higher than that in young (PD25) 2BS cells and significantly lower than that in senescent (PD58) cells (p < 0.01) (Fig. 1B). Considering that the signal of p16INK4a mRNA in 2BS/ASp16 results from both wild-type p16 mRNA and Asp16 mRNA, these results indicate that the introduction of ASp16 cDNA significantly suppressed the high expression of p16INK4a in senescent cells. Western blot results (Fig. 1C) also confirmed that the ASp16 significantly suppressed the expression of the wild-type p16INK4a in 2BS cells.
Finite Extension of Proliferative Life Span in 2BS/ASp16 Cells—The replicative senescence of normal human diploid cells is directly correlated to their number of PD rather than to the growth and metabolic time (1, 7, 10). After completing a finite number of divisions, cells enter permanent growth arrest. We compared the PD number of the 2BS/ASp16 with 2BS/p16, 2BS/neo, and the normal control 2BS cells. The results indicate that the life span of 2BS/ASp16 (PD70–75) is about 15–20 PD greater than the 2BS/neo and normal 2BS cells (PD55–60). In contrast, 2BS/p16 ceased cell division of 10–15 PD (PD40–45) earlier than the control cells. In addition, we also found that the 2BS/ASp16 cells maintained a growth rate similar to that of

![Figure 1](image1.png)

![Figure 2](image2.png)
young cells until they entered the growth arrest. All these results indicate that the introduction of ASp16 can enhance the replicative capacity, extend the proliferative life span, and finally delay the onset of senescence of 2BS cells.

**Inhibition of p16INK4a/CDK4/Cyclin D/pRB Pathway by ASp16**

It is known that p16INK4a is one of the key components of the RB pathway (8). p16 INK4a can inhibit the activity of CDKs, thereby blocking the entry of the proliferating cells to S phase through the p16INK4a/CDK4/cyclin D/pRB pathway. The function of p16INK4a is to maintain pRB in its hypophosphorylated active state. Therefore, ASp16 expression is expected to increase phosphorylated pRB by inhibiting p16 expression and by maintaining the hyperphosphorylation state of pRB.

**ASp16 Slows Down Telomere Shortening but Does Not Activate Telomerase**

As the previous data show, although it inhibited the pRB pathway of senescence, ASp16 expression did not result in the immortality of the cells. This indicates there must be other pathways of cell senescence that could not be bypassed. Telomere shortening is considered to be a molecular clock of cell senescence (7, 22, 25, 27). Therefore, it is another pathway that needs to be bypassed to escape senescence. As shown in Fig. 6A, the telomere-shortening process associated with cell senescence was significantly slowed down by ASp16 transfection whereas sustained overexpression of p16INK4a accelerated it (data not shown). This suggests that p16INK4a is involved in the telomere-shortening process. By inhibiting the p16 expression, ASp16 may slow down telomere shortening and delay the cell senescence process. Next, telomerase activity was determined for the 2BS/ASp16 cells using the telomere repeat amplification protocol assay (Fig. 6B). ASp16 did not activate observable telomerase activity. The 2BS/ASp16 cells also did not obtain the anchorage-independent growth ability, a common tumor cell phenotype (data not shown). These results indicate that 2BS/ASp16 cells did not result in the telomerase reactivation associated with immortalization or the tumor phenotypes and indicate that the delay of telomere shortening is not mediated by telomerase reactivation.

**Enhancement of the DNA Repair Capacity by ASp16**

Another reason for progressive shortening of telomeres is that breaks in telomere DNA induced by DNA damage cannot be repaired effectively in the absence of telomerase (22, 25, 27).

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**Figure 3. Flow cytometry analysis of 2BS/ASp16, A10 (PD50) (A); 2BS/neo, N5 (PD45) (C); and 2BS/p16, P2 (PD42) (E) compared with young (PD24) (B); middle-aged (PD45) (D); and senescent (PD56) (F) 2BS cells.**

**A**

- 2BS/ASp16, A10 (PD50)
- G1 46.4%
- S 37.5%
- G2/M 16.0%

**B**

- Young (PD24)
- G1 48.4%
- S 35.2%
- G2/M 16.4%

**C**

- 2BS/neo, N5 (PD45)
- G1 58.3%
- S 30.8%
- G2/M 10.9%

**D**

- Middle-aged (PD45)
- G1 56.4%
- S 32.6%
- G2/M 11.0%

**E**

- 2BS/p16, P2 (PD42)
- G1 80.7%
- S 9.9%
- G2/M 9.4%

**F**

- Senescent (PD56)
- G1 76.4%
- S 16.0%
- G2/M 7.7%
Therefore, we tried to determine whether there is any link between p16INK4a and the DNA damage and repair pathway. As shown in Fig. 7, UDS assays suggest that the DNA repair capacity of 2BS/ASp16 is similar to that of young cells but is higher than that of 2BS/neo and senescent cells (p < 0.01). This indicates that the introduction of ASp16 could enhance the DNA repair capacity of 2BS cells.

**DISCUSSION**

A growing body of evidence suggests a role for p16INK4a in cell senescence. Its loss or inactivation is correlated to the cell immortality (28). Given this postulated importance of p16INK4a in cell senescence, it is expected that inhibition of p16 INK4a would extend the proliferative life span of culture cells (11, 29–31). In this study, sense or antisense p16INK4a were introduced into early passage 2BS human diploid fibroblasts. Premature senescence, including irreversible cell growth arrest in G1 phase, irreversible senescent morphologic changes, and SA-β-gal expression, was imposed by overexpression of p16INK4a, whereas expression of the antisense p16INK4a delayed the onset of the cell senescence. The life span of 2BS/ASp16 is extended about 15–20 PDs compared with the untransfected 2BS or 2BS/neo control cells. The onset of many phenotypes associated with senescence, such as growth G1 arrest, accumulation of unphosphorylated pRB, SA-β-gal-positive, morphologic changes, telomere shortening, and the DNA repair capacity decline are all postponed. These data reinforce the important roles of p16INK4a in cell senescence and argue that the accumulation of p16INK4a is not a consequence of entry into senescence but rather it triggers the onset of senescence.

What is the mechanism by which p16INK4a programs the cell senescence process, and why does ASp16 extend the life span of 2BS cells? Immortalization of normal human cells in vitro has been obtained by transforming genes of DNA tumor viruses such as the SV-40 large T-antigen gene (32). These genes induce immortalization indirectly via a two-stage process. The first stage (M1) is a brief extension of life span associated with inactivation of the protein products of negative regulatory genes, including the Rb and p53, after which cells cease dividing and enter “culture crisis.” The second stage (M2) is the escape of rare clones from crisis, presumably as a result of some genetic changes that might be related to the acquisition of telomerase activity (47). It is well known that p16INK4a acts as
A negative regulator of cell cycle and cell proliferation. It is a key component of the p16INK4a/CDK4,6/cyclin D/pRB pathway. By inhibiting the CDK4,6/cyclin D complex, p16INK4a maintains pRB in its active state, which negatively regulates the cell cycle (8, 28). In our studies, overexpression of p16INK4a enforced a durable block of cell proliferation whereas ASp16 inhibited the cell arrest, and pRB phosphorylation was inhibited. This suggests that pRB is required in the p16-mediated senescence and that the life span of 2BS/ASp16 cells was extended by escape from M1 crisis, which is associated with inactivation of pRB. Beach and colleagues (49) used a similar strategy to express antisense p16INK4a or p19ARF (p14ARF in human) RNA in primary mouse embryonic fibroblasts (MEFs). The lifespan of MEFs was extremely extended, and a percentage of these cells eventually became immortal. Their studies suggest that the introduction of p16INK4a and/or p19ARF leads to immortalization in mouse (49) but not in human cells. Thus, the immortalization of MEFs by expressing antisense p16INK4a and/or p19ARF RNA (49) after bypassing M1 may not need to overcome the M2 barrier, whereas in human 2BS cells, overcoming of telomere shortening is required for the immortalization.

Some studies suggest that the increasing levels of p16INK4a may be caused by progressive telomere shortening (8, 33), but our data suggest p16INK4a may actually regulate the telomere-shortening process. Previous research in our lab suggested that p16INK4a introduction significantly induced telomere shortening. In this study, we also found that the introduction of ASp16 slowed telomere shortening, further supporting the role of p16INK4a in regulating telomere shortening. However, the underlying mechanism is still unknown. Telomerase appears to be important for telomere maintenance in many immortal cell lines (23, 34). Expression of telomerase in normal fibroblasts and retinal pigment epithelial cells results in a substantial extension of proliferative life span (35–38). To further explore the mechanisms by which ASp16 slows telomere shortening, we tested whether 2BS cells obtained telomerase activity after the introduction of ASp16, but no telomerase activity was detectable. Thus, the slow-down of telomere shortening is probably not achieved by activating telomerase even though some studies suggest a relationship between p16INK4a and telomerase (39). This may explain why 2BS/ASp16 cells were not immortal; the telomeres kept shortening progressively even though the process had been slowed down. Without sufficient telomerase activity, cells cannot escape the secondary stage of mortality (M2). Still, there may be other mechanisms to control the telomere shortening.

How does p16INK4a affect telomere shortening? Our studies suggest that the introduction of ASp16 delayed the decrease of the DNA repair capacity of 2BS cells (Fig. 7). In addition, previous studies from our laboratory also showed that the introduction of p16INK4a induced a decrease of DNA repair capacity. All these results suggest that p16INK4a correlates with DNA damage and repair. Therefore, there might be links among the DNA repair, telomere shortening, and the control of p16INK4a gene expression. p16INK4a may be a common mediator among several cell senescence models, such as DNA damage.
model, genetic model, and telomere model. However, it is worth emphasizing that the UDS assay is only a gross estimate of DNA-repair capacity. Some factors can affect the accuracy of the assay, including proliferative status. Further observations should be done, and assays that are more accurate need to be developed. In addition, it has been proposed that there may be more than one mitotic clock mechanism, and any of them may trigger the cell senescence. Some studies also show that there are alternative mechanisms such as gene recombination and transfer and retrotransposition for the maintenance and repair of telomeres (40–44). Thus, p16 may also influence the telomere shortening through these mechanisms.

In summary, there are several independent pathways that control the process of replicative senescence in human cells. The inactivation of one pathway can induce extension of cell life span but may not be sufficient to induce immortalization (45, 46). Cellular senescence in vitro may represent the aging process at the cellular level, and it is one possible mechanism of tumor suppression in vivo. Therefore, it may be possible to use ASp16 therapeutically in both aging and tumor suppression. Further insight into the molecular mechanisms by which ASp16 affects replicative senescence should greatly help in understanding and preventing aging and tumorigenesis.

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