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Research article

Telomerase positive human diploid fibroblasts are resistant to replicative senescence but not premature senescence induced by chemical reagents

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Abstract

Human diploid fibroblasts in tissue culture undergo replicative senescence after a finite number of divisions that is characterized by a permanent loss of their dividing potential. However, senescence-like phenotypes, including growth cessation, morphological changes, and appearance of senescence-associated β-galactosidae (SA-gal) activity, can be induced by treating early passage cells with C_6-ceramide, H_2O_2, LY294002, or trichostatin A. While there is convincing evidence that telomere shortening is causally related to replicative senescence, the role of telomere shortening in the chemical-induced premature senescence is unclear. Here we employed a normal human BJ cell strain and its telomerase-transfected counterpart, termed BJ-T cells, to examine whether active telomerase in BJ-T can block or delay the premature senescence induced by various chemicals and, if not, whether telomere shortening still occurs. We found that, although all four chemicals tested could induce growth arrest, and in some cases SA-gal activity, in both BJ and BJ-T cells, only H_2O_2 clearly caused an irreversible loss of dividing potential. H_2O_2 treatment did not inhibit the cellular telomerase activity, nor did it cause any appreciable telomere shortening in BJ-T cells. These results suggest that oxidative stress and other chemical reagents can target at sites unrelated to the telomere-associated clocking mechanism. Alternatively these chemicals may bypass the telomere length maintenance machinery and target at its downstream sites.

Abbreviations: SA-gal – senescence associated β-galactosidase; PDL – population doubling level; TK – thymidine kinase; TSA – trichostatin A

Introduction

Human diploid fibroblasts become senescent after undergoing a finite number of divisions in culture, a process referred to as replicative senescence. The inability of the senescent cells to initiate DNA synthesis in response to mitogenic stimulation is considered the hallmark of cellular aging. Two major theories have been advanced to address the cause of cellular aging. One proposes that progressive telomere shortening with cell division leads to senescence (Olovnikov 1996). The other states that cumulative oxidative stress, mainly reactive oxygene species (ROS) generated from mitochondria, may causes aging (reviewed in Beckman and Ames 1998).

Telomere shortening occurs in many cell types prior to senescence (e.g., Wright and Shay 2001; Sedivy 1998; Bryan and Cech 1999). Prevention of telomere shortening by the ectopic expression of telomerase gene leads to an extension of *in vitro* life span (Bodnar et al. 1998; Vaziri and Benchimol 1998). These studies strongly suggest that activation of telomerase can result in one-step immortalization and that the shortening of telomeres can serve as a molecular clocking mechanism for senescence.

Oxidative stress caused by H_2O_2 (Chen et al. 1994), Ras transfection (Serrano et al. 1997; Lee et al. 1999), or *tert*-butylhydroperoxide (Dumont et al. 2000) can shorten the life span of cells in tissue culture, a phenomenon termed premature senescence (Serrano et al. 1997). Other studies have shown that chemicals that interfere with certain signal transduction pathways can also affect the aging process. For example, it has been shown that ceramide, a component in sphingomyelin cycle (Venable et al. 1995), LY294002, an inhibitor of phosphatidylinositol 3-kinase (Tresini et al. 1998), and trichostatin A, an inhibitor of histone deacetylase (Ogryzko et al. 1996) can induce premature senescence.

In view of the causal relationship between telomere shortening and replicative senescence, it is reasonable to ask whether telomeric shortening also plays a key role in the premature senescence. We have taken two approaches to address this question. First, we examined the effects of these reagents on the telomere length in the treated human fibroblasts; second, we investigated whether the expression of human telomerase reverse transcriptase (hTERT) in the transfected normal cells can block or delay the premature senescence. Our study showed that telomere shortening did not occur when cells undergo chemically induced premature senescence. Moreover, the restoration of telomerase activity in normal human cells did not prevent the hTERT transfected cells from undergoing premature senescence. The possible connection between replicative senescence and chemically induced premature senescence is discussed.

Materials and methods

Cell culture and treatment

IMR-90 (human embryonic lung fibroblast) and SV40-transformed IMR-90 cells were obtained from NIA Aging Cell Repository at Coriell Institute for Medical Research (Camden, New Jersey). BJ (human foreskin fibroblastic cells) and BJ-T (BJ cells expressing human telomerase catalytic unit) cells were kind gifts from Drs J.R. Smith and J. Campisi, respectively. Cells were cultured in Dulbecco-modified Minimum Eagle's Medium supplemented with 10% fetal bovine serum (FBS) (Gemini Bio Products, California) under standard conditions.

Cells were treated with chemicals, including Trichostatin A (Calbiochem, San Diego, California), LY294002 (Alexis, San Diego, California), or C_6-ceramide (N-hexanoyl-D-sphingosine; Sigma, St. Louis, Missouri) at indicated concentrations. For H_2O_2 treatment cells were exposed to a complete medium containing H_2O_2 at different concentrations for only 2 h, washed and replenished with a fresh medium. Cell number counting and various biomarkers assays were performed at indicated times.

Reverse transcription-polymerase chain reaction (RT-PCR)

Confluent BJ-T cells (PDL 68) were treated with 300 μM H_2O_2 as described above. Total RNA were prepared and analyzed by RT-PCR as described (Matuoka and Chen 2000). A 25-cycle amplification was used for β-actin and thymidine kinase (TK) and a 30-cycle amplification for collagenase I. Primers used are: 5′-pGGGCCGTCTTCCCCTCCATCGTGG-3′/5′-pCCGTGGCCATCTCTTG CTCGAAGT C-3′ for β-actin; 5′-pAGCACAGAGTTGATGAGACGC-3′/5′-pGCTTCCTCTGGAAGGT CCCAT-3′ for TK; and 5′-pGCCAGTATGCACA GCTTTCCTCC-3′/5′-CAATTTTTCCT GCAGTTGAACCAG-3′ for collagenase 1.

Telomerase assay and telomere length analysis

Genomic DNA was prepared from cells with DNAzol (Molecular Research Center, Cincinnati, Ohio) and subjected to analysis of telomere length with TeloQuant (PharMingen, San Diego, California). The autoradiogram was analyzed with 'NIH Image' ver. 1.62. For telomerase activity, cellular proteins were extracted and analyzed by using TRAPeze Telomerase Detection kit (Intergen, Purchase, New York).

Results and discussion

The possible critical role of telomeric shortening poses two questions: (i) Does telomere length maintenance machinery hold the only key for controlling cell senescence? (ii) If there are other pathways for cell senescence, how do they integrate with the telomere maintenance machinery? Reagents such as TSA, LY942002, ceramide, and H_2O_2 have been reported in the literature to induce not only growth cessation but also certain senescence-like bio-markers before the cells reach Hayflick limit. Since these reagents target at different sites including histone deacetylase, phosphatidylinositol-3-kinase, sphingomyelin cycle,

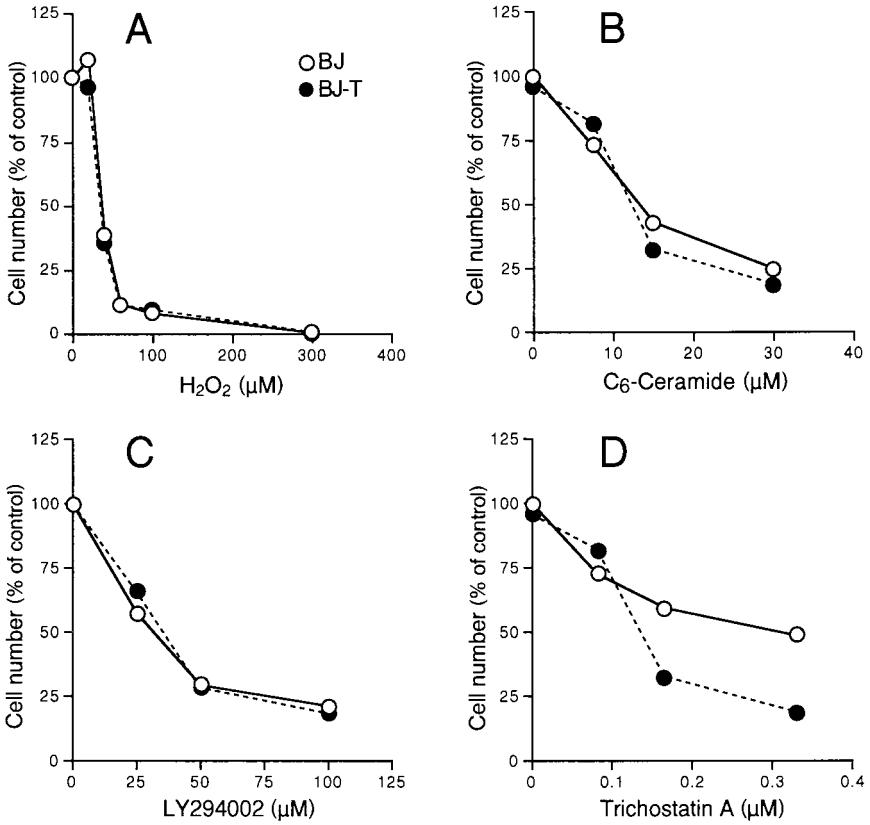

Figure 1. Effect of various chemical treatments on the growth of BJ (PDL 30) and BJ-T (PDL 59) cells. Cells were plated at a split ratio of 1 : 8. After overnight incubation, cells were treated with C_6-ceramide, LY294002 or TSA at indicated concentrations. For the oxidative stress treatment, cells were exposed to H_2O_2 for 2 h, washed and incubated in the fresh growth medium. The number of viable cells in each culture was determined four days after various treatments and compared with that of the control cultures.

and oxidative damages, it is possible that multiple pathways can independently lead to cellular senescence. Alternatively, it is possible that all these pathways integrate into the telomere maintenance machinery and that telomere shortening controls the ultimate senescence.

BJ is a normal foreskin fibroblastic cell strain that has a limited life span of about 90 divisions (Matuoka et al. 2001). The hTERT transfected BJ cell strain, termed BJ-T, remains normal with a diploid karyotype, but appears to be immortal (Kim et al. 1999). The availability of the BJ and BJ-T pair allows one to examine how the active telomerase activity in normal somatic cells may impact on cell physiology, including senescence. Using this pair of cell strains we first compared the effects of H_2O_2, C_6-ceramide, LY294002, and TSA on their growth. Figure 1A shows that the oxidative stress elicited by H_2O_2 produced an almost identical dose-dependent growth arrest in both BJ and BJ-T cells. Similarly, all the other three reagents also inhibited the growth of BJ and BJ-T cells to about the same degree at the concentrations tested (Figures 1B–D). The effective dosages of these reagents for growth arrest of both BJ and BJ-T cells were comparable to that reported previously for WI38 cells, another normal human fibroblastic cell strain (Venable et al. 1995; Ogryzko et al. 1996; Tresini et al. 1998).

Figure 2A shows that the senescent human fibroblasts displayed an enlarged and flattened morphology as compared to the thin and elongated morphology of young cells. Using this as a control we compared the effect of H_2O_2, C_6-ceramide, LY294002, and TSA on the morphology of BJ (PDL 13) and BJ-T (PDL 68) cells. Figure 2B shows that, while all four chemicals induced changes in cell shape, the morphology of H_2O_2- and TSA-treated cells resembled more closely that of the senescent cells. In contrast, the effect of LY294002 was not very prominent. Import-

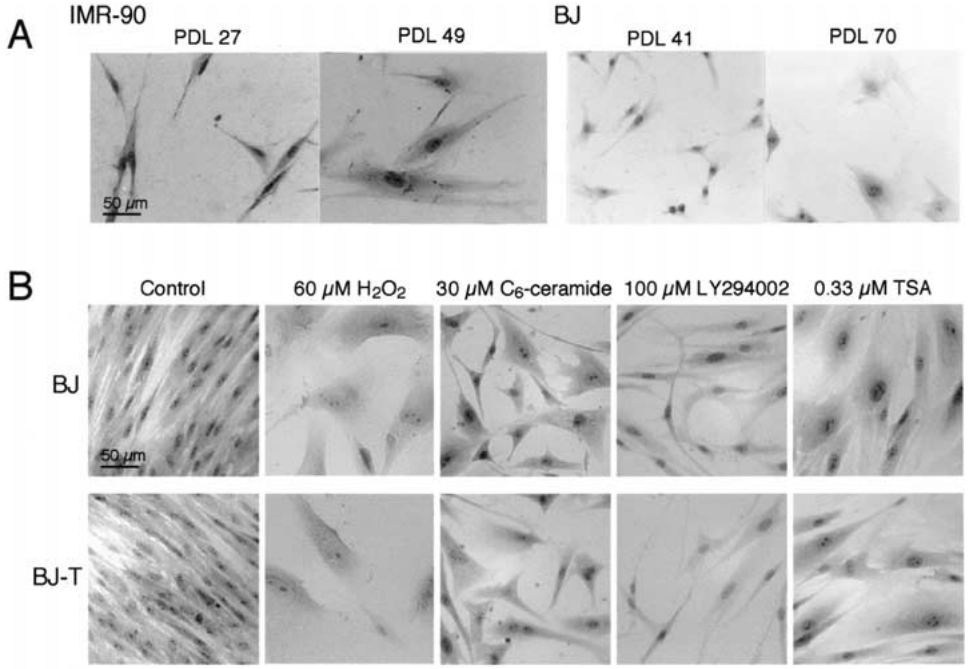

Figure 2. Effect of various chemical treatments on cell morphology. (A) Morphology of young (IMR-90 at PDL 27 and BJ at PDL 41) and senescent (IMR-90 at PDL 49 and BJ at PDL 70) cells. (B) Morphology of BJ and BJ-T cells. BJ and BJ-T cultures were treated with H_2O_2 (2 h exposure), C_6-ceramide, LY294002 or TSA for 4 days. The cells were fixed, stained with Giemsa and visualized under microscope.

antly, the morphological changes induced by H_2O_2, C_6-ceramide, LY294002, or TSA were indistinguishable between BJ and BJ-T cells. Thus, the presence of telomerase is not sufficient to block the action of any of these reagents.

We next examined whether the effects of these chemicals on growth arrest and morphological changes in BJ and BJ-T cells were reversible. Figure 3 shows that both BJ and BJ-T cells resumed growth after the removal of C_6-ceramide, LY294002, or TSA from the treated cultures and the proliferation rate of the BJ and BJ-T cells was restored to the control level by at least 50%. In contrast, a brief exposure of cells to H_2O_2 for 2 h was sufficient to yield an irreversible arrest of growth of both BJ and BJ-T cells. This result indicated the effects of C_6-ceramide, LY294002, and TSA were transient and only the H_2O_2 treatment led to an irreversible loss of dividing potential, a hallmark of cell aging.

It appears that H_2O_2 treatment can cause cells to undergo either cell death or premature senescence, depending on the PDL and cell density of the culture. Figure 4A shows that at low cell density the early passage (PDL 25) and late passage (PDL 45) cells responded differently to H_2O_2. Thus, after an

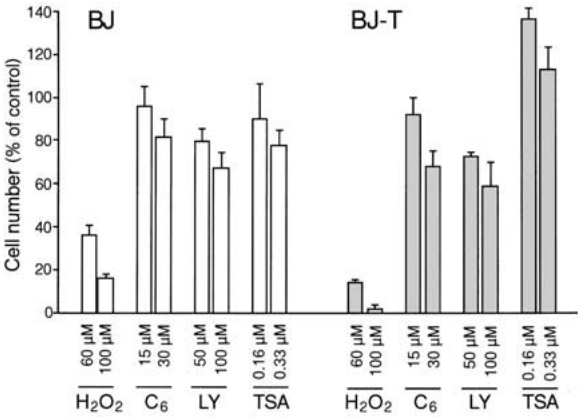

Figure 3. Reversibility of the growth arrest effects of various treatments. Cells were treated with various chemicals at indicated concentrations for 4 days as described in 'Materials and methods'. Cells were then washed with fresh growth medium and plated at the seeding density of 2×10^3 cells/cm^2. The cell number of the treated cultures was compared with that of the control four days after plating. The standard error of mean was estimated from two sets of triplicate experiments.

exposure of cells to H_2O_2 at a concentration of 100 μM or higher for 2 h, almost all early passage cells became apoptotic whereas the late passage cells

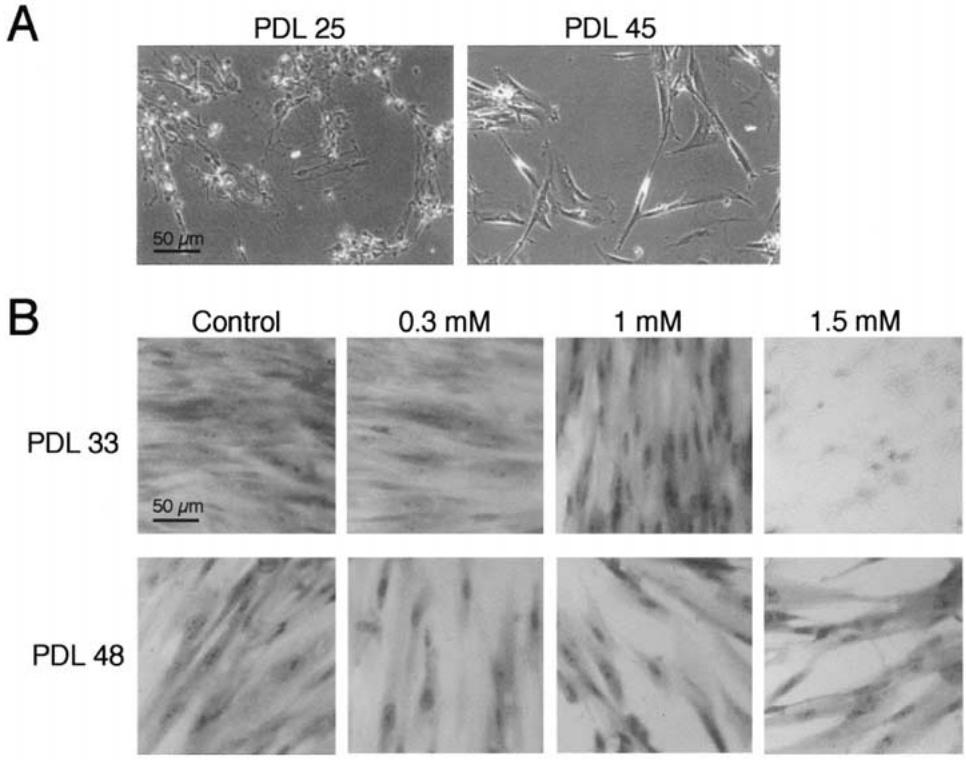

Figure 4. Senescent cells were resistant to H_2O_2-induced cell death. (A) Sparse cultures of young (PDL 25) and late passage (PDL 45) IMR-90 cells were treated with 300 μM H_2O_2 for 2 h, washed and incubated in fresh growth medium for 20 h and phase contrast photomicrographs were taken. (B) Confluent cultures of young and late passage cells were treated with H_2O_2 at 0.3, 1 and 1.5 mM for 2 h, washed, and incubated in fresh growth medium for 4 days and phase contrast photomicrographs were taken.

remained viable. In contrast, confluent cultures of both early and late passage human cells could withstand H_2O_2 treatment up to 1 mM without noticeable cell death (Figure 4B). However, at even higher concentrations of H_2O_2 (1.5 mM) the age-dependent difference in response to H_2O_2 became clear.

Since H_2O_2 caused an irreversible loss of dividing potential of the telomerase-positive BJ-T cells, we examined whether it also induced other senescence biomarkers, including SA-gal and the expression of thymidine kinase (TK) and collagenase. Figure 5A shows that H_2O_2 induced SA-gal activity in both BJ and BJ-T cells. Figure 5B shows that H_2O_2 suppressed the serum-induced TK gene expression, but enhanced the expression of collagenase in both BJ and BJ-T cells. These results indicated that, in addition to cell morphology, H_2O_2 could induce other senescence-associated biomarkers despite the presence of telomerase gene in BJ-T cells. In this connection, it is interesting to note that we have recently reported that nicotinamide rejuvenated the senescent cells by blocking the morphological changes associated with senescence (Matuoka et al. 2001). We have found that nicotinamide could also block the morphological changes in H_2O_2-treated BJ cells and BJ-T cells. However, nicotinamide could not reverse the loss of dividing potential due to either replicative senescence or premature senescence induced by H_2O_2 (Matuoka and Chen, unpublished data).

Since it is still possible that H_2O_2 induced senescence-like phenotypes in BJ-T cells by directly or indirectly inhibiting telomerase activity, we compared the telomerase activity in BJ-T cells before and after H_2O_2 treatment. Figure 6 shows that H_2O_2 did not inhibit the telomerase activity in BJ-T cells. As expected, telomerase activity was not detectable in BJ or IMR-90 cells. Finally, we examined whether telomere shortening occurred in BJ or BJ-T cells after H_2O_2 treatment. As a control we first showed that indeed the telomere restriction fragment (TRF) length of senescent IMR-90 cells (PDL 53) was shorter than that of presenescent IMR-90 cells (Figure 7A). The

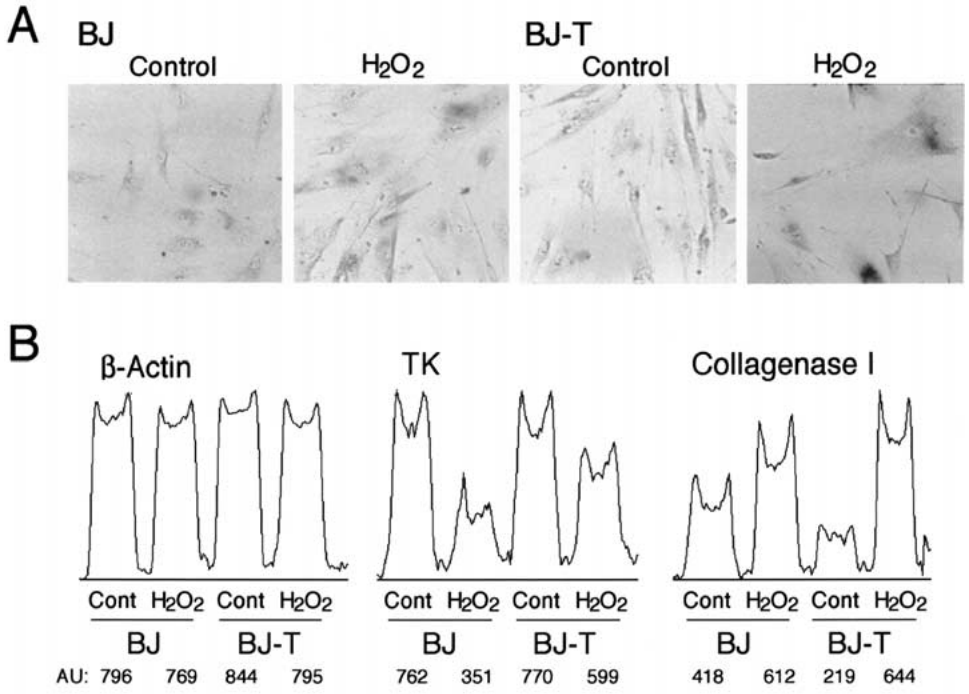

Figure 5. SA-gal activity and gene expression of BJ (PDL 25) and BJ-T (PDL 60) cells after treatment with H_2O_2. (A) Cells were exposed to H_2O_2 (300 μM) for 2 h, washed and incubated in fresh growth medium. Cells were stained for SA-gal activity 15 h after H_2O_2 treatment. (B) Confluent BJ (PDL 38) and BJ-T (PDL 68) cells were exposed to H_2O_2 (300 μM) for 2 h and then cultured in fresh growth medium. RNA samples were prepared and RT-PCR was performed as described in 'Materials and methods'. AU: arbitrary unit.

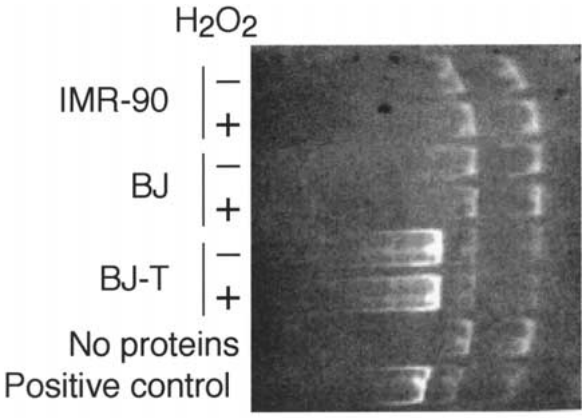

Figure 6. Effect of H_2O_2 treatment on telomerase activity in human fibroblasts. BJ (PDL 30), BJ-T (PDL 64) and IMR-90 (PDL 30) cells were treated with H_2O_2 and cultured in fresh growth medium for another 3 days. Telomerase activities were determined as described in 'Materials and methods'.

TRF length of either BJ or BJ-T cells was clearly not affected by the treatment of H_2O_2 over seven days, a period within which all the senescence-like phenotypes appeared (Figure 7B). These results indicated that H_2O_2 could induce premature senescence in cells without causing telomere shortening. Moreover, the presence of active telomerase activity in BJ-T cells could not block the action of H_2O_2 in inducing premature senescence. Our findings are consistent with the reports that the expression of catalytically active telomerase does not prevent Ras-induced premature senescence (Wei et al. 1999) and that telomere shortening does not occur in IMR-90 cells after the treatment with H_2O_2 (Chen et al. 2001).

On the other hand, Von Zglinicki et al. (1995) reported that hyperoxia accelerates the rate of telomere shortening and causes the cells to gradually gain senescent phenotypes prematurely. Xu et al. (2000) found that homocysteine increases telomere length lost per population doubling and accelerates endothelial cell senescence. Since these treatments do not lead to premature senescence without additional 10–15 divisions, it is likely that accelerated telomere shortening can occur during these divisions. In the case of H_2O_2 treatment, the senescence-like phenotypes appear in treated cells without any telomere shortening (Figure 7 and Chen et al. 2001), suggesting that these phenotypes, including permanent growth arrest, are not coupled to telomere length. It has been reported

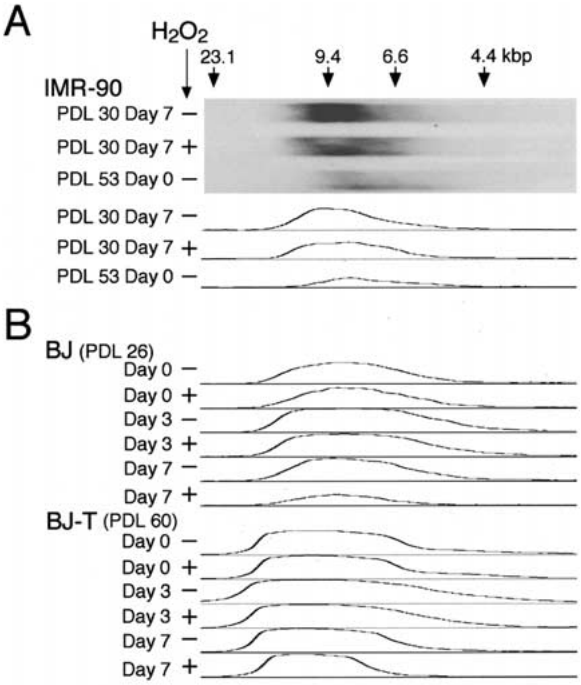

Figure 7. Telomere length of human diploid fibroblasts with passage and with H_2O_2 treatment. Confluent cultures of IMR-90 (A), BJ and BJ-T cells (B) were treated with or without 300 μM of H_2O_2 for 2 h (day 0), washed and cultured in the fresh growth medium for another 3 days (day 3). Then cells were subcultured at a split ratio of 1 : 8 for 4 days (day 7). Cells were then harvested at indicated time and genomic DNA prepared for telomere length assay.

that severe H_2O_2 treatment (300 μM in a serum-free medium) can lead to preferential accumulation of single-stranded breaks in the telomeric region, which may destabilize the telomeric complex (Petersen et al. 1998; von Zglinicki et al. 2000). Although we have not eliminated this possibility in the present study, we have carried out the H_2O_2 treatment in the presence of serum under much milder conditions.

Manipulation of life span by either chemical or genetic means may allow us to dissect and identify critical steps involved in the aging process. We have shown here that active telomerase cannot block the premature senescence induced by TSA, ceramide LY294002, or H_2O_2. Since active telomerase can block replicative senescence, but not chemical-induced premature senescence, it raises the possibility that the different pathways are involved in these two processes. Alternatively, it is possible that these reagents bypass the telomere clocking machinery and target at its downstream sites.

One of the key features of replicative senescence is the apparent global attenuation of G1/S genes (Chen 1997; Matuoka and Chen 1999). NF-Y, a CCAAT binding transcription factor, is involved in the regulation of a number of G1/S genes (Chen 1997). Since NF-Y binding activity is significantly attenuated during replicative senescence, probably due to a decrease in the subunit NF-YA, we have proposed that NF-Y may be causally involved in controlling cellular senescence (Good and Chen 1996). In this regard, it is of interest to note that H_2O_2 can directly inhibit NF-Y binding activity (Pang et al. 1996) and that p53 overexpression has been shown to suppress the NF-Y binding activity (Jung et al. 2001).

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