Uncoupling the Senescent Phenotype from Telomere Shortening in Hydrogen Peroxide-Treated Fibroblasts

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Normal human cells have a limited replicative potential and inevitably reach replicative senescence in culture. Replicatively senescent cells show multiple molecular changes, some of which are related to the irreversible growth arrest in culture, whereas others resemble the changes occurring during the process of aging *in vivo*. Telomeres shorten as a result of cell replication and are thought to serve as a replicometer for senescence. Recent studies show that young cells can be induced to develop features of senescence prematurely by damaging agents, chromatin remodeling, and overexpression of *ras* or the *E2F1* gene. Accelerated telomere shortening is thought to be a mechanism of premature senescence in some models. In this work, we test whether the acquisition of a senescent phenotype after mild-dose hydrogen peroxide (H_2O_2) exposure requires telomere shortening. Treating young HDFs with 150 μM H_2O_2 once or 75 μM H_2O_2 twice in 2 weeks causes long-term growth arrest, an enlarged morphology, activation of senescence-associated β -galactosidase, and elevated expression of collagenase and clusterin mRNAs. No significant telomere shortening was observed with H_2O_2 at doses ranging from 50 to 200 μM . Weekly treatment with 75 μM H_2O_2 also failed to induce significant telomere shortening. Failure of telomere shortening correlated with an inability to elevate p16 protein or mRNA in H_2O_2 -treated cells. In contrast, p21 mRNA was elevated over 40-fold and remained at this level for at least 2 weeks after a pulse treatment of H_2O_2 . The role of cell cycle checkpoints centered on p21 in premature senescence induced by H_2O_2 is discussed here. © 2001 Academic Press

INTRODUCTION

Aging is an inevitable consequence of life. In humans, aging is a risk factor for cancer and many other diseases. The causes of aging and its associated diseases are the subject of many recent studies. It appears that a large number of factors may play a role in the

overall aging process, from whole organismal wear and tear to changes at the cellular and molecular level. Among the changes observed are an accumulation of oxidative damage, reduced mitochondrial function, declined functionality of a particular cell type, changes in the composition of extracellular matrix proteins, altered gene expression, and reduction in telomere length. Despite numerous phenomena found to be associated with the process of aging, only a limited number of experimental models are available to study the biology of aging and its contribution to diseases. Normal human cells in tissue culture show a number of aging-related changes at the end of their replicative life span. Although these molecular changes are commonly referred to as senescence, replicative senescence usually defines a stable state at the end of the replicative life span of normal (i.e., nontumor) cells in culture. Replicative senescence has been used as an experimental model for studying aging and the relationship between aging and cancer [1–4].

Most tumor cells do not undergo senescence in culture. Studies from Pereira-Smith and Smith's group [5] have found that tumor cells regain the senescent program upon fusion with cells from a complementary group. The genotype of senescence is dominant over that of tumor cells because fusion of normal cells with tumor cells results in hybrids that have limited replicative life span [6, 7]. A distinct feature of normal somatic cells is their inability to synthesize telomeres at the ends of chromosomes. Shortening of telomeres occurs during the process of senescence *in vitro* and aging *in vivo*, while activation of telomerase is a hallmark for cellular immortality and occurs in many types of cancers.

Telomere shortening is thought to serve as a replicometer for senescence [8–10]. Telomeres, the simple repetitive DNA sequences (i.e., TTAGGG repeats for humans) at the ends of linear chromosomes, shorten between 50 and 150 bp per cell division in normal somatic cells [8, 9, 11]. This shortening is linearly proportional to the cumulative number of cell divisions during the process of replicative senescence [8, 9, 11]. Telomere shortening results from the inability of DNA

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polymerases to completely replicate the 3'-end of the lagging strand during DNA replication, leaving a shorter daughter strand. Most tumor cells or immortalized cells utilize the enzyme telomerase to synthesize telomeric repeats *de novo* onto the end of the 3'-strand to compensate for the incomplete DNA replication. However, most normal somatic cells do not contain functional telomerase, and therefore cell replication ultimately results in telomere loss [8–11]. The telomere hypothesis suggests that when telomeres reach a critical shortened length at one or more chromosome ends, a signal is generated to halt cell division and to cause the onset of the senescent phenotype [8–10]. It has been recently demonstrated that cellular senescence can be delayed or abolished by activating telomerase in normal human cells [12], providing strong evidence in support of the telomere hypothesis of replicative senescence.

It is thought that telomere shortening can trigger the activation of cell cycle checkpoints in replicatively senescent cells [13]. The p21 cyclin-dependent kinase inhibitor is elevated when HDFs are approaching replicative senescence [14, 15]. When cells are indeed senescent, an elevation of the p16 cyclin-dependent kinase inhibitor is observed [14, 16]. Traditionally, the senescent phenotype is observed after normal somatic human cells such as human diploid fibroblasts (HDFs) go through a certain number of cell divisions in culture. However, recent work has found that H_2O_2 , hyperoxia, inhibitors of histone deacetylase, hyperactive *ras* gene, and overexpression of the E2F1 transcription factor can all convert early passage HDFs to a state indistinguishable from replicative senescence [17–21]. Although activation of p21, p16, or both is found in certain models of premature senescence [20], hyperoxia (40% O_2)-induced premature senescence is accompanied by accelerated telomere shortening [13, 21], suggesting that critically shortened telomeres are necessary for the onset of the senescent phenotype under these circumstances. Regardless, it is not clear whether telomere shortening, elevation of p21, and elevation of p16 occur in all pathways leading to the senescent phenotype. Here we test whether the induction of a senescent phenotype by an acute or repetitive treatment of H_2O_2 in early passage IMR-90 cells is coupled to telomere shortening acceleration, elevation of p21, and elevation of p16.

MATERIALS AND METHODS

Cell culture and H_2O_2 treatment. IMR-90 cells [obtained at the population doubling level (PDL) 10.85 from the Coriell Institute for Medical Research, Camden, NJ] at PDL less than 30 were seeded at the density of 2×10^6 cells in 100-mm dishes containing 10 ml of Dulbecco's modified Eagle's medium (Life Science Technologies, Grand Island, NY) and 10% (v/v) fetal bovine serum (Life Science Technologies, Grand Island, NY). At 20 to 24 h after seeding, the

cells were treated with H_2O_2 for 2 h. For two 75 μM H_2O_2 treatments, the cells were split after being cultured for 1 week following the first treatment and treated again for 2 h with 75 μM H_2O_2 at 20 to 24 h after seeding. Replicative senescence was achieved by weekly subculture at a seeding density of 0.5×10^6 cells per 100-mm dish. The cells reached replicative senescence at PDL 54. For comparison studies, replicatively senescent cells were subcultured for 7 days before various measurements were made.

Measurement of DNA synthesis, SA β -gal activity, and cell volume. Cells were cultivated for 1 week after H_2O_2 treatment before being seeded into 24-well plates (1×10^5 cells per well) for bromodeoxyuridine (BrdU) incorporation and SA β -gal activity measurements. After a 48-h incubation in the presence of 10 μM BrdU, the cells were fixed in 5% formalin and stained for SA β -gal-positive cells as previously described [22]. Afterward, the cells were washed with 70% ethanol, and DNA was hydrolyzed by 4 N HCl. BrdU incorporated into the cells was detected with an anti-BrdU antibody (1:250 dilution, Sigma, St. Louis, MO) and an alkaline phosphate-conjugated secondary antibody. At least 300 cells from each group were scored under a phase-contrast microscope for BrdU- or SA β -gal-positive staining.

At 7 to 10 days after H_2O_2 treatment, the adherent cells were detached by trypsinization. Rounded cells were loaded onto a microslide field finder (Fisher Scientific, Pittsburgh, PA) for measuring the diameters of the cells that randomly landed on the grids under an inverted phase-contrast microscope. The cell volume was calculated using the equation for the volume of a sphere, $4/3 \times \pi \times \text{Radius}^3$.

Measurement of collagenase, clusterin, p21, and p16 mRNA levels by Northern blot. Cells were harvested with Trizol (Life Science Technologies) for isolation of total RNA. RNAs (20 μg /lane) were separated by formaldehyde denaturing gel and were transferred to a nylon membrane for hybridization with cDNA probes derived from collagenase-1, clusterin (both obtained from the American Type Culture Collection, Manassas, VA), p21, or p16 gene sequence. Random primer labeling method was used to synthesize cDNA probes as described [23]. The membranes were stripped or stored until the radioactivity was decayed for rehybridization with a different probe for comparative studies or with cDNA of 18S rRNA. Radioactive cDNA probes that are bound to the membrane generate autoradiography and allow for the determination of the abundance of the mRNA species. The densities of the bands were quantified using an Eagle Eye II Image System (Stratagene, La Jolla, CA).

Measurements of p16 protein level by Western blot. Cells were harvested at various time points after H_2O_2 treatment for Western blot analysis of p16 protein level using a protocol described previously [24]. After SDS-gel electrophoresis, proteins were transferred to PVDF membrane for blotting with an antibody against p16 (C-20 polyclonal from Santa Cruz Biotech, Santa Cruz, CA; clone Ab-2 from Neomarker, Fremont, CA, or clone G175-405 from Pharmingen, San Diego, CA). Secondary antibodies conjugated with horseradish peroxidase and enhanced chemiluminescence reaction were used to detect the bound primary antibodies [24]. The densities of the p16 bands were quantified using an Eagle Eye II Image System (Stratagene).

Measurement of the terminal restriction fragment (TRF) length. Cells were detached by trypsinization. Genomic DNA was prepared from cell pellets according to a standard protocol [11]. Briefly, cells were resuspended in 100 μl Tris-EDTA (TE) buffer, added to 1 ml DNA extraction buffer (100 mM NaCl, 10 mM Tris, 25 mM EDTA, pH 8, 0.5% SDS, and 0.1 mg/ml proteinase K), and digested at 50°C for 16 h. The cell lysate was then treated as described previously [11]. DNA was dissolved in TE buffer and heated at 50 to 55°C for 1 h. An aliquot (3–5 μg) of DNA was digested with *HinfI/RsaI* (2 units/ μg of DNA) and DNase-free RNase at 37°C for 16 h. Aliquots (1–2 μg) of digested DNA were separated on a 0.5% agarose gel in Tris-boric acid-EDTA buffer. The gels were dried for 30 min at 60°C, denatured (0.5 M NaOH, 1.5 M NaCl) for 15 min, and neutralized

(0.5 M Tris, pH 7.0, 1.5 M NaCl) for 15 min. Gels were prehybridized in 50 ml of Church and Gilbert hybridization solution at 37°C for 1 h. An aliquot (0.25 μ g) of a single-stranded telomeric oligonucleotide (TTAGGG)₃ was end-labeled using 50 μ Ci of [γ -³²P]ATP and 10 units of T4 polynucleotide kinase before being added to the prehybridization buffer. The gels were hybridized with the probe at 37°C for 16 h and washed with 0.5 $\times$ SSC, 0.1% SDS at 37°C (3 times, 10 min each). To determine the TRF length, a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) was used to quantify the position and strength of the radioactive signal in each of the lanes as described [11].

Statistics. The TRF lengths from duplicate experiments were averaged and plotted versus concentration of H₂O₂ or number of days. Regression analysis and analysis of variance of the resulting graphs determined whether the slope of the calculated line was significantly different from zero. A *P* value less than or equal to 0.05 would indicate a statistically significant change in TRF length with different H₂O₂ concentrations or time after treatment.

RESULTS

Induction of a Senescent Phenotype with Mild Doses or Repetitive Treatment of Oxidants

Exposing early passage HDFs (PDL <30) to sublethal doses of H₂O₂ for 2 h results in a prolonged inhibition of cell growth [17, 25]. H₂O₂ caused a dose-dependent inhibition of DNA synthesis in IMR-90 fetal lung diploid fibroblasts (Fig. 1A). H₂O₂ above 200 μ M (or 1 pmol/cell) induces apoptosis and necrosis [24]. With 150 μ M or lower concentrations, the majority of H₂O₂-treated cells remained attached, were capable of excluding trypan blue, and maintained an active metabolism. A single treatment of 150 μ M H₂O₂ abolished the ability of 95% of surviving cells to perform DNA synthesis (Fig. 1A). The cells could not replicate even when they were allowed to recover for 2 weeks (Table 1). The proportion of SA β -gal-positive cells increased with increasing concentrations of H₂O₂ (Fig. 1B). About 40% of the cells were positive for SA β -gal 1 week after 150 μ M H₂O₂ treatment. The percentage was somewhat close to that observed in replicatively senescent IMR-90 cells subcultured for 1 week (Fig. 1B). H₂O₂-treated early passage cells showed a senescent morphology (Fig. 1C) and an enlarged cell volume (Table 1).

With 75 μ M H₂O₂, treated cells could replicate an average of 0.35 population doublings over a week (Table 1), and 70% of these cells were incapable of synthesizing DNA 1 week after the treatment (Fig. 1A). About 30% of these cells were SA β -gal positive (Fig. 1B). The cells were enlarged, but to a lesser degree than that

induced by 150 μ M H₂O₂ or than that observed in replicatively senescent cells (Fig. 1C; Table 1). A second treatment with 75 μ M H₂O₂ 1 week after the first treatment completely eliminated the ability of the cells to replicate DNA (Fig. 1A; Table 1) and converted 100% of the cells to be SA β -gal positive (Fig. 1B). The cells appeared to show the morphology (Fig. 1C) and an enlarged volume similar to that of replicatively senescent cells (Table 1). Therefore, one treatment with 150 μ M H₂O₂ or two treatments with 75 μ M H₂O₂ can convert early passage IMR-90 cells to a state resembling replicative senescence.

Replicatively senescent cells show multiple molecular changes, some of which are related to growth arrest, whereas others are not [26–28]. For example, replicatively senescent cells elevate the expression of collagenase and clusterin genes [29, 30], which do not appear to be related to growth arrest per se. Like replicatively senescent cells, H₂O₂-treated early passage cells elevated the expression of collagenase and clusterin genes (Fig. 1D). This result indicates that H₂O₂-treated early passage cells likely resemble replicative senescent cells at the molecular level.

No Significant Shortening of Telomeres in Oxidant-Induced Premature Senescence

Telomeres are thought to serve as a replicometer for replicative senescence [8–10]. Induction of premature senescence by hyperoxia is thought to result from accelerated telomere shortening [13, 21]. To test whether telomere shortening occurs during the induction of premature senescence via acute or repetitive treatment of oxidants, we determined telomere length by measuring the length of TRF in IMR-90 cells. Cells were harvested immediately after a 2-h H₂O₂ treatment to determine whether the treatment can cause an immediate shortening of telomeres (presumably due to DNA damage), or were harvested 1 week after H₂O₂ treatment to determine whether onset of the senescent phenotype correlated with shortening of telomeres. With either set of samples, we did not detect any significant differences in TRF length with respect to the concentration tested immediately after H₂O₂ treatment (*P* = 0.14) or one week after H₂O₂ treatment (*P* = 0.22; Fig. 2). At 1 week after H₂O₂ treatment, 150 μ M H₂O₂-treated cells appeared to resemble senescent cells both morphologically and at the molecular level, yet did not

FIG. 1. H₂O₂-treated early passage IMR-90 cells develop a senescent phenotype. IMR-90 cells (PDL <25) were seeded at a density of 2 $\times$ 10⁶ cells in 100-mm dishes and were treated with H₂O₂ for 2 h at 20 to 24 h after seeding. For two 75 μ M H₂O₂ treatments, the cells were split 1 week after the first treatment for a repeated treatment. BrdU (final 10 μ M) was added to the cells for a 48-h incubation. The cells were harvested at 1 week after H₂O₂ treatment for determination of incorporated BrdU (A), SA β -gal activity (B), cell morphology (C), and collagenase or clusterin mRNA by Northern blots (D). The Northern blot data are expressed as ratios of densities of collagenase or clusterin bands to that of 18S rRNA analyzed by an Eagle Eye II Image System (D).

TABLE 1

H₂O₂ Treatment Inhibits Cell Replication but Causes Increases in Cell Volume^a

		Cell replication (population doubling/week)	Cell volume (μm ³ , ×10 ³)
1 week	Untreated	1.80 ± 0.07	6.4 ± 1.5
	75 μM H ₂ O ₂	0.35 ± 0.19	*25.7 ± 9.7
	150 μM H ₂ O ₂	≤0	*36.9 ± 12.1
2 weeks	Untreated	1.99 ± 0.02	10.1 ± 2.7
	75 μM × 2H ₂ O ₂	≤0	*83.3 ± 20.8
	150 μM H ₂ O ₂	≤0	*93.4 ± 24.9
	PDL 52	0	76.2 ± 25.2

^a The cells were treated as described in the legend to Fig. 1. Cell replication was determined by counting cell number using a Coulter counter. Cell volume was calculated after measurements of the diameter of 99 cells randomly in each group with a microscopic Field finder (Fischer Scientific).
* Statistically significant difference (*P* < 0.05) between the number and that of untreated cells by Student's *t* test.

show a senescent telomere length (10.9 kbp for H₂O₂-treated cells versus 9.1 kbp for replicatively senescent cells).

Because a one-time treatment with 75 μM H₂O₂ could not completely abolish cell proliferation and yet did not result in significant shortening of telomeres, we applied a second treatment of 75 μM H₂O₂ at 1 week after the first treatment to model chronic and repetitive oxidative stress. Although these cells showed a slight change in TRF length, it was not statistically significant (Fig. 3). To pursue this experiment further, we compared the TRF length in parallel cultures of cells maintained by weekly subculture with or without once-a-week treatment of 75 μM H₂O₂. The untreated group showed a significant (*P* < 0.0001) linear decrease in TRF length at the rate of 95 bp per population doubling, similar to previous reports [8], and the TRF length reached 9.1 kb at replicative senescence (Fig. 3). In contrast, weekly treatment with 75 μM H₂O₂ did not result in significant TRF shortening (*P* = 0.65; Fig. 3). Our results show that significant telomere shortening does not occur as a consequence of H₂O₂ treatment.

Elevation of p21 but Not p16 in H₂O₂-Induced Premature Senescence

Replicatively senescent cells show an elevation of p21 and p16 cyclin-dependent kinase inhibitors [14–16, 31]. Under certain circumstances, activation of p21 or p16 cell cycle checkpoints ultimately controls for the senescent phenotype, regardless of whether or not telomeres are shortened [13]. Our previous work indicates that H₂O₂ treatment causes prolonged elevation of p21 and sustained inhibition of Rb hyperphosphorylation [25]. Since p16 has been found to be elevated in repli-

cative senescence and in some models of premature senescence, we tested whether H₂O₂ treatment results in elevation of p16. Cells were collected at various time points after H₂O₂ treatment, and Western blots were performed. A 96-h time-course study found no eleva-

FIG. 2. Telomere length of IMR-90 cells treated with various H₂O₂ doses. Cells were harvested immediately or 1 week after 2-h H₂O₂ treatment for extracting genomic DNA. *HinFI/RsaI*-digested genomic DNAs were separated by gel electrophoresis, and the gel was hybridized to radiolabeled (TTAGGG)₃ as described under Materials and Methods. Duplicate experiments were performed and a representative gel is shown (A). M, molecular weight markers in kb. The average TRF lengths from the duplicate experiments are presented in the graph (B).

FIG. 3. Telomere lengths in long-term cultures of H₂O₂-treated vs. untreated cells. Cells were treated with 75 μM H₂O₂ for 2 h weekly (lanes 8–13) or left untreated (lanes 1–7) and cultured for the indicated number of days before TRF measurements. Duplicate experiments were performed, and a representative gel is shown (A). The average TRF lengths from the duplicate experiments are shown in graph B. The slope of the regression line for untreated cells (–95 bp/PD) was significantly different from zero ($P < 0.0001$), whereas that from the treated cells was nonsignificant ($P = 0.65$).

tion of p16 protein by H₂O₂ treatment at any time point tested (Fig. 4A). With samples harvested weekly, no elevation of p16 protein was observed at 1, 2, or 3 weeks after H₂O₂ treatment (Fig. 4B). These negative results were observed in 10 independent experiments with samples harvested at various time points after various doses of H₂O₂ treatment. Replicatively senes-

cent cells showed an average 11-fold elevation of p16 protein ($n = 5$; a representative gel is shown in Fig. 4C) compared with early passage cells. The average ratios of p16 band density from H₂O₂-treated cells compared with untreated cells immediately, 18 to 24 h, 44 to 48 h, 3 days, 7 days, or 14 days after H₂O₂ treatment are 1.1, 1.1, 1.5, 1.1, 0.9, 1.2, or 0.8, respectively ($n = 3$ or 7). Antibodies from two other sources confirmed the negative Western blot results of p16. In brief, no elevation of p16 protein was observed in H₂O₂-treated cells.

To further verify the negative results on p16, we performed Northern blot analysis to measure for the abundance of p16 mRNA. The cells were harvested immediately, 24, 32, 48, or 72 h after 150 μM H₂O₂ treatment for isolation of total RNAs, which were used for Northern blot analysis. The results failed to show elevation of p16 mRNA by H₂O₂ treatment (Fig. 5A). In comparison, we determined the abundance of p21 mRNA using the same blot. The results showed that p21 mRNA was elevated 50-fold within 24 h and remained more than 40-fold elevated for 72 h after H₂O₂ treatment (Fig. 5A).

Because 72 h is not long enough for the cells to develop all features of senescence, we determined the level of p16 mRNA using the cells that show features of senescence. Again, we used p21 mRNA as a comparison. Cells treated with 150 μM once or 75 μM H₂O₂ twice are indistinguishable from replicatively senescent cells by a number of measurements 1 week after H₂O₂ treatment (see first section of Results). However, no elevation of p16 mRNA was detected in these H₂O₂-treated cells (Fig. 5B), while the same blot showed about 40-fold elevation of p21 mRNA levels in H₂O₂-treated cells that displayed features of senescence (Fig. 5B). A dose-response curve was determined in an at-

FIG. 4. The level of p16 protein in H₂O₂-treated cells. IMR-90 cells (PDL <25) were seeded at the density of 2×10^6 cells in 100-mm dishes for 2-h treatment of 150 μM H₂O₂. Cells were harvested within 96 h (A) or 3 weeks (B) after H₂O₂ treatment for Western blot analysis (40 μg protein/lane). Replicatively senescent cells (C) were harvested 1 week after splitting and were used as a positive control (10 μg protein/lane).

FIG. 5. The level of p16 mRNA at various time points after H_2O_2 treatment. IMR-90 cells ($PDL \leq 25$) were seeded at the density of 2×10^6 cells in 100-mm dishes for 2-h treatment of $150 \mu M H_2O_2$. Cells were harvested within 72 h (A) or 1 week after the treatment (B) for isolation of total RNA and Northern blot analysis. Near-senescent ($PDL 52$) or senescent ($PDL 54$) were harvested 1 week after splitting and were used as positive controls (B). The densities of p16 or p21 bands were analyzed by an Eagle Eye II Image System and were corrected by the densities of 18S rRNA bands. The ratios were compared with those of untreated cells at 2 h (A) or 1 week (B) for determination of the fold induction.

tempt to detect elevation of 16 mRNA species by using doses around the range that induces premature senescence. Again, we detected elevation of p21 mRNA but not p16 mRNA species with any dose tested (Fig. 6).

DISCUSSION

This study demonstrates that induction of a senescent phenotype by low or mild doses of oxidants may not involve shortening of telomeres or elevation of p16 cyclin-dependent kinase inhibitor. Although early passage cells treated with one dose ($150 \mu M$) show many features of senescence, these cells did not show significant shortening of TRF length. Long-term culture with weekly $75 \mu M H_2O_2$ treatment failed to accelerate shortening of TRF length. The negative data on telomere shortening has redirected our focus to a role of cell cycle checkpoint proteins in the phenotype switch of H_2O_2 -treated early passage HDFs. However, although p21 protein and mRNA remain elevated for at least 2 weeks after a 2-h pulse treatment of H_2O_2 , no elevation of p16 protein or mRNA was observed in

H_2O_2 -treated cells within such a time frame. This leaves the possibility that p21, but not telomeres or p16, plays a critical role in premature senescence induced by H_2O_2 .

Telomere shortening may be one of a few mechanisms mediating the development of the senescent phenotype. It has been clearly demonstrated that cell replication causes telomere shortening due to the inability of DNA polymerase to completely replicate the 3'-ends of lagging strands in the majority of normal human somatic cells, which lack telomerase activity. As a result, replicatively senescent HDFs contain an average of 2.4-kb shorter telomeres compared with their early passage counterparts. Consistent with the telomere theory of senescence, activation of telomerase has been shown to be sufficient to bypass replicative senescence in normal human cells [12]. Von Zglinicki *et al.* [21] found that hyperoxia accelerates the rate of telomere shortening and causes cells to gradually gain a senescent phenotype prematurely. Under hyperoxic conditions, the cells remain capable of replicating, although

FIG. 6. The level of p16 mRNA in cells treated with various doses of H₂O₂. IMR-90 cells (PDL ≤ 25) were seeded at the density of 2×10^6 cells in 100-mm dishes for a 2-h treatment with H₂O₂ at indicated concentrations. The cells were harvested 1 week after H₂O₂ treatment for isolation of total RNA and Northern blot analysis. The data are expressed as described in Fig. 5.

at a slower rate than under normoxia [21]. Differing from the cells cultured under the hyperoxic condition, the majority (70%) of IMR-90 cells failed to synthesize DNA after one treatment of 75 μ M H₂O₂. Perhaps cell replication is indeed required for the telomeres to be shortened. Low levels of oxidative stress such as hyperoxia may accelerate the telomere-shortening rate in the presence of cell replication, presumably by causing DNA strand breaks in the telomere regions [32]. Nevertheless, the fact that low or mild doses of H₂O₂ can induce the senescent phenotype without causing significant shortening of telomeres supports a number of recent findings against telomere shortening as a universal mechanism of senescence. For example, Syrian hamster embryo cells contain telomerase and do not shorten telomeres by cell replication but undergo replicative senescence as a result of serial passage [33]. Although activating telomerase causes immortalization without apparent changes in the karyotype, the length of telomeres may not increase significantly in the telomerase active cells [34–38]. In fact, a few studies regarding the consequence of telomerase activation have found its function to be unrelated to telomere extension. For example, telomerase activation causes *c-myc* overexpression [39], a gene that is known to

cause hyperproliferation of mammalian cells. Taken together, it is likely that mechanisms other than telomere shortening can also trigger the onset of the senescent phenotype.

The senescent phenotype consists of at least three elements: growth cessation, morphological enlargement, and changes in gene expression. Cessation of growth itself, such as serum starvation of cells, will not induce a senescent phenotype. The fact that the phenotype is induced by either telomere shortening or damaging agents suggests that senescence-associated changes result from a surveillance system that over-views the integrity of the genome and the process of DNA replication. Vaziri *et al.* [13] recently reported that telomere shortening results in activation of cell cycle checkpoints, supporting the hypothesis that activation of cell cycle checkpoints ultimately controls for the induction of senescence features. In fact, activation of cell cycle checkpoints seems to be a common feature of premature senescence induced by a variety of stimuli, ranging from damaging agents and chromatin remodeling to overexpression of *ras* or the *E2F1* gene [17–21]. Nevertheless, although it is clear that the activation of cell cycle checkpoints is critical for the growth arrest of senescent or senescent-like cells, what controls the features of senescence beyond growth arrest remains unknown. Our recent work indicates that activation of Rb family proteins is important for developing senescent morphology [40]. Because p21 is elevated for at least 2 weeks after H₂O₂ treatment and is an upstream regulator of Rb, it is possible that p21 may participate in the control of senescent morphogenesis after H₂O₂ treatment. Regardless, whether p21 or Rb controls the expression of multiple senescence-associated genes has not been tested. Because a number of different stimuli can induce the senescent phenotype while inhibiting DNA synthesis and inducing cell cycle checkpoints, perhaps many features of senescence result from cessation of replication without cessation of other processes such as protein synthesis. In agreement with this hypothesis, *de novo* protein synthesis appears to be critical for senescent morphogenesis in H₂O₂-treated HDFs [40].

Changes in gene expression, such as increased expression of collagenase and clusterin, have been found to be associated with aging [30, 41]. Although replicative senescence has been used as a model for studying aging over the past 4 decades, whether cellular senescence indeed occurs *in vivo* during the process of aging has been a subject of much debate. An early study by Robbins *et al.* [42] failed to find senescent-like cells *in vivo* by examining the morphology of cells isolated from elderly donors. Using replicative potential as a measurement, Cristofalo's group [43] recently reported no significant correlation between senescence *in vitro* and aging *in vivo*. Although the senescent biomarker SA

β -gal was first reported to be present preferentially and selectively in the skin biopsy samples of elderly donors [22], works from other groups failed to detect SA β -gal-positive cells in tissues from elderly donors [44]. Regardless of whether cellular senescence occurs *in vivo*, the free radical theory of aging holds that aging results from random damage produced by the oxidant by-products of aerobic metabolism [45]. The level of oxidative damage accumulates during the process of aging [45]. Recent profiling of aging-associated genes found many oxidative stress-inducible genes in the tissues of aging mice [46, 47], supporting a role for oxidative damage perhaps by changing gene expression in aging. Given the fact that oxidants can induce changes in the expression of a large number of genes, it is possible that some of these changes play a critical role in aging and aging-associated diseases. The observed phenotypic switch that occurs within a well-defined time frame of H_2O_2 -treated cells gives us an advantage in studying the regulation of gene expression and the identification of critical control genes that might contribute to the process of aging or aging-associated disease at the molecular level.

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