Loss of Proliferative Capacity and Induction of Senescence in Oxidatively Stressed Human Fibroblasts*

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Cellular senescence can result from short, dysfunctional telomeres, oxidative stress, or oncogene expression, and may contribute to aging. To investigate the role of cellular senescence in aging it is necessary to define the time-dependent molecular events by which it is characterized. Here we investigated changes in levels of key proteins involved in cell cycle regulation, DNA replication, and stress resistance in senescing human fibroblasts following oxidative stress. An immediate response in stressed cells was dephosphorylation of retinoblastoma (Rb) and cessation of DNA synthesis. This was followed by sequential induction of p53, p21, and p16. Increase in hypophosphorylated Rb and induction of p53 and p21 by a single stress treatment was transient, whereas sustained induction or dephosphorylation were achieved by a second stress. Down-regulation of the critical DNA replication initiation factor Cdc6 occurred early after stress concurring with p53 induction, and was followed by a decrease in Mcm2 levels. A late event in the stress-induced molecular sequence was the induction of SOD1, catalase, and HSP27 coinciding with development of the fully senescent phenotype. Our data suggest that loss of proliferative capacity in oxidatively stressed cells is a multistep process regulated by time-dependent molecular events that may play differential roles in induction and maintenance of cellular senescence.

Normal human fibroblasts only have a limited proliferative capacity in vitro and will become senescent eventually (1). Although cellular senescence is mainly observed with cells in culture, several lines of evidence suggest that a similar process occurs in vivo and contributes to aging (Ref. 2 and references therein). Telomere shortening because of the end replication problem is believed to be the prime intrinsic cause that limits the cell proliferation capacity because critically shortened telomeres serve to activate the senescence process (3). However, various stresses such as DNA damaging agents, oxidative stress, and certain oncogene overexpression can also induce cellular senescence (4–7). The cell culture condition of 20% O2 is characterized. Here we show that oxidative stress-induced cellular senescence will allow us to study the progress of senescence-associated aging. Here we show that oxidative stress causes loss of proliferative capacity and induction of much lower in vivo physiological pressure of O2 (8–10). Thus telomere shortening may be driven to a large extent by stress, and telomere shortening by the end replication problem may set an upper limit to the replicative lifespan, which might only seldom be reached (11).

The free radical theory of aging proposes that reactive oxygen species produced in mitochondria cause damage to cellular macromolecules, thus resulting in a decline of cellular function and organism aging (12). This theory has gained strong support from studies that linked oxidative stress to longevity using model organisms including yeasts, nematodes, flies, and rodents (13, 14). Mutations that increase longevity are often associated with increased antioxidant activity and improved resistance to oxidative stress (15–17).

For the past decade we have studied the long term consequences of poor early growth using rodent models (18). One of our most striking observations is that the rate of growth in early life has profound effects on longevity. Fetal growth restriction followed by postnatal catch-up growth in male rats and mice results in reduced longevity (19, 20). Male rats most frequently die of renal failure (19). We have shown that the reduced longevity of fetally growth restricted, postnatally growth caught-up male rats is associated with accelerated shortening of telomeres in the kidney (21). We have suggested that this shortening is associated with accelerated senescence of renal cells with earlier renal failure and death. It seems unlikely that replicative senescence alone could lead to the degree of shortening observed and therefore telomere shortening consequent upon oxidative damage may be involved (21, 22). We have further speculated that telomeres not only function as a measure of the number of cell divisions undergone by telomerase-negative cells but also, by virtue of their G4G content, monitor oxidative damage by the accelerated telomere shortening that results (23).

The present study was undertaken to study a selection of key proteins involved in DNA damage response, cell cycle regulation, DNA replication licensing, and stress resistance in oxidatively senescing cells by exploiting a model cell line in vitro. Knowledge of the molecular markers of oxidative stress-induced cellular senescence will allow us to study the progress of cell senescence in vitro. It has been shown that early passage human fibroblasts can be induced into senescence prematurely by oxidative stress (4, 24, 25). Because the cells develop features of senescence within 7 days and the time course for developing senescent features is well defined, oxidatively stressed human fibroblasts provide a useful experimental model to study the underlying mechanism and markers of senescence-associated aging. Here we show that oxidative stress causes loss of proliferative capacity and induction of

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senescence in human fibroblasts by well defined sequential molecular events.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and H$_2$O$_2$ Treatment**—IMR-90 cells at population doubling (PD) 24.5 were obtained from the American Type Culture Collection (ATCC). The cells were grown in ATCC modified Eagle’s minimal essential medium supplemented with 10% fetal bovine serum. Cells for H$_2$O$_2$ treatment were prepared from exponential phase around PD30. H$_2$O$_2$ treatment was carried out 24 h after seeding by incubating $2 	imes 10^6$ cells in 100-mm dishes in 13 ml of the culture medium containing 600 $\mu$M H$_2$O$_2$ for 2 h. For a second H$_2$O$_2$ treatment the cells were split 1:3 after being cultured for 4 days following the first treatment and treated again for 2 h with 600 $\mu$M H$_2$O$_2$ 24 h after seeding. IMR-90 cells reached replicative senescence at PD59.

**BrdUrd Labeling and SA-β-gal Assay**—Cells were seeded and treated as described above in dishes containing autoclaved coverslips. For BrdUrd labeling, coverslips were transferred into a 6-well plate and incubated in 2 ml of the culture medium containing 10 $\mu$l BrdUrd for 2 h. Cells on coverslips were then washed with PBS, fixed in 4% paraformaldehyde in PBS, washed twice in PBS, permeabilized in 0.2% Triton X-100, and washed in PBS. DNA was denatured by incubation in 2 M HCl for 1 h followed by three washes with PBS. Coverslips were incubated for 1 h with fluorescein-conjugated mouse anti-BrdUrd monoclonal antibody (1:20 dilution; Alexis Biochemicals) and with DNA stain TOTO-3-iodide (0.5 $\mu$M; Molecular Probes), washed three times with PBS, and mounted in 90% glycerol, 10% PBS containing p-phenylenediamine (1 mg/ml; Sigma). For senescence-associated β-galactosidase (SA-β-gal) assay, cells on coverslips were washed twice in PBS, fixed for 5 min in 4% paraformaldehyde in PBS, washed three times in PBS, and incubated at 37 °C overnight in fresh SA-β-gal staining solution (1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactosidase (stock = 100 mg/ml of dimethylformamide)/ml of 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl in 40 mM citric acid/sodium phosphate, pH 6.0) (26). At least 500 cells from each time point were scored under microscope for BrdUrd- or SA-β-gal assay.

**Western Blotting**—Whole cell lysate was prepared by scraping cells in Laemmli buffer (0.12 M Tris, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol with protease inhibitors (mixture from Sigma)). Protein concentration was determined by the bicinchoninic acid (BCA) method (Sigma) with bovine serum albumin as a standard. 25 $\mu$g of proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Blots were probed with the following antibodies: anti-p53 antibody (ab7757, abcam), anti-p21 antibody (H-164, Santa Cruz), anti-p16 antibody (H-156, Santa Cruz), anti-Rb antibody (G3-245, BD Pharmingen), anti-Cdc6 antibody (180.2, Santa Cruz), anti-Mcm2 antibody (BM28, Transduction Laboratories), anti-Mcm5 antibody (27), anti-PCNA antibody (ab29, abcam), anti-catalase antibody (ab1877, abcam), anti-SOD1 antibody (FL-154, Santa Cruz), anti-HSF70 antibody (W27, Santa Cruz), and anti-HSP27 (H-77, Santa Cruz). The
bound primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), followed by enhanced chemiluminescence (Amersham Biosciences). The densities of the bands were quantified using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA).

Preparation of Nuclei and Cytosolic Extracts—Nuclear and cytoplasmic extracts were prepared by hypotonically swelling cells in ice-cold LS buffer (20 mM K-glutamate, 5 mM potassium acetate, 0.5 mM MgCl₂, 0.5 mM dithiothreitol), scrape harvesting, and Dounce homogenization as described previously (28) with the following minor modifications. Cytosolic supernatant was taken from the first spin (2,000 x g in a Whatman Micro-Centrifuge for 2 min) and respun at 10,000 x g for 10 min. Supernatant fractions were then aliquoted, snap-frozen, and stored in liquid nitrogen. Nuclear pellets from the first separating spin were resuspended in an equal volume of ice-cold SuNaSp solution (250 mM sucrose, 75 mM NaCl, 0.5 mM spermine tetrahydrochloride, 0.5 mM spermidine trihydrochloride, 3% bovine serum albumin) and respun at 2,000 x g for 2 min. The supernatant was discarded and the packed nuclear pellet was resuspended in an equal volume of SuNaSp solution, frozen, and stored at –80 °C.

In Vitro DNA Synthesis Assay—DNA replication reactions containing 30 µl of elongation buffer (60 mM KCl, 15 mM Tris-Cl, pH 7.4, 15 mM NaCl, 1 mM β-mercaptoethanol, 0.5 mM spermine tetrahydrochloride, 0.5 mM spermidine trihydrochloride), a buffered mixture of NTPs, dNTPs, and an energy regeneration system (yielding a final concentration of 0.25 µM biotin-16-dUTP (Roche Diagnostics). In vitro DNA replication reactions were performed in PBS (0.5 ml) and fixed for 3 h by adding an equal volume of 8% paraformaldehyde. After fixation, nuclei were spun through 30% sucrose/PBS onto poly-l-lysine-coated coverslips. All subsequent washing and staining steps were carried out in PBS, 0.2% Triton X-100, 0.04% SDS. Coverslips were washed, stained for incorporated biotin-16-dUTP with fluorescein-linked streptavidin (1:100 dilution, Amersham Biosciences) and for DNA with propidium iodide/RNase A (both at 50 ng/ml, Sigma), washed again, and mounted in vectashield (Vector Laboratories, Peterborough, UK). Confocal fluorescence microscopy of random fields of nuclei was performed on a Leica TCS DMRE confocal microscope. Images were collected, and merged pictures of the propidium iodide channel (red) and fluorescein channel (green) were obtained using Adobe Photoshop with standardized brightness- and contrast-enhanced operations of all samples. Images were printed, and the number of nuclei incorporating biotin-16-dUTP in vitro (yellow) and non-replicating nuclei (red) were counted. Routinely, 800–1000 nuclei were scored for each replication reaction and quantitated as percentages of the total number of nuclei that synthesized DNA in vitro.

RESULTS

Cessation of DNA Replication and Induction of Senescence in the H₂O₂-treated Cells—To understand molecular mechanisms underlying aging, we investigated key cell cycle regulatory proteins and stress-resistant factors in normal human diploid fibroblasts that were subjected to oxidative stress. We adopted H₂O₂ treatment to induce premature cellular senescence. Dose-response experiments showed that the cells could tolerate up to

FIG. 2. Changes in levels of cell cycle checkpoint proteins and phosphorylation of Rb in H₂O₂-treated cells. A, H₂O₂-treated IMR-90 cells were harvested at the indicated time points and whole cell lysates (25 µg of proteins) were analyzed by Western blotting using antibodies against p53, p21, and p16. Loading was revealed by Coomassie Blue staining. Indicated on the top are the times after H₂O₂ treatment and the number of H₂O₂ treatments. This experiment is representative of two independent experiments. B, changes in the phosphorylation state of Rb in the samples used for panel A were analyzed by Western blotting using an antibody against Rb. Hyperphosphorylated Rb and dephosphorylated Rb were analyzed by densitometry and the ratio of the two is shown.
600 μM H₂O₂ for 2 h with very few lethal effects under the conditions used in this study (data not shown). A small fraction of the cells that received a single H₂O₂ treatment were able to recover and re-enter the cell cycle. This was manifested by the gradual increase of cell number after the treatment. Indeed, about 20% of the treated cells incorporated BrdUrd 5 days after a first H₂O₂ treatment (Fig. 1A). We therefore reseeded the cells at a ratio of 1:3 4 days after the first treatment and performed a second treatment 24 h later. The second H₂O₂ treatment caused a permanent cell cycle arrest for over 95% cells. As shown in Fig. 1A, BrdUrd positive cells decreased to less than 4% after the second H₂O₂ treatment and eventually became undetectable 4 days later. Cells stressed with H₂O₂ twice eventually developed senescence phenotypic features including enlarged and flattened morphology and SA-β-gal activity. The fully developed phenotypic features appeared 4 days after the second H₂O₂ treatment when over 95% cells displayed SA-β-gal activity (Fig. 1B). This result shows that a second oxidative stress is necessary to induce a high incidence of premature senescence.

**Induction of Cell Cycle Checkpoint Proteins in the H₂O₂-treated Cells**—Among the cell cycle checkpoint proteins investigated, p53 was rapidly increased by oxidative stress (Fig. 2A). This was followed by the induction of p21, which is a target of p53 (Fig. 2A). Both proteins tended to return to basal level if the induction was caused by a single H₂O₂ treatment. Sustained induction of p53 and p21 was seen in the cells that received two H₂O₂ treatments (Fig. 2A). An elevated p16 level was only observed in the cells that received two H₂O₂ treatments and developed senescent phenotypic features (Fig. 2A).

Dephosphorylation of Rb was also induced by H₂O₂ treatment (Fig. 2B). The ratio of hyperphosphorylated Rb (pRb) to dephosphorylated Rb decreased from 53% in control cells to 43% in cells a half-hour after a 2-h H₂O₂ treatment (Fig. 2B). The ratio returned to the control level 5 days after the treatment. However, the second H₂O₂ treatment caused a sustained decrease in hyperphosphorylated Rb, which eventually became undetectable 1 day after the second treatment and remained dephosphorylated thereafter (Fig. 2B).

**Decrease of DNA Replication Proteins in the H₂O₂-treated Cells**—Cdc6, Mcm2, Mcm5, and PCNA are proteins involved in DNA replication (30). Apart from Mcm5 that remained largely unaffected, they generally decreased in the H₂O₂-treated cells (Fig. 3A). Cdc6 became undetectable in the cells that received two H₂O₂ treatments and had the senescent phenotype fully developed (Fig. 3A). However, a residual amount of Mcm2 and a substantial amount of PCNA remained detectable in the twice H₂O₂-treated cells (Fig. 3A).

**Effects of Oxidative Stress on Antioxidant Enzymes and Stress-response Proteins**—Levels of antioxidant enzymes SOD1 and catalase were not immediately affected by H₂O₂ treatment, but an increase in the levels of these two enzyme proteins was clearly detected in the stressed cells when senescent phenotypic features were fully developed (Fig. 3B). Levels of the stress response protein HSP70 did not change noticeably in the H₂O₂-treated cells, whereas HSP27 showed a slight increase
Molecular Events in Oxidatively Senescing Cells

Changes in levels of p53, p21, p16, and Mcm2 in replicatively senescing cells. Whole cell lysates (25 μg of proteins) from IMR-90 cells of early population doubling, near replicative senescence, and 4 weeks into replicative senescence were analyzed by Western blotting using respective antibodies. Actin is shown as a loading control. This experiment is representative of two independent experiments.

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in the treated cells that also showed high SA-β-gal activity (Fig. 3B).

Molecular Events in Replicatively Senescing Cells—To confirm that molecular events induced by oxidative stress are similar to those in replicatively senescing cells, we examined changes in p53, p21, p16, and Mcm2 in cells when they were in early population doubling (PD30), in a near senescent state (PD57), and in the fully established replicative senescent state (4 weeks into replicative senescence). As shown in Fig. 4, p53 and p21 increased substantially in the near replicative senescent cells but declined in the fully senescent cells. A significant increase in p16 was only observed in the fully senescent cells (Fig. 4). On the other hand, Mcm2 decreased substantially in the near replicative senescent cells and was undetectable in the fully senescent cells (Fig. 4). These data suggest that time-dependent changes in molecular events observed in the oxidatively senescing cells occur similarly in the replicatively senescing cells but over a much longer time frame.

Molecular Events in Early Time Points after H₂O₂ Treatment—Oxidative stress is a potential source of DNA damage. To investigate the immediate consequences of oxidative stress in the cells we examined molecular events in earlier time points after H₂O₂ treatment. Induction of p53 can be detected at the end of the 2-h H₂O₂ treatment (Fig. 5). The highest induction was observed 2 h later. Thereafter it declined gradually and returned to almost the basal level 5 days later. This rapid induction of p53 was repeated after a second H₂O₂ treatment (Fig. 5). Induction of p21 was detected 2 h later than that of p53 with the highest induction being seen 6 h after the 2-h H₂O₂ treatment (Fig. 5). Expression of p16 was not affected by H₂O₂ treatment during this early time course (Fig. 5). H₂O₂ treatment caused a decrease in the ratio of hyperphosphorylated Rb to dephosphorylated Rb in the cells. This change was detected in the cells that received treatment for only 1 h (Fig. 5). Densitometry analysis showed that the ratio decreased from 60% in the control cells to 46% in the 1-h H₂O₂-treated cells. Further decreases were seen in subsequent time points and the hyperphosphorylated Rb eventually became undetectable 10 h after the 2-h H₂O₂ treatment (Fig. 5). The hyperphosphorylated Rb reappeared a day after the treatment and returned to the control level 5 days later (Fig. 5). The second H₂O₂ treatment caused a similar change in the ratio in the cells at the end of the treatment (Fig. 5). In contrast, the DNA replication protein Mcm2 remained unchanged during the initial 24 h after the first H₂O₂ treatment with a slight decrease only being seen 5 days later and 2 h after a second H₂O₂ treatment (Fig. 5). Changes in levels of Cdc6 showed a similar pattern to that of Mcm2 (data not shown).

Cessation of DNA Replication Is a Rapid Responsive Event in the H₂O₂-treated Cells and Is Because of the Inhibition of DNA Elongation—H₂O₂ treatment caused a rapid cessation of DNA replication as demonstrated by a rapid decrease of BrdUrd positive cells in the oxidatively stressed cells (Fig. 6A). A 1-h H₂O₂ treatment caused an almost 10-fold decrease in DNA synthesis as compared with the untreated cells (83% BrdUrd positive cells). At the end of the 2-h H₂O₂ treatment BrdUrd positive cells decreased to 2.7%. A further decrease was observed 2 h later. A slight recovery was observed 4 h after the 2-h H₂O₂ treatment (Fig. 6A). Thus cessation of DNA replication was a rapid responsive event that preceded any detectable changes in protein levels of p53, p21, p16, Mcm2, and Cdc6 (see Fig. 5).

Rapid cessation of DNA replication in the H₂O₂-treated cells suggested that any ongoing DNA synthesis during S-phase must have been halted by the stress. To test this hypothesis we employed a cell-free in vitro DNA replication assay and compared DNA synthesis in elongation buffer using nuclei prepared from control and 2-h H₂O₂-treated cells. As shown in Fig. 6B, only 1.8% of nuclei prepared from H₂O₂-stressed cells were capable of DNA synthesis in contrast to the control in which about 10% of nuclei were synthesizing DNA (Fig. 6B). Note that the relatively low percentage of replicating nuclei compared with that detected by a 48-h BrdUrd labeling (Fig. 6A) is because of the short incubation period of the in vitro replication.
reaction (3 h). This suggests that elongation during S-phase was inhibited by H$_2$O$_2$ treatment.

**DISCUSSION**

We have established a unique rodent model in which longevity can be significantly increased or decreased by a minor manipulation of maternal diet (19, 20). A causative link between longevity and cellular senescence in vivo remains to be established. As a first step toward elucidating the molecular mechanisms underlying cellular senescence and such a profound effect of growth rate during fetal and postnatal development on aging, we used an *in vitro* human fibroblast model to study key proteins involved in cell cycle control, DNA replication, and stress resistance in oxidatively stressed cells. We demonstrated that around 20% of cells exposed to a single H$_2$O$_2$ treatment were able to recover and re-enter the cell cycle despite the initial abrupt cessation of DNA synthesis. This is consistent with an earlier report that showed that over 30% of the BrdUrd positive cells were detected in single H$_2$O$_2$-treated IMR-90 cells (24). Presumably this is because these cells were able to repair the DNA damage caused by a single H$_2$O$_2$ treatment. It is not clear as to why some cells are able to recover and re-enter the cell cycle while the majority become permanently arrested and eventually senesce. One possibility is the heterogeneity in DNA damage caused by H$_2$O$_2$ treatment. A high incidence of cell senescence was induced only by a second subcytotoxic H$_2$O$_2$ treatment. Indeed, cellular senescence in vivo is more likely to be induced by repeated oxidative insults. Mitochondria are major producers of reactive oxygen species and, at the same time, particularly susceptible to oxidative damage. In particular, damage to mtDNA results in faulty mitochondrial proteins, with a consequent decrease of electron transfer, leading to further production of reactive oxygen species and thus exposing cells to a vicious circle of oxidative stress (31).

In our time course study of oxidative senescence, we observed sequential inductions of cell cycle checkpoint proteins including p53, p21, and p16 with the induction of p16 coinciding with an increase in the activity of SA-$\beta$-gal. Increased p16 expression was also observed in replicative senescent cells. This is in keeping with cells undergoing replicative senescence in that p53 senses stresses, which then activates expression of p21 resulting in cell cycle arrest, whereas p16 is responsible for the maintenance of the senescent state (32, 33). The induced p53 and p21 by a single H$_2$O$_2$ treatment tended to return to basal levels. The transient induction of p53 may be because of the regulation of the p53-Mdm2 feedback loop. In the p53-Mdm2 feedback loop, elevated p53 induces Mdm2 transcription, which in turn binds p53 and promotes p53 degradation via the ubiquitin-proteasome pathway (34). Sustained elevations of p53 and p21 were observed only after the cells received a second treatment. This suggests that the p53-Mdm2 feedback loop may be disrupted by repeated oxidative stress. Louria-Hayon *et al.* (35) recently showed that PML (promyelocytic leukemia protein) is critical for the accumulation of p53 in response to DNA damage. PML protects p53 from Mdm2-mediated ubiquitination and degradation by prolonging the stress-induced phosphorylation of p53 on serine 20 (35). PML is essential for the formation of a subnuclear structure called PML nuclear bodies, which serve as an important meeting point for p53 regulation, including phosphorylation, acetyla-
tion, and de-ubiquitination (36). Several studies have indicated that PML is involved in the induction of premature cell senescence (37–39).

These changes in cell cycle checkpoint proteins in human fibroblasts in vitro upon oxidative stress could provide markers for detection of cells undergoing oxidative damage and thus becoming senescent in vivo. Melk et al. (40) recently showed that expression of mRNA for p16 was undetectable in most young rat kidneys, but rose 27-fold during growth and a further 72-fold during aging (40). p16 protein was localized to the nucleus and increased with age. Concomitantly, lipofuscin, an age-related pigment and SA-β-gal also increased with growth and aging (40). Similarly, strong correlation between the p16 level and age was detected in human kidney (41). It is evident, therefore, that aspects of molecular changes observed in senescent cells in vitro do occur in animals and humans in vivo.

It is believed that mitochondrial oxidant generation is one of many regulated processes that affect aging. Other factors such as antioxidant defenses, and the repair of oxidative damage are probably equally important (15). Superoxide dismutase (SOD) and catalase are antioxidant enzymes that convert superoxide to H₂O₂ and H₂O₂ to water, respectively (42). On the other hand, heat shock proteins are generally induced in response to many environmental stresses including heat, oxidizing conditions, and exposure to toxic compounds, making cells resistant to stress-induced cell damage (43). We found that increased expressions of SOD1, catalase, and HSP27 were only detected in the H₂O₂-treated cells when senescent features had fully developed. Although the underlying mechanism for the delayed stress response is not known, these stress responses may explain why senescent cells have increased tolerance to stresses and are resistant to apoptosis (44).

The present study also showed that H₂O₂-induced cell senescence is accompanied by the decline of several DNA replication proteins including Cdc6, Mcm2, and PCNA. Cdc6 and Mcm proteins are components of the DNA replication licensing complex, which is assembled during the G₁ phase, whereas PCNA is a DNA sliding clamp for replicative DNA polymerases, which is an essential component of the eukaryotic chromosomal DNA replisome during S-phase (30). These proteins undergo cell cycle-regulated synthesis, chromatin association, and proteolysis, thereby ensuring that DNA is replicated once and only once during a single cell division cycle (30, 45, 46). The absence of Cdc6 and decreased levels of Mcm2 and PCNA in the H₂O₂-induced senescent cells are probably a consequence of cell cycle arrest in the G₁ phase (24). The sequential down-regulation of Cdc6 and Mcm2 in the oxidatively stressed cells suggests that repression of DNA origin licensing is responsible for the loss of proliferative capacity upon oxidative stress. The presence of residual Mcm2 in cells that received two H₂O₂ treatments but its absence in replicative senescent cells again suggests that this protein is tightly correlated with cell proliferative capacity.

Indeed, we found that a small fraction of cells retained DNA replication potential even after receiving two H₂O₂ treatments (data not shown).

DNA damage rapidly activates DNA checkpoint machinery that stop a proliferating cell in the G₁, S, or G₂ phases of the cell cycle, contributing to the maintenance of genome integrity (47). Our cell-free in vitro assay indicated that the abrupt cessation of DNA replication caused by H₂O₂ treatment was because of inhibition of ongoing DNA elongation. This rapid inhibition was not because of the protelytic degradation of DNA replication proteins, as the protein levels of Cdc6, Mcm2, Mcm5, and PCNA were not significantly affected immediately after H₂O₂ treatment. Although H₂O₂ treatment stabilized p53 and caused subsequent induction of p21, these events only occurred after cessation of DNA replication had already occurred. It is therefore unlikely that p21 is directly involved in the inhibition of DNA replication under these conditions. Cicchillitti et al. (48) showed that a rapid inhibition of DNA synthesis in human umbilical vein endothelial cells (HUVEC) by H₂O₂ treatment was correlated with a rapid hypophosphorylation of the retinoblastoma family proteins Rb, p107, and p130 (48). This event did not require p53 or p21 and was not associated with cyclin/CDK down-modulation, but was rather dependent on the activity of protein phosphatase 2A (48). It was speculated that, upon cell treatment with H₂O₂, Rb family members might bind in their dephosphorylated form directly to the DNA replication origins thereby inhibiting their firing (48). Indeed, upon DNA damage by γ-irradiation, Rb binds to selected DNA replication origins, inhibiting DNA replication in S-phase (49). In this study we observed a decrease in the ratio of hyperphosphorylated Rb to hypophosphorylated Rb in the cells at the earliest time point (Fig. 5). It is conceivable, therefore, that dephosphorylation of Rb may play an important role in the immediate inhibition of DNA replication in the H₂O₂-treated cells. It also remains possible that Rb re-location from certain chromatin structures to chromatin locations that contain origins of DNA replication contributes to the rapid inhibition of DNA synthesis upon oxidative damage (49).

In conclusion, our study demonstrated that oxidative stress induced an array of molecular events. This involved increases and decreases in the levels of various proteins as well as changes in phosphorylation status. These events were time dependent and resulted in loss of proliferative capacity and ultimately the development of a senescent state that was indistinguishable from replicatively senescent cells. One of the initial events in this multistep process was the immediate inhibition of ongoing DNA elongation that is likely to be controlled by dephosphorylated Rb. Down-regulation of Cdc6 may contribute to the early response of cells to oxidative stress by inhibiting the pre-replicative complex from licensing during the G₁ phase. In contrast down-regulation of Mcm proteins appeared to constitute a late response in the process. Sustained loss of proliferative capacity and high induction of cellular senescence are achieved by a second oxidative stress that is presumably necessary to cause irreparable DNA damage in human fibroblasts.

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