Molecular analysis of H₂O₂-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G₁ arrest but not cell replication

Qin M. CHEN^{*1}, James C. BARTHOLOMEW[†], Judith CAMPISI[‡], Meileen ACOSTA[‡], Joshua D. REAGAN^{*} and Bruce N. AMES^{*}

^{*}Barker Hall, Division of Biochemistry and Molecular Biology, University of California, Berkeley, CA 94720-3202, U.S.A., [†]Division of Structural Biology, Melvin Calvin Laboratory, Berkeley National Laboratory, Berkeley, CA 94720, U.S.A., and [‡]Life Science Division, Building 70A, Berkeley National Laboratory, Berkeley, CA 94720, U.S.A.

Human diploid fibroblasts lose the capacity to proliferate and enter a state termed replicative senescence after a finite number of cell divisions in culture. When treated with sub-lethal concentrations of H₂O₂, pre-senescent human fibroblasts enter long-term growth arrest resembling replicative senescence. To understand the molecular basis for the H₂O₂-induced growth arrest, we determined the cell cycle distribution, levels of p53 tumour suppressor and p21 cyclin-dependent kinase inhibitor proteins, and the status of Rb phosphorylation in H₂O₂-treated cells. A 2-h pulse of H₂O₂ arrested the growth of IMR-90 fetal lung fibroblasts for at least 15 days. The arrested cells showed a G₁ DNA content. The level of p53 protein increased 2- to 3-fold within 1.5 h after H₂O₂ exposure but returned to the control level by 48 h. The induction of p53 protein was dose dependent, beginning at 50–75 µM and reaching a maximum at 100–250 µM. The induction of p53 did not appear to correlate with the level of

DNA damage as measured by the formation of 8-oxo-2'-deoxyguanosine in DNA. The level of p21 protein increased about 18 h after H₂O₂ exposure and remained elevated for at least 21 days. During this period, Rb remained underphosphorylated. The induction of p53 by H₂O₂ was abolished by the iron chelator deferoxamine and the protein synthesis inhibitor cycloheximide. The human papillomavirus protein E6, when introduced into the cells, abolished the induction of p53, reduced the induction of p21 to a minimal level and allowed Rb phosphorylation and entry of the cells into S-phase. The human papillomavirus protein E7 reduced the overall level of Rb and also abolished H₂O₂-induced G₁ arrest. Inactivating G₁ arrest by E6, E7 or both did not restore the replicative ability of H₂O₂-treated cells. Thus H₂O₂-treated cells show a transient elevation of p53, high level of p21, lack of Rb phosphorylation, G₁ arrest and inability to replicate when G₁ arrest is inactivated.

INTRODUCTION

Human diploid fibroblasts (HDFs) have been used as a model for studying replicative senescence [1–5]. These cells resemble most normal somatic cells *in vivo* in that they maintain a normal karyotype and undergo a limited number of cell divisions. In culture, these cells lose the ability to proliferate and reach senescence after serial passage. Senescent cells cannot replicate in response to physiological mitogens. We have found that senescent HDFs contain higher levels of oxidative DNA lesions compared with early passage cells [6], raising the possibility that oxidative damage may trigger the onset of cell-cycle checkpoints in senescent cells. Senescent cells contain an elevated level of the p21 cyclin-dependent kinase (CDK) inhibitor [7], underphosphorylated retinoblastoma tumour-suppressor protein Rb [8], reduced E2F activity [9], and are arrested in the G₁ phase of the cell cycle. Senescence in culture is thought to reflect processes related to aging *in vivo* and may evolve as a tumour suppression mechanism [1,3].

Cell-cycle checkpoints control the onset of DNA replication and mitosis in order to ensure the integrity of the genome [10]. The tumour suppressor protein p53 is a major component of cell-cycle checkpoints. A number of DNA damaging treatments cause increases in the level of p53 protein by post-transcriptional regulation [11] and in DNA sequence-specific binding by p53 [12]. Recent evidence suggests that p53 protein can be induced by

hypoxia [13] and depletion of ribonucleotide pools [14]. Thus multiple signals appear to control p53 induction.

The CDK inhibitor p21 can mediate the G₁ cell cycle arrest by elevated p53. The p53 protein activates transcription of the p21 gene [15,16], although activation of p21 can also be p53 independent [17,18]. The p21 protein inhibits the activity of CDKs, which phosphorylate Rb. Underphosphorylated Rb binds and inactivates the transcription factor E2F. Hyperphosphorylation causes Rb to release E2F and thereby permits E2F to activate the transcription of early S-phase genes. Thus induction of p53 may result in an elevation of p21 and underphosphorylation of Rb.

The human papillomavirus (HPV) type-16 proteins E6 and E7 are useful tools for probing the role of p53 and Rb in growth arrest. The E6 protein binds p53 and facilitates its degradation by ubiquitin-dependent proteolysis [19,20]. The E7 protein binds Rb and Rb-related proteins, thereby inactivating the function, including the negative regulation of E2F [19,20]. The E7 protein also enhances proteolytic degradation of Rb [21]. When introduced into cells, either E6 or E7 abolishes the G₁ arrest induced by DNA damaging agents, suggesting that both p53 and Rb proteins are essential for controlling this G₁ arrest [22–25]. However, in many of these studies, the G₁ arrest induced by DNA damage was transient and the cells eventually proceeded through the cell cycle.

It has been observed that HDFs arrest growth for a prolonged period in response to H₂O₂ [26]. The majority of H₂O₂-treated

Abbreviations used: BrdU, bromodeoxyuridine; CDK, cyclin-dependent kinase; DFAM, deferoxamine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HDF, human diploid fibroblast; HPV, human papillomavirus; oxo⁸dG, 8-oxo-2'-deoxyguanosine; PCNA, proliferating cell nuclear antigen; PDL, population doubling level.

¹ To whom correspondence should be addressed. Present address: Department of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ 85724, U.S.A.

HDFs develop a phenotype that shares features with replicative senescence, including the inability to replicate in response to a variety of growth factors, reduced activity of certain cell cycle-related enzymes and enlarged cell size [26]. The mechanism underlying this growth arrest is not understood at the molecular level, nor have the roles of p53, CDK inhibitors and Rb been elucidated. In the present study, we determine the cell cycle distribution, levels of protein p53, p21, p16 and Rb, and phosphorylation of Rb after treatment of the cells with sublethal concentrations of H_2O_2 .

MATERIALS AND METHODS

Cell culture

IMR-90 cells [obtained from the Coriell Institute for Medical Research (Camden, NJ, U.S.A.) at a population doubling level (PDL) of 10.5 were subcultured weekly at 0.5×10^6 cells per 100-mm Corning dish] containing 10 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Confluent cultures (6–7 days after subculture) were treated with H_2O_2 by adding it to the culture medium for 2 h, unless specified otherwise. After treatment, the cells were washed with PBS (37 °C) before harvesting, subculturing or incubating in fresh medium.

Measurement of DNA synthesis

Cells in 24-well (2 cm²/well) plates (seeded at 5×10^4 cells per well and grown for 6–7 days) were incubated with fresh DMEM containing 1% (v/v) FBS and 0.2% (w/v) BSA for the indicated intervals after H_2O_2 treatment. Cells were then replated at 2×10^3 cells per well for 48 h in fresh DMEM containing 10% (v/v) FBS and 10 μ M bromodeoxyuridine (BrdU). Incorporated BrdU was detected using an anti-BrdU antibody according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). Approx. 300 cells/well were scored for positive or negative staining. At least 3 wells were scored for each treatment.

Cell-cycle analysis

Cells were subcultured at a ratio of 1:3 after H_2O_2 treatment. Colchicine was added to a final concentration of 50 μ M for 8 or 24 h. The cells were treated with trypsin, collected by centrifugation and fixed in 25% ethanol containing 15 mM $MgCl_2$ at a density of 1×10^6 cells/ml. After RNase digestion (0.1 mg of RNase A and 2 units of RNase T1/ml at 37 °C for 1 h) and PBS wash, the cells were stained with 50 μ g/ml propidium iodide for at least 30 min before analysis by flow cytometry using an instrument as designed by Steinkamp et al. [27] with modifications to eliminate the sorting mode of operation. Cells were passed through a flow chamber at approx. 500 cells/s in a stream that intersected an argon-ion laser beam tuned to 488 nm. Emitted light collected at 90° from the laser beam and cell stream was passed through a band-pass filter centred at 610 nm. Signals from the photomultiplier were collected in an Oxford multi-channel analyser after analog to digital conversion. These data were converted into an Excel format for display and calculations.

Measurement of steady state levels of 8-oxo-2'-deoxyguanosine (oxo⁸dG) in DNA

Cells were harvested by trypsinization and cell pellets were suspended in extraction buffer [0.1 M Tris (pH 8.0)/0.1 M NaCl/20 mM EDTA/2 mM butylated hydroxytoluene/1 mM

deferoxamine/0.2% (v/v) Triton X-100]. After RNase and proteinase K digestion, DNA was extracted with phenol/chloroform and was digested to nucleosides by nuclease P1 and alkaline phosphatase [28]. The nucleosides or oxo⁸dG was analysed using HPLC with UV (for nucleosides) or electrochemical (for oxo⁸dG) detection [28] simultaneously.

Western-blot analysis

Cells were lysed [0.12 M Tris/HCl (pH 6.8)/2.4% (w/v) SDS/50% (v/v) glycerol] and the proteins were denatured by boiling for 5 min after the addition of an equal volume of SDS reducing buffer [0.5% Tris/HCl (pH 6.8)/10% (v/v) glycerol/2% (w/v) SDS/5% (v/v) 2-mercaptoethanol]. The protein concentration was determined by absorption at 280 nm using the Warburg-Christian method [29]. Bromophenol Blue [final concentration, 0.01% (w/v)] was added to the samples before loading equal amounts of protein on to each lane. Proteins were separated by SDS/PAGE using a mini-Protein II electrophoresis apparatus (Bio-Rad, Richmond, CA, U.S.A.) run at 10 mA constant current for the stacking gel and 15 mA constant current for the separating gel (12%, 8%, or 6% acrylamide for p21, p53, or Rb respectively). The separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) by electrophoresis overnight at 20 V using a mini trans-blot apparatus (Bio-Rad). The membrane was blocked with 5% (w/v) non-fat milk before incubating with anti-p21 (1:250 dilution; PharMingen, San Diego, CA, U.S.A.), anti-p53 (1:100 dilution; Oncogene Science, Uniondale, NY, U.S.A.) or anti-Rb (1:100 dilution; Santa Cruz Biotechnology, CA, U.S.A.) antibodies with 1% (w/v) BSA in Tris-buffered saline (10 mM Tris/HCl (pH 8.0)/150 mM NaCl) containing 0.05% (v/v) Tween-20 (TBST) for 2 h. The secondary antibody [anti-mouse IgG for p21 and p53 (Amersham, Arlington Heights, IL, U.S.A.), anti-rabbit IgG for Rb (Santa Cruz Biotechnology, CA, U.S.A.)] conjugated with horseradish peroxidase was used for detection using the Renaissance chemiluminescent reagent (NEN-DuPont, Boston, MA, U.S.A.) and X-OMAT Scientific Imaging film (Kodak, Rochester, NY, U.S.A.). Band intensities were quantified using an IS1000 imaging system (Alpha Innotech, Hayward, CA, U.S.A.).

Infection with recombinant HPV E6 or E7 retroviral constructs

The retroviruses carrying HPV E6 or E7 gene and the neo gene that confers G418 resistance were a gift from Dr. Denise Galloway (The Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.) [30]. Exponentially growing IMR-90 cells (PDL 16.8) in 100-mm dishes were infected with retroviruses carrying either the HPV E6 or E7 gene in 2 ml DMEM containing 10% (v/v) FBS and 4 μ g/ml polybrene. After 4 h at 37 °C, the medium volume was increased to 10 ml for overnight incubation. The medium was changed next day and cells expressing the constructs were selected by culturing in 500 μ g/ml G418.

RESULTS

The human fetal lung fibroblast cell strain IMR-90 is frequently used to study cellular senescence. A phenotype resembling senescence was previously shown to result from sublethal H_2O_2 exposure in human foreskin F65 fibroblasts, which have a relatively short replicative life span [26]. In the present work, we test whether IMR-90 cells undergo a long-term growth arrest in response to H_2O_2 . Early passage IMR-90 cells (PDL 20–28) were made quiescent by growth to confluence. As determined by Trypan Blue uptake, H_2O_2 at concentrations less than 550 μ M

Figure 1 H₂O₂ dose-dependent inhibition of DNA synthesis

Confluent IMR-90 cells (PDL 20–28) in 24-well plates were treated with various doses of H₂O₂ and BrdU incorporation was measured as described in the Materials and methods section. The data are the means $\pm$ S.D. of three samples.

Table 1 Percentage of cells not synthesizing DNA after H₂O₂ treatment

Confluent IMR-90 cells (PDL 20–28) in 24-well plates were treated with 300 μ M H₂O₂ for 2 h. The cells were subcultured for 48 h in the presence of 10 μ M BrdU for measurement of DNA synthesis, as described in the Materials and methods section. The values are the means $\pm$ S.D. of three independent experiments.

Day after treatment	BrdU-negative cells (%)	
	Control	H ₂ O ₂ treated
0	4.9 $\pm$ 0.2	46.9 $\pm$ 4.3
4	13.3 $\pm$ 1.0	67.1 $\pm$ 15.0
7	10.5 $\pm$ 3.4	59.2 $\pm$ 10.8
14	15.0 $\pm$ 2.6	59.0 $\pm$ 7.7

did not cause cell death (results not shown). H₂O₂ at sublethal concentrations caused a dose-dependent increase in the number of cells that did not synthesize DNA, as determined by BrdU incorporation (Figure 1). At 300 μ M H₂O₂, between 50% and 68% of cells did not synthesize DNA when subcultured immediately after treatment (Figure 1 and Table 1). More than 60% of cells remained incapable of synthesizing DNA when the culture was maintained for up to 14 days (Table 1). This suggests that the growth arrest caused by sublethal H₂O₂ concentrations may be irreversible.

Mammalian cell growth may be arrested in the G₁, S, or G₂/M phases of the cell cycle. The majority (50–70%) of quiescent cells treated with H₂O₂ did not incorporate BrdU (Figure 1 and Table 1), suggesting that H₂O₂ arrested cells in the G₁ phase of the cell cycle. The G₁ arrest was confirmed by flow cytometry. Quiescent cells (Figure 2A) treated with 550 μ M H₂O₂ were stimulated to proliferate by subculture. Untreated cells were distributed throughout the G₁, S and G₂/M phases by 20 h after subculture (Figure 2B). In contrast, H₂O₂-treated cells remained in G₁ (Figure 2C). This G₁ distribution was observed in H₂O₂-treated cells even 84 h after subculture (results not shown). To confirm that H₂O₂-treated cells arrested in G₁, we treated cells with the antimitotic drug colchicine, which inhibits the formation of microtubules and prevents mitosis. In cycling cells one expects an increase in the G₂/M peak in the presence of colchicine. Indeed, this occurred in untreated control cells (Figures 2D, 2F and 2H). In H₂O₂-treated cells, colchicine failed to cause an increase in the

G₂/M peak (Figures 2E, 2G and 2I). The result supports the conclusion that in H₂O₂-treated cells growth is arrested in the G₁ phase of cell cycle.

H₂O₂ is an oxidant and can be genotoxic. In the presence of iron, H₂O₂ is converted into reactive hydroxy radicals, which attack DNA and other biomolecules. Removing iron with the chelator deferoxamine abolished the H₂O₂-induced growth arrest [26], suggesting that formation of hydroxy radicals is required for the growth arrest. Hydroxy radicals may attack DNA, causing strand breaks and formation of oxidized bases, of which oxo⁸dG is a sensitive marker [31]. We determined the concentration of H₂O₂ that was required to induce DNA damage by measuring oxo⁸dG in DNA [28]. A significant increase ($P < 0.05$ using a one-tail Student's *t* test) in the steady-state level of oxo⁸dG in DNA was observed at H₂O₂ concentrations of 550 μ M or higher. Lower concentrations of H₂O₂ (50, 100 or 250 μ M) did not cause a statistically significant increase in the steady-state level of oxo⁸dG in DNA (Table 2). It is not clear whether the high variability of the results could account for the lack of significance or if there is a threshold for DNA damage. The data suggest that only high concentrations of H₂O₂ can cause detectable oxidative DNA damage.

An induction of p53 protein has been shown to mediate the G₁ arrest caused by DNA-damaging agents [11,32]. As determined by Western-blot analysis, H₂O₂ at a concentration as low as 75 μ M caused an increase in p53 protein (Figure 3A). This increase reached a maximum level, 3-fold above the untreated control level, at 100–250 μ M H₂O₂, and declined slightly at higher concentrations (550 μ M–1 mM). Even at the highest H₂O₂ concentration, however, the level of p53 was 1.5-fold higher than in untreated cells. Northern-blot analysis showed that the p53 mRNA level did not increase after 550 μ M H₂O₂ treatment (results not shown), consistent with a post-transcriptional mechanism of induction as reported previously [11]. The induction of p53 protein was inhibited by the iron chelator deferoxamine (Figure 3B), and by the protein synthesis inhibitor cycloheximide (Figure 3C). These results suggest that p53 can be induced by H₂O₂ at low concentrations, and that the induction is dependent on both hydroxyl radical formation and *de novo* protein synthesis.

Induction of p53 protein did not appear to be an immediate response. With 550 μ M H₂O₂, p53 protein did not increase until 1.5 h after the addition of H₂O₂, whereupon it remained elevated for the duration of the experiment (3 h) (Figure 3D). To determine whether this increase was prolonged, we analysed the p53 protein level at 18 h, 44 h and 3 days after treatment with 550 μ M H₂O₂. The level of p53 protein remained higher in H₂O₂-treated cells compared with that in untreated cells for up to 18 h but returned to the level in untreated cells by 44 h (Figure 4A). These data suggest that p53 elevation is transient.

Determination of the level of the p21 CDK inhibitor showed that, within 2 h, 100 μ M H₂O₂ caused a small elevation in the p21 protein level (results not shown). H₂O₂ at 550 μ M did not increase p21 within 2 h but caused an increase at 18 h after treatment and levels remained elevated for more than 21 days (Figure 4B). We concluded that treatment with H₂O₂ for 2 h caused a sustained elevation in p21 protein.

The p21 protein may control cell-cycle arrest by inhibiting the activity of CDKs which phosphorylate Rb. In untreated quiescent cells, hyperphosphorylated Rb was evident 18–24 h after serum stimulation (Figure 4C). Hyperphosphorylated Rb remained detectable up to 21 days later, as long as the cells were stimulated with serum (Figure 4C). The H₂O₂-treated cells failed to hyperphosphorylate Rb in response to serum (Figure 4C). Rb remained underphosphorylated in H₂O₂-treated cells despite serum stimulation, for up to 21 days (Figure 4C). Thus treatment with H₂O₂

Figure 2 Flow-cytometry analysis of the cell cycle distribution after H_2O_2 treatment

Confluent IMR-90 cells (PDL 20) in 100 mm dishes (Before) were treated with $550 \mu\text{M}$ H_2O_2 for 2 h. DNA content was analysed by flow cytometry 20 h later, as described in the Materials and methods section. Colchicine (CC) (final concentration $50 \mu\text{M}$) was added to the cells 8 h before harvesting. Solid lines indicate the results for control (ctr) or H_2O_2 -treated cells. Broken lines indicate the results for controls or H_2O_2 -treated cells incubated with colchicine. In H_2O_2 -treated cells, the solid and broken lines overlap. The data are from one representative experiment.

for 2 h prevented Rb hyperphosphorylation for prolonged intervals.

To explore the requirement for p53 and Rb in the H_2O_2 -induced G_1 growth arrest, we inactivated p53 or Rb function by expressing the HPV *E6* or *E7* gene. Cells expressing *E6*, *E7*, or both genes showed little changes in cell morphology. Cells expressing *E7* or *E6* and *E7* reached a higher saturation density than wild-type cells or cells carrying *E6*. We determined the levels of p53, p21, hyperphosphorylated Rb and G_1 arrest in cells

expressing *E6*, *E7* or both genes (designated *E6*, *E7* or *E6*+*E7* cells).

E6 cells showed reduced p53, whereas *E7* cells showed increased p53 (Figure 5A). The basis for the *E7*-dependent increase in p53 is unknown. H_2O_2 ($550 \mu\text{M}$) increased p53 protein in wild-type cells but failed to alter the p53 level in *E6* cells and caused a minor increase in the *E7* cells (Figure 5A). *E6*+*E7* cells showed a similar level of p53 to wild-type cells and the level of p53 did not change in response to H_2O_2 ($550 \mu\text{M}$) (Figure 5A). We

Table 2 Steady-state levels of oxo⁸dG in DNA from H₂O₂-treated cells

Early-passage IMR-90 cells were harvested after treatment with various concentrations of H₂O₂ for 2 h for analysis of oxo⁸dG in DNA as described in the Materials and methods section. The values are the means $\pm$ S.D. of three independent experiments. *, significantly different ($P < 0.05$) from untreated (0 μ M) cells using the one-tail Student's *t* test.

H ₂ O ₂ concentration (μ M)	oxo ⁸ dG/mg of DNA (pmol)
0	3.47 $\pm$ 1.37
50	4.29 $\pm$ 0.85
100	4.31 $\pm$ 0.36
250	3.71 $\pm$ 0.43
550	5.60 $\pm$ 1.43*
1000	6.19 $\pm$ 1.74*
2000	5.82 $\pm$ 1.72*

Figure 3 Induction of p53 protein in cells treated with H₂O₂

(A) Confluent IMR-90 cells (PDL 23.7) in 100-mm dishes were treated with H₂O₂ at various doses for 2 h. (B) H₂O₂ (100 μ M) in the absence or presence of 1 mM deferoxamine (DFAM) for 2 h. (C) H₂O₂ (550 μ M) in the absence or presence of 25 μ g/ml cycloheximide (CXM) for 2 h. (D) H₂O₂ (550 μ M) from 15 min to 3 h. The cells were harvested for Western-blot analysis of p53 protein as described in the Materials and methods section. (A) 75 μ g of protein, (B, C and D) 40 μ g of protein in each lane. The data are from one representative experiment.

concluded that H₂O₂ did not induce p53 in E6 cells and E6 + E7 cells but slightly increased p53 in E7 cells.

E6 cells contain a low basal level of p21 and H₂O₂ caused a slight increase in p21 in E6 cells (Figure 5B). E7 and E6 + E7 cells contain a higher level of p21 compared with wild-type cells and treatment with H₂O₂ caused a small increase in p21 in E7 cells but not in E6 + E7 cells (Figure 5B).

Finally, E6 cells retained the ability to hyperphosphorylate Rb despite H₂O₂ treatment (Figure 5C). E7 and E6 + E7 cells showed a reduced level of Rb compared with wild-type cells. E7 cells did not hyperphosphorylate Rb after H₂O₂ treatment but E6 + E7 cells showed some Rb hyperphosphorylation (Figure 5C). We concluded that E6 allows Rb hyperphosphorylation but E7 reduces the overall level of Rb protein.

The cell-cycle distribution was analysed after treating quiescent

Figure 4 Levels of p53, p21 and hyperphosphorylated Rb protein after H₂O₂ treatment

Confluent IMR-90 cells (PDL 25–28) were treated with 550 μ M H₂O₂ for 2 h. The cells were harvested immediately or were incubated in fresh medium containing 10% (v/v) FBS for 18 h, 44 h or 3, 7, 14 or 21 days before harvesting. For the 3–21 day time points (d), the cells were stimulated with 20% (v/v) FBS for 20–24 h before harvesting. The levels of (A) p53, (B) p21 and (C) Rb protein were determined by Western-blot analysis after 75 μ g (A) or 25 μ g (B and C) of protein was loaded on to each lane. The upper band in (C) indicates hyperphosphorylated Rb. The data are from one representative experiment.

Figure 5 Levels of p53, p21 and hyperphosphorylated Rb protein in E6, E7 or E6 + E7 cells

Confluent IMR-90 cells or IMR-90 cells expressing E6, E7 or E6 + E7 at similar PDL were treated with 550 μ M H₂O₂ for 2 h. The cells were harvested immediately to determine (A) p53 protein levels, or were incubated in fresh medium containing 10% (v/v) FBS for an additional 18–24 h to determine (B) the p21 protein level and (C) the phosphorylation status of Rb by Western-blot analysis (25 μ g of protein in each lane). The data are from one representative experiment.

E6, E7 or E6 + E7 cells with 550 μ M H₂O₂ (Figures 6A–6D). Unlike wild-type cells, which arrested in G₁ in response to H₂O₂ treatment (Figures 6I and 6Q), E6 cells showed a high proportion of S phase cells 24 h after H₂O₂ treatment (Figure 6J). The addition of colchicine to E6 cells caused an accumulation in G₂/M (Figure 6R), suggesting that E6 cells progressed through the S phase after H₂O₂ treatment. The E7 cells and E6 + E7 cells

Figure 6 Cell-cycle distribution of wild-type cells and E6, E7 or E6 + E7 cells after H₂O₂ treatment

Confluent IMR-90 cells or IMR-90 cells expressing E6, E7 or E6 + E7 at similar PDL (quiescent, before treatment) were treated with 550 μ M H₂O₂ for 2 h. DNA content was analysed by flow cytometry after subculture at a ratio of 1:3 into fresh medium containing 10% (v/v) FBS for 24 or 48 h. Colchicine (CC, final concentration 50 μ M) was added to cells 24 h before harvesting at 48 h. The data are from one representative experiment. ctr, control; HP, HPV-infected cells.

Table 3 Cell replication after H₂O₂ treatment

Wild-type (wt), E6, E7, or E6 + E7 cells at PDL 24–27 were treated with 550 μ M H₂O₂ for 2 h in 100-mm dishes. Cell number was determined within 5 days of H₂O₂ treatment by Coulter zM counter. The values are the means $\pm$ S.D. of three independent experiments.

Cell type	Cell division number	
	Control	H ₂ O ₂ -treated
wt	2.37 $\pm$ 0.15	0.46 $\pm$ 0.56
E6	2.23 $\pm$ 0.20	0.88 $\pm$ 0.15
E7	2.71 $\pm$ 0.13	0.70 $\pm$ 0.25
E6 + E7	2.50 $\pm$ 0.20	1.02 $\pm$ 0.67

showed distribution in G₁, S and G₂/M at 24 h after H₂O₂ treatment (Figures 6K and 6L), and showed an increase in the G₂/M peak after addition of colchicine (Figures 6S and 6T).

Therefore E6 or/and E7 can abolish the G₁ arrest induced by H₂O₂.

With senescent HDFs, the majority of the cells are arrested in the G₁ phase of the cell cycle. Inactivating G₁ cell-cycle checkpoints with simian virus (SV)-40 large T does not allow the cells to replicate, indicating that the mechanism of senescence is beyond the G₁ arrest [3]. Since H₂O₂-treated cells resemble senescent cells, we examined whether inactivation of G₁ arrest abolished senescent-like growth arrest by determining the replication ability of E6, E7, and E6 + E7 cells after H₂O₂ treatment. Cell replication was determined by increases in cell number. The results showed that E6, E7, and E6 + E7 cells were unable to replicate after H₂O₂ treatment (Table 3). Therefore inactivating G₁ checkpoints by E6, E7 or E6 + E7 does not restore the replication ability of H₂O₂-treated HDFs.

DISCUSSION

Sub-lethal concentrations of H₂O₂ cause a prolonged G₁ arrest in HDFs, a transient increase in p53 protein, a sustained elevation

of p21 and a sustained Rb underphosphorylation. HPV E6 abolished the induction of p53, reduced the elevation of p21 induction and allowed Rb hyperphosphorylation. HPV E7 reduced the level of Rb and presumably also disrupted the interaction between Rb and E2F. Both E6 and E7 abolished G₁ arrest but not loss of cell replication in response to H₂O₂. Our results are consistent with a model in which H₂O₂ induces an initial increase in p53, which is responsible for the initial induction of p21. Elevated p21 in turn inhibits the activity of CDKs and maintains Rb in an underphosphorylated state, thereby causing cells to arrest growth in G₁.

Cell replication is controlled by multiple mechanisms, in addition to the G₁ cell cycle checkpoint. Although E6, E7 or E6+E7 cells were not G₁ arrested, they could not replicate, suggesting that these cells may be arrested in G₂/M phase or may form an equilibrium of cell division and cell death. Our current studies involve testing whether programmed cell death, G₂/M arrest and G₁ arrest after the first cell cycle occur in E6, E7 or E6+E7 cells in response to H₂O₂. These mechanisms may complement the G₁ cell cycle checkpoint to prevent the transformation of oxidative damage to mutation or other genomic aberrations.

The molecular changes found in the present study suggest that H₂O₂-treated cells share some similarity with senescent cells at the molecular level. Both H₂O₂-treated cells and senescent cells contain a high level of p21 protein and underphosphorylated Rb [7,8]. However, in H₂O₂-treated cells p53 protein increased transiently within 2 days. Although it has been reported that the rate of p53 protein synthesis is higher in senescent cells than in the pre-senescent cells after serum stimulation [33], the early-passage quiescent and senescent cells contained similar steady-state levels of p53 mRNA and protein ([34] and J. Campisi, unpublished work). By this criterion, H₂O₂-treated cells differ from senescent cells.

The signal responsible for the increase in p53 and p21 in H₂O₂-treated cells remains to be elucidated. The induction of p53 and p21 by H₂O₂ did not correlate with the increase in the steady-state level of oxo⁸dG in DNA, suggesting that signals in addition to DNA damage may be responsible for p53 and p21 increases. The time course showed that p21 protein increased slowly but the elevation persisted after H₂O₂ treatment. E6 lowered the basal level of p21 protein and reduced the degree of p21 induction by H₂O₂, suggesting that p21 expression was at least partially p53 dependent. However, since the p53 level did not remain elevated, the sustained p21 level may be maintained by a p53-independent mechanism [17,18]. Therefore induction of p21 may be both p53 dependent and independent in HDFs.

The p21 protein increases in response to a variety of growth-inhibitory signals, such as DNA damage, nutrient deprivation, growth inhibitors and cell differentiation [35,36]. Inhibition of p21 expression by antisense mRNA or mouse germ-line inactivation results in a loss of G₁ arrest induced by nutrient deprivation or DNA damage [37,38]. The p21 protein is a universal inhibitor of CDKs [39]. In addition to preventing Rb hyperphosphorylation, p21 can inactivate E2F activity by Rb-independent mechanisms [9]. Furthermore, p21 binds to the proliferating cell nuclear antigen (PCNA), and inhibits PCNA-dependent DNA synthesis without affecting PCNA-dependent nucleotide excision repair [40,41]. All these functional aspects of p21 suggest that sustained elevation of p21 is critical for the prolonged G₁ arrest in H₂O₂-treated wild-type HDFs.

The induction of a prolonged G₁ arrest by H₂O₂ may require the cells to be in the early G₁ phase before the treatment. In agreement with our results, Di Leonardo et al. [42] showed that cells in early to middle G₁ phase enter a prolonged growth arrest

after γ -irradiation. Cells treated in the late G₁ phase (perhaps past the restriction point) entered S phase, and cells in S phase, when treated, progressed to G₂ and arrest before mitosis [43]. In contrast with the molecular changes in H₂O₂-treated cells, irradiation-induced G₁ arrest is associated with a long term elevation of both p53 and p21 [42,44].

In conclusion, the H₂O₂-induced growth arrest shared some features of the growth arrest induced by senescence and irradiation, namely sustained elevation of p21 and underphosphorylation of Rb. It differed from other forms of growth arrest in that p53 was only transiently elevated.

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