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Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells

(replicative cessation/morphology/DNA damage/p53 cell cycle checkpoint)

QIN CHEN AND BRUCE N. AMES*

Division of Biochemistry and Molecular Biology, 401 Barker Hall, University of California, Berkeley, CA 94720

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ABSTRACT **Human diploid fibroblast cells lose replicative potential after a certain number of population doublings. We use this experimental system to investigate the role of oxidative damage in cellular aging. Treating cells with H_2O_2 at <300 μM did not affect the viability of the majority of cells when judged by morphology, trypan blue exclusion, and protein synthesis. However, the treatment caused a dose-dependent inhibition of DNA synthesis. After a 2-hr treatment with 200 μM H_2O_2, the cells failed to respond to a stimulus of serum, platelet-derived growth factor, basic fibroblast growth factor, or epidermal growth factor by synthesizing DNA, and the loss of response could not be recovered by 4 days. Subcultivation showed that, as in senescent cells, division of the treated cells was inhibited. The life-time cumulative growth curve showed that the loss of replication due to H_2O_2 treatment was cumulative and irreversible. The H_2O_2 treatment decreased the number of the population doublings in the rest of the life span by 35.3 ± 10.3%. Enzymatic assays indicated that, like the cells in their senescent state, the treated cells were less able to activate ornithine decarboxylase and thymidine kinase. Furthermore, subcultivation after the H_2O_2 treatment showed that the cells developed the morphology of senescent cells. In conclusion, sublethal treatment of H_2O_2 "stunned" F65 cells and caused the cells to enter a state resembling senescence.**

One of the manifestations of aging is the accumulation of damage at both cellular and organismal levels. This damage is initiated by endogenous and exogenous toxicants. Oxidants appear to be the major source of damage and are produced during normal aerobic metabolism and inflammatory reactions (1, 2). High-dose exposure to oxidants may cause cytotoxicity and neoplastic transformation. We investigate here the role of oxidants in aging with human diploid fibroblast (HDF) cells as an experimental system.

HDF cells lose replicative capacity in culture after a specific number of population doublings (PDs) and have been widely used as an *in vitro* model of aging (3–6). Accompanying the loss of replicative potential, senescence is marked by several changes, including decreased activity of cell-cycle-related enzymes (9, 10) and decreased efficiency of protein synthesis and degradation (11–14). Nuclear and chromosomal aberrations are present at a higher frequency (15, 16). The content of 5-methylcytosine in DNA and the length of telomeres are decreased (17–19). Compared to their young counterparts, senescent cells are larger and grow to a lower saturation density (20). The degenerative changes suggest that senescence may result from accumulation of various types of damage.

The cause of replicative senescence has been examined at the molecular level. Senescent cells have intact DNA replication machinery and transduce most of signals during mitogenic stimulation (3, 4, 21, 22). The loss of proliferation correlates with selective depression of certain mitogenic events such as inability to phosphorylate the retinoblastoma gene product (23) and failure to express c-*fos*, *cdc2*, or cyclins (24–26). In addition, much experimental evidence suggests that loss of replication may result from the acquisition of factors that prevent proliferation (27–29). Some factors, such as terminin and senescent-associated gene, have been identified only in senescent cells (30, 31). Thus, although it seems that replicative senescence results from the down regulation of proliferative factors and the up regulation of antiproliferative factors, the mechanism of such alternation is not known.

We examine here the effect of sublethal oxidative damage to HDF cells. Replicative potential, morphology, and activity of cell-cycle-related enzymes were determined after treating the human skin fibroblast F65 cells with H_2O_2, an oxidant produced in aerobic metabolism and inflammation. We found that treating HDF cells with sublethal concentrations of H_2O_2 converts them to a state that resembles senescence. We describe here a model that can be used to determine the linkage of cellular damage, molecular response, and replicative cessation.

MATERIALS AND METHODS

Cell Culture. F65, the human cell line of diploid foreskin fibroblasts, was originally isolated at the Naval Bioscience Laboratory (Oakland, CA) from a clinically normal male. Cells were grown in the Dulbecco's modified Eagle's medium (DMEM; Irvine Scientific) supplemented with 10% (vol/vol) dialyzed fetal bovine serum (FBS; Sigma). Cells (under passage 20) were seeded at a density of 1×10^4 cells per cm^2 and were grown in 24-well plates (2 cm^2 per well) containing 10% dialyzed FBS at 1 ml per well. The cultures reached confluence after 5 days and were treated with H_2O_2 at day 7 for 2 hr under the same culture conditions.

Measurement of DNA and Protein Synthesis. Cells were incubated for 24 hr in DMEM with or without 10% FBS or individual growth factors (GIBCO) in 24-well culture plates containing [^{3}H]thymidine (50 Ci/mmol; 1 Ci = 37 GBq; from Amersham or 20 Ci/mmol from NEN) at 0.5 μCi per well or a ^{14}C-labeled mixture of amino acids (54.4 mCi/mol atom carbon from NEN) at 0.2 μCi per well. The labeling medium was washed out and the cells were fixed in the well with 10% (wt/vol) ice-cold trichloroacetic acid. After two additional washes with 10% trichloroacetic acid, one wash with 70% ethanol, and one wash with phosphate-buffered saline (PBS), the precipitates were dissolved in 0.1 M KOH. Trichloroacetic acid-insoluble radioactivity was determined by liquid scintillation chromatography.

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Abbreviations: HDF, human diploid fibroblast; PD, population doubling; FBS, fetal bovine serum; ODC, ornithine decarboxylase; TK, thymidine kinase; BSA, bovine serum albumin.
*To whom reprint requests should be addressed.

To visualize DNA-synthesis-competent cells, cells grown on a cover glass were labeled for 48 hr with 10 μM bromodeoxyuridine (BrdUrd). BrdUrd incorporated in DNA of cells was identified by immunocytochemical methods using anti-BrdUrd antibodies and secondary alkaline phosphate-conjugated antibodies according to the manufacturer's instructions (Boehringer Mannheim).

Determination of Morphology. Cells were grown on cover glasses in 24-well (2 cm^2 per well) or 6-well (10 cm^2 per well) plates. After fixing cells with 10% (vol/vol) formalin (Sigma) for 20 min and subsequent 70% ethanol for 5 min, the cover glasses were submerged in 0.1% Coomassie blue (Bio-Rad) for 10 min and then 0.4% Giemsa for 30 min as described (32).

Measurement of Ornithine Decarboxylase (ODC) and Thymidine Kinase (TK) Activities. Postconfluent cultures of F65 cells in 24-well plates were treated with 200 μM H_2O_2 for 2 hr. Cells were then split at a ratio of 1:5 according to the density into 100-mm dishes containing 10 ml of medium. After cultivation for 10 days, cells were serum-starved for 24 hr in DMEM containing 0.2% bovine serum albumin (BSA). FBS was then added to 10% for various time periods. At the end of the incubation, the cells were washed with PBS and scraped off the dishes. The cell pellets were collected after a brief centrifugation and were then resuspended in lysis buffer (40 mM Tris·HCl, pH 7.4/5 mM dithiothreitol/0.02% Tween 80). ODC activity was assayed with crude cell lysates by measuring the CO_2 released from ornithine at 37°C by 100 μg of total cellular proteins (33). TK activity was measured with 50 μg of soluble cellular protein (9) by incubating for 1 hr at 37°C with 1 μCi of [^{3}H]thymidine in 125 μl of 5 mM $MgCl_2$/5 mM ATP/2 mM dithiothreitol/40 μM thymidine/0.5 mg of BSA.

RESULTS

H_2O_2 is cytotoxic at high concentrations. Human dermal fibroblast F65 cells were tested for viability at 24–120 hr after a 2-hr treatment of H_2O_2 at various concentrations. As judged by trypan blue uptake and morphology 24 hr after the treatment, H_2O_2 at <300 μM had no adverse effect on cell survival. Cell number relative to untreated controls was unchanged 24 hr after treatment with 50–300 μM H_2O_2 but was up to 12% lower than controls between 48 and 120 hr. It is not yet known whether the small percentage of cell death is due to apoptosis or necrosis. However, the remaining adherent cells were viable since they were able to exclude trypan blue and exhibited normal morphology (Fig. 1*B*). The measurement of protein synthesis further verified the viability of the cells. The cells treated with H_2O_2 at <200 μM fully retained the ability to synthesize proteins (Fig. 1*C*).

The ability of the cells to synthesize DNA was determined after treatment of H_2O_2. With an increased concentration of H_2O_2, the ability to synthesize DNA decreased (Fig. 1*C*). H_2O_2 at 200 μM was chosen for the rest of the experiments since the treatment had little effect on viability and the rate of protein synthesis but abolished DNA synthesis.

The cells treated with 200 μM H_2O_2 lost the ability to synthesize DNA and to reenter the cell cycle in response to a variety of mitogens. As shown in Fig. 1 *A* and *B*, when DNA-synthesis-competent cells were scored by incorporation of BrdUrd, <1% of cells were able to synthesize DNA in response to serum stimulation after the treatment of H_2O_2. In addition, as measured by [^{3}H]thymidine uptake, the treated cells were unresponsive to a variety of growth factors including platelet-derived growth factor, basic fibroblast growth factor, and epidermal growth factor (Fig. 2*A*).

To determine whether the loss of proliferative response was temporary or irreversible, we determined the rate of DNA synthesis 0, 1, 2, or 4 days after the treatment. The cells did not recover their ability to synthesize DNA even 4 days after the treatment (Fig. 2*B*), suggesting that the loss of replicative capacity could be irreversible. The replicative capacity was further examined by subcultivating the cells for 16 days (Fig. 3) or by passaging the cells for the rest of their life span (Fig. 4).

The loss of DNA synthesis after H_2O_2 treatment suggests that the cells may exist in a state resembling senescence. The rate of cell division was determined by subcultivating the postconfluent cultures after treating with 200 μM H_2O_2 for 2 hr and was compared to that of near senescent cells. Fig. 3 shows that, like senescent cells, cells treated with H_2O_2 grew poorly. The growth potential of the treated cells was determined over their life span by continuous passage until no cell division was observed. Fig. 4 showed that the treated cells (one treatment of 200 μM H_2O_2) never recovered their ability to grow as fast as the control: 15.5 ± 3.8 PDs as compared to control cells that had 23.7 ± 3.6 PDs. A few cells that eventually grew up after growth arrest had reduced replicative potential, and fewer PDs than the untreated control, before they ceased replication completely. Therefore, the loss of replicative capacity due to H_2O_2 treatment is cumulative and irreversible.

ODC and TK activities have been used as enzymatic markers of proliferative capacity. Senescent cells are less able to activate ODC and TK in response to mitogens than are young cells (9). The activity of these two enzymes in response to serum stimulation was determined after subcultivating the H_2O_2-treated cells. As indicated in Fig. 5*A*, in controls, the

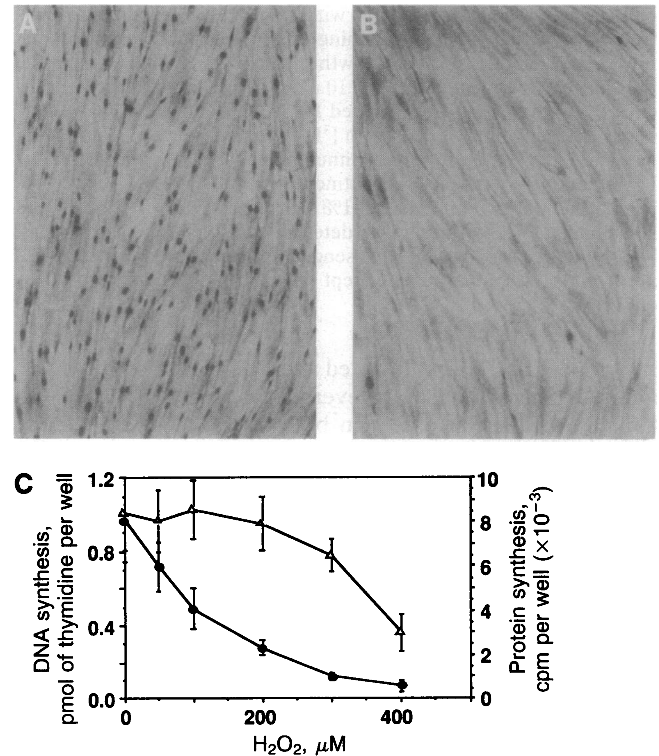

FIG. 1. Morphology and ability of control (*A*) and H_2O_2-treated (*B*) cells to synthesize DNA. Postconfluent cultures in 24-well plates were treated with H_2O_2 for 2 hr before they were switched to fresh DMEM containing 0.1% BSA for 24 hr. The cells were labeled with 10 μM BrdUrd in the medium containing 10% FBS for 48 hr and were stained. Pictures were taken under a light microscope after additional staining for 5 min with 0.05% Coomassie blue. (*C*) The effect of H_2O_2 pretreatment on protein and DNA synthesis in F65 cells. Postconfluent cultures in 24-well plates were treated with H_2O_2 for 2 hr before labeling with [^{3}H]thymidine or a ^{14}C-labeled mixture of amino acids in DMEM containing 10% dialyzed FBS for 24 hr to measure DNA (●) and protein (△) synthesis, respectively. Data are a summary of three experiments.

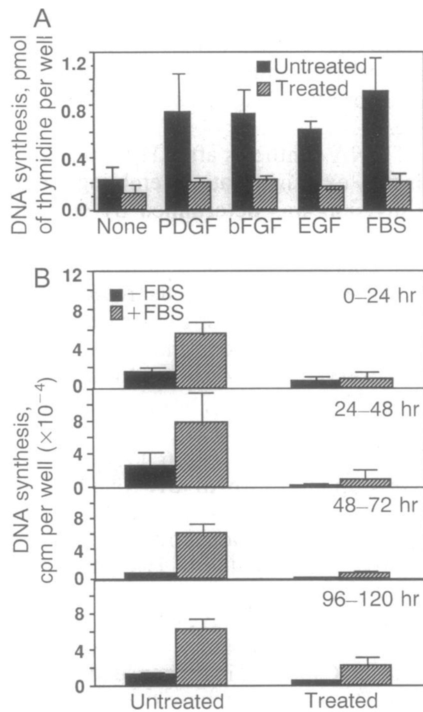

FIG. 2. Response of H_2O_2-pretreated F65 cells to synthesize DNA after stimulation with growth factors or serum. Postconfluent cultures of F65 cells were treated with 200 μM H_2O_2 for 2 hr. (*A*) Cells were placed in DMEM containing 0.1% BSA (control) or in the presence of platelet-derived growth factor (PDGF, 20 ng/ml), basic fibroblast growth factor (bFGF, 10 ng/ml), epidermal growth factor (EGF, 10 ng/ml), or 10% dialyzed FBS. DNA synthesis was determined after labeling of cells with [^{3}H]thymidine for 24 hr. (*B*) DNA synthesis in response to serum stimulation at 0, 24, 48, and 96 hr after H_2O_2 treatment. After a 2-hr treatment with 200 μM H_2O_2, cells were placed in DMEM containing 0.1% BSA until DNA synthesis was measured. DNA synthesis was determined after labeling cells with [^{3}H]thymidine for 24 hr in the absence or presence of 10% FBS. Data are from three experiments except with EGF, which are from two cultures.

activity of ODC was stimulated and reached a maximum 8 hr after serum addition. However, the activity of ODC was induced to a lesser extent in both senescent cells and the H_2O_2-treated cells. Furthermore, TK, the activity of which increased in a time-dependent manner in untreated cells in response to serum, showed a minimum elevation in either senescent or H_2O_2-treated cells (Fig. 5*B*). Thus, measurements of these enzyme activities indicated that H_2O_2-treated

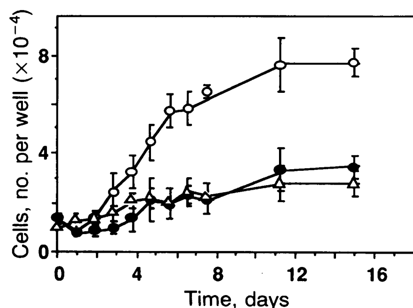

FIG. 3. Growth curves of normal (○), H_2O_2-treated (●), and near-senescent (△) F65 cells. Postconfluent cultures in 24-well plates were treated with 200 μM H_2O_2 for 2 hr. After trypsinization, the cells were then seeded into 24-well plates at a ratio of 1:5. Cells were grown in DMEM containing 10% dialyzed FBS. Medium was changed at days 1, 7, and 14.

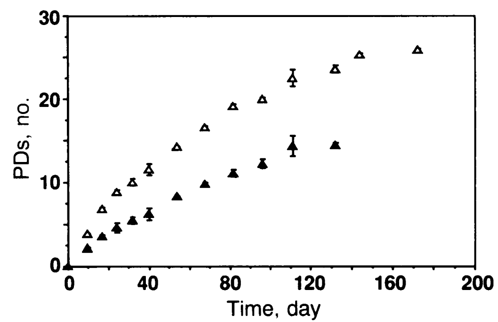

FIG. 4. Cumulative growth curve of normal (△) and H_2O_2-treated (▲) F65 cells. Postconfluent cultures in 24-well plates were treated with 200 μM H_2O_2 for 2 hr at day 0. The cells were seeded into 24-well plates at a ratio of 1:5 (day 0) for the first passage and were seeded at 5×10^3 cells per well for the rest of passages. PD for each passage was calculated as $\log_2 N/N_0$, where N is the cell number of harvestings and N_0 is the cell number of seedings. Each point is an average of triplicate samples.

cells and senescent cells appeared to behave in a similar manner.

Morphology is an important criterion for judging the senescence of human fibroblast cells. Senescent cells are enlarged and display reduced saturation density. When the cells were subcultivated after the H_2O_2 treatment, the cells started to develop the senescent morphology 3 days after subculture. A week later, the morphology of the treated cells resembled that of senescent cells (Fig. 6).

Much evidence suggests that H_2O_2 is activated by iron to form hydroxyl radicals through the Fenton reaction. Our data with catalase and the iron chelator deferoxamine mesylate, both of which protected cells from H_2O_2-induced replicative cessation (Table 1), suggest that indeed the loss of replicative capacity results from H_2O_2-derived hydroxyl radicals.

DISCUSSION

Sublethal H_2O_2 treatments "stun" F65 cells and induce a senescence-like growth arrest. Unlike cells that have undergone necrosis or apoptosis caused by cytotoxic doses of H_2O_2, stunned cells remain intact, retain the ability to synthesize protein, and display normal morphology but are unable to synthesize DNA in response to mitogens. If the cells are subcultivated, they display a senescent-like pheno-

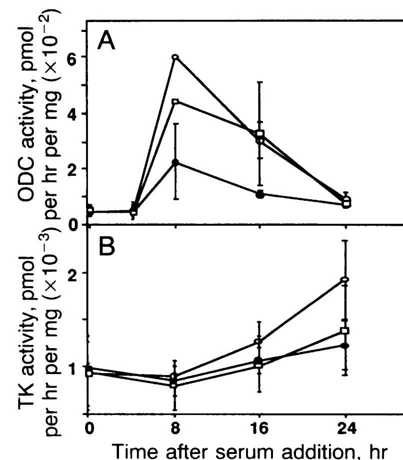

FIG. 5. ODC and TK activities in response to serum in normal (○), H_2O_2-treated (●), and senescent (□) F65 cells. Postconfluent cultures of F65 cells were treated with 200 μM H_2O_2 for 2 hr. The activity of ODC (*A*) or TK (*B*) was determined. Data are summary of three experiments.

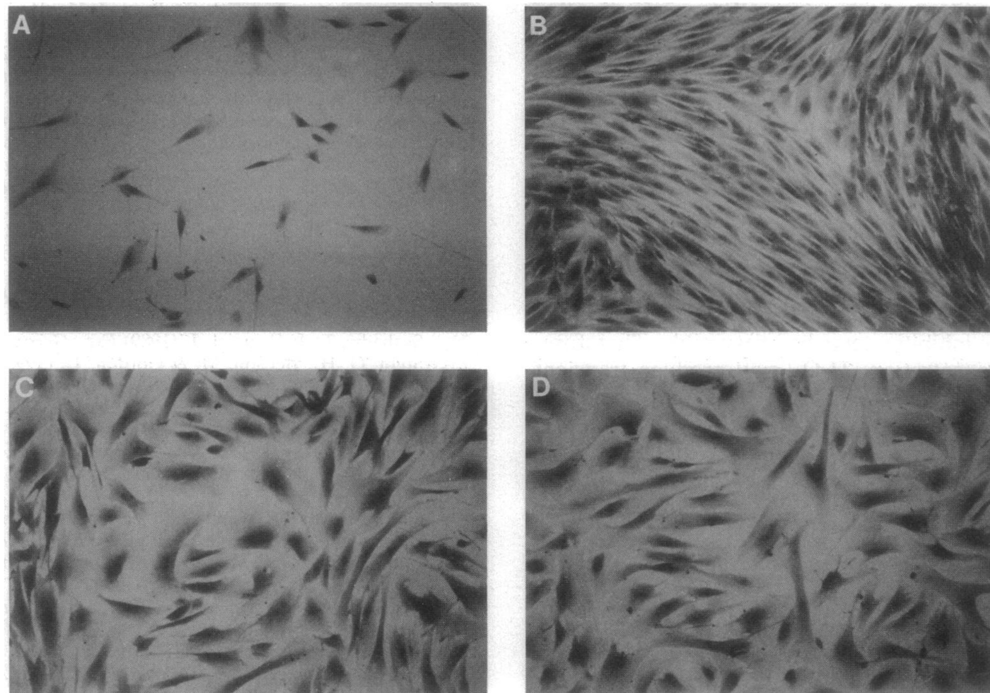

FIG. 6. Morphology of normal, H_2O_2-treated, and senescent F65 cells. Postconfluent cultures of F65 cells were treated with 200 μM H_2O_2 for 2 hr. The cells were seeded onto cover glasses at a ratio of 1:10. Pictures were taken under a light microscope. (*A*) Control untreated cells at day 3. (*B*) Control untreated cells at day 10. (*C*) H_2O_2-treated cells at day 10. (*D*) Senescent cells at day 10.

type, including loss of proliferative capacity, decreased activity of ODC and TK, and enlarged cell size. Although the stunned cells resemble senescent cells in several ways, we have not measured other senescence-specific markers that are not linked to proliferation.

The mechanisms causing replicative cessation by H_2O_2 are not known, though a plausible mechanism can be elaborated. Loss of replication may be initiated by lesions in DNA, although the role of other damaged cellular macromolecules cannot be ruled out. It is clear from this work that in oxidatively stunned cells, the mechanism involves hydroxyl radicals, since preventing formation of hydroxyl radicals from H_2O_2 with the iron chelator deferoxamine interfered with H_2O_2-induced replicative cessation (Table 1). Sublethal treatment of H_2O_2 caused a 30–100% increase of the adduct 8-oxo-2′-deoxyguanosine in DNA (unpublished data). This is about the same increase in the oxidative lesions seen in normal rat cells over their life time (1). H_2O_2 also causes single-strand breaks in DNA (34, 48). Although cells survived the insult, these lesions may constitute a signal that prohibits DNA and cell replication. That DNA lesions are likely to be the trigger is supported by the fact that methyl methanesulfonate, a DNA methylating agent, can also induce replicative cessation in F65 cells (unpublished data).

Table 1. Effect of catalase and deferoxamine mesylate on replicative cessation induced by H_2O_2

Treatment	Dose	PDs, no.	
		$-H_2O_2$	$+H_2O_2$
None		2.74 ± 0.06	−0.06 ± 1.04
Catalase	1000 units	2.55 ± 0.23	2.62 ± 0.23
Deferoxamine	2 mM	2.45 ± 0.22	2.21 ± 0.08

Postconfluent cultures in 24-well plates were treated with 200 μM H_2O_2 for 2 hr in the presence or absence of catalase or deferoxamine mesylate. Cells were seeded at a ratio of 1:8 into 24-well plates containing DMEM with 10% dialyzed FBS. Cell numbers were determined at day 1 (N_1) and day 7 (N_7). The number of cell divisions was determined as $\log_2 N_7/N_1$. Data are from three cultures.

A plausible mechanism to explain our results is that DNA lesions trigger replicative cessation through the G_1 to S phase checkpoint controlled by the tumor suppressor gene p53. Cells are arrested at G_1 or G_2 phase in the cell cycle after DNA damage (35–37). Lesions in DNA decrease transcription of many genes, including those that play important roles in cell proliferation and other cellular metabolism, while repair of such damage is coupled to and dependent on transcription (52, 53). Studies with radiation and carcinogens suggest that DNA lesions activate genes whose products suppress DNA replication and cell growth. Damaging DNA with UV irradiation causes an increase in the tumor suppressor gene p53 protein (37–39). It is likely that p53 induces transcription of genes that arrest the growth of cells at the G_1/S checkpoint, such as *gadd45* and WAF-1 (also called Cip 1 or sdi 1) (38, 54). Since WAF-1 is an inhibitor of cyclin-dependent kinase, the activity of which is required for G_1 to S phase transition, it may play a key role in growth arrest (55). In addition, p53 is associated with the replication and repair protein RPA (56, 57). When DNA damage occurs, RPA would bind to single-stranded DNA and release p53 (56, 57), which would in turn cause a block of cell division at the checkpoint thus preventing lesions being converted to mutations (M. Botchan, personal communication). It is also possible that p53 inhibits the promoter activity and transcription of TATA-controlled genes, including those required for cell proliferation, to block cell growth (41). The fact that in senescence the presence of functional wild-type p53 gene is required in HDF and primary cultures of mouse fibroblast cells (7, 8) supports the hypothesis that p53 may be involved in sensing lesions and communicating this information to the cell division checkpoint. It is known that p53 is involved in triggering apoptosis (40), so that the same sensing system may convert a higher level of DNA lesions to an apoptotic signal (45). In addition to the p53 gene, other negative growth regulatory genes, such as *gadd153* and *gadd45*, are highly induced by DNA damaging agents and may be critical in inhibiting the growth response (43).

The response to H_2O_2 varies depending on the level of damage. Cells can recover their ability to reenter into the cell cycle upon mitogenic stimulation after a lag period if the level of damage is suboptimal. For example, the cells treated with 100 μM H_2O_2 were unable to respond to mitogens for 1–2 days but recovered their ability to reenter the cell cycle upon mitogenic stimulation after 4 days. With the treatment of maximum noncytotoxic concentrations (e.g., 200 μM) of H_2O_2, as described in this study, the cells could not recover their ability to synthesize DNA in response to mitogens within the given time (Fig. 2*B*) and the reduction in replicative capacity was not restored during their life time (Fig. 4), suggesting that the inhibition may be irreversible. Therefore, depending on concentration, H_2O_2 may produce transient inhibition of DNA synthesis, senescence-like growth arrest, apoptosis, or necrosis.

The mortality or immortality of cells may also affect the cellular response to damage. Our preliminary experiments with IMR90 cells at various ages indicated that the response is age-dependent. A previous study (44) with ionizing radiation suggests that loss of growth potential occurs only in mid-late passage of HDF cells. It is possible that because F65 cells have a relatively short life span, they become senescent-like after the treatment. On the other hand, immortalized cells may respond to oxidants differently from HDF cells. Oxidants are mitogenic for several lines of mouse cells (42, 46, 47). With F65 HDF cells, we did not observe a mitogenic effect with any concentration of H_2O_2 tested (Fig. 1*C*). The capacity of cellular scavenger systems and repair systems could account for the differences in the response. Some immortalized cells miss certain checkpoints, which could be another factor.

Replicative cessation in response to DNA damage is an important defense mechanism against neoplastic transformation and cancer. Cancer occurs as a result of DNA lesions that are converted to mutations during cell division (49–51). Therefore, when damage occurs, cells inhibit DNA replication and cell division, providing the means to prevent mutation, heritable changes, and cancer. HDF cells in culture provide a system to study such defense mechanisms and to elucidate some of the mechanisms in normal senescence.

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