Cell Cycle Constraints on Peroxide- and Radiation-induced Inhibitory Checkpoints

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ABSTRACT

The growth of human skin fibroblasts was reduced in a dose-dependent manner after either treatment with hydrogen peroxide or exposure to ionizing radiation. Serum-starved cells were markedly responsive to the inhibitory properties of large doses of either agent at any time during the first 12–14 h after restitution. In contrast, when logarithmically growing cells were treated with hydrogen peroxide, a large percentage of G1 cells synchronously traversed S phase in a wave that appeared after a 3–4 h delay, with a population of these cells eventually arresting in late S and G2. An analogous compartment of cells exiting G1 was not obvious when logarithmically growing cells were treated with ionizing radiation alone. However, when irradiated cells were subsequently treated for 4 h with aphidicolin to depress ongoing DNA synthesis to the levels seen in cultures treated with peroxide, a similar pattern of cells synchronously exiting G1 was seen. Therefore, although cells between G1 and S had a marked sensitivity to the inhibitory effects of either peroxide or radiation, logarithmically growing cells in G1 between M and S were far less susceptible to either type of growth inhibition.

INTRODUCTION

A great deal of progress has been made in characterizing the multilevel response induced after the exposure of cells to either ionizing or UV irradiation, and a large number of both rapid and long-term processes modulated after radiation have been identified. Immediately after exposure, cells activate a large family of protein kinases that potentially play an important role in mediating the longer term cellular response. Among the well characterized kinases whose activity has been shown to increase are src (1), lyn (2), protein kinase C (3), and mitogen-activated protein kinase (4, 5). In addition, the activities of several unidentified tyrosine kinases are rapidly increased after irradiation (6). The signaling pathways activated by radiation in turn mediate an increase in the activity of a number of transcription factors, including AP-1 (7, 8) and nuclear factor kB (8, 9). The induction of nuclear factor kB after exposure to UV radiation is observed in enucleated cells (10), suggesting that its activity is independent of a signal induced directly by damaged DNA. An increase in the activity of these transcription factors, as well as others that have yet to be characterized, lead to a complex pattern of gene induction. Among the transcripts encoding genes with known functions that are induced after radiation are c-jun (7, 11), tumor necrosis factor α (12), ornithine decarboxylase (13), and metallothionein (14). In addition, differential screens have identified a large number of proteins (15) or RNAs (16) representing genes of unknown function the abundance of which was increased after ionizing radiation. The role that any of these gene products play in the protective or adaptive response of cells to radiation has not been described.

In addition, radiation induces a complex series of events leading to a cell cycle arrest or delay in both G1 and G2 (17, 18). Kastan et al. (19) first demonstrated the specific role of p53 in the induction of the G1 arrest seen after ionizing radiation, although a role of p53 in G2 arrests has recently been proposed as well (20). It appears that ionizing radiation induces the G1 arrest by modulating an inhibitor of the catalytic activity of cdk/cyclin complexes (21, 22), eventually leading to alterations in the pattern of retinoblastoma susceptibility gene product phosphorylation (23). The activation pathway that mediates the induction of p53 leading to cell cycle arrests is not known but might well involve a signal induced directly by DNA damage (24).

In this paper we investigated the kinetics of the cell cycle alterations induced by both ionizing radiation and hydrogen peroxide in normal precursors fibroblasts. We present evidence that the nature of the arrest is dependent both on the nature of the insult, as well as the position of the cell within the proliferative cycle.

MATERIALS AND METHODS

Cell Culture. HSF-55 cells, a strain originally derived from a human foreskin by Dr. David Chen, were maintained in α-MEM supplemented with 10% FCS, 50 units/ml penicillin, and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO2. Stock cultures were maintained in 100-mm tissue culture plates and were subcultured every 4 days. Vials of cells frozen at passage five were thawed at monthly intervals. All cells were found to be Mycoplasma free.

Mitogenesis. Cells to be used in growth curves were seeded at an initial density of 1 × 104 cells/dish in a 60-mm tissue culture dish for peroxide experiments or in a 25-cm² flask for radiation experiments. Twenty-four h after plating, the cultures were treated as described in the individual experiments. Fresh medium was added immediately after the treatment and every 48 h thereafter. Cell counts were determined 4 or 5 days after exposure, when the untreated control cells were approximately 80% confluent. Cells to be harvested for flow cytometry were seeded at an initial density of 3 × 104 cells/dish. Forty-eight h later, the plates were either rinsed in PBS and α-MEM containing 0.1% FCS was added to induce a G0 arrest or fresh α-MEM containing 10% FCS was added to maintain the cells in logarithmic growth. Cells were treated with peroxide or exposed to ionizing radiation after an additional 24-h incubation.

Flow Cytometry. Cells were removed from the plate with PBS containing 0.125% trypsin and 0.5 mM EDTA and fixed in 70% ethanol. The cells were then stained with mithramycin (25) and analyzed with a flow cytometer built at Los Alamos National Laboratory (Los Alamos, NM; Ref. 26). An argon laser tuned to a wavelength of 457 nm was used to excite the DNA/dye complex, and emission above 520 nm was collected. The DNA histograms were analyzed by using a commercial program purchased from Phoenix Flow Systems (San Diego, CA).

Treatments. Cells were exposed to hydrogen peroxide in a solution of calcium- and magnesium-free PBS at 37° for 30 min. At the end of the treatment, the plates were rinsed with PBS and fresh α-MEM was added. Cells were irradiated at room temperature with a Mark I Model 68A 137Cs γ high-dose irradiator (J. L. Shepherd and Associates) at a rate of 465 rads/min.

RESULTS

Inhibition of Cell Growth by Peroxide or Radiation. Sparse, logarithmically growing HSF-55 cells were treated with increasing concentrations of either ionizing radiation (Fig. 1A) or hydrogen peroxide (Fig. 1B), and the number of doublings the population underwent over the subsequent 5 days was determined. There was a
Similar results were seen when cellular proliferation was inhibited with peroxide instead of ionizing radiation, as shown in Fig. 3. Serum-starved cells were primarily arrested in G₀ (Fig. 3A), with 95% of the cells containing a 2n content of DNA. Twenty-four h after restimulation there was a large increase in both S phase (33%) and G₂ cells (16%) (Fig. 3B). When cells were treated with 100 mM peroxide either at the time of refeeding (Fig. 3C) or 12 h after stimulation (Fig. 3D) there were dramatic and equivalent decreases in the number of cells that exited G₀ and initiated DNA synthesis. There were 13% of cells in S+G₂ in culture treated with peroxide at the time of refeeding, whereas 9% of cells were in S+G₂ in cultures treated at 12 h.

These results indicated that cells treated with peroxide or exposed to ionizing radiation during essentially any portion of the G₀-G₁ prereplicative period were markedly prevented from entering S phase.

Alterations in DNA Distributions after Exposure of Logarithmically Growing HSF-55 Cells to Peroxide or Radiation. Irradiation of logarithmically growing cells with 800 rads caused marked changes in the distribution of DNA content, as shown in Fig. 4. At the time of exposure cells were distributed across the cell cycle with a pattern similar to that typically seen in logarithmically growing human fibroblasts, as shown in Fig. 4A. Although the distribution had not changed at 2-h postirradiation (Fig. 4B), by 4 h, cells had begun to accumulate in G₂ and were clearing out of early S phase (Fig. 4C). This pattern continued at 6 (Fig. 4D) and 9 (Fig. 4E) h, until by 12 h (Fig. 4F) there were only cells in G₁ and G₂ of the cell cycle. There was no additional change in the distribution either at 24 h (Fig. 4G) or up to 72 h, with no evidence of apoptosis at any time point as judged by a loss of cell number or accumulation of cells with less than a 2n content of DNA (data not shown).
A very different pattern of alterations in DNA distributions was seen when logarithmically growing cells were treated with 100 mM hydrogen peroxide. At the time of treatment, the cells had a DNA distribution characteristic of cells in log phase growth (Fig. 5A), with no changes observed during the first 2 h after treatment (Fig. 5B). By 4 h, cells began to accumulate in the G<sub>2</sub> compartment, and there was an emerging shoulder on the right-hand side of the G<sub>1</sub> peak (Fig. 5C). At 6 h a large percentage of cells had synchronously exited G<sub>1</sub> and were in early S phase (Fig. 5D). This population of cells was in mid to late S phase at 9 h after treatment with peroxide (Fig. 5E), eventually arresting in late S and G<sub>2</sub> by 12 (Fig. 5F) and 24 h (Fig. 5G). This pattern did not change over the next 24 h, again with no evidence of apoptotic cell death (data not shown). There were two major differences in the alterations in DNA distributions seen after treatment of cells with either peroxide or ionizing radiation. After treatment with peroxide there was evidence for the synchronous escape of large numbers of G<sub>1</sub> cells into S after approximately a 4-h delay. No such movement of G<sub>1</sub> cells into S was obvious from the single parameter analysis of DNA distributions seen in Fig. 4. In addition, although cells exposed to high levels of ionizing radiation arrested with a very clean G<sub>2</sub> block within 12 h after exposure (Fig. 4), a significant population of cells treated with high levels of peroxide remained blocked in late S phase even after 24 h of treatment (Fig. 5).

Inhibition of DNA Synthesis after Exposure to Peroxide or Radiation. The patterns of changes in DNA distributions seen after hydrogen peroxide are similar to those seen when cells are pulsed for 2–3 h with potent inhibitors of DNA synthesis such as aphidicolin (see below). Although it has been known for some time that radiation directly inhibits ongoing DNA synthesis (27), typically there is only a 50% inhibition of [3H]thymidine incorporation even at very high doses. Therefore, we wanted to test whether some of the differences in the alterations in the patterns of DNA distributions after exposure to either peroxide or radiation might be due to differences in the their ability to inhibit ongoing DNA synthesis in logarithmically growing cells.

As shown in Table 1, at 1 h after exposure of exponentially growing cells to 800 rads of ionizing radiation there was a 55% decrease in the amount of [3H]thymidine incorporated during a 30-min pulse. In contrast, there was a 96% reduction in the amount of thymidine incorporated a 1 h after cells were treated with 100 mM hydrogen peroxide. Thus, one potential difference in the phenotype induced by radiation or peroxide is the degree of inhibition of ongoing DNA synthesis.

The results in Table 1 raised the possibility that a large number of irradiated G<sub>1</sub> cells actually entered into S phase in a manner similar to what was seen after treatment with peroxide but that was never obvious in the DNA distributions because of the more moderate inhibition of DNA synthesis in irradiated S-phase cells. To test this possibility, the experiment described in Fig. 6 was performed. At the time of treatment (Fig. 6A), the cells were distributed throughout the cell cycle in a manner similar to that seen above. Control cells were then sham irradiated, whereas the treated cultures were exposed to 800 rads of ionizing radiation. All cultures were then incubated for 4 h in medium supplemented in 5 mg/ml aphidicolin to reduce the rates of DNA synthesis to levels seen in peroxide-treated cultures. Control cells treated with aphidicolin (Fig. 6B) had a DNA distribution somewhat different from the zero time samples. Because there was no inhibition of G<sub>2</sub> or M traverse in these cultures, these was a large loss of cells with a 4n content of DNA, and a subsequent gain (at a 2:1 ratio) of G<sub>1</sub> cells. This cell division makes it appear that there are fewer S-phase cells although there is no exit from S in the presence of aphidicolin. When control cultures were rinsed and incubated in drug-free medium for 2 h (Fig. 6C) there was a shoulder of cells leaving G<sub>1</sub> and entering S phase. By 4 h (Fig. 6D) there was a wave of cells in early S phase that was remarkably reminiscent of the patterns seen after incubation with peroxide (see above). When irradiated cells were incubated in medium supplemented with aphidicolin.

### Table 1 Inhibition of DNA synthesis after exposure to ionizing radiation or treatment with hydrogen peroxide

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]thymidine cpm (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63,750 (100)</td>
</tr>
<tr>
<td>Radiation</td>
<td>28,410 (44.5)</td>
</tr>
<tr>
<td>Hydrogen Peroxide</td>
<td>2,490 (3.9)</td>
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Fig. 5. Effects of hydrogen peroxide on the cell cycle distributions of cycling cells. Sparse, logarithmically growing HSF-55 cells were harvested at 0 h (A), or at 2 (B), 4 (C), 6 (D), 9 (E), 12 (F), or 24 (G) h after exposure to 100 mM hydrogen peroxide.
for 4 h (Fig. 6E) there was no change in the DNA distribution compared to zero time, because of the $G_2$ block and lack of subsequent cell division seen in irradiated cells. After the aphidicolin had been removed for 2 h (Fig. 6F), there was also a shoulder of cells beginning to exit $G_2$ and enter $S$ phase. A synchronous population that has entered early $S$ phase is clearly present in Fig. 6G. Therefore, there is clear evidence that a large percentage of $G_1$ cells exposed to very high levels of ionizing radiation can exit $G_1$ and enter $S$ phase. In addition, the marked differences between the alterations in DNA distributions seen after treatment with peroxide or exposure to ionizing radiation are not due to differences in the ability of damaged $G_1$ cells to enter $S$, but instead are due to differences in the magnitude of the inhibition of DNA synthesis.

**DISCUSSION**

The results presented here suggest that the type of regulatory checkpoint invoked after damage due to either radiation or hydrogen peroxide can be very complex and is quite dependent on the position of a cell within the cell cycle.

For the first 12–14 h after release from a $G_0$ arrest induced by serum starvation, cells were markedly inhibited by treatment with peroxide or exposure to ionizing radiation. These data are consistent with those reported by Dulic et al. (21) who observed a profound inhibition in cells exposed to radiation 6 h after release from a $G_0$ arrest induced by growth to high density. Our observations differ somewhat from those of Di Leonardo et al. (22) who found a period immediately before entry into $S$ phase during which cells lost their ability to be inhibited by radiation. While we observed a small diminution in the inhibition seen when cells were exposed to radiation at 12 h as compared to cultures irradiated at the time of restimulation, the effects were not nearly as large as those reported previously. These differences, however, are probably due to one of two possible explanations. The results of Di Leonardo et al. were obtained with cells exposed to 400 rads, whereas the experiments reported here utilized 800 rads. It is likely that the disparate results are due entirely to these differences in dosage levels. In addition, it is possible that subtle differences in the rate of entry into $S$ phase between the two cell strains used in the studies are responsible for a portion of the differences in radiation sensitivity.

The checkpoints invoked in logarithmically growing cells are much more complicated. At the levels of treatment used in these experiments there was an immediate cessation of cell division, and by 24 h after exposure to either radiation or peroxide there was a marked accumulation of cells in $G_2$. Although some of these effects might be due to the activation of cip1, the inhibitor of cdk/cyclin complexes that is induced after radiation (21, 22) and that is known to form complexes with cdc2/cyclin B (28), there well might be other levels of inhibition. When HeLa cells were irradiated during $S$ phase there was a repression of the accumulation of cyclin B mRNA, and cells exposed during $G_2$ failed to translate the cyclin B message that had accumulated (29). Therefore, the exact mechanism that leads to a $G_2$ arrest remains unclear.

As has been observed on several occasions (30) there was a 50% reduction approximately in the rate of thymidine incorporation after cells were exposed to ionizing radiation. In irradiated Chinese hamster ovary cells, the decrease in DNA synthesis seen after exposure to ionizing radiation was due to a specific inhibition of the initiation of replication with almost no effects on elongation (31). This observation accounts for fact that there is a biphasic effect of radiation on DNA synthesis, with a maximal inhibition of 50% in the rate of thymidine incorporation even at very high levels of exposure. Interestingly, treatment of cells with equitoxic levels of hydrogen peroxide causes a much more dramatic inhibition of DNA synthesis, with a repression of thymidine incorporation similar to what is seen when cells are treated with inhibitors such as aphidicolin. To produce such a large inhibition of DNA synthesis it would be predicted that peroxide will also inhibit elongation, unlike what has been seen after exposure to radiation. Experiments are under way to examine this possibility. The mechanism by which either radiation or peroxide might decrease DNA synthesis is not well understood. The activity of cyclin A/cdk2 is needed for traverse of $S$ phase (32, 33), and this complex might be a target for inhibition by cip1. The differences in the mechanism by which peroxide and radiation are postulated to inhibit DNA synthesis might be an important tool in describing processes regulating initia-
The data presented in this paper provide evidence that at least some of the irradiated cells traverse early S phase after the release from aphidicolin. The major point that can be derived from these data is that unlike the case with control cultures, where cells are dividing to provide mitogenically competent cells that are capable of entering S after release from aphidicolin, therefore, it is impossible to state whether or not the same number of control and irradiated G1 cells present at zero time traverse early S phase after the release from aphidicolin. The data presented in this paper provide evidence that at least some of the cells irradiated in G0 were capable of entering S phase, thus, suggesting that there is no immediate G0 block in even in cells with an intact p53 signaling network.

ACKNOWLEDGMENTS

Advice and comments from Drs. Bruce Lehnert and Paul Kraemer are acknowledged.

REFERENCES

22. Di Leonardo et al. (22) when cycling cells are exposed to 300–400 rad, where approximately 25% of the population remains viable, cells are delayed for up to 12 h in G2 but eventually become arrested in Gy2-G1. The data presented in this paper provide evidence that at least some of the cells irradiated in G0 were capable of entering S phase, thus, suggesting that there is no immediate G0 block even in cells with an intact p53 signaling network.
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