

DNA damage triggers a prolonged p53-dependent G₁ arrest and long-term induction of Cip1 in normal human fibroblasts

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The tumor suppressor p53 is a cell cycle checkpoint protein that contributes to the preservation of genetic stability by mediating either a G₁ arrest or apoptosis in response to DNA damage. Recent reports suggest that p53 causes growth arrest through transcriptional activation of the cyclin-dependent kinase (Cdk)-inhibitor Cip1. Here, we characterize the p53-dependent G₁ arrest in several normal human diploid fibroblast (NDF) strains and p53-deficient cell lines treated with 0.1–6 Gy gamma radiation. DNA damage and cell cycle progression analyses showed that NDF entered a prolonged arrest state resembling senescence, even at low doses of radiation. This contrasts with the view that p53 ensures genetic stability by inducing a transient arrest to enable repair of DNA damage, as reported for some myeloid leukemia lines. Gamma radiation administered in early to mid-, but not late, G₁ induced the arrest, suggesting that the p53 checkpoint is only active in G₁ until cells commit to enter S phase at the G₁ restriction point. A log-linear plot of the fraction of irradiated G₀ cells able to enter S phase as a function of dose is consistent with single-hit kinetics. Cytogenetic analyses combined with radiation dosage data indicate that only one or a small number of unrepaired DNA breaks may be sufficient to cause arrest. The arrest also correlated with long-term elevations of p53 protein, Cip1 mRNA, and Cip1 protein. We propose that p53 helps maintain genetic stability in NDF by mediating a permanent cell cycle arrest through long-term induction of Cip1 when low amounts of unrepaired DNA damage are present in G₁ before the restriction point.

[Key Words: Normal human diploid fibroblasts; gamma radiation; DNA damage; cell cycle control; p53; and cyclin-dependent kinase inhibitors (Cip1)]

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Alterations in genes involved in cell cycle control contribute to the genetic instability seen in many clinically important neoplasms by allowing cells to cycle under inappropriate conditions (Tlsty 1990; Wright et al. 1990; Livingstone et al. 1992; Yin et al. 1992). p53 is a cell cycle checkpoint protein governing progression of cells from G₁ into S phase (Kastan et al. 1991; Kuerbitz et al. 1992; Lane 1992; Livingstone et al. 1992; Yin et al. 1992). It helps preserve genetic stability by mediating either a G₁ arrest or apoptosis when conditions are suboptimal for DNA replication, thereby minimizing both the development of genetic lesions created by replication errors and their subsequent fixation in the genome. For example, gene amplification, one marker of genetic in-

stability, occurs in p53-deficient but not in normal cells during treatment with the anti-metabolite *N*-(phosphonacetyl)-L-aspartate (PALA) (Livingstone et al. 1992; Yin et al. 1992). The frequent loss of wild-type p53 (wtp53) function during human and rodent tumor progression, and the correlation between p53 loss and the emergence of cells exhibiting aneuploidy or structural chromosome alterations (Blount et al. 1993), implies that compromised regulation of the G₁-S transition by factors such as p53 contributes to tumor progression by increasing genetic instability (Hartwell and Weinert 1989; Hartwell 1992; Di Leonardo et al. 1994).

Treatment of cells with a variety of DNA-damaging agents, including ionizing radiation, topoisomerase inhibitors, and DNA intercalators, induces a p53-dependent G₁ arrest (Kastan et al. 1991, 1992; Kuerbitz et al. 1992; Kessis et al. 1993) or apoptosis (Clarke et al. 1993;

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Lowe et al. 1993a,b), depending on cell type. Recent evidence suggests that DNA double-strand breaks (dsbs) are the specific lesions required for p53 induction (Nelson and Kastan 1994). In addition, we have evidence that depletion of specific nucleotide pools leads to a p53-dependent G₁ arrest in the absence of DNA damage (S. Linke unpubl.). These studies suggest that p53 plays a major role as a cell cycle regulator in response to DNA damage or growth-limiting conditions.

The mechanism by which damaged DNA elicits the p53-mediated responses remains largely unknown. Treatment of cells with DNA-damaging agents leads to increases in p53 levels (Maltzman and Czyzyk 1984; Hall et al. 1993), nuclear localization (Fritsche et al. 1993), and sequence-specific DNA binding (Tishler et al. 1993). As a result, genes containing p53-binding sites, including *mdm2* (Price and Park 1994), the growth arrest and DNA-damage response gene *gadd45* (Kastan et al. 1992; Zhan et al. 1993), and the cyclin-dependent kinase (Cdk)-inhibitor *CIP1* (Xiong et al. 1993a; Dulic et al. 1994; El-Deiry et al. 1994) are activated transcriptionally. Cip1 forms inhibitory complexes with many different cyclins and their associated Cdks and may be a universal Cdk inhibitor (Harper et al. 1993; Xiong et al. 1993a, b). It has also been shown to complex with the replication factor proliferating cell nuclear antigen (PCNA) (Xiong et al. 1992). In addition, Cip1 mRNA levels are increased 10- to 20-fold in senescent cells compared with cycling cells and may contribute to maintaining the senescent phenotype (Noda et al. 1994).

The mechanism by which the p53-mediated cell cycle arrest limits genetic instability is also uncertain. One model proposes that the DNA damage-induced, p53-mediated G₁ arrest is transient, allowing sufficient time for DNA repair to occur before progression into S phase (Kastan et al. 1991; Lane 1992). This is analogous to the RAD9 cell cycle checkpoint protein that enables yeast to arrest transiently in G₂ to repair DNA damage prior to mitosis (Hartwell and Weinert 1989).

The experiments presented here were designed to characterize the response of normal human diploid fibroblast (NDF) strains to gamma radiation to gain further insight into how DNA damage-induced cell cycle arrest contributes to genetic stability. NDF strains were chosen for this study because tumor cell lines are likely to contain multiple mutations, independent of p53 status, that may affect radiation response and cell cycle regulation. We show that gamma irradiation of NDF causes a prolonged and most likely irreversible arrest characteristic of senescence. p53 protein levels were induced rapidly and remained above baseline level for ≥ 96 hr. This correlated with long-term induction of Cip1 mRNA and protein. We also show that NDF cells arrest when irradiated in early and mid-, but not late, G₁. The timing of the arrest suggests that the G₁ restriction point (Pardee 1989) may be a boundary after which cells are committed to S phase despite the presence of DNA damage. The arrest appears to follow single-hit kinetics, consistent with cytogenetic analyses indicating that cells destined to arrest contain, on average, one or very few unrepaired

DNA breaks. The induction of a prolonged arrest by low amounts of DNA damage provides an explanation of how p53 minimizes the occurrence of genetic alterations derived from chromosome breakage.

Results

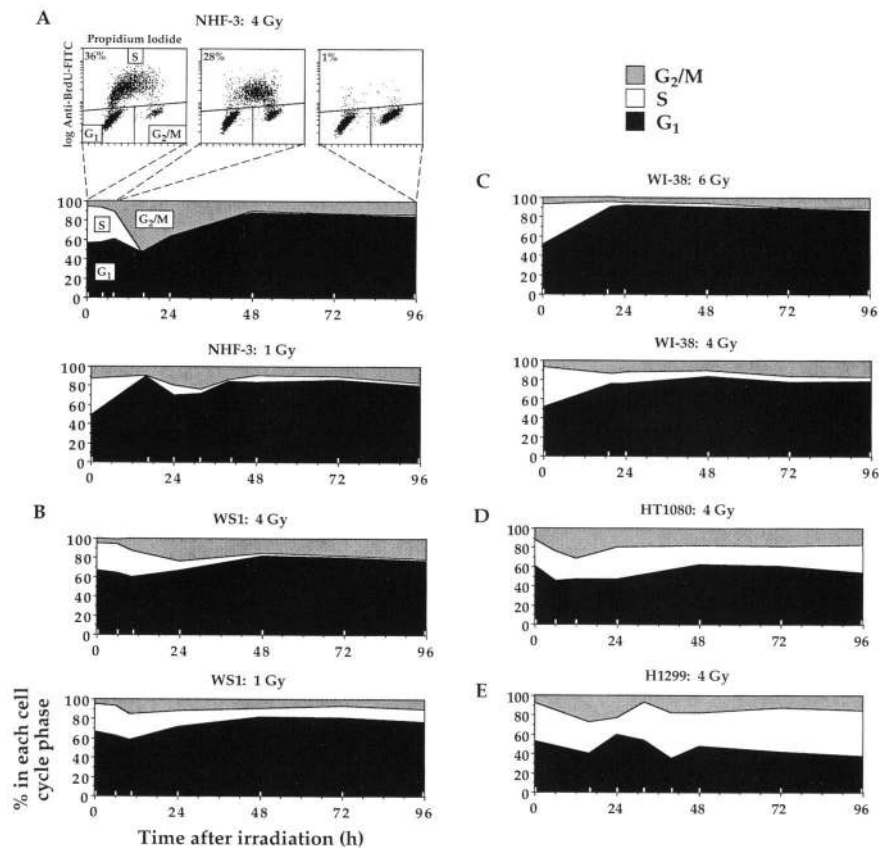
Gamma irradiation of asynchronous NDF causes a prolonged growth arrest

We tested the cell cycle response of NDF strains to gamma radiation to determine whether they undergo a transient G₁ arrest. Logarithmically growing, asynchronous populations of NDF were treated with 1–6 Gy gamma radiation. Cells were plated sparsely to prevent contact inhibition. Cell cycle distributions were assessed at various times after treatment by incorporation of bromodeoxyuridine (BrdU) during a 30-min pulse followed by bivariate flow cytometric analysis. Incorporation of BrdU indicates cells in S phase. The data are presented in the form of “area plots” in which the percentage of cells in each cell cycle phase is plotted against time (Fig. 1). Experiments were repeated at least twice, and representative experiments are shown.

Immediately after treatment with doses of ≥ 4 Gy, cells from all NDF strains analyzed began accumulating in the G₁ and G₂/M phases of the cell cycle and progression into S phase was slowed or halted (Fig. 1A–C). Consistent with previous work (Kastan et al. 1991, 1992; Kuerbitz et al. 1992), the S-phase fraction reached a minimum between 16 and 48 hr after treatment, and most cells arrested in G₁. The percentage of cells capable of incorporating BrdU was at background level (<3%) between 48 and 96 hr after irradiation (Fig. 1) and remained at background level after 1 and 2 weeks (data not shown). Almost all of the cells remained arrested through 3 weeks, and the few colonies that formed were small, had irregular morphologies, and 70% were unable to grow when replated (data not shown). We did not see extensive cell death or detect apoptotic cells by flow cytometric DNA content, terminal transferase assay, or DNA laddering. The irradiated cells were viable as determined by trypan blue exclusion and became enlarged and flattened, characteristic of senescent cells (Fig. 2B,E). Thus, doses of ≥ 4 Gy induced a prolonged, presumably permanent arrest in NDF.

At lower doses (1–2 Gy), cells from the NDF strains also accumulated in G₁ and G₂/M with a concomitant decrease in the percentage of S-phase cells (see Fig. 1A, B; data not shown for WI-38). However, small percentages of cells were able to incorporate BrdU at these lower doses. For example, at 1 Gy ~5% of NHF-3 cells appeared to traverse one or two additional cell cycles before arresting at 72 hr (Fig. 1A). Treatment of WS1 (Fig. 1B) and WI-38 (data not shown) with 1 Gy produced larger subsets of cells (typically 5–10%) that incorporated BrdU through 96 hr, but these fractions were substantially smaller than exponential S-phase levels (typically 25–30%) and did not expand back to exponential levels. This

Figure 1. Flow cytometric analysis of asynchronous NDF strains NHF-3, WS1, and WI-38 and p53-deficient cell lines HT1080 and H1299. Cultures were irradiated with the indicated doses of gamma radiation. Samples were pulse-labeled with BrdU at the times denoted with white hatch marks, fixed, stained with propidium iodide and anti-BrdU-FITC, and analyzed by flow cytometry. For the representative dot plots in *A*, G₁-phase cells have a 2N DNA content and no BrdU incorporation, whereas G₂- and M-phase cells have a 4N DNA content and no BrdU incorporation. S-phase cells have a DNA content ranging between 2N and 4N, depending on the degree of completion of DNA synthesis, and show BrdU incorporation. The data are presented in the form of area plots, where the percentages of cells in each cell cycle phase are plotted against time: (*A*) NHF-3; (*B*) WS1; (*C*) WI-38; (*D*) HT1080; and (*E*) H1299. Note that only three of the time points are shown as dot plots in *A*, corresponding with the area plot (percentages in S phase represent BrdU⁺ cells).



suggests that damage induced in the first cell cycle generated descendants whose replication was subsequently compromised.

The cell cycle progression of two p53-deficient cell lines, H1299 [lacking p53 expression; Bodner et al. 1992] and HT1080 [expressing mutant p53 (mtp53); A. Almasan, unpubl.; Maimets and Jenkins 1987; Anderson et al. 1994] was also assessed. Consistent with previous reports (Kastan et al. 1991, 1992; Kuerbitz et al. 1992), these cells did not arrest in G₁ in response to gamma radiation but, rather, continued to cycle after a delay in G₂ (Fig. 1D,E). In addition, pooled populations of the NDF strains NHF-3, WS1, and WI-38 infected with a retroviral construct containing an HPV16E6 gene, whose product directs the degradation of p53, also failed to arrest after gamma irradiation (S. Linke, unpubl.).

Gamma irradiation of G₀-synchronized NDF inhibits progression into S phase and causes a long-term G₁ arrest

We analyzed the effects of DNA damage on cells synchronized in G₀ by serum starvation to determine whether the cells remained in G₁ or exhibited delayed progression into S phase after treatment with gamma radiation. G₀-synchronized populations of NDF were irradiated at doses of 1–6 Gy just before, or immediately

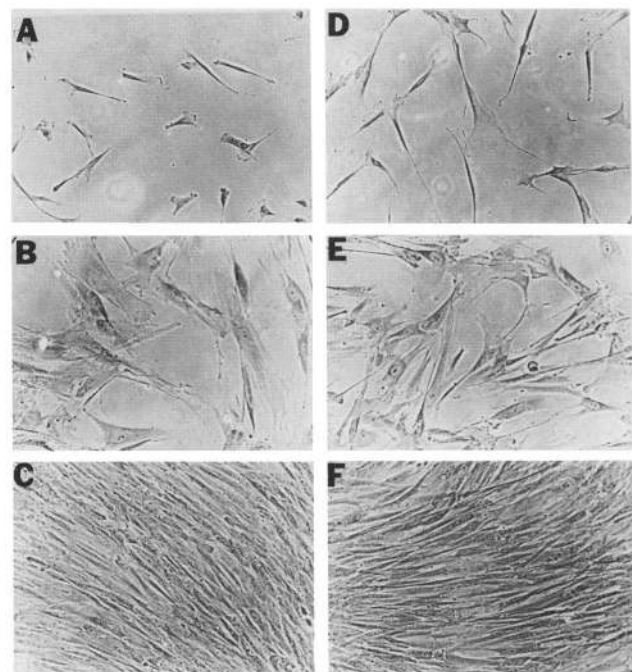


Figure 2. Morphology of NDF strains WS1 (*A–C*) and WI-38 (*D–F*). Cells were plated at 2×10^5 cells per 6-cm dish and either treated with 4 Gy gamma radiation or left untreated. (*A,D*) Untreated cells at 0 hr; (*B,E*) cells 96 hr after treatment with 4 Gy gamma radiation; (*C,F*) untreated cells after 96 hr.

after, release into complete medium, and cell cycle kinetics were assessed over 96 hr (Fig. 3). Unirradiated control cells began entering S phase 12–16 hr after addition of complete medium. In contrast, doses of ≥ 4 Gy prevented almost all cells from escaping G_0/G_1 over the 96-hr assay period. As with the asynchronous cultures, almost all of the irradiated cells became enlarged and flattened and were incapable of incorporating BrdU over the 96-hr time course (see Fig. 2B,E).

At lower doses (1–2 Gy), small percentages of cells incorporated BrdU (<6%). However, the cells began incorporating BrdU at the same time after release as the unirradiated controls, suggesting that they are not undergoing a G_1 delay or that the delay is shorter than the assay interval. The subsequent cell cycle behavior was different for each strain. The small BrdU⁺ fraction disappeared from the NHF-3 population by 48 hr after irradiation (Fig. 3A), whereas that present in WS1 (Fig. 3B) and WI-38 (data not shown) populations remained constant through 96 hr. Again, these small BrdU⁺ populations were substantially smaller than the untreated controls and did not expand over time, indicating that their replicative potential had been compromised.

We used a continuous BrdU-labeling method to determine the cell cycle effects of lower doses of gamma radiation administered in G_0/G_1 . G_0 -synchronized WS1 cultures were treated with 0.1, 0.25, 0.5, 0.75, 1, 2, and 4 Gy gamma radiation immediately before release into complete medium containing 10 μ M BrdU. Irradiation was done for 30 sec at distances to deliver the indicated doses. Cells were harvested 36 hr later and analyzed by flow cytometry. This assay provides a quantitative assessment of the percentage of cells that escape from the first G_0/G_1 , as all cells that enter S-phase during the

time course show BrdU incorporation. The data are plotted in Figure 4 as the logarithm of the percentage of treated relative to untreated cells that incorporated BrdU as a function of radiation dose. This plotting method is analogous to that used to analyze radiation survival curves. The data reveal an essentially straight line through all radiation doses >0.1 Gy. This straight-line relationship indicates that the G_1 arrest induced by gamma irradiation obeys single-hit kinetics. Consistent with the results of the BrdU-pulse experiments (see Fig. 3), a majority of the cells remained arrested at higher doses (>1 Gy) and did not appear to proceed beyond one cell cycle (data not shown). At lower doses (≤ 1 Gy), more cells escaped from G_1 , but those that did not escape remained in a prolonged arrest. In addition, the cells that escaped G_1 at low radiation doses appeared to undergo a G_2 delay, suggesting that they had gone through S phase with some damage (data not shown). Some of the cells irradiated with <1 Gy presumably continued to cycle as the plates became confluent by 96 hr after treatment.

Gamma irradiation prevents progression of NDF into S phase when administered in early to mid-, but not late, G_1

To assay the cell cycle effects of DNA damage administered at different times during G_1 , G_0 -synchronized WS1 cells were treated with 4 Gy gamma radiation at various times after release into complete medium. Cells were either pulse-labeled with BrdU for 30 min immediately before harvest or labeled continuously with BrdU beginning at the time of release and proceeding until harvest 36 hr later (Fig. 5). This experimental design enabled investigation of two parameters. First, it provided a direct

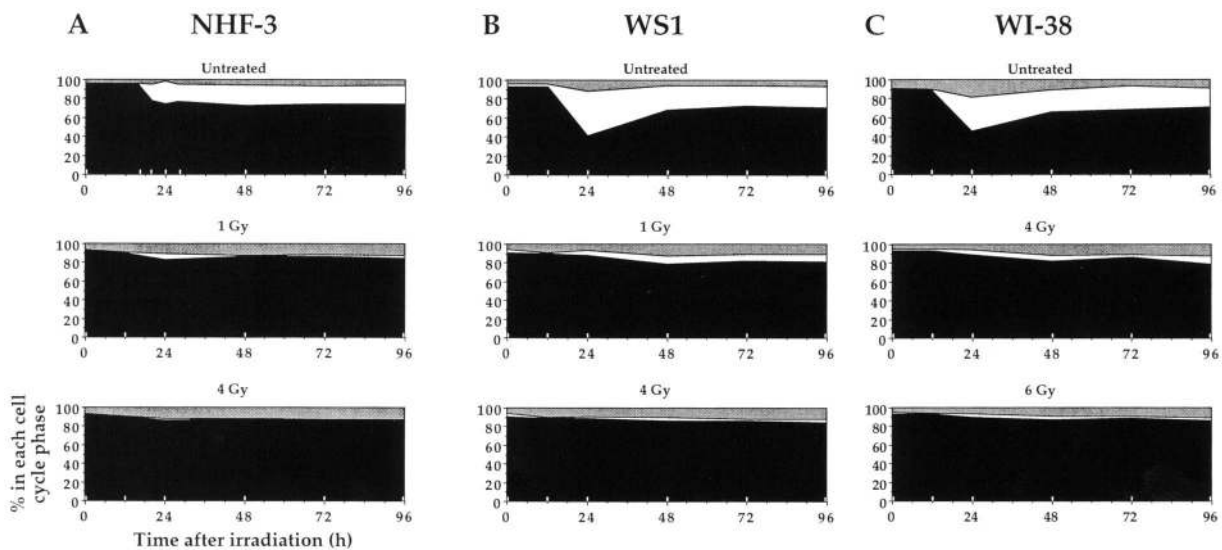


Figure 3. Flow cytometric analysis of G_0 -synchronized NDF strains NHF-3, WS1, and WI-38. Cultures were irradiated with the indicated doses of gamma radiation either immediately before or immediately after release into complete medium. Samples were pulse-labeled with BrdU at the times denoted with white hatch marks, fixed, stained with propidium iodide and anti-BrdU-FITC, and analyzed by flow cytometry. Area plots show the percentage of cells in each cell cycle phase plotted against time: (A) NHF-3; (B) WS1; and (C) WI-38.

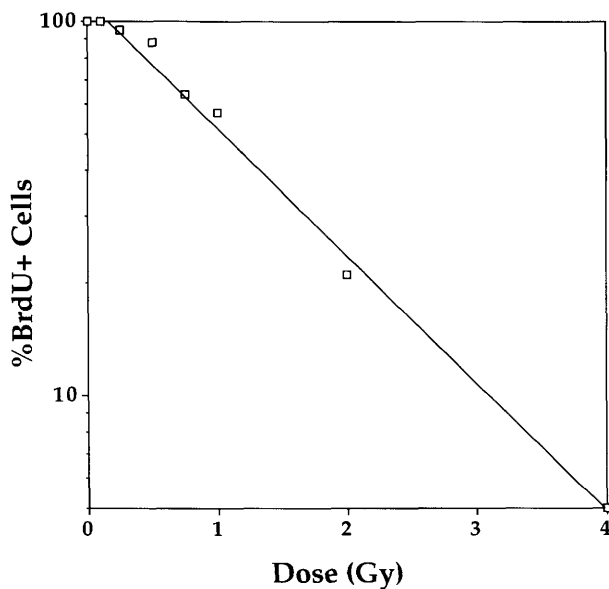


Figure 4. Flow cytometric analysis of G_0 -synchronized NDF strain WS1. Cultures were irradiated with 0.1, 0.25, 0.5, 1, 2, or 4 Gy of gamma radiation, continuously labeled with BrdU for 36 hr, and analyzed by flow cytometry. The graph shows the percentage of cells able to escape from the first G_0/G_1 phase relative to the untreated control at each dose.

way of determining whether cells damaged in G_1 delay their entrance into S phase. Second, it allowed us to determine whether there is a point in G_1 beyond which cells lose their ability to arrest in response to gamma radiation.

Unirradiated control cells began entering S phase ~16 hr after release into complete medium as determined by pulse-labeling (Fig. 5A). Continuous labeling showed that by 36 hr after release, 54% of the untreated cells had incorporated BrdU, indicating that they had escaped from G_1 into S (Fig. 5B). In contrast, few cells irradiated between 0 and 12 hr after release incorporated BrdU during the 36-hr continuous labeling (Fig. 5C), indicating that they were unable to escape from G_1 after treatment. However, when irradiated >12 hr after release, increasing percentages of cells incorporated BrdU (Fig. 5C). It is important to note that cells released from serum starvation progressed through G_1 at different rates. Under the conditions used in this study, the fastest progressing population of WS1 cells started entering S phase ~16 hr after release and continued to enter S phase for several more hours (Fig. 5A,B). Thus, the cells that entered S phase when irradiated >12 hr after release were presumably the fastest progressing population of cells. This indicates that cells that are within ~4 hr of entry into S phase are not subject to the gamma radiation-induced arrest response, even though they should have incurred the same level of DNA damage as cells irradiated earlier in G_1 .

Interestingly, the cells that entered S phase in cultures irradiated >12 hr after release did not undergo a detectable G_1 delay but did delay or arrest in the subsequent

G_2/M phase. The irradiated cells began incorporating BrdU at approximately the same time as the untreated control cells (16 hr), as determined by pulse-labeling (data not shown). During continuous labeling, irradiated cells accumulated in the first G_2/M phase after release (see 13- and 17-hr dot plots in Fig. 5C), whereas untreated cells progressed to the following G_1 (Fig. 5B). Thus, WS1 cells irradiated in the last 4 hr of G_1 appear to enter S phase without lag, but delay or arrest in the subsequent G_2/M .

A further indication that NDFs lose their ability to arrest in late G_1 in response to gamma radiation was obtained by analyzing cells synchronized with the plant amino acid mimosine. NHF-3 cells were synchronized in late G_1 with 200 μ M mimosine (Watson et al. 1991). Cells were irradiated with 4 Gy or left untreated, and the mimosine was then removed. Cell cycle analyses revealed that both the irradiated and untreated cells began incorporating BrdU ~2 hr after release from the mimosine block (data not shown), and equal percentages of cells incorporated BrdU at later times (see Fig. 6 for representative data 4 hr after release). The fact that progression of cells into S phase was not delayed or reduced by irradiation of mimosine-arrested cells lends further support to the proposal that cells in late G_1 fail to execute the damage-inducible arrest program that prevents replication of damaged DNA.

Cytogenetic analysis of DNA damage after gamma irradiation of NDF and p53-deficient cells

As shown above, even small doses of gamma radiation have profound effects on the cell cycle kinetics of NDF. We estimated the number of unrepaired dsbs required to induce the G_1 arrest by determining the number of aberrant chromosomes present in the preceding metaphase. Asynchronous cultures were treated with 1 or 4 Gy of gamma radiation and then placed immediately in colcemid to arrest the cells in the following mitosis. This protocol enabled us to determine the number and types of chromosome aberrations present in the first mitosis after treatment. The number of aberrant chromosomes detected under such conditions reflects both the initial amount of damage induced and the ability of the cells to repair the damage but does not address the accuracy of repair.

Table 1 shows the number and types of metaphase chromosome abnormalities detected in NHF-3 (NDF) and HT1080 (mtp53). As expected, the total number of unrepaired dsbs estimated from the observed aberrant metaphases increased linearly in both cell types as a function of dose. At 4 Gy, NHF-3 metaphases contained an estimated 0.59 dsbs per cell, whereas the same dose generated an estimated 0.99 dsb per cell in HT1080 cells, approximately four times the number estimated at 1 Gy. This dose was sufficient to arrest almost 100% of the NHF-3 cells in the next G_1 (see Fig. 1A), yet only 28% of the metaphases revealed abnormalities. Because of the nature of this assay, some unrepaired dsbs probably went undetected. However, when considered in the context of

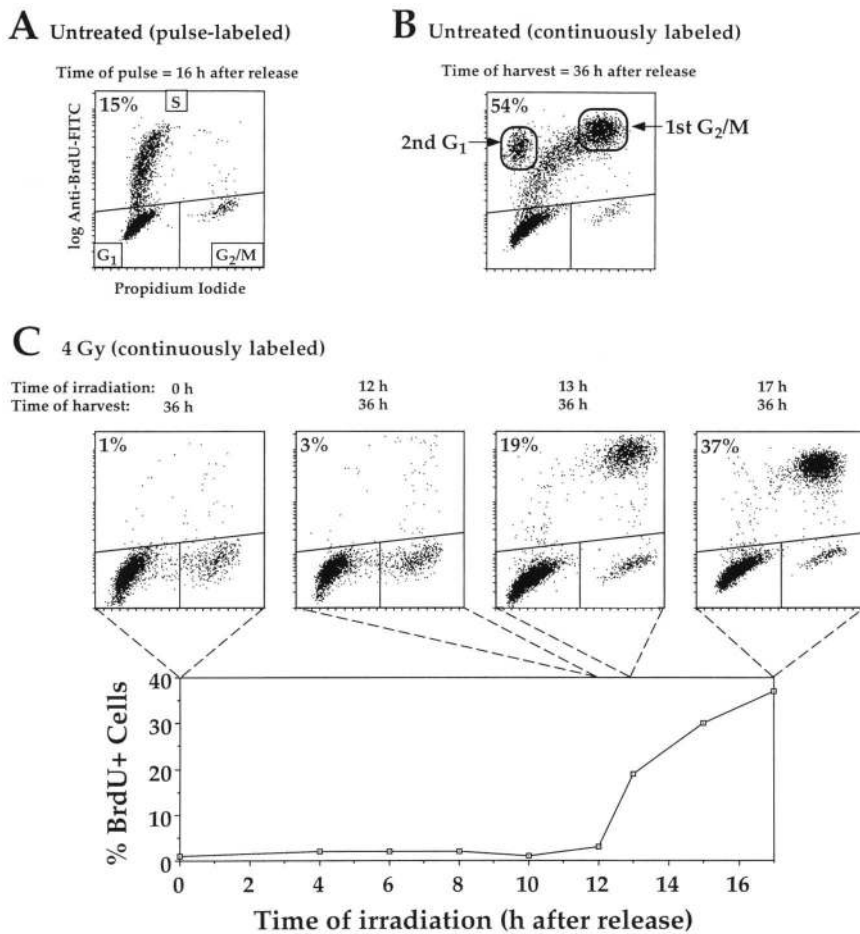


Figure 5. Flow cytometric analysis of NDF strain WS1 synchronized by serum starvation and treated with 4 Gy gamma radiation at various times after release into G₁. Samples were either pulse-labeled or continuously labeled with BrdU, fixed, stained with propidium iodide and anti-BrdU-FITC, and analyzed by flow cytometry. Percentages in dot plots represent BrdU⁺ cells. (A) Untreated cells pulse-labeled 16 hr after release; (B) untreated cells continuously labeled for 36 hr. Cells in the first G₂/M and second G₁ phase after release contain a 4N and 2N DNA content, respectively, but generate a signal above those in the first G₁ and previous G₂/M phase attributable to continuous incorporation of BrdU during their traverse through S phase. (Note that the number of events in the second G₁ phase was divided by two in determining the percentages, as these cells have divided.) (C) Percentage of BrdU⁺ cells treated with 4 Gy gamma radiation at the indicated times after release, continuously labeled with BrdU for 36 hr after release. Representative dot plots show cells arrested in G₁ or delayed in the first G₂/M phase after release.

the results represented above, these data suggest that only a small number of breaks present in early G₁ may be sufficient to induce and sustain a G₁ arrest. They also indicate that the absence of a wtp53 pathway in HT1080 cells does not reduce their repair capacity significantly.

Induction of p53 and Cip1 in NDF after gamma irradiation

Recent data on the correlation between p53 transcriptional activation of the Cdk inhibitor Cip1 and cell cycle arrest (El-Deiry et al. 1993; Harper et al. 1993) prompted us to study the possibility that these molecular events are involved in the long-term arrest of NDF in response to gamma radiation. Asynchronous populations of WS1 (NDF) and H1299 (lacking p53 expression) were treated with 4 Gy gamma radiation, and protein extracts or total RNA were isolated over 96 hr for Western or Northern blotting, respectively. WS1 p53 protein levels increased approximately threefold within 2–3 hr after irradiation and remained above baseline over the 96-hr time course (Fig. 7A). WS1 *CIP1* mRNA levels increased fivefold within 24 hr and remained high through 96 hr (Fig. 7B), correlating with similar increases in Cip1 protein levels (Fig. 7C). H1299 *CIP1* mRNA levels were very low and

remained unchanged after irradiation (Fig. 7B). H1299 Cip1 protein levels were extremely low and, if present at all, were not induced by treatment (Fig. 7C).

Discussion

The experiments presented in this report explore the effects of DNA damage on the cell cycle kinetics of NDF. Both asynchronous and synchronized NDF showed a prolonged and apparently irreversible arrest resembling senescence after gamma irradiation. p53 protein levels were induced within 2–3 hr of irradiation, and Cip1 mRNA showed strong induction by 24 h after treatment with a corresponding increase in Cip1 protein levels that were sustained throughout the 96-hr analysis. These data support a model in which DNA damage leads to elevation of p53 levels, which then initiates the arrest response through transcriptional activation of the Cdk inhibitor Cip1 (Dulic et al. 1994; El-Deiry et al. 1994). Cip1 may sustain the arrest by remaining associated with various G₁ cyclins, Cdks, and PCNA in inhibitory complexes. We also found that cells arrest when irradiated in early and mid-, but not late, G₁. This is consistent with previous reports showing that overexpression of p53 leads to arrest in G₁ phase (Martinez et al. 1991;

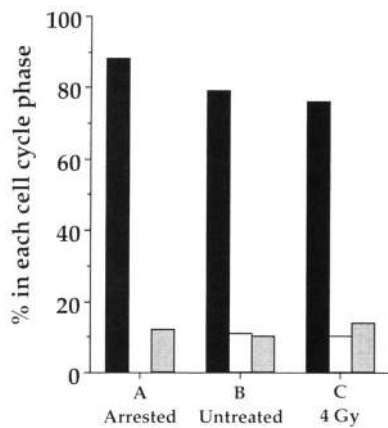


Figure 6. Flow cytometric analysis of mimosine-synchronized NDF strain NHF-3. Cultures arrested in 200 μ M mimosine were treated with 4 Gy gamma radiation or left untreated. The mimosine was then washed off and replaced with drug-free medium. Samples were pulse-labeled with BrdU at various times after release, fixed, stained with propidium iodide and anti-BrdU-FITC, and analyzed by flow cytometry. The graph shows the percentages of cells in each cell cycle phase: (A) mimosine-arrested; (B) mimosine-arrested and released 4 hr without treatment; and (C) mimosine-arrested and released 4 hr after treatment with 4 Gy gamma radiation. (Solid bar) G₁; (open bar) S; (stippled bar) G₂/M phase.

Lin et al. 1992). The timing of the arrest suggests that the G₁ restriction point (Pardee 1989) may be a boundary after which cells are committed to S phase despite the presence of DNA damage.

It has been proposed that p53 helps maintain genetic stability by initiating a transient G₁ cell cycle arrest to allow time for repair of damaged DNA before entry into S phase (Kastan et al. 1991; Lane 1992), analogous to the yeast RAD9 checkpoint (e.g., see Hartwell and Weinert 1989). Kastan et al. (1991) found that gamma irradiation (0.5–4 Gy) of the myeloid leukemia cell line ML-1 (containing wtp53) caused a rapid transient induction of p53 protein with a coincident decrease in the percentage of S-phase cells and an increase in the percentage of G₁ and

G₂/M cells within 16 hr. By 24 hr after treatment, cells began re-emerging in S, and within 72 hr had reached normal asynchronous levels, suggesting that the G₁ arrest was transient.

In contrast, the cell cycle data on NDF presented in this report suggest a permanent rather than a transient cell cycle arrest model. Consistent with previous reports, we observed a decline in the S-phase population of asynchronous NDF cultures after irradiation. However, rather than re-emerging in S phase, the cells entered a prolonged G₁ arrest (Fig. 1). Although these cells were not undergoing DNA replication or division, they were viable as determined by trypan blue exclusion and had enlarged, flattened morphologies (Fig. 2; Di Leonardo et al. 1994) characteristic of senescent cells. This suggests that irradiation may be inducing premature senescence. The same was true for NDF irradiated in G₀ or early to mid-G₁ (Figs. 3 and 5). Importantly, in the G₀-synchronized populations exposed to lower doses of gamma radiation (1–2 Gy), the cells capable of incorporating BrdU did so at the same time after release as the untreated controls. Thus, even at low doses, we found no evidence of a prolonged G₁ delay for DNA repair after irradiation.

This raises questions about the specific role played by p53 in the cellular response to DNA damage in different cell types and whether one can generalize from the example provided by myeloid leukemia tumor lines. In contrast to the previous study (Kastan et al. 1991), we found that ML-1 cells undergo apoptosis after gamma irradiation (A. Di Leonardo, unpubl.). This is consistent with other studies showing that DNA-damaging agents induce apoptosis in most cells of hematopoietic lineage containing wtp53 (Walker et al. 1991; Clarke et al. 1993; Levy et al. 1993; Lotem and Sachs 1993; Lowe et al. 1993b; O'Connor et al. 1993; Ryan et al. 1993; Yonish-Rouach et al. 1993; Radford 1994; Radford and Murphy 1994; Radford et al. 1994), as does overexpression of wtp53 (Yonish-Rouach et al. 1991). It has also been shown that wtp53 plays an essential role in the radiation-induced apoptosis of murine intestinal epithelial stem cells (Merritt et al. 1994).

Table 1. Chromosomal aberrations in NHF-3 and HT1080 after gamma irradiation

Cell type treatment	Aberrant metaphases ^a	CTB ^b	CTE ^c	Chr.E ^d	DM ^e	Total breaks ^f	Average breaks
NHF-3							
1 Gy	8/100	3	2	2	2	15	0.15
4 Gy	28/100	3	2	6	20	59	0.59
HT1080							
1 Gy	10/100	1	10	1	1	25	0.25
4 Gy	29/100	7	15	13	18	99	0.99

^aNumber of aberrant metaphases/number of observed metaphases scored as described by Savage (1976).

^bChromatid breaks.

^cChromatid exchanges (including non-sister chromatid interchanges such as triradials and quadriradials).

^dChromosome exchanges.

^eDouble minute chromosomes.

^fTotal number of unrepaired dsbs in all cells, estimated from the numbers and types of observed aberrant metaphases. The aberrations scored as CTE, Chr.E, and DM are multiplied by two in calculating the total number of breaks, because two breaks are necessary for their formation.

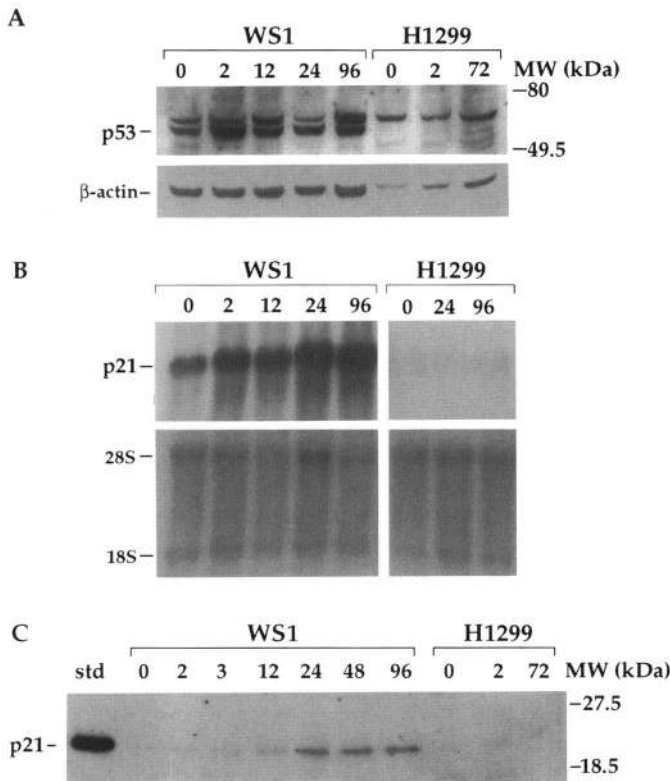


Figure 7. Northern and Western analyses of WS1 (NDF) and H1299 (lacking p53 expression). Numbers designate time in hours after treatment with 4 Gy gamma irradiation. (A) Western probed with anti-p53 antibody DO-1 and then with anti- β -actin as an internal control. (B) Northern analysis of total RNA (20 μ g per lane) probed with Cip1 and then with rRNA probe p11A-2 as an internal control. (C) Western probed with anti-Cip1 antibody. (std) p21 protein control.

One prediction of the p53 transient arrest/repair model is that cells with a normal p53 response pathway, which normally do not undergo damage-induced apoptosis, should be more radio resistant than p53-deficient cells. Consequently, it has been proposed that the high radiosensitivity and genetic instability exhibited by ataxia-telangiectasia (AT) fibroblasts arises from their failure to induce p53 (Kastan et al. 1992). However, numerous studies suggest that there is either no correlation between p53 status and radiosensitivity (Brachman et al. 1992; Carr et al. 1992; Jung et al. 1992; Murnane and Schwartz 1993; Slichenmyer et al. 1993) or that p53-deficient cells are actually more radio resistant than cells with a wtp53 pathway (Chang et al. 1987; Arlett et al. 1988; Su and Little 1992a,b; Bristow et al. 1994; Di Leonardo et al. 1994; McIlwrath et al. 1994). We reported previously that the NDF strains tested in this study were 5–10 times more radiosensitive than the p53-deficient cell lines as determined by colony formation. The few NDF colonies that developed were very small, and the cells had irregular, flattened morphologies similar to senescent cells (Di Leonardo et al. 1994). This is consistent with the cell cycle data presented here, in which most of

the normal fibroblasts underwent a prolonged arrest after gamma irradiation, and those that did cycle appeared to be compromised replicatively. Our cytogenetic observations indicate that NDF and cell lines with mutated p53 genes accumulate approximately the same amount of DNA damage after irradiation. Thus, the differences in radiosensitivity between cells with normal and inactivated p53 DNA damage pathways is not likely to reflect the amount of damage they sustained, but rather the integrity of their p53-mediated arrest mechanisms.

In light of the data summarized above, alternatives to the transient arrest/repair model should be considered. As one alternative, we propose that the p53 pathway preserves genetic integrity by mediating either apoptosis or a permanent arrest, depending on the cell type, when irreparable DNA damage is present in early to mid G_1 . This model is consistent with our cell cycle data and with the data showing that cells with a functional p53 pathway are more radiosensitive than p53-deficient cells. Either response would prevent the emergence of variants with structural chromosome changes that could contribute to tumor progression. The absence of a normal p53 pathway would create a permissive environment for the proliferation of cells with genomic alterations induced by chromosome breakage. Our data imply that cells damaged during late G_1 , S, G_2 , and M progress to the next G_1 before p53 can mediate an arrest. During their transit through the cell cycle, ample time exists for DNA repair, such as in the elongated G_2 phase observed after irradiation of both wtp53- and p53-deficient cells. If the damage cannot be repaired completely in one cell cycle, if the damage is misrepaired and breakage occurs in a later mitosis, or if there is insufficient time for complete repair, p53 may mediate either a permanent arrest or apoptosis upon entry of the cell into a subsequent G_1 .

The radiation dose and cytogenetic data presented here, along with previous genetic studies, provide a consistent picture that the amount of unrepaired DNA damage required to initiate the arrest is very low. The log-linear plot relating the fraction of cells able to enter S phase at each radiation dose revealed a straight line. By analogy with target theory applied to radiation survival curves (Hall 1994), this result indicates that a single "event," probably an unrepaired dsb, precipitates the arrest. Interestingly, the almost complete absence of a shoulder in this plot is reminiscent of the radiation survival curves reported for radiosensitive cell lines containing mutations in a variety of genes thought to be involved in DNA repair (e.g., see Hall 1994). This encourages the speculation that gamma radiation-induced cell cycle arrest in NDF may result from the induction of a type of dsb that is not readily repaired, or that the cell lacks the capacity to repair such lesions in early or mid- G_1 . Furthermore, our cytogenetic analyses showed that cells destined to arrest in G_1 had an average of only one broken chromosome in the metaphase preceding arrest. Although precise quantitative data are difficult to obtain by such an assay, the cytogenetic observations are in remarkably good agreement with the conclusion drawn from the radiation dose experiment that one or a few

unrepaired dsbs trigger the p53-dependent G₁ arrest mechanism.

Another line of evidence that a stringent arrest response can be triggered by one or a few unrepaired breaks derives from previous studies on gene amplification. Presumably, gene amplification can be initiated by small amounts of DNA damage, perhaps a single unrepaired dsb (Windle and Wahl 1992; Di Leonardo et al. 1994), generating dicentric chromosomes or acentric fragments. However, amplification is not detected in NDF unless p53 is inactivated (Livingstone et al. 1992; Yin et al. 1992; Schaefer et al. 1993; White et al. 1994). Taken together, these data indicate that p53 mediates a prolonged arrest when even small numbers of unrepaired breaks are present in G₁, effectively preventing the emergence of genetic variants. On the other hand, similar amounts of damage in p53-deficient cells do not lead to G₁ arrest, and continued cycling enables perpetuation of genetic damage (e.g., see Marder and Morgan 1993).

p53 levels were induced rapidly and remained above baseline level for ≥ 96 hr. The initial increase has been proposed to result from post-translational protein stabilization (Kastan et al. 1991). Recent data indicating that p53 regulates its own promoter positively (Deffie et al. 1993) suggest that the sustained induction may derive from autoregulation. Our data are consistent with a previously proposed model that p53 accumulation after DNA damage initiates the arrest response through induction of the Cdk inhibitor Cip1 (Dulic et al. 1994; El-Deiry et al. 1993, 1994), although additional factors may be involved. Activation of cyclin-Cdk complexes in late G₁, particularly cyclin E-Cdk2 and cyclin D-Cdk4, is a key event in the commitment to S phase (Dulic et al. 1992; Koff et al. 1992). Elevated Cip1 has been shown to decrease the activity of a variety of Cdks normally active in late G₁ (Harper et al. 1993; Xiong et al. 1993a; Dulic et al. 1994; El-Deiry et al. 1994) and to prevent the phosphorylation of pRb (Harper et al. 1993; Dulic et al. 1994). Furthermore, Cip1 is highly expressed in senescent fibroblasts (Noda et al. 1994). We found that Cip1 levels remained elevated for ≥ 96 hr after irradiation. Thus, inactivation of Cdks by sustained expression of p21 may be the biochemical mechanism underlying the observed long-term cell cycle arrest.

There are at least two potential explanations for the failure of cells irradiated in late G₁ to arrest, despite the presence of a wtp53 pathway. First, DNA damage may not lead to induction of p53 or Cip1 in late G₁. Second, p53 and Cip1 could be induced, but the induction may be too late to inactivate the Cdks involved in progression into S phase. For example, Rb may already have been phosphorylated by activated G₁ Cdks, allowing release of E2F before p53 and Cip1 induction. As a result, E2F-dependent transcriptional activation of genes required for entry into and progression through S phase would continue unabated (Goodrich et al. 1991; Hinds et al. 1992; Nevins 1992; Dulic et al. 1994).

The sensitivity of the arrest mechanism to low amounts of DNA damage, and the resemblance of the arrested cells to those undergoing senescence, encour-

ages the speculation that the p53 pathway may also be part of the clock involved in natural senescence. It has been proposed that the absence of telomerase activity in normal somatic cells contributes to senescence as a result of decreasing telomere length at each cell division (Greider 1990; Allsopp et al. 1992). Eventually, chromosomes with shortened or absent telomeres may resemble dsbs or may fuse to form dicentric chromosomes that subsequently would break during mitosis to create dsbs. Failure to repair such dsbs would signal the p53-dependent arrest system to prevent further cell division.

Our studies show that gamma irradiation of NDF in early to mid-G₁ causes a long-term induction of the Cdk inhibitor Cip1 and a prolonged growth arrest. Data accumulated during the past year indicate that mutations in genes other than p53 can also inactivate the G₁ response to DNA damage or metabolite limitation. For example, we and others showed that several cell lines with wtp53 genes do not arrest in response to DNA damage or uridine biosynthesis inhibitors (Livingstone et al. 1992; Di Leonardo et al. 1994). These cell lines probably contain mutations in factors that act upstream or downstream of p53. Importantly, two other Cdk inhibitors have been cloned recently: Apparently, p27^{kip} is involved in the G₁ arrest induced by transforming growth factor- β (TGF- β) (Koff et al. 1993), whereas the signals activating the recently described p16 (Serrano et al. 1993; Kamb et al. 1994; Nobori et al. 1994) have not been elucidated. Clearly, it will be important to assess whether such factors participate in the p53-mediated arrest/death response pathway.

Materials and methods

Cell culture

WS1 (embryonic skin, ATCC CRL 1502), WI-38 (embryonic lung, ATCC CCL 75), and NHF-3 (foreskin, provided by Dr. M. Cordeiro-Stone, University of North Carolina, Chapel Hill) are NDF strains. HT1080 (fibrosarcoma-containing mtp53; ATCC CCL 121) and H1299 (non-small cell lung carcinoma lacking p53 expression because of a 5' intragenic deletion; provided by Dr. D. Carbone, University of Texas Southwestern Medical Center, Dallas) are human p53-deficient cell lines. All cell strains and lines were maintained in Earle's minimal essential media (MEM) supplemented with 1 \times nonessential amino acids (GIBCO) and 10% dialyzed fetal bovine serum (dFBS; Hyclone) at 37°C in a humidified atmosphere containing 7% CO₂. NDF strains were obtained at ~ 30 population doublings and experiments were carried out between population doubling 30 and 50. All cells were split 1:5 approximately every 3 days at 70% confluence for maintenance.

Treatments

Asynchronous cells were split 1:2 to 1:5 24 hr before treatment. For G₀ synchronization by serum starvation, cells were grown to $\sim 80\%$ confluence and then switched into MEM + 0.1% dFBS for 48 hr before treatment and release into complete medium. G₀-synchronized cells were split 1:3 to 1:8 before irradiation. Cells were irradiated at room temperature with a ⁶⁰Co gamma irradiator (Gammabeam 150-C) at a distance of 40 cm at a rate

of 3.8 Gy/min (1 Gy = 100 rads), except as noted otherwise. Two methods of irradiation were used with no significant differences. Cells were trypsinized just before treatment and irradiated in suspension in 15-ml tubes, followed by immediate replating. Alternatively, adherent cells were irradiated in their flasks. Test irradiations were also done using different media, dose rates, and cell concentrations with no significant differences. Mimosine (1000 \times stock solution; Sigma) was prepared in PBS at a concentration of 200 mM.

Flow cytometric cell cycle analysis

Cell cycle distribution was analyzed using flow cytometry as described previously (Yin et al. 1992). Briefly, cells were pulse-labeled in 10 μ M BrdU (Sigma) for 30 min (except as noted otherwise), harvested, and fixed in 70% ethanol until the day of analysis. Cells were treated with 0.1 M HCl containing 0.5% Triton X-100 to extract histones, followed by boiling and rapid cooling to denature the DNA. Cells were then incubated with anti-BrdU-FITC (Boehringer-Mannheim) and counterstained with propidium iodide-containing RNase. Samples were run on a Becton-Dickinson FACScan using color compensation when appropriate. Data analysis was done using SunDisplay3 (J. Trotter, Salk Institute Flow Cytometry Lab, La Jolla, CA). Cellular debris and fixation artifacts were gated out, and the G₁, S, G₂/M, and second G₁ fractions were quantified, depending on the labeling method.

Determination of morphology

Cells (2 \times 10⁵) were seeded on coverslips in 6-cm dishes. Twenty-four hours later, the cells were treated with 4 Gy gamma radiation or left untreated. At the indicated times, cells were fixed in 0.1% glutaraldehyde/2% formalin for 5 min, washed, and stained with 2.5% Giemsa for 10 min.

Metaphase aberrations

Asynchronous cell populations were treated with 1 or 4 Gy gamma radiation as indicated above; 2 \times 10⁶ cells were seeded in 75-cm² flasks. Cultures were then incubated in 0.1 μ g/ml of colcemid (GIBCO) for 24 hr and mitotic cells were harvested. Cells were swollen for 10 min in 75 mM KCl at 37°C, fixed with three changes of methanol/acetic acid (3:1), and dropped onto clean glass microscope slides. Slides were stained in 2.5% Giemsa for 10 min, and metaphase spreads were scored for chromatid breaks, chromatid exchange, chromosome exchange (dicentric), and double minutes (Savage 1976; Di Leonardo et al. 1993).

Western blot analysis

Cells were lysed in 50 mM Tris (pH 8), 120 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 2 mM EDTA, and 0.4% NP-40 with 100 μ g/ml PMSF and 10 μ g/ml each of chymostatin, leupeptin, and antipain. Protein concentrations were determined by a modified Lowry method (Bio-Rad). Fifty micrograms of the lysates were resolved by 10% SDS-PAGE, and electroblotted to nitrocellulose (Schleicher & Schuell) under standard conditions (Ausubel et al. 1994). Electroblotted gels were stained with Coomassie blue, and membranes were reversibly stained with Ponceau S to verify equal loading and transfer. Membranes were then probed with rabbit polyclonal anti-Cip1 (provided by Dr. J.W. Harper, Baylor College of Medicine, Houston, TX) or anti-p53 DO-1 (Ab-6; Santa Cruz Biotechnology) and anti- β -actin (Sigma). Detection was done by enhanced chemiluminescence according to manufacturer's instructions (Amersham), and

quantification was done by densitometry (LKB Enhanced Laser Densitometer).

Northern blot analysis

Total cellular RNA was isolated using a guanidine isothiocyanate/cesium chloride method (Ausubel et al. 1994). Twenty micrograms of each sample was resolved on a 1% MOPS-formaldehyde gel. Electrophoresis, transfer to nylon membranes, hybridization, and autoradiography were done by standard techniques (Ausubel et al. 1994). Membranes were hybridized with Cip1 (provided by Dr. S.J. Elledge, Baylor College of Medicine; Harper et al. 1993) and rRNA probe p11A-2 (Wahl et al. 1983) labeled with [³²P]dCTP by the random primer method (Ausubel et al. 1994). Bands were quantified using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

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