Control of Radiation-induced G1 Arrest by Cell-Substratum Interactions

Donna M. Gadbois,^2 E. Morton Bradbury, and Bruce E. Lehnert

Cell & Molecular Biology Group, Life Sciences Division, MS M888, Los Alamos National Laboratory, Los Alamos, New Mexico 87545 [D. M. G., E. M. B., B. E. L.], and Department of Medical Biology and Chemistry, University of California Davis, Davis, California 95616 [E. M. B.]

ABSTRACT

Ionizing radiation has been reported to cause an irreversible, senescence-like G1 arrest in human fibroblasts, which is accompanied by elevated p21CIP1 amounts. In further support of a senescence-like arrest, we show that expression of p53 and cyclin D1 is elevated in γ-irradiated, arrested fibroblasts. However, we also demonstrate that the arrest is reversible if the irradiated cells are trypsinized and replated, which may implicate cellular-extracellular matrix interactions in cell cycle control after irradiation.

INTRODUCTION

Radiation-induced cell cycle checkpoints are thought to provide cells with additional time to repair DNA damage before further traversing the cell cycle. Delay in G1 phase after exposure to ionizing irradiation is characteristic of cells that contain a wild-type p53 pathway (1, 2). The p53 protein is induced after γ-ray irradiation and is functionally a transcription factor for p21CIP1, which mediates the G1 arrest by inhibiting cdk2/cyclin E kinase activity (3). Until recently, it was thought that cells transiently pause in G1 phase after irradiation, but then cells with repaired DNA damage reenter the cell cycle. However, it has now been reported that fibroblasts arrest irreversibly in G1 phase after irradiation (4). This arrest was called senescence-like because it was irreversible, because of morphological similarities to senescent cells, and because cellular p21CIP1 amounts were elevated, which is an indicator of senescence (5). In agreement with the studies of Di Leonardo et al. (4), our flow cytometric studies showed that γ-irradiation ultimately causes a G1 arrest of HSFs until at least 44 h after irradiation (6). These data are perplexing because colony survival assays show that, at the doses used in our studies, many of the fibroblasts are capable of growth and should not arrest permanently in G1/G0 (6). Therefore, we have now undertaken studies to analyze the differences between flow cytometric and colony assays to determine the basis of the prolonged radiation-induced G1 arrest detected using flow cytometry. We found that the G1 arrest is, in fact, reversible if cell-substratum interactions are perturbed, which implicates the involvement of cell surface-extracellular matrix interactions in the control of the longevity of radiation-induced G1 arrest in HSFs.

MATERIALS AND METHODS

Cell Culture and Irradiations. Human diploid fibroblast strain 55 (HSF) was derived from neonatal foreskin and was cultured at 37°C under 5% CO2/air in α-MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 100 μg/ml streptomycin sulfate (Life Technologies, Inc.), and 100 units/ml penicillin (Life Technologies, Inc.). Mycoplasma-free cells from passages 6—12 were used in these studies.

For γ-ray exposures, cells were seeded into 75-cm^2 tissue culture flasks at an initial density of 5 × 10^5 cells/cm^2 30 h before irradiation. A Mark I model 68A high-dose-rate 137Cs source chamber irradiator (J. L. Shepherd and Associates) was used for the γ-ray exposures. The Mark I instrument delivers a beam of 0.66 MeV γ-rays. Cells were irradiated with 2 or 4 Gy at a dose rate of 99 cGy/min at room temperature. All cells, including unirradiated controls, were kept at room temperature for the length of the longest exposure time. Medium was replaced with culture medium containing 10 μM BrdUrd (Sigma, St. Louis, MO) at indicated times after irradiation.

Flow Cytometric Analyses of the Cell Cycle. Cells were harvested after radiation treatment using trypsin. The protocol used to stain the cells for the flow cytometric analyses of DNA content and incorporation of BrdUrd has been described elsewhere (7). Briefly, cells fixed in 70% ethanol were stained at room temperature for a minimum of 30 min in PBS containing 0.5 μg/ml HO (Calbiochem, San Diego, CA), 5 μg/ml MI (Pfizer Corp., Groton, CT), and 5 mM MgCl2 at a cell concentration of 7.5 × 10^5 cells/ml.

A two-laser flow cytometer (8) was used for the cell cycle analyses with one laser operating in the UV region (333.6—363.8 nm) and one tuned to 457.9 nm. The lasers were separated by 250 μm to provide sequential excitation and analysis of each fluorochrome (i.e., HO and MI, respectively). HO emissions were measured over a 400—495-nm range, and MI fluorescence was measured above 495 nm. The electronic gains were adjusted so that the BrdUrd-negative G1 peaks of the HO- and MI-DNA content histograms were initially in the same channel number, and the fluorescence signals were then compared electronically on a cell-by-cell basis (8). Such comparisons reflect the quenching of the HO fluorescence by BrdUrd, which is proportional to cellular BrdUrd content in cells that had synthesized DNA during the labeling period (7).

p53 and Cyclin D1 Analyses. For the p53 and cyclin D1 analyses, postirradiated and unirradiated cells were harvested by trypsinization and washed two times with PBS, and cell pellets were stored at −70°C until processed further. Frozen cell pellets were suspended in lysis buffer (50 mM Tris(hydroxymethyl)aminomethane (pH 7.9), 150 mM NaCl, 20 mM EDTA, 0.5% NP40, 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 μg/ml soybean trypsin inhibitor, 20 mM sodium fluoride, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 1 mM DTT) and incubated on ice for 30 min. Cells were lysed mechanically, and the supernatants were collected after centrifugation at 14,000 × g for 30 min at 4°C. Equal amounts of protein as determined by the Bio-Rad D@

RESULTS

γ-Irradiation Causes a Prolonged G1 Arrest in HSFs. We and others have used flow cytometric assays to show that γ-irradiation causes an apparently irreversible G1 arrest in HSFs (4, 6). However, we were compelled to question the interpretation of these data because colony survival assays showed that the 2- or 4-Gy doses used in our study resulted in 48 or 17% survival, respectively (6). One difference between flow cytometric cell cycle analyses and colony-forming assays is that proliferation in the flow cytometric assay is measured during the first 2 days after irradiation, whereas survival in a colony assay is scored after a much longer postirradiation period, e.g., 7—10 days. Therefore, we assayed for cell proliferation at longer times after
γ-irradiation to determine if the surviving cells in colonies result from cell growth that commences after 44 h postirradiation. HSFs were irradiated with 2 or 4 Gy of γ-rays, and then cellular proliferation was assayed by measuring BrdUrd incorporation during 20-h pulses up to 136 h postirradiation. Most of the cells did not contain BrdUrd and therefore remained arrested in G1 or G2 phase throughout the experiment (Fig. 1, boxes). The cells outside of the boxes in Fig. 1 were proliferating and therefore incorporated BrdUrd during the 20-h pe-

Fig. 1. Determination of proliferation of HSFs at long times after γ-irradiation. HSFs were irradiated with 2 or 4 Gy of γ-rays. Medium was replaced with fresh medium containing 10 μM BrdUrd 20 h before harvest times at 44, 64, 91, 112, or 136 h after irradiation. Fixed cells were stained with MI and HO, and 20,000 cells were analyzed for DNA content using flow cytometry (7). HO fluorescence (Y axis) decreases with increasing BrdUrd incorporation. MI fluorescence (X axis) is minimally affected by BrdUrd incorporation and therefore represents the cell cycle phase. The boxed regions contain nonproliferating G1 or G2 cells, which did not incorporate BrdUrd. Cells outside the boxed regions are cycling cells, which incorporated BrdUrd. The percentage of cycling cells is shown in each panel.
Subculture of Irradiated HSFs Results in Increased Cellular Proliferation. Another difference between flow cytometric and colony assays is that proliferation in the flow assay is determined by measuring BrdUrd incorporation into the DNA of cells that are left on the same culture dish after irradiation, whereas in a colony assay, cells are irradiated, then removed from the dish using trypsin, and then replated. Therefore, we determined the effect of replating on the growth potential of irradiated cells using the BrdUrd flow assay. Cells were irradiated, immediately harvested using trypsin, and then replated. To eliminate the complicating effect of cell density, the cells were replated at the original density, e.g., cells irradiated in one flask were harvested and replated in another flask of equal surface area. There was no evidence of a substantial decrease in the number of cells plated due to cell death after irradiation. This experiment demonstrated that there was a dramatic increase in proliferation after replating (Fig. 2). Cells that were arrested in G1 phase or G2 phase after irradiation did not incorporate BrdUrd and are shown in the boxed areas. If cells were not replated after being irradiated, only 15% of cells were cycling 64 h after 2 Gy of γ-irradiation, and only 3% of cells were cycling at 64 h after 4 Gy of γ-irradiation (Fig. 2, 44/64 h panel). However, if cells were trypsinized and replated immediately after irradiation, 61% of the cells were cycling 64 h after 2 Gy of γ-irradiation, and 30% of the cells were cycling 64 h after 4 Gy of γ-irradiation (Fig. 2, 44/64 h replate panel). Therefore, trypsinizing and replating the cells after irradiation rendered the cells capable of growth.

Subculture of Irradiated, G1-arrested HSFs Causes Reversal of Growth Arrest. We next determined if the cells that arrest in G1 phase after γ-irradiation have growth potential. HSFs were irradiated with 2 or 4 Gy of γ-rays and kept in culture for 44 h to arrest cells in G1 or G2 phase, and then the cells were trypsinized and replated. Subculturing restored growth capability to a major proportion of the cells (Fig. 3). Cells that remained arrested in G1 or G2 phase throughout the experiment are contained in the boxed regions of Fig. 3. By 20 h after replating, cells had entered S-phase in both the 2- and 4-Gy samples (Fig. 3, arrows in 20 h panels). The cells continued to cycle through the period of 44 and 68 h after replating and, in fact,
Expression of p53 and Cyclin D1 after Irradiation. The p53 protein is induced after γ-ray irradiation and acts as a transcription factor to increase the expression of p21\(^{\text{CIP1}}\), which causes G\(_1\) arrest by inhibiting cdk2/cyclin E kinase (3). Elevated p21\(^{\text{CIP1}}\) protein levels accompany the prolonged radiation-induced arrest in fibroblasts (4).

This supports the idea that the radiation G\(_1\) arrest in fibroblasts is senescence-like because increased amounts of p21\(^{\text{CIP1}}\) have been demonstrated in senescent cells and, in fact, p21\(^{\text{CIP1}}\) was isolated independently as Sdi1, an inhibitor of growth in senescent cells (5).

The amount of p53 has also been shown to increase with age in hematopoietic cells (9). Therefore, we determined whether increased p53 amounts correlate with the prolonged γ-ray-induced arrest in HSFs. We found that p53 was induced by 2 h after irradiation and remained elevated at least 35 h postirradiation, whereas p53 amounts in control cultures remained low through this time period (Fig. 4).

Senescent cells also contain unusually high amounts of cyclin D1 that form inactive complexes with cdk2 (10, 11). Hence, we measured the amount of cyclin D1 in γ-irradiated HSFs. Cyclin D1 was not induced at early times after irradiation, but increased by 26 h postirradiation and remained unusually high at 35 h postirradiation (Fig. 4).

A sustained increase in both p53 and cyclin D1 supports the idea of a senescence-like arrest in irradiated HSFs. However, as shown above, the arrest is not truly senescence because it can be reversed by trypsinization and cell replating.

Expression of p53, p21\(^{\text{CIP1}}\), and Cyclin D1 after Subculture of Radiation-arrested HSFs. As shown above, subculturing radiation-arrested HSFs causes the cells to reenter the cell cycle and undergo at least two rounds of DNA replication. Therefore, we next determined whether the elevated amounts of p53, cyclin D1 (Fig. 4), and p21\(^{\text{CIP1}}\) (4) that were observed during the prolonged radiation arrest decrease as the cells resume growth after replating. HSFs were irradiated with 2 or 4 Gy of γ-rays and kept in culture for 44 h to arrest cells in G\(_1\) or G\(_2\) phase, and then the cells were trypsinized and replated.

The change in expression pattern of p53, p21\(^{\text{CIP1}}\), and cyclin D1 was similar for either the 2- or 4-Gy dose (Fig. 5). The amount of p53 and p21\(^{\text{CIP1}}\) decreased as cells commenced proliferation but then began to

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**Fig. 3.** Reversal of γ-ray-induced G\(_1\) arrest in HSFs by trypsinization and replating. HSFs were irradiated and allowed to arrest in G\(_1\) or G\(_2\) phase for 44 h after irradiation. Cells were then harvested using trypsin, replated in medium containing 10 μM BrdUrd, and then harvested 20, 44, or 68 h later. BrdUrd analysis was done as in Fig. 1. The boxed regions contain nonproliferating G\(_1\) or G\(_2\) cells. Cells outside the boxed regions are cycling cells, which have incorporated BrdUrd. The arrows in the 20 h bivariate plots point to cells that have escaped the G\(_1\) arrest and entered S-phase by 20 h after replating. The small arrows in the 68 h bivariate plots point to BrdUrd-labeled G\(_1\) cells that have completed one round of DNA replication after replating. The large arrows in the 68 h bivariate plots point to BrdUrd-labeled G\(_1\) cells that have completed two rounds of DNA replication after replating.

**Fig. 4.** Western analyses of cellular p53 and cyclin D1 protein amounts after irradiation. Equal amounts of protein were loaded in each lane, and efficient transfer of protein to the nitrocellulose membrane was ensured by Ponceau S staining of the membrane. Monoclonal antibodies were used to detect p53 protein in whole-cell extracts of HSFs at 0, 1, 26, or 35 h in control cells or 2, 4, 26, or 35 h after irradiation with 4 Gy of γ-rays.
increase by 32 h after subculture, which raises the question of whether the irradiated cells are on a normal growth pathway after they begin to proliferate or whether they will undergo a premature growth arrest compared to an unirradiated population. A change in the amount of cyclin D1 was not an indicator of growth potential in the subcultured HSFs because the amount of cyclin D1 remained constant throughout the experiment. It is possible that cyclin D1 amounts remained constant but that cyclin D1 activity changed because it became complexed with a different cdk subunit. Additional experiments will be required to clarify these issues.

DISCUSSION

Cells respond to radiation by delaying at checkpoints in the G1 or G2 phase of the cell cycle (2, 9, 12). In the context of DNA repair, the checkpoint delays are widely believed to provide cells with more time to repair DNA damage before continued proliferation to preserve genome integrity in daughter cells (1, 13). Based on findings with immortalized or nonadherent/anchorage-independent cells, the checkpoints have been considered to represent only transient delays, followed by reentry into the cell cycle after DNA damage is repaired. However, these ideas were challenged when it was shown that irradiation causes fibroblasts to ultimately arrest in the G0/G1 phase (4). It was suggested that the delays do not allow for all DNA damage to be repaired and that even a very small amount of DNA damage (0.15 breaks/cell at a dose of 1 Gy and 0.59 breaks/cells at a dose of 4 Gy) would cause fibroblasts to arrest permanently in G1 phase (4). However, the idea that residual DNA damage remains in all cells and is the trigger for the G1 arrest in fibroblasts is questionable because colony survival assays in our studies show that many of the cells are capable of growth and should not arrest permanently in G0/G1 (6). A major distinction between the flow cytometric assay and the colony assay is the manner in which the fibroblasts are treated after irradiation. In a flow cytometric assay, cells are irradiated, left attached to the culture dish, and then assayed for growth at later times. In a colony assay, cells are irradiated and immediately treated with trypsin to remove the cells from the culture dish, the cells are replated, and the ability to form colonies is assessed. Indeed, when we repeated the flow cytometric assay in a manner similar to that of the colony assay, we found that the trypsin treatment restored growth potential to a major portion of the irradiated fibroblasts and thus showed that the previously reported apparently terminal state of arrest is, in fact, reversible. Furthermore, we found that trypsin treatment reverses the arrest up to at least 44 h after irradiation.

Therefore, although there are similarities between senescence and the prolonged G1 arrest caused by γ-irradiation (e.g., cellular morphology and patterns of p53, p21, and cyclin D1 expression), the radiation-induced arrest is not truly senescence because it is reversible by disruption of anchorage attachment. This suggests that cell-extracellular substrate attachments are involved in the radiation-induced arrest observed in fibroblasts. Perhaps the initial delays observed...
postirradiation of fibroblasts are due to DNA damage, whereas the prolonged G1 arrest is due to perturbations that can be reversed by disruption of cell surface adhesion receptors with trypsin. The idea that the prolonged arrest in fibroblasts is not caused by residual DNA damage is supported by the observation that arrested fibroblasts contained an average of only 0.15 breaks/cell after a dose of 1 Gy and only 0.59 breaks/cell after a dose of 4 Gy of γ-rays (4).

One candidate for a signal transduction system that could potentially be disrupted to reverse a radiation G1 arrest is the integrin pathway. Anchor-dependent cells contain membrane-bound integrin receptors, which have traditionally been thought to control cell migration and structure but are now known to play an important role in growth regulation, signal transduction, and differentiation (14—17). Integrin receptors may transmit growth-regulatory signals through the binding of intracellular signal-transducing kinases such as focal adhesion kinase and IRS-1 (18—20) and the activation of MAP kinase (21—24). Also, alternatively spliced β1c integrin caused a G1 arrest in 10T1/2 cells, which demonstrates a direct link to cell cycle control (25). Further evidence for regulation of cell cycle progression by integrin attachment to extracellular matrix was presented in studies of anchor-dependent growth of fibroblasts (26, 27). Growth of anchor-dependent fibroblasts in suspension caused the cells to arrest in late G1 phase, concomitant with inhibition of cdk2/cyclin E kinase activity by p21cip1. It was postulated that growth factors present in the medium stimulate fibroblasts maintained in suspension to progress to the late cdk2/cyclin E G1 restriction point but that a working anchorage-dependent integrin signal transduction pathway is required for the activation of cdk2/cyclin E kinase, subsequent Rb hyperphosphorylation, and progression into S-phase. As mentioned, γ-irradiation also causes cells to arrest in G1 phase by p21cip1 inhibition of cdk2/cyclin E kinase (3). Therefore, there are distinct parallels between anchorage-dependent and radiation-induced G1 arrest in that both are associated with the inhibitory cdk2/cyclin E/p21cip1 complex. The difference between the two types of arrest is that in irradiated fibroblast cultures, the arrest is irreversible until cell-substratum disruption and subsequent cell reattachment. It is tempting to speculate that radiation-induced alterations in integrin expression or integrin-substratum interactions mechanistically result in a persistent inhibition of cdk2/cyclin E kinase activity and, thus, inhibition of cell cycle progression even after the initial response to DNA damage. For example, fibroblasts normally express integrin β1; however, ectopic expression of an alternatively spliced integrin β1 (i.e., integrin β1c) causes a G1 phase arrest in 10T1/2 fibroblasts (25). If expression of an inhibitory integrin, such as integrin β1c, is involved in radiation-induced cell cycle arrest in fibroblasts, then trypsinization and replating the irradiated cells might restore expression of the proliferative form of the integrin. Alternatively, cell shape and perhaps cytoskeletal alterations that occur as a result of releasing adherent cells may influence the cell cycle arrest.

REFERENCES


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