Cyclin B Expression in HeLa Cells during the G2 Block Induced by Ionizing Radiation

Ruth J. Muschel, Hong Bing Zhang, George Iliaikis, and W. Gillies McKenna
Departments of Pathology and Laboratory Medicine [R. J. M.] and Radiation Oncology [H. B. Z., W. G. M.], University of Pennsylvania, Philadelphia, Pennsylvania 19104, and Department of Radiation Oncology [G. J.], Thomas Jefferson University, Philadelphia, Pennsylvania 19107

ABSTRACT
After exposure to ionizing radiation, eukaryotic cells undergo a division delay which is reflected by increased time spent in the G2 portion of the cell cycle. Recent information identifies increased levels of mitotic cyclins as key biochemical events initiating mitosis. In HeLa cells cyclin B mRNA and protein levels have been shown to increase in G2 and to decrease after division is completed. Cyclin B protein binds to cdc2, resulting in histone kinase activity which is necessary for the initiation of mitosis. Accordingly, we chose to investigate how cyclin B mRNA and protein levels were perturbed by irradiation in order to gain further understanding of the mechanisms by which irradiation leads to a division delay. Our experiments revealed at least two effects on cyclin B regulation which might contribute to the division delay: (a) when HeLa cells were irradiated in S phase, there was a delay in the accumulation of cyclin B mRNA; (b) when cells were irradiated in G2 phase, at a time when mRNA levels were increasing, a division delay was induced which coincided with a markedly lowered level of cyclin B protein despite high levels of the mRNA.

INTRODUCTION
There is evidence to suggest that the G2 delay induced by ionizing radiation contributes to the ability of the cells to survive irradiation. Some cell lines from patients with ataxia telangectasia are highly sensitive to ionizing radiation and do not undergo a G2 delay after radiation exposure (1, 2). Rad9 mutant cells of Saccharomyces cerevisiae are more sensitive to radiation than normal cells and also do not undergo a division delay after irradiation (3). Caffeine, a drug which renders cells sensitive to irradiation, reduces or abolishes the radiation-induced G2 delay (4, 5). Conversely, we have shown that rat cells transformed by the H-ras and v-myc oncogenes are highly resistant to radiation and have G2 delays which are considerably prolonged when compared to the radiation-sensitive, parental cells (6, 7). This information indicates that the G2 delay is a component of the response of cells to ionizing radiation and that modulation of this response may contribute to relative radiation sensitivity. Since the development of irradiation resistance by cancer cells may be one of the key reasons for treatment failure, an understanding of this phenomenon could have considerable clinical importance.

The entry of cells in G2 phase into M phase is induced by an activity called maturation- or mitosis-promoting factor which has been identified as a complex between cdc2 kinase and a series of proteins known as mitotic cyclins (8–22). Cyclin A, in HeLa cells, appears to be complexed to a related but different protein (23). This pattern is similar to that seen in Xenopus oocytes (17), but in HeLa cells the levels of both the protein and the mRNA for cyclins A and B oscillate in this fashion. This is unlike the situation in oocytes of either Xenopus or clams in which cyclin message levels are stable in early divisions, since they result from preformed maternal RNA, and the protein levels alone appear to oscillate. Since the initiation of mitosis appears to be triggered by the synthesis of cyclins regulated, at least in part, in mammalian cells by the level of mRNA in G2 phase, we chose to investigate how this process might be altered in cells undergoing a G2 delay induced by ionizing radiation.

The answer to this query appeared to depend upon the phase of the cell cycle in which the cells were irradiated. We found evidence for altered timing of cyclin B expression both by the delayed synthesis of cyclin B mRNA when cells were irradiated in S phase and by the absence of accumulation of cyclin B protein in cells irradiated in G2 phase when message levels were already increasing. Thus, at least two mechanisms result in lowered amounts of cyclin B in mammalian cells after radiation exposure.

MATERIALS AND METHODS
Synchronization of HeLa Cells. HeLa cells (1 × 10⁶) were plated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum on 100-mm tissue culture dishes and cultured at 37°C in a CO₂ incubator. The cells were synchronized using a modification of the procedure developed by Heintz et al. (24). After 2 days, 100 μM thymidine was added to the medium for 12 h. It was washed out and the cells were cultured for another 18 h. Then, 5 μg/ml aphidicolin was added for 12 h and washed out. Thirty min after release from the aphidicolin the experimental cells were exposed to 10 Gy X-rays using a Siemens therapeutic X-ray machine operated at 250 kV, 15 mA with a 2-mm Al filter (effective energy 70 kV). The control cells were mock irradiated for the same time. Immediately after irradiation and at various times thereafter, cells were harvested for RNA content analysis and for extraction of RNA. Distribution of cells through the cell cycle was measured in each experiment by flow cytometry using a Partek flow cytometer after fixation of the cells in 70% ethanol (25). The methods used were as described by Gohde et al. (25), and calculation of the percentage of cells in each phase of the cell cycle was performed using software supplied by Partek.

RNA Blotting. Total cellular RNA was extracted using the commercial adaptation (RNAzol; Cinna/biotex, Houston, TX) of the method of Chomczynski and Sacchi (26). RNA (10 μg) was denatured with formaldehyde and formamide and electrophoresed through a 1% agarose gel containing formaldehyde. The RNA was transferred to a Zeta
probe filter (S and S) by capillary blotting in 20× SSC. After the filter was baked for 2 h at 80°C, it was prehybridized in 5× SSC, 100 μg denatured herring sperm DNA, and 4× Denhardt’s solution (2% bovine serum albumin, 2% Ficoll, and 2% polyvinylpyrrolidone) and then hybridized in the same solution at 67°C overnight using a cyclin B probe made by random primer labeling of the 1.6-kilobase cyclin B cDNA (very generously supplied by Drs. Pines and Hunter) to approximately 5 × 10^6 cpm/μg DNA and then washed with 2× SSC followed by 0.5× SSC at 65°C. Filters were exposed at −70°C with an intensifying screen using XAR-5 film and developed at 3−5 days. Filters were reprobed with probes made from a human β-actin cDNA (kindly supplied by R. Taub) or by a rat ribosomal 18S clone (supplied by the laboratory of Dr. Schmickel). Extent of hybridization was quantified using an LKB densitometer equipped with GelScan software.

Immunoblotting. Immunoblots were performed on samples derived from 5 × 10⁴ cells as described by Pines and Hunter (15). Filters were exposed at −70°C to Kodak XAR film with an intensifying screen. An LKB laser scanning densitometer equipped with GelScan software was used to quantify the intensity of the cyclin bands on the autoradiograph.

RESULTS

To examine the effect of irradiation in S phase, HeLa cells were synchronized using a double block, thymidine, followed by aphidicolin. Thirty min after the aphidicolin was washed out, the cells, now in S phase, were irradiated with 10 Gy X-rays. In the unirradiated controls, cells began to enter (i.e., phase until after 24 h. It should be noted that the controls did not reach late S phase or G2/M until 9 h longer than the subsequent time the cells remained in G2 phase. In contrast, cells irradiated at the start of S phase did not enter G1 phase until after 24 h. It should be noted that the irradiated cells did not reach late S phase or G2/M until approximately 3 h after the controls. The delay in S phase is also an expected component of the radiation-induced division delay but is shorter than the subsequent time the cells remained in G2/M. Thus, the irradiated cells remained in G2 for at least 9 h longer than the controls. Nonetheless, in spite of more than 80% of the cells in the population accumulating in G2, cyclin B mRNA remained at low levels. To determine whether equivalent amounts of RNA were present in each lane, the blots were reprobed with a ribosome 18S probe and with a β-actin probe, since β-actin mRNA levels are known not to vary through the cell cycle and are known to be unaffected by X-rays (15, 28, 30). Signals from these probes indicated that the amounts of total RNA and β-actin mRNA were similar at each time point (data not shown). Since the yields of RNA were similar from equal numbers of irradiated and unirradiated cells, radiation does not result in a generalized depression in cellular RNA. Thus, the delay in G2 is associated with an absence of accumulation of cyclin B mRNA when HeLa cells are irradiated in early S phase.

These results led us to investigate the effect of radiation on cyclin B mRNA levels when cells are irradiated in G2 phase where cyclin B mRNA is known to accumulate. HeLa cells were again synchronized using a thymidine, aphidicolin block. Nine h after release from aphidicolin, 76% of cells were in late S or G2/M phases and 6 Gy X-rays was administered to the cells. This dose resulted in a G2 delay lasting 7.5 h (Fig. 2B). This was defined as the difference between the times in the control and irradiated cultures for 50% of the cells originally in G2 phase to exit from M phase into G1. In the controls the cells had entered G1 phase within 2.5 h from time 0 (Fig. 2A), whereas in the irradiated cultures 9 h was required to reach this point (Fig. 2B). RNA harvested from these cells was used in RNA blotting. In the controls, the cyclin B mRNA level was maximal at 3 h and declined thereafter, correlating with the percentage of cells in G2/M phase as predicted from the results of Pines and Hunter (15). In the irradiated cells the levels also began to increase and peaked at 2 h. The level did not quite reach that seen in the unirradiated controls; nevertheless, it was more than an order of magnitude higher than that seen in cells in early S phase. In the irradiated cells the level of mRNA decreased slowly during the 6-h period that the cells remained in G2 block and then declined more rapidly as the cells exited from the block.

In parallel experiments to those measuring mRNA, protein levels were also measured. One of these experiments is shown in Fig. 3. In this experiment HeLa cells were synchronized and irradiated in G2/M phase with 6 Gy X-rays in a protocol identical with that in Fig. 2, and samples were analyzed with...
Fig. 2. Cyclin B mRNA levels after irradiation in G1 phase. This experiment was carried out as in Fig. 1 except that 9 h were allowed to elapse after release from the aphidicolin block before irradiation. A: ○, level of cyclin B mRNA (in arbitrary units as in Fig. 1); ●, percentage of cells in G1 as in Fig. 1. B: ○, level of cyclin B mRNA; ●, percentage of cells in G2 plotted against the time after radiation with 6 Gy. C and D, photographs of the corresponding autoradiograms from which the data plotted in A and B were derived.

immunoblotting using an anti-cyclin B antibody. In the unirradiated controls the levels of cyclin B protein began to increase rapidly after a 1-h lag. This increase exactly corresponded to the percentage of cells in G2/M as determined by flow cytometry. As the cells entered G1, the levels of cyclin B protein decreased rapidly. In the irradiated cells, however, it is apparent from Fig. 3B that the levels of cyclin B protein remained at the low levels seen at the time for 4–5 h, even though at least 60% of the cells were in G2/M throughout this time and while ample amounts of cyclin B mRNA were present (cf. Fig. 2B to Fig. 3B). The levels of cyclin B protein in the irradiated cells did not begin to increase until after 5–6 h, corresponding to the beginning of the exit of the cells from the radiation-induced G2 block.

DISCUSSION

These experiments indicate that exposure of HeLa cells to ionizing radiation results in aberrant expression of cyclin B. This appears to result from a two-tiered system in that after radiation in S phase, the accumulation of cyclin B mRNA is delayed, thereby making cyclin B protein synthesis impossible, while in G2 phase after the mRNA is present, there is a marked diminution in the level of detectable cyclin B protein. In both cases the ultimate effect is a decreased amount of cyclin B at a point in the cell cycle when mitosis would normally have been triggered.

Lock and Ross (27) examined cdc2 H1 kinase activity in G2-enriched CHO cells after exposure to etoposide and in an asynchronous culture after exposure to γ-irradiation. Etoposide induces an irreversible G2 block in CHO cells. In both the drug and the radiation-treated cells, they found a reduction in the cdc2 kinase activity. Although they speculated that inhibitors of the enzyme complex might account for this result, our results suggest that, at least in the case of the irradiated cells, the absence of cyclin B, whose presence in the cdc2 complex is required for H1 kinase activity, may also contribute to this effect. Our experiments lead to the hypothesis that ionizing radiation induces a division delay, at least in part, through insufficient levels of cyclin B.

Although DNA-damaging agents result in the induction of
increased mRNA levels of several genes, including c-fos, metallothioneins, heat shock transcripts, type I collagenase, β-polym-erase, and a series of 20 different cDNAs called “ODI” (DNA damage inducible) sequences, it does not appear that most of these are specifically affected by X-irradiation (28-32). Overall, levels of RNA do not alter and Fornace et al. (31) have shown that the levels of particular mRNAs, β-polym-erase, α-actin, and HSP70 mRNA are not altered in CHO cells after 40 Gy X-rays, a dose considerably higher than those used in this study.

Levels of many proteins have been shown to be unaffected by irradiation. There have been some attempts to determine specific proteins whose expression is altered by radiation. Overall, protein synthesis does not appear to be altered by ionizing radiation at the doses under consideration here. Holland et al. (33) labeled cells with [35S]methionine and then compared one-dimensional gel electrophoresis from cells radiated in G2 to control G0 cells. While the protein profile was essentially identical between the two, there was one band at Mr 170,000 whose synthesis was diminished by radiation. The effect of radiation on cells in plateau phase has also been examined. Boothman et al. (34) examined protein synthesis after radiation in plateau phase human cells. Although the great majority of proteins as assessed by two-dimensional gel electrophoresis was unchanged in amounts, eight spots increased in intensity after irradiation and two decreased. Thus, it is likely that the expression of a number of genes in addition to cyclin B is affected by X-rays, but the identification of cyclin B as a gene product dramatically decreased by ionizing radiation is particularly interesting in light of the known effects of radiation on the cell cycle and because of the information which implicates cyclin B in the control of cell cycle progression.

There is evidence that protein synthesis is required during the exit from G2 delay (35-37). If cells are irradiated and treated with puromycin D, a protein synthesis inhibitor, they do not exit from the G2 delay, but if treated with actinomycin D, an RNA synthesis inhibitor, they will enter division. Thus, after irradiation in G2 phase, protein synthesis, but not RNA synthesis, is required for cells to complete mitosis (37). These data are consistent with our observation that after irradiation in G2 mRNA for cyclin B is present but that cyclin B protein levels do not increase until the G2 block ends. Thus, a protein synthesis inhibitor but not an RNA synthesis inhibitor would be predicted to prevent the exit from the G2 delay based on these data.

These experiments do not prove that the G2 delay seen after radiation is solely mediated through altered metabolism of cyclin B, but the data are entirely consistent with alterations in cyclin B expression having a major role in the induction of G2 delay. This information implicates cyclin B as a likely candidate for a role in the induction of G2 delay and identifies cyclin B as a molecule whose expression is markedly altered after exposure to ionizing radiation. Further work will be required to allow an understanding of the mechanisms through which irradiation results in alterations in expression of cyclin B.

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