Down-Regulation of Cell Cycle Control Genes by Ionizing Radiation

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Abstract

The cellular responses to ionizing radiation include growth arrest, DNA repair, and lethality. However, little is known about the signaling events responsible for these responses. The present studies have examined the effects of ionizing radiation on the expression of genes involved in cell cycle control. The results demonstrate that the treatment of asynchronous cells with 20 Gy ionizing radiation is associated with transient down-regulation of the cdc2, cyclin A, cyclin B, and cdc25 genes. This effect was associated with transient induction of the c-jun gene. RNA stability studies demonstrate that the down-regulation of gene expression following ionizing radiation exposure is at least in part due to a decrease in transcript half-life. Other studies were performed with elutriated cells enriched for populations in G1 and S phases. Treatment of G1 enriched cell populations with 10 Gy resulted in a selective decrease in cyclin B mRNA levels, whereas this effect on cyclin B expression was less pronounced at 5 Gy and undetectable at 1 Gy. Similar results were obtained with S phase enriched cells. Taken together with clonogenic survival studies, these findings indicate that down-regulation of cell cycle control gene expression is associated with lethality, whereas lower doses of ionizing radiation have little, if any, effect on the expression of these genes. The findings also suggest that DNA damage may activate signaling events which regulate expression of cell cycle control genes.

Introduction

The responses of eukaryotic cells to ionizing radiation include growth arrest, DNA repair, and lethality (1). However, the molecular mechanisms responsible for these effects are unknown. Recent studies have demonstrated that the cellular response to ionizing radiation involves the induction of immediate early response gene expression (2–4). Immediate early response genes that encode transcription factors include the jun (c-jun, jun-B, and jun-D; Refs. 5–9), fos (c-fos, fos-B, fra-1; Refs. 10–12), and EGR (EGR-1, EGR-2; Refs. 13–15) families. Treatment of diverse cell types with x-rays is associated with increases in c-jun, jun-B, and c-fos mRNA levels (2, 3). These effects are mediated at least in part by activation at the transcriptional level (2). Similar findings have been obtained for the EGR-1 gene (3). Moreover, recent results have demonstrated that ionizing radiation activates DNA binding activity of the nuclear factor kβ (NF-κβ) and increases expression of this gene (4). The products of these different classes of early response genes function as transducers of nuclear signals and therefore may couple early biochemical signals induced by X-rays to longer-term changes in gene expression.

Several checkpoints in cell cycle progression control growth in response to diverse positive and negative signals (16). Ionizing radiation, for example, slows growth by inducing delays in both S and G2 phases of the cell cycle. The available evidence indicates that G2 arrest is necessary for repair of DNA damage before entry into mitosis. Indeed, treatment of cells with caffeine or the protein kinase inhibitor 2-aminopurine overcomes G2 delay following DNA damage and increases chromosomal fragmentation (17, 18). Genetic studies in yeast have identified certain genes that are important in the control of G2 and entry into mitosis. For example, mutations in the rad9 gene of Saccharomyces cerevisiae have demonstrated that the RAD9 protein controls G2 arrest induced by DNA damage (19, 20). Mutants at the rad9 locus are unable to delay entry into mitosis following exposure to DNA damaging agents. The replication of damaged chromosomes is associated with mutations and lethality. In contrast to RAD9, which is not required for cell cycle progression, the onset of mitosis is controlled by a complex of the serine/threonine protein kinase p34cdc2 and cyclin B (21–23). p34cdc2 or related kinases appear to be essential for G2-S and G2-M transitions in both yeast and mammalian cells (21–25). Transformed cells may bypass the requirement for cdc2 activity in G1-S transition (26), although it is not known whether cells can enter mitosis without this kinase. Studies in fission yeast have also demonstrated that the cdc25 gene product, p80cdc25, regulates mitosis by dephosphorylation of p34cdc2 and thereby activation of the cdc2/cyclin B complex (27–29).

Although these findings in eukaryotic cells have provided certain insights into the regulation of G2 delay, little is known about the control of genes coding for these cell cycle regulatory proteins, particularly in response to DNA damage. The present studies have examined the effects of ionizing radiation on cdc2, cyclin A, cyclin B, and cdc25 gene expression. The results demonstrate that X-ray exposure is associated with down-regulation of these genes and that this effect occurs at least in part by posttranscriptional mechanisms.
Fig. 1. Effects of ionizing radiation on expression of cell cycle control genes. U-937 cells were treated with 20 Gy ionizing radiation (XRT) and maintained in culture for the indicated times. Total cellular RNA (20 μg) was hybridized to the 32P-labeled cdc2, cyclin A, cyclin B, cdc25, c-jun, and actin (not shown) probes. Densitometric scanning of the signals and normalization to that for actin demonstrated the following decreases in expression at 6 h: cdc2, 91%; cyclin A, 92%; cyclin B, 76%; and cdc25, 80%. Similar results were obtained in two separate experiments.

Results

U-937 myeloid leukemia cells were initially used to study the effects of ionizing radiation on levels of cdc2, cyclin A, cyclin B, and cdc25 expression. Transcripts for each of these genes were detectable in asynchronous U-937 cell populations (Fig. 1). Exposure to 20 Gy ionizing radiation was associated with a partial down-regulation of cdc2, cyclin A, and cyclin B mRNA levels which was apparent after 1–3 h (Fig. 1). This effect was transient, and, by 12–24 h, the expression of these genes had returned to near baseline levels. A similar down-regulation of cdc25 mRNA to nearly undetectable levels was also found at 3–4 h. Moreover, expression of this gene returned to that in control cells by 12–24 h (Fig. 1). These findings were in contrast to the transient induction of c-jun transcripts and indicated that this down-regulation was not a nonspecific response to ionizing radiation. The effects on gene expression were not limited to U-937 cells in that similar results were obtained with the HL-60 cell line (Fig. 2). In these studies, maximal down-regulation of the cdc2, cyclin A, and cyclin B genes was found at 4–6 h. However, in contrast to U-937 cells, we were unable to detect cdc25 transcripts in HL-60 cells. Nevertheless, the effects of ionizing radiation on expression of the cdc2 and cyclin genes in HL-60 cells were transient and occurred in the absence of changes in actin mRNA levels (Fig. 2).

Although these findings indicated that ionizing radiation down-regulates the expression of cell cycle control genes, further studies were performed to determine whether these effects are controlled at the transcriptional or posttranscriptional levels. Using nuclear run-on assays (2), we were unable to detect sufficient signals to determine relative transcriptional rates of the cdc2, cyclin A, cyclin B, or cdc25 genes in untreated cells (data not shown). Consequently, the sensitivity of that assay was below that needed to determine the effects of ionizing radiation on the transcription of these genes. However, mRNA stability studies were feasible and demonstrated effects at the posttranscriptional level. For example, the half-life of cdc2 transcripts in actinomycin D-treated cells was >180 min (Fig. 3A). In contrast, treatment with 20 Gy ionizing radiation was associated with a decrease in this half-life to 108 ± 6 min (mean ± average deviation of two determinations) (Fig. 3A). Similar findings were obtained for cyclin A, cyclin B, and cdc25 transcripts. For example, the half-life of cyclin A transcripts was decreased from >180 min in control cells to 107 ± 11 min following irradiation (Fig. 3B). Cyclin B transcripts had half-lives of >180 and 99 ± 11 min in control and irradiated cells, respectively (Fig. 4A). Moreover, the half-life of cdc25 mRNA was >180 min in control cells, and
this half-life was decreased to 88 ± 14 min after irradiation (Fig. 4B). Taken together, these results indicated that ionizing radiation down-regulates cdc2, cyclin A, cyclin B, and cdc25 mRNA levels at least in part by posttranscriptional mechanisms.

In order to determine whether the down-regulation of gene expression was associated with alterations in cell cycle phase distribution, we performed similar studies with enriched populations of G1 and S phase cells. There was a progressive increase in cdc2, cyclin A, cyclin B, and cdc25 expression as control G1 cells progressed through the cell cycle (Fig. 5). Similar findings were obtained for cdc2, cyclin A, and cdc25 transcripts in G1 cells exposed to 10 Gy ionizing radiation (Fig. 5). In contrast, there was a decrease in cyclin B gene expression (Fig. 5). Cell cycle distribution studies demonstrated accumulation of cells in S phase through 12 h and then an increase in G2-M cells (Fig. 6). Comparable results were
obtained with S phase enriched cells. Exposure of these cells to ionizing radiation was associated with down-regulation of cyclin B expression (Fig. 7). Moreover, whereas there was a transient accumulation in S phase, these cells underwent a marked G2 arrest at 12-20 h (Fig. 8). These findings indicated that the G2 arrest in response to ionizing radiation is preceded by selective decreases in cyclin B expression.

Additional studies were performed to determine whether down-regulation of cyclin B expression corresponds to G2 arrest at lower doses of ionizing radiation. There was no detectable effect on cdc2 or cdc25 mRNA levels when G1 enriched cells were exposed to doses of 5 and 1 Gy (Fig. 9). In contrast, exposure of these cells to 5 Gy, but not 1 Gy, was associated with a partial decrease in cyclin A expression at 24 h and in cyclin B expression at 6, 12, and 24 h compared to that obtained for control cells (Fig. 9). Despite the partial decline in cyclin expression at 5 Gy and the absence of an effect at 1 Gy, exposure to both of these doses resulted in a significant accumulation of cells in G2-M (Fig. 10). Although treatment of these cells with 5 Gy ionizing radiation was associated with 4-5 logs of cell killing in clonogenic survival studies, a dose of 1 Gy resulted in only a 25-30% decrease in colony formation.

Discussion

The response of mammalian cells to genotoxic agents is of fundamental importance to issues such as (a) resistance to ionizing radiation and anticancer drugs, (b) mutagenesis, and (c) lethality. Certain insights are available regarding different aspects of this response. In particular, cells undergo growth arrest following DNA damage and delay G2 phase for repair of lesions before entering mitosis. The signaling events responsible for these effects remain unclear, although it is not apparent that multiple proteins are involved in control of the eukaryotic cell cycle. Indeed, activation of the p34 cells/cyclin B complex by the product of the cdc25 gene is required for entry into mitosis (27-29). Consequently, decreased expression of any one of these genes in response to DNA damage could account for G2 delay. Whether the 34 cells/cyclin B complex may function in other aspects of cell cycle control, recent work has indicated that cyclin A complexes, perhaps involving p34 cells or related proteins, may be involved in S phase regulation (30). Thus, down-regulation of cyclin A expression might be associated with S phase growth arrest. Other studies have confirmed that cyclin A has a distinct role in cell cycle progression and, in particular, entry into mitosis (31).

Recent studies have demonstrated that cdc2 kinase activity is decreased when asynchronously dividing CHO cells are exposed to 8 Gy ionizing radiation (32). This effect was transient, as kinase activity recovered with accumulation of cells in G2 (32). Although these and other findings with etoposide suggested that loss of cdc2 kinase activity represents one of the early responses of cells to DNA damage, little is known about the events responsible for this effect. Other recent work has demonstrated that cyclin B expression is down-regulated by treatment of HeLa cells with 10 Gy X-rays (33). The present work was performed to determine the effects of ionizing radiation on the regulation of cdc2, cyclin A, cyclin B, and cdc25 gene expression. The results demonstrate that treatment of asynchronous cultures with 20 Gy is associated with a rapid and transient down-regulation in their mRNA levels. This down-regulation appeared to be specific for these genes in that the c-jun gene was transiently induced during the same period.

Fig. 5. Down-regulation of gene expression by ionizing radiation in G1, enriched cell populations. Cells in logarithmic phase were subjected to elutriation. The G1 fraction was isolated, seeded at 2 x 10^5 cells/ml in complete medium, and left untreated or exposed to 10 Gy ionizing radiation (XRT). Total cellular RNA (20 μg) was isolated at the indicated times and hybridized to the cdc2, cyclin A, cyclin B, and cdc25 probes. Hybridization to the actin probe demonstrated equal loading of the lanes.

Fig. 6. Cell cycle distribution of G1, enriched cells treated with ionizing radiation. The G1 fraction was isolated as described in the legend to Fig. 5 and left untreated (O) or treated with 10 Gy ionizing radiation (O). Flow cytometry was performed at the indicated times. The results (mean ± standard deviation of two experiments) are expressed as the percentage total cells in G1, S, and G2-M phases.
This dose of radiation was also associated with a transient arrest of cell cycle progression and at least 6 logs of cell kill in clonogenic survival experiments (data not shown). Thus, these effects on gene expression are detectable in lethally irradiated cells. At least part of the decrease in cdc2, cyclin A, cyclin B, and cdc25 transcripts was related to posttranscriptional mechanisms as demonstrated by mRNA stability studies. Transcriptional effects may also play a role, although we were unable to explore this possibility in nuclear run-on assays. Nonetheless, these findings appear to represent the first demonstration that DNA damaging agents down-regulate multiple cell cycle control genes and that this effect is mediated by posttranscriptional mechanisms.

We also found that down-regulation of the cdc2, cyclin A, cyclin B, and cdc25 genes in asynchronous cultures is related to dose of ionizing radiation (data not shown). Consequently, other studies were performed at lower doses using elutriated cells to relate changes in gene expression with cell cycle arrest. Whereas doses of 10 Gy were predominantly associated with down-regulation of cyclin B gene expression in enriched populations of G1 and S phase cells, this effect was less pronounced at 5 Gy. These doses were associated with marked accumulations of cells in G2. Moreover, whereas a dose of 1 Gy also resulted in G2 arrest, there was no detectable effect on levels of cdc2, cyclin A, cyclin B, or cdc25 gene expression. These findings suggest that at doses of radiation (1 Gy) that induce relatively little lethality, other mechanisms must be responsible for G2 delay, whereas higher and more lethal doses (5–20 Gy) inhibit expression of these genes and may prevent cells from completing the transition from G2 to mitosis. Regulation at the mRNA level, especially when involving several genes that contribute to G2 control, would ensure the inability of cells to survive. In contrast, in the event of DNA damage which is potentially repairable, expression of the cdc2, cyclin B, and cdc25 genes would be needed for entry into mitosis. As such, other mechanisms, conceivably at the posttranslational level and perhaps involving a RAD9-like homologue, might be responsible for controlling p34cdc2/cyclin B kinase activity and thereby G2 delay. Indeed, recent studies in yeast have shown that the wee1 protein kinase is required for radiation induced delay in G2-M (34). In this regard, we have hybridized a human wee1 probe (35) to the RNAs from synchronized cells used in the present studies and have found no detectable change in expression of this gene during the cell cycle or following exposure to ionizing radiation (data not shown).

Little is known about the induction of proteins by ionizing radiation which may protect against mutagenesis and cell killing. Two-dimensional gel electrophoresis has been used to demonstrate X-ray induced increases in eight polypeptides with apparent molecular weights of 126,000 to 275,000 (36). Although these proteins appear to represent a specific response to ionizing radiation, their function remains unclear. Ionizing radiation has also been shown to induce the DNA binding of a protein translocated to the nucleus, although the role of this potential transcription factor is also unclear (37). Other studies have demonstrated that the synthesis of proliferating cell nuclear antigen and coregulated peptides is inhibited by ionizing radiation (38). However, the mechanisms responsible for this effect remain unknown. There is also no evidence that the gadd45 gene, which is
strongly induced by X-rays in mammalian cells, is part of a negative regulatory pathway involved in the repair of mutagenic or lethal damage (39). Thus, although ionizing radiation induces changes in the expression of a variety of genes and their products, none of these events has been attributed to an activity analogous to that of RAD9 in yeast. Moreover, whereas the RAD9 protein has homology to a segment of the DNA binding domain of the transcription factor encoded by c-jun (20), the absence of detectable c-jun transcripts at doses of 1 Gy ionizing radiation (data not shown) suggests that, under these conditions, c-jun is not responsible for G2 arrest.

Materials and Methods

Cell Culture. U-937 cells were grown in RPMI 1640 medium containing 10% FBS1 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mm L-glutamine. HL-60 cells were grown as described (2). Cells were irradiated at room temperature using a Gammacell 1000 (Atomic Energy of Canada, Ltd., Ontario, Canada) with a 197Cs source emitting at a fixed dose rate of 14.3 Gy/min as determined by dosimetry. Control cells were exposed to the same conditions but not irradiated. Clonogenic survival studies were performed by plating up to 5 × 10⁴ cells in RPMI 1640 medium containing 0.33% agarose and 10% FBS. The number of colonies (greater than 40 cells) was determined after 7 days.

Centrifugal Elutriation and Cell Cycle Analysis. Cells in exponential growth phase and after treatment with ionizing radiation were subjected to centrifugal elutriation using the JE-5.0 elutriation system (Beckman, Inc., Palo Alto, CA). Approximately 2 × 10⁶ cells were applied to the standard chamber (1600 rpm at 27°C) using a digital flow controller (Cole-Palmer Instruments, Inc., Chicago, IL). The calibrated pump speed was increased from 10 to 30 ml/min. Enriched G1 and S phase cell populations were elutriated in 100 ml RPMI 1640 medium containing 1% FBS. Aliquots (1 ml) were fixed by adding 2 ml ice-cold methanol for 60 min on ice. After centrifugation, the supernatant was removed, and the cell pellet was incubated with 500 μl RNase (200 units/ml) and 500 μl propidium iodide buffer in the dark at room temperature for 30 min (40). The stained cells were analyzed for DNA content on a FACSscan (Becton Dickinson, San Jose, CA) using CellFIT cell cycle analysis software.

Preparation of RNA and Northern Blot Hybridization. Total cellular RNA was isolated by the guanidine thiocyanate-cesium chloride technique (41). Total cellular RNA (20 μg) was subjected to electrophoresis in 1% agarose-2.2 M formaldehyde gels, transferred to nitrocellulose paper, and hybridized to one of the following 3²P-labeled DNA probes: (a) the 471-base pair Kpnl/BglII cdc2 insert of pCDC2 plasmid (42); (b) the 2.2-kb EcoRI cyclin A insert in the pGEM4Z plasmid (43); (c) the 1.6-kb BamHI cyclin B insert in the pGEM4Z plasmid (43); (d) the 2.0-kb EcoRI cdc25 insert of pBSKl plasmid (28); (e) the 1.8-kb BamHI/EcoRI c-jun complementary DNA (44); and (f)

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1 The abbreviations used are: FBS, fetal bovine serum; kb, kilobase(s).
the pA1 plasmid containing a 2.0-kb PstI insert of the chicken β-actin gene (45).

References


