DNA-dependent Protein Kinase Does Not Play a Role in Adaptive Survival Responses to Ionizing Radiation

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We previously demonstrated that exposure of certain human tumor cells to very low chronic doses of ionizing radiation led to their enhanced survival following exposure to subsequent high doses of radiation. Survival enhancement due to these adaptive survival responses (ASRs) ranged from 1.5-fold to 2.2-fold in many human tumor cells. Furthermore, we showed that ASRs result from altered G2 checkpoint regulation, possibly mediated by overexpression of cyclin D1, proliferating cell nuclear antigen (PCNA), and the X-ray induction of cyclin A. Because cyclin D1 and PCNA proteins are components of many DNA synthetic and repair processes in the cell, we tested the hypothesis that preexposure of cells to low doses of ionizing radiation enabled activation of the DNA repair machinery needed for survival recovery after high-dose radiation. We examined the role of DNA break repair in ASRs using murine cell deficient (i.e., severe combined immunodeficiency [SCID]) or proficient (i.e., parental mouse strain [CB-17]) cells in DNA-dependent protein kinase catalytic subunit (DNA-PKcs) expression and DNA double-strand break repair. DNA-PKcs is a nuclear serine/threonine protein kinase that is activated by DNA breaks and plays a key role in double-strand break repair. DNA-PKcs also phosphorylates several nuclear DNA-binding regulatory transcription factor proteins (e.g., Sp1 and p53), which suggests that DNA-PKcs may play a role in regulating transcription, replication, and recombination as well as DNA repair, after radiation. Therefore, we exposed confluent SCID or CB-17 cells to low priming doses of ionizing radiation (i.e., 5 Gy) and compared the survival responses of primed cells to those of unprimed cells after an equitoxic high-dose challenge. Low-dose-primed SCID or CB-17 cells demonstrated 2-fold enhanced survival after a high-dose challenge compared to that of unprimed control cells. These data suggest that expression of the catalytic subunit of DNA-PKcs (expressed in CB-17 not SCID cells) and the presence of active double-strand break repair processes (active in CB-17, deficient in SCID cells) do not play a major role in ASRs in mammalian cells. Furthermore, we present data that suggest that DNA-PKcs plays a role in the regulation of the G2/M cell cycle checkpoint following extremely high doses of ionizing radiation. — Environ Health Perspect 106(Suppl 1):301–305 (1998).

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Introduction

Adaptive survival responses (ASRs) are observed when cells become resistant to cytotoxic agents after previous exposure to low doses of the same (1–4) or different agents. ASRs have been observed in many cell types, including neoplastic (4) and normal (5–7) human cells, but the mechanisms underlying the emergence of such damage-induced resistance phenotypes remains obscure.

We recently found that ASRs were regulated by altered G2 cell-cycle checkpoint events occurring at an A point in which the involvement of cyclin A was suggested (4). We discovered that elevated proliferating cell nuclear antigen (PCNA) and cyclin D1 levels correlated in human cells with the ability of the cell to demonstrate ASRs (4). The fact that cells primed with low doses of ionizing radiation appeared to enter the cell cycle earlier after release by low-cell density than untreated confluent control cells confirmed that altered G2 cell-cycle checkpoint regulation controlled ASRs (4). Although we demonstrated that such ASRs were the result of new transcripts and translation, we also demonstrated that the DNA binding of many common transcription factors (including Sp1, retinoblastoma control proteins [RCPs], p53, nuclear factor-κB, egr-1, AP-1, Oct-1, CREB, GRE, AP-2, and AP-3) remained unchanged in untreated, primed (i.e., low-dose irradiated), or high-dose challenged human cells that demonstrated highly efficient ASRs (8).

After characterizing ASRs in various human cancer cells after exposure to ionizing radiation (4,8,9), we decided to use model cell systems to explore the mechanisms behind ASRs. Since DNA double-strand break repair is probably the single most important determinant in radiation sensitivity, we explored the role of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) in ASRs by comparing severe combined immunodeficiency (SCID) cells, which do not express DNA-PKcs protein, and parental mouse strain (CB-17) cells, which express normal levels of the DNA double-strand break repair component [C-R Yang et al., unpublished data; (10)]. Our data indicate that DNA-PKcs plays no role in ASRs since low-dose ionizing radiation-induced resistance was apparent in both cell lines. Further cell cycle analyses using flow cytometry did reveal, however, that DNA-PKcs plays a role in G2/M cell-cycle checkpoint responses after very high-dose ionizing radiation exposures. Major differences between SCID and CB-17 cells in the overall number of cells in G2/M and differences in the length of time that damaged cells delayed in G2/M following ionizing radiation were observed. These data suggest a dual role for DNA-PKcs in repair and cell-cycle regulation.

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Abbreviations used: ASRs, adaptive survival responses; CB-17, parental mouse strain; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; pRB, retinoblastoma protein; RCPs, retinoblastoma control proteins; SCID, severe combined immunodeficiency.
Materials and Methods

Cell Culture Conditions

Fibroblast cell lines derived from an SCID mouse and the corresponding CB-17, from which SCID mice were derived, were provided by M. Brown (Stanford University, Stanford, CA) (10). Both cell lines were grown in Dulbecco's Modified Eagle's medium, supplemented with 10% fetal bovine serum, penicillin 6-k (100 U/ml), and streptomycin sulfate (50 U/ml) in a 10% CO₂–90% air atmosphere at 37°C. Analyses of these cell lines indicated that both express mutant p53 protein (i.e., high levels of p53 protein were found in the nucleus of each cell and DNA binding as measured by DNA band shift analyses was not observed), both express the retinoblastoma protein (pRb), and each cell line exhibits high levels of unphosphorylated Ku70–Ku80 heterodimeric DNA end-binding activity. SCID cells did not express DNA-PKcs enzyme activity or protein levels (or at least expressed levels that were not detected by either assay) (C-R Yang et al., in preparation).

Cell Irradiation and Clonogenic Survival Assessments

SCID and CB-17 cells were plated onto 100-mm² tissue culture dishes and fed every third day until confluent, with both sets of cells obtaining an 85 to 90% arrest in G₀/G₁, as determined by flow cytometry (4,11,12). The cell lines did not vary significantly in their doubling times or plating efficiencies, and each cell line demonstrated approximately 42% efficiency in plating. Once confluent, low-dose (priming) or high-dose (challenging) ionizing radiation exposures were applied using a Phillips X-ray generator as described (13,14). For low-dose priming exposures, cells were treated with 5 cGy at 24-hr intervals if multiple priming doses were used. In a previous report (4) we demonstrated that a single priming dose of ionizing radiation was equivalent to repeated multiple priming doses. Cells (primed or unprimed) were also exposed to a high-dose challenge to yield equitoxic survival (i.e., approximately 10% survival in nonadapted cells). As expected, we found that SCID cells were much more sensitive to ionizing radiation than CB-17 cells. A dose of 250 cGy to SCID cells was equivalent in terms of clonogenic survival to a 500-cGy dose to CB-17 cells. Four hours after exposure cells were trypsinized, serially diluted in 5-fold increments, and plated on 60-mm² tissue culture dishes for clonogenic survival assessment. After 14 days of growth, cells were rinsed twice with phosphate-buffered saline (PBS) and stained with a 0.2% crystal violet solution in methanol. Colony-forming ability was then scored by counting normal-appearing colonies (i.e., those containing 100 or more normal-appearing cells) as previously described by Boothman et al. (14), with error values indicating the standard deviation for each group. Experiments were conducted three times and duplicate cultures from each manipulation were obtained for each experiment.

Flow Cytometry

Cells were treated with or without various doses of ionizing radiation as described above. For flow cytometric analyses, cells were washed twice with ice-cold PBS (150 mM NaCl, 50 mM KH₂PO₄, pH 7.4), trypsinized, and washed with a Tris/saline solution (10 mM Tris [pH 7.0] and 50 mM NaCl). Cells were then fixed in 90% ethanol-Tris/saline and stored at 4°C for up to 7 days. Samples were prepared for cell-cycle analyses by washing once with phosphate-citric acid buffer (0.2 M Na₂HPO₄ and 0.1 M citric acid [pH 7.8]) and staining with a solution containing 0.2% NP-40, RNase A (7,000 U/ml), and 33 µg/ml propidium iodide for 10 min. In our studies the phosphate citric acid wash has been a critical step for accurate assessment of apoptotic cell populations (15). Stained nuclei were analyzed for DNA-propidium fluorescence using a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA) at a laser setting of 36 mW and an excitation wavelength of 488 nm. Resulting DNA distributions were analyzed for the proportion of cells in apoptosis and for cells in G₀/G₁, S, and G₂/M phases of the cell cycle. Both SCID and CB-17 cells were 100% diploid at the beginning and end of each experiment, and in each case over 7000 events or cells were modeled using the program Modfit (Verity Software House, Topsham, ME). To visualize alterations in cell-cycle distributions, supralethal doses of ionizing radiation (i.e., 10–15 Gy) were used. Experiments were conducted three times and duplicate cultures from each manipulation were obtained in each experiment.

Results

Survival Assessments

When treated with low priming doses of ionizing radiation (5 cGy), both SCID and CB-17 cells demonstrated greater survival (125 ± 9% and 137 ± 15%, respectively) than those in unirradiated cultures (Table 1). This increase in colony-forming ability was highly reproducible in these two cell lines and has been observed with all cell lines that subsequently demonstrate ASRs to ionizing radiation (4,8,9). The plating efficiencies (~42%) and growth rates of each uniradiated cell line were very similar.

Both SCID and CB-17 cells also demonstrated efficient ASRs following to either one or two low-dose priming exposures of ionizing radiation. As expected, unprimed SCID or CB-17 cells treated with approximately equitoxic high challenging doses of ionizing radiation (i.e., 250 cGy for
SCID, 500 cGy for CB-17) demonstrated similar surviving fractions (approximately 9–12% survival) (Table 1). Exposure of CB-17 cells to a high-dose challenge of irradiation resulted in 12 ± 5% survival. CB-17 cells demonstrated a nearly 2-fold increase in survival (i.e., 22% ± 3%) after exposure to 5 cGy priming and a subsequent high-dose challenge (Table 1). Interestingly, CB-17 cells exposed to two priming doses followed by a high-dose challenge also demonstrated a 2-fold increase in survival (20% ± 7%) compared to cells receiving only a high-dose challenge. It is not clear why CB-17 cells demonstrated low survival following two priming doses of ionizing radiation alone (58 ± 2%), yet still demonstrated efficient ASRs (Table 1).

SCID cells treated with a high dose of equitoxic, ionizing irradiation (250 cGy) showed similar survival levels (i.e., 9 ± 1%) to those demonstrated for CB-17 cells (Table 1). SCID cells exposed to one priming dose (5 cGy) followed by a high-dose challenge of ionizing radiation showed 21 ± 3% survival. Cells receiving two priming doses followed by a high-dose challenge showed 18% ± 6% survival, indicating that as with CB-17 cells one priming dose of ionizing radiation was equivalent to multiple doses of radiation in terms of establishing ASRs. Unlike CB-17 cells, SCID cells demonstrated only a slight decrease in survival (i.e., 90 ± 11%) after two priming doses (Table 1).

To investigate the possibility that ASRs were the result of altered cell-cycle distribution or cell viability between cell types [other than minor fluctuations in the G0/G1 portion of the cell cycle as previously described (4)], we analyzed cell-cycle population differences in primed or unprimed confluent SCID or CB-17 cells 4 to 30 hr after a high-dose challenge of ionizing radiation (250 cGy for SCID, 500 cGy for CB-17). Analyses of multiple experiments in which confluence-arrested SCID or CB-17 cells were treated with various doses of ionizing radiation revealed no measurable differences between unirradiated and irradiated cells (regardless of dose) in the cell cycle (i.e., cells in G0/G1, S, G2/M, and the overall percentage of apoptotic cells) when cells were maintained in confluence arrest (data not shown). Both unirradiated and irradiated populations (24 hr posttreatment) were 90% in G0/G1 under the conditions of our experiments. We are currently examining longer time points after radiation to ascertain if more dramatic cell-cycle changes occur; however, results similar to those described here were previously published (4,9).

**Role of DNA-PKcs in Cell-cycle Regulation after Ionizing Radiation**

In an attempt to determine the effect of DNA-PKcs expression (and therefore the presence or absence of double-strand break repair) on cell-cycle regulation after ionizing radiation exposure, log-phase SCID and CB-17 cells were treated with various doses of X-rays; samples were taken at 4, 16, and 28 hr. Changes in cell-cycle distribution were then monitored by flow cytometry, as described in "Materials and Methods." The data in Figure 1A to E (for CB-17) and Figure 2A to E (for SCID) indicate that

**Figure 1.** Cell cycle regulation in CB-17 mouse cells, which express normal levels of the DNA-dependent DNA-PKcs after various doses of ionizing radiation. Log-phase CB-17 mouse cells, which express normal levels of DNA-PKcs, were plated at low densities. Twenty-four hours later, cells were irradiated with various doses of ionizing radiation and allowed to grow. Changes in cell-cycle distribution were monitored using flow cytometry as described in "Materials and Methods." Doses of ionizing radiation to which CB-17 cells were exposed were as follows: (A) 0.0 Gy; (B) 2.5 Gy; (C) 5.0 Gy; (D) 10 Gy; and (E) 15 Gy (1 Gy is equivalent to 100 cGy, the units used in the text). At certain data points, error bars were smaller than the symbol used.
SCID cells (which lack DNA-PKcs expression) arrest only transiently in G2/M after ionizing radiation, particularly at higher doses (>1000 cGy) of ionizing radiation. In contrast CB-17 cells (which express normal levels of DNA-PKcs) demonstrated efficient (>60%) and a more prolonged (>30 hr) G2/M cell-cycle checkpoint, particularly at the higher doses (>1000 cGy) of ionizing radiation. No apparent G0/G1 arrest was observed in CB-17 cells (Figure 1D, 1E) at the higher doses of irradiation as observed in SCID cells (Figure 2D, 2E), probably because of the rather potent G2/M cell cycle arrest and because both cell lines express mutant p53 levels. These data are consistent with the lack of G1 cell-cycle checkpoint function of mutant p53 in various human and rodent cells (16-18). The S phase population of both cell types was reduced at these higher doses of radiation (Figure 1D, 1E and Figure 2D, 2E) consistent with cell-cycle progression and subsequent arrest at G2/M. These data indicate not only that the expression of DNA-PKcs is involved in double-strand break repair, but that DNA break repair may play a role in the G2/M cell-cycle checkpoint. Although there were only slight changes in apoptosis within and between cell lines, additional experiments currently are being performed to examine later time points that may indicate a role for DNA-PKcs in the regulation of apoptosis.

**Discussion**

Our data demonstrate that the DNA double-strand break repair protein, DNA-PKcs, does not play a major role in adaptive survival responses in mammalian cells. SCID cells, which lack expression of DNA-PKcs [(10,19,20); Yang et al., in preparation], demonstrated efficient and pronounced (2-fold) ASR-mediated increased resistance to ionizing radiation due to prior 5 cGy priming. Our data demonstrate that a single low dose of ionizing radiation can induce ASRs in mouse cells and that multiple priming doses are not necessarily required. These data are consistent with our previous results (4,9).

Although DNA-PKcs does not play a role in ASRs, it may play a role in the G2/M cell-cycle checkpoint similar to the recently described role of the human mutL homologue protein in this cell-cycle checkpoint (21). Data in Figures 1 and 2 demonstrate that genetically matched, irradiated cells (i.e., CB-17 cells that express DNA-PKcs and SCID cells that lack this protein) differ significantly in the extent and length of time spent in the G2/M checkpoint following very high doses of ionizing radiation (i.e., 10 Gy). Only a small proportion of SCID cells demonstrated a very transient G2/M cell-cycle arrest, whereas a much larger proportion of CB-17 cells appeared to delay much longer at this cell-cycle checkpoint. Assuming that SCID and CB-17 cells received the same initial DNA damage (10,19), (i.e., cells were treated with equitoxic doses of X-rays), the difference in cell-cycle regulation at this checkpoint could be a major determinant in overall cell sensitivity to ionizing radiation. The persistent presence of DNA double-strand breaks and the lack of cell-cycle blockage at the G2/M cell-cycle checkpoint before chromosome segregation would be
disastrous to cell viability in SCID cells. It should be noted, however, that this phenomenon was only observed at very high doses of ionizing radiation. Later time points may be needed to observe difference among these cells at lower doses.

According to conventional theory, the presence of double-strand breaks is thought to lead to G2/M cell-cycle checkpoint arrest. In the case of SCID cells, however, the data indicate the possible existence of DNA double-strand break tolerance that would be sufficient to explain the dramatic decrease in cell survival of these cells compared to that for their genetically matched partner, CB-17 cells. Our data indicate the possibility of a prominent role for DNA-PKcs in the G2/M cell-cycle checkpoint, which may exist to allow repair before mitotic segregation. Further experimentation is under way to explore links between DNA-PKcs, its absence, and/or apoptosis. We speculate that SCID cells may be similar to preapoptotic cells, in which DNA-PKcs is cleaved by radiation- or DNA damage-induced apoptotic proteases (22). The lack of expression of DNA-PKcs in SCID cells may, therefore, predispose the cell for commitment to undergo an apoptotic death. Therefore, DNA-PKcs may not only be involved in double-strand break repair and cell-cycle regulation; it actually may be a pivotal player as an apoptotic protector against programmed cell-death responses that occur after ionizing radiation.

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