

Induction of Stress Genes by Low Doses of Gamma Rays

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Using cells of a human myeloid tumor cell line (ML-1), we have detected induction of several stress-responsive genes by doses of γ rays below 50 cGy. We found a linear dose-response relationship for induction of *CDKN1A* (formerly known as *CIP1/WAF1*) and *GADD45* mRNA levels over the range of 2-50 cGy, with no evidence of a threshold for induction. Although exposures to 2 and 5 cGy did not result in any detectable reduction in cloning efficiency or increased apoptosis in ML-1 cells, these exposures did produce a transient delay of cells in the phases of the cell cycle in addition to the observed up-regulation of *CDKN1A* and *GADD45*. The relative induction of genes such as *CDKN1A* by radiation doses that produce little toxicity indicates that surviving cells do contribute significantly to the observed stress responses. These studies should provide insight into the molecular responses to physiologically relevant doses that cannot necessarily be extrapolated from high-dose studies. © 1999 by Radiation Research Society

INTRODUCTION

Studies of changes in gene expression after exposure to ionizing radiation have led to insights into diverse aspects of cell biology. For instance, the tumor suppressor TP53 (formerly known as p53) has been found to contribute significantly to stress responses to ionizing radiation (reviewed in ref. 1). The induction of such genes as *GADD45* (2, 3), *CDKN1A* (formerly known as *CIP1/WAF1*) (4), *MDM2* (5), *BAX* (6-8), *MCL1* (9) and *BCLX* (10) have been found to be dependent on wild-type TP53 function. These genes in turn play important roles in such processes as DNA repair, progression of cells through the phases of the cell cycle, and cell death by apoptosis. In addition to up-regulating TP53 and its effector genes, exposure to ionizing radiation also results in a down-regulation of *MYC* (11, 12). While high levels of *MYC* stimulate the activity of TP53, the TP53-effector gene *MDM2* promotes its down-regulation, forming an autoregulatory loop for control of the TP53 response. Most of these studies of gene induction and regulation, however, have been carried out using very high doses of radiation, the response to which does not necessarily predict responses to more biologically relevant doses.

Transcriptional responses to doses of ionizing radiation with relatively little effect on cell survival have not been as well investigated, although small variations in the levels of expression of several genes have been detected at lower doses. A dose of 50 cGy reportedly reduced expression of the gene encoding β - and γ -actin (13) and induced *Rb1* and H4 histone (14) in Syrian hamster embryo cells, while a decrease in *Myc* and increase in *Jun* was detected in these cells after a dose as low as 6 cGy (14). In cells of a transformed human lymphoblast cell line, activation of NF- κ B has been reported after exposure to as little as 10 cGy (15), along with induction of *FOS*, *JUN*, *MYC* and *HRAS* in the range of 25 cGy-2 Gy (16). The induction by a dose of 25 cGy of *PBP74*, a member of the HSP70 gene family, has also been reported in cells of two human cancer cell lines (17). None of these studies, however, have demonstrated a dose-response relationship for gene induction by low radiation doses, and no systematic quantitative studies have been reported. These observations in cell lines of diverse species and tissue of origin and varying TP53 status have also been difficult to relate to the more in-depth studies using higher doses.

We have used cells of the human myeloid cancer cell line ML-1 for the current study, because of its intact TP53 pathway (18) and its strong stress-gene induction profile as shown in previous high-dose experiments (8, 19, 20). For instance, using cDNA microarray hybridization technology, we have recently identified 30 induced or suppressed sequences in ML-1 cells not previously known to be responsive to radiation (12). When we screened for induction of these genes in a panel of 12 human cancer cell lines, ML-1 was the only cell line to exhibit radiation induction of all the genes tested. In the same study, we also demonstrated for the first time the ionizing radiation responsiveness and TP53 regulation of *ATF3*, one of the genes for which the low-dose radiation response is characterized here. Using a quantitative approach, we have now defined a dose-response relationship in the range of 2-50 cGy for the induction of *CDKN1A*, *GADD45*, *MDM2*, *ATF3* and *BAX* by γ rays. Induction of these genes in the low-dose range showed a slightly different dependence on time after irradiation from that observed at higher doses and was accompanied by a transient disturbance in progression of cells through the phases of the cell cycle, but no measurable

effects on cell survival or the induction of apoptosis at the lowest doses assayed.

MATERIALS AND METHODS

Cell Culture and Treatment

The human myeloid leukemia cell line ML-1 has wild-type TP53 function (18). The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 45 min) fetal calf serum and 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified, 5% CO₂ atmosphere in a 37°C incubator. Cells were irradiated at approximately 5.1 cGy/min with total doses of 2–50 cGy using a Mark I-68 ¹³⁷Cs source (J. L. Shepherd and Associates, Inc.) with lead attenuators in place. The dosimetry of the source was confirmed by exposing TLD monitors (Landauer Inc.) in the same configuration used for irradiations of cells with the range of doses used. Even at the lowest doses, the calculated absorbed dose (Landauer special dosimetry services) varied by less than 3% from the expected dose (data not shown). Due to the nature of sparsely ionizing radiation such as γ rays, it is highly unlikely that any cells in the exposed population will remain unirradiated at even the lowest doses used in this study. Clonogenic survival was determined by plating cells immediately after irradiation in 96-well microtiter plates at between 2 and 5000 cells/well. Plates were incubated and scored for colony formation after 21 days. Plating efficiencies and surviving fractions were calculated using the method of Furth *et al.* (21).

Determination of Apoptosis

Cells were incubated for 1, 2 or 3 days after irradiation, then fixed in methanol and stained with DAPI solution as described previously (22). An Olympus fluorescence microscope was used to score nuclei exhibiting characteristic morphological features of apoptosis, and results were expressed as the number of apoptotic nuclei divided by the total number of nuclei counted.

Flow Cytometry

Cells were fixed in 70% ethanol 0, 8, 10, 12 and 24 h after irradiation, treated with RNase at 37°C, and then stained with propidium iodide. Samples were analyzed using a Becton Dickinson FACScan and cell cycle distributions were fitted using the Cell Quest data analysis program.

Measurement of Gene Induction

Irradiated cells were incubated at 37°C for 1, 2, 3 or 4 h, and RNA was extracted using a modified guanidine thiocyanate method (23). Serial dilutions of RNA were immobilized on nylon membranes, hybridized with cDNA probes at 55°C in a buffer containing 50% formamide (Hybrisol I, Oncor), and washed under standard conditions as described previously (24). The amount of hybridization was quantified on a PhosphorImager (Molecular Dynamics), and relative signal levels, normalized to the polyA content of each sample, were determined using the RNA-Think program (24). This approach ensures that the values for relative RNA levels are directly proportional to RNA abundance, and differences of 1.5-fold or more can be measured reliably (24, 25). Results obtained with this sensitive approach also agreed well with those obtained by determinations of RNase protection (26).

RESULTS

Cells of ML-1, a human myeloid leukemia cell line, showed a survival response after exposure to ¹³⁷Cs γ rays similar to that of other human cell lines of myeloid or lymphoid lineage (Fig. 1), which are typically more radiosensitive than cell lines derived from other types of tissue. The survival curve had no apparent shoulder and predicted a *D*₀

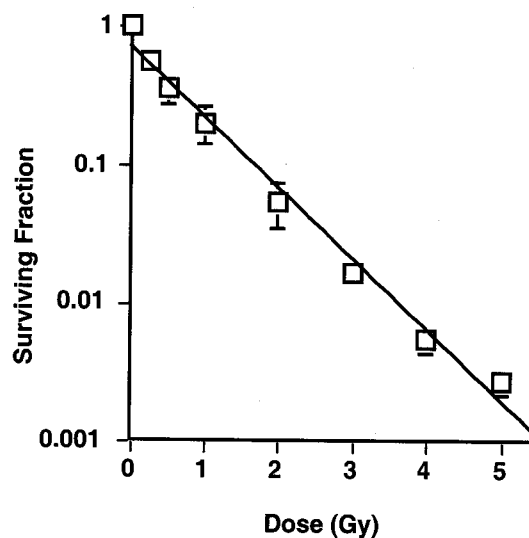


FIG. 1. Clonal survival of ML-1 cells exposed to ¹³⁷Cs γ rays. The *D*₀ is approximately 73 cGy. Points are the average of five independent experiments, and error bars are standard errors. Linear regression of the data was used to fit the curve shown.

of approximately 73 cGy. The changes in plating efficiency induced by doses of 2, 5 and 10 cGy were so low as not to be measurably different from fluctuations in the colony formation assay (*P* > 0.1). In four independent experiments, the mean plating efficiency of untreated controls was 0.129 ± 0.069 , while the mean plating efficiency for all doses combined (2, 5 and 10 cGy) was 0.127 ± 0.058 .

High doses of ionizing radiation efficiently induce rapid apoptosis in ML-1 cells (8). At lower, relatively nontoxic doses, however, very little apoptosis was measurable. Neither end-labeling of DNA breaks (27) nor filter elution (28), an assay for early events in the pathway to apoptosis, detected increased apoptosis in cells irradiated with 25 cGy or less (data not shown). A slight increase in apoptosis was detected, however, by scoring morphology of DAPI-stained cells. Two days after irradiation, a trend of increasing apoptosis with increasing dose emerged between 2 and 25 cGy, although due to experimental fluctuations, this increase was significant only at the *P* < 0.01 level (paired *t* test) after 25 cGy (Fig. 2). In contrast, cultures treated with 2.5 Gy showed over 40% morphologically apoptotic cells 2 days after irradiation. No further increases in the fraction of cells undergoing apoptosis were observed at times later than 2 days after treatment for any of the doses tested.

In contrast to their effect on apoptosis, doses below 25 cGy significantly and reproducibly perturbed cell cycle progression (Fig. 3) in a dose-dependent manner. Even the lowest dose tested, 2 cGy, caused a transient reduction of the S-phase fraction. This effect was significant only at 12 h after irradiation with 2 cGy, while higher doses resulted in larger and more rapid decreases in the S-phase fraction of the population (Fig. 3). By 24 h postirradiation, the populations treated with doses of 25 cGy or below had all recovered their normal cell cycle distributions, while cells

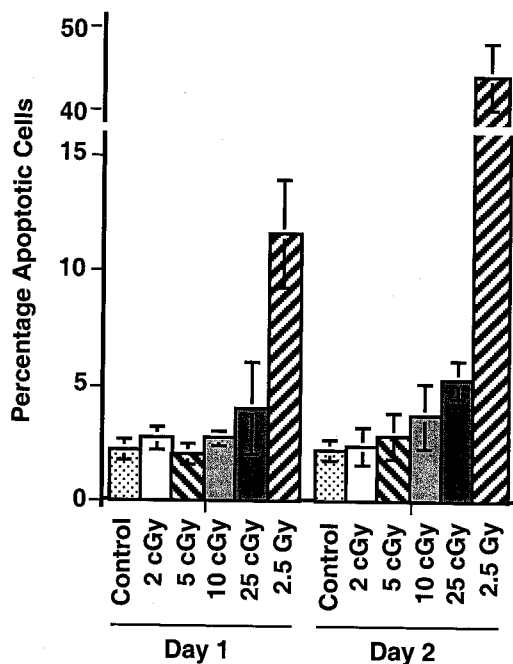


FIG. 2. Apoptotic cells scored by morphology after DAPI staining either 1 or 2 days after radiation treatment. Results are the average of three or four independent experiments in which at least 250 cells were scored for each point. Error bars are standard errors.

treated with the higher dose of 2.5 Gy still showed a profoundly arrested profile with very few ($6.6 \pm 1.9\%$) S-phase cells.

In previous studies employing high radiation doses,

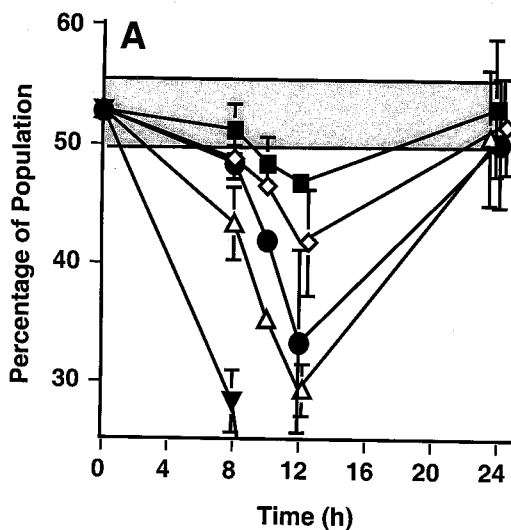
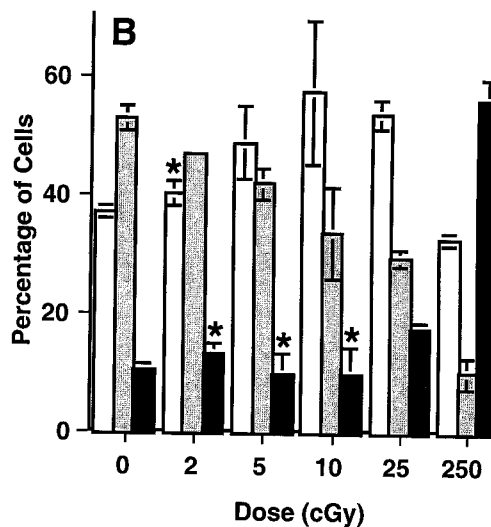


FIG. 3. Panel A: Fraction of S-phase cells in the population at various times after irradiation of exponentially growing ML-1 cells with ^{137}Cs γ rays: 2 (\blacksquare), 5 (\diamond), 10 (\bullet) or 25 cGy (\triangle) or 2.5 Gy (\blacktriangledown). Points are the mean of three independent experiments, and error bars are standard deviations. The points for 12 and 24 h have been offset slightly to make the error bars easier to distinguish. The shaded area represents the range of S-phase fractions in untreated control populations and is the mean \pm the standard deviation of the control measurements at all times. Panel B: Cell cycle distribution at the time of maximum perturbation (12 h) for the low-dose treatments. For each dose, the percentage of cells in G_1 phase is represented by the open bar, cells in S phase by the gray bar, and cells in G_2 phase by the black bar. The values shown are the mean of three independent experiments, and error bars are standard deviations. Where error bars are not visible, the standard deviation was ≤ 0.76 . An asterisk indicates a point not significantly different from the control at or below the $P < 0.005$ level.

CDKN1A, *GADD45*, *MDM2*, *ATF3* and *BAX* were all found to be induced in ML-1 cells with kinetics typical of that of early response genes (12). After treatment with 20 Gy γ rays, these genes reached maximal induction 4 h after treatment, then declined rapidly, reaching basal levels by 24 h. The induction of *CDKN1A* and *GADD45* (Fig. 4A) by low doses of γ rays peaked more rapidly than that by supralethal doses, reaching maximum levels 2 to 3 h postirradiation, and in most cases already beginning to decline after 4 h.

The maximal induction of *CDKN1A* and *GADD45* was approximately linear between 2 and 50 cGy and showed no indication of a threshold for gene induction (Fig. 4B). Significant induction over basal control levels was found for both genes at all doses. A more gradual dose-dependent response was found for the other genes tested. *MDM2*, *BAX* and *ATF3* were also induced between 2 and 3 h after low doses of radiation, but 50 cGy resulted only in a two- to threefold increase in the expression of these genes over control levels (Fig. 4B). The lower inducibility of these genes by low doses of radiation was consistent with their induction by high doses, which was considerably less than that of *CDKN1A* and *GADD45* (12). The maximal induction appeared linear between 5 and 50 cGy for *MDM2* and between 10 and 50 cGy for the other genes. Below these doses, induction fell off sharply, dropping below the limit of accurate quantification by this technique, previously shown to be approximately 1.5-fold (24, 25).

A broader dose range was next tested for the induction



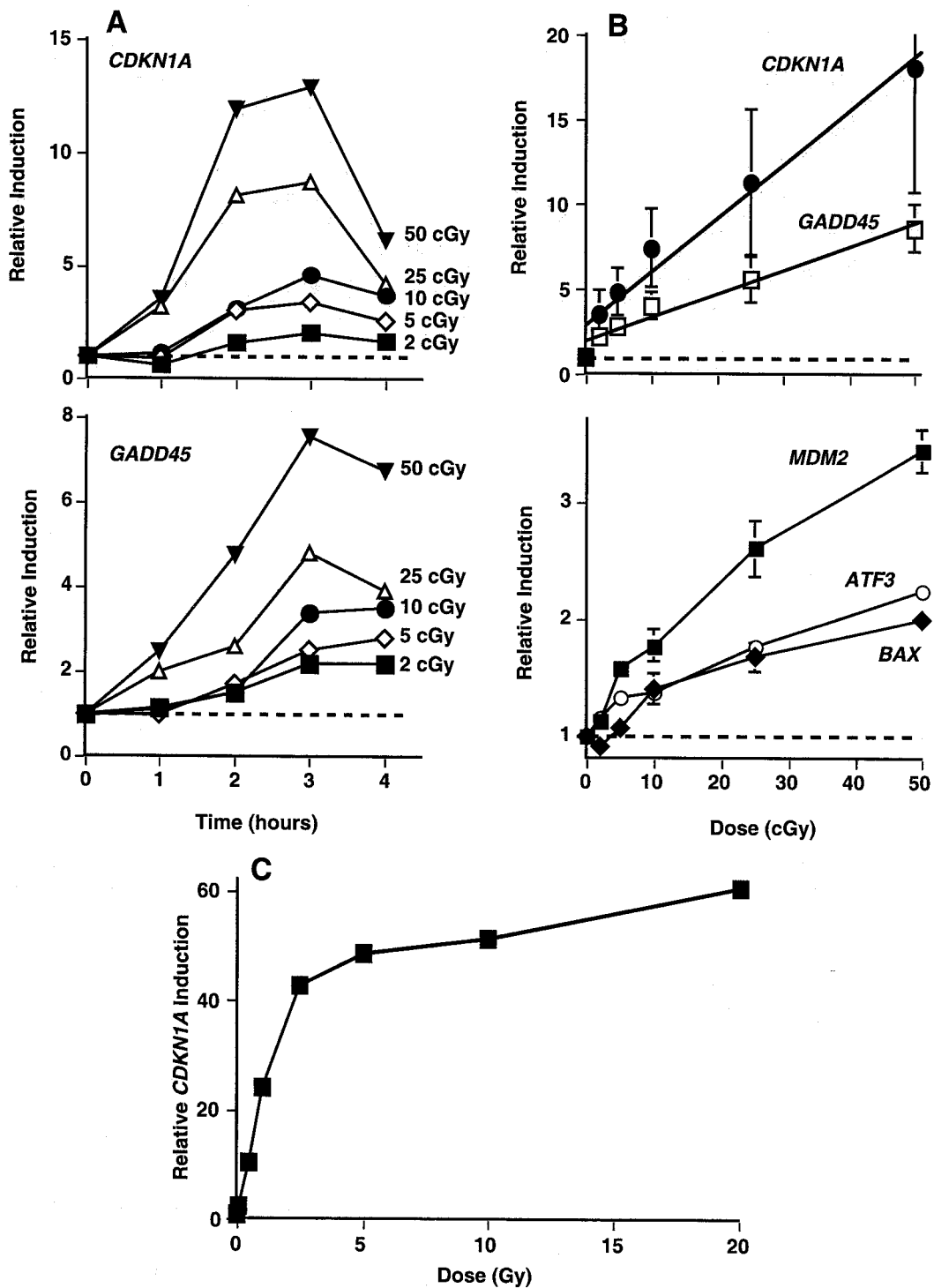


FIG. 4. Panel A: Time course of induction of *CDKN1A* and *GADD45* transcripts by 2 (■), 5 (◇), 10 (●), 25 (△) or 50 cGy (▼) of γ rays. Data from a representative experiment are shown. The dashed line indicates the basal level in untreated controls. Panel B: Maximal induction of *CDKN1A* (●), *GADD45* (□), *MDM2* (■), *ATF3* (○) and *BAX* (◆) by low doses of γ rays. Points are the average of four independent experiments, and error bars are standard errors. The dashed line indicates the basal level in untreated controls; solid lines were fitted by linear regression through the data. Panel C: Dose response of maximal induction of *CDKN1A* across a broader range of doses. Data are shown from a representative experiment. The dashed line indicates the basal level in untreated controls.

of *CDKN1A* to determine the point at which the induction response begins to saturate. Induction continued to increase in a dose-dependent manner up to approximately 2.5 Gy (Fig. 4C). Beyond this point, the dose-dependent increase in relative induction leveled off sharply, although a trend of increased induction appeared to continue up to 20 Gy, the highest dose tested.

DISCUSSION

Recent developments in high-throughput screening technologies (29, 30) have ignited unprecedented interest in the field of differential mRNA expression. While previous studies of stress-gene induction by supralethal exposures to DNA-damaging agents have been useful in elucidating damage response pathways and cellular biochemical defects, transcriptional responses to lower exposures remain largely unexplored. Furthermore, the less toxic an exposure is, the greater the potential for development of late effects, such as carcinogenesis, from damage sustained in surviving cells. It is clearly important to understand the effects of low-dose exposures in the range of those sustained from medical applications or industrial accidents. Many of our assumptions about low-dose effects, however, have been based on extrapolations from effects measured at high doses.

The extrapolation of data gathered at high doses to predict effects at low doses can be fraught with difficulties. In particular, it cannot necessarily be assumed that the dose-response relationship observed at high doses applies to the entire dose spectrum. For example, an unexpected effect on cytotoxicity of radiation doses below 30 cGy in several human cell lines has recently been documented using two independent methods for very accurate quantification of cell surviving fractions (31, 32). Low doses of ionizing radiation were found to be more toxic per centigray than were higher doses. Extrapolation from survival at high doses would not have predicted the low-dose hypersensitivity revealed by the more accurate methods. This response may reflect an induction of radioresistance, perhaps through inducible DNA repair, which requires a certain threshold dose to be triggered in these cell lines. The recently reported association between induction of *PBP74* transcription by 25 cGy and low-dose hypersensitivity in cells of several human cell lines (17) supports the likelihood of a transcriptional basis for this phenomenon and would be consistent with induction of repair. Understanding the mechanism(s) underlying such induced resistance could have broad implications in areas from risk assessment to cancer treatment.

Low-dose hypersensitivity and induced radioresistance may be related to the adaptive response to ionizing radiation, another potentially important physiological effect of low-dose exposures. Exposure to a "priming" or "adapting" dose, usually in the range of 1–25 cGy, has been reported to reduce the effects of a subsequent higher "challenging" dose (reviewed in ref. 33). Radioadaptation was first

documented convincingly for protection against radiation-induced chromosomal aberrations in human lymphocytes (34–37). After similar irradiation protocols, modification of cytotoxicity (38, 39) and protection against mutation induction (40, 41) were also described.

The phenomena of low-dose hypersensitivity and radioadaptation raise the question of the requirement for a minimum threshold dose to induce a transcriptional response. The threshold effect is a key question for modeling low-dose effects from results obtained at high doses, as the existence of a threshold implies that a dose can be identified below which exposure carries no risk of response (at least for the end point under consideration). The experiments showing low-dose hypersensitivity have been interpreted to indicate a threshold, usually in the neighborhood of 25–30 cGy, which is required to activate inducible repair. Doses below this threshold are proportionately more toxic than doses which trigger the putative repair system. Radioadaptive protection against cytogenetic aberrations or cell killing, however, has been shown to occur after doses as low as 1–2 cGy, indicating a much lower or even no threshold for induction of this effect, and perhaps a distinct mechanism of action for the two phenomena.

While the genes monitored in this study have not been implicated in induced repair, we have shown changes in mRNA levels in response to radiation in the dose range relevant to this phenomenon. Our present experiments revealed no indication of a threshold for the induction of the genes studied here. *CDKN1A* and *GADD45* were significantly induced by as little as 2 cGy with an approximately linear dose response through 50 cGy. For *MDM2*, *ATF3* and *BAX*, however, the possibility of a threshold for induction could not be distinguished from the noise of the assay. An additional complication in the interpretation of these data is the fact that it is currently unknown whether a threshold exists for biological significance of relative gene expression levels. It is likely that no generalized answer to this question will apply to all genes in all cell lines or cell types.

While we found no evidence of a minimum threshold for induction of *CDKN1A*, there clearly was an upper limit to the linear increase in induction, as the induction by 50 cGy was about half that previously observed at 20 Gy. In experiments covering a wider range of doses, from 10 cGy to 20 Gy, we found a saturation of induction in the neighborhood of 2.5 Gy. At this dose, less than 5% of the cells will retain clonogenic viability. The coincidence of saturation for gene induction with nearly complete cell killing raises the question of whether surviving cells contribute to the transcriptional response after even low doses of ionizing radiation, or if gene induction occurs only in lethally damaged cells. We therefore calculated the relative induction per lethally damaged cell which would be required to produce the inductions observed in the treated population as a whole, if the measured gene induction were due entirely to the response of lethally damaged, reproductively dead cells

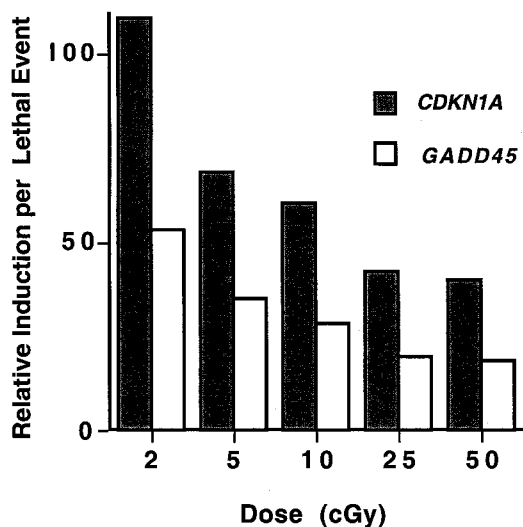


FIG. 5. Relative induction of *CDKN1A* and *GADD45* that would be required per lethal event if the gene induction measured at low doses occurred only in lethally damaged cells. Nonsurviving fractions for 2, 5 and 10 cGy were calculated from the survival curve in Fig. 1. If only lethally damaged cells contributed to the observed gene induction, the induction per lethal event might be expected to be a constant for all doses. As this is not the case, it suggests that surviving cells do contribute significantly to gene induction.

(Fig. 5). For the doses where surviving fractions could not be measured directly, they were predicted from the survival curve (Fig. 1). In this model, each cell responding to 2 cGy would have to produce a relative induction of *CDKN1A* greater than 100-fold over the basal level to result in the observed induction of approximately 3.5-fold across the whole population. This relative induction per responding cell would then have to decrease with increasing dose through 50 cGy. Furthermore, as the proportion of the population suffering lethal damage increased further, the induction per responding cell would level out, then begin to increase again at doses above 5 Gy. A similar relationship was also calculated for the dose response of *GADD45* induction (Fig. 5). If the measured gene induction were due entirely to the response of lethally damaged cells, the relative induction per lethal event would be expected to remain constant with changing dose and surviving fraction, so the relative induction of cell killing in an irradiated population would not appear to account for the observed increases in mRNA levels.

The contribution of cells which survive the treatment to the gene inductions measured at low doses would also be consistent with the disturbances in cell cycle progression observed at all doses tested. Even with 2 cGy, there was a transient decrease in the number of S-phase cells in the population. This decrease corresponded to a larger proportion of the population than the predicted nonsurviving fraction. Perturbation of the cell cycle has been reported before in human lymphocytes at doses as low as 2 cGy (42), and by decays of tritium in cellular DNA as low as 0.1 dpm per cell (43). We have now shown a transient G₁-phase

delay that follows a consistent trend in both dose response and temporal kinetics. While the lowest dose used, 2 cGy, resulted in a brief accumulation of cells in G₁ phase, accumulation of cells in G₂ phase appeared to require a higher dose. It may be interesting to note that *CDKN1A*, the transcript most strongly induced by low doses of γ rays, is known to be a major mediator of G₁-phase arrest (44, 45). Thus our observed G₁-phase delay would be consistent with this induction of *CDKN1A*. A complete understanding of low-dose effects clearly cannot come solely from high-dose experiments, but will require further careful studies in the low-dose range.

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