

# Differential Responses of Stress Genes to Low Dose-Rate $\gamma$ Irradiation

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## Abstract

In the past, most mechanistic studies of ionizing radiation response have employed very large doses, then extrapolated the results down to doses relevant to human exposure. It is becoming increasingly apparent, however, that this does not give an accurate or complete picture of the effects of most environmental exposures, which tend to be of low dose and protracted over time. We have initiated direct studies of low dose exposures, and using the relatively responsive ML-1 cell line, have shown that changes in gene expression can be triggered by doses of  $\gamma$ -rays of 10 cGy and less in human cells. We have now extended these studies to investigate the effects on gene induction of reducing the rate of irradiation. In the ML-1 human myeloid leukemia cell line, we have found that reducing the dose rate over three orders of magnitude results in some protection against the induction of apoptosis, but still causes linear induction of the p53-regulated genes *CDKN1A*, *GADD45A*, and *MDM2* between 2 and 50 cGy. Reducing the rate of exposure reduces the magnitude of induction of *CDKN1A* and *GADD45A*, but not the magnitude or duration of cell cycle delay. In contrast, *MDM2* is induced to the same extent regardless of the rate of dose delivery. Microarray analysis has identified additional low dose-rate-inducible genes, and indicates the existence of two general classes of low dose-rate responders in ML-1. One group of genes is induced in a dose rate-dependent fashion, similar to *GADD45A* and *CDKN1A*. Functional annotation of this gene cluster indicates a preponderance of genes with known roles in apoptosis regulation. Similarly, a group of genes with dose rate-independent induction, such as seen for *MDM2*, was also identified. The majority of genes in this group are involved in cell cycle regulation. This apparent differential regulation of stress signaling pathways and

outcomes in response to protracted radiation exposure has implications for carcinogenesis and risk assessment, and could not have been predicted from classical high dose studies.

## Introduction

There is growing interest in quantification of the biological effects of low doses of ionizing radiation, particularly when they are delivered at low dose rate, a situation relevant to most environmental exposures. Increasing use of nuclear power increases occupational exposures and the risk of industrial accidents, while fears of radiological terrorism have also recently come to the fore. Long-term radiation exposure effects are also an important issue for such projects as the planned manned mission to Mars. The effects of these protracted low dose-rate exposures cannot be entirely predicted by extrapolation from large acute exposures. Early split-dose experiments that used dose fractionation to mimic low dose rates found increased cellular survival compared with single acute exposures (1). This sparing effect was confirmed in later low dose-rate experiments, and related to the DNA repair capacity of cell lines. While wild-type parental cell lines showed markedly increased survival with dose protraction, repair-deficient mutants including mouse lymphoma LY-S (2), *xrs5* and *xrs6* (3), *irs20* (4), and fibroblasts from AT patients (5) showed little or no dose-rate effects for survival. Dose-rate effects on mutation and cell transformation have also been studied as end points with possible relevance to long-term risk in humans. While some studies have seen low dose-rate protection against mutation induction both *in vivo* (6) and *in vitro* (7), other investigations have revealed inverse dose-rate effects for mutation induction. In this latter case, decreasing the dose rate actually resulted in increased mutagenesis in both somatic (8–10) and germ cells (11). This phenomenon has been linked to windows of extreme sensitivity in the cell cycle (12, 13). Despite such studies, insufficient data are currently available to fully understand the impact these low dose-rate phenomena may have on human health in terms of carcinogenesis or other end points. Meanwhile, risk assessment predictions must continue to be based largely on studies of high dose acute exposures, such as those experienced by the survivors of Hiroshima and Nagasaki.

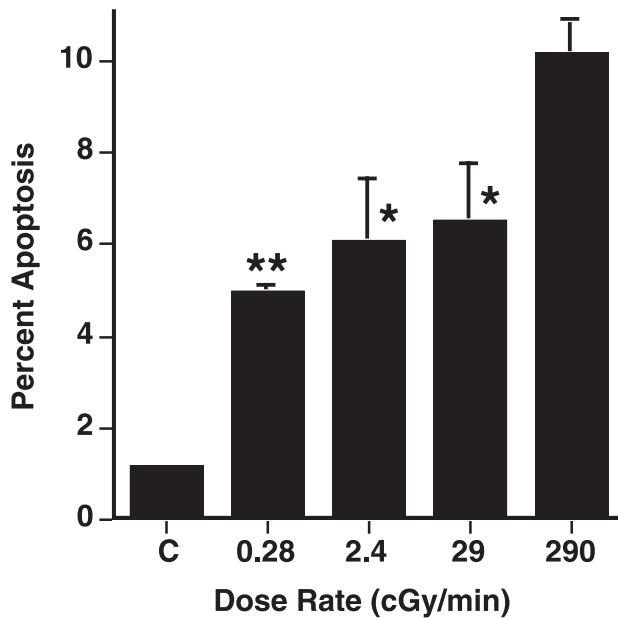
As low dose-rate irradiation can have diverse effects on end points of potential relevance to human risk, examination of gene induction changes in the context of dose rate may reveal early indicators of differential signal transduction pathway activation. A limited number of studies have begun to address

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**FIGURE 1.** Percentage of ML-1 cells scored as apoptotic by 4',6'-diamidino-2-phenylindole staining 48 h after completion of treatment with 0.5 Gy  $\gamma$ -rays delivered at different dose rates. C indicates the level of apoptosis in untreated control cultures. Columns, means of three experiments; bars, SE. Apoptosis induced by low dose-rate irradiation was significantly different from that induced by acute exposure (\* $P < 0.05$ ; \*\* $P < 0.001$ ).

this question. Low dose-rate exposure of human fibroblasts has been shown to induce expression of *GADD45A* (14), although this induction was not compared to that by acute exposure. In another study using five human glioblastoma cell lines, transcript levels of *IL-1 $\beta$*  and *IL-6* were decreased by low dose-rate exposures, and increased by acute exposure (15). Both these studies, although using low exposure rates, used high total accumulated doses, between 3.5 and 10 Gy, to obtain the reported effects. In contrast, a study of fractionated *in vivo* irradiation of mice reported significant increases in *Hsp70* gene expression in several tissues at total accumulated doses of 0.5 Gy and less (16). While such results are intriguing, they do not yet present a coherent picture of gene expression changes at low dose rates, or how these may differ from the changes seen with acute exposures.

In previous studies of gene expression, we have found the human myeloid cancer cell line ML-1 to be extremely responsive to ionizing radiation. Similar to other myeloid and lymphoid cell lines, it has shown robust induction of genes associated with rapid onset apoptosis, as well as numerous p53-dependent genes in high dose studies (17–20). We were also able to demonstrate a dose-response relationship for the induction of five genes (*CDKN1A*, *GADD45*, *MDM2*, *ATF3*, and *BAX*) in response to acute  $\gamma$ -ray exposures between 2 and 50 cGy (21). We have now extended these studies to investigate dose-response relationships in the same dose range over three orders of magnitude of dose rate.

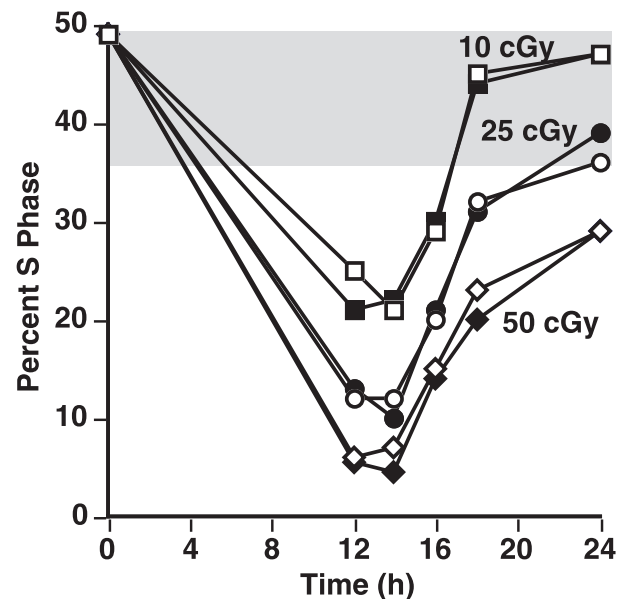
## Results

We first investigated the induction of apoptosis in ML-1 cells exposed to 0.5 Gy ionizing radiation delivered at dose

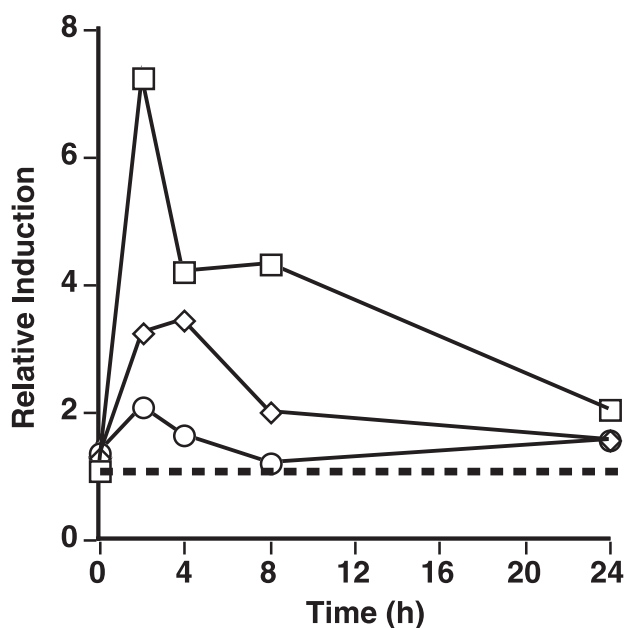
rates varying over three orders of magnitude. While significant induction of apoptosis was observed in all treated cultures 24 h after completion of exposure, only slight differences were observed between the different rates of irradiation. In contrast, at 48 h after treatment (Fig. 1), the cultures treated at 29 or 2.4 cGy/min showed significantly less apoptosis than the acute exposure ( $P < 0.05$ ). Similarly, the cultures treated at 0.28 cGy/min also showed significantly less apoptosis than the acutely exposed cultures ( $P < 0.001$ ).

Next, we examined the effect of lowering the rate of exposure on cell cycle progression. Cultures of ML-1 cells were irradiated to 10, 25, or 50 cGy at either 290 or 0.28 cGy/min, then monitored by flow cytometry at intervals up to 24 h after the end of treatment. The results are illustrated as the percentage of the population in S phase (Fig. 2). It can be seen clearly that while the extent and duration of cell cycle delay increase with increasing dose, the rate of dose delivery has no modifying effect.

As gene expression following DNA damaging stress is a dynamic temporal process, it was important to understand any differences in the timing of gene expression induced by the different dose-rate exposures. At the lowest dose rate used in these studies, 0.28 cGy/min, it takes nearly 3 h to deliver a dose of 50 cGy, and as we had previously found maximal gene expression to occur at 2–3 h after exposure to low doses of acute  $\gamma$ -rays (21), we were concerned that the length of treatment might affect our ability to compare gene induction at the various dose rates. Fig. 3 illustrates the time course of induction of several common stress genes after irradiation with 50 cGy X-rays at 0.28 cGy/min. Although there is a slight elevation over the untreated controls in the levels of most of



**FIGURE 2.** Effect on ML-1 cell cycle progression of irradiation at low dose rate. The percentage of cells in S phase is plotted at various times following the conclusion of low dose irradiation delivered at either 2.9 Gy/min (filled symbols) or 0.0028 Gy/min (open symbols). The shaded area indicates the normal range of S phase observed in untreated cells through the course of the experiment.



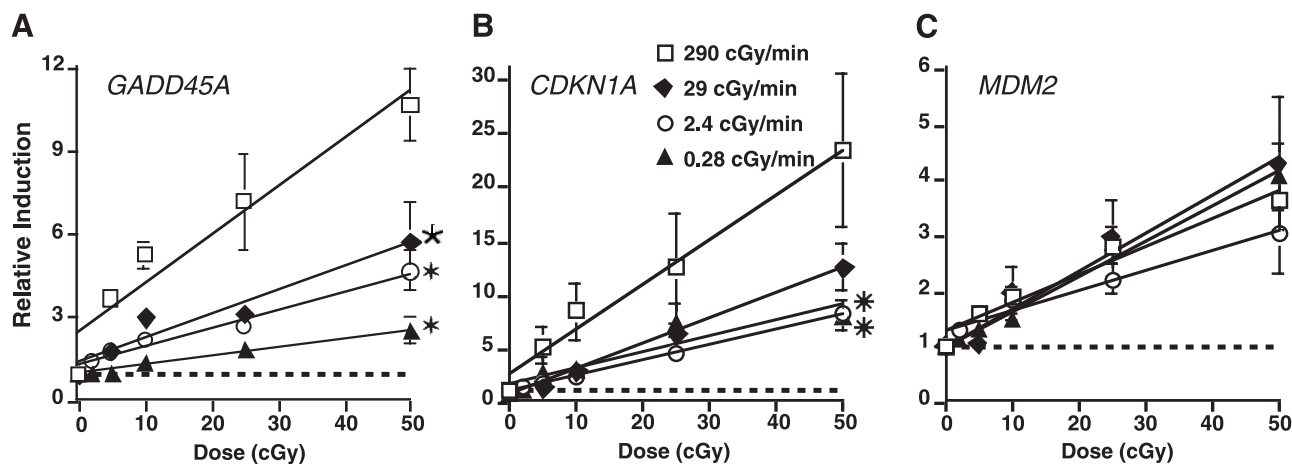
**FIGURE 3.** Induction of *CDKN1A* (□), *MDM2* (◇), and *GADD45A* (○) in ML-1 cells by 50 cGy X-rays delivered at 0.28 cGy/min. Time is measured from the end of the irradiation period. Dashed line, level of gene expression in the un-irradiated control culture. A similar temporal pattern of gene induction was observed in TK6 cells (data not shown).

these genes at time zero (the end of treatment), these genes all show maximal expression around 2 h after the completion of dose delivery. As this is consistent with the maxima previously obtained with acute irradiations, we chose 2 h from the end of irradiation as the time point for subsequent studies of gene induction with all the dose rates employed.

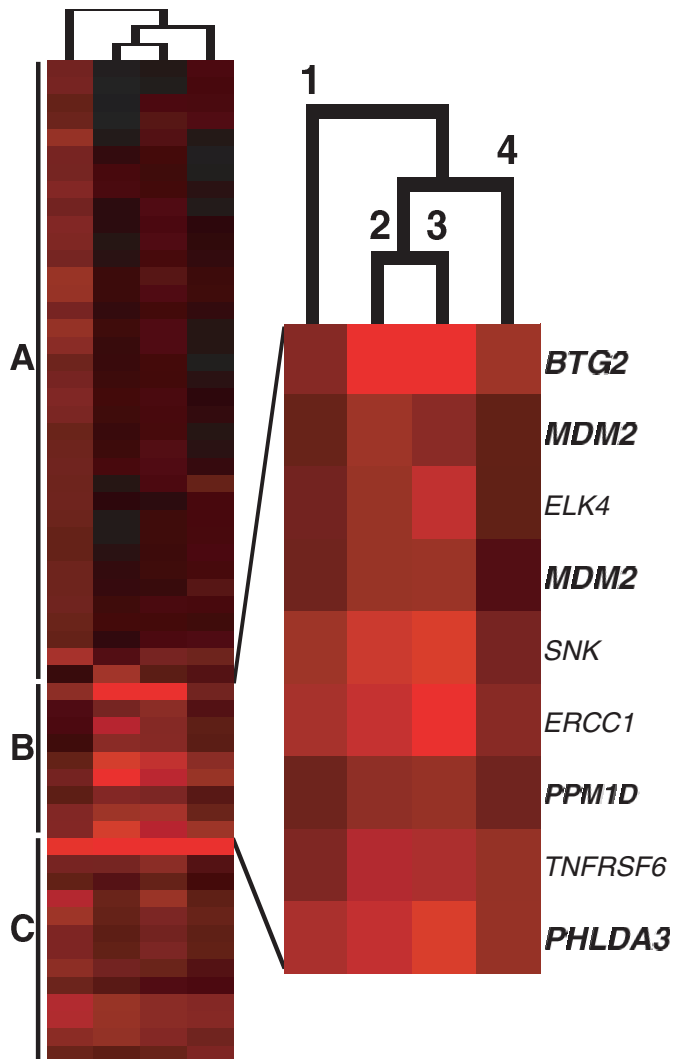
The dose response between 2 and 50 cGy for induction of *CDKN1A*, *GADD45A*, and *MDM2* was next determined in ML-1 cells. In all experiments, RNA was harvested 2 h after the

end of irradiation when differences in gene expression had been determined to be at or near maximal levels. Graded doses of radiation produced essentially linear increases in expression of these three genes at all four dose rates tested (Fig. 4), but a dose-rate effect was observed only for *GADD45A* and *CDKN1A*. Both these genes were induced to lesser levels by irradiation at decreasing dose rates. For *GADD45A* (Fig. 4A), this effect was marginally significant between the acute (290 cGy/min) and 29 cGy/min dose rates ( $P < 0.02$ ), and highly significant between acute and either 2.4 or 0.28 cGy/min exposures ( $P < 0.0001$ ). The same trend was also seen for induction of *CDKN1A* (Fig. 4B), but in this case, only the 2.4 and 0.28 cGy/min dose rates produced a dose response significantly different from the acute exposure ( $P < 0.01$ ). In contrast, neither a trend of dose-rate effect nor any significant differences in induction by acute versus low dose-rate exposure was evident for *MDM2* ( $P > 0.1$ , Fig. 4C).

Microarray hybridization was performed on RNA samples harvested 2 h after the delivery of 50 cGy at each of the four dose rates being used. First, we looked at genes showing high signal-to-noise ratios and good quality hybridization on all arrays, and significant up- or down-regulation in at least one experimental condition. This yielded a set of 268 genes from the set of 6727 genes on the microarrays. Examination of these results indicated that the majority of genes responding to 50 cGy do so to a greater extent when it is delivered as an acute exposure. Most genes responding to this relatively low dose were induced, with only 8 genes being down-regulated. This data were further filtered to include only genes induced at least 2-fold by one or more of the exposure conditions, resulting in a set of 58 genes. When cluster analysis was applied to this data (Fig. 5), the acute exposure was identified as being most different from any of the low dose-rate exposures, having a separate branch on the dendrogram. Three clusters of genes showing different dose-rate effects were also evident, as illustrated in Fig. 5. Genes in the region marked A were induced above 2-fold only by the acute exposure, while genes



**FIGURE 4.** Dose-response relationships for induction of *GADD45A* (panel A), *CDKN1A* (panel B), and *MDM2* (panel C) by  $\gamma$ -rays delivered at 290 cGy/min (□), 29 cGy/min (◆), 2.4 cGy/min (○), and 0.28 cGy/min (▲). Points, means of at least four independent experiments; bars, SE. RNA levels were measured at 2 h after the end of exposure. Dose-response curves with slopes significantly different from that of the acute exposure are indicated by asterisks; \* $P < 0.02$ ; \*\* $P < 0.0001$ ; \*\*\* $P < 0.01$ . Dashed line, base-line level of expression in untreated cells.



**FIGURE 5.** Clustergram of genes induced at 2 h after the end of treatment with 50 cGy  $\gamma$ -rays delivered at either (1) 290 cGy/min, (2) 29 cGy/min, (3) 2.4 cGy/min, or (4) 0.28 cGy/min. Array data have been filtered to include only induced genes with significant hybridization above background, area greater than 20 pixels, and overall quality greater than 0.5 as determined by analysis with ArraySuite 2.1. The *dendrogram* at the top of the figure indicates the relative relationships between the four different treatment conditions as determined by the Clustering Online program [National Human Genome Research Institute (NHGRI)]. The *darker red squares* indicate low or absent gene induction, while the *brighter the red*, the larger the relative gene induction. The genes in the region marked **A** generally show a significant response only to acute exposure. Region **B** is a cluster of genes exhibiting dose rate-independent regulation, while the genes in region **C** show a marked dose rate-dependent regulation. An *enlargement* of the candidates for dose rate-dependent regulation is also shown with the genes examined in greater detail listed in *boldface*. (See also Table 1.)

in region C were induced by the lower dose rates, but in a rate-dependent fashion. As would be expected, *CDKN1A*, a gene shown in this report to be dose-rate responsive (Fig. 4B), was in this cluster. A third gene cluster, B, was also identified, in which genes were robustly induced by both acute and low dose-rate exposures with no obvious trend of dose-rate effect. As might be expected from the results shown in Fig. 4C, the *MDM2* gene fell in cluster B, where it was represented by two different clones from the microarray.

Several of the genes thus identified by microarray and cluster analysis as having a pattern of low dose-rate behavior similar to that of *MDM2* were subjected to further study using repeated experiments with a range of doses and the more quantitatively sensitive single probe hybridization. As would be predicted from the behavior of *MDM2*, both *BTG2* and *PHLDA3* showed linear dose responses for gene induction between 2 and 50 cGy, but no difference in the magnitude of induction with decreased rates of irradiation. The two broad patterns of gene response we have found at low dose rates can be clearly illustrated by plotting the gene induction by a single

dose as a function of the rate of dose delivery. *CDKN1A* and *GADD45A* show an obvious trend of increased magnitude of gene induction with increasing dose rate (Fig. 6A). In contrast, there is no discernable relationship between exposure rate and induction of *MDM2*, *BTG2*, or *PHLDA3* (Fig. 6B).

## Discussion

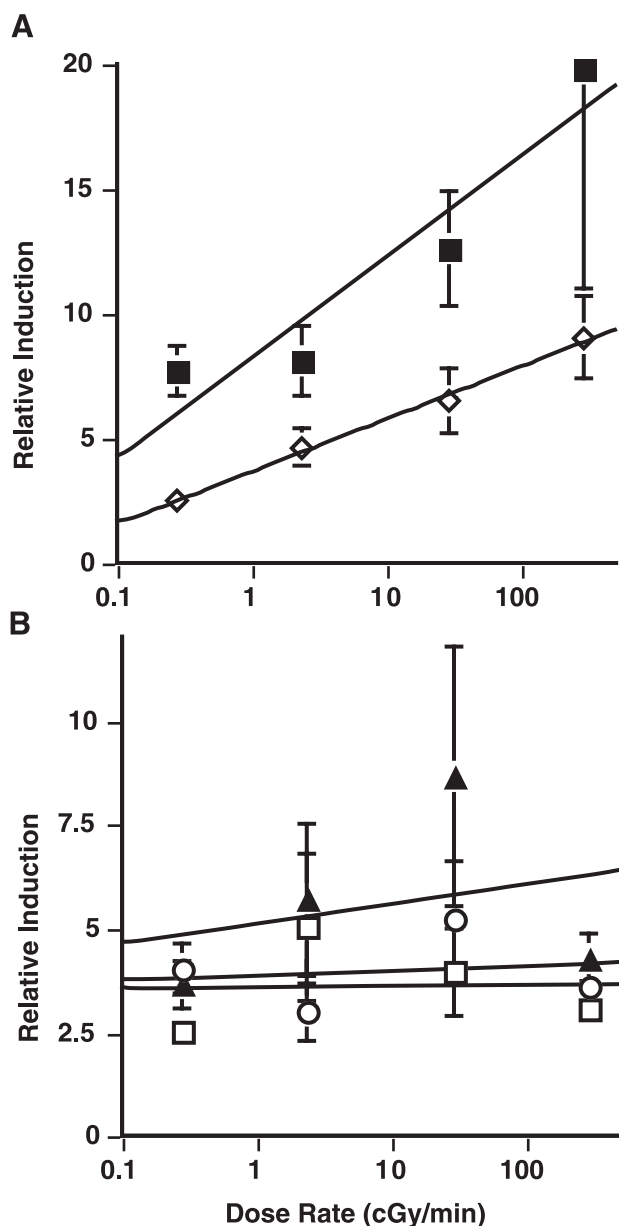
This study for the first time compares the acute and low dose-rate induction of *CDKN1A*, *GADD45A*, *MDM2*, *BTG2*, and *PHLDA3*, as well as many other genes by microarray, following exposure to low cumulative doses of low LET radiation. While the dose-response relationship for induction of *CDKN1A*, *GADD45A*, and *MDM2* appeared linear between 2 and 50 cGy, a significant decrease in the slope of the dose-response curve with decreasing dose rate was seen only for *CDKN1A* and *GADD45A*. This pattern of steady decrease in gene induction with decreasing exposure rate would be consistent with previously reported dose-rate effects on cell survival, mutation, and transformation. As the dose rate

approaches that of background irradiation, the expression of all genes, including those like *MDM2*, must drop to the level of background expression seen in the controls. It would be interesting to see how low the dose rate can be dropped before the induction of genes such as *MDM2* disappears.

To address the question of whether *MDM2* was unique in its lack of dose-rate effect on low dose induction, we used cDNA microarray analysis to identify other genes that behaved similarly to *MDM2* under conditions of low dose-rate irradiation. Consistent with our earlier findings using acute

delivery of high doses of ionizing radiation (20, 23), the majority of genes responding in any of the tested conditions were up-regulated. This effect has appeared particularly pronounced in cell lines with wild-type p53 status (24) such as ML-1. The majority of the genes up-regulated in this study either responded only to the acute exposure or showed a pattern of response similar to that seen for *CDKN1A* and *GADD45A*, suggesting that for the majority of genes responding to low doses of ionizing radiation, a protective dose-rate effect does, in fact, apply. However, hierarchical clustering analysis did also identify a subset of genes with apparent regulation similar to that of *MDM2*. Subsequent quantitative experiments with two of the most highly induced genes in this cluster, *BTG2* and *PHLDA3*, confirmed the lack of any significant dose-rate effect within the studied range of three orders of magnitude. Looking only at the 50-cGy dose, there appears to be a trend among the low dose-rate exposures for *PHLDA3*, while the acute response is identical to that of the lowest dose rate used (Fig. 6B). When the entire dose response from 2 to 50 cGy is taken into account, however, the only significant deviation from the slope of the acute dose response is for the 29-cGy/min dose rate, and this is only significant at the  $P < 0.025$  level. While it may be tempting to speculate that this represents an inverse dose-rate effect on gene induction, such as has been seen for mutation induction (6, 8, 10), it is likely the result of small fluctuations in the time and magnitude of peak induction occurring in repeated experiments.

In addition to the separation of two broad groups of induced genes with different dose-rate effects, low dose-rate irradiation of ML-1 cells also revealed a separation between two physiological cellular responses. We observed a clear dose-rate effect on the induction of apoptosis, while in contrast, the cell cycle delay induced by 0.28 cGy/min was identical to that induced by acute irradiation. It was therefore of interest to examine the known functional roles of genes in the two robustly induced gene clusters identified in Fig. 5. Table 1 presents these genes along with functional annotations derived from LocusLink (National Center for Biotechnology Information). While both clusters contain genes with roles in a number of diverse processes, it is striking that the majority of genes in the dose rate-independent group have roles in the regulation of proliferation or cell cycle progression, the parameter that showed similar dose rate-independent behavior. Likewise, the majority of genes in the dose rate-dependent cluster play roles in apoptosis, the parameter shown to be sensitive to reduction in dose rate. While the single probe hybridization results (Fig. 4A) clearly place *GADD45A* in this latter category, it does not appear in Table 1 due to poor hybridization on the microarrays. This gene is known to play roles in many processes, including DNA repair, cell cycle progression, and apoptosis (25, 26). However, the correspondence between gene function and dose-rate effect is not absolute, as for example, *CDKN1A* showed a clear dose-rate protective effect, but is known to be a prominent mediator of  $G_1$  arrest. Despite such exceptions, the broad behavior of these two clusters of genes is consistent with the physiological parameters measured, and suggests qualitatively different mechanisms of response to low and high dose-rate irradiation that could never have been predicted from high dose-rate studies alone.



**FIGURE 6.** Gene induction 2 h after the end of treatment with 50 cGy  $\gamma$ -rays expressed as a function of the rate of dose delivery. A clear trend of protection with decreasing dose rate is seen for *CDKN1A* (■) and *GADD45A* (◇) (panel A). In contrast, there is no effect of dose rate on *MDM2* (○), *BTG2* (□), or *PHLDA3* (▲) induction (panel B).

**Table 1. Summary of Genes in Clusters C (Dose Rate-Responsive) and B (Dose Rate-Independent) from Fig. 5**

Gene Name	Apoptosis	Cell Cycle/ Proliferation	Other
<i>Dose rate-responsive genes</i>			
CDKN1A		+	
Killer/DR5	+		
TRID	+		
Apoptosis (APO-1) antigen 1	+		
<i>Homo sapiens</i> TRAIL receptor 2	+		
5' Nucleotidase (CD73)			DNA
<i>Homo sapiens</i> TRAIL receptor 2	+		
XPC			DNA
TNFSF9	+		
MAP17			Extracellular
Schwannomin interacting protein 1			Cytoplasmic
Leukemia inhibitory factor			Cytokine
Prolactin receptor			Embryogenesis
<i>Dose rate-independent genes</i>			
BTG2		+	
MDM2		+	
ELK4		+	
MDM2		+	
Serum-inducible kinase SNK		+	
ERCC1			DNA
PPM1D		+	
TNFRSF6	+		
PHLDA3			Embryogenesis

Note: Broad functional classification (LocusLink annotation) of genes involved in apoptosis or regulation of proliferation and cell cycle progression are indicated by a "+." Most genes in the dose rate-responsive cluster have roles in the regulation of apoptosis, a parameter found to also be responsive to variations in dose rate, while most genes in the dose rate-independent cluster have roles in cell cycle progression, which was shown not to vary with dose rate. The last column indicates genes with known roles in other processes, including DNA metabolism and repair (DNA).

While the present findings suggest that low dose-rate irradiation may be a useful probe for teasing apart some of the intertwined signaling events resulting from low dose-rate irradiation, the differential effects of low dose-rate irradiation on cell cycle progression and the induction of apoptosis may also have serious implications for the risk of carcinogenesis from such exposures. If cells damaged by protracted exposures escape apoptosis, but undergo normal cell cycle arrest, it is possible that critically damaged cells that would normally be eliminated may be more likely to misrepair and continue proliferating. Such a situation could both increase the early DNA damage and mutational events escaping surveillance, and enhance tumor promotion by increasing the probability of survival of cells with accumulating damage or mutations in an environment of continued low dose-rate radiation exposure.

The finding that some genes show a dose-rate effect while others do not also has implications for the potential usefulness of gene induction as a biomarker for radiation exposure. Recent fears of a possible "dirty bomb" detonation or similar scenarios have spurred the search for biomarkers that could be used to rapidly assess radiation exposure status in large, potentially exposed, populations. Identification in an easily biopsied tissue, such as peripheral blood lymphocytes (PBL), of a radiation-associated gene response signature could be one approach to such biomonitoring. Once informative genes are identified, they could feasibly be incorporated into rapid assays utilizing such techniques as real-time quantitative PCR. As an initial step in this direction, we previously have shown linear dose responses

for the induction of several genes, including the dose rate-sensitive *CDKN1A* and *GADD45A*, in human PBL irradiated *ex vivo* with low doses of acute  $\gamma$ -rays (23). Such dose rate-sensitive genes might be expected to show less prominent induction by environmental exposures occurring at low dose rates, while more robust responses may still be obtained from genes insensitive to dose-rate effects. In light of the dichotomous dose-rate responses reported here, it will be interesting to extend the PBL studies to low dose-rate exposures. If similar dose rate-sensitive and -insensitive responses can be identified in this system, and ultimately confirmed *in vivo*, comparison of these two aspects of the gene induction profile could provide a more robust indicator of both absolute dose and the rate of exposure, and hence, a more accurate assessment of risk.

## Materials and Methods

### Cell Culture and Treatment

The human myeloid leukemia cell line ML-1 has wild-type p53 function (27), and was grown in RPMI 1640 supplemented with 10% heat-inactivated (56°C for 45 min) FCS and 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin in a humidified, 5% CO<sub>2</sub> atmosphere in a 37°C incubator. Cells were irradiated at dose rates of 2.4, 29, or 290 cGy/min to total doses of 2–50 cGy using a Mark I-68 <sup>137</sup>Cs source (J.L. Shepherd and Associates, Inc., San Fernando, CA) with lead attenuators in place. The lowest dose rate used, 0.28 cGy/min, was obtained with a Torrex120 X-ray machine (Torr X-ray Corp., Van Nuys, CA) running at 3 mA with 13 mm aluminum filtration. For treatment with this lowest dose rate, 25 mM HEPES buffer was included in the culture medium, and cultures were irradiated in sealed flasks. When the pH was checked at the end of the irradiation, there was no measurable difference between the low dose-rate-irradiated cultures and cultures kept in the incubator. During irradiation, the cultures were kept warm with the use of sand pre-warmed to 37°C. For experiments with all the different dose rates, mock-irradiated controls were treated identically to the irradiated cultures with the exception of the actual radiation exposure to control for any variation in temperature, CO<sub>2</sub> levels, etc. The dosimetry of both sources was confirmed by exposing TLD monitors (Landauer, Inc., Glenwood, IL) in the same configuration used for cellular irradiations to 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 dose expected (data not shown). Due to the nature of sparsely ionizing radiation such as X- and  $\gamma$ -rays, it is highly unlikely that any cells in the irradiated population will remain unexposed at even the lowest doses used in this study.

### Determination of Apoptosis

Cells were incubated for 24 or 48 h after the end of irradiation, then fixed in methanol, and stained with 4',6-diamidino-2-phenylindole solution as previously described (28). An Olympus fluorescent microscope with the appropriate filter set was used to score nuclei exhibiting characteristic morphological features of apoptosis, and results were expressed as the number of apoptotic nuclei over the total number of nuclei counted.

### Flow Cytometry

Cells were fixed in 70% ethanol for 0, 12, 14, 16, 18, and 24 h after the end of irradiation, treated with RNase at 37°C, then stained with propidium iodide. Samples were analyzed using a Becton Dickinson FacScan (Becton Dickinson, San Jose, CA) 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 0, 2, 4, 8, or 24 h following the end of treatment, and RNA was extracted using a modified guanidine thiocyanate method (29). 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, Gaithersburg, MD), and washed under standard conditions as previously described (30). Hybridization was quantitated on a phosphorimager (Molecular Dynamics, Piscataway, NJ), and relative signal levels, normalized to the poly(A) content of each sample, were determined using the RNA-Think program (30). With this approach, the values for relative RNA levels are directly proportional to RNA abundance, and differences of 1.5-fold or more can be reliably measured (31, 32). Results obtained with this sensitive approach also agreed well with those obtained by RNase protection determinations (33).

### Microarray Analysis

Eighty to 100 µg of whole-cell RNA were labeled and hybridized to 6727 element microarrays as described previously (20). In brief, probes were prepared by PCR amplification of IMAGE consortium clones and arrayed on poly-L-lysine-coated glass slides. Fluorescently labeled cDNA was prepared from control and  $\gamma$ -irradiated ML-1 whole-cell RNA by a single round of reverse transcription (Superscript II, Invitrogen, Carlsbad, CA) in the presence of fluorescent dUTP (Cy3 dUTP or Cy5 dUTP, NEN, Boston, MA). Probes and targets were hybridized together for 16–18 h in  $3\times$  SSC at 65°C in the presence of blocking agents human CoT1 DNA, yeast tRNA, and polydeoxyadenine. Hybridized slides were washed at room temperature twice in  $0.5\times$  SSC, 0.01% SDS for 5 min and once in  $0.06\times$  SSC for 5 min. Cy3 and Cy5 fluorescences were scanned using a laser confocal scanner (Agilent Technologies, Palo Alto, CA), and images were analyzed using the ArraySuite 2.1 extensions (Y. Chen, NHGRI) in the IPLab program (Scanalytics, Inc., Fairfax, VA) to calibrate relative ratios and develop confidence intervals for their significance (34). The ratios were normalized to those of a set of 88 internal controls (35) with a theoretical ratio of 1.0. The variance in the housekeeping set was used to determine the significance of expression changes following irradiation. Cluster analysis of the microarray data was performed using the Clustering Online program of the NHGRI.

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