Gene Expression Profiling of Breast, Prostate, and Glioma Cells following Single versus Fractionated Doses of Radiation

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Abstract

Studies were conducted to determine whether gene expression profiles following a single dose of radiation would yield equivalent profiles following fractionated radiation in different tumor cell lines. MCF7 (breast), DU145 (prostate), and SF539 (gliosarcoma) cells were exposed to a total radiation dose of 10 Gy administered as a single dose (SD) or by daily multifractions (MF) of 5 × 2 Gy. Following radiation treatment, mRNA was isolated at 1, 4, 10, and 24 h and processed for cDNA microarray analysis. To determine the influence of the tumor microenvironment on gene expression, one cell type (DU145) was evaluated growing as a solid tumor in athymic nude mice for both radiation protocols. Unsupervised hierarchical cluster map analysis showed significant differences in gene expression profiles between SD and MF treatments for cells treated *in vitro*, with MF yielding a more robust induction compared with SD. Several genes were uniquely up-regulated by MF treatment, including multiple IFN-related genes (*STAT1, G1P2, OAS1, OAS3, G1P3, IFITM1*) and *TGF-β*–associated genes (*EGR1, VEGF, THBS1, and TGFB2*). DU145 cells grown *in vivo* exhibited a completely different set of genes induced by both SD and MF compared with the same cells exposed *in vitro*. The results of the study clearly show distinct differences in the molecular response of cells between SD and MF radiation exposures and show that the tumor microenvironment can significantly influence the pattern of gene expression after radiation exposures. [Cancer Res 2007;67(8):3845–52]

Introduction

Radiation is commonly used in cancer management, with ~40% to 60% of all cancer patients receiving radiation treatment. Conventionally, radiation is delivered to the tumor in multiple 2-Gy fractions (Monday–Friday) for 5 to 7 weeks to total radiation doses of 50 to 75 Gy. However, for specific applications, large single radiation doses have been used, as is the case for gamma knife exposures, where median single doses of 25 Gy are delivered to the tumor locally (1). Multifraction radiation treatment is considered to be advantageous for tumor control because (*a*) greater nonrepairable damage is induced per unit dose in tumor cells, (*b*) tumor reoxygenation can occur between each fraction, and (*c*) redistribution of clonogenic tumor cells into more radiosensitive portions of the cell cycle can transpire as treatment progresses (2). Cell survival assays, extensively used in radiobiology research, have provided valuable information; however, cells do not die immediately following radiation exposure (3). Hence, surviving cells and even cells destined to die continue to interact with the internal/external environment through the induction of various signaling pathways and/or gene expression alterations leading to the production of various cytokines, hormones, and growth factors. How (or if) these complex interactions ultimately influence cell survival and tissue response to radiation is an intense area of study.

At the molecular level, a number of genes have been shown to be responsive to radiation exposure. The role of p53 (normal and mutated) following single versus multifraction radiation treatment has been studied extensively. Cells with normal p53 block in G_1 after radiation (4). When radiation doses are split into multiple fractions, it is possible that cell cycle redistribution could occur, thus altering the inherent radiosensitivity with time. For example, Scott et al. (5) compared PC-3 prostate cells (p53 null) with cells transfected with a vector containing a temperature-sensitive normal p53 gene and given single versus multiple 2-Gy fractions. No difference in survival was shown for either cell line exposed to single radiation doses. However, when the p53 gene was functional, the survival after 3 × 2-Gy fractions was increased compared with the same cell line with an nonfunctional p53 gene (5). Conversely, Haas-Kogan et al. (6) found that a mutated p53 gene in glioblastoma cells showed increased survival after multiple 2-Gy fractions compared with the same cells transfected with a normal p53 gene. Finally, Dahlberg et al. (7) found that a series of eight tumors with varying degrees of radiocurability isolated from patients had a response to fractionated radiation treatment that was independent of the p53 status of each cell line. In several cell lines, there was no difference in survival if the total radiation exposure was given as a single large dose or if the dose was fractionated into 2-Gy fractions (7). However, some of the tumors were much less affected by fractionated therapy compared with a single large dose. Although controversy remains as to the precise role of p53 in the radiation response, these studies and others suggest that p53 status can influence radiation sensitivity, particularly for multifractioned radiation delivery.

Although p53 is but one radiation-responsive gene, there are doubtless other genes that may also contribute to the radiation response. Response may differ depending on whether cells are exposed to single versus fractionated radiation doses. In the current study, gene expression profiles were compared in three human tumor cell lines exposed to single or multifraction radiation

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doses. Additionally, one of the cell lines was also grown as a solid tumor and exposed to the same radiation protocols, enabling a comparison of the radiation response of the same cells growing *in vitro* or *in vivo*.

Materials and Methods

Cell culture. Three cancer cells, MCF7 (breast carcinoma), DU145 (prostate carcinoma), and SF539 (gliosarcoma), were maintained as exponentially growing cultures in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were plated into 150-mm dishes (2×10^6) and incubated 3 days and then treated with a single dose (SD) of 10 Gy or a multifraction (MF) schedule consisting of five daily fractions of 2 Gy. Total RNA was extracted from untreated or treated cells at 1, 4, 10, and 24 h after irradiation for cDNA microarray analysis. After SD, mRNA was extracted for various times after radiation; however, for MF, mRNA was extracted after the last 2-Gy fraction at 1, 4, 10, and 24 h. Three independent cultures were used for cDNA microarray experiment. Cell survival was assessed in parallel by the clonogenic assay as previously described (8). Cells were irradiated with an Eldorado 8 ^{60}Co teletherapy unit (Theratronics International Ltd., Kanata, Ontario, Canada) at dose rates between 200 and 250 cGy/min.

Animal studies. Female athymic NCR *nu/nu* mice (6–7 weeks of age) were purchased from Taconic Farms (Germantown, NY). All animal experiments were carried out on a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animal Resource (1996), National Research Council. DU145 cells (10^6) were injected s.c. in the right hind leg. When tumors reached a diameter of 0.8 to 0.9 mm, animals were divided into three groups (two animals per group): control, SD, and MF. SD and MF employed the same doses/schedule as used for the *in vitro* studies. Selective tumor irradiation was accomplished by placing animals in customized Plexiglas jigs that allowed for immobilization of the right leg without the use of anesthetics. Special care was taken to avoid irradiation of other body parts using lead shields specifically designed for the jigs. Tumors were irradiated using a Therapax DXT300 X-ray irradiator (Pantak, Inc., East Haven, CT) using 2.0 mm Al filtration (300 kVp) at a dose rate of 1.9 Gy/min. Animals were euthanized at 1, 4, 10, and 24 h after irradiation, tumors were removed, and RNA was extracted as described below. For each time point, RNA from two tumors were pooled and processed. Two complete replicate experiments were done.

Probe labeling and microarray hybridization. Methods for RNA extraction, probe labeling reactions, and microarray hybridization were as described previously (8). The microarray slides used for the experiments contained 7,680 human cDNA clones, and the details of this array and the methods for microarray fabrication were described previously (9).

Statistical analysis. The experiments were conducted under normal laboratory conditions over a 1-year period. Samples were collected immediately after IR treatment at designated times for time course patterns, whereas the replicates were measured at time intervals of about 1 to 3 months. The reproducible time course patterns of such data set are time tested and robust. Initial unsupervised analysis by multidimensional scaling revealed clustering of samples by replicates rather than by time points (data not shown). Hence, the correlations between complete time course patterns of replicates were used for statistical validation. Pairwise correlation coefficients of the time course patterns consisting of control, 1, 4, 10, and 24 h of the replicates were calculated. Heat maps of genes having altered expressions at least by 1.5-fold in 24-h time course were generated using Eisen Cluster and Treeview software 1.

Results

Survival. A SD exposure of 10 Gy reduced survival of the MCF7, DU145, and SF539 cells to 0.31%, 0.42%, and 1.6%, respectively. Survival following MF (5×2 Gy) was higher than SD as expected because of repair of sublethal damage between fractions. Survival following MF was 3.7% for MCF7, 6.1% for DU145, and 13% for the SF539 cells. Survival data were in agreement with results published on the surviving fraction at 2 Gy for 35 of the NCI 60 cell line panel, including MCF7, DU145, and SF539 cells (10).

Global microarray response. The three cell lines responded to SD and MF with a large numbers of changes in genes at the 1.5- and 2-fold threshold levels within the 24-h time course (Table 1). A comparison of the time course changes in the global expression patterns by multidimensional scaling analysis revealed differences rather than similarities among the cell lines as well as between the SD and MF regimens (Fig. 1*A*). MCF7 cells responded to either SD or MF with approximately equal numbers of gene changes; whereas SF539 cells had the highest difference between the numbers of SD and MF responding genes. The majority of the up- or down-regulated genes remained in the same direction under either SD or MF protocols. The highest percentage of up-regulated genes was observed by MF in the MCF7 cell line. Gene ontology terms of these gene sets analyzed by the Expression Analysis Systematic Explorer software identified cell proliferation as highly populated both by SD and MF treatment in MCF7 cells, but not for either DU145 or SF539 cells. The number of genes up-regulated by at least 2-fold, common to all three cell lines, was found to be small (13 out of 463) and are listed in Table 2. Seven of the 13 genes were identified as IFN-associated genes.

Table 1. Number of genes altered as a function of time after radiation treatment

Cell line	Treatment type	1.5-fold		2-fold	
		Number of genes	% Up*	Number of genes	% Up*
MCF7	Single	1,229	66	274	75
	Fractionated	1,049	70	242	87
SF539	Single	611	67	114	67
	Fractionated	1,162	56	402	66
DU145	Single	614	49	60	43
	Fractionated	881	45	127	53
DU145 *in vivo*	Single	688	50	90	62
	Fractionated	598	64	90	51

*Up- or down-regulation was assessed by the highest fold change in 24 h time course.

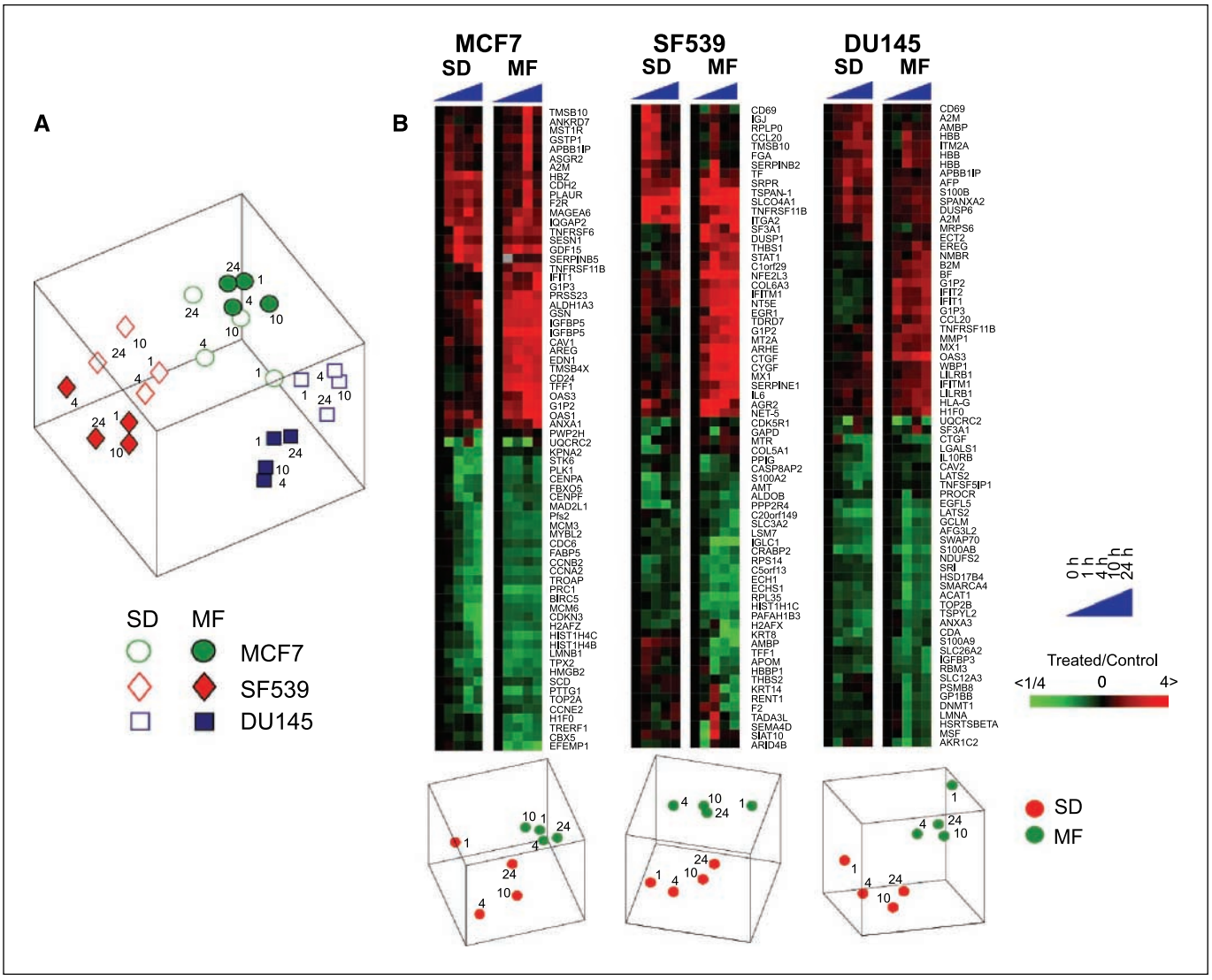

Figure 1. *A,* multidimensional scaling (MDS) of 5,608 genes (with no missing values) after 1, 4, 10, and 24 h of SD or MF exposure in the MCF7, SF539, or DU145 cells. Distances between points represent 1−Pearson correlation coefficient. *B,* the time-dependent changes in the top 70 genes up- and down-regulated for the combined SD or MF treatments were selected for each cell line (MCF7, SF539, and DU145), and unsupervised hierarchical cluster maps were derived using the Pearson's correlation coefficients for the gene similarity metric. Beneath each cluster map is the MDS plot of the 70 genes comparing the SD and MF postradiation exposure response.

p53 and cell cycle response. MCF7 cells are known to respond to DNA-damaging agents with a vigorous p53 response and cell cycle delays (8). For validation purposes, gene expression data were analyzed to determine if SD or MF influenced genes associated with p53 in the three cell lines. Complete sequencing of the *p53* gene is known for all three cells lines used in our studies. MCF7 and SF539 contain wild-type *p53*, whereas DU145 has a C-T base change at position 223 (11). MCF7 and SF539 cells exhibited very different postradiation cell cycle changes (11). In fact, SF539 exhibited a cell cycle response similar to cells having a mutated p53 (no G_1 block, G_2 block). Thus, SF539 cells would not be expected to show regulation of *p53*-regulated genes after radiation exposure. Time-dependent changes of four known *p53*-related genes, *GDF15, SESN1, XPC,* and *BTG2* were examined for each cell line. MCF7 cells showed a clear time-based expression of these genes after either SD or MF exposures with maximum ratios with respect to untreated controls coming at 4 h postradiation (average ratio increase for all four genes: 3.2 versus 2.4 for SD and MF, respectively), whereas the expression of these genes was essentially unchanged over time in the DU145 or SF539 cells (Supplemental Fig. S1). Thus, *p53* genes responded similarly in MCF7 cells to either the SD and MF protocols, although survival was some 10-fold increased after the MF regimen (0.31% SD versus 3.7% MF).

Global gene expression by hierarchical cluster maps. To detect patterns of similarity between the three cell lines, the top 35 genes up-regulated with expression ratios (comparing treated to untreated controls) greater than 2-fold and the top 35 genes down-regulated with ratios <0.5 were chosen for unsupervised hierarchical analysis for each individual cell line by combining the data for both the SD and MF conditions (Fig. 1*B*). Mean maximal increase of the 35 genes analyzed (including genes induced by either SD and MF) was 4.0 for MCF7, 6.0 for SF539, and 2.4 for the DU145 cells. For down-regulation, there was less effect after

Table 2. Genes up-regulated by 2-fold or greater after radiation exposure *in vitro* in the MCF7, SF539, and DU145 cells

Name	Accession number	MCF7*	SF539*	DU145*
OAS3 †	NM_006187	5.78	2.99	4.32
G1P3 †	NM_002038	4.04	2.91	3.05
TNFRSF11B	NM_002546	3.79	4.91	2.45
G1P2 †	NM_005101	3.78	3.97	3.07
A2M	NM_000014	3.29	2.51	2.28
PROPERDIN	NM_001710	3.24	3.12	2.40
IFIT2 †	NM_001547	2.90	2.58	2.71
HBB	NM_000518	2.71	2.76	2.70
IFITM1 †	NM_003641	2.62	3.77	2.26
LIPC	NM_000236	2.46	2.02	2.02
LGALS3PB †	NM_005567	2.29	2.52	2.07
CD69	NM_001781	2.21	3.89	2.16
BST2 †	NM_004335	2.18	4.88	2.04

*Maximal gene induction compared with untreated controls over the 24-h period after either SD or MF protocols.
† Genes up-regulated by IFN.

radiation because the average minimum ratio for the 35 genes was 0.45 for the MCF7 cells, 0.44 for the SF539 cells, and 0.45 for the DU145 cells. Among the 35 up-regulated genes in the three cell lines, there were only two common genes, *TNFRSF11B* (osteoporgerin) and *G1P2* (*ISG15* ubiquitinlike modifier), whereas there were no common genes down-regulated. MCF7 cells had a cluster of p53-related genes (*TNFRSF6, SESN1, GDF15,* and *SERPINB5*), which were regulated by both the SD and MF schedules. No p53-related genes were detected in either the SF539 or DU145 cells. No genes were up-regulated by only SD and not by MF. However, there were genes predominantly up-regulated by MF and not by SD for all three cell lines. For MCF7 cells, these included the genes *G1P2, OAS3, OAS1, G1P3, IGFBP5, AREG, EDN1,* and *TFF1*. For SF539 cells, these included the genes *G1P2, IFITM1, MX1, C1orf29, STAT1, THBS1*, and *IL-6*, and for DU145 cells, these included the genes *G1P2, OAS3, IFIT1, MX1, IFIT2,* and *MMP1*. There were no obvious differences between the SD and MF for down-regulated genes. As discussed above, a significant number of genes related to cell cycle and growth were down-regulated by both SD and MF for MCF7 cells (Supplemental Fig. S2). Examples of these genes included *CCNB2, CCNA2, CCNA2, PLK,* and *STK6*. Cell cycle and growth genes were not particularly altered by radiation in either the SF-539 cells or the DU145 cells (Supplemental Fig. S2).

DU145 *in vivo* gene expression. Given that cells grown in mice as solid tumors may be under very different environmental conditions, radiation-induced gene expression was also evaluated for DU145 tumors exposed to SD and MF protocols identical to those used for the *in vitro* studies. Figure 2*A* shows the multidimensional scaling analysis of the time response of 6,018 genes after either SD or MF exposure for *in vitro* or *in vivo* DU145 cells. There was no overlap between any of the four conditions, indicating that the gene response was highly dependent on the type of exposure (SD versus MF) and also on what environment the cells were growing in (*in vitro* versus *in vivo*). Genes identified by the SD or MF treatment under *in vitro* conditions were also used to create an unsupervised hierarchical cluster map facilitating comparison between the *in vitro* and *in vivo* models. Figure 2*B* shows that genes that exhibited changes >2-fold up or down selected under the *in vitro* treatment changed very little when the DU145 cells were grown and irradiated as solid tumors. The *in vivo* induction for the 35 top genes identified as a result of the *in vitro* exposure indicated, on average, only a 1.5-fold change in the ratio (compared with 2.4 after *in vitro* exposure). Likewise, for the 35 down-regulated genes identified after the *in vitro* exposure, an average ratio change of 0.78 was found after *in vivo* exposure (compared with 0.45 after *in vitro* exposure). Figure 2*C* shows the top 35 up- or down-regulated genes selected for unsupervised hierarchical cluster analysis after combining results from the SD and MF exposures for the DU145 grown *in vivo*. The average gene induction ratio for the 35 up-regulated genes was 3.0, whereas the average gene ratio was 0.40 for the 35 down-regulated genes. The fate of *in vivo* genes was followed in the *in vitro* exposed DU145 cells as well. These *in vivo* genes, for the most part, showed little expression differences between SD and MF exposures in the *in vitro* DU145 cells. However, striking differences were observed after *in vivo* exposure between the SD and MF exposures with genes up-regulated by SD exposure and down-regulated by MF (Fig. 2*C, cluster I*). A cluster of genes down-regulated by SD exposure and not by MF exposure (Fig. 2*C, cluster II*) and genes up-regulated by MF but not SD (Fig. 2*C, cluster III*) was observed.

IFN-related gene regulation. IFN (α, β, and γ)-related genes were predominantly up-regulated by MF exposure in all three cell lines under the *in vitro* growth conditions and were further examined. First, eight genes known to be regulated by the IFNs were selected (*STAT1, G1P2, G1P3, IFITM1, IFIT1, MX1, OAS1,* and *OAS3*), and results from SD versus MF were compared for each individual cell line and for the DU145 tumor. Maximal induction over the 24-h time course was chosen and plotted for each gene. Time-dependent changes were noted for all these genes, increasing the confidence that these genes were responding to the radiation treatments (Supplemental Fig. S3). Figure 3*A* to *C* shows that MF exposure increased these genes by 2- to 4-fold, whereas after SD exposure, no changes were observed in all three cell lines. MCF7 and SF539 cells showed the largest increases, whereas the DU145 cells exhibited more modest increases; moreover, MF treatment of DU145 tumors also exhibited increases in the *IFN*-related genes (1.4–2.0-fold; Fig. 3*D*).

TGF-β–associated gene expression. It was observed that *COL6A1, THBS1*, vascular endothelial growth factor (*VEGF*), and transforming growth factor *(TGF)-β2* were up-regulated in the SF539 cells, particularly after MF exposure, suggesting the possibility that the TGF-β pathway may be activated in these cells after radiation. Table 3 lists 15 genes, which have been found to have some association with a response to TGF-β, including *TGF-β2*. SF539 cells showed the strongest up-regulation of these genes after either SD or MF exposure (average ratio after MF exposure, 2.5; Table 3). SF539 cells had *TGF-β2* up-regulated maximally at the 10-h point, but was still elevated at 24 h (2.3 at 10 h versus 2.0 at 24 h). Genes with the highest expression ratios after radiation such as *IL-6* (12.5), *EGR-1* (5.4), *CYR61* (4.4), and *THBS1* (3.4) are associated with the TGF-β pathway (12). Although some elevation was noted for the SD exposure, in many cases, this was only at a single time point in contrast to the MF exposure where the increase occurred over multiple time

points. Importantly, the maximal increase found in the SF539 cells for most of these genes occurred at the 24-h time point. In MCF7 cells, only *EGR1* and *IGFBP5* were increased, whereas most of the collagen genes were not increased (average ratio for collagen genes, 1.6 for SD and 1.9 for MF). Neither *TGF-β1, TGF-β2*, nor *TGF-β3* were altered after radiation; however, *TGFBR2* and *TGFBR3* did increase in a time-dependent manner. None of the TGF-β/SMAD genes in DU145 cells were increased after either SD or MF exposure (average ratio for genes, 1.1 for SD and 1.2 for MF).

Discussion

The aim of this study was to determine whether the manner in which radiation is delivered either as a single large dose (SD) or as multiple small doses (MF) would alter the postirradiation gene expression response. Specifically, we sought to compare and contrast radiation-induced gene expression following a 10-Gy dose delivered as SD or MF for three human tumor cell lines that possess differences in radiosensitivity. In addition, this study aimed to determine the role of the cell growth environment in influencing the transcriptional response after radiation. The study clearly shows that (*a*) there are significant differences in the gene response depending on how the radiation is delivered; (*b*) fractionating the radiation dose leads to a more robust induction of genes than does a large single doses; (*c*) there was a small subset of identical genes up-regulated by MF in three very different tumor types; and (*d*) after radiation, DU145 xenografts exhibited a completely different set of genes induced than were identified under *in vitro* growth conditions.

As expected, there was a significant difference in survival between SD and MF, the latter exhibiting less cell killing primarily due to the repair of sublethal damage between doses. One clear factor in the present study was that the survival after MF (2 × 5 Gy) was some 10 times higher than with the SD protocol, and thus, the gene response is much more likely to represent cells that are viable than with the SD exposure, where cell killing may be the dominant feature. No significant changes in DNA repair genes were observed among the cell lines despite differences in inherent radiosensitivity. The reason(s) for a lack of correlation is unclear; however, it could be that differences in various signaling pathways for DNA damage repair underlie inherent radiosensitivity (not evaluated in the present study), or that genes that may be involved in inherent radiosensitivity were not included on cDNA array used for these studies.

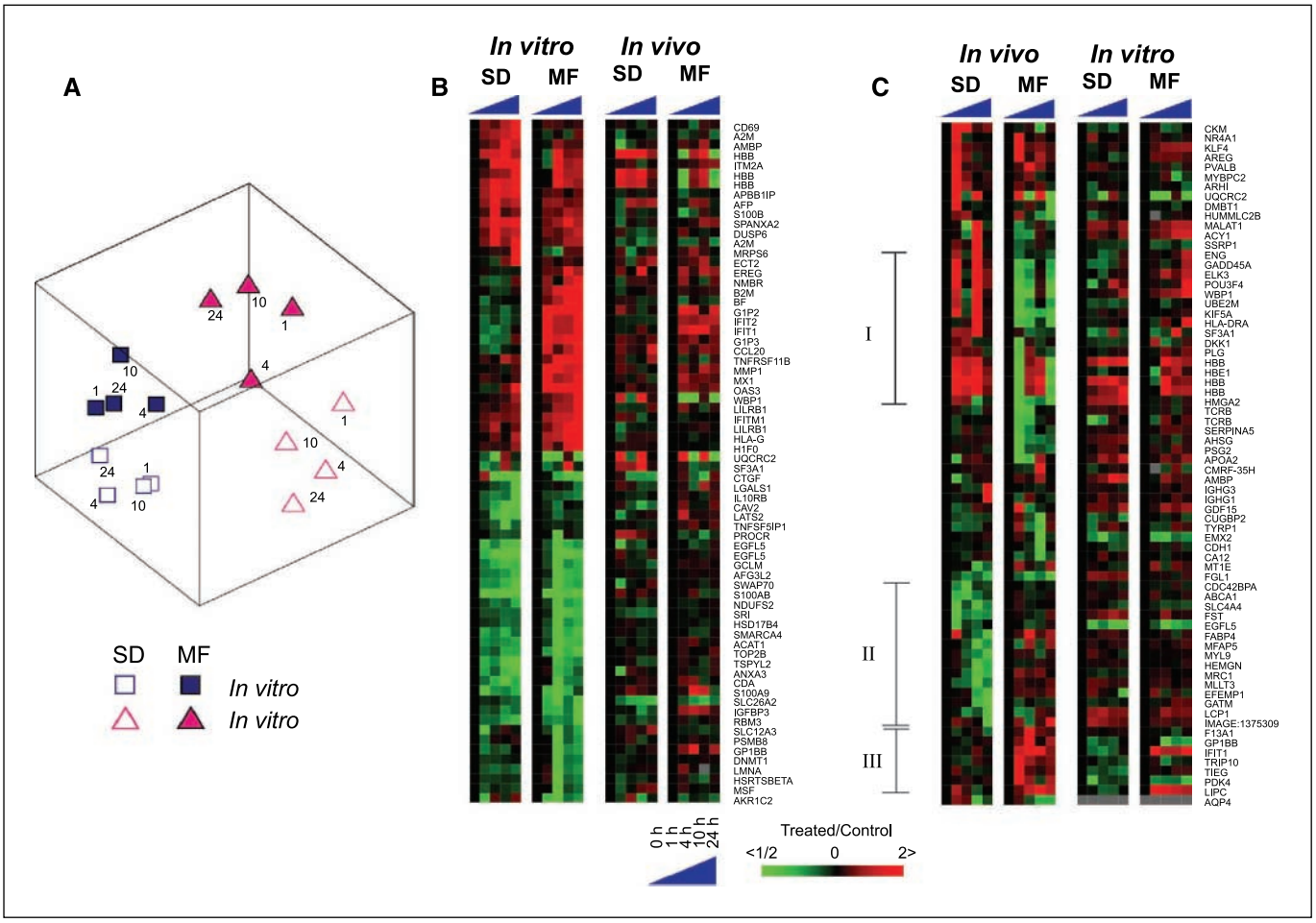

Figure 2. *A,* MDS of 6,018 genes (with no missing values) in DU145 cell line *in vitro* and *in vivo*. Distances between points represent 1−Pearson correlation coefficient. *B,* time-dependent changes in the top 70 genes up- and down-regulated were selected from the DU145 cells irradiated under *in vitro* conditions, and these same genes were analyzed for DU145 cells irradiated under *in vivo* conditions (both by SD and MF protocols) using unsupervised hierarchical cluster analysis. *C,* time-dependent changes in the top 70 genes up- or down-regulated were selected under *in vivo* radiation conditions, and these same genes were also analyzed in cells irradiated under *in vitro* conditions using unsupervised hierarchical cluster analysis.

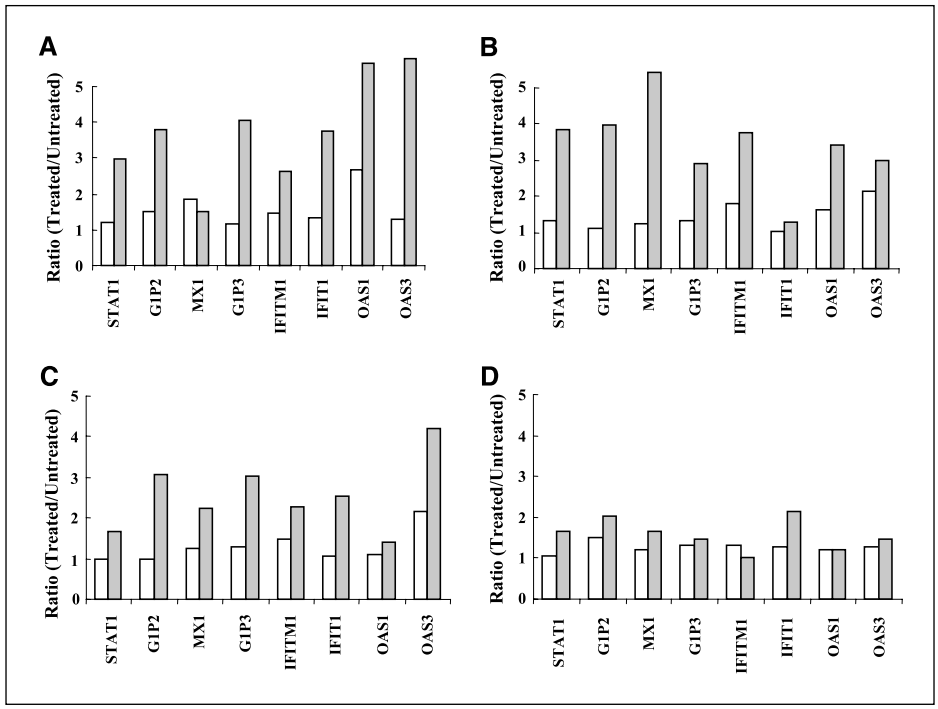

Figure 3. Eight IFN-associated genes, *STAT1, G1P2, MX1, G1P3, IFITM1, IFIT1, OAS1*, and *OAS3*, were selected, and the time point with the greatest ratio increase (compared with the untreated controls) was plotted for both SD and MF exposures. □, cells irradiated under the SD protocol; ■, cells irradiated using the MF protocol. *A,* MCF7 cells; *B,* SF539 cells; *C,* DU145 cells, *in vitro*; *D,* DU145 tumors, *in vivo*.

Inclusion of the MCF7 cell line was important for two reasons: (*a*) radiation is frequently part of the breast cancer treatment, and (*b*) MCF7 cells have a normal p53 and provide a measure of validation of the arrays used in this study. We have shown previously that MCF7 cells respond to other DNA-damaging agents (H_2O_2, menadione, and *t*-butylhydroperoxide) by up-regulation of a vigorous *p53* response using cDNA arrays (8). As other reports have shown, and as was observed in this study, radiation also induced a vigorous *p53* response (13). There was remarkable similarity between SD and MF with respect to *p53* and cell cycle–related genes in MCF7 cells. The MF protocol exhibited a lower induction of *p53*-related genes than the SD, consistent with the finding that MF survival was ~10-fold higher compared with SD (13). Neither SD nor MF treatment resulted in expression of *p53*-related genes for the SF539 and DU145 cells. This observation was expected for DU145 cells, which have a mutated *p53* (11). SF539 cells, on the other hand, have a normal *p53*;

Table 3. TGF-β/SMAD-associated genes induced by either SD or MF exposure in MCF7, SF539, or DU145

Gene	Accession number	MCF7 SD	MCF7 MF	SF539 SD	SF539 MF	DU145 SD	DU145 MF
CTGF	NM_001901	1.8	1.8	1.6	2.5	1.4	1.7
CYR61	NM_001554	1.1	1.4	1.3	4.4	1	1.4
COL6A3	NM_004369	2.1	2.1	1.6	3.3	1.5	1.5
COL6A1	NM_001848	1.6	1.6	1.4	2.1	1	1.3
COL6A2	NM_001849	2	1.3	1.6	2.9	1	1.2
COL4A1	NM_001845	1	1	1.3	2.5	1	1
TGFB1I4	NM_006022	1.5	1.0	1.3	1.9	1.2	1.2
TGFB2	NM_003238	1.3	1.4	1.3	2.3	1.9	1.0
THBS1	NM_003246	1.5	1.9	1.3	3.4	1	1
VEGF	NM_003376	1.2	1.2	1.6	2.4	1.1	1.3
VEGFC	NM_005429	1.6	1.9	1.2	1.9	1	1.2
EGR1	NM_001964	1.6	2.6	1.9	5.4	1	1.4
SERPINE1	NM_000602	1.7	2.7	1.8	3.4	1.4	1.5
PLAT	NM_000930	1.7	2.1	1.7	3.1	1.1	1.6
SMAD3	NM_005902	1.5	2.4	1.2	1.7	1	1.1
IGFBP5	NM_000599	2.0	3.4	1.3	1.2	1.3	1.2
IL-6	AF372214	1.4	1.5	1.9	12.5	1.3	2.1
TGFBR2	NM_003242	1.4	1.7	1.2	1.2	1.1	1.1
TGFBR3	NM_003243	1.6	1.9	1.7	1.4	1.0	1.0
Median		1.6	1.8	1.4	2.5	1.1	1.2

however, previous studies with this cell line indicate that the *p53* pathway is not responsive to radiation (11).

Radiation-induced gene expression patterns varied substantially between SD and MF. Taken in the context of the relevance of cell culture models to the treatment of tumors in the radiation oncology clinic, MF may be a more useful approach to identify genes and/or gene families potentially important to radiation cancer treatment. An excellent example of this was the selective induction after MF exposure of *IFN*-related genes in each cell line examined, which was not evident after SD treatment (at least over the 24 h analyzed). This group of genes has been implicated in inflammation and may be associated with radiation resistance through the induction of *STAT1* (14, 15). In fact, *STAT1* was identified as significantly up-regulated after MF radiation in MCF7, SF539, and the DU145 cells (Fig. 2*A–C*). The expression array data indicated that the response was robust because multiple *IFN*-induced genes (which are known to be transcriptionally activated by *STAT1*; refs. 14, 16) were identified in the cell lines examined. Induction of the *IFN*-based genes was not only up at a single time point but changed continuously throughout the times analyzed (Supplemental Fig. S3). Thus, the time-based analysis provides additional confidence that these genes were, in fact, influenced by MF. Although MF treatment was a strong inducer of *STAT1*, other investigators have shown that *STAT1* can be up-regulated by single fraction doses of radiation (17). Clave et al. showed that two IFN-related genes 1-8 d (*IFITM2*) and 9-27 (*IFITM1*) were induced in p53-deficient leukemic KG1a cells 24 and 48 h after 3 Gy radiation (18). Kita et al. (15) showed that RSa cells (human fibroblasts), which had elevated levels of 9-27 (*IFITM1*), were more radioresistant than RSa cells, which had no *IFITM1* expression. Khodarev et al. (14) showed that head and neck tumors, made resistant to radiation exposure of the parent cell line to 8 × 5 Gy as xenografts, followed by isolation of the surviving cells, had overexpressed mRNA of *STAT1α*, *STAT1β*, and other *IFN*-related genes. Finally, Sreekumar et al. (17) showed that STAT1 protein was elevated in LoVo colon carcinoma cells 24 h after a 6-Gy radiation exposure. At least one mechanism of *STAT1* elevation has been studied, and it seems that IFN-γ is not responsible because several reports have not found elevated IFN-γ after ionizing radiation (17). However, whether IFN-α or IFN-β is elevated by radiation has not been addressed. Thus, from the above studies, it is clear that in a number of different cell types, both *STAT1* mRNA and protein are elevated after exposure to radiation. The results of the present study confirm these reports and show that MF treatment is a much more robust means of elevating STAT1 and the *IFN*-related genes.

The consequences of *STAT1* elevation after radiation exposure could have profound effects on both normal and tumor cells. As mentioned, *STAT1* elevation in head and neck tumor cells leads to a more radiation-resistant response (14). It is intriguing that a recent report indicated that *STAT1* directly interacts with the ATM protein following DNA damage and, thus, may directly participate in the repair of DNA damage (19). On the other hand, *STAT1* elevation in tumor-associated macrophages has been linked with the suppression of T cell–mediated immune responses in tumors (20). *STAT1* knock-out mice are extremely sensitive to infection by microbial pathogens and viruses (21), and *STAT1* elevation is associated with increased apoptosis in a number of cell types (22). IFN-γ, in combination with retinoic acid, resulted in a strong induction of *STAT1* in breast cancer cells with an associated increase in cell killing (23). Thus, it is possible that elevation and activation of *STAT1* by radiation could affect directly the radiation sensitivity of tumor cells and also modify the immunologic surveillance system of the normal host cells. Drugs capable of altering *STAT1* levels, such as curcumin and fludarabine (22), have been reported to enhance radiosensitivity of tumor cells (24), suggesting that the *STAT1* pathway could be an interesting target to both improve radiosensitivity.

The TGF-β signaling pathway was strongly activated in the SF539 cells as a consequence of the radiation exposure (Table 3) and was most prominent after MF. The candidate gene most likely responsible for the TGF-β response was *TGF-β2*, which increased, in a time-dependent manner, after MF exposure (Table 3). SF539 cells are classified as human gliosarcoma cells (25), and it is interesting that the rat 9L gliosarcoma (like human gliomas) secretes TGF-β (26). Human gliomas are known to secrete both TGF-β1 and TGF-β2 (27). MCF7 cells had several genes up-regulated after radiation, which have been reported to be responsive to TFG-β (*IGFBP5* and *EGR1*; Table 3), and *TGF-β1* has been reported to increase in MCF7 cells 48 to 72 h after radiation exposure (28). *TGFβR2* and *TGFβR3* also increased in a time-dependent manner in MCF7 cells, but were not in either the SF539 or DU145 cells. DU145 cells were completely unresponsive with regard to the TGF-β pathway (Table 3), consistent with reports that later stage, androgen-independent, prostate tumor cell lines are resistant to the effects of TGF-β (29).

The results of this study clearly showed that genes elevated after radiation *in vitro* did not necessarily change when DU145 cells were exposed to radiation *in vivo*, suggesting that the tumor microenvironment (hypoxia, nutrient deficiencies, blood flow, etc.) can exert a major influence on genes that responded to radiation under *in vitro* conditions. In addition, hypoxia reduces the radiation effectiveness by 2.5- to 3-fold; hence, a 10-Gy exposure *in vivo* may only approximate a 2- to 3-Gy exposure under *in vitro* growth conditions. A completely different set of genes was identified for SD and MF in DU145 cells irradiated *in vivo* compared with DU145 cells irradiated *in vitro* (Fig. 2*B*). What was surprising was that there was greater differences between the SD and MF protocols for DU145 cells irradiated *in vivo* (Fig. 2*B*). Fig. 2*B* (*cluster I*) shows genes up-regulated after SD exposure but down-regulated after MF exposure. This pattern was not apparent for the cells irradiated under *in vitro* conditions. There was no discernible pattern in the genes identified after *in vivo* exposure, but *HBB* (hemoglobin β chain) was altered by both the SD and MF procedures, perhaps due to the presence of hypoxia. However, *HBB* was also somewhat elevated by radiation in the DU145 cells irradiated *in vitro*.

In summary, gene profiles after ionizing radiation exposure can vary extensively depending on whether the dose is delivered as a SD versus MF and whether the cells are grown under *in vitro* or *in vivo* conditions. MF radiation exposure was shown to alter several genes, selectively providing the opportunity to explore molecular target-directed interventions to enhance the tumor response to radiation.

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