

Human *In vivo* Radiation-Induced Biomarkers: Gene Expression Changes in Radiotherapy Patients

Sally A. Amundson,¹ Marcy B. Grace,² Christopher B. McLeland,² Michael W. Epperly,³ Andrew Yeager,³ Qimin Zhan,³ Joel S. Greenberger,³ and Albert J. Fornace, Jr.¹

¹Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland; ²Biological Dosimetry Team, Armed Forces Radiobiology Research Institute, Bethesda, Maryland; and ³University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Abstract

After initially identifying potential biomarkers of radiation exposure through microarray studies of *ex vivo* irradiated human peripheral white blood cells, we have now measured the *in vivo* responses of several of these biomarker genes in patients undergoing total body irradiation. Microarray analysis has identified additional *in vivo* radiation-responsive genes, although the general *in vivo* patterns of stress-gene induction appear similar to those obtained from *ex vivo* white blood cell experiments. Additional studies may reveal correlations between responses and either diagnosis or prognosis, and such *in vivo* validation marks an important step in the development of potentially informative radiation exposure biomarkers.

Introduction

Molecular biological markers of radiation response could potentially be of use for monitoring the progress of radiation therapy, and even for predicting outcome early in a treatment regimen. Biomarkers of radiation exposure could also be an important tool for triage of individuals in potentially exposed populations after a radiologic accident or “dirty bomb” incident (1). Most currently available radiation exposure biomarkers, such as those based on cytogenetic assays, do not provide the rapid results, however, that are optimal for such situations; therefore, there is increasing interest in the development of rapid, automation-capable, noninvasive tests for radiation exposure (reviewed in ref. 2). Microarray hybridization analysis provides an attractive avenue for the discovery of potentially informative radiation-exposure gene expression profiles. Once validated, a set of biomarker genes could be developed into a simple rapid assay on a sensitive quantitative real-time PCR (QRT-PCR) multiplex platform (3). Initial studies of *ex vivo* irradiated human peripheral white blood cells supported such an approach, documenting dose-response relationships with little variability among donors for a number of genes (4) and confirming the feasibility of multiplex QRT-PCR in this system (3). To develop a useful gene expression biomonitor, however, human gene expression changes occurring in response to irradiation *in vivo* must be measured directly. Patients undergoing total body irradiation (TBI) in preparation for allogeneic or autologous hematopoietic stem cell transplantation provide a suitable test population for such *in vivo* studies. The information gathered in such studies may also contribute to a future increased mechanistic understanding of individual response to therapy. We now report *in vivo* induction of expression

of the *CDKN1A*, *GADD45A*, and *DDB2* genes in a set of TBI patients, along with the identification of additional potential *in vivo* exposure marker genes.

Materials and Methods

Patients and RNA. Patients undergoing TBI at the Pittsburgh Cancer Institute were recruited into this study with informed consent for participation in University of Pittsburgh Cancer Institute (UPCI) Protocol 91–32. Blood was drawn into sodium citrate, frozen immediately in liquid nitrogen, and held at -80°C until processing. Samples were drawn within 2 hours before the initial radiation treatment, then 6 hours after the first 1.5-Gy fraction, at 24 hours, and at the same intervals after subsequent fractions (one patient only). Two daily 1.5-Gy fractions of X-rays were administered at 10 to 12 cGy/min 6 hours apart with a Siemens 10MV linear accelerator on 4 successive days. None of the patients had prior exposure to any genotoxic treatment for at least 2 weeks before the start of radiotherapy, although diagnosis and white blood cell differentials varied (Table 1). RNA was extracted from whole blood with two rounds of Trizol reagent (Invitrogen, Carlsbad, CA) and subsequent purification on RNeasy columns (Qiagen, Valencia, CA) in accordance with the instructions of the suppliers. Samples were monitored on the Agilent 2100 Bioanalyzer, and only patients yielding sufficient quantities of nondegraded RNA were included in this study.

Microarray Hybridization. One hundred micrograms of whole-cell RNA was labeled and hybridized to 6485-element cDNA microarrays [Gene Expression Omnibus (GEO) accession no. GPL1217], as described previously (5). In brief, probes were prepared by PCR amplification of Integrated Molecular Analysis of Genomes and their Expression (IMAGE) consortium clones and arrayed on poly-L-lysine-coated glass slides. Fluorescently labeled cDNA was prepared from whole-cell RNA by a single round of reverse transcription with Superscript II (Invitrogen) in the presence of fluorescent dNTP (Cy3 dUTP or Cy5 dUTP, Amersham Biosciences, Piscataway, NJ). Probes and targets were hybridized together for 16 hours in $3\times$ SSC at 65°C in the presence of the blockers human CoT1 DNA, yeast tRNA, and polydeoxyadenine. Hybridized slides were washed and then scanned with a laser confocal scanner (Agilent Technologies, Palo Alto, CA), and images were analyzed with the use of the ArraySuite 2.1 extensions [Dr. Y. Chen, National Human Genome Research Institute (NHGRI)] in the IPLab program (Scanalytics Inc., Fairfax, VA; refs. 6, 7). Expression ratios were normalized to those of a set of 88 internal controls (8) with a theoretical ratio of 1.0. The variance in the housekeeping set was used to determine the significance of expression changes after treatment. Minimum Information About a Microarray Experiment (MIAME)-compliant intensity, quality, and normalized ratio data for this series of experiments has been deposited in the Gene Expression Omnibus (GEO) database maintained by the National Center for Biotechnology Information (accession no. GSE1366). Uncentered Pearson clustering was done with tools developed by the Division of Computational Bioscience of the Center for Information Technology and the Cancer Genetics Branch of the National Human Genome Research Institute at the NIH.⁴

Quantitative Real-time PCR. Primer and probe sets for target genes were chosen with the aid of Primer Express (Applied Biosystems, Inc., Foster City, CA) and Oligo6 (Molecular Biology Insights, Inc., Cascade, CO), as described previously (3). FAM, HEX, and Texas Red were used as fluorochrome

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Note: S. Amundson is currently in the Center for Radiological Research, Columbia University Medical Center, New York, New York.

Requests for reprints: Sally Amundson, Columbia University, Center for Radiologic Research, 630 West 168th Street VC11-215, New York, NY 10032. Phone: 212-305-3911; Fax: 212-305-3229; E-mail: saa2108@columbia.edu.

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⁴ Internet address: <http://arrayanalysis.nih.gov>.

Table 1 Patient diagnoses and white blood cell differentials

| Diagnosis | Age | Gender | WBC differential before start of TBI (%) | | | | | | | Blasts |
|-----------|-----|--------|--|-------|-------|------|------|-----|--|--------|
| | | | Lymphs | Neuro | Bands | Mono | Baso | EOs | | |
| NHL* | 41 | M | 10 | 38 | 3 | 26 | | | | 18 |
| ALL | 44 | M | 28 | 62.6 | 0.4 | 7.7 | 0.4 | 1.4 | | |
| AML | 24 | M | 51 | 48 | | | | 1 | | |
| AML | 50 | F | 8 | 32 | 18 | 42 | | | | |
| HD | 26 | F | 6 | 88 | 0.1 | 5 | | 0.2 | | |
| MM | 51 | M | 7 | 80 | 3 | 2 | | 8 | | |
| MM | 66 | M | 10 | 80 | 0.7 | 10 | | 0.2 | | |
| MM | 52 | M | 8 | 70 | 15 | 6 | | | | |

Abbreviations: WBC, white blood cell; Lymphs, lymphocytes; Neuro, neutrophils; Mono, monocytes; Baso, basophils; EO, eosinophils; Blasts, blast cells; NHL, non-Hodgkin's lymphoma, ALL, acute lymphoblastic leukemia, AML, acute myeloid leukemia, HD, Hodgkin's disease, MM, multiple myeloma.

* RNA from this patient was used for the microarray analysis.

reporters for the hydrolysis probes, with their corresponding dark quencher (Black Quencher, Biosource International Laboratories, Inc., Camarillo, CA). One microgram of RNA was reverse transcribed with random hexamer primers, and 20 ng of the resulting cDNA were used per duplex PCR reaction on the iCycler iQ (Bio-Rad, Inc., Hercules, CA). Standard BR18S primers and an original probe were used for the internal control (3). Reaction conditions, data collection, and analysis with the iCycler iQ software, and construction of calibration curves were all as described previously (3). The cycle number at which the fluorescent signal crosses the detection threshold was denoted the threshold cycle (C_T). Gene target C_T values were normalized to the 18S ribosomal subunit internal standard with the formula $\Delta C_T = \text{target } C_T - \text{internal standard } C_T$. Relative gene expression levels after irradiation were similarly obtained from comparison with the preirradiation control C_T used as the reference standard (9). Each PCR reaction was run in triplicate in two or three independent experiments to obtain mean and SEMs of the relative expression values.

Results and Discussion

Microarray hybridization was carried out with RNA extracted from whole peripheral blood of a non-Hodgkin's lymphoma patient after the first two fractions of TBI. Because of the increasing ablation of white blood cells by treatment, insufficient RNA for microarray analysis was recovered after subsequent fractions. Replicate hybridizations in which the control and treated samples were labeled with the opposite fluorochrome (fluorochrome switching) gave generally consistent responses, and hierarchical clustering of the data from genes that were significantly altered after treatment (6) revealed a number of gene expression patterns (Fig. 1). A subset of genes was induced after the first fraction and then appeared to recover to their normal control levels, remaining unresponsive, or even decreasing after the second fraction. Interestingly, many of the genes with this pattern of rapid induction and damping, including *HSPCB*, *DNAJA1*, *FKBP4*, *HSPA1L*, *AHSA1*, and *HSPA8*, are involved in the heat shock response (marked by black bars in Fig. 1). This may suggest a coordinate regulation of these genes during the initial phases of the *in vivo* response to ionizing radiation. Using the Expression Analysis Systematic Explorer (EASE) analysis (10), we found genes for heat shock proteins to be significantly overrepresented among up-regulated genes on the arrays (EASE score, $P < 0.00973$; Fisher exact test, $P < 0.000443$), when compared with their representation among all hybridizing genes on the microarray. Other up-regulated genes showed a dose-responsive trend, with modest induction after the first fraction and increased induction after the second. Two of these genes that were not previously known to be radiation responsive, Fc fragment of IgG receptor 1a (*FCGR1A*) and chemokine (C-X-C) motif ligand 10 (*CXCL10*), were selected for further study, along with *CDKN1A*, *DDB2*, and *GADD45A*, which we had previously identified as radiation responsive in our *ex vivo* white blood cell irradiation studies (1, 4). The microarray results for these genes were confirmed

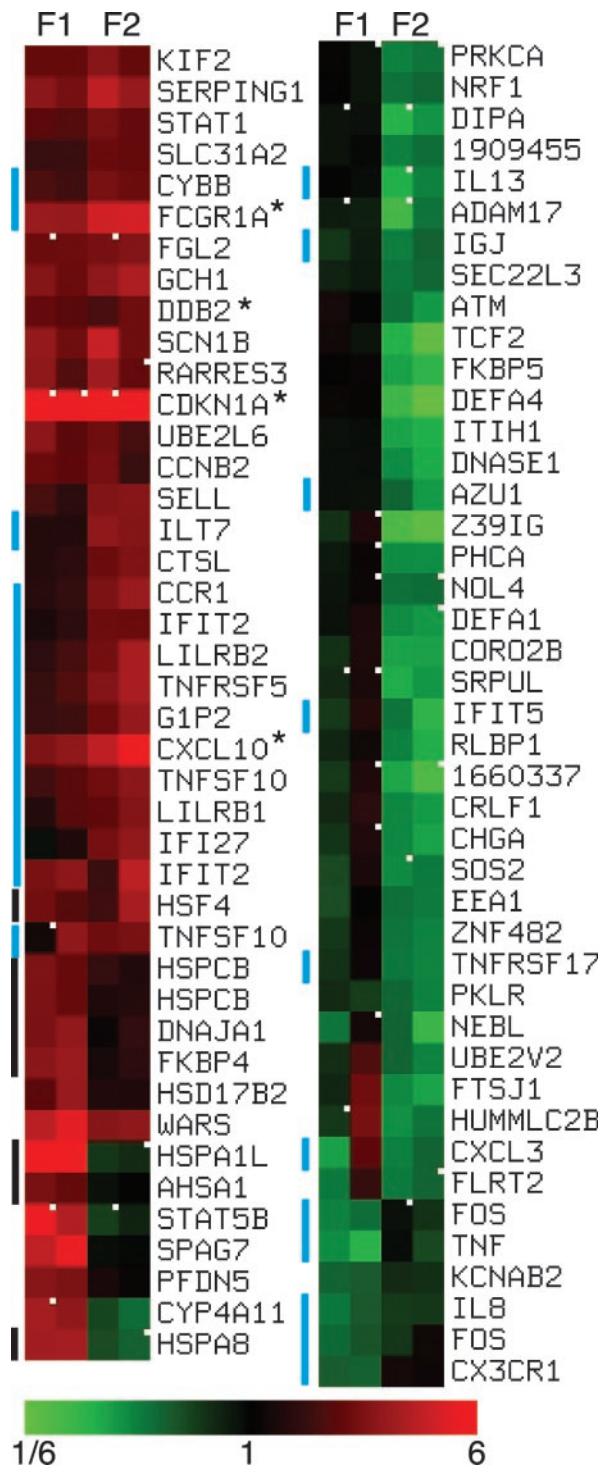
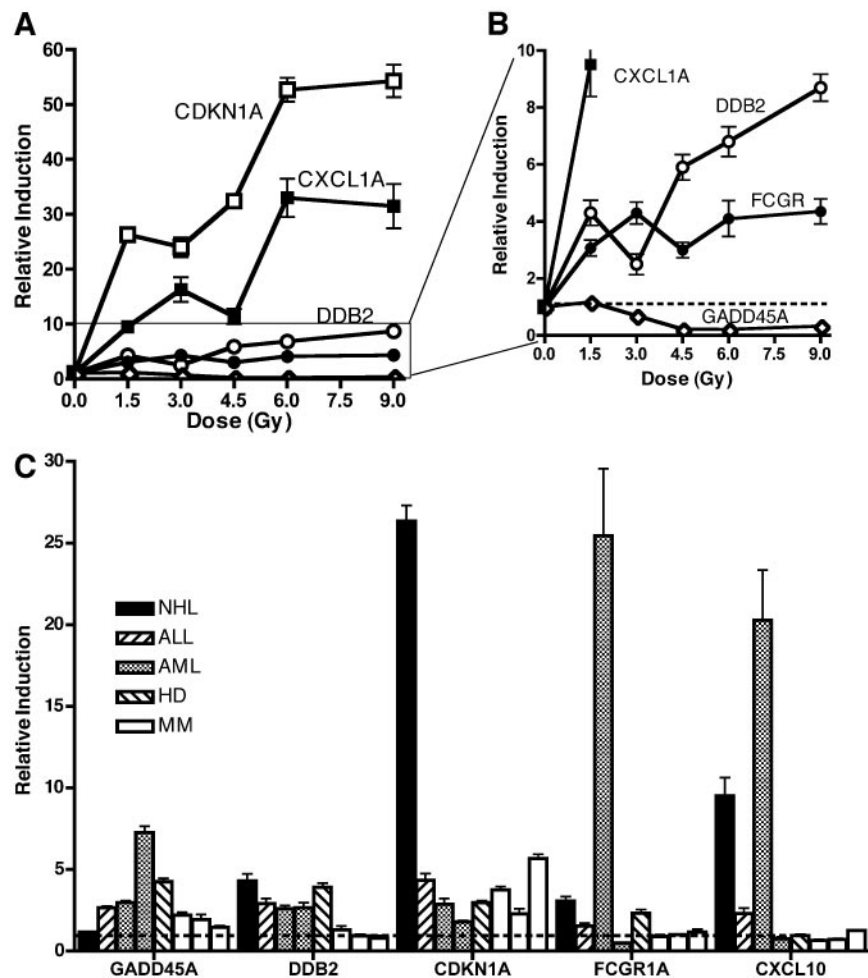


Fig. 1. Hierarchical clustering of genes regulated by *in vivo* irradiation. Illustrated are genes significantly up- or down-regulated in two or more microarray hybridizations with minimum red or green intensity of 500 and minimum mean quality of 0.5. White dot in the corner of a square, individual measurements with quality < 0.4 . Selection criteria, quality metrics, and significance determination from microarray housekeeping gene variance were as described previously (6, 7). F1, RNA harvested six hours after the first 1.5-Gy fraction. F2, RNA harvested after the second fraction, 24 hours after the beginning of treatment. The two columns represent repeated hybridizations with the control labeled with Cy5 (first column), then with Cy3 (second column). Color scale at the bottom of the figure, the relative expression after treatment relative to the preexposure control. Genes are listed by their Human Genome Organization (HUGO)-approved names, or by their IMAGE clone identification when HUGO names have not been assigned. *, genes studied by QRT-PCR. Black bars and blue bars, functional annotation of selected overrepresented Gene Ontology categories as determined by EASE (10). Black bars, heat-shock response (EASE score, $P < 0.00973$; Fisher exact test, $P < 0.000443$). Blue bars, immune and inflammatory responses (EASE score, $P < 6.8 \times 10^{-12}$; Fisher exact test, $P < 1.31 \times 10^{-12}$).

Fig. 2. Measurements by QRT-PCR. **A**, response through the course of TBI treatment of a non-Hodgkin's lymphoma patient. Data points are the average of two (*DDB2*) or three (all other genes) independent PCR runs carried out in triplicate for a total of six or nine determinations. Error bars, SEMs. The slopes of the dose response line for all of the genes were found to be significantly non-zero: *CDKN1A* (\square) $P = 0.0002$; *CXCL10* (\blacksquare) $P = 0.001$; *DDB2* (\circ) $P = 0.0002$; *FCGR1A* (\bullet) $P = 0.0035$; *GADD45A* (\diamond) $P = 0.0041$. **B**, the same data graphed as in **A**, with the Y axis expanded so that relative inductions of ≤ 10 -fold can be more clearly seen. Dashed line, the level of basal expression before the start of TBI. **C**, relative gene induction 6 hours after the first 1.5-Gy fraction of TBI in the original non-Hodgkin's lymphoma (NHL) patient and in seven additional patients diagnosed with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), Hodgkin's disease (HD) or multiple myeloma (MM). Columns, the mean of two or three independent triplicate PCR reactions; error bars, SEM. Dashed line, gene expression levels before the start of TBI.



by QRT-PCR (Table 2). The two techniques revealed the same pattern of gene expression changes after irradiation, although the QRT-PCR results for *CDKN1A* and *CXCL10* revealed the microarray signal compression often observed with high levels of differential expression (5).

Whereas relatively few genes were down-regulated after the initial radiation fraction, a strong down-regulation response was apparent after the second fraction. In contrast to our prior findings with irradiated cell lines (ref. 11 and manuscript in preparation),⁵ there is not a significant involvement of cell cycle regulatory genes among *in vivo* radiation-repressed genes. This may be a result of the quiescent nature of the unstimulated white blood cells used in this study in contrast to the transformed cell lines used in prior studies. Instead, the predominant functional gene ontology classification of both up- and down-regulated genes identified in this *in vivo* study was immune response (category overrepresented by EASE, $P < 6.8 \times 10^{-12}$; Fisher exact test, $P < 1.31 \times 10^{-12}$) and more specifically inflammatory response (EASE $P < 9.7 \times 10^{-6}$; Fisher exact test, $P < 1.3 \times 10^{-6}$). Genes with these classifications are indicated by the blue bars in Fig. 1. Such broad differences in the functional categories of responding genes highlight the contrast between whole-organism stress responses and *in vitro* responses and emphasize the importance of *in vivo* studies.

The expression of the genes in Table 2 was measured by QRT-PCR beyond the range of the microarray data to follow their responses through six fractions of TBI treatment. Whereas expression of *DDB2*,

CDKN1A and *CXCL10* (Fig. 2A) continued to increase with accumulating dose through the course of irradiation, *FCGR1A* expression leveled off above 3 Gy of accumulated dose (Fig. 2B). In contrast, *GADD45A*, which appeared unresponsive in the microarray data through the first two fractions, actually decreased after the later TBI fractions. Although we have observed consistent radiation-induction of this gene in previous *ex vivo* white blood cell studies (1, 4) and in many p53 wild-type cell lines (12), a subset of p53 wild-type cell lines does not induce *GADD45A* after ionizing radiation (13), and the stress-response regulation of this gene is known to be complex. The p53 signaling pathway was functional in this patient, however, because expression of two other p53-regulated genes, *DDB2* and *CDKN1A*, was induced. It should also be noted that the initial basal level of *GADD45A* in this patient was extremely high and within the range of the postinduction levels seen among the other patients.

Using QRT-PCR, we were able to compare the response of the set

Table 2 Comparison of microarray and real-time PCR measurements

| Gene | Dose 1 | | Dose 2 | |
|----------------|--------|---------|--------|---------|
| | Array* | QRTPCR† | Array* | QRTPCR† |
| <i>GADD45A</i> | 0.95 | 1.17 | 1.8 | 0.69 |
| <i>CDKN1A</i> | 5.4 | 26.0 | 7.5 | 24.0 |
| <i>DDB2</i> | 2.0 | 4.3 | 2.0 | 2.5 |
| <i>FCGR1A</i> | 2.9 | 3.1 | 4.4 | 4.3 |
| <i>CXCL10</i> | 2.5 | 9.5 | 5.5 | 16.3 |

* Mean of two determinations.

† Mean of nine determinations (six for *DDB2*).

⁵ Manuscripts in preparation.

of five genes with the first fraction of TBI in an additional seven patients with a range of different diagnoses (Table 1). Even this fairly small sampling revealed considerable heterogeneity in responses (Fig. 2C), perhaps, because of confounding factors not controlled for *in vivo*, or because of specific signal transduction abnormalities associated with the various disease states. It should be noted, however, that the majority of white blood cells contributing to the gene expression profiles are not themselves cancer cells. Nonetheless, some diagnosis-specific gene expression response trends were observed. For instance, the three multiple myelomas showed quite similar responses to each other and up-regulated only *CDKN1A* and, to a much lesser extent, *GADD45A*. All of the genes examined showed variability in responses among individuals, although *CDKN1A* was induced in all of the patients, and *DDB2* was induced to very similar levels in all of the patients except the three nonresponding multiple myelomas. *CDKN1A* is known to be an important effector of the p53-mediated G₁ arrest in response to many stresses (14). *DDB2*, on the other hand, links p53 response and DNA nucleotide excision repair (15), although its specific role in ionizing radiation response is less clear. These two genes were among our most promising exposure biomarkers from the initial *ex vivo* radiation-response work, and, despite some apparent heterogeneity of individual responses to *in vivo* irradiation, these remain the best of the tested candidates. Because both of these genes, as well as *GADD45A*, are regulated by p53 in response to ionizing radiation, this suggests a prominent role for the p53 pathway in the emerging gene expression biomarker signature. Variations in p53 response may also have implications for individual risk. The observed variations in overall individual responses substantiates our earlier prediction that individual genes would not have great value as either exposure or diagnostic biomarkers, but that the overall expression pattern of a set of genes would likely be more informative.

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