

Human *In vivo* Dose-Response to Controlled, Low-Dose Low Linear Energy Transfer Ionizing Radiation Exposure

Zelanna Goldberg,¹ David M. Rocke,² Chad Schwietert,¹ Susanne R. Berglund,¹ Alison Santana,¹ Angela Jones,¹ Jörg Lehmann,¹ Robin Stern,¹ Ruixiao Lu,³ and Christine Hartmann Siantar⁴

Abstract Purpose: The effect of low doses of low-linear energy transfer (photon) ionizing radiation (LDIR, <10 cGy) on human tissue when exposure is under normal physiologic conditions is of significant interest to the medical and scientific community in therapeutic and other contexts. Although, to date, there has been no direct assessment of the response of human tissue to LDIR when exposure is under normal physiologic conditions of intact three-dimensional architecture, vasculature, and cell-cell contacts (between epithelial cells and between epithelial and stromal cells).

Experimental Design: In this article, we present the first data on the response of human tissue exposed *in vivo* to LDIR with precisely controlled and calibrated doses. We evaluated transcriptional responses to a single exposure of LDIR in the normal skin of men undergoing therapeutic radiation for prostate cancer (research protocol, Health Insurance Portability and Accountability Act-compliant, Institutional Review Board-approved). Using newly developed biostatistical tools that account for individual splice variants and the expected variability of temporal response between humans even when the outcome is measured at a single time, we show a dose-response pattern in gene expression in a number of pathways and gene groups that are biologically plausible responses to LDIR.

Results: Examining genes and pathways identified as radiation-responsive in cell culture models, we found seven gene groups and five pathways that were altered in men in this experiment. These included the Akt/phosphoinositide-3-kinase pathway, the growth factor pathway, the stress/apoptosis pathway, and the pathway initiated by transforming growth factor- β signaling, whereas gene groups with altered expression included the keratins, the zinc finger proteins and signaling molecules in the mitogen-activated protein kinase gene group. We show that there is considerable individual variability in radiation response that makes the detection of effects difficult, but still feasible when analyzed according to gene group and pathway.

Conclusions: These results show for the first time that low doses of radiation have an identifiable biosignature in human tissue, irradiated *in vivo* with normal intact three-dimensional architecture, vascular supply, and innervation. The genes and pathways show that the tissue (*a*) does detect the injury, (*b*) initiates a stress/inflammatory response, (*c*) undergoes DNA remodeling, as suggested by the significant increase in zinc finger protein gene expression, and (*d*) initiates a "pro-survival" response. The ability to detect a distinct radiation response pattern following LDIR exposure has important implications for risk assessment in both therapeutic and national defense contexts.

Although there is increasing concern and interest on the effects of low-dose low-linear energy transfer ionizing radiation (LDIR) in humans, particularly with respect to secondary radiation carcinogenesis following therapeutic radiation, there

exists no direct evidence that doses in the range of 1 to 10 cGy have any biological effects whatsoever. Studies in this regard have long been stymied by ethical constraints that prohibit the intentional radiation of healthy tissue to simply examine the

Authors' Affiliations: ¹Department of Radiation Oncology, University of California Davis Cancer Center, Sacramento, ²Division of Biostatistics, ³Department of Statistics, University of California Davis, Davis, and ⁴Lawrence Livermore National Laboratory, Livermore, California

Received 11/30/05; revised 2/18/06; accepted 3/6/06.

Grant support: Office of Biological and Environmental Research, U.S. Department of Energy (DE-FG03-01ER63237), a Campus Laboratory Collaboration grant from UCOP, the Air Force Office of Scientific Research FA955006-0132 (Z. Goldberg, S. Berglund), the National Cancer Institute grant P30 CA093373-04 (D.M. Rocke), the National Institute of Environmental Health Sciences grant P42-ES04699 (D.M. Rocke), and the University of California Davis Health Systems (D.M. Rocke). The

work of C. Hartmann Siantar and J. Lehmann was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract W-7405-ENG-48.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Zelanna Goldberg, Department of Radiation Oncology, University of California Davis Cancer Center, 4501 X Street, Suite G-149, Sacramento, CA 95817. Phone: 916-734-8172; Fax: 916-454-4614; E-mail: zgoldberg@ucdavis.edu.

© 2006 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-05-2625

response to ionizing radiation. Attempts to address this information gap have approached the issue from two different angles.

Epidemiologic studies of exposed populations have evaluated global population exposure and health outcomes of the group, usually, although not exclusively, malignancy (1–5). A careful review of these studies reveals that, at the lowest exposure levels, no increase in detectable risk is observed. Whether this is a true threshold or simply an inherent limitation of the epidemiologic tools is the subject of vigorous debate.

The other approach to determining any potential biological activity in response to LDIR has been through classic *in vitro* cell biology, and more recently, molecular biology using genomics to characterize the response pattern. Classic end points such as cell lethality have not been sensitive enough to show any biological effects from such low dose exposures. Using genome-wide techniques on cell culture models, evidence of transcriptional changes following LDIR exposure has been shown. However, such responses are often subtle and inconsistent across models (6, 7).

Cell culture data of primary human keratinocytes and fibroblasts exposed to low or high doses of ionizing radiation have suggested a different response profile under each of these sets of circumstances (6, 7). Another group has examined brain samples from whole animal exposures and showed a transcriptomic response profile to LDIR that was qualitatively different than that seen at higher doses (8). Each of these studies has identified three distinct groups of transcripts: those that were altered by high- and low-dose exposures, those that were unique to high-dose, and those unique to the low-dose exposures. This suggests the possibility of a low-dose radiation response that is biologically dissimilar to higher doses of radiation.

While these results are intriguing, it remains unknown how applicable they are to the actual human response because the experiments were conducted on single cell layer, single cell type *in vitro* models, which fail to accommodate the known importance of cell-cell communication in radiation response. Furthermore, data suggesting that transcriptional changes of key response genes, e.g., protein kinase C, were markedly different in sensitivity and isoform when ionizing radiation was delivered *in vitro* or *in vivo* in a mouse model. This highlights the necessity for actual human data to begin assessment of the human responses to LDIR (9).

The studies described herein begin to address this information gap. We have developed a scientifically valid and ethically compliant model for direct evaluation of LDIR effects in normal human tissue, as well as a statistically validated tool to evaluate the response. Using genomic evaluation, with a priori selection of transcripts potentially sensitive to radiation-induced changes, we have (a) established the first data set of human responses to well-defined doses of LDIR, (b) developed a new methodology to address the natural heterogeneity of response in an unselected patient population, (c) developed a benchmark data set of transcript responses to LDIR in human skin, the first line of defense to radiation exposure from natural, medical, or terrorist sources, and (d) showed that although the *in vitro* evaluation of LDIR effects has identified genes and pathways activated by such exposure, it does not characterize the response in tissue. Although *in vitro* assays do serve an important role in the support of translational radiation response models, they cannot evaluate true radiation response in humans.

Materials and Methods

Patients. Men from the clinical practice of the first author, who are undergoing therapeutic radiation for the treatment of their early stage prostate cancer, were approached for possible trial participation. Informed consent was obtained (Institutional Review Board–approved, Health Insurance Portability and Accountability Act–compliant). At the time of patient treatment planning for their therapeutic ionizing radiation, an extra computed tomography scan was obtained with the patient in the treatment position. This scan was similar to the one used for therapeutic treatment planning purposes but was without any contrast material and with a slab of tissue-equivalent bolus material over the areas where biopsies were to be taken. The research scan was used for protocol treatment planning. The bolus material on the skin ensured that the region was in electronic equilibrium, reducing the radiation-absorbed dose uncertainty in the sample. The biopsy site was determined by PEREGRINE Monte Carlo dose calculations (10). On the first day of treatment, biopsy points were identified, and marked on the patient skin. TLD and/or MOSFET microdosimeters were placed on the marked sites to confirm the accuracy of the dose delivered. Biopsies were done and the samples were placed in RNeasy lysis buffer (Qiagen, TX) until processed. Each man underwent four full skin thickness punch biopsies (3 mm diameter cores) of the normal abdominal skin. The first biopsy was prior to any ionizing radiation and served as the control sample. The other three samples were obtained 3 hours following ionizing radiation at sites determined to have received 1, 10, or 100 cGy point dose at the skin surface. Biopsies were stored at -20°C until further processing.

Tissue disruption. Each biopsy sample was loaded into a Lysing Matrix D tube (Qiagen, Irvine, CA) containing 1 mL of a guanidine thiocyanate solution (5.1 mol/L guanidine thiocyanate, 50 mmol/L sodium citrate, 50 mmol/L EDTA, and 0.5% β -mercaptoethanol). Each sample underwent three rounds of bead beating in the Fastprep beadbeater instrument at a setting of 6 m/s (model 120A, Qiagen). Samples were kept on ice between each pulverization step. After tissue disruption, the samples were centrifuged at $12,000 \times g$ for 5 minutes at 4°C . The supernatant was removed and transferred to a new 1.5 mL Eppendorf tube.

RNA extraction and quantitation. An equal volume (1 mL) of phenol chloroform (5:1, pH 4.7) containing 0.5% *n*-lauroylsarcosine was added to each sample and incubated for 5 minutes at room temperature (Sigma, St. Louis, MO). The samples were centrifuged at $12,000 \times g$ for 10 minutes at 2°C . The aqueous phase was separated from the organic phase containing DNA and protein. An additional phenol chloroform (5:1, pH 4.7) extraction was done for further purification. One milliliter of isopropanol and 200 μL of 3 mol/L sodium acetate was added to each sample for precipitation and stored overnight at -20°C . The precipitated RNA samples were cleaned up using RNeasy columns (Qiagen, CA) by following the manufacturer's cleanup procedure. Modifications to the procedure included passing the RNA through the binding column twice to increase the binding efficiency and elution of the RNA with RNA storage solution (Ambion). The Turbo DNA-Free kit was used to remove any residual DNA contamination (Ambion). RNA integrity was verified with the Molecular Devices SpectraMax plus. The RNA samples averaged 1.6 μg of total RNA and the 260:280 nm ratios were between 1.7 and 1.9. The RNA was stored at -80°C .

Amplification and labeling. The RNA was processed for the Affymetrix arrays using their one-cycle *in vitro* transcription labeling kit (Affymetrix, Santa Clara, CA). Approximately 500 μg of mRNA was reverse-transcribed using a T7-Oligo(dT) promoter primer in the first-strand synthesis. Second-strand cDNA synthesis was done and the resulting double-stranded cDNA was purified. This cDNA served as a template in the *in vitro* transcription reaction. The *in vitro* transcription reaction was carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analogue/ribonucleotide mix. The biotinylated cRNA targets were then cleaned up and fragmented using standard

Affymetrix materials and protocols. The cRNA was hybridized to the Human Genome U133 Plus 2.0 arrays.

Data set selection. To address the complexity of the data which would be generated by the Affymetrix GeneChips; a priori, we selected genes and pathways to be examined. These genes or gene pathways were chosen based on the published literature in radiation response, mostly, but not exclusively, at higher doses. To address the variable temporal response between individuals, any gene in which there was evidence in the literature to justify it as a radiation-responsive gene was evaluated, as was that entire gene pathway. This was to capture the data from all of the sample cohorts who were evaluated (biopsies obtained) at a fixed point in time after ionizing radiation exposure, recognizing that the rate of the transcriptional response would vary across the population. Below, we review the chosen genes/pathways and their rationales.

Ionizing radiation has been shown to activate nuclear factor κ B through the degradation of inhibitor- κ B. Therefore, the signaling pathways upstream of this (extracellular signal-regulated kinase and mitogen-activated protein kinase) were also considered in the pathways analysis (11). Cell cycle-modulating genes such as cyclins, cdc25 phosphatases, GADD45, ATM, and ATR as well as the chk-1 and chk-2 transcripts were also examined. The TP53 pathway, with upstream and downstream signaling and effector transcripts, was examined, as was transforming growth factor- β and the ubiquitons (12, 13). Growth factors, such as basic fibroblast growth factor, and its downstream targets (RAS/MEK/mitogen-activated protein kinase/RSK) were assessed, as well as other apoptosis-related paths and survival signaling pathways (Akt/phosphoinositide-3-kinase, growth factors such as tumor necrosis factor, epidermal growth factor receptor, vascular endothelial growth factor, and their downstream effectors Ras, Raf-ERK) were evaluated (14–18). DNA repair genes, in particular, the Rad 52 epistasis group, have been reported by some to be radiation-responsive, although others have questioned this relationship given the constituent expression of DNA repair proteins (6, 19). Inflammatory mediators such as cyclooxygenase-2, prostaglandin E₂, and interleukins have also been reported (20). We also selected some tissue-specific genes with structural functions (i.e., keratins) given that we anticipated some tissue reorganization in response to ionizing radiation. This type of response was reported by Ding et al. with increased expression of cytoskeleton components, ANLN and KRT15, from normal human fibroblasts (6). DNA damage/remodeling via topoisomerase and zinc finger proteins has also been reported and hence these transcripts were also examined (21) along with genes involved in cell-cell signaling, such as GRAP2 and GPR51, and cytoskeleton, such as ANLN and KRT15.

Therefore, there were seven preidentified gene pathways analyzed. They include the Akt/phosphoinositide-3-kinase pathway (14, 18, 22, 23), chemokine pathway (9, 24), fibronectin pathway (14, 24, 25), growth factor/insulin pathway (9), inflammation pathway (9, 14, 20, 22, 24, 26), stress/apoptosis pathway (9, 13, 14, 20, 22–24, 27), and the transforming growth factor- β /cyclin/ubiquitin pathway (11, 13, 14, 18, 22, 24, 25, 28). There were 19 gene groups analyzed (see Table 1).

Statistical methods. The data for this analysis consist of 32 Affymetrix HGU133 Plus 2.0 GeneChips, four from each of eight patients, at doses of 1, 10, and 100 cGy, as well as a preexposure control at 0 cGy. The HGU133 Plus 2.0 contains 1,354,896 probes divided into 54,675 probe sets, which have been summarized using the GLA expression index (29), although the results are very similar if RMA is used instead (30).

The analysis looked for dose-response patterns that were linear in dose either on the original cGy scale, or using a modified log dose (MLD) in which the positive exposures are coded as 0, 1, and 2, and the 0 exposure is coded as -1 , as if it were 0.1 cGy. Because we expected (and found) variability between individuals as to timing of response on pathways and on the type of transcript within classes of genes, we did an analysis in which we aggregated results across individuals and transcripts in order to amplify possibly weak signals, without inducing false-positives.

Full details are given in Rocke (31) and Rocke et al. (32), but in summary, for each probe set and each individual, we conducted a linear regression and computed the t statistic testing for whether there is up-regulation, down-regulation, or neither. The t score will be positive if there is a trend towards up-regulation, and negative if there is an opposite trend. We examined the entire collection of t scores for a given gene group or pathway to determine the possibility of a weak signal in the direction of up-regulation or in the direction of down-regulation. We did this by testing the hypothesis that the collection of t scores had a mean of zero using the one-sample t test or a median of zero using the Wilcoxon rank-sum test. If this was rejected, it indicated that there was a trend towards up-regulation with dose of the gene group or pathway if the t scores are biased in a positive direction or towards down-regulation if the t scores are biased in a negative direction. Because of possible correlations in these test statistics, the most reliable P values are provided by resampling by repeatedly sampling random groups of transcripts of the same size. The empirical P value is the fraction of cases in the resampled gene sets in which the test statistic was more significant than the actual test statistic from the gene group or pathway. These were always two-sided P values.

Results

The Affymetrix HGU133 Plus 2.0 array platform shows the advantages and disadvantages of whole genome assays. The greatest advantage of genome-wide assays is the comprehensiveness. Not only are the genes represented on the Affymetrix chip, but each gene is represented by multiple exons. The disadvantage is that with such a large number of probe sets, there will be many apparently significant changes that occur strictly by chance, and to control for this requires a very high bar for declaring differential expression to be significant. The model that uses all 32 expression values for each probe set and tests for significant patient effects and dose-response effects would perhaps be sensitive enough if the response were highly consistent in time across individuals, but if the response is varied or diffuse, this may not yield significant results. In the 54,765 probe sets, and using the methods of Rocke (31), we found no cases in which the overall dose-response coefficient is significant at a 5% FDR level, and 11 where the FDR-adjusted significance level is better than 10% (31, 33, 34). The patient effect, however, was significant at the 5% FDR-adjusted level for 13,514 of the 54,675 probe sets, and significant at the 10% FDR-adjusted level for 18,605 of the probe sets. This shows how important the individual variation is in gene expression in radiation response. Thus, we chose to analyze the results using the methods of Rocke et al. (32) and using the gene groups and pathways identified in the literature, as described in Materials and Methods above (32).

Tables 1 and 2 show the results for the preidentified gene groups and pathways. Of the 19 gene groups, 7 have empirical $P < 0.0125$ on either the regression on dose or the regression on MLD, using either the t test or the Wilcoxon test, a number far exceeding chance (the expected number out of 19 is <1 , and the probability of getting 7 significant by chance is <0.0003). The largest effects are for the BCL-6 group, keratins, protein disulfide isomerases, S100, and zinc finger proteins.

For the pathways, five of seven pathways show significant difference at the 0.0125 level in dose or MLD using either the t test or Wilcoxon test, with an expected number $\ll 1$, and the probability of having five or more significant being ~ 0.00006 . The effect is strongly exemplified by transforming

Table 1. Results for preidentified gene groups

Gene group	No. of probe sets	Direction of effect	ToTS <i>t</i> , <i>P</i> value (dose/MLD)	ToTS <i>W</i> , <i>P</i> (dose/MLD)	Empirical <i>t</i> , <i>P</i> (dose/MLD)	Empirical <i>W</i> , <i>P</i> (dose/MLD)
BAG	9		0.1480 0.0251	0.0397 0.0332	0.1865 0.0195	0.0460 0.0420
BCL 2	47		0.2389 0.6898	0.6906 0.8323	0.3410 0.7160	0.7400 0.9960
BCL 6	8	Up	0.0005 0.0091	0.0005 0.0039	0.0005 0.0035	0.0000 0.0020
BMPs	7		0.2181 0.5623	0.2227 0.5434	0.2705 0.6275	0.2360 0.5440
BMP receptor	8		0.4613 0.6515	0.7660 0.3828	0.5320 0.6815	0.7360 0.4480
Cyclins	123		0.1200 0.1347	0.3530 0.2394	0.2370 0.1165	0.3400 0.3560
Cytokines	86	Up	0.0169 0.0184	0.0013 0.0074	0.0365 0.0140	0.0020 0.0260
GADD 45	6		0.0563 0.0247	0.0668 0.0380	0.0555 0.0175	0.0820 0.0500
Heat shock	58		0.7280 0.5784	0.6620 0.3377	0.7725 0.6030	0.7100 0.2740
Interleukin	147		0.9877 0.6330	0.4346 0.9583	0.9935 0.6395	0.5540 0.8080
Keratins	101	Down	0.0020 2.99E-14	7.21E-15 2.20E-16	0.0070 0.0000	0.0000 0.0000
Mitogen-activated protein kinase	131	Up	0.0020 0.0143	0.0025 0.0164	0.0105 0.0140	0.0040 0.0440
Protein disulfide isomerase	8	Down	0.0139 0.0121	0.0240 0.0064	0.0150 0.0070	0.0420 0.0000
RAD 51	10		0.4673 0.0411	0.2794 0.1495	0.5340 0.0365	0.3220 0.1900
S100	21	Down	0.0144 0.0001	0.0015 0.0001	0.0020 0.0000	0.0002 0.0000
Serine/threonine kinase	73		0.3329 0.3778	0.9555 0.9997	0.4425 0.4090	0.9340 0.8860
Tumor necrosis factor	109		0.8158 0.8300	0.2513 0.8376	0.8710 0.8505	0.3520 0.6220
Topoisomerase	14		0.2022 0.0541	0.1399 0.1561	0.2490 0.0500	0.1960 0.2520
Zinc finger	799	Up	2.68E-05 4.17E-06	7.79E-5 2.20E-16	0.0135 0.0000	0.0000 0.0000

NOTE: The empirical *P* values are based on simulations of 2,000 trials for the *t* test and 1,000 trials for the Wilcoxon (*W*) test. Rows in bold are gene groups for which there is good evidence of differential expression. Other gene groups with borderline significance include BAG, GADD 45, RAD51, and topoisomerase. In each cell, the upper *P* value is for regression on dose, and the lower *P* value for the regression on MLD.

growth factor- β /cyclin/ubiquitin pathway activated in response to stress/apoptosis, inflammation, growth factor/insulin, and Akt/phosphoinositide-3-kinase pathways. Of the seven preidentified gene pathways analyzed, five showed a significant dose-response up-regulation to LDIR exposure. The two that did not were the chemokine and fibronectin pathways.

There were 19 gene groups analyzed, of which seven showed radiation-responsive differential gene expression. The groups that were up-regulated include BCL6 (14), cytokines (9, 23, 25, 28), mitogen-activated protein kinases (14, 20, 23, 24, 35), and

zinc finger proteins (27). Those that were down-regulated include the keratins (25), the protein disulfide isomerases (15), and S100 (36). Those that did not change significantly included BAG (37), BCL2 (38), BMPs (39, 40), BMP receptors (40), cyclins (41, 42), GADD45 (43), heat shock (41, 44), interleukins (45), RAD51 (19), serine/threonine kinases (46, 47), tumor necrosis factor- β (48), and topoisomerases (21, 49). The radiation-responsive gene groups and pathways identified in our human biopsy studies have key regulatory roles in cell cycle arrest, DNA repair, inflammation, and apoptosis.

Table 2. Results for preidentified pathways

Pathway	No. of probe sets	Direction of effect	ToTS <i>t</i> , <i>P</i> (dose/MLD)	ToTS <i>W</i> , <i>P</i> (dose/MLD)	Empirical <i>t</i> , <i>P</i> (dose/MLD)	Empirical <i>W</i> , <i>P</i> (dose/MLD)
Akt/phosphoinositide-3-kinase pathway	99	Up	0.0014 0.1333	0.0002 0.0006	0.0050 0.1335	0.0020 0.0020
Chemokine pathway	79		0.2716 0.8565	0.4047 0.2649	0.3895 0.8730	0.4780 0.4080
Fibronectin pathway	196		0.0206 0.0497	0.0649 0.0169	0.0990 0.0455	0.1020 0.0460
Growth factor/insulin pathway	208	Up	0.0001 0.5044	0.0072 0.0083	0.0015 0.5405	0.0100 0.0220
Inflammation pathway	78	Up	0.0365 0.0687	0.0013 0.1187	0.0785 0.0810	0.0020 0.1780
Stress/apoptosis pathway	151	Up	0.9343 0.0359	0.0739 0.0029	0.9500 0.0420	0.0400 0.0070
Transforming growth factor-β/ cyclin/ubiquitin pathway	355	Up	0.0003 0.0014	0.0005 0.0012	0.0095 0.0005	0.0000 0.0010

NOTE: The empirical *P* values are based on simulations of 2,000 trials for the *t* test and 1,000 trials for the Wilcoxon (*W*) test. Rows in bold are pathways for which there is good evidence of differential expression. In each cell, the upper *P* value is for regression on dose, and the lower *P* value for the regression on MLD.

In most cases, gene groups and pathways that were significant over all patients were also significant for individual patients, although this did not always occur, which is consistent with the assumption of interindividual variability in response. In many cases, the individual tests were not significant, consistent with the lower statistical power of a test based on one individual, and in only a few cases was the effect significant in the opposite direction. This overall consistency gives additional confidence in the realism of the results. Details are given in Table 3.

As noted above in Materials and Methods, all expression data were analyzed using a model of linear dose-response. Thus, significant results indicate linear response in expression up (or down) with dose over the range of 1 to 100 cGy. Those gene groups or pathways that were not statistically significant on a

linear model had variable responses across dose and could not be modeled by a linear fit.

The microarray data from this study are available in Minimum Information About a Microarray Gene Experiment-compliant format from the National Center for Biotechnology Information Gene Expression Omnibus repository and include the 32 CEL files and the probe set summary data for all 32 arrays and 54,675 probe sets in a Microsoft Excel file, as well as the experimental metadata. The data sets defining the gene groups and pathways, and the programs in the R language that were used to process the data are available as supplementary files from the Journal web site. All of these files are also available on the second author's web site, <http://www.idav.ucdavis.edu/~dmrocke>.

Table 3. Results for individual patients for significant gene groups and pathways

Gene group or pathway	Direction of effect	No. of patients up-regulated	No. of patients down-regulated	No. of patients not significant
BCL 6	Up	4	0	4
Cytokines	Up	3	1	4
Keratins	Down	8	0	3
Mitogen-activated protein kinase	Up	2	0	6
Protein disulfide isomerase	Down	0	2	6
S100	Down	0	3	5
Zinc finger	Up	6	1	1
Akt/phosphoinositide-3-kinase pathway	Up	5	0	3
Growth factor/insulin pathway	Up	4	0	4
Inflammation pathway	Up	4	1	3
Stress/apoptosis pathway	Up	3	1	4
Transforming growth factor- β /cyclin/ubiquitin pathway	Up	3	0	5

Discussion

Ionizing radiation is a naturally ubiquitous toxicant. Background radiation is now supplemented through most peoples' lives by industrial exposures and medical exposures for diagnostic and/or therapeutic reasons. Furthermore, in the era of intensity-modulated radiation therapy, larger volumes of normal tissue are exposed to low doses of ionizing radiation. There is also a newly elevated threat from radiologic terrorism, driving the need to determine the biological effects, if any, of low levels of radiation exposure in the human.

By devising a dosimetrically sound sampling method on individuals who are otherwise healthy but receiving therapeutic localized radiation for early stage prostate cancer, we have developed a new model system whereby direct evaluation of radiation effects can be determined. This model is ethically sound, Institutional Review Board–approved, and allows real-time sampling of human tissue after *in vivo* radiation exposures of precisely calibrated radiation doses. This model is therefore unique in allowing the evaluation of whole tissue effects when exposure is under normal physiologic conditions.

Biopsies were obtained from patients 3 hours after an acute exposure to LDIR. This time point was chosen for practical considerations. More recently, emerging data have shown that low dose-response may be better defined at a later time point (6, 7, 35). Although none of the published data have examined an *in vivo* human model, it is reasonable to question whether a greater response may be seen after a longer interval. If so, this would suggest that secondary tissue effects, i.e., cellular responses to signaling from neighboring cells may be as important in tissue response to LDIR as the initial radiation deposition within the cells. Studies to better characterize the temporal response to LDIR in tissue, in a separate cohort of men, are ongoing and will be reported separately.

Using genome-wide analysis, preselected targeted genes, and pathways from *in vitro* studies, we have shown that human skin initiates a transcript program to enhance survival whereas simultaneously undergoing an inflammatory and stress response. Whether these pathways are all activated in the same cells within the tissue, or represent responses from different tissues (i.e., epithelial versus stromal versus vasculature) cannot be determined from this data set. Laser capture dissection of the biopsied tissue for separate analysis of each cell type can be undertaken now that benchmark data have been compiled showing that low-dose ionizing radiation is biologically active in a definable way in the human. These data were analyzed on the assumption of a linear dose-response. Nonlinearity at the lowest doses cannot be excluded.

The tissues sampled and examined were evaluated as whole tissue. Therefore, the genomic signature is a summative evaluation of the responses of thousands of cells of several lineages, containing both epithelial and stromal cells. Some of the cells would represent lineages that are classically “radiation-sensitive” whereas others were radiation-resistant. Although this model does not allow precise mechanistic intervention, it does offer a comparative advantage in giving us tissue level response which will be the defining level of human response—i.e., the tissue level response is more than just the summative whole of individual cell responses. The doses examined in this study, and those of interest to the radiation risk community are well below those that are frankly cytotoxic. Therefore, the tissue

response will inevitably be an amalgamation of the primary response of the cell and the modulating effects of the cells surrounding it, both of the same lineage and others. This amalgamated response therefore incorporates the bystander response and represents the organismal response to the radiation insult. Aside from better determining the extent of true human response to low doses of ionizing radiation, it also provides a potential biosignature for evaluating skin exposure to low doses of ionizing radiation, well below doses expected to produce symptomatic radiation effects.

We have devised a methodology whereby an individual biopsy from these same sample types can be processed for both RNA and protein with both collected for analysis. This will allow a matched genomic and proteomic evaluation.⁵ These evaluations are ongoing, but will further extend our ability to define the tissue level response of human skin to low doses of ionizing radiation.

Defining the effects of low-dose radiation in humans requires a model with diverse genetic background with the subsequent statistical tool development to identify the effect of the agent above the background diversity of the study population. Therefore, we did these benchmark studies on an otherwise unselected population and devised statistically rigorous tools to address human complexity. Limitations of a sample set consisting of males with early stage prostate cancer are a clear lack of gender diversity and a limited age range. Radiation sensitivities in other populations, specifically the young and fetus' *in utero*, cannot be extrapolated from these data. This data set included samples from six Caucasians and two African-Americans.

The skin forms the first barrier of the body to radiation exposure and is an easy tissue to biopsy for scientific evaluation, clinical studies and potential field application for population screening for radiation exposure. It is a dynamic, complex system with multiple cell types. Because only healthy skin was biopsied (not previous scars or cancerous lesions), this radiation profile may represent a generalizable biosignature. The age distribution within the patient population sampled does not allow an analysis of age effects, and, as noted above, gender effects cannot be excluded. Relating this transcriptomic signature to a functional tissue end point for human health is part of an ongoing project.

These benchmark data show for the first time that low doses of radiation have an identifiable biosignature in human tissue, irradiated *in vivo* with normal intact three-dimensional architecture, vascular supply, and innervation. The genes and pathways show that the tissue (a) detects the injury, (b) initiates a stress/inflammatory response, (c) undergoes DNA remodeling, suggested by the significant increase in genes for zinc finger proteins being expressed, and (d) initiates a “pro-survival” response.

Acknowledgments

We thank the patients who volunteered and all of the radiation therapists in the Department of Radiation Oncology, University of California Davis Cancer Center, UCDCS. The authors are grateful for the helpful comments from the referees of the original version of this article.

⁵ Berglund S.R., Schwietert C.W., Jones A., Stern R.L., Lehmann J., and Goldberg Z. Optimized methodology for dual extraction of RNA and protein from small human skin biopsy, submitted for publication.

References

1. Boice J, Jr. Study of health effects of low-level radiation in USA nuclear shipyard workers. *J Radiol Prot* 2001;21:400–3.
2. Brooks AL. Developing a scientific basis for radiation risk estimates: goal of the DOE Low Dose Research Program. *Health Phys* 2003;85:85–93.
3. Cohen BL. Test of the linear no-threshold theory of radiation carcinogenesis in the low dose, low dose rate region. *Health Phys* 1995;68:157–74.
4. Ghiassi-nejad M, Mortazavi SM, Cameron JR, Niroomand-rad A, Karam PA. Very high background radiation areas of Ramsar, Iran: preliminary biological studies. *Health Phys* 2002;82:87–93.
5. Kostyuchenko VA, Krestinina L. Long-term irradiation effects in the population evacuated from the east Urals radioactive trace area. *Sci Total Environ* 1994;142:119–25.
6. Ding LH, Shingyoji M, Chen F, et al. Gene expression profiles of normal human fibroblasts after exposure to ionizing radiation: a comparative study of low and high doses. *Radiat Res* 2005;164:17–26.
7. Franco N, Lamartine J, Frouin V, et al. Low-dose exposure to γ rays induces specific gene regulations in normal human keratinocytes. *Radiat Res* 2005;163:623–35.
8. Yin E, Nelson DO, Coleman MA, Peterson LE, Wryobek AJ. Gene expression changes in mouse brain after exposure to low-dose ionizing radiation. *Int J Radiat Biol* 2003;79:759–75.
9. Varadkar PA, Krishna M, Verma NC. Dose-dependent differential expression of protein kinase C isozymes in mouse lymphocytes after γ irradiation *in vivo* and *ex vivo*. *Radiat Res* 2003;159:453–7.
10. Lehmann J, Stern RL, Daly TP, et al. Dosimetry for quantitative analysis of low dose ionizing radiation effects on humans in radiation therapy patients. *Radiat Res* 2006;165:240–7.
11. Li N, Karin M. Ionizing radiation and short wavelength UV activate NF- κ B through two distinct mechanisms. *Proc Natl Acad Sci U S A* 1998;95:13012–7.
12. Barcellos-Hoff MH. How tissues respond to damage at the cellular level: orchestration by transforming growth factor- β (TGF- β). *BJR Suppl* 2005;27:123–7.
13. MacCallum DE, Hall PA, Wright EG. The Trp53 pathway is induced *in vivo* by low doses of γ radiation. *Radiat Res* 2001;156:324–7.
14. Gu Q, Wang D, Wang X, et al. Basic fibroblast growth factor inhibits radiation-induced apoptosis of HUVECs. II. The RAS/MAPK pathway and phosphorylation of BAD at serine 112. *Radiat Res* 2004;161:703–11.
15. Prasad SC, Soldatenkov VA, Kuettel MR, et al. Protein changes associated with ionizing radiation-induced apoptosis in human prostate epithelial tumor cells. *Electrophoresis* 1999;20:1065–74.
16. Sartor CI. Epidermal growth factor family receptors and inhibitors: radiation response modulators. *Semin Radiat Oncol* 2003;13:22–30.
17. Shonai T, Adachi M, Sakata K, et al. MEK/ERK pathway protects ionizing radiation-induced loss of mitochondrial membrane potential and cell death in lymphocytic leukemia cells. *Cell Death Differ* 2002;9:963–71.
18. Sartor C. Mechanisms of disease: radiosensitization by epidermal growth factor receptor inhibitors. *Nat Clin Pract Oncol* 2004;1:22–30.
19. Bishay K, Ory K, Olivier MF, et al. DNA damage-related RNA expression to assess individual sensitivity to ionizing radiation. *Carcinogenesis* 2001;22:1179–83.
20. Tessner TG, Muhale F, Schloemann S, et al. Ionizing radiation up-regulates cyclooxygenase-2 in I407 cells through p38 mitogen-activated protein kinase. *Carcinogenesis* 2004;25:37–45.
21. Forrester HB, Radford IR. Ionizing radiation-induced chromosomal rearrangements occur in transcriptionally active regions of the genome. *Int J Radiat Biol* 2004;80:757–67.
22. Dent P, Yacoub A, Contessa J, et al. Stress and radiation-induced activation of multiple intracellular signaling pathways. *Radiat Res* 2003;159:283–300.
23. Kumar P, Miller AI, Polverini PJ. p38 MAPK mediates γ -irradiation-induced endothelial cell apoptosis, and vascular endothelial growth factor protects endothelial cells through the phosphoinositide 3-kinase-Akt-Bcl-2 pathway. *J Biol Chem* 2004;279:43352–60.
24. Lee SA, Dritschilo A, Jung M. Impaired ionizing radiation-induced activation of a nuclear signal essential for phosphorylation of c-Jun by dually phosphorylated c-Jun amino-terminal kinases in ataxia telangiectasia fibroblasts. *J Biol Chem* 1998;273:32889–94.
25. Kim AH, Lebman DA, Dietz CM, et al. Transforming growth factor- β is an endogenous radioresistance factor in the esophageal adenocarcinoma cell line OE-33. *Int J Oncol* 2003;23:1593–9.
26. Zimmermann JS, Kumpf L, Kimmig B. Variability of individual normal tissue radiation sensitivity. An international empirical evaluation of endogenous and exogenous response modifiers. *Strahlenther Onkol* 1998;174:16–9.
27. Ahmed MM. Regulation of radiation-induced apoptosis by early growth response-1 gene in solid tumors. *Curr Cancer Drug Targets* 2004;4:43–52.
28. Pajonk F, McBride WH. Ionizing radiation affects 26S proteasome function and associated molecular responses, even at low doses. *Radiat Res* 2001;115:203–12.
29. Zhou L, Rocke D. An expression index for Affymetrix GeneChips based on the generalized logarithm. *Bioinformatics* 2005;21:3983–9.
30. Irizarry RA, Bolstad BM, Collin F, et al. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 2003;31:e15.
31. Rocke DM. Design and analysis of experiments with high throughput biological assay data. *Semin Cell Dev Biol* 2004;15:708–13.
32. Rocke DM, Goldberg Z, Schweitert C, Santana A. A method for detection of differential gene expression in the presence of inter-individual variability in response. *Bioinformatics* 2005;21:3990–2.
33. Reiner A, Yekutieli D, Benjamini Y. Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* 2003;19:368–75.
34. Benjamini Y, Hochberg Y. Controlling the false discovery rate—a practical and powerful approach to multiple testing. *J R Stat Soc Ser B-Methodological* 1995;57:289–300.
35. Amundson SA, Fornace AJ, Jr. Monitoring human radiation exposure by gene expression profiling: possibilities and pitfalls. *Health Phys* 2003;85:36–42.
36. El-Naaman CG-SB, Mansouri A, Grigorian M, et al. Cancer predisposition in mice deficient for the metastasis-associated Mts1 (S100A4) gene. *Oncogene* 2004;23:3670–80.
37. Chen X, Shen B, Xia L, et al. Activation of nuclear factor κ B in radioresistance of TP53-inactive human keratinocytes. *Cancer Res* 2002;62:1213–21.
38. An J, Xu QZ, Sui JL, Bai B, Zhou PK. Silencing of DNA-PKcs alters the transcriptional profile of certain signal transduction genes related to proliferation and differentiation in HeLa cells. *Int J Mol Med* 2005;16:455–62.
39. Lee JH, Lee YM, Park JW. Regulation of ionizing radiation-induced apoptosis by a manganese porphyrin complex. *Biochem Biophys Res Commun* 2005;334:298–305.
40. Pohl F, Hassel S, Nohe A, et al. Radiation-induced suppression of the Bmp2 signal transduction pathway in the pluripotent mesenchymal cell line C2C12: an *in vitro* model for prevention of heterotopic ossification by radiotherapy. *Radiat Res* 2003;159:345–50.
41. Shankar B, Sainis KB. Cell cycle regulators modulating con A mitogenesis and apoptosis in low-dose radiation-exposed mice. *J Environ Pathol Toxicol Oncol* 2005;24:33–43.
42. Pond CD, Leachman SA, Warters RL. Accumulation, activation and interindividual variation of the epidermal TP53 protein in response to ionizing radiation in organ cultured human skin. *Radiat Res* 2004;161:739–45.
43. Snyder AR, Morgan WF. Gene expression profiling after irradiation: clues to understanding acute and persistent responses? *Cancer Metastasis Rev* 2004;23:259–68.
44. Kang CM, Park KP, Cho CK, et al. Hspa4 (HSP70) is involved in the radioadaptive response: results from mouse splenocytes. *Radiat Res* 2002;157:650–5.
45. Rho HS, Park SS, Lee CE. γ Irradiation up-regulates expression of B cell differentiation molecule CD23 by NF- κ B activation. *J Biochem Mol Biol* 2004;37:507–14.
46. Contessa JN, Hampton J, Lammering G, et al. Ionizing radiation activates Erb-B receptor dependent Akt and p70 S6 kinase signaling in carcinoma cells. *Oncogene* 2002;21:4032–41.
47. Khodarev NN, Park JO, Yu J, et al. Dose-dependent and independent temporal patterns of gene responses to ionizing radiation in normal and tumor cells and tumor xenografts. *Proc Natl Acad Sci U S A* 2001;98:12665–70.
48. Zeise E, Weichenthal M, Schwarz T, Kulms D. Resistance of human melanoma cells against the death ligand TRAIL is reversed by ultraviolet-B radiation via downregulation of FLIP. *J Invest Dermatol* 2004;123:746–54.
49. Agner J, Falck J, Lukas J, Bartek J. Differential impact of diverse anticancer chemotherapeutics on the Cdc25A-degradation checkpoint pathway. *Exp Cell Res* 2005;302:162–9.