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Antibody-Based Screen for Ionizing Radiation-Dependent Changes in the Mammalian Proteome for Use in Biodosimetry

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Abstract

In an effort to identify proteomic changes that may be useful for radiation biodosimetry, human cells of hematological origin were treated with ionizing radiation or mock-irradiated and then harvested at different times after treatment. Protein lysates were generated from these cells and evaluated by Western blotting using a panel of 301 commercially available antibodies targeting 161 unique proteins. From this screen, we identified 55 ionizing radiation-responsive proteins, including 14 proteins not previously reported to be radiation-responsive at the protein level. The data from this large-scale screen have been assembled into a public website (http://labs.fhcrc.org/paulovich/biodose_index.html) that may be of value to the radiation community both as a source of putative biomarkers for biodosimetry and also as a source of validation data on commercially available antibodies that detect radiation-responsive proteins. Using a panel of candidate radiation biomarkers in human cell lines, we demonstrate the feasibility of assembling a complementary panel of radiation-responsive proteins. Furthermore, we demonstrate the feasibility of using blood cell-based proteomic changes for biodosimetry by demonstrating detection of protein changes in circulating cells after total-body irradiation in a canine model.

INTRODUCTION

Although most radiation injuries to humans in the past 50 years have been due to accidents (1), there is mounting concern that radioactive materials could be used as weapons for radiological terrorism. In fact, the Strategic National Stockpile (SNS) assembled a Radiation Working Group that developed a consensus document for evaluation, triage and medical management of patients with acute radiation exposure (1), and efforts are under way to establish methods and materials to be deployed in the event of a nuclear attack or accident (2,3).

Moderate exposure to ionizing radiation can be survived with dose-appropriate medical intervention, and hence rapid triage based on biodosimetry is essential for saving lives and optimizing deployment of resources in the event of a nuclear catastrophe. For example, victims exposed to <1 Gy will survive without clinical intervention, whereas there are no known life-saving treatments for victims receiving whole-body exposures of >10 Gy. For whole-body doses of between 2 and 10 Gy, there is survival benefit with medical intervention (1–4). Depending on the exposure, the intervention may be minimal, involving simply supportive care, or extensive, requiring interventions such as stem cell

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. Summary of screening results. <http://dx.doi.org/10.1667/RR1638.1.S1>

transplantation (4). Hence prudent deployment of medical resources and optimal provision of medical care require accurate dosimetry in individual patients.

Developing procedures for evaluation, triage and medical management of exposed individuals is complicated by uncertainties concerning the nature of exposure. Exposures can be whole- or partial-body, external or internal, and they can range greatly in dose rate and in radiation quality. Physical dosimetry would be essentially impossible. Only biodosimetry has the potential to quantify individual exposures for triage for dose-appropriate medical intervention.

Although there are several methods for radiation biodosimetry (e.g. time to onset of vomiting, lymphocyte depletion kinetics), the gold standard is cytogenetic analysis (e.g. chromosome aberrations, micronuclei) of peripheral blood lymphocytes (5–9). This provides a highly accurate measure of exposure that also has the potential to distinguish exposures to different LETs and can be used even when there are partial-body exposures, due to the continual mixing of lymphocytes in the blood. Major limitations are that the assays take 2–3 days and require culture of cells in laboratories (i.e., the assays cannot be deployed in the field). Hence the potentially large numbers of individuals that would be affected (or panicked) by a nuclear attack or large-scale accident cannot be screened effectively. Thus there is significant need for an easily administered (field-deployable), quantitative, sensitive diagnostic test for radiation exposure.

This has led to a search for alternative markers of exposure including gene expression profiles (10–18). Gene sets derived from experimental mouse models and/or pre-transplantation patients have been shown to be accurate (82% sensitivity, 75% specificity) in predicting irradiated and nonirradiated human samples (16). With these promising results, work continues to develop transcript-based assays that can predict actual dose at different times postirradiation.

An alternative and complementary approach is to use the proteome to monitor molecular changes after irradiation. A recently published literature review identified 173 human proteins that either are post-translationally modified or are altered in abundance after irradiation (19). These alterations in protein profiles reflect phenomena such as activation of DNA repair networks, arrest of cell cycle progression, and apoptosis (20).

ATM, a PI(3)K (phosphatidyl-inositol-3-OH kinase)- like kinase, is the key signaling kinase in response to radiation-induced DNA damage and phosphorylates a wide range of substrate proteins including ATM itself, H2AX, CDKN1A, NBS1, BRCA1, BRCA2, SMC1, p53 and MDM2 (21). Many of these proteins are modified and/or assembled in nuclear foci associated with sites of DNA damage in a dose- and time-dependent manner (22,23) at clinically relevant dose ranges, and hence these proteomic changes may be useful for biodosimetry.

While there is an extensive body of literature on proteomic changes in response to radiation, experiments were done in a wide variety of cell lines and tissues and under many different experimental conditions. We have undertaken a standardized Western blot-based screen of 301 commercially available antibodies using a common set of cell lines of hematological origin treated with clinically relevant doses of radiation (1 to 12 Gy). We have evaluated these responses between 1 and 24 h postirradiation, the critical period for triage for medical intervention. This screen has generated a large data set that we have assembled on a public website (http://labs.fhcr.org/paulovich/biodose_index.html) to be shared with the biodosimetry community. This data set may be of use to the radiation community both as a source of putative biomarkers and also as a source of detailed protocols and validation data on commercially available antibodies that detect radiation-responsive proteins. Finally, we

demonstrate that radiation-induced proteomic changes can be detected in circulating cells after total-body irradiation (TBI) in the well-established canine model, supporting the feasibility of using the proteome for *in vivo* biodosimetry.

METHODS

Cell Culture

Cells of the lymphoblast cell lines GM06990, GM07057, GM10333, GM10832, GM10834, GM10860, GM03187 and GM01526 were obtained from the Coriell Institute (Camden, NJ) and grown at 37°C in 95% air/5% CO₂ in RPMI 1640 medium supplemented with 15% heat-inactivated FBS, 100 U/ml of penicillin, and 100 µg/ml streptomycin.

Cells of the canine cell line CLGL90 (provided by Dr. Beverly Torok-Storb, FHCRC) were grown at 37°C and 95% air/5% CO₂ in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin, and 100 µg/ml streptomycin.

Blood Samples

Human whole blood samples were obtained from healthy donors under IRB protocol nos. 3942 and 6074 as approved by the Fred Hutchinson Cancer Research Center's Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque gradient. Briefly, whole blood was diluted with an equal volume of PBS, layered over a half volume of room temperature Ficoll-Hypaque (density = 1.077), and centrifuged at 900g for 20 min. PBMCs were harvested from the Ficoll-plasma interface with a plastic transfer pipette, treated with Red Cell Lysis Buffer (catalog no. FP2301300, Fisher Scientific, Pittsburgh, PA), and washed twice with PBS. Cells were expanded by activation with either 10 U/ml PHA (catalog no. 10576-015, Invitrogen, Carlsbad, Ca.) and 10 U/ml IL-2 (PeproTech, Rocky Hill, NJ) or 50 µl/10⁶ cells Dynabeads® CD3/CD28 T Cell Expander (catalog no. 111-31D, Invitrogen, Carlsbad, CA) and 10 U/ml IL-2 in RPMI 1640 medium supplemented with 15% heat-inactivated FBS, 100 U/ml of penicillin, and 100 µg/ml streptomycin and cultured for 6 to 8 days at 37°C in 95% air/5% CO₂.

Canine whole blood samples were obtained before and after TBI by phlebotomy under IACUC protocol no. 1668 as approved by the Fred Hutchinson Cancer Research Center's Institutional Review Board. PBMCs were isolated as above and immediately lysed as described below.

Ex Vivo Irradiation and Preparation of Protein Lysates

Before irradiation, cells were suspended at 10⁶ cells/ml in fresh medium in T-25 flasks and allowed to equilibrate at 37°C, 95% air/5% CO₂ for 36 to 40 h. Flasks were irradiated in a Nordion Gammacell irradiator at 7.2 Gy/min. Flasks were returned to the incubator for the indicated times. Cells were harvested in prechilled tubes, aliquots were removed for counting, and cells were spun down and washed two times in an equal volume of ice-cold PBS. Cells were counted with a Beckman Coulter Z1 Particle Counter. Cells were also visualized under the microscope in the presence of Trypan Blue to confirm cell numbers and cell viability. Cells were lysed at 10⁸ cells/ml in the appropriate buffer (described below) containing protease and phosphatase inhibitors (Sigma, St. Louis, MO, catalog nos. P2850, P5726, and P8340). All lysates were stored in liquid nitrogen and thawed on ice. Lysates were used within 6 weeks. Lysis buffers included: whole cell extraction buffer (150 mM NaCl, 20 mM b-Glycerophosphate, 10 mM NaF, 1 mM EDTA, 0.5% Triton X-100, 0.005% Tween 20, filter sterilized), nuclear and cytoplasmic lysate buffer (NE-PER Buffer, catalog no. 78833, Fisher Scientific), Laemmli buffer (Catalog no., 161-0737, Bio-Rad, Hercules,

CA), 10× RIPA buffer (Catalog no. 9806, Cell Signaling Technology, Danvers, MA) diluted in sterile water.

Western Blots

Cell lysates (0.5×10^6 cells/lane, ~50 µg) were adjusted to 1× NuPAGE® LDS Sample Buffer containing NuPAGE® Sample Reducing Agent (catalog nos. NP0007 and NP0004, Invitrogen) and heated to 98°C for 5 min before being subjected to SDS-PAGE on tris-acetate or bis-tris novex gels (Invitrogen). Proteins were transferred to 0.45-µm nitrocellulose membranes using an XCell II™ Blot Module (Catalog no. EI9051, Invitrogen). Membranes were placed in 50-ml conical tubes (Falcon 352070, BD) and blocked for 1 h in SuperBlock (catalog no. 37515, Pierce, Rockford, IL) with 0.1% Tween20 (Sigma) on a rotisserie rotator (catalog no. M107625Q, Barnstead/Thermolyne, Dubuque, IA) at room temperature. The blocking agent was aspirated away and primary antibody was added at the appropriate dilution and incubated overnight at 4°C in 1× PBS, 10% SuperBlock and 0.1% Tween 20. Membranes were washed with 2×10 ml 1× PBS, 0.1% Tween 20. HRP-conjugated horse anti-mouse (catalog no. 7076, Cell Signaling Technology), goat anti-rabbit (catalog no. 7074, Cell Signaling Technology), or mouse anti-goat (catalog no. 305-035-003, Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies were diluted (per the manufacturer's recommendation) in 1× PBS, 10% SuperBlock and 0.1% Tween 20, added to the membrane, and incubated 1 h at room temperature on a rotisserie rotator. Secondary antibody was aspirated away and the membrane was washed with 2×10 ml 1× PBS, 0.1% Tween 20, 5 min/wash. Then 1× LumiGLO substrate (catalog no. 7003, Cell Signaling Technology) was added and incubated 5 min at room temperature on a rotisserie rotator. The membrane was then exposed to film (CL-XPosure, Pierce), developed, scanned and digitized. Although Western blot data do not provide absolute quantification of protein signals, relative signal intensities can be estimated through the use of internal controls. We ran a series of gels, each containing the same standard lysate (see Fig. 5A). After digitization of the film image, ImageQuant TL software (GE Healthcare, Piscataway, NJ) was used to measure pixel intensity to calculate the total signal of the relevant band from each lane. Local background corrections were used. Care was taken to ensure proper loading of the gel and proper length of exposure of the film to ensure that the film was not saturated. The signal from each lane was normalized to the signal in the control lane. These normalized values were then plotted for each antibody at the indicated times postirradiation (see Fig. 5B).

RESULTS

Overall Design of Study

The purpose of this screen was to identify radiation-responsive proteomic changes that are induced in the clinically relevant (i.e. potentially treatable) dose range (≤ 10 Gy) within 24 h of exposure. The ultimate goal was to identify a panel of radiation-responsive protein changes that may be useful for biodosimetry.

A total of 301 commercially available antibodies were screened by Western blot analysis to identify and characterize radiation-induced changes in human cells. As described below, multiple cell lysate buffers were used to maximize the chances of observing radiation-responsive proteins. We chose to use human lymphoblastoid cell lines for our initial screen for several reasons: (1) Large quantities of cells can be grown with ease, allowing large batches of lysate to be harvested for the screen; (2) a large body of research has demonstrated an intact proteomic radiation response in human lymphoblastoid cell lines; (3) hematotoxicity is the major cause of death from radiation exposures in the clinically treatable dose range; and (4) for the down-stream application of dosimetry, blood cells are

easily obtained through phlebotomy. To ensure that radiation-responsive proteomic changes were not an artifact of immortalization of lymphoblastoid cell lines, a subset of responsive proteins was confirmed in human peripheral blood mononuclear cells (PBMCs; see below).

The rationale for choosing the radiation type (γ rays), exposure type (pulse or continuous), and dose rates was as follows: (1) The radiation dose from prompt neutrons dissipates rapidly as one moves from the hypocenter (24), such that the vast majority (~98%) of the total dose comes from γ rays at distances where potential survivors will be located. In addition, radiological terrorism from a “dirty” bomb or a contaminated food supply is probably more likely than a nuclear bomb, and many of the currently available sources of radiation material for “dirty” bombs emit γ rays. (2) A single burst of exposure was chosen for the initial experiments; in the event of a nuclear explosion, most of the total dose from γ rays (both prompt primary and secondary) occurs over a relatively short time (24). In the case of Hiroshima and Nagasaki, there was little contribution to the dose beyond 40 s (24). (3) Although our dose rates will not match the delivery rate from a nuclear explosion, different types of radiological terrorism (i.e. dirty bomb, contaminated food supply) may be at a similar or slower rate of exposure than we have chosen. Of course, other forms of radiation and exposure may contribute to biological effects; future validation studies will need to examine these various parameters before accurate biodosimetry can be performed.

The overall design of the screen is shown in Fig. 1. Briefly, all 301 antibodies were initially screened against lysates derived from human lymphoblastoid cell lines treated with 10 Gy or mock-irradiated and harvested at different times between 0 and 24 h postirradiation. As a technical quality control (QC) measure, the Western blot was repeated for 42 of the 110 antibodies that showed radiation-responsive changes; all 42 results were reproduced successfully. As a second QC measure, 104 antibodies were also confirmed in a completely independent lymphoblastoid cell line; all results were reproduced successfully. Finally, a subset of 25 antibodies that showed robust radiation-dependent responses with minimal non-specific bands were confirmed in freshly harvested human peripheral blood mononuclear cells (PBMCs) irradiated or mock-irradiated *ex vivo* and harvested at multiple times out to 24 h. All results are displayed on the website and summarized in Supplementary Table 1.

Standardized Conditions

The scale of this screen (301 antibodies, multiple radiation doses, multiple times, multiple cell types/lines, and multiple lysate buffers) necessitated the production of a large amount of protein lysate for Western blotting and hence obviated detailed optimization of working conditions for individual antibodies. Therefore, considerable effort was put into developing robust standard operating protocols for generating cell lysates and Western blots to ensure reproducible results that worked well for most antibodies throughout the large-scale screen. For example, we optimized a generalized Western blot protocol after evaluating different parameters including transfer membranes, blocking agents, wash conditions, secondary antibodies and chemiluminescence substrates. We settled on conditions (see Methods section and detailed protocols on the website) that gave us an optimal signal-to-noise ratio for a wide variety of primary antibodies. While more optimized conditions may be obtained for individual antibodies, we found that our generalized conditions worked well and increased the likelihood of detecting radiation-induced changes for the antibodies in aggregate.

We also tested a variety of lysis buffers including RIPA, Laemmli, an ELISA-compatible whole cell buffer, and a commercial kit that enriched for cytoplasmic or nuclear proteins (NE-PERS; see Methods). No single buffer was optimal for all proteins (Fig. 2). As expected, chromatin-associated proteins (e.g., H2AX and Smc1) were best extracted with higher detergent levels such as Laemmli or NE-PERS, while non-chromatin proteins (e.g.,

Chk2, P53, Rad17) were best extracted with more gentle buffers such as whole cell lysis buffer or the cytoplasmic buffer in the NE-PERs kit.

Summary of Antibodies Screened

We screened 301 different commercially available antibodies. Many antibodies were selected based on either previous documentation of their radiation responsiveness or their known involvement in pathways and processes associated with the DNA damage response (e.g., apoptosis, cell cycle control). These 301 antibodies map to 161 unique proteins. (Supplementary Table 1). In the cases where we had multiple antibodies to the same protein, they typically recognized different post-translational modifications (PTM) or different epitopes on the protein. In some cases we screened multiple antibodies to the same PTM to evaluate the relative quality of the antibodies for Western blotting. More than 675 Western blots were generated during the screen.

Of the 301 antibodies, 144 recognized total protein (pan), 126 recognized specific phosphorylated sites, six recognized specific acylated sites, seven recognized phospho-motifs (e.g. phospho-ATM/ATR motif), two recognized only their non-phosphorylated epitope, and 16 recognized either their target protein's pro-form and/or activated cleaved form (Fig. 3A). Antibodies were obtained from 13 different companies with three-quarters of the antibodies obtained from three vendors (Fig. 3B). The majority of the antibodies (78%) were derived from rabbits with about a third of the rabbit antibodies from rabbit monoclonal cell lines. Twenty percent of the antibodies were derived from mice and seven antibodies were raised in either goats or sheep (Fig. 3C). We counted the number of non-specific bands (up to a maximum of 10) for each blot. For our Western blotting conditions, rabbit monoclonal lines gave the least number of non-specific bands while goat polyclonal antibodies gave the largest number of non-specific bands (Fig. 3D).

Results of the Screen and Summary of Website

The results of the 301 antibody screen are as follows: (1) 110 different antibodies that map to 55 unique proteins showed a radiation-responsive change (Table 1); (b) 153 antibodies detected a band at the expected molecular weight but did not detect a radiation-dependent change; and (c) 38 antibodies failed to detect the target protein (i.e., no band was observed at the expected molecular weight). The phospho-motif antibodies (e.g. phospho- ATM/ATR motif) detected two to six different radiation-dependent changes per antibody. Twenty-one proteins that were previously identified as radiation responsive in the literature were non-responsive in our screen (ADAM9, AQP3, ARHGDI, BAX, BID, CASP8, CDKN2A, DCLRE1C, ERBB2, FAS, FMR1, GADD45A, GSS, MAPK6, MAPK8, NPM1, PCNA, RAD51, RAF1, RPS6KA1, SOD2). This discrepancy is most likely due to either failure of the antibody in our screen or perhaps differences between the cell types or conditions examined in the various studies.

The data from more than 675 Western blots generated in this study have been assembled on a public website (http://labs.fhcrc.org/paulovich/biodose_index.html). On the Biodosimetry project home page (Fig. 4A), the results are indexed both by protein name and by antibody. On the protein index page (Fig. 4B), the protein name, gene symbol, protein precursor molecular weight, and a link to the protein SwissProt ID are provided. For each protein, the index page links to a page (Fig. 4C) providing a brief protein or gene description, bioinformatics links to both protein and gene-based websites, and a link to the Western blot data for that specific target (Fig. 4D–G), including specific information and links regarding membrane number, antibody catalog number, target protein, target site, precursor molecular weight, a link to the source company, antibody type and antibody dilution used. Additional links from the home page (Fig. 4A) include protocols, cell lines and links to vendors and

resources. Finally, a master spreadsheet summarizing the experiments performed for each protein/antibody is available as Supplementary Table 1 and also as a downloadable file from the website.

The 55 Radiation-Responsive Proteins

The 55 radiation-responsive proteins are shown in Table 1. Of the changes observed, 29 showed up-regulation and 26 showed down-regulation relative to the nonirradiated sample. An additional 12 proteins, not included in Table 1, were classified as marginal due to conflicting data and warrant further investigation (CDC27, CDC42, CDK6, DAP, DDB1, EIF4B, ESR1, HGS, HIST4H4, HTT, NFKBIA, RAD21).

Of the 55 radiation-responsive proteins in our screen, 34 were previously identified in a recent comprehensive literature review by Marchetti *et al.* (19). Of these 34 proteins, 14 were previously identified in blood cell types by Western blotting, while the remaining 20 proteins were identified in other tissues or cell types. Of the 21 proteins identified in our screen but not identified in the literature review of Marchetti *et al.*, seven were described elsewhere in the literature previously as responding to ionizing radiation. Hence our screen identified an additional 14 radiation-dependent proteomic changes that have not been described previously at the protein level, although some (e.g., GSK3A, PHLDA3, PLK1) have been identified at the transcript level (10) (Table 1).

Response Profiles for Radiation-Modulated Proteins

Although a single molecular change (e.g., appearance of a phosphoprotein) may be used to make the binary distinction between an exposed and an unexposed individual, it is highly unlikely that a single molecular change will be sufficient for accurate, detailed biodosimetry. For example, given the dose and time dependence of radiation-induced changes, use of the level of an radiation-responsive phosphoprotein to estimate exposure will be possible only if the exact time of exposure is known. However, a panel of markers whose activation profiles are complementary in both the dose and time dimensions would have better discriminatory capability, perhaps even in the absence of knowledge of the timing of the event. We demonstrated the feasibility of this approach by semi-quantitative Western blotting (Fig. 5). By comparing the relative expression levels across the panel of markers, we found a unique signal pattern for each of the doses tested at each of the four times evaluated. For example, while phospho-Chk2^{T68} is induced after exposure to 1 Gy, ace-P53 requires 3 Gy for significant induction. Further, radiation-dependent signals decay at different rates. For example, Phospho- Rad17^{S645} reaches maximal activity by 3 h and remains at a steady level through at least 16 h while acyl-P53^{K382} peaks near 8 h and is significantly reduced by 16 h.

In Vivo Results

Our screen was performed on cultured (and cycling) blood-derived cells irradiated *ex vivo*, whereas we ultimately want to perform dosimetry measurements on blood cells collected after *in vivo* exposures. Hence, for these proteomic changes to be useful for dosimetry, they must also occur in circulating blood cells after an *in vivo* exposure. As an initial test to determine whether radiation-responsive proteins identified after *ex vivo* radiation could also be detected after *in vivo* radiation, we used a large animal model, the dog. The canine model of radiation exposure and hematopoiesis is highly predictive of clinical outcomes in humans (25–28). Hence, although ultimately any new diagnostic will need to be validated in humans, the dog is an attractive large animal model for pilot testing and initial characterization of new diagnostics for detailed biodosimetry, since it will be ethically unfeasible to obtain data from human *in vivo* exposures over a wide range of doses and times (we are limited for human studies to rare accidental and well-characterized exposures or to therapeutically determined doses and times).

First, we identified a panel of antibodies that exhibited cross-reactivity to the orthologous canine proteins by Western blot analysis of canine PBMCs irradiated *ex vivo*. Of 14 antibodies selected from our screen, eight showed cross-reactivity with the orthologous canine proteins (Fig. 6A). Of these eight antibodies that cross-reacted with the canine protein, five antibodies [p-DNAPK^{T2609}, 53BP1 (pan; band shift and reduction), p-Smc1^{S957}, p-Smc1^{S966} and p-TP53^{S392}] revealed the expected proteomic change in response to radiation in the canine cells (Fig. 6A).

We next determined whether the radiation-induced phosphorylation of Smc1p observed in the canine cells irradiated *ex vivo* (Fig. 6A) could also be detected in PBMCs derived from animals after 1 Gy TBI. Whole blood samples were collected from the animal before TBI and 2 h after TBI. PBMCs were isolated, protein lysates were prepared, and the lysates were subjected to Western blot analysis. As shown in Fig. 6B, phosphorylation of Smc1 at two different sites is easily detected in the blood samples collected after TBI, supporting the feasibility of using the proteomic response of circulating cells for biodosimetry.

DISCUSSION

Feasibility of Proteomic-Based Biodosimetry

The following observations strongly support the feasibility of protein-based biodosimetry: First, the cellular response to ionizing radiation is rapid (onset within seconds-minutes), dose-dependent and time-dependent. Second, different radiation-responsive proteins show diverse activation profiles (such as different activation thresholds or kinetics of induction and disappearance), suggesting that a panel of complementary protein changes could be assembled to perform dosimetry in both the time and exposure dimensions. Third, the large number of potential radiation-induced proteomic changes that could be assembled into a biodosimetry panel will likely enhance the specificity of the dosimetry since many potential confounding conditions during a nuclear event (e.g., a stress response, trauma) would be unlikely to activate a broad panel of radiation-responsive proteins. Fourth, radiation-induced proteomic responses can be detected in blood samples drawn after TBI (Fig. 6B). Fifth, protein-based diagnostic formats such as the ELISA are widely used in clinical diagnostics to measure targets in the ng/ml to pg/ml range. ELISA-based assays are used extensively as clinical diagnostic tests (29), and hence there is significant precedent for the success of such assays to be commercialized as diagnostics.

Development of a new diagnostic test is a lengthy process, and this study is only the beginning. To translate these radiation-responsive protein changes into a biodosimetry panel, quantitative assays must be developed. The ELISA is an attractive option given its current use in diagnostics (e.g. home pregnancy tests), generally high sensitivity (pg/ml), and ease of distribution and use. Once quantitative assays are constructed and characterized, a tremendous amount of validation will need to be performed before the test can be deployed for biodosimetry. For example, humans are highly variable with respect to their intrinsic radiation sensitivity, as demonstrated by the severe radiation sensitivity of individuals who lack Atmp and are afflicted with ataxia telangiectasia. Hence it will be important to characterize this variation in population studies and to determine how this variation correlates with radiation toxicity. Additionally, biodosimetry is complicated by uncertainties concerning the nature of exposures, which can be whole- or partial-body, external or internal, and can range greatly in dose rate and in radiation quality; future validation studies will need to examine these various parameters before accurate biodosimetry can be performed. Indeed, it may make good sense to prioritize for assay development those radiation-induced changes that display the most consistent responses, such as those that give comparable kinetic and intensity patterns over as wide a range of dose rates as possible.

Biology of the Radiation Response

A dramatic cascade of kinase activity occurs in response to radiation, as supported by a recent large-scale study that identified ~700 proteins phosphorylated by mammalian cells after irradiation (30). Hence it is not surprising that of the 14 novel radiation-responding proteins identified in this screen, six are protein kinases (EEF2K, IRAK4, PLK1, SKP1, SYK and ZAP70) and one is a kinase-associated cyclin (CCNE2). One of these radiation responders, eukaryotic elongation factor 2 kinase (eEF2k), phosphorylates and inactivates eEF2, leading to inhibition of translation (31). This kinase can be inactivated by the p70S6 kinase (RPS6KB1), which is involved in G¹ cell cycle progression (32) and is in turn phosphorylated in a radiation-dependent manner [reviewed in refs. (19,33)].

Proteins more directly involved in DNA metabolism are also on the list of 14 novel radiation-responsive proteins. For example, TOP2A is a type II topoisomerase (TOP2A), a class of enzymes that can convert to a DNA nuclease when cells encounter oxidative stress (34). Additionally, CASP7 is known to cleave the PARP1 gene product (35), which is a known radiation-dependent response (36); both CASP7 and cleavage of PARP were detected in this screen.

Although most published studies of proteomic responses of human cells to radiation have been done with transformed or immortalized cell lines, it is possible that the responses of immortalized cells do not reflect the normal physiological response of un-immortalized cells in their normal physiological milieu. In fact, one could argue that the likelihood that there are artifacts in cell lines is high, given that the very processes that result in immortalization affect pathways known to be involved in cell cycle and growth regulation, and some are directly involved in the DNA damage checkpoint (37). Despite these concerns, our results demonstrate that many of the radiation responses initially studied in immortalized cells are relevant to normal physiology. For example, all 25 antibodies (15 unique proteins) that we screened in both lymphoblastoid cell lines and PBMCs demonstrated perfect qualitative concordance in the radiation profiles across the two cell types. Additionally, our initial results of blood samples drawn after TBI in the canine model (Fig. 6B) demonstrate that at least some of these responses will be present *in vivo*. Whether quantitative dosimetry and kinetics of the response will differ between *in vivo* and *ex vivo* irradiated cells remains to be seen and must be investigated once quantitative assays are available.

Website Resource

In addition to identifying a panel of potentially useful proteins for biodosimetry, this data set may also provide a valuable resource for the community by providing validation data and working protocols for antibodies of interest. For example, a major obstacle for basic research is a lack of validated affinity reagents. Often an antibody is not available for a target of interest. Even when antibodies are available, they are generally poorly validated and often do not work for the desired application. Frequently, laboratories purchase several antibodies from multiple sources and hope that one will work. The National Cancer Institute is currently working to address this issue, creating a “shared proteomics reagents resource” through which it will coordinate efforts in characterization, validation and annotation of antibody reagents (38). The envisioned end product will be a publicly accessible virtual repository of antibodies and associated validation data. A similar project has recently emerged from the European community (39). We note that as part of this study, we have generated antibody validation data that will be of use to the radiation research community. Specifically, our large antibody screen (1) validates a subset of commercial antibodies (and identifies poor performers) for Western blot detection of proteins relevant to the radiation response, (2) provides working conditions for using these antibodies in Western blotting, including a determination of the most compatible cell lysate conditions, and (3) documents

the dose and time dependence of radiation-induced proteomic changes in human cells. Thus we hope that the website described in this study will be a useful resource for the radiation research community.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1. Overview of the screen and results; 301 commercially available antibodies were screened by Western blotting to identify those that revealed robust radiation (IR)-responsive protein changes in the clinically relevant dose range.

FIG. 2.

The ability to visualize a radiation response is dependent on the protein lysate buffer used. Cells were lysed in the indicated lysis buffers and protein from the equivalent of 0.5×10^6 cells was run out in each lane except for the samples extracted with Laemmli buffer, which contain protein from 0.25×10^6 cells. Membranes were immunoblotted with the indicated antibodies. Lysis buffers include: L, $1 \times$ Laemmli; W, whole cell lysis buffer; P, pellet from W extracted with $1 \times$ Laemmli; N, Nuclear; C, cytoplasmic. The N and C lysates were generated with NE PERS kit as described in the Methods section.

FIG. 3.

Summary of antibodies used in the screen. Panel A: Distribution of antibodies by class; numbers of antibodies indicated after each class label. Panel B: Source of antibodies by vendor; numbers of antibodies indicated after each vendor. Panel C: Distribution of antibody types; numbers of antibodies indicated after each type. Panel D: Average number of non-specific bands by antibody type. The maximum number of bands per antibody was limited to ten. Error bars equal one standard deviation.

FIG. 4.

Screen shots of the public website. Panel A: The biosimetry project home page with an arrow indicating the link to protein index page. Panel B: The protein index page with an alphabetical listing of proteins and the arrow indicating the link to the p53 protein page. Panel C: The p53 protein page with associated information, links, and an arrow indicating the link to the Western blot data page. Panel D: The p53 Western blot data page displaying an index of antibodies and an arrow indicating the link to the data for a specific antibody located on the same page. Panel E: The relevant regions of multiple blots for a single antibody. The arrow indicates that the image is a link to the full Western blot image page. Panel F: The full Western blot image page contains a table listing all of the antibodies that were screened that day as well as the dilution used and a link to the protocol used. The arrow indicates the link to a full-sized image of the blot located lower down on the same page (panel G).

FIG. 5.

The time and dose dependence of a panel of radiation-responsive proteins. Panel A: Representative Western blots of whole cell lysates from lymphoblastoid cell lines cultures irradiated with different doses and harvested at different times. Panel B: 3D representation of semi-quantitative Western blot data. The Western blots were digitized and band intensities were normalized to the 1 Gy, 1 h control on each blot (as described in the Methods section). For each indicated time after irradiation (3, 8, 16 or 24 h), there is a unique expression profile across the panel of markers for any dose (0, 1, 3, 6, 9, 12 Gy).

FIG. 6.

Western blots of the *ex vivo* and *in vivo* canine radiation response. Panel A: Human (Hs) and Canine (Cf) cells were irradiated or mock-irradiated and whole cells lysates were prepared 2 h postirradiation. Lysates were subjected to Western blotting with the indicated antibodies. Panel B: Canine whole blood was drawn before and 2 h after (*in vivo*) total-body irradiation. PBMCs were isolated by Ficoll gradient and whole cell lysates were then subjected to Western blotting with the indicated antibodies.

Fifty-five Radiation-Responsive Proteins

TABLE 1

Gene symbol	Protein name	SwissProt ID	Radiation response type	PTM(s)	Radiation response direction	Reference (PMID), if reported previously ^a
AKT1	RAC-alpha serine/threonine-protein kinase	P31749	Phosphorylation	pS473	up	Reviewed in 17050475
ATF2	Cyclic AMP-dependent transcription factor ATF-2	P15336	Phosphorylation	pT71	up	Reviewed in 17050475
ATM	Serine-protein kinase ATM	Q13315	Phosphorylation	pS1981	up	Reviewed in 17050475
BCL2L1	Bcl-2-like protein 11	O43521	Protein Level		down	16865280
BRCA1	Breast cancer type 1 susceptibility protein	P38398	Phosphorylation	pS1524	up	Reviewed in 17050475
BRCA2	Breast cancer type 2 susceptibility protein	P51587	Protein Level		down	12530132
CASP7	Caspase-7	P55210	Cleavage	cleaved D198	up	This Study
CCNB1	G2/mitotic-specific cyclin-B1	P14635	Protein Level		down	Reviewed in 17050475
CCND1	G1/S-specific cyclin-D1	P24385	Protein Level		down	Reviewed in 17050475
CCNE2	G1/S-specific cyclin-E2	O96020	Protein Level		up	This Study
CDC2	Cell division control protein 2 homolog	P06493	Protein Level		down	Reviewed in 17050475
CDC25C	M-phase inducer phosphatase 3	P30307	Phosphorylation	pS216	up	Reviewed in 17050475
CDKN1A	Cyclin-dependent kinase inhibitor 1	P38936	Protein Level		up	Reviewed in 17050475
CHEK1	Serine/threonine-protein kinase Chk1	O14757	Phosphorylation	pS317, pS296	up	Reviewed in 17050475
CHEK2	Serine/threonine-protein kinase Chk2	O96017	Phosphorylation	pS16, pS33/35 pT68	up	Reviewed in 17050475
CREB1	CREB1_HUMAN	P16220	Phosphorylation	pS133	up	15073328
EEF2K	Elongation factor 2 kinase	O00418	Protein Level		down	This Study
EGFR	Epidermal growth factor receptor	P00533	Phosphorylation	pY1197	down	Reviewed in 17050475
EIF2S1	Eukaryotic translation initiation factor 2 subunit 1	P05198	Phosphorylation	pS51	down	This Study
ENO1	Alpha-enolase	P06733	Protein Level		up	Reviewed in 17050475
ENO2	Gamma-enolase	P09104	Protein Level		down	Reviewed in 17050475
FANCD2	Fanconi anemia group D2 protein	Q9BXW9	Mobility Shift		up	Reviewed in 17050475
FRAP1	FKBP12-rapamycin complex-associated protein	P42345	Phosphorylation	pS2448	down	15940265
GSK3A	Glycogen synthase kinase-3 alpha	P49840	Phosphorylation	pS21	up	This Study
H2AFX	Histone H2A.x	P16104	Phosphorylation	pS139	up	Reviewed in 17050475
HIST3H3	Histone H3.1t	Q16695	Phosphorylation	pSIO	down	11059758
HSPB1	Heat shock protein beta-1	P04792	Phosphorylation	pS78, pS82	down	This Study
IRAK4	Interleukin-1 receptor-associated kinase 4	Q9NWW3	Protein Level		down	This Study

Gene symbol	Protein name	SwissProt ID	Radiation response type	PTM(s)	Radiation response direction	Reference (PMID), if reported previously ^a
JUN	Transcription factor AP-1	P05412	Phosphorylation	pS63	up	Reviewed in 17050475
LYN	Tyrosine-protein kinase Lyn	P07948	Phosphorylation	pY507	down	Reviewed in 17050475
MAP2K1	Dual specificity mitogen-activated protein kinase 1	Q02750	Phosphorylation	pS221	up	Reviewed in 17050475
MAPK1	Mitogen-activated protein kinase 1	P28482	Phosphorylation	pT202/pY204	up	Reviewed in 17050475
MAPK3	Mitogen-activated protein kinase 3	P27361	Phosphorylation	pT202/pY204	up	Reviewed in 17050475
MDM2	E3 ubiquitin-protein ligase Mdm2	Q00987	Phosphorylation	pS166	down	Reviewed in 17050475
MYC	Myc proto-oncogene protein	P01106	Phosphorylation	pT58/pS62	up	Reviewed in 17050475
NBN	Nibrin	O60934	Phosphorylation	pS343	up	Reviewed in 17050475
PARP1	Poly (ADP-ribose) polymerase 1	P09874	Cleavage	cleaved D214	up	8358726, 17428792
PHLDA3	Pleckstrin homology-like domain family A member 3	Q9Y5J5	Protein Level		up	This Study
PLK1	Serine/threonine-protein kinase PLK1	P53350	Protein Level		down	This Study
PPM1D	Protein phosphatase 1D	O15297	Protein Level		up	17685927, 9177166
PRKDC	DNA-dependent protein kinase catalytic subunit	P78527	Protein Level		up	Reviewed in 17050475
RAD17	Cell cycle checkpoint protein RAD17	O75943	Phosphorylation	pS645	up	Reviewed in 17050475
RB1	Retinoblastoma-associated protein	P06400	Phosphorylation	pS780, pT821	down	Reviewed in 17050475
RELA	Transcription factor p65, NFkappaB p65	Q04206	Phosphorylation	pS536	down	Reviewed in 17050475
RPS6KA1	ribosomal protein S6 kinase, 90 kDa, polypeptide 1	Q15418	Phosphorylation	pT359/pS363	down	Reviewed in 17050475
RPS6KB1	Ribosomal protein S6 kinase beta-1	P23443	Phosphorylation	pT389, pT421/pS42	down	Reviewed in 17050475
SKP1	S-phase kinase-associated protein 1	P63208	Protein Level		down	This Study
SMAD1, 5, 9	Mothers against decapentaplegic homolog 1	Q15797	Phosphorylation	pS463/465	down	This Study
SMC1A	Structural maintenance of chromosomes protein 1A	Q14683	Phosphorylation	pS957, pS966	up	Reviewed in 17050475
SYK	Tyrosine-protein kinase SYK	P43405	Phosphorylation	pY525/526	down	This Study
TOP2A	DNA topoisomerase 2-alpha	P11388	Protein Level		down	This Study
TOPBP1	DNA topoisomerase 2-binding protein 1	Q92547	Protein Level		up	Reviewed in 17050475
TP53	Cellular tumor antigen p53	P04637	multiple PTMs	pS6, pS15, pS20, pS37, pS392, ace-K382	up	Reviewed in 17050475
TP53BP1	Tumor suppressor p53-binding protein 1	Q12888	Protein Level		down	Reviewed in 17050475
ZAP70	Tyrosine-protein kinase ZAP-70	P43403	Phosphorylation	pY319	down	This Study

^aProteins annotated in the reference column as “This Study” have not been reported previously in the literature as radiation responsive.