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Identification and Quantitation of Biomarkers for Radiation-Induced Injury via Mass Spectrometry

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Abstract

Biomarker identification and validation for radiation exposure is a rapidly expanding field encompassing the need for well-defined animal models and advanced analytical techniques. The resources within the consortium, Medical Countermeasures Against Radiological Threats (MCART), provide a unique opportunity for accessing well-defined animal models that simulate the key sequelae of the acute radiation syndrome and the delayed effects of acute radiation exposure. Likewise, the use of mass spectrometry-based analytical techniques for biomarker discovery and validation enables a robust analytical platform that is amenable to a variety of sample matrices and considered the benchmark for bio-molecular identification and quantitation. Herein, we demonstrate the use of two targeted mass spectrometry approaches to link established MCART animal models to identified metabolite biomarkers. Circulating citrulline concentration was correlated to gross histological gastrointestinal tissue damage and retinoic acid production in lung tissue was established to be reduced at early and late time points post high dose irradiation. Going forward, the use of mass spectrometry-based metabolomics coupled to well-defined animal models provides the unique opportunity for comprehensive biomarker discovery.

Keywords

Biomarkers; radiation damage; mass spectrometry; animal models; bioassay; gastrointestinal tract; exposure; radiation; laboratory animals; plasma; radiation damage; tissue; body; whole body irradiation

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INTRODUCTION

The potential risk associated with deliberate and/or accidental radiological catastrophes highlights the urgent need for medical preparedness and effective medical countermeasures (MCM). To date, there are no approved U.S. Food and Drug Administration (FDA) MCM to treat severely irradiated personnel. The National Institute of Allergy and Infectious Diseases (NIAID)-sponsored Consortium, Medical Countermeasures Against Radiological Threats (MCART), is charged with developing MCM to treat the key sequelae of the acute radiation syndrome (ARS) and the delayed effects of acute radiation exposure (DEARE).

MCART's cornerstone in developing MCM is establishment of well-defined animal models that accurately mimic ARS and DEARE (MacVittie 2012). Within the framework of these well-defined animal models is the identification of biomarkers that accurately delineate radiation-induced injury. An ideal biomarker for radiation injury will display a tethered relationship not only between radiological dose (dose dependence) but also dose over time (temporal dependence) and dose over time over intervention (mitigating response dependence). Of noted interest, identification of prognostic biomarker(s) have the potential to provide early indication of radiation-induced syndromes prior to the onset of organ-specific damage and could be used prospectively for rapid, mass screening (i.e., triage) subsequently following a radiological event (Blakely et al. 2005; Riecke et al. 2010). The identification of radiation-linked biomarkers should encompass not only prognostic signatures but also diagnostic signatures as well. In other words, where prognostic biomarkers have the early stage predictive power to be clinically relevant especially in the case of a mass radiological event, diagnostic biomarkers reflecting the real-time biological status have the potential to provide mechanistic details underpinning the biology behind radiation-induced injury.

To date, a number of biomarkers associated with radiation-induced tissue damage have been identified spanning the -omics spectrum including genomics (gene expression) (Hande et al. 2003; Su et al. 2010), transcriptomics (DNA damage and repair) (Iliakis et al. 2003), proteomics (protein profiles) (Barrett et al. 1982; Belli et al. 2002; Bertho et al. 2001; DeBell et al. 1987; Richter et al. 2001; Ward 1988), and metabolomics (metabolites) (Johnson et al. 2011, 2012; Lanz et al. 2009; Lutgens et al. 2003; Tyburski et al. 2008, 2009). In the context of a systems biology prospective (Fig. 1), metabolites, which are generally referred to as biological molecules having a molecular mass less than 1,000 Daltons (Da), represent the end product of cellular processes resulting from genetic and environmental influences (Fiehn 2002). This connection between upstream transcriptomic and proteomic regulation of downstream metabolomic response enables metabolomic expression to be correlated to phenotypic outcome. Consequently, identification of metabolite biomarkers has received considerable attention from a variety of disciplines including those involved in radiation research (Coy et al. 2011; Patterson et al. 2010).

The search for metabolite biomarkers has been achieved via the use of a variety of analytical platforms (Putri et al. 2013). In particular, the use of mass spectrometry-based instrumentation has yielded a sensitive and robust platform not only for biomarker discovery but also for validation (Dettmer et al. 2007). Figure 2 describes of a mass spectrometry-based biomarker discovery and validation platform. Briefly, the initial discovery phase of biomarker identification utilizes an un-targeted mass spectrometry approach that is augmented by the complimentary use of mass spectrometry imaging (MSI) and mass spectrometry profiling. MSI will be discussed in detail in the companion article and the remainder of this article will focus on the use of mass spectrometry profiling for both targeted and discovery approaches. Mass spectrometry profiling refers to the use of mass spectrometry as a means to identify differentially expressed biomarkers from control versus

perturbed conditions. Within mass spectrometry profiling, a targeted approach when prior knowledge is available can be pursued or an untargeted discovery approach is available for identification of potential targets (Fig. 2).

In this article, we will focus on two targeted biomarker projects using quantitative mass spectrometry and end with discovery-based mass spectrometry metabolomics. The first project involves the detection of citrulline in mouse blood plasma. Citrulline, a non-DNA encoded amino acid, is a metabolite end product of glutamine metabolism in small bowel enterocytes (Rabier and Kamoun 1995; Wu et al. 1994). Citrulline is found as a free amino acid in blood plasma and other physiological fluids. The principal source of circulating citrulline is the small intestine and, consequently, plasma concentration is proportional to intestinal cell mass (Wakabayashi et al. 1995, 1991). Because of this relationship, plasma citrulline has been identified as a potential biomarker for radiation-induced small bowel injury (Lutgens et al. 2004; Lutgens and Lambin 2007). We will describe a specific project in which we quantified plasma citrulline from control and irradiated mice to shed light on the connection between citrulline concentration and gastrointestinal (GI) ARS. Next, we will present the use of quantitative mass spectrometry for identification of retinoic acid as a potential biomarker for radiation-induced lung injury corresponding to DEARE. Retinoic acid (RA), the active metabolite in Vitamin A metabolism, is a small molecule metabolite associated with many physiological processes including development, nervous function, immune response, cell proliferation and differentiation, and reproduction (Maden 2000; Mark et al. 2006; Wolf 1984). In the lung, RA is a key signaling molecule for lung development and regeneration of lung alveoli (Maden 2004). RA production is reduced under conditions of cellular stress (e.g., oxidative stress and inflammation) (Kane and Napoli 2010) similar to what is seen in lung injury following radiation exposure. Here we demonstrate the connection between endogenous RA levels and radiation-induced lung injury immediately following radiation exposure and 26 weeks post-irradiation. Finally, we highlight the use of radiation metabolomics for identifying differentially expressed metabolites from plasma between control and irradiated samples with the potential of identifying radiation exposure biomarkers.

MATERIALS AND METHODS

Plasma Citrulline Experiments

Mice—All procedures were certified according to the UK Animal (Scientific Procedures) Act 1986. Male C57BL/6 mice, aged eight to ten weeks were purchased from Harlan UK and allowed to acclimatize for two weeks prior to irradiation. C57BL/6 have been the standard mouse strain used in these studies.

Husbandry—All mice were held in individually ventilated cages (IVCs) in a specific pathogen free (SPF) barrier unit. A twelve hour light:dark cycle was maintained with lights being turned on at approximately 0700 hours and off at approximately 1900 hours. There was a constant room temperature of 21 ± 2 °C and a mean relative humidity of $55\% \pm 10\%$. The animals received 2018 extruded rodent diet (Harlan) and sterile acidified water (ad libitum) from time of arrival and throughout the study. Animals were identified by ear punches in cages labeled with the appropriate information necessary to identify the study, dose, animal number and treatment groups.

Supportive Care—Levofloxacin (0.67 g L^{-1}) was administered in the drinking water from day one (24 hours) post irradiation and was also added to water to wet the food and generate mash that was supplied within the cage to allow for easier access by mice

potentially weakened from radiation exposure. Levofloxacin was administered to mice until mice were euthanized.

Irradiation—Animals were irradiated at 15:00 ± one hour (15 animals per radiation dose per study). Irradiation was performed using a Pantak HF320 X-ray set (Agfa NDT Ltd, Reading, UK), operated at 300 kV, 10 mA. The X-ray tube has additional filtration to give a radiation quality of 2.3 mm Copper half-value layer (HVL). Mice were restrained in a compartmentalized Perspex (plexiglass) jig, positioned at a distance of 700 mm below the focus of the X-ray tube. Each rectangular box is divided to provide twelve ventilated restraints, each holding one animal. Thus, twelve mice were irradiated simultaneously. Dosimetry was checked every two months during which time it has remained within ± 1% of the original value. QA and control procedures were performed prior to and during each irradiation to confirm dose and energy output remain within range. Animals received total-body irradiation (TBI). Irradiation was delivered at a dose rate of 79.5 cGy min⁻¹.

Health Status Monitoring—All animals were weighed and their well-being inspected daily from the initiation of treatment to the end of the study. Any animal demonstrating more than 15% weight loss was considered unwell and humanely euthanized if the weight loss was sustained at greater than 20% for 24 hours and mice also demonstrated signs of a moribund state (withdrawn behavior, reduced body temperature as judged by feeling cool to touch, lack of grooming and dehydration as judged by a persistent skin tent on pinching). During the peak periods of diarrhea incidence and general decline in well-being, animals were inspected several times during each 24 hour period.

Generation of plasma—Five mice per group were deeply anaesthetized with isoflurane and blood collected via cardiac puncture. The mice were then terminated by cervical dislocation. Blood was collected into 1.5 mL K₃ EDTA-coated tubes, immediately placed on ice and centrifuged at 5000 *g* for 5 minutes, at 4 °C within 30 minutes. After centrifugation, the plasma was transferred by sterile pipette into pre-labelled vials and immediately frozen on dry ice.

Intestinal Histopathology—For crypt survival, following fixed time point euthanasia, the small intestine was removed from each animal and Carnoys fixed. The intestines were ‘bundled’ prior to embedding in order to obtain the ideal orientation of the crypts (Merritt et al. 1992). From each mouse a series of small lengths of intestine, approximately 0.5 cm long, were placed within a loop of surgical Micropore tape, and the tape tightened to immobilize the lengths. This allows the alignment of many pieces of fixed intestine alongside each other like a series of logs, so that in every section from a mouse there are several well-orientated cross sections. Each paraffin block generated was then sectioned to provide one slide per block, each slide containing two non-serial sections that were stained with hematoxylin and eosin (H&E). The number of surviving and regenerating crypts per intestinal circumference was scored and the average per mouse and per group determined. A surviving crypt was defined as one that had ten or more tightly packed strongly H&E stained cells (excluding Paneth cells). Only regions that were orientated correctly and did not contain Peyers patches were scored (Peyers patches influence both the number of crypts in a normal circumference and the ability of a crypt to survive insult). The size of surviving crypts varied, influencing the likelihood of observing a surviving crypt in cross section, so a size correction factor was applied to reduce this error, based on the widths of the crypts (Potten et al. 1981). The corrected number of crypts was calculated according to the following equation:

$$\text{Corrected number of crypts} = (\text{mean width in control mice} / \text{mean width in irradiated mouse}) \times \text{mean number of surviving crypts in the irradiated mouse}.$$

Chemicals and Materials—Citrulline and arginine were purchased from Sigma-Aldrich (St. Louis, MO, USA). D₄-Citrulline (4,4,5,5-D₄, 95%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). LC-MS grade acetonitrile, water, ammonium acetate, and formic acid were purchased from Fisher Scientific (New Jersey, NJ, USA).

Sample Preparation—10 µL of internal standard (D₄-Citrulline) was added to 100 µL of plasma followed by 30 s of mixing. 500 µL of acetonitrile was then added followed by 60 s of mixing and centrifugation (10 min at 10,000 rpm). 500 µL of supernatant was transferred to a new container and evaporated to dryness under nitrogen at 30 °C. The residue was reconstituted with 100 µL of water/acetonitrile (1:1 v/v) with 0.1% formic acid.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)—LC-MS/MS analyses were performed on a TSQ Quantum Ultra Triple Stage Quadrupole Mass Spectrometer coupled to an Ultimate 3000 RS Liquid Chromatogram system (Thermo Scientific, San Jose, CA). The LC separation was performed on a Kinetex HILIC column (4.6×100 mm, 2.6 µm) (Phenomenex, Torrance, CA) operated at 30 °C. Solvent A consisted of 0.1% formic acid and 10 mM ammonium acetate in water. Solvent B consisted of 0.1% formic acid in acetonitrile. The gradient program was 0.0–0.5 min, 70% B; 0.5–1.0 min, gradient to 10% B; 1.0–3.5 min, 10% B; 3.5–4.0 min, gradient to 70% B; 4.0–6.0 min, 70% B. The flow rate was to 0.6 mL/min during all separation steps and injection volume was 10 µL. Detection was performed in the positive-ion mode and the electrospray ionization (ESI) source parameters were as follows: spray voltage, 3000; capillary temperature, 325 °C; sheath gas pressure, 60; ion sweep gas pressure, 0.2; capillary offset, 10; tube lens offset, 86. Selected reaction monitoring (SRM) was used for mass detection with the following transitions: Citrulline (*m/z* 176.2 → 70.2) and D₄-Citrulline (*m/z* 180.2 → 74.2). Data collection and analysis was performed by Xcalibur V 2.1 (Thermo Scientific, San Jose, CA).

Method Validation—Calibration standards were generated by spiking known concentrations of standards into neat solution (water/acetonitrile 1:1 with 0.1% formic acid) and non-irradiated mouse plasma. The added concentrations in the calibration curves ranged from 0.5 µM to 100 µM. Quality control (QC) samples were prepared separately from the calibration standards ranged in concentrations from 1.0 µM to 100 µM. Calibration curves were obtained by plotting the peak area ratio of the standard to the internal standard against the concentration and linear regression curves were calculated to check the linearity of the method. Interferences from arginine were investigated to ensure detection of citrulline was selective and sensitive. Baseline separated of citrulline (*t_R* = 3.0 min) and arginine (*t_R* = 3.2 min) was achieved for neat standards and plasma samples. Arginine was determined not to interfere with citrulline quantitation by generating calibration curves with and without the presence of arginine. The calibration curve slopes were not significantly different (F test; *P*=0.31). Limit of detection (LOD) and limit of quantitation (LOQ) were determined as signal-to-noise ratio of 3 and 10, respectively.

Experimental Parameters for mice—Plasma was collected from groups of five mice at two time-points and a range of radiation doses, or un-irradiated controls. Mice were euthanized 4 or 6 days following 8, 9, 10, 11, 12, 13, 14, or 15 Gy.

Lung Tissue Retinoic Acid Experiments

Animals—Female C57L/J, CBA/J, and C57BL/6J mice were purchased at 10–12 weeks of age (~20 g) from Jackson Labs (Jackson Labs, Bar Harbor, ME). Animals were housed five per cage at the Duke Genome Science Research Building, a barrier facility, and provided food and water ad libitum. All experiments were performed with prior approval from Duke University.

Irradiation—Animals were allowed to acclimate to the facility for 1–2 weeks prior to radiation exposure. Animals were anesthetized (100 mg kg⁻¹ ketamine; 10 mg kg⁻¹ xylazine) and randomized to groups to receive a single, uniform dose of 15 Gy delivered to the whole lung at a dose rate of 69 cGy min⁻¹ (320-kVp, HVL = 1.00 mm Cu; Precision X-ray Inc., North Branford, CT). Sham-irradiated mice were included as controls.

Health Status Monitoring—Mice were closely monitored for signs of respiratory distress over the first 6 months post-radiation, including hypomotility, hunched backs, or tachypnea. Criteria for euthanasia included a combination of animal observation (lethargy, hunchback, ruffled fur) and/or body weight loss > 15–20%. Mice with extreme respiratory distress were euthanized by barbiturate overdose (>250 mg kg⁻¹). All surviving mice were euthanized at a pre-determined time point of 182 days. A separate cohort of animals were treated identically, but were euthanized at 24 hours post-radiation by barbiturate overdose. At the time of necropsy, lung tissue mass and pleural fluid content were measured.

Lung Histopathology—Histopathology studies also provided supporting secondary endpoint data. Tissue was either snap frozen in liquid nitrogen (right lung) or formalin fixed and paraffin embedded for histology. Paraffin embedded tissue was stained with H&E for assessment of inflammatory cells, alveolar capillary distension or congestion, the presence of hyaline membranes, and alveolar wall thickness or with Masson's trichrome stain to assess the degree of fibrosis.

Retinoids and Retinoid Extraction—Retinoids were purchased from Sigma (St. Louis, MO) and handled under yellow light. Tissue samples were harvested from mice and frozen at –80 °C until extraction. Retinoids were extracted from homogenized tissue by a two-step liquid-liquid extraction as described previously (Kane and Napoli 2010; Kane et al. 2005, 2008; Pierzchalski et al. 2013). Retinoids were quantified in extracted samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for retinoic acid (RA) isomers (Kane and Napoli 2010; Kane et al. 2005, 2008). RA isomers had the following LC-MS/MS retention times: all-*trans*-RA (atRA), 7.1 min; 9-*cis*-RA (9cRA), 6.8 min; 9,13-di-*cis*-RA (9,13dcRA), 6.6 min; and 13-*cis*-RA (13cRA), 6.3 min (Pierzchalski et al. 2013). Lung RA content was normalized per g tissue and data is expressed as mean ± SEM.

Experimental Parameters for Mice—Mouse lung tissue was collected at time of necropsy. The control group consisted of N=5 and these mice did not receive irradiation. The 24 hour and 180 day time points consisted of 5 mouse lungs each at a radiological dose of 15 Gy.

RESULTS

Detection of citrulline from mouse plasma

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a sensitive and robust method for quantitating small molecule metabolites from a variety of physiological fluids, including plasma (Xiao et al. 2012). Liquid chromatography (LC) is a powerful separation technique used to separate complex solution-based sample mixtures by a combination of the molecule's size and chemistry. The LC separation technique facilitates quantitation of metabolites by reducing sample complexity and allowing metabolite separation prior to detection. Following LC separation, molecules within the sample are ionized (i.e., conversion of neutral molecules to gas-phase ions), mass detected, and quantified via mass spectrometry. In particular, a specific tandem mass spectrometry experiment called selected reaction monitoring (SRM) is used for highly sensitive and selective quantitation. The

coupling of chromatographic separation to mass detection via the LC-MS/MS platform provides highly confident identification and quantitation of metabolites.

LC-MS/MS detection of citrulline from a variety of animal plasma has been demonstrated previously (Gupta et al. 2011; Jaisson et al. 2012; Shin et al. 2011; Wang et al. 2010a). Adopting a similar strategy, we established a LC-MS/MS method for detection of plasma citrulline from control (age-matched, non-irradiated) and irradiated mice. The basic analytical set-up can be seen in Figure 3 (targeted approach). Briefly, mouse plasma was collected followed by sample preparation primarily involving addition of internal standard and protein precipitation. The sample was then analyzed by LC-MS/MS where citrulline was selectively separated, detected, and quantified.

Figure 4A displays plasma citrulline concentrations for control, day 4, and day 6 cohorts following the range of radiation exposures. Our reported control mouse plasma citrulline concentration at 41.6 μM was in good agreement with literature (Brown et al. 2011; Gupta et al. 2011; Shin et al. 2011). For the day 4 cohort of samples, doses of 8, 9, and 10 Gy recorded about a 50–60% decrease in plasma citrulline concentration when compared to control samples. At higher radiological doses (11–15 Gy) there was approximately a 90% reduction in plasma citrulline concentration, as compared to control samples. By day 6 there was a complete recovery of circulating citrulline levels at the lower radiation doses of 8–10 Gy (e.g., the control value was 41.6 μM and day 6 at 9 Gy was 42.2 μM). At 11 and 12 Gy citrulline values remained low at 17.6 and 15.7 μM representing < 50% of the control value. At the highest doses of 13–15 Gy there was little to no recovery in citrulline levels.

Circulating plasma citrulline almost exclusively originates from production in small bowel enterocyte cells (Rabier and Kamoun 1995; Wakabayashi et al. 1995). Consequently, plasma citrulline concentration is decidedly dependent on intestinal cell mass. Furthermore, rapidly proliferating small intestinal epithelium is acutely prone to radiological exposure (Potten 1990). Thus, we investigated the connection between plasma citrulline concentrations and histopathological alterations in the small intestine following radiation exposure. The histopathology of the small intestine was assessed via hematoxylin and eosin (H&E) staining and calculation of crypt survival (Fig. 4B and 4C). Figure 4B shows the pathology of the mouse small intestine under control conditions (no irradiation), day 4 post-irradiation (11 Gy), and day 6 post-irradiation (11 Gy). The pathology illustrates the noticeable loss of the epithelium at day 4 post-irradiation consistent with clonogen cell death and the interruption of enterocyte production (Booth et al. 2012). By day 6 post-irradiation, rapid regeneration from the surviving clonogens began to restore the epithelium. Small intestinal crypt survival, as measured via corrected crypt number, was determined over the same dose response range as the citrulline measurements (8–15 Gy) (Fig. 4C) in order to further correlate the two. As might be expected, crypt survival was radiation dose dependent at both day 4 and day 6 post-irradiation with crypt number decreasing pseudo-exponentially. However, the day 6 crypt numbers were consistently higher than day 4, indicative of intestinal regeneration at this time-point (as illustrated by the 11 Gy H&E images; Fig. 4B). Comparing the day 4 citrulline data (Fig. 4A) to the day 4 crypt survival data (Fig. 4C) revealed a similar radiation dose response relationship from 8–11 Gy. However, at higher doses the citrulline levels did not correlate with the increasing crypt loss. Crucially, these higher doses are associated with the acute radiation syndrome in the gastrointestinal system (i.e., there was no correlation with GI-ARS on day 4 post irradiation).

By day 6, citrulline concentrations were markedly restored following 8–12 Gy. At these lower radiation doses this relative increase was greater than the relative increase in crypt survival between days 4 and 6. Indeed at 8 Gy the levels were higher than control. This was consistent with an initial sub-lethal loss of crypts, and subsequent recovery of the mucosal

mass (crypt number $\times$ crypt size). In other words, following the lower radiation exposures the reduced number of surviving crypts actively overproduced cells in order to restore barrier function. At the higher (GI-ARS) radiation doses, however, there was no increase in citrulline levels. In effect, the circulating levels of citrulline did not mirror the regeneration of a small number of intestinal crypts.

Detection of retinoic acid from lung tissue

Similar to the detection of citrulline from blood plasma, the detection of retinoic acid from lung tissue was accomplished via a LC-MS/MS platform. Development of the LC-MS/MS platform for retinoic acid quantitation was highly sensitive (62.5 attomol lower limit of detection) and uniquely robust (250 attomol to 10 pmol dynamic range) for endogenous retinoids from serum and tissue (Kane et al. 2008). Refer to Figure 3 (targeted approach) for workflow. Briefly, mouse lung tissue was collected followed by a two-step acid/base extraction procedure primarily involving tissue homogenization, addition of internal standard, and protein precipitation. The sample was then analyzed by LC-MS/MS where retinoic acid was selectively separated, detected, and quantified. The experimental details involved lung tissue from three mouse strains (CBA/J, C57BL/6J, and C57L/J) that were either control (no irradiation; N=5 for all three strains) or exposed to 15 Gy (whole-thorax lung irradiation (WTLI); N=5 for all three strains). Time points for harvesting the lung tissue were 24 hours and 180 days post-irradiation.

Figure 5 displays retinoic acid concentrations from mouse lung for control and 15 Gy for all three mouse strains at 24 hours post-irradiation and 180 days post-irradiation. The early time-point of 24 hours represents a time point where lung tissue damage is not evident in traditional histological staining. The late time point at 180 days post-irradiation represents a time point where strain specific mature clinical symptoms (including pneumonitis, fibrosis or combination of both) are phenotypically expressed. Strain differences in pulmonary response to radiation were first recognized by Down and Steele (1983) and have been well-characterized since that time (Jackson et al. 2010, 2011, and 2012). The three strains chosen represent variations in latency and phenotypic expression of radiation-induced lung damage: strain CBA/J is characterized with pneumonitis, strain C57BL/6J is associated with fibrosis, and strain C57L/J is representative of both pneumonitis and fibrosis (Jackson et al. 2012).

All three strains recorded a measurable and significant decrease in endogenous retinoic acid concentration 24 hours post-irradiation (Fig. 5A) compared to the corresponding age-matched strain-specific control. Conversely, these data can be visualized as percent retinoic acid remaining post 24 hours after a radiological dose of 15 Gy compared to the age-matched strain-specific control (Fig. 5B). Here, we see CBA/J and C57BL/6J mice had less than 50% of its endogenous RA remaining upon radiation exposure. C57L/J mice, although more modest, still recorded a 25% decrease in RA concentration. RA concentration was also measured at 180 days post-irradiation (Fig. 5C). Across all three strains, RA concentration was significantly down-regulated, as measured in lung tissue, compared to the corresponding age-matched strain-specific control. Once again, these data can be expressed via percent RA remaining 180 days post-irradiation (Fig. 5D). All three strains registered approximately a 70% decline in RA production compared to age-matched strain-specific controls.

The effects of lung injury from high-dose irradiation can be seen in Masson's Trichrome staining of lung tissue from strain C57L/J at 15 Gy at 125 days post-irradiation (Fig. 6). The non-irradiated control lung (Fig. 6A) displayed normal histopathology with a well-defined lattice structure of bronchioles and thin-walled alveoli. Similarly, lung tissue, even subjected to high dose irradiation, displayed "normal" Masson's Trichrome histopathology at 24 hours post-irradiation (refer to Fig. 6A for analogous image). In contrast, the histopathology of

lungs imaged at progressed time points (e.g., 125 days post-irradiation) exhibited a high degree of pneumonitis (Fig. 6B) and pulmonary fibrosis (Fig. 6C). These lung tissues were characterized by severe airway obstruction, alveolar edema, perivascular lymphocyte cuffing, and extreme alveolar inflammation (Jackson et al. 2012). The degree and extent of pneumonitis and fibrosis in mouse lungs are strain specific yet across all these strains, mice exhibit DEARE that is typically not evident until lung tissue is damaged. Accordingly, if the extent of radiation exposure could be predicted by detection of a biomarker(s) then possible therapeutic intervention could treat the long term effects of radiation-induced lung injury.

DISCUSSION

Identification of biomarkers that accurately model and quantitatively assess radiation-induced injury are of vital importance. These sought-after biomarkers are clinically pertinent both for a triage emergency in the event of a radiological catastrophe and for effective, sustainable oncogenic treatment in the case of clinically prescribed radiotherapy. In both cases, radiation-induced injuries can have severe consequences and accessible biomarkers provide a unique opportunity to predict, evaluate, and treat tissue damage caused by radiation exposure. It is unlikely that one biomarker will be the catch-all biomarker for the spectrum of radiological doses and time points that effect the key sequelae of the acute radiation syndrome (ARS) and the delayed effects of acute radiation effects (DEARE). A multiparametric approach, as suggested by Riecke et al (2010), seems the most likely scenario. In addition, to further emphasize the potential need for a panel of biomarkers, the identification of organ/tissue-specific biomarkers which may reveal localized radiation-induced damage may not accurately represent the totality of the radiation exposure. In other words, a panel of biomarkers representing radiological dose and time across the ARS and DEARE for a range of sequelae may allow for a more comprehensive understanding of radiation exposure than one might get from a single or localized organ/tissue-specific biomarker.

We have detailed the use of a targeted mass spectrometry based approach for quantitating and evaluating two identified biomarkers for radiation exposure. The first of which was linking citrulline concentration to histopathology for assessment of GI ARS. Citrulline has been identified as a biomarker for radiation-induced small intestine tissue damage from both chemotherapy (Papadia et al. 2007) and radiotherapy (Lutgens and Lambin 2007) and has also been associated with enterocyte mass assessment in villous atrophy disease (Crenn et al. 2003), Crohn's disease (Papadia et al. 2007), follow-up on small bowel transplantation (Pappas et al. 2001), gut permeability following ethanol exposure (Rendon et al. 2012) and enterocyte functional mass in HIV patients (Cynober et al. 2009). The aforementioned evidence linking citrulline concentration to small intestinal dysfunction and the known association of GI injury to radiation has led to a community-wide suggestion of citrulline as a biomarker for radiation-induced GI tissue damage, although this has not been rigorously tested against the GI-ARS time-course. The use of a plasma citrulline assay for assessing radiation exposure in animal models has been carried out in mice (Fu et al. 2009; Gupta et al. 2011) and baboons (Hérodin et al. 2012), but to date there has yet to be a published report specifically linking circulating citrulline concentration to GI ARS for a well-defined radiation exposure animal model. We pursued the detection and quantitation of citrulline from plasma for control and irradiated mice from a well-defined radiation exposure animal model (Booth et al. 2012) covering the radiological dose and time-points associated with GI ARS.

The citrulline data was analyzed in the context of matching relevant histological benchmarks. Plasma citrulline concentration and crypt survival both displayed a dose response relationship, although the former dose response was less evident at high GI-ARS

radiation doses. Citrulline levels appeared to reflect enterocyte mass, but may be more reflective of the presence of a function differentiated cell population (i.e., absorptive villus cells) than regenerating clonogens or crypts or even mucosal barrier. Clearly a regenerating tissue must first undergo rapid cell production, restore barrier function, and then restore functional absorptive activity. Thus, lower radiation doses where functional cellularity was rapidly restored appeared to correlate with citrulline levels, but following higher radiation doses where functional differentiated cells were not present there was little correlation. It was therefore significant that citrulline may be a biomarker indicative of small intestinal functionality and mechanism of action of any potential mitigator.

Shifting gears to the targeted mass spectrometry approach for identifying retinoic acid (RA) as a biomarker for radiation exposure, we focused on the delayed effects of acute radiation exposure for lung injury. Here, we concentrated on detection and quantitation of retinoic acid from tissue. It was demonstrated RA concentration in lung tissue was significantly reduced upon high dose irradiation across three phenotypically diverse mice strains. Although tissue is not a readily accessible sample matrix and this inherent drawback is not suitable to rapid, high-throughput screening, it does provide some unique advantages over circulating biomarkers. Detection of a biomarker from the prospective and/or damaged tissue provides a well-established connection that the biomarker is intimately associated with the tissue of interest. This in turn allows the connection of the biomarker to other cellular and molecular processes within that tissue which paves the way for a greater understanding of the mechanism of action. Case in point is identification of RA as a biomarker for lung injury from radiation exposure. It's not likely detection of RA from lung tissue will ever be used clinically as we do not currently know of a rapid, minimally invasive technique for lung tissue extraction but understanding why and how RA is reduced in the lungs upon radiation exposure highlights mechanistic details with potential therapeutic intervention. RA concentration in the lungs is reduced prior to visible lung tissue damage as evident in Figure 4 and 5. In this particular case, the potential restoration of RA by a mitigating RA therapeutic could off-set and/or thwart lung injury from radiation exposure.

In light of the two targeted approaches for identifying radiation exposure biomarkers, several questions are left unanswered. In the case of citrulline, current literature, including our data here within, provide sound evidence linking circulating citrulline concentration at specific radiological doses and time points to radiation exposure. Yet, what is not known is whether circulating citrulline concentration can be used in a mass casualty situation to determine radiological dose (i.e., biodosimetry), dose over time, and dose over time with intervention. What's more, the use of circulating citrulline concentration as a predictive measure for radiation exposure prior to the on-set of tissue damage is yet to be established. With regards to these unanswered questions, the use of well-defined animal models that accurately model radiation exposure is paramount to designing experiments for biomarker validation. Analogous to citrulline, the potential use of RA as a biomarker for radiation exposure displayed promise yet questions concerning its practicality and validation still require on-going and future research effort. For example, can endogenous levels of RA be linked to other radiation sensitive tissue (e.g., gastrointestinal tract) and can RA in tissue be linked to circulating biomarker(s) for routine, rapid detection.

The use of targeted approaches for identifying radiation exposure biomarkers can only shed light on known targets. This effectively limits the discovery phase of biomarker identification to what has already been discovered. It is with this caveat that radiation metabolomics carries unique promise for metabolite biomarker discovery. The field of metabolomics in general has received considerable attention recently and is the latest addition in the -omics revolution. Metabolomics, as described in a number of topical reviews including radiation (Coy et al. 2011; Patterson et al. 2010), is the impartial,

concurrent identification and quantitation of the complete metabolome of a particular system of interest. The metabolome is characterized by 1000s of endogenous metabolites that represent the end products of an organism's cellular and molecular processes. In the case of radiation metabolomics, the ultimate goal is identification of a panel of radiation exposure-linked physiologically accessible biomarkers that have been validated in well-defined animal models for prognostic and diagnostic outcomes of radiological dose, temporal set-points, and mitigating response dependency. The daunting task of comprehensive characterization of a system-wide metabolome is a major challenge requiring robust animal models, cutting-edge instrumentation, and sophisticated bioinformatics. This being the case, the field of metabolomics is pushing forward with numerous research groups across the globe making substantial in-roads into solving some of the challenges presented by characterization of system-wide metabolomes (Wang et al. 2010b; Zhang et al. 2012). It is within this context that MCART is actively pursuing the use of mass spectrometry based radiation metabolomics for identification and validation of radiation exposure biomarkers.

CONCLUSION

The identification and validation of metabolite biomarkers for radiation-induced injury have the potential to be both clinically relevant (e.g., triage and treatment in a mass radiological event) and mechanistically productive (e.g., in terms of understanding of the mechanism of action for radiation injury). Mass spectrometry-based techniques were used to evaluate and identify targeted biomarkers that are pertinent to acute and delayed effects of radiation injury. Circulating citrulline levels were linked to gross histological changes in gastrointestinal tissue and endogenous levels of retinoic acid were determined to be significantly reduced in lung tissue receiving high doses of radiation at early and delay time points. Although the use of targeted biomarker approaches for radiation exposure yielded important information for identification and validation, the use of un-targeted, discovery based metabolomic approaches hold the future for the panel of biomarkers that are needed for comprehensive coverage of the expansive effects of acute radiation syndrome and delayed effects of acute radiation exposure. Likewise, the use of well-defined animal models that accurately portray radiation exposure is paramount to designing experimental approaches for biomarker identification and validation.

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System Biology Perspective

Figure 1.

System Biology Perspective. Metabolites are the end products of the complex interactions of the genome, transcriptome, and proteome resulting from genetic and environmental influences. In effect, metabolite appearance and abundance is a direct reflection of cellular processes responsible for phenotypic expression.

Figure 2.
Mass Spectrometry-Based Biomarker Discovery Platform.

Figure 3.

Mass Spectrometry-Based Biomarker Workflow. First, a well-defined animal model is chosen representing control versus perturbed samples. Next, the sample matrix which contains the analyte(s) of interest are procured for both control and perturbed samples. At this stage depending if the target analyte is known then the targeted approach (left side) is pursued. Here, an LC-MS/MS platform is used to separate, detect, and quantitate the analyte of interest from control and perturbed samples generating raw data (e.g., extracted ion chromatogram), calibration parameters, and data visualization. The end point being quantitation of the targeted analyte ideally produces differential expression between control and perturbed samples. A discovery approach can also be pursued using a very similar

workflow (right side). Similar to the targeted workflow, the discovery workflow involves a LC-MS/MS platform with the primary difference being the detection scheme. In this case, the electronic signal from numerous analytes are detected in an un-biased manner. This generates large data sets which are filtered via differential expression algorithms. The potential outcome here is identification of a panel of analytes that are differentially expressed in control versus perturbed samples. Of note, the discovery approach involves several additional steps for proper identification and quantitation of “discovered” analytes. Also of note, LC is one of several separation techniques, including gas chromatography and capillary electrophoresis, that can be coupled to mass spectrometry-based detection schemes.

Figure 4.

Correlation of plasma citrulline concentration to gastrointestinal histopathology for mice with and without irradiation. A.) Plasma citrulline concentration (μM) for control (non-irradiated) and irradiated mice at dose ranges of 8 to 15 Gy from day 4 and day 6 post-irradiation. Citrulline was quantified by LC-MS/MS. Values are means of $N=5$ mice/group. All data are $\pm$ SEM. B.) H&E stained cross sections of small intestines from control (non-irradiated), 11 Gy at day 4 post-irradiation, and 11 Gy day 6 post-irradiation. Image was recorded at $\times 5$. C.) Calculation of crypt survival as measured by corrected crypt number (see Methods) for control (non-irradiated), and at dose ranges of 8 to 15 Gy from day 4 and day 6 post-irradiation.

Figure 5.

Endogenous RA is depleted in the lung of irradiated mice. A.) 24 h after 15 Gy WTLI radiation exposure in CBA/J, C57BL/6J, and C57L/J strains. * = $p < 0.0406$ vs. non-irradiated control. B.) Data from figure 5A represented at percent retinoic acid remaining 24 h after 15 Gy normalized to age-matched strain-specific control. C.) 180 d after 15 Gy WTLI radiation exposure in CBA/J, C57BL/6J, and C57L/J strains. * = $p < 0.0219$ vs. non-irradiated control. D.) Data from figure 5C represented at percent retinoic acid remaining 180 d after 15 Gy normalized to age-matched strain-specific control. RA was quantified by LC-MS/MS. Values are means of $N=5$ mice/group. All data are $\pm$ SEM.

Figure 6.

Comparison of histopathologic morphology for control lung and lung exposed to high dose radiation at 125 days post-irradiation. A.) Masson's Trichrome stain of control lung tissue. B.) Masson's Trichrome stain of irradiated (15 Gy) lung tissue from C57L/J highlighting pneumonitis. C.) Masson's Trichrome stain of irradiated (15 Gy) lung tissue from C57L/J highlighting fibrosis.