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Proteomics of nonhuman primate plasma after partial-body radiation with minimal bone marrow sparing

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Abstract

High-dose radiation exposure results in organ-specific sequelae that occurs in a time- and dose-dependent manner. The partial body irradiation with minimal bone marrow sparing model was developed to mimic intentional or accidental radiation exposures in humans where bone marrow sparing is likely and permits the concurrent analysis of coincident short- and long-term damage to organ systems. To help inform on the natural history of the radiation-induced injury of the partial body irradiation model, we quantitatively profiled the plasma proteome of non-human primates following 12 Gy partial body irradiation with 2.5% bone marrow sparing with 6 MV LINAC-derived photons at 0.80 Gy min⁻¹ over a time period of three weeks. The plasma proteome was analyzed by liquid chromatography-tandem mass spectrometry. A number of trends were identified in the proteomic data including pronounced protein changes as well as protein changes that were consistently upregulated or downregulated at all time points and dose levels interrogated. Pathway and gene ontology analysis were performed; bioinformatic analysis revealed significant pathway and biological process perturbations post high dose irradiation and shed light on underlying mechanisms of radiation damage. Additionally, proteins were identified that had the greatest potential to serve as biomarkers for radiation exposure.

Keywords

biological indicators; radiation damage; partial body radiation

INTRODUCTION

High-dose radiation exposure results in organ-specific sequelae including the hematopoietic acute radiation syndrome (H-ARS) and the gastrointestinal acute radiation syndrome (GI-ARS). Delayed effects of acute radiation exposure (DEARE) follow the ARS and are characterized by multi-organ injury including pulmonary injury (MacVittie et al. 2012). The

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Conflicts of Interest:

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partial body irradiation (PBI) with minimal, 2.5% or 5%, bone marrow (BM) sparing non-human primate (NHP) model was developed to mimic intentional or accidental radiation exposures in humans. Such exposures are likely to include bone marrow sparing and to permit the concurrent analysis of coincident short- and long-term damage to organ systems in a time and dose dependent manner (MacVittie et al. 2012). Previous publications by our team have described this PBI model, the analysis of resulting tissue injury, as well as its use for medical countermeasure development (MacVittie et al. 2012; Jones et al. 2015a; MacVittie et al. 2015; Shea-Donohue et al. 2016; Cohen et al. 2017; Prado et al. 2017; Carter et al. 2019; Cohen et al. 2019; Farese et al. 2019; MacVittie et al. 2019a; MacVittie et al. 2019b; Parker et al. 2019a; Parker et al. 2019b; Parker et al. 2019c). Here we sought to further characterize the natural history of radiation-induced injury in NHP via determination of differential protein expression in plasma. A more in-depth characterization of the natural history of radiation-induced damage in NHPs exposed to PBI with minimal BM sparing increases the usefulness of the model for medical countermeasure development as understanding of the natural history of an injury is a general expectation of drug development under the FDA Animal Rule (FDA 2015). Additionally, plasma is a minimally invasive biofluid that can inform on health and has been pursued for radiation biomarker development (Ossetrova and Blakely 2009; Bazan et al. 2014; Ossetrova et al. 2014b; Singh et al. 2016; Byrum et al. 2017; Blakely et al. 2018; Ossetrova et al. 2018).

Proteomics is able to measure hundreds to thousands of proteins at a time and identifies a subset of proteins that changes significantly between groups. The comparative proteomic approach is a powerful tool for the discovery of mechanism(s) of injury along the natural history of radiation-induced damage. Comparative proteomic analyses can have additional utility toward identifying novel radiation biomarkers. Previous mass spectrometry-based untargeted proteomic profiling after radiation has been conducted in plasma or serum of mouse (Huang et al. 2019a), rat (Magic et al. 1995; Sun et al. 2020), NHP (Rithidech et al. 2009; Byrum et al. 2017), and patients undergoing radiotherapy (Menard et al. 2006; Widlak et al. 2015; Ouerhani et al. 2019). Targeted proteomic profiling of specific proteins has also been assayed in mice (Liu et al. 2019). Mass spectrometry-based approaches have been complemented by immunoassay methods focusing on detection of specific proteins in the mouse (Ossetrova and Blakely 2009; Ossetrova et al. 2014a; Ossetrova et al. 2018; Sproull et al. 2019), rat (Sun et al. 2020), NHP (Ossetrova NI 2007; Blakely et al. 2010; Ossetrova et al. 2014b; Blakely et al. 2018) as well as human (Hayashi et al. 2003; Bazan et al. 2014). Most of these animal studies utilized total body irradiation; a systematic proteomic analysis of plasma in the PBI model with minimal BM sparing is lacking.

To help inform on the natural history of the PBI model, we quantitatively profiled the plasma proteome of NHPs following 12 Gy PBI with 2.5% bone marrow sparing (PBI/BM2.5) over a time period of three weeks. Further bioinformatics analysis revealed significant pathway and biological process perturbations post high dose irradiation and shed light on underlying mechanisms of radiation damage. Additionally, we sought to identify proteins with the potential to serve as biomarkers for radiation exposure.

MATERIALS AND METHODS

Radiation animal model.

All animal procedures were conducted in accordance with the NIH guidelines for the care and use of laboratory animals and experiments were performed with prior approval from the University of Maryland Institutional Animal Care and Use Committee (IACUC). Plasma obtained from EDTA- anticoagulated peripheral blood of male rhesus macaques (*Macaca mulatta*) samples were provided by the laboratory of Thomas J. MacVittie, University of Maryland School of Medicine, Department of Radiation Oncology (Baltimore, MD). Description of the animal models, including radiation exposure and dosimetry, medical management (supportive care and health monitoring), as well as collection of plasma and tissue have been previously described (MacVittie et al. 2012). NHP were exposed to 12 Gy partial body irradiation with 2.5% bone marrow sparing (PBI/BM2.5) with 6 MV LINAC-derived photons at 0.80 Gy min⁻¹. Bone marrow sparing was accomplished with tibiae outside the beam field. Plasma was obtained from a natural history study where timed euthanasia was planned at day 4, 8, 15, and 21 after radiation. Additional plasma samples were obtained from animals that were euthanized according to criteria specified in the IACUC protocol. We grouped animals at day 8 and 9, day 11 and 12, and day 21 and 22 to increase the numbers of n at the timepoints across our study. Plasma from two non-irradiated animals were used as baseline controls. Thirty-two plasma samples were analyzed in total with the numbers of NHP at each time point (n) as follows: day 0 (baseline), n=2; day 4, n=4; day 8-day 9, n=11; day 11-day 12, n=7; day 15, n=5; day 21–22, n=3.

Proteomic sample preparation.

Two of the most abundant plasma proteins albumin and immunoglobulin G, were depleted by immunoprecipitation using high-capacity anti-albumin and anti-IgG resin cartridges following manufacture's instruction (R & D Systems). The immunodepleted plasma were washed, reduced, alkylated and trypsinolyzed on filter as described previously (Wisniewski et al. 2009; Erde et al. 2014) and previously used by us (Defnet et al. 2019; Kim et al. 2019).

Liquid chromatography-tandem mass spectrometry data acquisition.

Tryptic peptides were separated on a nanoACQUITY UPLC analytical column (BEH130 C18, 1.7 µm, 75 µm x 200 mm, Waters) over a 165-minute linear acetonitrile gradient (3 – 40%) with 0.1 % formic acid on a Waters nano-ACQUITY UPLC system and analyzed on a coupled Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer as previously described (Williamson et al. 2016; Defnet et al. 2019; Kim et al. 2019). Full scans were acquired at a resolution of 240,000, and precursors were selected for fragmentation by collision induced dissociation (normalized collision energy at 35 %) for a maximum 3-second cycle.

Liquid chromatography-tandem mass spectrometry data analysis.

Tandem mass spectra were searched against a UniProt *Macaca mulatta* reference proteome using Sequest HT algorithm described previously (Eng et al. 2008) and MS Amanda algorithm developed by Dorfer *et al.* (Dorfer et al. 2014) with a maximum precursor mass

error tolerance of 10 ppm. Carbamidomethylation of cysteine and deamidation of asparagine and glutamine were treated as static and dynamic modifications, respectively. Resulting hits were validated at a maximum false discovery rate of 0.01 using a semi-supervised machine learning algorithm Percolator developed by Käll *et al.* (Kall et al. 2007). Label-free quantifications were performed using Minora, an aligned AMRT (Accurate Mass and Retention Time) cluster quantification algorithm (Thermo Scientific, 2017). Protein abundance ratios between groups were measured by comparing the MS1 peak volumes of peptide ions, whose identities were confirmed by MS2 sequencing as described above.

Bioinformatic and statistical analysis.

Pathway and gene ontology analysis were performed with Qiagen Ingenuity and DAVID databases, as described by Kramer *et al.* and Huang *et al.*, respectively (Huang et al. 2009; Kramer et al. 2014). Proteins showing at least a 2-fold change (FC) with a false discovery rate (FDR) adjusted ANOVA *p*-value < 0.05 were considered significantly changed and used for further analysis. Fisher's exact *p*-values of < 0.01 were used in the gene ontology analyses to identify biological processes associated with observed protein changes. Ingenuity Pathway Analysis (IPA) analysis was used to predict canonical pathways according to the proteins that were significantly different using a Benjamini-Hochberg corrected Fisher's exact test *p*-value < 0.001 with a non-zero absolute activation z-score.

Retinoid analysis.

Plasma samples were stored at -80 °C until processed. Only glass containers, pipettes, and syringes were used to handle retinoids. Extraction of retinoids was performed under yellow lights using a two-step liquid-liquid extraction that has been described in detail previously using 4,4-dimethyl-retinoic acid as an internal standard for retinoic acid (RA) and retinyl acetate as an internal standard for retinol and total retinyl ester (RE) (Kane et al. 2005; Kane et al. 2008b; Kane and Napoli 2010; Jones et al. 2015b). Briefly, for the extraction of retinoids, 1 ml of 0.025 M KOH in ethanol was added to 100 µL plasma followed by addition of 10 ml hexane to the aqueous ethanol phase. Where sample was limiting, lesser volumes of plasma were extracted down to 30 µL for this study. The samples were vortexed and centrifuged for 1 to 3 min at 1000 rpm in a Dynac centrifuge (Becton Dickinson) to facilitate phase separation and pellet precipitated protein. The hexane (top) phase containing nonpolar retinoids (retinol and RE) was removed. 4 M HCl (65 µl) was added to the remaining aqueous ethanol phase, samples were vortexed, and then polar retinoids (RA) were removed by extraction with a second 10 ml aliquot of hexane as described above. Organic hexane phases were evaporated under nitrogen while heating at approximately 30 °C in a water bath (model N-EVAP 112, Organomation Associates, Berlin, MA, USA). All samples were resuspended in 60 µl acetonitrile.

Levels of RA were determined by liquid chromatography-multistage tandem mass spectrometry (LC-MRM³) which is an LC-MS/MS method utilizing two distinct fragmentation events for enhanced selectivity (Jones et al. 2015b). RA was measured using a Shimadzu Prominence UFLC XR liquid chromatography system (Shimadzu, Columbia, MD) coupled to an AB Sciex 6500+ QTRAP hybrid triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) using atmospheric pressure chemical ionization (APCI)

operated in positive ion mode as previously described (Jones et al. 2015b). For the LC separation, the column temperature was controlled at 25 °C, the autosampler was maintained at 10 °C and the injection volume was typically 20 µL. All separations were performed using an Ascentis Express RP-Amide guard cartridge column (Supelco, 50 × 2.1 mm, 2.7 µm) coupled to an Ascentis Express RP-Amide analytical column (Supelco, 100 × 2.1 mm, 2.7 µm). Mobile phase A consisted of 0.1 % formic acid in water, and mobile phase B consisted of 0.1 % formic acid in acetonitrile. Endogenously occurring retinoid isomers including all-trans RA, 9-*cis* RA, 13-*cis* RA, and 9,13-di-*cis* RA are resolved using a gradient separation at a flow rate of 0.4 mL min⁻¹ with gradient conditions described previously (Jones et al. 2015b). The APCI source conditions and MRM³ detection parameters were as previously described where the MRM³ transition for RA was m/z 301.1 → m/z 205.1 → m/z 159.1 and for 4,4-dimethyl RA was m/z 329.2 → m/z 151.2 → m/z 100.0 (Jones et al. 2015b).

Levels of retinol and total RE were quantified via HPLC-UV according to previously published methodology (Kane et al. 2008a; Kane and Napoli 2010). Retinol and RE were resolved by reverse-phase chromatography (Zorbax SB-C18, 4.6 × 100 mm, 3.5 µm) on a Waters Acquity UPLC H-class system and were quantified by UV absorbance at 325 nm. Analytes were separated at 1 ml min⁻¹ with a gradient separation as described previously with a typical injection volume of 30 µl.

The amount of RA, retinol and total RE was normalized per mL of plasma. Data are expressed as mean ± SEM with statistical significance assessed with a two-sided, unpaired student's t-test as compared to the baseline value.

RESULTS

Study design.

The plasma proteome was assayed after radiation exposure as part of a natural history study in NHP after 12 Gy PBI/BM2.5. Plasma samples were interrogated at various times after radiation up to 21 days after exposure, including 4, 8/9, 11/12, 15, and 21/22. Sampling occurred from NHP at timed euthanasia as planned at day 4, 8, 15, and 21 after radiation as well as from NHP that were euthanized for cause according to pre-defined euthanasia criteria (MacVittie et al. 2012).

Expression of proteins most changed after radiation.

To identify the plasma proteins that are most altered after radiation, we determined the differential protein expression using mass spectrometry-based analyses. Out of a total of 418 proteins identified from the plasma samples by nano-flow UPLC coupled high resolution tandem mass spectrometry, 98 plasma proteins showed significant alterations. Heatmaps representing the greatest changes in protein expression after radiation were made, where we used an FDR of 0.01, a protein expression FC > 2-fold and the FDR corrected ANOVA *p*-value cut-off of $p < 0.05$ to identify changes in protein expression (Fig. 1). Many proteins showed strong and consistent changes over the times interrogated.

Canonical pathways altered by radiation.

To determine the impact of protein changes towards defining the mechanism of injury, IPA analysis was used to predict canonical pathways according to the proteins that were significantly different (Kramer et al. 2014). We focused on canonical pathways to understand key biological events post-irradiation (Table 1). The calculated z-score is a statistical measure of the match between expected relationship direction from published literature and observed gene expression from the experimental dataset. It was used to infer likely activation states of canonical pathways based on comparison with a model that assigns random regulation directions. We used a z-score absolute value above zero and a Benjamini-Hochberg corrected p -value < 0.001 as criteria for inclusion in Table 1. Four canonical pathways were significantly altered by radiation according to these criteria including acute phase response signaling, LXR/RXR activation, coagulation system, and intrinsic prothrombin activation pathway.

Proteins showing a consistent elevation or depression in expression after radiation.

Proteins that have a consistent response across a larger window of time may have diagnostic utility for identifying exposed populations. As such, we identified proteins with either a consistent elevation or depression in expression after radiation. The majority of proteins (95/98) were consistently altered across days post-irradiation, *i.e.*, had the condition that all observed changes were activating or inhibiting over the time period of our study were identified (Fig. 1). Criteria used to identify these proteins was a FC > 2 and FDR corrected ANOVA p -value < 0.05 for at least one condition while the rest of conditions showed changes in the same direction. Protein levels of 57 proteins were consistently elevated whereas protein levels of 38 proteins were consistently reduced. We noted that among the proteins that were upregulated > 5 -fold were CASS4, CRP, and SAA1. Other proteins of note with consistent upregulation included APOA2, APOA4, APOM, CP, HP, HPX, SOD1, SOD3, ITIH3, ITIH4, SERPINA3, and LRG1.

We then looked for biological processes that were enriched in all the proteins that were consistently activated or depressed post-irradiation (Table 2). The inclusion criteria we used were a protein FC > 2 , FDR corrected ANOVA p -value < 0.05 and significant biological process by Fisher's exact test $p < 0.01$. We found that biological processes related to acute phase response, cholesterol metabolism and lipoprotein metabolism, and superoxide radical removal were consistently overrepresented (Fisher's exact test $p < 0.01$).

Proteins related to RA.

A number of proteins were identified that were associated with RA whose dysregulation was consistent with an observed decrease in RA including: POSTN, TIMP1, APOA2, CD163, SOD1 (Fig. 1). Plasma RA was quantified and found to be reduced 27% from baseline at day 4 and steadily continued to decline to 48% reduced at day 21–22 after radiation (Fig. 2a). Vitamin A (retinol), the substrate for RA biosynthesis, as well as circulating retinyl esters were also measured and found to be unchanged after radiation over the time course of this study (Fig. 2b, Fig. 2c). The plasma chaperone for vitamin A, retinol-binding protein 4, was also unchanged after radiation in this study (data not shown).

Proteins that have biomarker potential

Some proteins showed strong and consistent responses or time-dependent changes in protein abundances in plasma (Fig. 1). Both CRP and SAA1 showed strong responses to radiation in our study over most time points until day 15 when responses started to decline (Fig. 1). Interestingly, CASS4 (Crk associated substrate 4), a novel candidate biomarker, showed a strong and time-dependent response over all time points, with the lowest response of ~7-fold increase observed at day 4 and the highest response of ~700-fold increase observed at day 21–22 (Fig. 1). Forty-nine additional proteins showed consistent upregulation with potential utility to inform on radiation exposure (Fig. 1).

DISCUSSION

In this study, we quantitatively profiled the plasma proteome of NHPs following PBI/BM2.5 at 12 Gy over a time period of three weeks. We depleted the two most abundant proteins in plasma, albumin and immunoglobulin G (IgG), prior to analysis. Proteins whose abundance in plasma were significantly altered were identified using tandem MS/MS by matching peptide fragmentation patterns. We used the comparative proteomic approach and bioinformatic pathway analysis to further inform on mechanisms of injury that occur post-radiation. Previous publications have described this PBI model (MacVittie et al. 2012) and the analysis of resulting tissue injury (Jones et al. 2015a; Shea-Donohue et al. 2016; Cohen et al. 2017; Prado et al. 2017; Carter et al. 2019; Cohen et al. 2019; MacVittie et al. 2019a; MacVittie et al. 2019b; Parker et al. 2019a; Parker et al. 2019b; Parker et al. 2019c). Here we sought to further characterize the natural history of radiation-induced injury in NHP via determination of differential protein expression in plasma. Additionally, we considered the proteins yielded by our data towards supporting efforts to identify proteins that could have utility as biomarkers to identify exposed populations, and may be useful during drug development under the FDA Animal Rule, or inform on medical management of radiation-induced injuries post-exposure. The plasma proteomic data from NHP after 12 Gy PBI/BM2.5 reported here show some similarities in the canonical pathway dysregulation as compared to canonical pathways dysregulated in murine lung (after whole-thoracic lung irradiation, WTLI) and murine ileum (after total body irradiation, TBI) as part of studies previously reported by us (Huang et al. 2019b; Huang et al. 2019c). The acute phase response pathway is one of the most significantly activated pathway in NHP plasma, which is also consistent with our previous results of mouse ileum. The acute phase response pathway is known to show rapid response to a variety of inflammatory events including infection, tissue injury, trauma, neoplastic growth, immunological disorders and homeostasis restoration (Moshage 1997). The LXR/RXR (liver X receptor/retinoid X receptor) pathway is also one of the top activated pathways post-irradiation in NHP plasma, which is consistent with widespread dysregulation of lipid homeostasis that was previously reported in various tissues and plasma in both mouse and NHP (Carter et al. 2015; Carter et al. 2017; Jones et al. 2017a; Jones et al. 2017b; Carter et al. 2019; Jones et al. 2019a; Jones et al. 2019b) and is similar to proteomics data reported previously in both murine ileum and murine lung (Huang et al. 2019b; Huang et al. 2019c). LXR/RXR pathway upregulates the efflux of cholesterol in human intestinal cells and plays a vital role in intestinal high-density lipoprotein production and cholesterol absorption (Murthy et al. 2002). The LXRs are important

regulators that regulate expressions of genes involved in efflux and excretion of cholesterol and lipid homeostasis in tissues including the liver and intestine (Hong and Tontonoz 2014), and respond to inflammatory cytokine signaling through lipid-sensing (Venteclef et al. 2011). It was likely that damage to the liver and intestine by ionizing radiation contributed to the significant activation of the LXR/RXR pathway. In addition, the coagulation system and intrinsic prothrombin activation pathways were likely activated as response to tissue vascular damage. All four of the canonical pathways in Table 1 were also reported in the top 5 dysregulated canonical pathways in NHP plasma after lower doses of TBI (6.7, 7.4 Gy) (Byrum et al. 2017).

Ionizing radiation induces reactive oxygen species (ROS) and free radicals that lead to DNA, protein, and lipid membrane damage as well as disruption of critical metabolic processes, which have been linked to carcinogenesis, fibrosis, inflammation, genomic instability, and acceleration of the onset of degenerative tissue injury processes. They are thought to be a major cause of radiation-induced cell and tissue injury (Spitz and Hauer-Jensen 2014). We observed significant activation of superoxide radical degradation process in our NHP model as indicated by significant overexpression of superoxide dismutases (SOD1, SOD3) found in plasma. Cells respond to increased ROS by overexpressing various antioxidants, including superoxide dismutases, glutathione, and catalase. Superoxide dismutase catalyzes superoxide ions to hydrogen peroxide, which is then degraded to oxygen and water by catalase (Petkau et al. 1976). We observed a significant upregulation of SOD1 as well as a non-significant increase in catalase, consistent with activation of antioxidant defense mechanisms. SOD1 and catalase were also among a targeted panel of proteins related to Nrf2 that were quantified by mass spectrometry-based targeted proteomics using parallel reaction monitoring from bone marrow after 6 Gy TBI in mouse in an effort to characterize biomarkers for radiation exposure (Liu et al. 2019).

Among the proteins that showed strong and consistent responses or time-dependent changes in protein abundance in plasma, three proteins displayed particularly attractive attributes as potential biomarkers, CRP, SAA1, and CASS4. Two of them, CRP and SAA1, are fast reactants of acute phase response which were also proposed as candidate biomarkers for radiation injury in previous studies (Ossetrova and Blakely 2009; Bazan et al. 2014; Nylund et al. 2014; Ossetrova et al. 2014b; Widlak et al. 2015; Byrum et al. 2017; Blakely et al. 2018; Ossetrova et al. 2018; Sproull et al. 2019). Nine out of the 49 additional protein have been noted in previous radiation biomarker reports including C9, CP, FGA, ITIH3, HP, HPX, LRG1, SERPINA3, and SOD1 (Magic et al. 1995; Hayashi et al. 2003; Hayashi et al. 2005; Ossetrova and Blakely 2009; Bazan et al. 2014; Nylund et al. 2014; Ossetrova et al. 2014b; Widlak et al. 2015; Byrum et al. 2017; Blakely et al. 2018; Ossetrova et al. 2018; Liu et al. 2019; Ouerhani et al. 2019; Sproull et al. 2019; Moertl et al. 2020; Sun et al. 2020). Bone marrow derived protein Flt-3L that has been proposed as a biomarker was not detected in our data (Ossetrova et al. 2014b). For proteins that showed biomarker potential in the data provided by this natural history study, future studies to assess their responsiveness according to dose and their response after lower radiation doses would be needed.

The most strongly upregulated proteins in NHP plasma after 12 Gy PBI/BM2.5 were the radiation-responsive plasma proteins, CRP and SAA, which have been reported previously

as rapidly upregulated within hours after radiation and as strong biomarker candidates (Ossetrova and Blakely 2009; Bazan et al. 2014; Nylund et al. 2014; Ossetrova et al. 2014b; Widlak et al. 2015; Blakely et al. 2018; Ossetrova et al. 2018; Sproull et al. 2019). Serum amyloid A1 protein (SAA1) is a major acute phase responder and is an apolipoprotein of the HDL complex. It is synthesized and secreted by the liver as well and is known to increase during acute-phase response due to acute bacterial infection, trauma, inflammatory and autoimmune disease, and neoplasia (De Buck et al. 2016). Elevated levels of SAA1 have been observed after radiation exposure in mouse (Ossetrova and Blakely 2009; Ossetrova et al. 2018; Sproull et al. 2019), NHP (Rhesus macaques and baboons) (Ossetrova et al. 2014b; Blakely et al. 2018), patients after radiotherapy (Bazan et al. 2014), and radiation accident victims (Nylund et al. 2014). CRP is one of the known biomarkers of systemic inflammation. It is produced in liver in response to acute inflammation (Schmidt-Arras and Rose-John 2016). However, CRP is rapidly metabolized, which makes persistently elevated levels of CRP a biomarker of continuing chronic inflammation. Elevated levels of CRP have been observed in mouse (Ossetrova et al. 2014a), NHP (Byrum et al. 2017), atomic-bomb survivors (Hayashi et al. 2003), and radiotherapy recipients (Rodriguez-Gil et al. 2014; Widlak et al. 2015). CRP and SAA1 have been investigated in murine models as biomarkers for radiation injury for up to 1 week post-radiation (Ossetrova and Blakely 2009; Ossetrova et al. 2014a; Ossetrova et al. 2014b; Ossetrova et al. 2018; Sproull et al. 2019). In NHP, CRP and SAA protein biomarkers were proposed as early biomarkers for radiation exposure assessment and tissue injury up to one-week post-irradiation (Ossetrova et al. 2014b). In NHP studies using TBI at various doses between 1 and 8.5 Gy, CRP alone was shown to have utility in radiation dose prediction, which was improved by combining it with the measurement of SAA, IL-6, and FLT3L (Ossetrova et al. 2014b). Additionally, SAA was part of a three protein panel that has shown utility in humans as a point of care diagnostic to detect clinically significant levels of radiation exposure (Bazan et al. 2014).

We also observed a rapid and strong time-dependent response of CASS4 which has been less characterized in radiation models. CASS4 has roles in thrombosis and fibrosis, which could make it a promising prognostic biomarker of radiation injury. CASS4 is a FAK interacting protein that activates PTK2/FAK1 signaling and regulates cell adhesion, spreading, migration and motility (Singh et al. 2008). Overexpression of CASS4 also activates Akt signaling and inhibited e-cadherin in a NSCLC cell model (Li et al. 2016). It was reported that CASS4 was involved in cystic fibrosis severity and progression (Wright et al. 2011). CASS4 was also implicated in thrombosis by signaling platelet activation and aggregation (Zimman et al. 2014).

RA is a master regulator of gene expression mainly through ligand-activated control of transcription mediated through retinoic acid receptors (RAR) in the nucleus (Germain et al. 2006). RA was previously shown to be reduced in the murine lung (Jones et al. 2014; Huang et al. 2019b) and murine ileum (Huang et al. 2019c) after radiation and that a number of proteins associated with RA were dysregulated (Huang et al. 2019b; Huang et al. 2019c). Here, plasma RA was reduced after radiation in the same NHP assayed for protein changes (Fig. 2a). The decrease ranged from a 27% decrease at day 4 to a 48% decrease at day 21–22. A 48% decline in plasma RA is similar to the decrease in RA that we observed in an NHP model of SIV infection where there was significant infection-induced GI damage

(Byrareddy et al. 2016). Consistent with depleted RA, we observed a decrease in direct targets of RA; POSTN is a HOXA1 target gene where HOXA1 is a direct RA target (Martinez-Ceballos et al. 2005). TIMP1 is a key regulator of metalloproteinase activity including MMPs and ADAMs (Masciantonio et al. 2017). We have previously observed decreased TIMP1 levels when RA levels were low and remodeling was increased (data not shown). Moreover, expressions of SOD1 and SOD3 were significantly upregulated as shown in Fig. 1. SOD1 has been shown to be directly regulated by RA signaling (Zhu et al. 2016). In tamoxifen-induced RARalpha gene deletion mice, increased ROS and reduced SOD1 were rescued by RARalpha, which suggests that impairment of cardiac RARalpha signaling plays an important role in pathological stimuli-induced cardiac remodeling (Zhu et al. 2016). RA treatment of primary hippocampal neurons reduced ROS and enhanced SOD1 supporting the antioxidant defense (Ahlemeyer et al. 2001). Here, we measured a depletion of plasma RA which may hinder being able to mount the most robust antioxidant defense after radiation. KRT1 mRNA is destabilized in the presence of RA (Aldehlawi et al. 2020); our observed increase in KRT1 is consistent with depleted RA. RA represses CD163 (Chen et al. 2019); our observed increase in CD163 is consistent with depleted RA. RBP4, the plasma chaperone for vitamin A (retinol) was unchanged consistent with the unchanged levels of retinol (Fig. 2b). APOM, which was upregulated in the plasma, has been shown to bind retinol, all-trans-RA and 9-*cis*-RA, albeit with apparent dissociation constants several orders of magnitude higher (low μ M) than retinoid-binding proteins like RBP4 (low nM) (Ahnstrom et al. 2007).

In addition to vitamin A signaling, our data reveal radiation also disrupts other essential nutrient signaling. Radiation induced changes to a number of acute phase proteins that are involved in metal homeostasis and signaling, including CP, HP, HPX, SOD1, and SOD3. CP is the main copper-containing plasma protein that also has the ability to oxidize Fe(II) (Ramos et al. 2016). This systemic ferroxidase activity is thought to mediate iron efflux from cells (Vashchenko and MacGillivray 2013), play a role in the oxidative inactivation of NO (Shiva et al. 2006), as well as the oxidative inactivation of some biogenic amines, including serotonin (Wrona and Dryhurst 1991). Correspondingly, we observed an overexpression of CP and a decrease in plasma serotonin after radiation; serotonin results are reported in another manuscript in this issue (Kumar et al. 2020). Intracellular and extracellular Cu/Zn superoxide dismutases, such as SOD1 and SOD3, and CP help neutralize ROS (Goldstein et al. 1979). Upregulation of CP was identified in a proteomics study in rats intended to establish biomarkers for radiation exposure (Sun et al. 2020), as well as in patients undergoing radiotherapy (Widlak et al. 2015). Other proteins consistent with dysregulation of iron homeostasis are HP and HPX. HP binds free hemoglobin and allows degrading enzymes to access hemoglobin while preventing adverse biochemical and physiologic effects of extracellular hemoglobin (Schaer et al. 2014). HPX is a plasma protein with high affinity for heme. HPX binding to heme prevents its pro-oxidant, pro-inflammatory effects and promotes detoxification (Schaer et al. 2014). HP and HPX are acute phase proteins which are induced in inflammatory conditions to minimize tissue injury and facilitate repair (Tolosano and Altruda 2002). HP upregulation was observed in the serum proteome of patients that was interrogated to understand radiation-induced mechanisms of damage that can lead to normal tissue damage (Ouerhani et al. 2019). HPX

upregulation was observed in the proteomics of EVs secreted from irradiated isolated peripheral blood mononuclear cell (PBMCs) after up to 6 Gy (Moertl et al. 2020). Both HP and HPX upregulations were observed in patients undergoing radiotherapy (Widlak et al. 2015).

Consistent with LXR/RXR dysregulation and disruption of lipid homeostasis, we observed significant upregulation of APOA2, APO4, and APOM which are plasma apolipoproteins. We previously observed upregulation of APOA2 in murine lung and ileum after radiation (Huang et al. 2019b; Huang et al. 2019c). RA has been shown to repress APOA2 in a hepatic cell line (Mamoon et al. 2014). Here, upregulation of APO2 is consistent with our measured depletion of endogenous RA (Fig. 2.). APOA4, APOM are involved in lipid transport whereas APOA4 has also displayed anti-oxidant properties (Xu and Dahlback 1999).

ITIH3, ITIH4, and SERPIN3A are protease inhibitors present at elevated levels in plasma in our study that have also been noted as changed in the human serum proteome after radiation (Ouerhani et al. 2019) and in the plasma of patients undergoing radiotherapy (Widlak et al. 2015). ITIH3 has been reported as a marker for gastric cancer detection (Chong et al. 2010) and ITIH4 correlated with higher stages of liver fibrosis (Sira et al. 2014). LRG1 is a plasma glycoprotein that was upregulated in our study and has been associated with lung cancer and inflammatory conditions (Serada et al. 2010; Guergova-Kuras et al. 2011; Serada et al. 2012) and observed in patients undergoing radiotherapy (Widlak et al. 2015).

CONCLUSION

In summary, we used a mass spectrometry-based proteomics approach to interrogate plasma in order to characterize the natural history of the NHP PBI/BM2.5 model and to assess the potential for plasma proteins to be a minimally invasive assessment of ionizing radiation-induced alterations. Cellular defense processes such as acute phase response, LXR/RXR activation, cholesterol and lipoprotein metabolism, superoxide radical degradation, and coagulation were represented as the main physiologic responses of the body to ionizing radiation injury according to changes in the proteomic profile. CRP, SAA1, and CASS4 were shown to be the most promising proteins for biomarker utility for radiation injury according to their robust response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of proteins most changed in plasma after radiation.
LC-MS/MS proteomic analysis of NHP plasma after 12 Gy PBI/BM2.5. Minimum 2-fold change of expression for at least one condition and FDR adjusted ANOVA p -value < 0.05.

Figure 2. Plasma RA is reduced after radiation.

Retinoid quantification in plasma after 12 Gy PBI/BM2.5. Radiation days after radiation dose are notated. (a.) RA, (b.) retinol, (c.) total RE. Data are mean $\pm$ standard deviation, * $p < 0.05$ using student's t -test between groups as compared to control (0). pmol/mL: picomole/milliliter; nmol/mL: nanomole/milliliter.

Table 1.
Canonical pathways altered by radiation.

Criteria for pathway changes were non-zero absolute activation z-score and Benjamini-Hochberg corrected Fisher's exact test p -value < 0.001 . Derived from LC-MS/MS proteomic analysis of NHP plasma after 12 Gy PBI/BM2.5.

Canonical Pathways	B-H p -value	Activation z-score	Proteins
Acute Phase Response Signaling	1×10^{-20}	3.0	HPX, ITIH3, C4BPB, APOA2, C9, SERPINA3, CP, FGG, C5, C4A/C4B, HP, ORM1, ITIH4, SAA1, CRP, FGB, FGA, AGT
LXR/RXR Activation	5×10^{-15}	3.1	KNG1, HPX, APOM, APOA4, APOA2, C9, CETP, C4A/C4B, ORM1, ITIH4, SAA1, FGA, AGT
Coagulation System	$< 5 \times 10^{-14}$	0.3	KNG1, F10, F9, PROS1, FGG, SERPINA5, FGB, FGA, F13B,
Intrinsic Prothrombin Activation Pathway	$< 5 \times 10^{-11}$	1.1	KNG1, F10, F9, PROS1, FGB, FGA, F13B, FGG

Table 2.
Biological processes altered by radiation.

Criteria for biological process enrichment was Fisher's exact test p -value < 0.01 . Derived from LC-MS/MS proteomic analysis of NHP plasma after 12 Gy PBI/BM2.5.

Biological Process	Fold Enrichment	P -value	Proteins
complement activation	126	$< 5 \times 10^{-6}$	CFB, C8A, C5, C1S
positive regulation of cholesterol esterification	126	< 0.0005	APOA2, AGT, APOA4
high-density lipoprotein particle assembly	126	< 0.0005	APOA2, APOM, APOA4
removal of superoxide radicals	84	< 0.001	SOD1, SOD3, APOA4
fibrinolysis	76	< 0.001	FGG, FGB, PROS1
reverse cholesterol transport	76	< 0.001	APOA2, APOM, APOA4
acute-phase response	63	< 0.001	ORM1, SAA1, ITIH4
lipoprotein metabolic process	54	< 0.005	APOA2, APOM, APOA4
cholesterol efflux	33	< 0.005	APOA2, APOM, APOA4
retina homeostasis	33	< 0.005	B2M, SOD1, KRT1
cellular iron ion homeostasis	23	< 0.01	HPX, SOD1, CP
negative regulation of plasma lipoprotein particle oxidation	252	< 0.01	APOM, APOA4