Published in final edited form as:

Health Phys. 2010 February ; 98(2): 186–195. doi:10.1097/HP.0b013e3181b17cbd.

THE URINE PROTEOME FOR RADIATION BIODOSIMETRY: EFFECT OF TOTAL BODY VERSUS LOCAL KIDNEY IRRADIATION

Mukut Sharma^{§,†,‡,*}, Brian D. Halligan[#], Bassam T. Wakim[¤], Virginia J. Savin^{§,†}, Eric P. Cohen^{§,†,‡}, and John E. Moulder^{‡,**}

[§] Nephrology Division, Department of Medicine, Medical College of Wisconsin, Milwaukee, WI

[†] Kidney Disease Center, Medical College of Wisconsin, Milwaukee, WI

[‡] Center for Medical Countermeasures against Radiological Terrorism, Medical College of Wisconsin, Milwaukee, WI

[#] Biotechnology and Bioengineering Center, Medical College of Wisconsin, Milwaukee, WI

[¤] Protein and Nucleic Acid Facility, Medical College of Wisconsin, Milwaukee, WI

^{**} Radiation Oncology, Medical College of Wisconsin, Milwaukee, WI

Abstract

Victims of nuclear accidents or radiological terrorism are likely to receive varying doses of ionizing radiation inhomogeneously distributed over the body. Early biomarkers may be useful in determining organ-specific doses due to total body irradiation (TBI) or partial body irradiation. We used liquid chromatography and mass spectrometry to compare the effect of TBI and local kidney irradiation (LKI) on the rat urine proteome using a single 10 Gy dose of X-rays. Both TBI and LKI altered the urinary protein profile within 24 hours with noticeable differences in Gene Ontology categories. Some proteins including fetuin-B, tissue kallikrein, beta-glucuronidase, vitamin D-dependent calcium binding protein and chondroitin sulfate proteoglycan NG2 were detected only in the TBI group. Some other proteins including major urinary protein-1, RNA binding protein 19, neuron navigator, Dapper homolog 3, WD repeat and FYVE domain containing protein 3, sorting nexin-8, ankyrin and aquaporin were detected only in the LKI group. Protease inhibitors and kidney proteins were more abundant (fraction of total scans) in the LKI group. Up/Uc ratio and urinary albumin abundance decreased in both TBI and LKI groups. Several markers of acute kidney injury were not detectable in either irradiated group. Present data indicate that abundance and number of proteins may follow opposite trends. These novel findings demonstrate intriguing differences between TBI and LKI, and suggest that urine proteome may be useful in determining organ-specific changes caused by partial body irradiation.

Keywords

Biodosimetry; kidney; total body irradiation; partial body irradiation; radiological terrorism; nuclear accidents; urine proteome; biomarkers

*Correspondence: Mukut Sharma, M4160, Nephrology/CVC/MEB, 8701 Watertown Plank Road, Milwaukee WI 53226, Phone: 414-456-4894, Fax: 414-456-6675, msharma@mcw.edu.

AUTHOR NOTE

Supplementary Material containing detailed results can be accessed at <http://proteomics.mcw.edu/>.

INTRODUCTION

Nuclear accidents or terrorist initiated radiological events may expose large numbers of people to doses of radiation ranging from innocuous to immediately lethal. Numerous variables including the type of radiation, weather conditions, location and total or partial body exposure would determine the actual radiation doses received by the victims. Victims affected by non-lethal doses of radiation may remain asymptomatic, and possibly develop chronic diseases long after the radiation event. Chaos and confusion combined with injuries due to blast and/or burns may complicate dose determination and impede medical care. Rapid high-throughput assessment of radiation dose will be needed to alleviate anxiety, initiate early intervention and to prevent loss of organ function and mortality (Jarrett 1999; Ricks et al. 2002; Mathouse and Jackson 2007).

A simple, convenient and self-administered initial assessment of radiation dose would be valuable for initial triage, planning further diagnosis, treatment, mitigation and follow up. A first step towards developing a user-administered test would be to identify radiation-induced molecular changes including the biochemical modifications of molecules in blood or urine e.g., nucleic acids, proteins, lipids or carbohydrates.

Urine is routinely used in clinical practice for diagnosis and follow-up. Sufficient quantities of urine can be obtained non-invasively and inexpensively without dependence on medical professionals. The urine proteome is composed of filtered plasma proteins and, proteins from the kidneys and other genitourinary organs. We hypothesize that the urine proteome is a useful source of biomarkers to develop dosimetry of ionizing radiation. Radiation-induced changes in tissue function and plasma protein composition may immediately alter the urinary protein profile without causing immediate proteinuria or other overt signs of disease. Early changes in the plasma- or kidney-derived urinary proteins may be useful in developing a self-administered test for early triage. The urine proteome is orders of magnitude smaller than the plasma proteome and urine samples can be conveniently processed for high throughput proteomic analysis.

In a rat model of radiation nephropathy, proteinuria appears as the first pathophysiological indicator of renal injury several weeks after TBI (Moulder and Fish 1989). Early signs of tissue injury are not known, suggesting subtle change(s) that progress to proteinuria and organ failure after considerable latency. In this regard we have shown that a single dose of 10 Gy X-rays to rats alters glomerular albumin permeability within one hour suggesting altered characteristics of the filtration barrier (Sharma et al. 2001, Sharma et al. 2006). Glomerular functional changes are accompanied by ultrastructural changes such as reduced capillary diameter. Immediate effects of radiation on the urine proteome included altered protein profiles and increased protein count without increase in protein concentration at 24 hours post-TBI (Sharma et al. 2008).

Renal injury due to TBI or partial body irradiation such as localized kidney irradiation (LKI) may cause different effects on the urine proteome. Additionally, homogeneous or inhomogeneous exposure would determine the irradiated organs, metabolic changes and cellular responses to radiation. Present studies were planned to compare the urine proteome at 24-hour after TBI or LKI using a 10 Gy single dose of X-rays. Liquid chromatography-mass spectrometry results showed differences between TBI and LKI groups with regard to protein profiles, protein abundance and number.

MATERIALS AND METHODS

Animals

The study used eight week-old male WAG/RijCmcr rats bred and maintained in a barrier facility at the Translational Biomedical Resource Center, Medical College of Wisconsin, Milwaukee. This facility is fully accredited by the American Association of Accreditation of Laboratory Animal Care (AAALAC). The protocols used in the study had been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) and the Biohazard Committee of the Medical College of Wisconsin, Milwaukee.

X-Irradiation

Conscious animals were held in a ventilated plexiglass restrainer for TBI (n=3) or LKI (n=5). Irradiation was done using a 320 kVp X-RAD orthovoltage machine (Precision X-ray Inc.) with a half value layer of 1.4 mm Cu according to detailed radiation dosimetry described previously (Cohen et al. 2007). TBI was delivered with a posterior-anterior field. LKI was done with parallel-opposed lateral fields; the field size was large enough to account for the uneven positioning of the kidneys. Rats received 10 Gy X-rays in one fraction at a dose rate of 1.73 Gy per minute for TBI or 1.43 Gy per minute for LKI. Control (n=3) rats were held in plexiglass restrainers and sham irradiated for the same duration.

Urine collection

Following irradiation, rats were placed individually in metabolic cages for 24 hours and urine was collected in clean plastic tubes. Fresh urine samples were centrifuged (3000xg) and the supernatant was used to determine total urinary protein (Up) using a reagent kit based on Lowry's method (BioRad, Hercules, CA) and urine creatinine (Uc) using a method based on Jaffe reaction (Teco Diagnostics, Anaheim, CA).

Liquid chromatography-mass spectrometry (LC-MS/MS) analysis of urinary proteins

Sample preparation and analysis were performed as previously described (Yu et al. 2007). Briefly, freshly collected urine samples were mixed with bis-acrylamide, ammonium persulfate and TEMED to obtain proteins in a 15% polyacrylamide gel matrix. Gels were washed with 7% acetic acid in 40% methanol, followed by 50% acetonitrile in water and finally with 50mM ammonium bicarbonate in 50% acetonitrile. Gels were vacuum dried and proteins were trypsinized (1µg trypsin) in 20 mM ammonium bicarbonate (pH 8.0), extracted with 70% acetonitrile in 0.1% formic acid and dried. Dried samples were suspended in 6M guanidine HCl and 1mM DTT in 5 mM potassium phosphate (pH 6.5), purified by C₁₈ Zip tip (Millipore) chromatography and collected in sample vials for mass spectrometry. Samples were dried and re-suspended in 0.1% formic acid with 5% acetonitrile in MS water. Since the present method does not involve iso-electric focusing for sample fractionation as before (Sharma et al. 2008), it is likely that ion suppression by abundant proteins such as major urinary protein may limit the detection of low abundance proteins. Sample fractionation using iso-electric focusing prior to LC-MS/MS would permit detection of low abundance masses but such an approach for a large number of samples would be uneconomical and time consuming. A combination of two approaches to process samples (i.e., an initial in depth analysis of pooled samples followed by comparison of selected fractions from individual samples) may be an efficient approach that needs to be tested.

Samples were analyzed using an automated nano-flow liquid chromatography/mass spectrometry (nano LC-MS/MS). Peptide mixtures were separated on a C18 reverse phase column (PicoFrit capillary column, 75µm×10cm, New Objective) using a linear gradient of Solvent A (0.1% formic acid in 5% acetonitrile) and Solvent B (0.1% formic acid in 95%

acetonitrile) at a flow rate of 1 μ l per min for 180 minutes. The eluate from the column was electrosprayed at a voltage of 1.75 kV. Mass spectrometry was carried out using an LTQ mass spectrometer (Thermo-Fisher, Waltham, MA).

Data Analysis

MS/MS spectra peak lists (.dta files) were extracted from the instrument-produced RAW data files using the *extract_ms* program (version 3) (ThermoFisher, Waltham, MA) with the following key parameters: B 600, T 3500, M 1.4, S1 G1, E 250. The MS/MS data was searched against the rodent subset (Taxon 9989) of the UniProt database (release 54.0) with a version of the *Sequest* (version 27, release 12) program adapted for use on the JS-20 processor (IBM, Armonk, NY) in a cluster environment (ThermoFisher, Waltham, MA) using the following key parameters: Enzyme Trypsin [KR], peptide tolerance 2.5 AMU, fragment ions tolerance 1.0 AMU, differential modifications: M 16, C 57. The search results (.out files) were parsed and the peptide and protein probabilities calculated using the *Epitomize* program (<http://proteomics.mcw.edu/visualize>) and the protein hits with probability scores > 0.6 from each search were saved. Data from the LKI, TBI or control animals were combined using the *Visualize* program (<http://proteomics.mcw.edu/visualize>) and revised protein probability scores calculated based on the observation of proteins in multiple runs and proteins with a probability score of 0.60 retained. Quantitative comparisons were carried out using only proteins with a probability score of > 0.9 and 2 or more unique peptides and using only spectra with a global False Discovery Rate (FDR) of less than 5% (Wu et al. 2006). Combined samples from each group (Control, TBI and LKI) were compared using *Visualize* and the statistical significance of normalized ratios for individual protein abundances between sample groups was determined using the G test (log likelihood).

Visualize was used for quantitative Gene Ontology (GO) analysis for the categories Cellular Localization, Molecular Function and Biological Process (Harris et al. 2004). In order to avoid multiple counting of spectra that can be assigned to more than one protein, we adopted the following strategy.

For each spectrum, the GO terms for every protein that could have generated the peptide that best matched the spectrum were collected. Each GO term was assigned a fractional value based on the number of proteins assigned to the spectrum and the total number of GO terms assigned to each of the proteins for each of the three GO categories. Thus, for each spectrum, the sum of all GO terms from all proteins equaled 1 for each of the three GO categories. Expressing proteins as fractional scans minimizes repeat count and provides a more accurate estimate of protein abundance than a simple scan count.

Statistical analysis

The statistical significance of normalized ratios for individual protein abundances between sample groups was determined using the G test for log likelihood (Hendrickson et al. 2006). Unpaired Student's *t*-test was used for comparing normalized fractions (fraction of total scans) of proteins or Up/Uc ratios.

Protein Annotation

To examine the changes in protein abundance in biological classes of proteins, lists of rodent proteins were obtained by querying the UniProt database (<http://www.uniprot.org>) for each protein group from the rodent taxon, e.g. query="kidney"+taxonomy:9989 or from the literature (Bairoch, 2005). The lists of protein hits of rodent proteases, acute phase proteins, apolipoproteins, kallikreins, kidney injury proteins, kidney expressed proteins, protease

inhibitors, SERPINS and serum proteins used are presented in Supplementary Material accessible at <http://proteomics.mcw.edu/>.

RESULTS

Urine protein (Up) and creatinine (Uc) ratios (Up/Uc) were calculated (means $\pm$ SD) for Control, TBI and LKI groups. The Up/Uc ratio of the Control group (14.25 $\pm$ 0.27) was not different from that of the LKI group (12.53 $\pm$ 3.4 vs. Control, NS) but was higher than that of the TBI group (9.3 $\pm$ 2.4 vs. Control, P <0.05).

The Venn diagram provides a convenient overview of the distribution of proteins in different groups based on the number of scans without rigorous analysis (Fig. 1). The total number of proteins was in the order: LKI (273) > Control (255) > TBI (188). All groups shared 119 (28.9%) proteins. The Control group shared 14 (3.4%) and 39 (9.5%) proteins with the TBI and LKI groups, respectively. The TBI and LKI groups shared 12 (2.9%) proteins. Proteins unique to the Control, TBI or LKI groups were 83 (20.1%), 42 (10.2%) and 103 (25.0%), respectively.

Proteins assigned to cellular compartments terms extracellular spaces, membranes, integral to membrane and cytoplasm constituted most of the urinary proteins in all groups (fraction of scans >0.1) (Fig. 2A). Most other terms constituted <0.05 fraction of total scans. Thus, there was no difference between Control, TBI and LKI groups with regard to proteins from major cellular compartments.

Proteins assigned to molecular function terms transporter activity and pheromone binding were more abundant in the LKI group (LKI > TBI or Control; fraction of scans >0.2) (Fig. 2B). Proteins assigned to terms protein binding, antigen binding, endopeptidase inhibition, serine-type endopeptidase inhibitor, heparin binding and actin binding were TBI > LKI or Control. Proteins assigned to molecular function terms calcium binding, metal binding, growth factor binding, lipid binding (binding proteins), chymotrypsin and trypsin (hydrolases) were Control > TBI or LKI.

Proteins assigned to GO category biological process terms transport, hypersensitivity were more abundant in the LKI group (LKI > TBI or Control; fraction of scans >0.2) (Fig. 2C). Proteins annotated to terms proteolysis and peptidolysis, cell adhesion, acute phase response, muscle development, glutathione synthase, superoxide metabolism, response to oxidative stress, removal of superoxide development, angiogenesis were TBI > LKI or Control (fraction of scans >0.05). Proteins annotated to carbohydrate metabolism, vasodilation and digestion were Control > TBI or LKI (fraction of scans >0.05).

Table 1 summarizes data from Fig. 2A–2C and shows proteins that were detected predominantly or exclusively in the TBI or LKI group.

A comparison of fractional scans of proteins assigned to terms proteases and protease inhibitors in the TBI, LKI and Control groups is shown in Fig. 3. Proteases decreased in both LKI and TBI groups (Control > LKI or TBI) but the difference was not significant (P <0.1 vs. Control). Protease inhibitors increased in the TBI group compared to the Control or LKI group (TBI > Control or LKI; P <0.005 vs. TBI).

A comparison of fractional scans of proteins annotated as kidney proteins, kidney injury proteins, acute phase proteins and the major urinary protein (MUP) is shown in Fig. 4. Proteins assigned to term kidney proteins were more abundant in the LKI group compared to the Control or TBI group (P <0.001). MUP increased in the LKI group compared to the

Control ($P < 0.05$). Proteins assigned to terms kidney injury and acute phase did not constitute an appreciable fraction of the total scans in any group.

Figure 5 shows that the number of proteins (protein count) under terms proteases, protease inhibitors and kidney proteins was TBI > LKI > Control.

Results on serine protease inhibitors (SERPINS), acute kidney injury (AKI) biomarkers, albumin and acute phase proteins are included in Supplementary Materials accessible at <http://proteomics.mcw.edu/>.

DISCUSSION

Ionizing radiation is generally not delivered or absorbed uniformly. Additionally, radiation affects tissues in a dose and time dependent manner. In the kidney, glomeruli, tubules, interstitium and the vasculature differ with regard to sensitivity and/or response to ionizing radiation (Rose 1987; Sharma et al. 2001; Yammani et al. 2002). A better understanding of the effects of homogeneous or inhomogeneous irradiation is important in countermeasure strategies. This subject is receiving increasing attention indicated by a recent AFRR workshop (http://www.afri.usuhs.mil/pb_rad_workshop/index.shtml). Dosimetry of TBI or partial body irradiation would depend upon application of various technologies on suitable samples. Biofluids including plasma, urine, saliva, sweat and tears are potential sources of biomarkers for radiation dosimetry.

Urinalysis provides valuable diagnostic and prognostic information in modern evidence-based medicine. Low abundance plasma and renal proteins in the urine are gaining increasing recognition as potential biomarkers. Carefully prepared urine samples can be fractionated using HPLC, 2-dimensional electrophoresis or capillary electrophoresis followed by mass spectrometry and computational data analysis (Thongboonkerd 2007; Zurbig and Mischak 2008). Special analytical tools such as peptide libraries, lectins or phosphoprotein specific techniques are used to obtain in depth information on low abundance proteins (Boschetti et al. 2008; Moon et al. 2008; Gonzales et al 2008).

Urine proteomics is advancing from discovery phase to biomarker validation in clinical studies on several diseases including steroid resistant nephrotic syndrome (Traum 2008), polycystic kidney disease (Hogan et al. 2009), acute renal failure (Ho et al. 2008), allograft failure (Quintana et al. 2008), pre-eclampsia (Buhimschi et al. 2008), and contrast-induced nephropathy (Bennett et al. 2008). Early biomarkers of radiation nephropathy are not known but the nature and severity of radiation injury prohibits inclusion of human subjects in studies on biomarker discovery. Lower mammalian species such as rodents may provide preliminary information for future verification using higher species (e.g., dogs). Therefore, we are using an established model of radiation nephropathy to determine changes in the urinary protein profiles.

We compared the effect of TBI and LKI on Up/Uc ratio as an indicator of renal function. TBI caused a significant decrease in the Up/Uc ratio ($P < 0.05$) and the effect of LKI was similar but not significant. These results support our previous findings of a decreased Up/Uc ratio at 24 hour post-TBI (Sharma et al. 2008). Decreased total urinary protein may be caused by reduced renal blood flow (Rose 1987) due to vascular and neurohumoral changes. Increased tubular reabsorption may also decrease urinary albumin. In parallel studies, using confocal microscopy, we found that in the puromycin aminonucleoside (PAN) model of minimal change disease, tubular reabsorption of filtered albumin increases immediately while proteinuria appears at about seven days after PAN injection (McCarthy et al. 2005).

We compared the TBI, LKI and Control groups to determine the number of proteins and to identify unique proteins in each group. LC-MS/MS results were searched against a rodent proteomic database and the high confidence hits determined ($<5\%$ FDR, see Methods). The total number of proteins in the LKI group was higher than in the TBI group. This observation is in parallel with the decreased Up/Uc in the TBI group and suggests differences between the two irradiated groups (Fig. 1).

We annotated the LC-MS/MS results using the Gene Ontology (GO) algorithm for the categories cellular compartment, molecular function and biological processes using a probability score of 0.6 for stringent selection and fractional scan to minimize multiple counting of proteins retained for comparison (see Methods). GO analysis indicates that urinary proteins in LKI, TBI and Control groups were derived from all terms assigned to category cellular compartments (Figure 2A). A large number of proteins from the membrane compartments is consistent with proteins being shed into the urine by the kidney or being transferred from the plasma. Increased levels of nuclear or cytoplasmic proteins may arise from increased cellular apoptosis.

GO analysis of terms under molecular function (Fig. 2B) and biological processes (Fig. 2C) showed differences between the LKI and TBI groups. Briefly, LKI increased the abundance of proteins termed transport function and transportation pathway. TBI resulted in increased abundance of most other classes of proteins (see Results). We speculate that these changes are determined by differences in tissue vasculature and perfusion, cellular function and proliferation, and neurohumoral factors.

These interesting and unexpected differences between the TBI and LKI are summarized in Table 1. These novel findings also underscore the growing interest in organ-specific effects of ionizing radiation and suggest the possibility of discovering biomarkers of radiation injury. While a complete understanding of the molecular mechanism of injury and the significance of protein markers will require extensive studies, we note that LKI involves a low dose irradiation of the circulating blood within the kidneys. Renal circulation receives approximately 20% of the cardiac-output. During LKI, the fraction of the total blood in the kidney is free of systemic effects of radiation. In contrast, TBI-induced systemic changes are included in the renal circulating. These differences in plasma irradiation may invoke different physiological responses to maintain homeostasis following TBI or LKI.

Next, we compared proteins annotated in GO terms proteases, protease inhibitors, SERPINS, kidney proteins, MUP, acute phase proteins, serum proteins and albumin (see Methods). Proteases and protease inhibitors constitute a considerable part of the urine proteome. Expressed as a fraction of total scans (fractional scans), abundance of Proteases showed a decrease in the irradiated groups (Control $>$ TBI or LKI). However, protease inhibitors showed a significant increase in the TBI group compared with the Control or LKI groups ($P<0.005$) (Fig. 3). Further analysis of Protease inhibitors showed that SERPINS increased in the TBI group compared to the LKI or Control group ($P<0.01$, data not shown). SERPINS are among the largest and most diverse families of protease inhibitors and 36 SERPINS have been identified in humans. SERPINS play an important role in homeostasis both as protease inhibitors and as non-inhibitory regulators of proteins. A large number of processes including coagulation and inflammation depend upon SERPINS and their deficiency is associated with several diseases (Silverman et al. 2001).

Kidney proteins and MUP (alpha-2 μ -globulin, MUP_RAT) were more abundant in the LKI than in the TBI or Control groups ($P<0.001$) (Figure 4). MUP is a member of the lipocalin family of proteins and is characteristically abundant in the urine and blood of male rodents. MUP is secreted into plasma principally by liver cells, filtered through glomeruli and

reabsorbed by proximal tubules. MUP binds and transports fatty acids and pheromones (Cavaggioni and Mucignat-Caretta 2000). Increased urinary MUP in the LKI, but not in the, TBI further distinguishes the two irradiated groups and suggests decreased tubular reabsorption of certain proteins.

Differences among LKI, TBI and Control groups with regard to the number of proteins (protein count) of proteases, protease inhibitors and kidney proteins are highlighted in Fig. 5. Greater numbers of proteases, protease inhibitors and kidney proteins were identified in the TBI vs. LKI or Control. This is in contrast to expression of results as fractional scans in Figs. 3 and 4 showing decreased abundance of proteases (Control > LKI or TBI). Thus, greater numbers of proteins may be present despite lower abundance. Such differences between protein count and abundance may be important considerations in strategies for biomarker discovery.

GO terms acute phase and kidney injury proteins did not change after TBI or LKI (Fig. 4). Early renal effects of irradiation may share common mechanisms with acute kidney injury (AKI, acute renal failure, ARF) which is associated with a high incidence of morbidity and mortality. Increased serum creatinine is the classical marker of AKI but several molecules constitute a growing list of putative biomarkers of AKI (Waiker and Bonventre 2008;Devarajan 2008). New biomarkers of acute kidney injury namely, kidney injury molecule-1 (KIM), N-acetyl-Beta-D-glucosaminidase, neutrophil gelatinase-associated lipocalin (NGAL) and interleukin-18 (IL-18) were not detectable in the control, TBI or LKI groups. Increased levels of circulating protein fetuin-B were detected only in the TBI group. Previously, we noted a TBI-induced increase in urinary cystatin-C (Sharma et al. 2008). Fetuin and cystatin-C are circulating proteins that are members of the same super family and are also considered biomarkers of AKI (Devarajan 2008). These findings suggest likely differences between the mechanisms and biomarkers of AKI and radiation injury.

Present studies also show a 40% decrease in serum albumin scans in the TBI group compared to a modest 15% decrease caused by LKI. These findings support previous results that showed a 50% decrease in albumin scans following TBI (Sharma et al. 2008). These noticeable differences between TBI and LKI contribute to the idea that systemic effects of radiation contribute to observed differences between TBI and LKI.

In summary, present studies suggest that TBI and LKI cause noticeable changes in the urine proteome within 24 hours after irradiation. Gene Ontology annotation of LC-MS/MS results showed differences between the effects of TBI and LKI on the abundance and number of proteins in several GO groups. The urine proteome may be useful in advancing our understanding of the differences between homogeneous, inhomogeneous and organ-specific irradiation and, lead to biomarker discovery for radiation dosimetry.

Acknowledgments

Financial support

This work received financial support from the NIAID/NIH Center for Medical Countermeasures against Radiological Terrorism, Medical College of Wisconsin, Milwaukee, Wisconsin (NIAID/NIH U19-AI067734) through a Pilot Project Grant award (MS). This work was also supported by the NHLBI Proteomics Center contract NIH-N01 HV-28182.

We thank Dr. R.S. Reddy, D.V.M., Ph.D., Marylou Mader and Cherie Plate for expert technical support. We thank Brian L. Fish (Project Manager) and Yvonne Morauski (Program Coordinator), Center for Medical Countermeasures against Radiological Terrorism (CMCR) for excellent managerial support. We thank Nadine Halligan for critical review of the manuscript. We thank the Protein and Nucleic Acid Facility, and the Biotechnology and Bioengineering Center, Medical College of Wisconsin, WI, for facilitating the LC/MS-MS

work. Some of the results were presented at the Workshop on Partial-Body Radiation Diagnostic Biomarkers and Medical Management of radiation Injury, AFRRI, Washington DC, May 5–6, 2008.

References

- Bairoch A, Apweiler R, Wu CH, Barker WC, Boeckmann B, Ferro S, Gasteiger E, Huang H, Lopez R, Magrane M, Martin MJ, Natale DA, O'Donovan C, Redaschi N, Yeh LS. The Universal Protein Resource (UniProt). *Nucleic Acids Res* 2005;33(Database issue):D154–159. [PubMed: 15608167]
- Bennett MR, Ravipati N, Ross G, Nguyen MT, Hirsch R, Beekman RH, Rovner L, Devarajan P. Using proteomics to identify preprocedural risk factors for contrast induced nephropathy. *Proteomics Clin Appl* 2008;2:1058–1064. [PubMed: 18953418]
- Boschetti E, Giorgio Righetti P. Hexapeptide combinatorial ligand libraries: the march for the detection of the low-abundance proteome continues. *Biotech* 2008;44:663–665.
- Buhimschi IA, Zhao G, Funai EF, Harris N, Sasson IE, Bernstein IM, Saade GR, Buhimschi CS. Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. *Am J Obstet Gynecol* 2008;199:551.e1–551.e16. [PubMed: 18984079]
- Cavaggioni A, Mucignat-Caretta C. Major urinary proteins, alpha(2U)-globulins and aphrodisin. *Biochim Biophys Acta* 2000;1482:218–228. [PubMed: 11058763]
- Cohen EP, Fish BL, Sharma M, Li XA, Moulder JE. The role of the angiotensin II type-2 receptor in radiation nephropathy. *Translational Res* 2007;150:106–115.
- Devarajan P. Proteomics for the investigation of acute kidney injury. *Contrib Nephrol* 2008;160:1–16. [PubMed: 18401157]
- Gonzales PA, Pisitkun T, Hoffert JD, Tchapyjnikov D, Star RA, Kleta R, Wang NS, Knepper MA. Large-scale proteomics and phosphoproteomics of urinary exosomes. *J Am Soc Nephrol* 2008;20:363–379. [PubMed: 19056867]
- Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, Foulger R, Eilbeck K, Lewis S, Marshall B, Mungall C, Richter J, Rubin GM, Blake JA, Bult C, Dolan M, Drabkin H, Eppig JT, Hill DP, Ni L, Ringwald M, Balakrishnan R, Cherry JM, Christie KR, Costanzo MC, Dwight SS, Engel S, Fisk DG, Hirschman JE, Hong EL, Nash RS, Sethuraman A, Theesfeld CL, Botstein D, Dolinski K, Feierbach B, Berardini T, Mundodi S, Rhee SY, Apweiler R, Barrell D, Camon E, Dimmer E, Lee V, Chisholm R, Gaudet P, Kibbe W, Kishore R, Schwarz EM, Sternberg P, Gwinn M, Hannick L, Wortman J, Berriman M, Wood V, de la Cruz N, Tonellato P, Jaiswal P, Seigfried T, White R. The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Res* 2004;32(Database issue):D258–261. [PubMed: 14681407]
- Hendrickson EL, Xia Q, Wang T, Leigh JA, Hackett M. Comparison of spectral counting and metabolic stable isotope labeling for use with quantitative microbial proteomics. *Analyst* 2006;131:1335–1341. [PubMed: 17124542]
- Ho J, Lucy M, Krokhn O, Hayglass K, Pascoe E, Darroch G, Rush D, Nickerson P, Rigatto C, Reslerova M. Mass spectrometry-based proteomic analysis of urine in acute kidney injury following cardiopulmonary bypass: A nested case-control study. *Am J Kidney Dis* 2008;53:584–595. [PubMed: 19070948]
- Hogan MC, Manganelli L, Woollard JR, Masyuk AI, Masyuk TV, Tammachote R, Huang BQ, Leontovich AA, Beito TG, Madden BJ, Charlesworth MC, Torres VE, Larusso NF, Harris PC, Ward CJ. Characterization of PKD protein-positive exosome-like vesicles. *J Am Soc Nephrol*. Jan 21;2009 Epub ahead of print PMID: 19158352.
- Jarrett, DG. Medical management of radiological casualties. Armed Forces Radiobiology Research Institute; Bethesda, MD: 1999.
- Manthous CA, Jackson WL Jr. The 9-11 Commission's invitation to imagine: a pathophysiology-based approach to critical care of nuclear explosion victims. *Crit Care Med* 2007;35:716–723. [PubMed: 17255868]
- McCarthy, ET.; Reddy, RS.; Isaacs, F.; Sharma, J.; Sandoval, R.; Dunn, K.; Molitoris, B.; Sharma, R.; Savin, VJ.; Sharma, M. Novel techniques demonstrate the effect of puromycin aminonucleoside (PAN) on glomerular protein permeability and protection by clofibrate or 20-hydroxyeicosatetraenoic acid (20-HETE). Abstract for the ASCI/AAP Joint Meeting; Chicago, IL USA. April 15–17; 2005.

- Metzger J, Schanstra JP, Mischak H. Capillary electrophoresis-mass spectrometry in urinary proteome analysis: current applications and future developments. *Anal Bioanal Chem* 2008;393:1431–1442. [PubMed: 18704377]
- Moon PG, Hwang HH, Boo YC, Kwon J, Cho JY, Baek MC. Identification of rat urinary glycoproteome captured by three lectins using gel and LC-based proteomics. *Electrophoresis* 2008;29:4324–4331. [PubMed: 18956432]
- Moulder JE, Fish BL. Late toxicity of total body irradiation with bone marrow transplantation in a rat model. *Int J Rad Onc Biol Phys* 1989;16:1501–1509.
- Quintana LF, Sole-Gonzalez A, Kalko SG, Bañon-Maneus E, Sole M, Diekmann F, Gutierrez-Dalmau A, Abian J, Campistol JM. Urine proteomics to detect biomarkers for chronic allograft dysfunction. *J Am Soc Nephrol* 2008;20:428–435. [PubMed: 19056874]
- Pellmar, TC.; Prasanna, PGS.; Blakely, WF. Biomarkers for partial body irradiation and organ-specific injuries. *BioDose* 2008; Dartmouth, NH. September 7–10; 2008.
- Ricks, RC.; Berger, ME.; O'Hara, FM. The medical basis for radiation-accident preparedness: the clinical care of victims. *Proceedings of the Fourth International REAC/TS Conference on the Medical Basis for Radiation-Accident Preparedness*; March 6–8, 2001; Orlando, Florida. Boca Raton: Parthenon Pub. Group; 2002.
- Rose, BD. Pathophysiology of renal disease. McGraw-Hill Professional; 1987. p. 366–368.
- Sharma M, Sharma R, Ge XL, Fish BL, McCarthy ET, Savin VJ, Cohen EP, Moulder JE. Early detection of radiation-induced glomerular injury by albumin permeability assay. *Rad Res* 2001;155:474–480.
- Sharma M, McCarthy ET, Sharma R, Fish BL, Cohen EP, Savin VJ, Moulder JE. Arachidonic acid metabolites mediate the radiation-induced increase in glomerular albumin permeability. *Exp Biol Med* (Maywood, NJ) 2006;231:99–106.
- Sharma M, Halligan BD, Wakim BT, Savin VJ, Cohen EP, Moulder JE. The urine proteome as a biomarker of radiation injury. *Proteomics-Clinical Applications* 2008;2:1065–1086. [PubMed: 19746194]
- Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnell E, Salvesen GS, Travis J, Whisstock JC. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J Biol Chem* 2001;276:33293–33296. [PubMed: 11435447]
- Thongboonkerd V. Practical points in urinary proteomics. *J Proteome Res* 2007;6:3881–3890. [PubMed: 17824635]
- Traum AZ. Urine proteomic profiling to identify biomarkers of steroid resistance in pediatric nephrotic syndrome. *Expert Rev Proteomics* 2008;5:715–719. [PubMed: 18937561]
- Waikar SS, Bonventre JV. Biomarkers for the diagnosis of acute kidney injury. *Nephron Clin Pract* 2008;109(4):c192–197. [PubMed: 18802367]
- Wu B, Guan Z, Zhao H. Parametric and nonparametric FDR estimation revisited. *Biometrics* 2006;62:735–744. [PubMed: 16984315]
- Yammani RR, Sharma M, Seetharam S, Moulder JE, Seetharam B. Loss of albumin and megalin binding to renal cubilin results in albuminuria in rats exposed to total body irradiation. *Am J Physiol Regul Integr Comp Physiol* 2002;283:R339–346. [PubMed: 12121845]
- Yu H, Wakim B, Li M, Halligan B, Tint GS, Patel SB. Quantifying raft proteins in neonatal mouse brain by 'tube-gel' protein digestion label-free shotgun proteomics. *Proteome Sci* 2007;5:17. [PubMed: 17892558]
- Zurbig P, Mischak H. Capillary electrophoresis coupled to mass spectrometry for biomarker discovery and diagnosis of kidney diseases. *Contrib Nephrol* 2008;160:107–126. [PubMed: 18401165]

Fig. 1.

The Venn diagram presents an overview of LC-MS/MS results. These numbers are derived from raw protein counts. About one third of all proteins (28.9%) were present in all groups. Proteins uniquely present in the Control, TBI and LKI groups were 20.1%, 10.2% and 25.0%, respectively. Differences between the TBI and LKI groups suggest tissue-specific effects of ionizing radiation.

Figure 2A

Figure 2B

Figure 2C

Fig. 2.

Fig. 2A. Proteins assigned to cellular compartments extracellular spaces, various membrane compartments, nucleus, lysosomes, endoplasmic reticulum, actin filament, cytosol, plasma membrane, intermediate filament, soluble fraction and golgi stack were most abundant in all groups and higher in the Control group compared with the TBI or LKI groups.

Fig. 2B. Proteins assigned to molecular functions transporter activity, pheromone binding, growth factor binding, epidermal growth factor receptor binding, heme binding were in the order LKI > Control > TBI. Proteins assigned molecular functions hydrolase activity, alpha amylase activity, glycosyl binding, endopeptidase activity, actin binding and structural molecule binding activity were in the order TBI > LKI > Control.

Fig. 2C. Proteins assigned to biological process transport, Type 1 hypersensitivity, oxygen transport, iron ion homeostasis and glutathione biosynthesis were in the order LKI > TBI > Control.

Fig. 3. Proteases and protease inhibitors in Control, LKI and TBI groups are presented as percent of total scans. Proteases were detected in the order Control > LKI > TBI. Protease inhibitors were detected in the order TBI > Control $\geq$ LKI. Increased abundance of protease inhibitors suggests activation of proteases in the blood.

Fig. 4.

Proteins grouped as kidney proteins, kidney injury proteins, acute phase proteins and major urinary protein based on UniProt classification are shown. Kidney proteins in the LKI group were significantly different from Control (P <0.001 vs. LKI) and TBI groups (P <0.001 vs. LKI). MUP in the LKI was significantly increased compared with Control (P <0.05). Kidney injury proteins and acute phase proteins constituted very small fractions of the total.

Fig. 5.
The protein count (protein number) of proteases, protease inhibitors and kidney proteins increased in the urine proteome following irradiation in the order TBI > LKI Control.

Table 1

Protein detected in the TBI or LKI group

Proteins in the TBI and LKI groups were compared. Table summarizes the proteins that were significantly different between the TBI or LKI group. Proteins identified only in the TBI or LKI group are listed in column titled Group. Detailed list of proteins in each group is presented as Supplementary Material (<http://proteomics.mcw.edu/>).

Protein	Accession	Description
Proteins exclusively present in TBI group (p<0.05 vs. LKI)		
FETUB_RAT	Q9QX79	Fetuin-B precursor (IRL685).
BGLR_RAT	P06760	Beta-glucuronidase precursor (EC 3.2.1.31).
KLK3_RAT	P15950	Glandular kallikrein-3, (submandibular, Tissue kallikrein, S1 kallikrein) (EC 3.4.21.35)
CALB1_RAT	P07171	Calbindin (Vitamin D-dependent calcium-binding protein, avian-type)(Calbindin D28)
CSPG4_RAT	Q00657	Chondroitin sulfate proteoglycan 4 precursor (Chondroitin sulfate proteoglycan NG2)
Proteins exclusively present in LKI group (p<0.05 vs. TBI)		
MUP_RAT	P02761	Major urinary protein precursor (MUP) (Alpha-2u-globulin)
RBM19_MOUSE	Q8R3C6	Probable RNA-binding protein 19 (RNA-binding motif protein 19).
NAV1_MOUSE	Q8CH77	Neuron navigator 1 (Pore membrane and/or filament-interacting-like protein 3).
MUPL_MOUSE	P11588	Major urinary protein 1 precursor (MUP 1).
RAI14_RAT	Q5U312	Ankycorbin (Retinoic acid-induced protein 14)
DACT3_MOUSE	Q0PHV7	Dapper homolog 3.
WDFY3_MOUSE	Q6VNB8	WD repeat and FYVE domain-containing protein 3
MUP_RATRT	P84855	Major urinary protein (MUP, Alpha-2u-globulin, Alpha(2)-euglobulin)(Fragments).
SNX8_MOUSE	Q8CFD4	Sorting nexin-8.
AQP1_RAT	P29975	Aquaporin-1 (AQP-1, Water channel protein for RBC and kidney proximal tubule).