MALDI and LCMS Protein Biomarkers of Ionizing Radiation

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INTRODUCTION

• The goal of this project was to identify dose-related protein biomarkers of ionizing radiation (IR) with mass spectrometry, specifically, biomarkers of the 2 Gy threshold dose for radiation sickness.
• A civilian nuclear power plant accident or a terrorist nuclear event on U.S. soil could result in a vast number of casualties.
• Some of the victims would be hospitalized because of acute signs and symptoms, such as vomiting, burns and pain requiring immediate care.
• Some victims would appear symptom free, but may have received $\geq 2$ Gy; total body radiation (TBI). **They would develop radiation sickness in the next 6-24 hours.**
• Initial mass spectrometry (MS) experiments demonstrated radiation dose-related albumin and other induced proteins in radiosensitive murine buccal mucosa and tongue tissue.
 METHODS

- Forty Swiss Webster mice, 10 control and 30 experimental.
- The anesthetized animals received TBI, with a low linear energy transfer (LET) photon beam (Linac 23 MV linear accelerator) as follows: 1Gy(10 mice), 2Gy(10 mice) and 3Gy(10 mice), in groups of five.
- Sucrose 30%, 5cc was infused (intracardiac/femoral perfusion) under terminal anesthesia.\(^1\) The sucrose cryoprotected the tissue from freezer artifact (disruption of tissue architecture by ice crystals).\(^2\)

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METHODS

• Fresh paraformaldehyde fixative 3.7% perfusion (3cc) followed the sucrose. The tongue and heart were removed after perfusion.

• The organs were placed in 3.7% paraformaldehyde for one hour (10:1 solution:tissue, V/V) at 4°C. The short immersion time fixed the tissue without completely cross-linking all tissue proteins. Cross-linking interferes with both matrix assisted laser desorption ionization (MALDI), MALDI imaging (IMS), and liquid chromatography mass spectrometry (LCMS).

• The tissue was then immersed in sucrose 30% (10:1 solution:tissue, V/V) at 4°C until equilibration (tissue sinks to the bottom of the sucrose solution). The sucrose immersion provided further cryoprotection.

CRYOSECTION PREPARATION

- The samples were placed in a tissue mold filled with tissue freezing medium (TFM). TFM supports the tissue and prevents cutting artifacts.\(^5\)
- The tissue mold was placed on a small rapid-freeze disk in the cryostat.
- The mold was immersed in liquid nitrogen-cooled isopentane which flattened the block and improved tissue sectioning.
- Contiguous one-micrometer (µm) serial sections were obtained for histology, IMS, and protein extraction for LCMS.

A section was applied to a stainless steel (glass slide sized) MALDI conductive plate.

The plates were immersed in a methanol bath for 15 minutes followed by immersion in a xylene bath for 15 minutes.

The purpose of methanol and xylene baths was to improve IMS by defatting the tissue, and removing all TFM, a polymer that interferes with MALDI.

A protein calibrant mixture (insulin, cytochrome C, apomyoglobin, aldolase and BSA) covered with uniform matrix was used for MALDI and IMS calibration.

Sinapic acid (matrix) was applied uniformly to the tongue tissue for IMS.

MALDI TOF TOF MASS SPECTROMETER
BAROCYLER EXTRACTION

- A contiguous section was obtained for protein extraction and LCMS analysis for protein identification.
- The section was immersed in 900 µl of ammonium bicarbonate 100 mM in a pressure cycling device (Barocycler) pulse tube.
- The proteins were extracted with 35,000 psi alternating pressure for about 30 seconds. 
- The extracted samples were centrifuged for about 5 minutes at 15 G then lyophilized to a volume of 50 µl.

TRYPSIN DIGEST

- Trypsin, 1 µl (1 µg/µl), was added to the 50 µl sample and maintained at 37°C overnight.

- The trypsin digest was analyzed with LCMS for protein biomarker identification.
Nanoflow LCMS

- High resolution MS\(^n\) analysis
- Wide dynamic range
- High sensitivity
- Stable splitless nanoflow
- Information-based acquisition
IMS & LCMS

- IMS(images) were obtained from tissue sections of normal, 1Gy, 2Gy and 3Gy irradiated tongue.
- LCMS spectra were obtained from normal, 1, 2 and 3 Gy irradiated tongue samples; normal and 2 Gy irradiated heart samples.
RESULTS

• Hematoxylin & eosin (H&E) were used to stain tissue sections post 1Gy TBI.

• The samples demonstrated progressive increase in tissue destruction. The corresponding IMS tissue change was the identification of albumin (not seen in the tissue IMS of the normal control).

• At 2 Gy there was increased peripheral tissue damage of the spicules on H&E, and a corresponding increase in peripheral albumin in the IMS images.

• At 3 Gy the peripheral tissue damage of the spicules and the central damage of the tongue was severe on the H&E sections. The albumin was now virtually absent in the periphery and concentrated in the center of the IMS image.
H&E OF NORMAL MURINE TONGUE

Histopathology of normal murine tongue 10X, longitudinal section. Note the well defined epithelial cornified spicule layer and basal cells.

Histopathology of normal murine tongue, 63X longitudinal section. Note the well defined epithelial cornified spicule layer and basal cells.
Normal 33781.1 Da Double Charge Albumin

IMS of normal murine tongue, longitudinal section. Note scattered foci of residual Albumin (red) within tongue vessels.
MURINE TONGUE H&E SECTION OF 1Gy TBI

H&E section 10X one hour post 1 Gy TBI. Note destructive changes in the cornified spicules and edema in the basal layers.

H&E section 63X one hour Post 1 Gy. Note swelling of basal cells and edema of sub-basal layer.
1Gy 33,322.7 Da Double Charge Albumin

MALDI image of murine tongue one hour post 1 Gy TBI, longitudinal section. Note peripheral foci of Albumin
MURINE TONGUE H&E SECTION OF 2Gy TBI

H&E section 10X of murine tongue one hour post 2 Gy TBI. Note progressive disruption of the cornified spicule layer, and increased edema of the sub-basement membrane layer.

Post 2 Gy H&E section 63X. Note marked “smudging” and edema of the basal cells.
2Gy 33,551.1 Da Double Charge Albumin

IMS, note marked increase in peripheral foci of Albumin compared to the post 1 Gy IMS (Red).
MURINE TONGUE H&E SECTION OF 3GY TBI

H&E section 10X of murine tongue one hour post 3 Gy TBI. Note progressive destructive changes and virtual complete loss of the cornified spicule layer corresponding to the loss of peripheral Albumin foci in the MALDI image; increased edema and damaged architecture of the center of the tongue, compared to the 2 Gy H&E section.

Post 3 Gy H&E section 63X. Note severe damage at the base of the spicules, chromatin debris, and edema compared to the 2 Gy H&E.
IMS of murine tongue one hour post 3 Gy TBI. Note loss of peripheral albumin and increase in central foci of Albumin. This is due to tissue loss at the periphery and increased destruction of the center of the tongue. Albumin (Red) compare with the post 2 Gy image.
RESULTS

• IMS tongue
  • Albumin was demonstrated in the 1Gy, 2Gy and 3Gy sections.
  • Hemoglobin Subunit α in the IMS post 1 Gy.
  • Fatty Acid-Binding Protein Adipocyte in the IMS image post 2 Gy.
  • Hemoglobin α Chains in the IMS image post 3 Gy.

• LCMS tongue
  • Albumin was demonstrated in the contiguous tissue section extracts of 1Gy, 2Gy and 3Gy.

• LCMS heart
  • Protein identified in the normal and post 2 Gy cardiac tissue was identical, Actin, alpha cardiac muscle 1 OS=Mus musculus
LCMS spectrum tongue normal
LCMS spectrum tongue post 1 Gy

(1) Spec #1 \([BP = 399.2, 549.0]\)
LCMS spectrum tongue post 2 Gy
LCMS spectrum tongue 3 Gy
Mascot search of proteins in normal murine tongue.

No albumin was identified.
Mascot search of proteins in 1GY murine tongue

Albumin appears at the end of the list and corresponds to the findings in the MALDI image one-hour post 1 Gy. The 1 Gy IR induced proteins are listed in Table 1.
Mascot search of proteins in 2GY murine tongue

Albumin appeared in the list as Q3TVO3_mouse. Albumin and the proteins post 2 Gy listed in Table 1 are IR dose related biomarkers.
Mascot search of proteins in 3GY murine tongue one hour post 3 Gy TBI. Albumin appeared in the list as Q3TVO3_mouse. Albumin and the other proteins identified post 3 Gy listed in Table 1 are IR dose related biomarkers.
Normal Mouse Tongue

Mouse Tongue 1hr post 1 GY
Q3TV03 - Mus: albumin 1 - full insert sequence.
Q9CXH5 - Mus: hemoglobin, beta adult major chain
PO1942 - Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Mouse Tongue 1hr post 2 GY
Q3TV03 - Mus: albumin 1 - full insert sequence.
PO1942 - Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Mouse Tongue 1hr post 3 GY
Q3TV03 - Mus: albumin 1 - full insert sequence.
PO1942 - Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Q9CY06 - Mus: hemoglobin, beta adult major chain Symbol: Hbb-b1
P02088 - Mus: HBMS Hemoglobin subunit beta
Q3UJH8 - Mus: glutamate oxaloacetate transaminase 1 EC=2.6.1.1
P11404 - Mus: fatty acid-binding protein, heart FABPH.
P04117 - Mus: FABPA Fatty acid-binding protein, adipocyte (AFABP) also (Adipocyte lipid-binding protein) (ALBP)
S02654; S00195 Mus: malate dehydrogenase DEMSMC - (EC 1.1.1.37), cytosolic – mouse, L-lactate dehydrogenase
Q3UBW7 - Mus: transferrin

Q9CY54 - Mus: hemoglobin, beta adult major chain Symbol: Hbb-b1

Q9JJ20 - Mus: 14-3-3 protein sigma; Sfn MGI 1891831 Synonyms: Mme1 EMBL AAF36093.1
Q64475 Mus: histone H2B type 1-B (h2B-143)H2B1B_
Q9Z1R9 Mus: - trypsinogen 16

BAC33789 - Mus: Sgol1 shugoshin-like 1 [(S. pombe) (AK049517.1)]

Q9Z1R9 - Mus: - trypsinogen 16

Q9CY12 - Mus: hemoglobin, beta adult major chain
Q3USS4 - Mus: glial fibrillary acidic protein
Q3TWV0 - Mus: vimentin
JC4030 - Mus: DnaJ-like protein MTJ1
Q9QZ05 (E2AK4) Mus: eukaryotic translation initiation factor 2-alpha kinase 4 EC=2.7.11.1
DISCUSSION

• The goal of this project was to determine dose-related IR induced protein.

• Initial MALDI and LCMS, experiments in mice demonstrated radiation dose related albumin and induced proteins in radiosensitive buccal mucosa and tongue tissue.\(^8\)

• For this study, the initial experiments were expanded to include evaluation of induced protein changes in the tongue and heart of mice exposed to 1-3 Gy IR.

• Heart tissue was chosen to illustrate that total body radiation affected other organs which were less radiosensitive than the tongue.

DISCUSSION

• Histologic examination of the murine tongue post 1, 2, and 3 Gy demonstrated:
  - progressive epithelial keratin spicule destruction with swelling of the epithelial basal cell layer.
  - progressive loss of the integrity of arteriole endothelium with opening of the tight junctions between endothelial cells and leaking of serum (albumin) first into the periphery and then into the center of the tongue.
  - increasing edema is a function of arteriole integrity failure.
  - loss of peripheral structure.
  - disorganization of the central anatomy of the tongue.

• This correlated with serial IMS sections that demonstrated the progressive increase in albumin and other proteins that were found at increasing IR doses.
CONCLUSION

• MALDI, IMS, and LCMS identified IR induced dose-related proteins.
• The identification of these IR dose-related biomarkers is novel.
• These protein biomarkers of IR could distinguish the 2 Gy threshold dose level for radiation sickness.
• This study is proof of principle that MALDI and LCMS can identify dose-related protein biomarkers of IR that can be used in tongue test strips for nuclear disasters.
THANK YOU