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Comparative analysis of the serum proteome for biomarker discovery to reveal hepatotoxicity induced by iron ion radiation in mice

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ABSTRACT

Aims: Proteomic analysis of serum biomarkers to determine liver toxicity after exposure to cosmic radiation has not been performed previously. This study was to identify serum biomarkers associated with hepatotoxicity following exposure to iron ion radiation.
Main methods: Male mice were whole-body irradiated with a 2 grayunit (Gy) iron ion beam, and after 3 months, serum and liver samples were collected. Two-dimensional electrophoresis (2-DE) was used to separate the identified serum proteins, and matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-TOF-TOF) was performed to identify differentially expressed proteins. Enzyme-linked immunosorbent assays and immunoblotting were applied to evaluate protein expression, and immunohistochemistry and immunofluorescence were used to investigate protein localization. Real-time polymerase chain reaction (PCR) was performed to confirm altered gene expression.
Key findings: A total of 11 spots that showed differential expression were screened and identified as seven proteins. Of these, six proteins were in the same bioinformatics network and included complement component 3, serum amyloid P-component, apolipoprotein E, alpha-2-macroglobulin, fibrinogen alpha chain, and fibrinogen gamma chain. All of these proteins are synthesized by the liver, and may play an important role in liver toxicity. We also confirmed the mRNA transcription, and found that mRNA expression of the six identified proteins increased in the liver in irradiated mice.
Significance: These results suggest that these proteins may be potential biomarkers of hepatotoxicity in astronauts enduring long space missions.

1. Introduction

It is important to determine whether exposure to cosmic radiation induces disease or toxicity in astronauts and space travellers [1]. The long duration of space missions is particularly dangerous and astronauts are at risk of exposure to high background levels of ionizing radiation [2]. This exposure causes oxidative damage, which induces DNA lesions, cancer, cell death [3] and other adverse effects [4]. Protons and ions with a high atomic number and energy particles are the main source of chronic whole body radiation in astronauts during space missions [5, 6], and the high density of heavy ions causes complex clustered DNA damage, which is more difficult to repair than electromagnetic radiation-induced damage due to x-rays and γ-rays [7]. Thus, carbon ions are the preferred heavy ions used in cancer radiotherapy [8]. Moreover, iron ions have aroused special interest in researchers [9], as ^{56}Fe is the most important ion during cosmic radiation exposure [10]. Therefore, in future long-term space missions, protection strategies against the possible harmful effects of cosmic radiation are necessary.

Thus, it is important to ascertain whether exposure to cosmic radiation induces disease or toxicity so that we can understand the possible effects on the health of astronauts and space travellers. The liver biopsies can induce potential complications and patient discomfort.

Abbreviations: IIR, iron ion radiation; 2-DE, two-dimensional gel electrophoresis; MALDI-TOF-TOF, matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry; HIRFL, Heavy Ion Research Facility in Lanzhou; CBB, Coomassie Brilliant Blue; TBS, Tris buffer saline; HRP, horseradish peroxidase; C3, complement component 3; A2M, alpha-2-macroglobulin; APCS, serum amyloid P-component; APOE, apolipoprotein E; FGA, fibrinogen, alpha polypeptide; FGG, fibrinogen, gamma polypeptide.

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Therefore, diagnostic markers for liver disease or toxicity have important research significance, as in the future they could influence daily clinical practice by driving clinical decision-making [11]. Comparative proteome analysis is a new and emerging technology for the discovery of new serological biomarkers for early diagnosis of chronic diseases and the identification of disease-specific proteins or peptides for defining the onset, progression and prognosis of human diseases [12]. Analysis of serum with proteomics has been successfully applied to the analysis and characterization of changes in protein expression in liver disease [13], ovarian cancers [14] and Alzheimer's disease [15]. A 2-DE profile of serum has been used to identify diagnostic biomarkers of liver disease [16].

Knowledge of the biological effects of cosmic radiation exposure is mainly from in vitro studies using human or animal cells and in vivo research with animal models from ground-based experiments at accelerators [17]. In this study, we used a comparative proteomics approach based on a 2-DE reference map to detect alterations in protein expression in the serum of Swiss–Webster mice following exposure to iron ion radiation (IIR), and to identify biomarkers of liver toxicity. The results reveal the underlying molecular mechanisms of liver disease or toxicity following exposure to IIR.

2. Materials and methods

2.1. Animals

Healthy male Swiss-Webster mice aged 3 weeks and weighing 12–18 g were purchased from Lanzhou Medical College (Lanzhou, China) and randomly divided into the control and iron ion irradiation (2 Gy) groups ($n = 6$ in each group). Three mice per cage were maintained in polycarbonate cages at a temperature of 22 ± 2 °C and humidity of 60 ± 10% in cellulosic fiber cages with a 12 h light/dark photoperiod. All animals had free access to deionized water in glass bottles with a rubber stopper and rodent feed (Keaoxieli Laboratory Animal Feed, Beijing, China). All animals were subjected to the same laboratory conditions for 1 week prior to the experiments and were killed by cervical dislocation. However, control animals were kept in the animal house until killed and did not undergo irradiation, and represented the 0 Gy group. All experiments were approved by the Institutional Animal Care Committee [18].

2.2. Irradiation procedure and animals treatment

4-week-old male mice were whole-body irradiated with a 2 Gy iron ion beam at 140 MeV/U and 31.3 keV/μm (at the beam entrance), to give a dose of approximately 0.5 Gy/min, at the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China). Mice from each group were killed at 3 months after irradiation. Blood was collected and centrifuged at 2000 ×g at 4 °C for 15 min. Serum was stored at −80 °C until analysis. Livers of 3 mice were removed and were fixed in 4% paraformaldehyde solution (4 g paraformaldehyde in 100 mL PBS) and embedded in paraffin blocks, and livers of the other 3 mice were frozen immediately in liquid nitrogen and stored at −80 °C until analysis.

2.3. Histological analysis of hepatic tissues

Serial 4-μm-thick slices were prepared and stained with hematoxylin and eosin (H&E). Each stained section observed and took figures with a light microscope (Nikon 80i, Japan). Three fields were randomly selected from each section. Three sections were examined in each mouse.

2.4. 2-DE, image analysis and MALDI-TOF/TOF MS analysis

Each 10 μL serum sample was dissolved in 350 μL rehydration buffer and loaded, then separated by 17 cm Immobiline IPG strips at pH 3–10 (Bio-Rad Laboratories, Hercules, CA, USA) on an IPGphor isoelectric focusing system (IEF) using the following program: 14 h at 50 V; 1 h at 250 V; 1000 V for 1 h; 9000 V for 6 h; 9000 V for 8 h. After focusing, the equilibrium buffer (2% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 8.8), 6 M urea, and 30% glycerol) with dithiothreitol (DTT) and iodoacetamide was used to equilibrate the strips for 15 min, respectively. The strips then underwent 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for the second dimension. After electrophoresis, gels were stained with Coomassie Brilliant Blue (CBB)-R250 overnight and destained with ultrapure water three times for 10 min. Three 2-DE gels were prepared for each sample and were scanned with an Epson scanner and stored as TIF files. PDQuest 8.0 software (Bio-Rad) was used to detect and match spots. Spots were selected if the difference in protein quantity was ≥1.5-fold (normalized spot volume) between control and irradiated groups. Protein spots were carefully excised from 2-DE gels, destained, washed, and then digested for 13 h with modified sequencing grade trypsin (Roche, Mannheim, Germany) [19,20]. MS and MS/MS data for protein identification were obtained using a MALDI-TOF-TOF instrument (4800 proteomics analyzer; Applied Biosystems). Instrument parameters were set using the 4000 Series Explorer software (Applied Biosystems).

2.5. Extracted and measured protein concentration of hepatic tissues

Hepatic tissues was treated with a lysis buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, 2% (w/v) DTT in the presence of 1% (w/v) protease inhibitor cock-tail (Sigma Chemical, St. Louis, MO, USA) [20]. Protein concentration was measured by the Bio-Rad Bradford protein assay with bovine serum albumin (Sigma) as a standard.

2.6. C3 levels of serum and hepatic tissues

Concentrations of C3 in serum and hepatic tissues were measured using ELISA kits (Elabscience Co., Wuhan, China).

2.7. Immunoblotting

Forty micrograms of hepatic tissue protein and 3 μL serum were electrophoresed by 12% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (PVDF) (Roche). The membranes were blocked with 5% skim milk (TBS with 5% nonfat milk powder) for 1 h and then incubated overnight with the primary antibodies in TBS/5% nonfat milk powder overnight at 4 °C. The primary antibodies were polyclonal anti-APCS (Cat. no. BS6527), anti-APOE (Cat. no. BS5614), anti-A2M (Cat. no. BS60859) and anti-β-actin (Cat. no. AP0060) (1:1000, Bioworld technology, Inc., Ltd., Nanjing, China), anti-FGA (Cat. no. bs-7548R) and anti-FGG (Cat. no. bs-6895R) (1:500, Boaoseng Biotechnology, co, Ltd., Beijing, China), β-actin was used as a protein control to normalize volume of liver protein expression and β-tubulin (Cat. no. sc-9104) (1:1000, Santa Cruz, CA, USA) was used as a protein control to normalize volume of serum protein expression. After washing membranes 3 times (10 min each) with TBS, immunoreactivity was detected using an enhanced chemiluminescent HRP substrate kit (Cat. no. BLH01S050; Bioworld) and images were captured using a FluorChem 2 imaging system (Alpha Innotech, San Lean-dro, CA, USA). Protein bands from scanned images were quantified using the Quantity One 4.5.2 image analysis software (Bio-Rad). Data were corrected for background and expressed as optical density (OD/mm^2).