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Proteomic analysis of rat plasma with experimental autoimmune uveitis based on label-free liquid chromatography–tandem mass spectrometry (LC–MS/MS)

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ABSTRACT

Uveitis is a severe autoimmune eye disease that can cause intraocular inflammation even lead to severe vision loss, and the occurrence of uveitis can be closely associated with abnormal expression of proteins. However, the abnormally expressed proteins involved in uveitis are not well identified. Using liquid chromatography–tandem mass spectrometry technique, we examined the alterations in proteomic expression profiling in rat plasma specimens related to experimental autoimmune uveitis (EAU) *versus* normal samples. In addition, the experimental verification was further performed using enzyme-linked immunosorbent assay (ELISA) for abnormally expressed proteins in EAU rat plasma. The results indicate that 62 proteins were upregulated and 106 proteins were downregulated in plasma from EAU rats compared with those in saline-treated samples. In the meantime, we observed that the plasma level of complement component 3 in EAU rats was upregulated *versus* saline-treated rats (from 92.32 μg/mL to 168.92 μg/mL), whereas the level of interleukin-1 receptor accessory protein was downregulated (from 1120.97 pg/mL to 798.39 pg/mL), and these results were highly in agreement with those of mass spectrometry determination. Taken together, our results indicate that liquid chromatography–tandem mass spectrometry analysis possesses a good resolution for peptides in plasma, and the findings will provide the baseline plasma dataset for EAU rats and the relevant information can contribute to future studies on the understanding the mechanism of uveitis.

1. Introduction

Uveitis is a severe autoimmune eye disease that can lead to intraocular inflammation. Either uncontrolled or inadequately treated posterior uveitis will result in permanent damage in various ocular tissues, and eventually causes irreversible visual loss. As an autoimmune disease, uveitis is commonly involved in intraocular inflammation associated with the aberrant expression of various cytokines, which are usually mediators of immunologic and inflammatory responses, such as interleukin (IL)-1 [1,2], IL-2 [3,4], IL-4 [3,4], IL-6 [2], IL-10 [2,3,5], IL-17 [6,7], IL-21 [8,9] interferon (IFN)-gamma [2–4], and tumor necrosis factor (TNF)-alpha [3,10,11]. In the meantime, the development and pathogenesis of uveitis are also associated with the activation of intracellular signaling pathways including p38 mitogen-activated protein kinase (MAPK) [12,13], nuclear factor (NF)-κB [14,15], toll-like receptor [16,17] signaling pathways and so on. Nevertheless, the change of proteomic profiling that associated with uveitis is still unclear, and the detailed molecular pathogenesis needs to be addressed.

Abbreviations: LC–MS/MS, label-free liquid chromatography–tandem mass spectrometry; EAU, experimental autoimmune uveitis; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; NF, nuclear factor; MS, mass spectrometry; DE, dimensional electrophoresis; IRBP, interphotoreceptor retinoid-binding protein; IL-1RAcP, IL-1 receptor accessory protein; CFA, Complete Freund's Adjuvant; ppm, parts per million; FDR, false discovery rate; SD, standard deviation; BPI, base peak intensity.

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Proteomic analysis allows the identification of aberrantly expressed proteins in diseased individuals *versus* normal ones. Also, it can provide an informative basis for assigning observed biological processes to specific signaling pathways. Mass spectrometry (MS)-based proteomic analysis could efficiently identify and quantify proteins in a global and unbiased manner and highlights the cellular processes that are altered between such systems, and thus it has quickly been a beneficial strategy model for unbiased large-scale protein investigation.

Recent developments in proteomic profiling provide a unique opportunity for studying uveitis pathogenesis and latency. Using two-dimensional electrophoresis (2-D E) and micro liquid chromatography–tandem mass spectrometry (LC–MS/MS) techniques, 20 spots were identified in vitreous bodies of endotoxin-induced uveitis rats, and 18 of these spots were members of the crystallin family [18]. The analysis on mitochondrial proteomics in experimental autoimmune uveitis (EAU) oxidative stress revealed that the presence of mitochondrial-specific oxidative stress-related proteins in the early EAU retina along with the down-regulation of ATP synthase was involved in the mechanism of stress-related retinal damage [19].

Currently, the EAU model has extensively been used to study the immune mechanism and to delineate the tissue damage related to intraocular inflammation. Lewis rats, which are susceptible to the occurrence of uveitis, are commonly used as a model animal. Herein, we used Lewis rats to induce EAU with interphotoreceptor retinoid-binding protein (IRBP) peptide emulsion, and further explored the effect of uveitis on the proteomic profiling of plasma from rats with experimental autoimmune uveitis by using label-free LC–MS/MS analysis. Our results will facilitate the understanding of alteration in proteomic profiling in plasma from EAU rats determined by LC–MS/MS and the possible mechanism in the development of uveitis.

2. Materials and methods

2.1. Animals

Female Lewis rats (6–8 weeks, 160–180 g) were purchased from Vital River Laboratory Animal Co., Ltd. (Beijing, China). All animal experiments were approved by the Laboratory Animal Care and Use Committee of Shandong University of Traditional Chinese Medicine. In the meantime, animal care and use were in accordance with the guidelines of Care and Use of Laboratory Animals published by China National Institute of Health and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Induction of experimental autoimmune uveitis in rats

Experimental autoimmune uveitis (EAU) was induced by interphotoreceptor retinoid-binding protein (IRBP) peptide (1177–1191, ADGSSWEGVGVVPDV) emulsified in Complete Freund's Adjuvant (CFA, Sigma–Aldrich, St. Louis, MO, USA) in female Lewis rats. IRBP peptide was synthesized and purified by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (China). For the preparation of IRBP emulsion, 100 μg of IRBP peptide was dissolved in 2.5 mg/mL of CFA supplemented with 100 μg of *Mycobacterium tuberculosis* (strain H37RA, Difco, Detroit, MI, USA) in a total volume of 150 μL. In the present study, a total volume of 150 μL of IRBP emulsion was injected subcutaneously in every rat in both footpads; meanwhile, control groups of rats ($n = 6$) were injected with the same volume of saline.

2.3. Preparation of proteomic samples

On day 0, 6 healthy rats were separately immunized using 150 μL IRBP emulsion and another 6 healthy rats were separately injected the same volume of saline. On day 11 post-immunization, EAU rats were sacrificed and plasma in every EAU rat was isolated using EDTA-anticoagulated tube. In the meantime, plasma in saline-injected rats ($n = 6$) was set as controls. Both EAU and control samples (2 mL blood for each sample) were centrifuged at $2000 \times g$ at 4 °C for 15 min and plasma was then treated to enrich low abundance proteins according to the literature [20]. Briefly, all plasma samples were treated with acetone containing 10% of trichloroacetic acid and kept at −20 °C for 90 min, and plasma samples were then centrifuged at $16{,}000 \times g$ at 4 °C for 20 min. After discarding the supernatant, the pellet was rinsed using cold acetone and kept on the ice for 15 min, followed by centrifugation at $16{,}000 \times g$ at 4 °C for 20 min. To obtain a trusted result, all procedures were repeated 3 times. Finally, quantitative determination of every plasma sample was measured using a micro-spectrophotometer (K5600, Beijing Kaiao Technology Development Co., Ltd., China) at 280 nm.

2.4. LC–MS/MS analysis

Prior to LC–MS/MS analysis, 200 μg of protein from plasma was digested using a filter-aided sample preparation (FASP) method [21] for every sample. Finally, the digested proteins were solved in 0.1% formic acid (100 μL). All samples were analyzed by Q-Exactive (Thermo Scientific) on a Thermo Scientific Easy-nLC 1000 system equipped with an Easy-spray column (C18, 2 μm, 100 Å, 75 μm × 50 cm) maintained at 35 °C. Peptides were separated with a 240 min gradient procedure using a flow rate of 250 nL/min.

The mobile phase consisted of 0.1% formic acid plus 2% acetonitrile in water (A) and 0.1% formic acid in acetonitrile (B). In order to fully separate the target peptides in plasma in both saline-treated rats and EAU rats, the gradient elution program was set as follows: the concentration of (B) in the mobile phase was 3% at the initial, and then a linear increase from 3% to 8% (B) from 0 min to 8 min, followed by a linear increase from 8% to 20% (B) from 10 min to 200 min, and a linear increase from 20% to 30% (B) from 200 min to 220 min, further a linear increase from 30% to 90% (B) from 220 min to 225 min, finally 90% constant 90% (B) from 225 min to 240 min.

Raw files were generated using the data dependent top-20 experiment. Resolution for MS survey scan was set to 70 K and 17.5 K for MS/MS survey scan. A dynamic exclusion of 30 s was used to discover low abundance proteins. Scan range for MS scan was 300–1800 Th, and MS/MS spectra generated with NCE 27 were set to scan from 100 Th.

2.5. Database search

Database search was carried out by using Proteome Discoverer 1.3 software (Thermo Fisher Scientific) with SEQUEST search engine against Swiss-Prot rat database. Proteome Discoverer software is a comprehensive tool for proteomic data analysis. It supports multiple dissociation techniques, quantitation technologies, and database search algorithms for more confident and comprehensive protein identification and characterization. The Proteome Discoverer Daemon facilitates automated data analysis for greater productivity. With regard to SEQUEST, it correlates uninterpreted tandem mass spectra of peptides with amino acid sequences from protein and nucleotide databases. SEQUEST will determine the amino acid sequence and thus the protein(s) and organism(s) that correspond to the mass spectrum being analyzed.

In the present study, the precursor mass tolerance was 15 parts per million (ppm) and 20 millimass units (mmu) for fragment ions.