



Comparative serum proteome expression of osteonecrosis of the femoral head in adults

Re-Wen Wu^{a,1}, Feng-Sheng Wang^{b,c,1}, Jih-Yang Ko^{a,c,*}, Ching-Jen Wang^{a,c}, Shin-Long Wu^b

^a Department of Orthopedic Surgery, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Taiwan

^b Department of Medical Research, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Taiwan

^c Graduate Institute of Clinical Medical Science, Chang Gung University College of Medicine, Kaohsiung, Taiwan

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ABSTRACT

Osteonecrosis of the femoral head (ONFH) is a skeletal disorder characterized by ischemic deterioration, bone marrow edema and eventually femoral head collapse. The systemic regulation of ONFH in adult patients has not been examined. Serum proteomic is an innovative tool that potentially detects simultaneous expressions of serum proteins in pathological contexts. We compared the serum proteome profiles of 11 adult patients with ONFH (3 females and 8 males) and 11 healthy volunteers (3 females and 8 males). The proteins in the aliquots of sera were subjected to isoelectric focusing, two-dimensional gel electrophoresis and silver staining. The protein spots were matched and quantified using an imaging analysis system. The differentially expressed protein spots were subjected to in-gel trypsin digestion. The peptide mass fingerprints were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF) and a bioinformation search. We found that ONFH patients showed significantly higher abundances of kininogen 1 variant, complement factor C3 precursor, and complement factor H and lower levels of antithrombin III chain B, apolipoprotein A-IV precursor, and gelsolin isoform α precursor. These proteins of interest were reported to modulate thrombotic/fibrinolytic reactions, oxidative stress, vessel injury, tissue necrosis or cell apoptosis in several tissue types under pathological contexts. Taken together, the occurrence of ONFH was associated with various serum protein expressions. Our high-throughput serum proteomic findings indicated that multiple pathological reactions presumably occurred in ONFH.

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Introduction

Osteonecrosis of the femoral head (ONFH) is a joint disorder complicated by pain and disability of the lower extremity and is one of the leading causes for total hip arthroplasty [1]. Intense ischemia necrosis in bone tissue, marrow cavity edema, and subsequent femoral head collapse characterize the progression of this skeletal disease [2]. Excessive corticosteroid administration [3], alcohol abuse [4], thrombotic disorders [5], collagen II gene mutations and endothelial nitric oxide gene polymorphisms [6,7] are potent risk factors of ONFH.

Intense bone tissue deterioration [8], perturbation of the osteogenic/adipogenic activity of bone marrow mesenchymal cells [9,10], and promotion of osteoblast/osteocyte apoptosis [11] are linked to the pathogenesis of corticosteroid- and alcohol-mediated ONFH. In experimental animal models, glucocorticoid treatment has been reported to promote bone resorption that accelerates the microarchitecture deterioration and impairs the mineral density of the femoral head in rabbits [12]. Administration of

supraphysiological doses of glucocorticoids has been found to inhibit blood flow to the femoral head [13] and induce endothelial damage and thrombosis of osseous vessels in pigs [14]. These findings imply that multiple pathological reactions may be involved in the development of ONFH.

Proteomic technology is an emerging tool for detecting the simultaneous expression of various proteins in serum, fluid, cell cultures, and tissue in pathological conditions (reviewed in Ref. [15]). This technology has recently been employed to screen potent bioactive molecules underlying the pathogenesis of arthritis and osteopenia. The proteome expressions in serum, joint fluid, and bone tissue reportedly correlate to the severity of rheumatoid arthritis [16], the autoimmune response in ankylosing spondylitis [17], the loss of cartilage integrity in osteoarthritis [18] and estrogen loss-induced osteoporosis in experimental animals [19]. However, the changes of serum protein expressions in adult patients with ONFH detected by proteomic technologies have not been tested.

In this study, we employed serum proteome technology which was integrated with isoelectric focusing, two-dimensional gel electrophoresis, image match, mass spectrometry, and bioinformation match to assess the simultaneous expression of several serum proteins in adult patients with ONFH and to characterize the associations between differentially expressed proteins and the incidence of ONFH.

* Corresponding author. Department of Orthopedic Surgery, Kaohsiung Chang Gung Memorial Hospital, 123, Ta-Pei Road, Niao-Sung, Kaohsiung 833, Taiwan.

E-mail addresses: wangfs@ms33.hinet.net (F.-S. Wang), kojy@adm.cgmh.org.tw (J.-Y. Ko).

¹ The first two authors contribute equally to this work.

Materials and methods

Patients

This study was approved by the Institutional Review Board of the hospital, and informed consent was obtained from all patients. Eleven patients (3 females and 8 males) were enrolled in the ONFH group. The exclusion criteria for sampling were history or evidence of metabolic bone diseases including hyper- or hypoparathyroidism, Paget's disease, renal osteodystrophy, and presence of cancers with bone metastasis. For the control group, 11 healthy volunteers (3 females and 8 males) were enrolled. Five milliliters of peripheral venous blood was drawn from each patient in outpatient department or prior to general anesthesia for total hip replacement, processed to collect serum and then stored at -80°C till analysis.

Isoelectric focusing and gel electrophoresis

Serum protein concentrations were measured by using a Bio-Rad Protein Reagent Kit (Bio-Rad Laboratories Inc, Hercules, CA). Isoelectric focusing (IEF) and two-dimensional electrophoresis of serum proteins were performed according to the manufacturer's instructions. Briefly, aliquots of serum (250 μg) in 250 μl rehydration buffer containing 2% CHAPS, 1% dithiothreitol, 0.5% IPG (pH 4–7), 8 M urea, and bromophenol blue were loaded onto immobiline™ strips (pH 4–7, 13 cm; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The strips were hydrated and then isoelectric focused at 32,000 V/h, 20°C using an Ettan™ IPGphor II/3 isoelectric focusing unit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After IEF, the strips were equilibrated in a buffer containing 50 mM Tris–HCl (pH 8.8), 30% glycerol, 2% SDS, 0.25% iodoacetamide and 8 M urea and were loaded onto the sample well in the 10% SDS-polyacrylamide gels. Second-dimension electrophoresis was performed at 110 V, 4°C for 16 h. Each specimen was subjected to IEF and gel electrophoresis in duplicate.

Silver staining

After electrophoresis, the gels were silver-stained according to the manufacturer's instructions (Amersham Pharmacia). Briefly, the gels were fixed (mixture of 10% glacial acetic acid and 40% methanol), sensitized (mixture of 0.5% glutaraldehyde, 0.2% sodium thiosulphate, 5.6% sodium acetate and 30% ethanol), rinsed with de-ionized water, and then silver-stained (0.25% silver nitrate and 0.01% formaldehyde). The protein spots in the gels were developed in 0.25% sodium carbonate and 0.01% formaldehyde.

Gel imaging

The stained polyacrylamide gels were scanned using an Amersham ImageScanner (Amersham BioScience Inc.). The gel images and spot patterns were matched and analyzed using Bio-Rad Proteomewer 2-D Analysis Software Version 4.0 (radius limit: 4; intensity limit: 2000; contrast limit: 50; border contrast: 0.2; active spots intensity warning limit: 5000; Bio-Rad Laboratories Inc, Hercules, CA) according to the manufacturer's instructions.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

The spots of interest were manually excised, washed with de-ionized water, de-stained (1% $\text{K}_3\text{Fe}(\text{CN})_6$ and 1.6% $\text{Na}_2\text{S}_2\text{O}_3$), rehydrated with 100% acetonitrile, reduced with 50 mM NaHCO_3 in acetonitrile then trypsin digested (20 ng/ml trypsin; Sigma-Aldrich, St Louis, MO) at 37°C for 16 h. The digestion products were extracted with 1% trifluoroacetate in acetonitrile. Aliquots of the digestion products were then loaded onto AnchorChip followed by MALDI-TOF assessment using an Ultraflex™ TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) according to the manufacturer's instructions. The peptide mass data were submitted to the NCBI and the SwissPort database using MASCOT search engines. The matched peptides having the highest MASCOT score (≥ 65) and a peptide sequence coverage 20% of the matched peptide were considered as potent candidates [19].

Statistical analysis

All values were expressed as mean $\pm$ standard error. A P value of <0.05 was considered statistically significant. Correlations between the differential expression of serum protein with ONFH were analyzed with Spearman's correlation test. Power values of the differential serum protein abundance ranged from 0.8332 to 0.9229 under $\alpha = 0.05$.

Results

Patients

Eleven patients with ONFH (age, 41.5 ± 3.3 years; range, 29–65 years) and eleven healthy volunteers (age, 41.0 ± 3.6 years old; range, 27–66 years) were enrolled. There was no significant difference in the age ($p = 0.927$) between the ONFH and healthy volunteer groups. Five and six ONFH patients had a history of alcohol consumption and corticosteroid treatment, respectively. Two patients had both the history of alcohol and corticosteroid intake. Two patients neither had the history of alcohol consumption nor corticosteroid intake. The severity of ONFH was evaluated according to the Ficat and Arlet staging system [1]. Six, one and four patients had stage II, stage III and stage IV ONFH, respectively. Patients with stage III and stage IV ONFH underwent total hip arthroplasty.

2-DE profiles

Image analyses showed that approximately 230 spots (228 ± 13 spots in the ONFH group vs. 220 ± 7 spots in the healthy control group, $p = 0.600$; Fig. 1) could be detected in the gels. The number of spots detected in the ONFH group and healthy volunteer groups was reproducible.

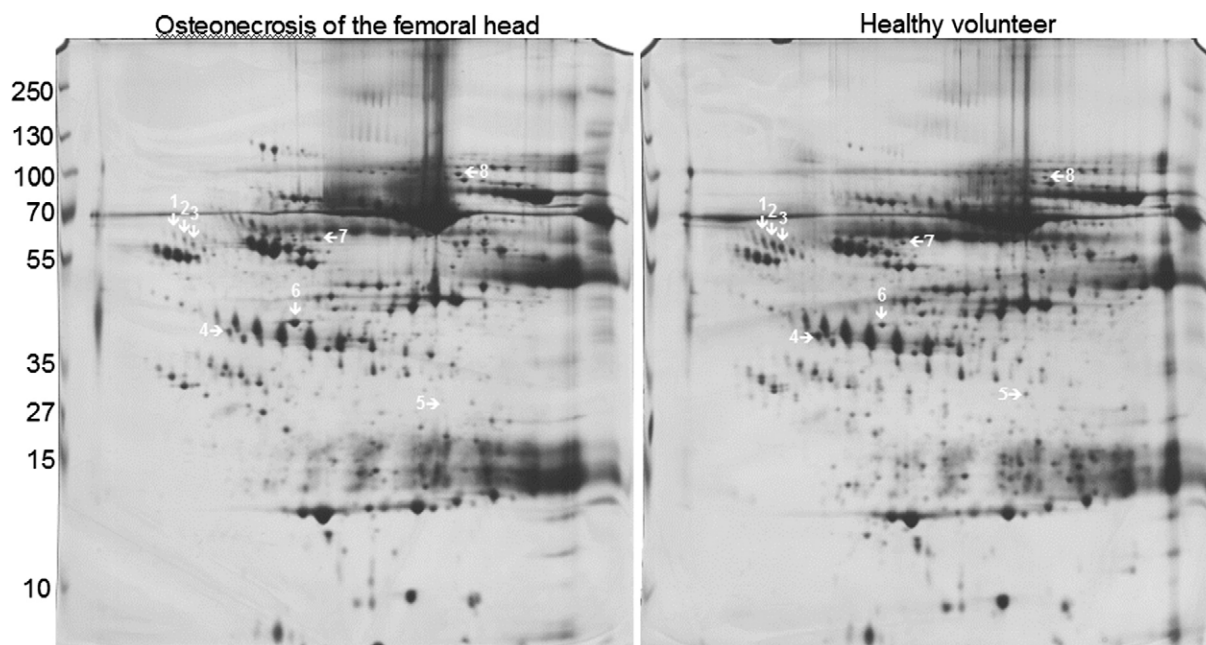


Fig. 1. Representative two-dimensional gel electrophoretograms of serum proteins in a patient with ONFH and an age- and gender-matched healthy volunteer; 250 μg of sera was subjected to IEF (pH 4–7), SDS-polyacrylamide gel separation and silver staining. The number in the left lane indicates the molecular weight (kDa) of each protein standard.

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