Evaluation of a Pretreatment Method for Two-Dimensional Gel Electrophoresis of Synovial Fluid Using Cartilage Oligomeric Matrix Protein as a Marker

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Received: November 2, 2011 / Revised: December 30, 2011 / Accepted: January 2, 2012

Osteoarthritis (OA) is the most common rheumatic pathology. One of the major objectives of OA research is the development of early diagnostic strategies such as those using proteomic technology. Synovial fluid (SF) in OA patients is a potential source of biomarkers for OA. The efficient and reliable preparation of SF proteomes is a critical step towards biomarker discovery. In this study, we have optimized a pretreatment method for two-dimensional gel electrophoresis (2DE) separation of the SF proteome, by enriching low-abundance proteins and simultaneously removing hyaluronic acid, albumin, and IgG. SF samples pretreated using this optimized method were then evaluated by 1DE and 2DE separation followed by immunodetection of cartilage oligomeric matrix protein (COMP), a known OA biomarker, and by the identification of 3 proteins (apolipoprotein, haptoglobin precursor, and fibrinogen D fragment) that are related to joint diseases.

Keywords: Osteoarthritis, synovial fluid, two-dimensional gel electrophoresis, biomarker, cartilage oligomeric matrix protein, hyaluronic acid

Osteoarthritis (OA) is a degenerative joint disease that is characterized by progressive cartilage destruction and bone changes, accompanied by synovial inflammation [5]. A major objective for OA research is the development of early diagnostic technologies, including those based on genomics, proteomics, and metabolomics [7, 15]. Proteomic tools have been used to discover diagnostic or therapeutic biomarkers in OA [2, 9]. Successful separation of proteins is acknowledged to be a critical step in OA proteomics [11].

Synovial fluid (SF) is a potential source of OA biomarkers because it is derived directly from the joint disease site [4]. Hyaluronic acid, a high molecular weight glycosaminoglycan, is the major constituent of SF (1–3 mg/ml) and forms a complex with protein components [3]. Enzymatic digestion of hyaluronic acid facilitates the proteomic analysis of SF [17]. However, protein concentrations in SF range from ng/ml (e.g., protein biomarker) to mg/ml scales (e.g., albumin and IgG) [5], and the verification and quantification of protein OA biomarkers in SF using two-dimensional polyacrylamide gel electrophoresis (2DE) are therefore challenging [6]. Enriching low-abundance proteins and simultaneously removing highly abundant ones in SF would greatly facilitate the proteomic study of SF.

In this study, we systematically compared and optimized SF pretreatment methods, particularly the removal of hyaluronic acid, albumin, and IgG. The pretreated SF samples were then evaluated both by 1DE and 2DE immunodetection of cartilage oligomeric matrix protein (COMP) [8], a known OA biomarker, and by the identification of 3 proteins that are related to joint diseases. Because COMP is a biomarker for cartilage turnover and its level is elevated in the SF of OA patients [14], it can serve as a marker for evaluating SF preparations from OA patients.

MATERIALS AND METHODS

Clinical SF Samples

Human SF samples were obtained from 5 OA patients whose ages ranged from 39 to 64 years. Informed consent was obtained from each patient. The Institutional Review Board (IRB) of Ajou University approved all procedures. SF samples were centrifuged at 3,000 rpm for 20 min at 4°C, and supernatants were stored at –80°C in 1 ml aliquots until use.

Digestion of Hyaluronic Acid in SF Samples

SF sample contains highly viscous hyaluronic acid, which can be removed by treatment with hyaluronidase [6]. To prepare hyaluronidase stock solution, SHSE buffer (60 mM NaOAc, 1 mM EDTA; pH 6.0) was added to 1 vial of hyaluronidase at a final concentration of 1,300 units/ml. Hyaluronic acid digestion was carried out at 37°C...
Depletion of Albumin and IgG in SF Samples by Affinity Chromatography

To deplete albumin and IgG in SF samples, hyaluronidase-treated or untreated SF samples were loaded onto the Aurum Serum Protein Mini Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. To determine the extent of nonspecific binding to the spin affinity columns, the resin used for removal of albumin and IgG was washed with elution buffer and collected for further study.

Two-Dimensional Gel Electrophoresis (2DE)

Protein concentrations in pretreated or untreated SF samples were measured using the 2D Quant kit (GE Healthcare, Waukesha, WI, USA) according to the manufacturer’s instructions. To determine the extent of nonspecific binding to the spin affinity columns, the resin used for removal of albumin and IgG was washed with elution buffer and collected for further study.

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Western Blotting and 2DE Immunodetection of COMP

Quantitative detection of COMP in SF samples was performed in duplicate using ELISA according to the recommendation of the manufacturer (Abcam, Cambridge, MA, USA). For Western blotting, samples containing 5 µg of proteins were separated on pre-made SDS polyacrylamide gels (Bio-Rad), and then transferred onto PVDF membranes (Bio-Rad). For 2DE immunodetection, samples containing 50 µg of proteins were separated by IEF (7 cm IPG strip) and then on pre-made SDS polyacrylamide gels (Bio-Rad) for the second dimension, and finally transferred onto PVDF membranes (Bio-Rad). PVDF membranes were treated with blocking solution (PBS, 5% BSA, and 0.1% Tween-20) for 1 h, and washed with PBS containing 0.1% Tween-20 for 30 min. PVDF membranes were probed with anti-COMP antibody (Abcam) and alkaline phosphatase-conjugated anti-mouse IgG (Sigma, St. Louis, MO, USA). Bound antibodies were visualized using the chromogenic substrate for alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT; Sigma).

RESULTS AND DISCUSSION

Effect of Hyaluronic Acid Removal from SF on 2DE Proteome Resolution Profiles

The high concentration of hyaluronic acid in SF makes it difficult to analyze the SF proteome by two-dimensional liquid chromatography-coupled tandem mass spectrometry because of the high viscosity of hyaluronic acid [6]. The SF of OA patients had bubbles (Fig. 1A) and was viscous, suggestive of high hyaluronic acid content. When the SF was treated with hyaluronidase to remove hyaluronic acid, the bubbles disappeared (Fig. 1B) and the viscosity of SF was significantly reduced. Next, we compared the proteome separation profiles of untreated and hyaluronidase-treated
EVALUATION OF A PRETREATMENT METHOD FOR 2DE OF SYNOVIAL FLUID

SF samples from one OA patient were treated as described in Materials and Methods, and separated on 2DE gels. (A) Untreated SF proteome, (B) hyaluronidase-treated SF proteome, (C) albumin/IgG-depleted SF proteome, and (D) hyaluronidase-treated and albumin/IgG-depleted SF proteome.

Fig. 2. Two-dimensional gels of SF samples, stained with silver nitrate.

SF samples from a single OA patient were treated as described in Materials and Methods, and separated on 2DE gels. (A) Untreated SF proteome, (B) hyaluronidase-treated SF proteome, (C) albumin/IgG-depleted SF proteome, and (D) hyaluronidase-treated and albumin/IgG-depleted SF proteome.

Variability in 2DE Separation Profiles of SF Samples from Different OA Patients

Next, to assess the variability in 2DE separation profiles of SF samples from different OA patients, we performed 2DE separation of SF samples from 4 different OA patients using the optimized pretreatment. As shown in Fig. 3A–D, SF proteomes were slightly different among the 4 patients, suggesting that the SF proteome might be strongly related to OA progression and/or severity [12, 16]. Unfortunately, we cannot speculate whether these proteome changes are specifically related to OA without information on OA severity and comparison data with normal SF. Therefore, 3
spots with increased or decreased expression in the 4 proteomes were selected and identified by peptide mass fingerprinting (Table 1). These 3 spots were apolipoprotein [1], haptoglobin precursor [1, 10, 12], and fibrinogen D fragment B [13]. Details of these identified proteins are shown in Table 1. These 3 proteins are related to rheumatoid arthritis (RA), OA, and ankylosing spondylitis (AS), indicating that all these proteins are involved in joint disease.

**Western Blotting and 2DE Immunodetection of COMP**

Because normal SF could not be obtained for control experiments, we instead decided to use a known biomarker protein, COMP, to validate our optimized SF pretreatment method. First, we detected the presence of COMP in the SF of an OA patient by Western blotting, using a primary antibody against COMP. As shown in Fig. 4A, we confirmed that the SF contained COMP at levels compatible with immunodetection. Next, proteins in the SF were separated by 2DE (Fig. 4B) and detected using the COMP primary antibody (Fig. 4C). These studies showed that COMP was clearly detected on 2DE gels (arrow in Fig. 4C). Even though a control was not studied as mentioned above, this observation indicates that the SF pretreatment method developed in this study can serve as a good preliminary step for OA biomarker discovery from the SF proteome.

In conclusion, we optimized a pretreatment method for 2DE separation of the SF proteome. Enriching low-abundance proteins and simultaneously removing highly abundant ones are important for the proteomic analysis of SF. In particular, the removal of hyaluronic acid, albumin, and IgG is recommended for successful 2DE separation.

**Table 1.** Three representative proteins from human OA identified in the SF proteome.

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein</th>
<th>Access. No.</th>
<th>MW (kDa)</th>
<th>pI</th>
<th>No. matched peptides</th>
<th>Seq. cov. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOA1</td>
<td>Apolipoprotein</td>
<td>P02647</td>
<td>30.8</td>
<td>5.56</td>
<td>15</td>
<td>37</td>
</tr>
<tr>
<td>HPT</td>
<td>Haptoglobin precursor</td>
<td>P00738</td>
<td>45.9</td>
<td>6.13</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>RFD</td>
<td>Fibrinogen D fragment, chain B</td>
<td>P02675</td>
<td>36.3</td>
<td>7.08</td>
<td>18</td>
<td>47</td>
</tr>
</tbody>
</table>
Our pretreatment method for 2DE was successfully evaluated using COMP, a known OA biomarker protein, and by the identification of 3 proteins related to joint disease from pretreated SF. Further human studies using our optimized 2DE pretreatment technique are necessary to provide information on individual variability, relationship to gender and OA disease stage, as well as in the early diagnosis and monitoring of OA.

Acknowledgments

This study was supported by a grant of the Korean Health Technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A091120) and by the Priority Research Centers Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2011-0022978).

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