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NanoLC/mass spectrometry-based proteomic analysis of serum and synovial fluid samples from osteoarthritis patients

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Introduction

Osteoarthritis (OA) is the most common rheumatic pathology, characterized mainly by cartilage degradation [1]. Despite its high prevalence, the diagnosis methods currently available are limited and lacked of sensitivity. Therefore, there is a considerable interest pointed in the characterization of new specific OA biomarkers in biological fluids. In this work we set up a nLC-MALDI-MS method for OA biomarker search in complex mixtures, with the aim of obtaining a standardized protocol for serum and synovial fluid (SF) protein profiling.

Methods

a) Sample preparation and immunodepletion: Serum and SF samples were obtained from OA patients and control donors. Prior to protein depletion, SF was treated with hyaluronidase. The 20 most abundant proteins were removed from crude serum and SF using the Immunodepletion column ProteoPrep® 20 (Figure 1), according to manufacturer's instructions (Sigma Aldrich). Depletion of abundant proteins was checked by SDS-PAGE separation of the proteins and identification in a 4800 MALDI-TOF/TOF system (ABI). For both serum and SF depleted samples, protein concentration was determined using a nanoDrop instrument (Fisher Thermo Scientific, USA).

b) Pre-fractioning of the samples: For serum samples, digestion of the depleted fractions was done with trypsin, and the peptides obtained were fractionated by strong cation exchange (SCX) liquid chromatography in a HP 1200 system (Agilent), using a PolySulfoethyl column (PolyLC). SF proteins were separated by SDS-PAGE, using 10% acrylamide gels. The gel lanes were divided into 12 sections, excised and proteins were in-gel digested with trypsin following standard procedures.

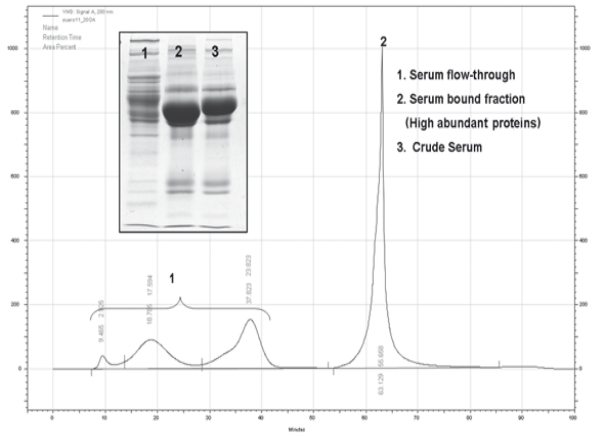

Figure 1. *Chromatogram of the affinity depletion of the 20 most abundant proteins from human plasma. Inset shows a SDS-PAGE of the different fractions. 1. Serum flow-through fraction (low abundant proteins); 2. Serum bound fraction (high-abundant proteins); 3. Untreated serum.*

c) NanoLC-MS/MS analysis: The serum and SF peptide fractions were desalted using PorosR2 home-made columns (ABI). Each dried fraction was re-dissolved and injected on a precolumn (PepMap100), and peptides were desalted and loaded to a C18 column (Integrafit C18, Proteopep™ II, New Objetive, USA) to perform the separation. Fractions were collected from the NanoLC, mixed with matrix and spotted onto a MALDI plate using a MALDI Spotter/Micro-Fraction Collector (SunChrom). MS spectra were acquired for each fraction in a 4800 MALDI-TOF/TOF instrument (ABI).

d) Protein Identification and Database Search: Protein identification was carried out using the ProteinPilot software v2.0 (ABI). Only proteins identified with at least 99.9% confidence or a ProtScore of 1.3 were reported. A Global False discovery Rate (FDR) of 1% was calculated independently with PSPEP software. SwissProt database was used to determine the putative cellular function of the identified proteins.

Results

We have standardized two alternative protocols for the proteomic analysis of human biological fluids, one employing liquid chromatography and the other SDS-PAGE as pre-fractionation method.

Using the first approach, we were able to identify 105 different proteins in human serum from OA patients. These proteins are involved mainly in the immune response (33%), proteolysis (22%) and transport (29%) processes (Figure 2). An efficient depletion of the 20 most abundant proteins was verified, as only 2 of these proteins could be identified in the samples. Nevertheless, still a number of other typically abundant serum proteins such as complement proteins and apolipoproteins were found.

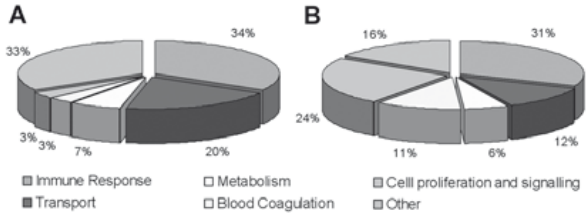

Figure 2. *Functional classification of proteins identified in (A) serum and (B) SF from OA patients.*

By the second approach, we identified 88 different proteins in SF samples from OA donors. As expected, many of these (76%) were also found in serum. Some of those had been previously suggested as putative OA biomarkers, such as Gelsolin, Fibronectin, Clusterin or Serum Amyloid P protein. As happened with serum, the most abundant functional group of proteins identified in SF was involved in the immune response (Figure 2), but we also found in this case many proteins related with metabolism, transport, cellular proliferation and signalling. Interestingly, we found in the group of 21 proteins specifically identified in SF a number of proteins that are directly involved in cartilage extracellular matrix (ECM) synthesis, that are listed in Table 1.

Conclusions

We have standardized two approaches for the identification of proteins in human biological fluids after depletion of the 20 most abundant proteins of plasma. These procedures have allowed us to describe the protein profiles of serum and SF from OA patients. Despite the high similarities of both profiles, we found more joint-specific proteins in SF. Our data point out the usefulness of these approaches for OA biomarker discovery, although further studies employing stable isotope labelling will profit from quantification of the potential markers that have been identified in this work.

References

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Table 1. *Proteins identified specifically in SF from OA patients that are directly involved in cartilage ECM synthesis.*

Acc. No.|Abbr.	Name	Cellular role
P01033|TIMP1	Metalloproteinase inhibitor 1	Prevents ECM degradation
P16112|PGCA	Aggrecan core protein	ECM component
P49747|COMP	Cartilage oligomeric matrix protein	ECM component
Q15113|PCOC1	Procollagen C-endopeptidase enhancer 1	Collagen synthesis
Q92954|PRG4	Proteoglycan-4	ECM component
Q9H9S5|FKRP	Fukutin-related protein	Glycoprotein synthesis
Q9NQ79|CRAC1	Cartilage acidic protein 1	Cartilage component

Functional Proteomics: Beads –based array system for Biomarker Discovery

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Despite the immense progress in Molecular Biology and Genetics, only a small fraction of the proteome is understood at biochemical level. Currently, a development of new methodological strategies in high-throughput format is needed for applications in Proteomics, such as Biomarker and Drug Discovery studies. These new methodologies must be able to analyze simultaneously hundreds or thousands of proteins in order to evaluate functionality, stability, interactions, relative abundance, post-transduction modifications, etc.

Our group has developed a microspheres array (Bead-based Array System, BBAS) that allow the simultaneous analysis of numerous sera and intracellular proteins. The method consist of having different populations of spheres, colored by surface- labeling with different fluorescence dyes and coupled with different antibodies against target proteins. A wide range of available dyes could potentially be used to generate complex color codes that are analyzed by standard flow cytometres.

In the experimental process a lysate of B cells is fractioned by size exclusion chromatography (FPLC) and incubated with a 1300 populations array. Each population has a code of dyes and is specific for a particular target protein. The labeling of these target proteins with phycoerythrin (PE) allows the detection by flow cytometry.

This methodology is capable of giving information about the amount of protein present in each fraction, in addition to protein state (soluble, membrane protein, monomeric vs multimeric, phosphorylated, coupled with other proteins in functional complexes …).