Journal of Cellular and Molecular Pharmacology

Mini Review **Open Access**

Pharmacoproteomic Assessment of Three Sulfated Molecules on Human Osteocyte Receptors and Extracellular Proteomes

Francisco J. Blanco*

Osteoarticular and Aging Research Lab, Proteomics Unit, ProteoRed/ISCIII, Rheumatology Division, INIBIC-CHU A Coruña, As Xubias 84, 15006 A Coruña, Spain

Abstract

The CS employed in scientific studies is generally derived from animal sources by extraction and purification processes and is mainly obtained from bovine, porcine, chicken, or marine cartilage. From these, bovine CS is the most often used *in vitro* and in clinical trials Although beneficial effects of orally administrated CS in OA patients have been reported caution should be exercised in the study or use of different CS formulations, because the species or tissue of origin could result in great differences in CS structural organization or disaccharide composition. Probably for this reason, recent meta-analysis and large scale clinical trials have demonstrated variable effects on OA symptoms, yielding conflicting result.

The differentially labeled co-resolved proteins within each gel were imaged at 100 dots/inch resolution using a DIGE Imager scanner (GE Healthcare). Cy2-, Cy3-, and Cy5-labeled images of each gel were acquired at excitation/emission values of 488/520, 523/580, and 633/670 nm, respectively [1-15].The gels were scanned directly between the glass plates, and the 16-bit image file format images were exported for data analysis. After imaging for CyDyes, the gels were removed from the plates and stained with colloidal Coomassie following standard procedures.

Introduction

Semi-automated image analysis was performed with Progenesis SameSpots V3.2 software (Nonlinear Dynamics). Image quality control was first performed to identify saturated spots. Multiplexed analysis was selected for DIGE experiments, and a representative gel image was chosen as reference. Spots were detected, and their normalized volumes were ranked on the basis of analysis of variance *p* values, fold changes, and statistical power (which reflect our confidence in the ability of the experimental data to find the differences that do actually exist).

Subjective heading

The gel spots of interest were manually excised and transferred to microcentrifuge tubes. Samples selected for analysis were in-gel reduced, alkylated, and digested with trypsin according to the method of Sechi and Chait). The samples were analyzed using the MALDI-TOF/TOF mass spectrometer 4800 Proteomics Analyzer (ABSCIEX, Framingham, MA) and 4000 Series Explorer™ software (ABSCIEX). Data Explorer version 4.2 (ABSCIEX) was used for spectra analyses and generating peak picking lists. All of the mass spectra were internally calibrated using autoproteolytic trypsin fragments and externally calibrated using a standard peptide mixture (Sigma-Aldrich). TOF/TOF fragmentation spectra were acquired by selecting the 10 most abundant ions of each MALDI-TOF peptide mass map (excluding trypsin autolytic peptides and other known background ions.

Syphilis is an infection caused by Treponema pallidum. Usually, *T.* pallidum is transmitted through sexual intercourse. In addition, syphilis greatly increases the risk of infection and transmission of acquired immune deficiency syndrome . In recent years, the global incidence of syphilis has increased because of the ability of *T.* pallidum to evade host immune defenses and spread from the initial site of infection to other organs and tissues. Hence, it is also termed a "stealth pathogen. How *T.* pallidum overcomes the immune response and damages tissue is incompletely understood. Explaining the pathogenesis and immune mechanism of action of *T.* pallidum has become a key link to controlling syphilis.

Discussion

The monoisotopic peptide mass fingerprinting data obtained by MS and the amino acid sequence tag obtained from each peptide fragmentation in MS/MS analyses were used to search for protein candidates using Mascot version 2.2 from Matrix Science. Peak intensity was used to select up to 50 peaks/spot for peptide mass fingerprinting and 50 peaks/precursor for MS/MS identification. Tryptic autolytic fragments, keratin, and matrix-derived peaks were removed from the data set used for the database search. The searches for peptide mass fingerprints and tandem MS spectra were performed in the UniProt knowledgebase (2010_09 release version, August 10, 2010), by searching in the UniProtKB/Swiss-Prot database, containing 519,348 entries. Fixed and variable modifications were considered (Cys as *S*-carbamidomethyl derivate and Met as oxidized methionine, respectively), allowing one trypsin missed cleavage site and a mass tolerance of 50 ppm. For MS/MS identifications, a precursor tolerance of 50 ppm and MS/MS fragment tolerance of 0.3 Da were used. Identifications were accepted as positive when at least five peptides matched and at least 20% of the peptide coverage of the theoretical sequences matched within a mass accuracy of 50 or 25 ppm with internal calibration. Probability scores were significant at *p* < 0.01 for all matches. The intracellular localization of the identified proteins was predicted from the amino acid sequence using the PSORT II program.

***Corresponding author:** Francisco J. Blanco, Osteoarticular and Aging Research Lab, Proteomics Unit, ProteoRed/ISCIII, Rheumatology Division, INIBIC-CHU A Coruña, As Xubias 84, 15006 A Coruña, Spain, E-mail: frci56jk@gmail.com

For SILAC experiments, freshly isolated chondrocytes were recovered and plated at low density in SILAC Dulbecco's modified Eagle's medium lacking arginine and lysine and supplemented with 10% dialyzed FBS, 4.5 g/liter glucose, 2 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. In the case of light media, standard l-lysine and l-arginine were added, whereas in the heavy media, isotope-labeled l-lysine ($^{13}C_6$) and isotope-labeled l-arginine ($^{13}C_6,^{15}N_4$) were used. Cell expansion was carried out as described previously by our group ([15]). Chondrocytes were used at week 3 in primary culture (P1) when 100% of labeling was reached. The cells were washed thoroughly to remove abundant serum proteins and incubated for 48 h in serum-free medium supplemented with 200 μg/ml of CS1, CS2, or CS3.

Extracted peptide mixtures were desalted and concentrated through a C18 microcolumn (NuTip, Glygen) and finally eluted from the C18 bed using 70% ACN, 0.1% TFA. The organic component was removed by evaporating in a vacuum centrifuge, and the peptides were resuspended in 2% ACN, 0.1% TFA. 5 μl were injected into a reversed phase column (Integrafit C18, Proteopep™ II; New Objective) for nano-flow LC analysis, using a Tempo nanoLC (Eksigent) equipped with a SunCollect MALDI Spotter/Micro-Fraction Collector (SunChrom Wissenschaftliche Geräte GmbH). LC eluate microfractions were mixed with MALDI matrix (3 mg/ml α-cyano-4-hydroxycinnamic acid in 70% ACN and 0.1% TFA, containing 10 fmol/μl angiotensin as an internal standard) and deposited onto an Opti-TOF LC MALDI target plate (1534-spot format, ABSciex) with a speed of one spot/15 s. Mass spectrometry analysis was performed on a 4800 MALDI-TOF/TOF instrument (ABSciex) with a 200 Hz repetition rate (Nd:YAG laser). MS full scan spectra were acquired from 800 to 4000 m/z. A total of 1500 laser shots were accumulated for each TOF-MS spectrum at an optimized fixed laser setting.

Chondroitin sulphate is a symptomatic, slow-acting medication for osteoarthritis that is commonly used to treat the illness, which is marked by articular cartilage degeneration. However, little is known about how it works, and recent large-scale clinical trials have yielded mixed outcomes in terms of symptom relief. We used two complimentary proteomic techniques to investigate the changes in the intracellular proteome and secretome of human articular cartilage cells treated with three distinct chemicals, each of which had a different origin or purity. Each brand of CS was given 200 g/ml to osteoarthritic cells The DIGE and stable isotope labelling with amino acids in cell culture techniques were used to conduct quantitative proteomics experiments, which were then analysed. The DIGE analysis of chondrocyte whole cell extracts revealed 46 locations that were differentially modulated between conditions in our study: 15 were modulated by, and 46 were modulated by We were able to identify 104 distinct proteins after doing the experiment on a subset of chondrocyte-secreted proteins..

The DIGE and stable isotope labelling with amino acids in cell culture techniques were used to conduct quantitative proteomics experiments, which were then analysed. The DIGE analysis of chondrocyte whole cell extracts revealed 46 locations that were differentially modulated between conditions in our study: 15 were modulated by, and 46 were modulated by We were able to identify 104 distinct proteins after doing the experiment on a subset of chondrocyte-secreted proteins.

A major goal of modern therapeutic techniques is to effectively prevent structural harm. Multiple chemicals, medicines, and nutraceuticals have been studied in vitro and/or in vivo for their beneficial benefits. The recent decade has seen a lot of research into symptomatic slow-acting medicines for osteoarthritis The usage of chondroitin sulphate, either alone or in combination with glucosamine sulphate, is on the rise all over the world. CS is a glycosaminoglycan that is found in abundance in numerous connective tissues, including cartilage. In vitro investigations demonstrate that CS works, despite the fact that its mechanisms of action are unknown. Inhibits the expression of IL-1-induced metalloproteinases and prostaglandin E2, avoiding cartilage injury. Stimulates the formation of proteoglycans by chondrocytes and synoviocytes and inhibits the expression of IL-1-induced metalloproteinases and prostaglandin E2. Simultaneously, CS has been demonstrated to improve various OA-related degenerative processes affecting the synovial tissue and subchondral bone. We recently conducted a gel-based proteomic analysis employing normal chondrocytes activated with IL-1 and treated with alone or in conjunction with GS to better understand the molecular pathways behind. However, because of variances in molecular composition, tissue of origin, purity, and production/purification methods, the quality of the formulations is poorly regulated, which may have an impact on the therapeutic outcome. Tat recently examined protein modulations of variables such as prostaglandin from three distinct manufacturers and sources, and assessed them using an ELISA test. Invitrogen provided the culture media and foetal bovine serum. Costar provided the culture flasks and plates, while GE Healthcare provided the DIGE materials. Materials Silantes provided Dulbecco's modified Eagle's medium, dialyzed FBS, and amino acids. All additional chemicals and enzymes were purchased from Sigma-Aldrich unless otherwise stated. BD Biosciences provided a monoclonal antibody against human manganese-superoxide dismutase. The horseradish peroxidase-conjugated secondary is the same as the primary.

Two-dimensional Western blot analyses were performed following standard procedures. Briefly, 20 μg of secreted proteins were loaded onto 7-cm pH 3–11 NL IPG strips for first dimension separation, and then resolved using standard 10% SDS-PAGE. The separated proteins were then transferred to PVDF membranes (Immobilon P, Millipore Co., Bedford, MA) by electroblotting and probed with specific antibody against SOD2. Immunoreactive bands were detected by chemiluminescence using corresponding horseradish peroxidase-conjugated secondary antibodies and ECL detection reagents (GE Healthcare) and then digitized using the LAS 3000 image analyzer. Equivalent loadings were verified by Ponceau Red staining after transference.

Each experiment was repeated at least three times. The statistical significance of the differences between mean values was determined using a two-tailed t test, considering significant p values ≤ 0.05. In the proteomic analyses, normalization tools and statistical package from SameSpots and ProteinPilot software were employed. We considered statistically significant only those changes with a p value of ≤0.05 and a ratio ≥1.2 (or ≤0.83), while exhibiting high statistic power (>0.8, in the SameSpots analysis) or low error factor of the quantification (<2, in the ProteinPilot analysis). Where appropriate, the results are expressed as the means ± S.E.

Conclusion

The Rheumatology Service at Complejo Hospitalario Universitario Corua offered osteoarthritic cartilage from patients undergoing total joint replacement. Before surgery, the patients gave their informed permission. According to the American College of Rheumatology's OA classification criteria, patients of both genders over the age of 40 were included in the study. The study was given the green light by the local ethics commission.

Progenesis SameSpots V3.2 software was used to do semi-automated picture analysis. To identify saturated regions, image quality inspection was initially done. For DIGE investigations, multiplexed analysis was used, and a typical gel picture was chosen as a reference. Spots were found and their normalised volumes were sorted using analysis of variance p values, fold changes, and statistical power which reflects our confidence in the experimental data's ability to uncover differences that do exist.

Chondrocytes were obtained from three pathological OA cartilages and primary cultured. After the first passage, the cells were treated with 200 μg/ml of each brand of CS for 48 h. Untreated cells were used as control (*CTL*). Chondrocytic proteins were extracted and labeled with the corresponding Cy dyes, following the scheme shown in the *table*. The samples were then mixed and resolved on six independent DIGE gels. Three fluorescence images were obtained from each gel and subjected to image analysis using SameSpots software.

CS, like other natural macromolecules, has a complex structure that is known to change with the source tissue, organ, and species Its commercial manufacture relies on bovine, porcine, chicken, or cartilaginous fish (such as shark and skate) by-products, in particular cartilage, as raw material. CS from different sources contains disaccharides possessing sulfate groups in different positions and in different percentages within the polysaccharide chains Moreover, extraction and purification processes may introduce further modifications of the structural characteristics and properties and may led to extracts more or less rich in chondroitin sulfate, having a variable grade of purity because of the presence of polluting side-products, like other glycosaminoglycans, such as heparin, heparan sulfate, dermatan sulfate, and hyaluronic acid. Additionally, chondroitin is administered orally during therapy, and bioavailability and pharmacokinetic parameters have been reported to change depending on its structural characteristics and origin As a consequence, the low quality chondroitin sulfate generally present in nutraceuticals would be unable to exert comparable pharmacological effects to those of the pharmaceutical grade chondroitin sulfate. *Use of CS for in Vitro Studies*

Although the oral bioavailability of CS is acceptable (15–24%), ~90% is depolymerized or degraded either in plasma or the joints Many *in vitro* studies have used high and variable concentrations of CS, ranging from 12.5 to 2000 μg/ml but generally 200 μg/ml or lower. The relationship between *in vitro* pharmacological studies and what may be expected *in vivo* is therefore a matter that has still to be resolved for some target actions. This is particularly evident because, in general, only CS has been investigated for effects *in vitro*, and virtually no information is available on the mixture of depolymerized or degradation products that are known to exist in inflamed joints Nevertheless, these *in vitro* investigations (principally using chondrocytes or cartilage explants of bovine or human origin) have provided insight into the likely actions of CS including increased synthesis of proteoglycan, hyaluronic acid, and aggregan, blockade of proteoglycan degradation by interleukin-1 and other pro-inflammatory cytokines and metalloproteinases, prevention of oxyradical formation, modulation of chondrocyte signaling pathways.

Acknowledgement

Conflict of Interest

The authors declare that they are no conflict of interest.

References

1. Qin J, Li R, Raes J(2010) A human gut microbial gene catalogue established by metagenomic sequencingNature.464: 59-65.

2. Abubucker S, Segata N, Goll J(2012) Metabolic reconstruction for metagenomic data and its application to the human microbiome. PLoS Comput Biol 8.

3. Hosokawa T, Kikuchi Y, Nikoh N (2006) Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. PLoS Biol 4.

4. Canfora E E, Jocken J W, Black E E (2015) Short-chain fatty acids in control of body weight and insulin sensitivity. Nat Rev Endocrinal 11: 577-591.

5. Lynch SV, Pedersen(2016) The human intestinal microbiome in health and disease. N Engl J Med 375: 2369-2379.

6. Araújo APC, Mesak C, Montalvao MF(2019) Anti-cancer drugs in aquatic environment can cause cancer insight about mutagenicity in tadpoles. Sci Total Environ 650: 2284-2293.

7. Barros S, Coimbra AM, Alves N(2020) Chronic exposure to environmentally relevant levels osimvastatin disrupts zebrafish brain gene signaling involved in energy metabolism. J Toxic Environ Health A 83: (3) 113-125.

8. Ben I,Zvi S, Kivity, Langevitz P(2019) Hydroxychloroquine from malaria to autoimmunity.Clin Rev Allergy Immunol 42 (2) : 145-153, 10.1007/s12016-010-8243.

9. Bergqvist Y, Hed C, Funding L (1985) Determination of chloroquine and its metabolites in urine a field method based on ion-pair. ExtractionBull World Health Organ 63 (5): 893.

10. Burkina V, Zlabek V, Zamarats G (2015)Effects of pharmaceuticals present in aquatic environment on Phase I metabolism in fish. Environ Toxicol Pharmacol 40 (2) : 430-444.

11. Cook JA, Randinitis EJ, Bramson CR(2006) Lack of a pharmacokinetic interaction between azithromycin and chloroquin. Am J Trop Med Hyg 74 (3) : 407.

12. Davis SN, Wu P, Camci ED, Simon JA (2020) Chloroquine kills hair cells in zebrafish lateral line and murine cochlear cultures implications for ototoxicity .Hear Res 395: 108019.

13. De JAD Leon C(2020) Evaluation of oxidative stress in biological samples using the thiobarbituric acid reactive substances assay. J Vis Exp (159): Article e61122.

14. Dubois M, Gilles MA, Hamilton JK(1956) Colorimetric method for determination of sugars and related substances.Anal. Chem 28 (3): 350-356.

15. Ellman GL, Courtney KD, Andres V (1961) Featherston A new and rapid colorimetridetermination of acetylcholinesterase activityBiochem. Pharmacol 7 (2): 88-95.