Identification of a panel of serum protein markers in early stage of sepsis and its validation in a cohort of patients

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Original Article

Keywords

ELISA; Mass spectrometry analysis; Proteome; Sepsis; Septic shock; Serum markers

Abstract  Background: Sepsis is a life-threatening illness with a challenging diagnosis. Current serum biomarkers are not sensitive enough for diagnosis. With the aim of finding proteins associated with sepsis, serum protein profile was compared between patients and healthy donors and serum classical inflammatory proteins were analyzed in both groups. Methods: Serum protein profiles were characterized by two-dimensional electrophoresis (2DE). Identification of the proteins was carried out by mass spectrophotometry and their validation was performed by Enzyme-Linked-Immunosorbent Assay (ELISA) in a cohort of 85 patients and 67 healthy donors. Seven classical inflammatory proteins were analyzed in the same cohort by ELISA: interleukin-2 receptor α-chain (sCD25), scavenger receptor cysteine-
Introduction

Pathogenesis of sepsis is poorly understood and remains one of the major health problems of the current medicine, due to its high associated mortality and its elevated economic burden at intensive care units. Physicians usually make their diagnosis based on a set of clinical symptoms but diagnosis is challenging even following the guidelines published by Surviving Sepsis Campaign Consortium and Society of Critical Care Medicine Consensus Conferences.

Sepsis has been recently re-defined as a life-threatening organ dysfunction caused by a deregulated host response to infection. In this context, sepsis and infection are different as sepsis implies both a deregulated host response and the presence of organ dysfunction. Clinical criteria for identifying septic patients by using Sequential (Sepsis related) Organ Failure Assessment (SOFA) have recently been published. An increase of SOFA is associated with sepsis, and septic shock is defined as a subset of sepsis in which despite fluid resuscitation, the patient requires vasopressors to maintain arterial pressure ≥65 mm Hg and serum lactate ≥2 mmol/L. Despite these criteria, clinical presentation of sepsis/septic shock is often heterogeneous and symptoms are usually influenced by many factors such as host susceptibility or the virulence and bioburden of the pathogen. An accurate and early diagnosis is crucial for prognosis and prompt therapy based on supportive care and antibiotics.

Although some markers (C-reactive protein, procalcitonin, among others) are currently in use in clinical practice for sepsis, they are not sensitive enough for diagnosis. Furthermore, due to the complexity of the sepsis disease a single biomarker may not reflect accurately a septic patient’s status. In this context, definition of specific markers panels may help the physician to improve clinical decisions for septic patients.

Analysis of specific protein patterns occurring in biological fluids has a great relevance for both understanding the pathogenesis and definition of disease markers. Among biological fluids, serum is one of the best candidates and may provide greater knowledge about molecular alterations caused by sepsis leading to characterization of biomarkers related to its diagnosis.

Methods

This study was carried out at Hospital Universitario Cruces between 2011 and 2015. Blood samples were obtained from venous catheter in 85 patients (31 females, 54 males; median age: 62 years-old) diagnosed with sepsis/septic shock according to diagnosis criteria set by the International Sepsis Definitions Conference (sepsis associated with organ dysfunction and/or tissue hypoperfusion, despite adequate volume resuscitation, requiring vasopressors) during the first hours after diagnosis, which means in early stage of sepsis. Blood samples were taken through Vacutainer venepuncture in 67 healthy donors (23 females, 44 males; median age: 50 years-old). Samples were allowed to clot at room temperature and were centrifuged at 2000 rpm for 20 min. Separated serum was stored at -80 °C until analysis. Patients were monitored until day 60 after sepsis. Five patients died due to sepsis during the first 15 days and other five patients were dead for other reasons during the monitoring time.

Protocol was approved by Ethics Committee of the Basque Country and all the patients provided written informed consent. Serum samples from healthy donors were obtained from the Basque Center for Transfusion and Human Tissues. All the samples were initially processed and stored until analysis in Basque Biobank for Research in accordance with...
ethical principles stipulated for research with human beings. Study was performed according to Spanish Law (Biomedical Research and Protection of Personal Data) and Declaration of Helsinki.

In the initial proteomic study, five healthy donors (median age: 51 years-old) and five patients (median age: 58 years-old) affected with sepsis/septic shock were included. Blood samples from patients were obtained during the first 24–48 h after diagnosis. Clinical features of the patients enrolled in proteomic assessment are summarized in Table 1. Four patients were diagnosed with septic shock and one with sepsis. In two patients, infections were confirmed by microbiological analysis. Initial antibiotic treatment was administered to all patients in the emergency department. The five patients showed organ dysfunction, and three of them presented dysfunction of two or more organs. However, all these patients survived.

For proteomic analysis, Proteominer Large Capacity Kit™ (Bio-Rad Laboratories, Hercules CA, USA) was applied to the ten serum samples in order to remove high-abundance proteins such as albumin or IgG, because they tend to mask those in lower abundance.14

Beginning with 2D-assay, protein samples (100 μg protein each) were dissolved separately in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.2% ampholytes and bromophenol blue) including DeStreak TM (GE Healthcare, Piscataway, NJ, USA) and ampholytes (Bio-Rad). In the next step, samples were loaded individually in 11 cm pH 4–7 gradient IPG strips (Immobiline DryStrips, GE Healthcare) to carry out the first dimension in Protean IEF-Cell (Bio-Rad). Each IPG strip was submitted to the following IEF program: passive rehydration for 14 h; 2 h at 150 V; 2.5 h at 8,000 V; 8,000 V until 25,000 V/h; 24 h at 500 V. After first dimension, samples were loaded individually in 11 cm pH 4–7 gradient IPG strips (Immobiline DryStrips, GE Healthcare) to carry out the first dimension in Protean IEF-Cell (Bio-Rad). Each IPG strip was submitted to the following IEF program: passive rehydration for 14 h; 2 h at 150 V; 2.5 h at 8,000 V; 8,000 V until 25,000 V/h; 24 h at 500 V. After first dimension, strips were equilibrated with buffer-I (6 M Urea, 0.375 M Tris–HCl, 2% SDS, 20% Glycerol, 2% DTT) with gentle shaking for 5 min (twice), followed by a second equilibration with buffer-II (6 M de Urea, 0.375 M Tris–HCl, 2% SDS, 20% Glycerol, 2.5% Iodoacetamide) with gentle shaking for 10 min (twice).

For the second dimension, IPG strips were placed in contact with commercial acrylamide gels (Criterion XT Precast Gel, Bio-Rad) for protein separation according to their molecular weight. Gels were subjected to electrophoresis at 90 mA for 3 h, until the bromophenol blue front reached the bottom of the gels. Gels were stained overnight with SYPRO Ruby Protein Gel Stain (Bio-Rad) and scanned by Image Scanner (Typhoon Trio, GE Healthcare). Image analysis was performed with LUDESI Redfin-3 software (www.ludesi.com). Spots with at least a 1.2-fold abundance change and p < 0.05 were accepted as statistically significant and selected for protein identification.

Mass spectrometry analysis were performed as described elsewhere,15 with minor modifications. Protein digestion was conducted following the protocol described by Shhevchenko et al.,16 with minor variations. Gel spots were digested with trypsin overnight at 37°C. After digestion, supernatants were collected and acidic peptides were extracted with trifluoroacetic acid. Samples were dried out in a Christ RVC C2-25 speed vac concentrator (Christ GmbH, Osterode, Germany) and submitted to MALDI-TOF/TOF analysis as described in Anitua et al.15 When no confident identification was obtained with MALDI-TOF/TOF, peptides were analyzed in an LTQ Orbitrap XL ETD mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA) coupled to a nanoACQUITY UPLC System (Waters, Manchester, UK). A 30 min linear gradient of 3–50% acetonitrile was used for the chromatographic runs. Collision-induced dissociation was used for the generation of fragmentation spectra. Precursors with charge states of 2 and 3 were specifically selected and excluded from further analysis during 60 s using the dynamic exclusion feature. Searches were conducted against human Swissprot/Uniprot database with Mascot search engine (www.matrixscience.com), under standard search parameters and tolerances for each of the mass spectrometers used. Carbamidomethylation of cysteines and oxidation of methionines were considered as fixed and variable modifications respectively.

Deregulated proteins found in serum by the proteomic study were validated by ELISA assays in cohorts of 85 patients and 67 healthy donors. Given the importance of inflammatory processes in sepsis,17 the same serum samples from septic patients and healthy individuals were tested for a set of seven inflammatory proteins by ELISA assays: hemeoxigenase-1 decycling (HO-1), interleukin-6 (IL-6), interleukin-18 (IL-18), soluble intercellular adhesion-molecule-1 (sICAM-1), soluble interleukin-2 receptor alpha-chain (sCD25), soluble scavenger receptor cysteine-

<table>
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<tr>
<th>Table 1</th>
<th>Clinical features of septic patients included in proteomic study.</th>
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<td>Patient 4</td>
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</table>

a APACHE (Acute Physiology and Chronic Health Evaluation).
b SOFA (Sequential Organ Failure Assessment).
Results

Two-dimensional gels profile of healthy and septic patients

Protein extracts from serum samples obtained from five healthy volunteers and five septic patients were loaded individually onto the 2DE system. Differential protein 2DE gel profiles between healthy donors and septic patients are shown in Fig. 1. More than 600 spots were detected in each gel, however, only 22 were considered as significantly deregulated between healthy donors and patients (1.2-fold abundance change and p < 0.05) by computational analysis performed with LUDESI Redfin-3 software.

In order to identify proteins differentially expressed in septic patients, spots were excised manually and subjected to digestion before submission to mass spectrometry analysis. A total of 20 out of the 22 spots considered as significantly deregulated were successfully identified by MALDI-TOF/TOF and LC-MS. The results for their deregulation and identification are summarized in Table 2.

These results revealed the identity of seven different deregulated proteins: Antithrombin-III (AT-III), Apolipoprotein-E (Apo-E), Clusterin (CLUS), Complement factor H-related-1 (FRH1), Filaggrin, Hemoglobin subunit beta (Hb-β) and Serum amyloid A-1 (SAA-1). All these proteins were related to inflammation and immune system, with the exception of Filaggrin, which is associated with epidermis and its presence in the gels was probably due to contamination. For this reason, this protein was excluded from the following validation process.

ELISA assay: Validation of proteomic assay and study of a set of inflammatory proteins

Immune related proteins identified in the proteomic study (AT-III, Apo-E, CLUS, FHR1, Hb-β and SAA-1) were analyzed in a cohort of 85 patients and 67 healthy donors by ELISA assays. Fifty percent of the proteins identified in the proteomic experiment were validated when patients and controls were compared: AT-III, CLUS and SAA-1. Results are shown in Table 3. Consistent with the results obtained in 2DE, SAA-1 showed higher values in patients’ serum, whereas serum concentration of AT-III and CLUS was significantly lower in septic patients. However, FRH1, ApoE and Hbβ did not show significant differences in serum concentration levels between patients and controls when they were validated in a cohort of 85 patients and 67 healthy donors by ELISA assays.

Gene ontology analysis

Finally, a functional analysis of the ten proteins significantly deregulated in septic patients (AT-III, CLUS, SAA-1, HO-1, IL-6, IL-18, sICAM sCD25, sCD163 and sFAS) was also analyzed. The results are shown in Table 3 and as can be noted, all the seven inflammation-related serum factors analyzed by ELISA were shown to be significantly more abundant in patients’ serum.

In a next step, we evaluated if these ten deregulated proteins (AT-III, CLUS, SAA-1, HO-1, IL-6, IL-18, sICAM sCD25, sCD163 and sFAS) were different between dead patients and survivors, and no significant differences were found between both groups (data not shown).

Discussion

Characterization and proper diagnosis of sepsis patients remains a challenge in current medicine. Complications derived from septic processes very often have deleterious consequences for patients and high economic costs. Thus, a better knowledge of the molecular mechanisms underlying sepsis may allow better diagnosis tools and the development of new therapies against the disease. Serum appears to be a suitable candidate for monitoring the sepsis process, since its properties make it an affordable biofluid that can be analyzed with ease and low cost procedures.

2DE-based differential proteomics provides a better understanding of the disease though the discovery of novel disease markers. In this work we present the analysis and validation of differentially expressed proteins upon sepsis.
Twenty differential spots were successfully identified, and seven different proteins accounted for their deregulation, revealing that the same protein was present in many of these spots. This is a common phenomenon in 2DE-technology, since different post-translational modifications of proteins, such as glycosylation, phosphorylation, among many others or proteolytic processing, may alter their electrophoretic properties. Thus, different forms of a protein may form different spots. Therefore, our results suggest some kind of modification for AT-III, CLUS, and FHR1. Elucidating the nature of these modifications, however, surpasses the scope of this study.

The proteomic approach allowed us to identify seven proteins, all of them related to inflammation and immune system, except Filaggrin. Taking into account that samples came from human serum and Filaggrin is associated with epidermis, its presence may respond to a contaminated spot. The big score and number of peptides of Filaggrin could be explained by the fact that contaminants, often relatively more abundant than proteins of interest, render more detectable peptides. For this reason, Filaggrin was excluded from the validation process.

The other six identified proteins were potentially interesting in sepsis research and the differential expression in septic serum was validated for three of them. The inherent complexity of the serum 2DE-protein patterns may in part explain this result. As mentioned before, 2DE has the power of resolving protein isoforms when these have different isoelectric points or molecular weights. Therefore, some of the results obtained by 2DE may be ascribed to certain modifications of a protein rather than to its total amount. Thus, when analyzing the whole amounts of these proteins, such as in ELISA assays, it is not surprising that some of the results may not converge, since different subsets of the protein may be analyzed in each of them. This could be the case of proteins with complex 2D-patterns, such as FHR1.

Nonetheless, the 2DE analysis led us to robust validation of deregulation of three proteins in a large cohort of patients: AT-III, CLUS and SAA-1. In our study, serum

Figure 1. Gels from controls (up) and patients (down). Numbers correspond to the spots accepted as statistically significantly deregulated between controls and septic patients group (1.2-fold abundance change and p < 0.05).
In addition to the proteomic analysis, seven inflammatory proteins (HO-1, IL-6, IL-18, sCD25, sCD163, sICAM-1 and sFas) were characterized and validated by ELISA in the same cohort of individuals used for the validation of the proteomic results. The main reason for the lack of inflammation-related proteins within the 2DE results is that they are very likely out of the limit of detection of our experimental setup and their detection was only affordable using a targeted approach such as ELISA. Statistical analysis revealed a significantly increased presence of these seven proteins in patients’ serum. The increase in sFAS, HO-1 and sICAM-1 in septic patients is in accordance with previous studies pointing in the same direction.\textsuperscript{33–35} The assessment of sCD25 and sCD163 is already used in the diagnosis and follow-up of Hemophagocytic Lymphohistiocytosis (HLH) disease activity,\textsuperscript{36} and given that HLH and septic patients share many clinical features, their use in sepsis diagnosis should not be discarded.\textsuperscript{37} In fact, recent studies suggest that sCD163 and sCD25 could be promising markers in sepsis research,\textsuperscript{38,39} and our results support this hypothesis. Moreover, the pro-inflammatory cytokines IL-6 and IL-18 were significantly increased in the septic patient group. This result is in agreement with their role in sepsis, already pinpointed previously.\textsuperscript{40}

Finally, Gene Ontology analysis of the combined results from both proteomics and ELISA revealed that the ten proteins included in the study (AT-III, CLUS, SAA-1, HO-1, IL-6, IL-18, sCD25, sCD163, sICAM-1 and sFas) are at least partially biologically connected as a group, and play a role mainly in the activation of the immune response, inflammatory response and response to wounding. As these markers are not sepsis specific, and changes in serum levels may be observed in other diseases (e.g. cancer), further

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\textsuperscript{a} Spot: number of spot in the 2DE gel.
\textsuperscript{b} Analysis: mass spectrometric analysis method providing the identification.
\textsuperscript{c} Ratio: sepsis/control spot volume ratio.
\textsuperscript{d} Accession: accession number for that protein in Uniprot knowledgebase.
\textsuperscript{e} Name: protein name in Uniprot knowledgebase.
\textsuperscript{f} Score: Mascot score.
\textsuperscript{g} # Peptides: number of identified peptides matching to the protein.
\textsuperscript{h} MW [kDa]: molecular weight of the protein expressed in kDa.
comparative studies would be very interesting in the future.

This work provides an interesting insight into the discovery of sepsis biomarkers based on the identification of a panel of deregulated serum proteins in patients diagnosed with sepsis. We show a set of ten proteins (AT-III, CLUS, SAA-1, HO-1, IL-6, IL-18, sCD25, sCD163, sICAM-1 and sFas) that seem to be crucial in the pathogenesis and may potentially work as a complementary tool for the physician in the diagnosis of sepsis. The correlation of these ten markers with other biomarkers and clinical features, and clinical validation follow-up are needed for a more comprehensive characterization. In addition, further analyses should provide a better understanding of the role of these proteins in the pathophysiology of the disease and may increase the list of molecules susceptible of being helpful for sepsis diagnosis and treatment.

Conflicts of interest

None of the authors has any conflicts of interest to declare.

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References


Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jmii.2016.12.002.