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Sepsis Plasma Protein Profiling with Immunodepletion, Three-Dimensional Liquid Chromatography Tandem Mass Spectrometry, and Spectrum Counting

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Sepsis is a systemic, often fatal inflammatory response whose biochemical pathways are not fully understood and with no single biomarker capable of its reliable prediction. Increased interest in protein profiling to reveal fundamental biochemical events as well as disease diagnosis has grown considerably, largely due to advances in mass spectrometry and related front-end technologies. In this study, patients with sepsis and systemic inflammatory response syndrome (SIRS) were examined using plasma protein profiling following immunodepletion treatment to remove the most abundant proteins, serum albumin, transferrin, haptoglobin, anti-trypsin, IgG, and IgA. These proteins cause significant signal suppression, and their removal allows for lower abundance proteins to be examined through improved ion signal. Analyses after immunodepletion were performed using 3-dimensional reverse phase/strong cation exchange/reverse phase liquid chromatography with electrospray ion trap mass spectrometry (3D LC−MS/MS) and spectrum counting for comparative quantitation. The results revealed a major theme in immune system activity, including activation of the complement and coagulation pathways. Additionally, lipid transport may prove to be important in distinguishing sepsis from SIRS. Specifically, significant multi-fold changes were observed in 10 proteins and are now being investigated for the early diagnosis of sepsis.

Keywords: sepsis • protein profiling • plasma • tandem mass spectrometry • liquid chromatography

Introduction

Sepsis is a systemic inflammatory response to overwhelming infection or trauma and the leading cause of death in non-coronary ICU patients,[1] with over 700 000 cases diagnosed each year in the US.[2,3] The innate host response to infection leading to sepsis can be caused by bacteria, fungi or yeast, trauma, and conditions such as cancer, pneumonia, or a compromised immune system, where the infection becomes amplified and ultimately dysregulated.[4−7] Excessive or inappropriate inflammatory reactions ensue, and this loss of homeostasis is characterized as an uncontrolled cascade of coagulation, impaired fibrinolysis, and inflammation.[8−11]

At present, the clinical response to sepsis diagnosis includes broad spectrum antibiotics, direct fluid injections, medication to restore normal blood pressure, surgical removal of the infected source, nutritional support, and individual support of failing organs. Numerous compounds are currently being investigated for the treatment of sepsis, including corticosteroid therapy and recombinant human activated protein C (rhAPC; drotecogin alfa, Xigris) to reduce mortality.[12,13]

Although many of these therapeutic approaches are effective, the primary limitation in treatment is diagnosing sepsis at an early stage. For example, the disadvantage with culture-based pathogen identification in sepsis diagnosis is that the results require 24−48 h and clinical symptoms can also occur in the presence of a negative culture. To this end, several molecules have been investigated in the pursuit of a sepsis biomarker, such as procalcitonin (PCT),[14−16] several interleukins[7,17] including IL-6, IL-1ra, IL-8, tumor necrosis factor TNFα,[7,17] and C-reactive protein (CRP).[18−20] However, as yet, there is no definitive biomarker that can universally identify sepsis, and it is likely that a suite of markers will be needed. Therefore, our aim was to design a platform capable of identifying a single or a small group of proteins as an early indicator for sepsis.

One approach to identifying biomarkers is to monitor plasma protein changes. However, the challenge associated with plasma analysis is that it is the most complex human-derived proteome, containing other tissue proteomes as subsets,[14] with more than 10 000 proteins present. The 26 most abundant proteins account for 99% (wt %) of total plasma protein, with

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albumin alone making up over 50% of the total plasma protein.[21] The extraordinary dynamic range of plasma protein, spanning more than 10 orders of magnitude, proves a great challenge in the investigation of low-abundance small molecule biomarkers (typically in the ng/mL range or lower) such as cytokines and hormones.[21] Strategies for the enrichment of low-abundance proteins in tissue samples have included laser microdissection and other mechanical methods, as well as subcellular fractionation.[22,23] Recently, the development of affinity chromatography methods for the enrichment of low-abundance proteins in biological fluids have proven to be effective.[23,24] In this study, the multiple affinity removal system (Agilent Technologies) was used to remove the six most abundant human plasma proteins (albumin, transferrin, haptoglobin, anti-trypsin, IgG, and IgA) to improve the identification and achieve semiquantitation of lower abundant plasma proteins.

Two additional challenges in protein biomarker discovery are the separation of the hundreds of thousands of peptides generated from the complex digested protein mixture, and the quantitative comparison of biological samples with this extreme complexity. Although 2-dimensional strong cation exchange/reverse phase (SCX-RP) liquid chromatography coupled with tandem mass spectrometry has proven to be a powerful method for peptide separation and identification in highly complex mixtures, it still cannot meet the challenges presented by biofluids such as plasma.[25] Our novel approach to addressing the first challenge of sample complexity was to utilize the high resolving power of reverse phase separation by combining a 2-dimensional on-line fractionation column to the analytical reverse phase column. Further, we applied spectrum counting for semiquantitative analysis. Here, we report the investigation of plasma samples from 25 patients with systemic inflammatory response syndrome (SIRS) and 25 sepsis patients, for the presence of protein biomarkers using this approach.

Experimental Section

Plasma Samples. Plasma samples from 25 SIRS and 25 sepsis patients (150 μL) were provided by BD (Becton, Dickinson and Company). The diagnoses were made based on the clinical standard for both SIRS and sepsis patients.[5−7] Briefly, SIRS is described as a common inflammatory response to a wide variety of insults, and sepsis is defined as SIRS with a confirmed infection, leading to dysregulation of the immune system. Equal volumes of plasma (50 μL) were taken randomly from individual patient samples from the same disease state (either sepsis or SIRS) for the creation of six pools (three sepsis and three SIRS), each containing a total of 20 individual samples. This approach was necessary as using 3D LC−MS/MS, the analysis of individual patient samples would have been extremely time and cost intensive. The goal was to identify proteins common to each pooled dataset that were either up-regulated or down-regulated in the early stages of sepsis, ultimately allowing for the identification of protein biomarkers.

Immunodepletion. The pooled plasma samples were purified using the Agilent Multiple Affinity Removal System (5185−5985, 4.6 mm × 50 mm, Agilent Technologies). This immunodepletion column is based upon affinity purified polyclonal antibody technology,[26−28] containing six types of antibodies to specifically remove six target proteins: albumin, transferrin, haptoglobin, anti-trypsin, IgG, and IgA. The six antibodies are oriented on the surface of solid beads and chemically cross linked via the FC (fragment crystallizable) region resulting in a stable, nonleaching product, with the material packed in an HPLC column format.

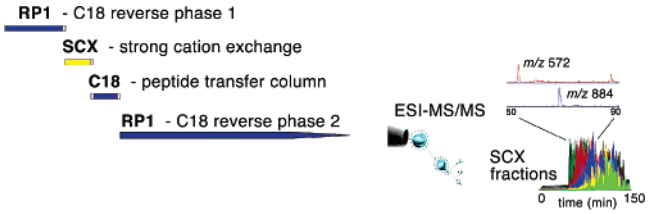

Figure 1. Schematics of reverse phase (RP)−strong cation exchange (SCX)−reverse phase (RP) 3D liquid chromatography separation. (Right) Total ion chromatograms (TIC) and extracted ion chromatograms (EIC) of individual peptides generated from the 3D separation.

A 25 μL aliquot from each plasma sample was diluted 5 times with column loading buffer A (Agilent Technologies, #5185−5987) and followed by centrifugation at 12 000 rpm for 10 min. The column was attached to a capillary HPLC pump and equilibrated with buffer A at a flow rate of 0.5 mL/min for 10 min. The diluted plasma sample was then injected onto the column, and the column was washed with buffer A at a flow rate of 0.25 mL/min. The first 1.5 mL flow-through containing low-abundance plasma proteins was collected. The retained proteins were then removed by elution using buffer B (Agilent Technologies # 5185−5988) at a flow rate of 0.5 mL/min for 7 min. Retained proteins were checked by 2D-Gel and no other proteins except these six binding proteins have been found.[26,28] The column was either stored or reused. The protein concentrations of samples before and after immunodepletion were measured by Coomassie protein assay reagent kit (Pierce #23200).

Protein Digestion. A 2 mg sample of protein from the immunodepletion column was collected for each pooled plasma sample. The proteins were concentrated by Vacufuge (eppendorf) to 0.25 mL (4 mg/mL protein) and denatured in 8 M urea (ACROS) for 10 min. The proteins were reduced and alkylated using 5 mM D,L-dithiothreitol (Sigma) at 37 °C for 30 min and 10 mM iodoacetamide (Sigma) at 37 °C for 30 min, respectively. The samples were then diluted 4 times to a final concentration of 2 M urea using 100 mM ammonium hydrogen carbonate (Fluka). The proteins were digested with trypsin (Promega) at the ratio of 1:50 (w/w) overnight and followed by the second trypsin digestion under the same conditions. The protein digestion efficiency was checked with a Coomassie protein assay reagent kit.

Three-Dimensional Chromatography and Sample Loading. Using this approach, the peptide mixture was prefractionated by the first reverse phase column (RP1) based on hydrophobicity and then each fraction was then further fractionated by the SCX column based on the peptide ion strengths. The final high-resolution separation was performed on the second reverse phase (RP2) column by a shallow reverse phase gradient that was determined by the first reverse phase (RP1) fractionation gradient (Figure 1).

Approximately 2 mg of digested protein was harvested from each pooled sample and 0.5 mg was then subjected to 3D LC−MS/MS analysis (Figure 1) using an Agilent 1100 LC/MSD Trap system coupled directly to an Agilent 1100 nanopump and a microautosampler. Using an in-house constructed pressure cell, 5 μm Zorbax SB−C18 packing material (Agilent Technologies) was packed into a 500 μm ID 1/32" OD PEEK tubing (Upchurch Scientific). A 10-cm section was cut off to form the first

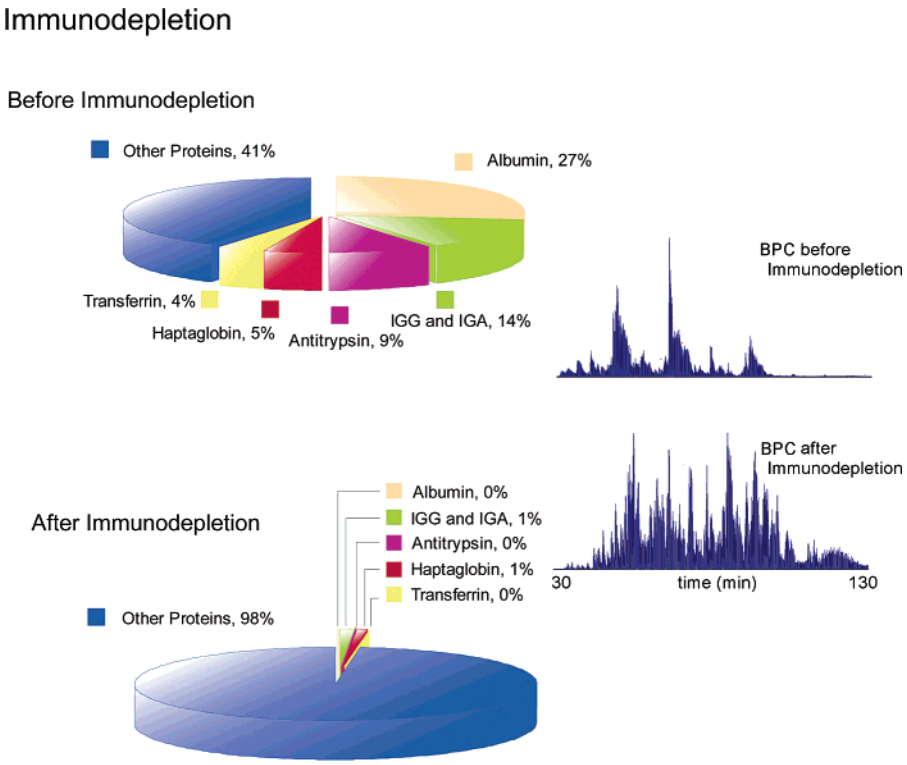

Figure 2. Percentage of validated MS/MS spectra from the 6 target proteins before and after the immunodepletion column. The typical abundance of the top 6 most abundant proteins is 85−90%.

dimension RP column (RP1). A similar column (500 μm ID, 4 cm length) packed with 5 μm PolySulfoethyl (Western Analytic Production) packing material was used as the SCX column. A second C18 column 4 cm in length and 250 μm ID was used as the trap column. A zero dead volume 1 μm filter (Upchurch, M548) was attached to the exit of each column for column packing and connecting. A fused silica capillary (100 μm ID, 360 μm OD, 20 cm length) packed with 5 μm Zorbax SB−C18 packing material (Agilent Technologies) was used as the analytical column (RP2). One end of the fused silica tubing was pulled to a sharp tip with the ID smaller than 5 μm using a Sutter P-2000 laser puller (Sutter Instrument Company, Novato, CA). The peptide mixtures were loaded onto the first C18 column (RP1) using the same in-house pressure cell. To avoid sample carry-over, a new set of the four columns was used for each sample. To maintain good reproducibility for quantitation, each of the above four columns was packed to the exact same length for every 3D experiment. The 3D LC separation consisted of two HPLC pumps, four micro- and nanoflow LC columns constructed in-house, together with a switch valve. A 0.5 mg sample of each digested protein sample was loaded onto the first dimension reverse phase (RP) column for every analysis. Up to 5 RP fractions and up to 8 strong cation exchange (SCX) fractions were eluted sequentially from the loading column to the analytical column for high-resolution peptide separation. All fractionation and separation methods were identical for the samples within the same batch. The runtime for each fraction was about 2.5 h and total runtime for each sample was about 3 days. A scan range of 200−2000 m/z was employed in the positive mode.

Results and Discussion

Immunodepletion: Removal of High Abundance Proteins. LC−ESI/MS signal suppression from high-abundance species is a significant obstacle in analyzing complex biofluids such as plasma. Therefore, to enhance the observation of low-abundance protein biomarkers, immunodepletion was performed to remove the six most abundant plasma proteins (albumin, transferrin, haptoglobin, anti-trypsin, IgG, and IgA). The first step in the plasma sample analyses was to validate this protein removal approach using the immunodepletion column. This validation was accomplished using the Coomassie protein assay, where it was determined that ~85% of total protein was removed from the original plasma sample after immunodepletion. The binding capacity of the column for human serum albumin was 1.4 mg, and a single column was typically used over 500 times without significant loss of efficiency. The results from 3D LC−MS/MS experiments also showed a dramatic decrease of the total number of MS/MS spectra from the six most abundant proteins (from 59 to 2%) after immunodepletion (Figure 2). The lower abundance proteins could now be readily identified because the corresponding number of peptides per protein increased dramatically. This increase in the amount of data generated on lower abundance proteins was beneficial for relative quantitation using the spectrum counting method[29] (Table 1).

Protein Identification from 3D LC−MS/MS. The use of two-dimensional (2D) strong cation exchange-reverse phase (SCX-RP) chromatographic separations before MS analysis has enabled the extension of LC−MS/MS methodologies for protein identification to mixtures of high complexity. Complex tryptic digests, such as from plasma, are first separated into fractions using SCX followed by RP chromatography (known as 2D LC−MS/MS). A step gradient of salt concentrations is used to elute different peptide fractions from the SCX resin onto the RP material, after which RP chromatography is performed without affecting the other peptides still bound to the SCX resin. The resulting nano-RP LC column eluent is electrosprayed directly

Table 1. Partial List of Identified Proteins Before and After the Immunodepletion of Human Plasma[a]

# MS/MS spectra		proteins observed
before immunodepletion	after immunodepletion	6 most abundant plasma proteins
1958	0	serum albumin precursor
673	8	alpha-1-antitrypsin precursor
356	3	IGHG3 protein (IGG)
346	77	haptoglobin-2 precursor
299	17	serotransferrin precursor
73	35	Ig alpha-1 chain C region (IGA)
before immunodepletion	after immunodepletion	other plasma proteins
256	739	complement C3 precursor
78	138	vitamin D-binding protein precursor
12	46	histidine-rich glycoprotein precursor
7	34	complement C6 precursor
0	10	lipopolysaccharide-binding protein precursor (LBP)
0	5	similar to profilaggrin − human (fragments)
0	4	protein kinase A anchoring protein Ht31

[a] The numbers represent validated MS/MS spectra from the identified proteins. Notice the dramatic decrease in MS/MS spectrum numbers from the 6 target proteins.

into the mass spectrometer, making this method an efficient means of transferring the ions into the mass analyzer, with a higher tolerance of complex mixtures. Using this "MudPIT" methodology (multi-dimensional protein identification technology),[30] hundreds to over a thousand of unique proteins have been identified from a whole cell lysate in a single 2D LC−MS/MS experiment. Additionally, recent studies have also indicated that this technique possesses a greater dynamic range than that obtained using 2D gel electrophoresis, enabling the detection of lower abundance proteins.[30]

Both RP one-dimensional (1D) LC−MS/MS and 2D strong cation exchange SCX/RP LC−MS/MS proved to be inadequate for this plasma protein biomarker study, generating 60−70 proteins and approximately 300 proteins, respectively. Additionally, the use of SCX as the first step and RP in the second step is not sufficient for the identification of most of the proteins present in a complex mixture such as plasma. It was therefore necessary to develop a more sophisticated technique using an entirely automated 3D LC−MS/MS process.

For this reason, we have implemented a 3D LC−MS/MS approach similar to one recently described.[25] Briefly, an initial reverse phase separation (RP1) separates peptides based on hydrophobicity, the subsequent SCX further separates the peptides using a salt gradient, and finally the second RP2 enables high-resolution separation of the SCX fractions (Figure 1). Using an entirely automated 3D LC−MS/MS process, approximately 150 000 MS/MS spectra were collected over the period of 3 days for each experiment, resulting in over 10 million MS/MS spectra. Representative total ion chromatograms (TICs) and extracted ion chromatograms (EICs) of individual peptides are shown in Figure 1. The sharp chromatogram peaks in the EICs demonstrate the high resolving power of the reverse phase separation.

Data Analysis. The second challenge of quantitative analysis was addressed using a database search software package, Spectrum Mill MS Proteomics Workbench (version 2.7, Agilent Technologies), for protein identification and semiquantitation. MS/MS spectra were extracted from the raw data using SpectrumMill's Xtractor. A total of more than 10 million MS/MS spectra were collected. MS/MS spectra with a sequence tag length of 1 or less were considered as poor spectra and discarded, and the remaining MS/MS spectra were searched against the National Center for Biotechnology Information (NCBI) nonredundant protein database (NCBI-nr human 11/06/2003, 97027 sequences) limited to human taxonomy. The enzyme parameter was limited to full tryptic peptides with a maximum miscleavage of 2. All other search parameters were set to SpectrumMill's default settings (carbamidomethylation of cysteines, ±2.5 Da tolerance for precursor ions, ±0.7 Da tolerance for fragment ions, and a minimum matched peak intensity of 50%).

The false positive rate was estimated by searching one 3D dataset against a combined forward-reverse database (NCBI-nr human 11/06/2003, 97027 sequences).[31] A total of 4294 spectra and 107 proteins were autovalidated. Among them, 16 spectra and 12 proteins were from the reverse database. Thus, the false positive rate of our filtering criteria was 0.75/% spectra and 22% protein. The false positive rate for proteins with a minimum unique peptide of 2 was 0.19/% spectra and 2.8% protein. Only proteins with at least 2 unique peptides were selected for relative quantitative analysis.

SpectrumMill grouped the proteins with the same set or subsets of unique peptides together to minimize protein redundancy. The number of identified proteins reported in SpectrumMill is the number of identified "protein groups" rather than the number of identified protein sequences in the database. Spectrum counting was used for relative protein quantitation. The number of valid MS/MS spectra from each protein was normalized to the total MS/MS spectra number of each dataset. Samples were divided into two patient groups, SIRS vs sepsis. The Z-test was used for statistical analysis. The Z-scores (Δ/stdev) of each protein were calculated between those 2 groups, and proteins with Z-scores above 2 were considered to be biomarker candidates. The candidate list was further filtered by relative standard deviation (<100%), absolute MS/MS spectrum number (≥10), unique peptide number (≥2) and manual inspection to remove obvious false hits such as keratin.

The Spectrum Mill Workbench output produced 2810 protein entries across the three pools. The total spectra numbers were normalized across all pools and entries with a distinct sum tag score greater than 13. The Genbank Accession numbers for each hit were cross referenced with their corresponding Entrez Gene ID using the gene2accession table from ftp://ftp.ncbi.n-

Table 2. Biocarta and KEGG Pathways Represented in the Output Data[a]

cat. system	term	count[b]	%[c]	*p* value
KEGG_PATHWAY	complement and coagulation cascades	25	24	2.25E−33
BIOCARTA	intrinsic prothrombin activation pathway	8	7	5.81E−09
BIOCARTA	complement pathway	7	6	1.04E−06
BIOCARTA	classical complement pathway	6	5	2.70E−06
BIOCARTA	alternative complement pathway	5	4	2.86E−05
BIOCARTA	lectin induced complement pathway	5	4	8.36E−05
BIOCARTA	extrinsic prothrombin activation pathway	4	3	8.54E−04
BIOCARTA	acute myocardial infarction	4	3	1.44E−03
KEGG_PATHWAY	regulation of actin cytoskeleton	8	7	8.59E−03
KEGG_PATHWAY	focal adhesion	7	6	4.48E−02
KEGG_PATHWAY	ECM-receptor interaction	4	3	7.31E−02

[a] The data indicates participation of the complement and coagulation systems. Cytoskeleton elements such as actin were also highly represented in this data set and may play an important role in distinguishing sepsis from SIRS. [b] Number of proteins in the uploaded list from that particular pathway. [c] Percentage of matched input protein # over total input protein #.

Table 3. Gene Ontology Categories Over-Represented in This Data[a]

term	count[b]	%[c]	*p* value
acute-phase response	18	17	5.07E−28
response to pest, pathogen or parasite	34	33	5.70E−26
complement activation	14	13	9.01E−20
blood coagulation	16	15	1.13E−17
serine-type endopeptidase inhibitor activity	16	15	1.52E−17
wound healing	16	15	2.96E−17
lipid transport	12	11	1.76E−13
humoral immune response	14	13	2.56E−11
immune response	39	37	4.73E−11
humoral defense mechanism (sensu vertebrata)	12	11	1.61E−10
inflammatory response	12	11	3.87E−08

[a] Similar to the pathway output, compliment and coagulation activity is highly represented in this data set. The major theme of the data is immune system activity. Additionally, lipid transport (apolipoproteins) is a functional process that may prove to be important in distinguishing sepsis from SIRS. [b] Number of proteins in the uploaded list from that particular pathway. [c] Percentage of matched input protein # over total input protein #.

lm.nih.gov/gene/DATA. Only proteins with a gene ID were included for further analysis (484 remaining entries). Because the goal of this study was to find proteins that varied between the two conditions, Sepsis to SIRS ratios were calculated using the normalized total spectra numbers. Where SIRS > sepsis, the ratio was calculated using 1/(Sepsis/SIRS). If either number was zero (so a ratio could not be calculated) the value was tagged SEPSIS+ or SIRS+ as appropriate. Gene quantities would be expected to converge or diverge as a septic event approached; therefore, the range of ratio values across time points was calculated for each round, and protein entries were included if they had a range >1.5 or contained SEPSIS+ or SIRS+ at any time point. This left 151 remaining entries constituting 103 unique gene ID's.

The 103 proteins (Entrez Gene ID's) were uploaded into DAVID 2.1 (Database for Annotation, Visualization, and Integrated Discovery)[32] (http://david.abcc.ncifcrf.gov/main.htm). All 103 genes were recognized by DAVID. The canonical pathways contained in the data were examined by selecting output from Biocarta and KEGG pathways. Any pathways containing at least 2 genes from the list of 103 and having a probability score (*p*-value) ≤ 0.1 were included. (Table 2). Additionally, the molecular functions and biological process inherent in the data set were examined by outputting any "over-represented" gene ontology categories (Table 3). The ontologies were filtered to include only those with a level 5 distinction (most specific gene ontologies) and >10% of the input gene list. Only ontologies from the molecular function or biological processes were incorporated (http://www.geneontology.org/).

The canonical pathways identified by DAVID are shown in Table 2. Each pathway was over-represented in this data set,

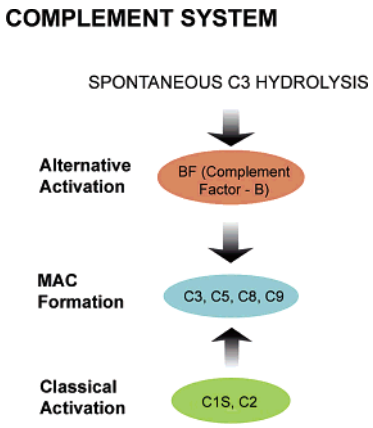

Figure 3. Involvement of the classical and alternative complement cascades in differentiating sepsis from SIRS patients. The proteins listed are those found in this study.

implying that they contained more proteins from the data than would be expected by chance. The results indicate a significant focus on both the complement and coagulation cascades, known to play a major part in sepsis.[9,11]

The Complement Pathway. The complement pathway consists of a complex series of over thirty plasma proteins that are part of the immune response, providing a critical defense against infection. Figure 3 shows the identified proteins from this study in the complement cascade. Activation of the complement system lyses bacterial cells, forms chemotactic peptides (C3a and C5a) that attract immune cells, and increases phagocytotic clearance of infecting cells. Additionally, the complement pathway can result in increased permeability of

Table 4. Up-Regulated and Down-Regulated Proteins in the Plasma of Sepsis Patients Compared to SIRS Patients

Up-regulated
complement component C4
C-reactive protein precursor
plasminogen precursor [contains: angiostatin]
apolipoprotein A-II precursor (Apo-AII) (ApoA-II)
plasma protease C1 inhibitor precursor (C1 Inh) (C1Inh)
transthyretin precursor (prealbumin) (TBPA) (TTR) (ATTR)
serum amyloid P-component precursor (SAP) (9.5S alpha-1-glycoprotein)
Down-regulated
apolipoprotein A−I precursor (Apo-AI)
antithrombin-III precursor (ATIII) (PRO0309)
serum amyloid A-4 protein precursor (constitutively expressed serum amyloid A) (C−SAA)

vascular walls and inflammation. Most complement proteins exist in plasma as inactive precursors that cleave and activate each other in a proteolytic cascade leading ultimately to the formation of the membrane attack complex (MAC), which causes lysis of cells. MAC formation may be activated by three pathways distinct in the initiation of the proteolytic cascade but share most of their components: the classical pathway, alternative pathway, and membrane attack pathway. Here, the classical and alternative pathways are discussed. The classical pathway is activated by the recognition of foreign cells by antibodies bound to the surface of the cells. In these data, proteins C1S and C2 were unique to this pathway. Proteolysis is triggered in the alternative pathway by the spontaneous activation of C3 convertase from C3. Complement Factor-B (Protein BF, properdin) was found in the data presented here and is unique to the alternative activation pathway. Additionally the proteins C3, C5, C8, and C9, discovered in the plasma samples in this study, are common to all methods of complement activation.

The Coagulation Pathway. Activation of coagulation is a normal component of the acute inflammatory response, and disorders of coagulation are common in sepsis. Tissue factor production is increased and leads to the activation of both the intrinsic and extrinsic prothrombin activation pathways. In this study, the data strongly indicated participation of the intrinsic prothrombin activation pathway (Figure 4). Briefly, blood coagulation or clotting takes place in 3 essential phases. First is the activation of a prothrombin activator complex, followed by the second stage of prothrombin activation. The third stage is clot formation as a result of fibrinogen cleavage by activated thrombin. The prothrombin activation complex is formed by two pathways, each of which results in a different form of the prothrombin activator. The intrinsic mechanism of prothrombin activator formation begins with trauma to the blood or exposure of blood to collagen in a traumatized vessel wall. Although the extrinsic pathway was identified by DAVID, it appeared to be included because of the overlapping proteins PROS, SERPINC1, Thrombin, and Fibrinogen. The data also contained SERPING1, KNG, KLKB1, and F9, which are all uniquely involved in the formation of the prothrombin activator complex specific to the intrinsic prothrombin activation pathway. The inclusion of gene ontologies in Table 3 covering both complement and coagulation also further support the role of these pathways in distinguishing sepsis from SIRS samples.

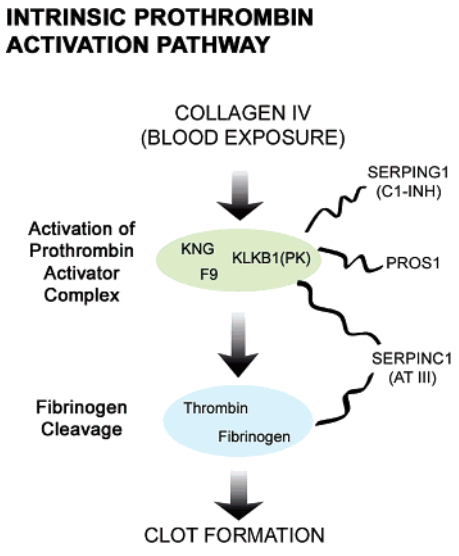

Figure 4. Involvement of the intrinsic prothrombin activation pathway in differentiating sepsis from SIRS patients. The proteins listed are those found in this study.

Protein Biomarker Candidates. Overall, seven proteins showed a common increase in the plasma from sepsis patients in all three batches, whereas three proteins showed a common decrease, making a total of 10 potential biomarker candidates (Table 4). The possibility of nonspecific protein binding to the immunodepletion column, which could cause losses of the lower-abundance proteins, was investigated by randomly analyzing the samples twice without immunodepletion. Even in the absence of immunodepletion, these 10 proteins were still identified as strong biomarker candidates. This lack of nonspecific protein binding to the immunodepletion column has also been shown by Echan and colleagues.[28]

Interrogation of the data using DAVID had shown the complement and coagulation pathways to be over-represented, suggesting that they could play an important role in distinguishing sepsis from SIRS. These findings were supported by some of the proteins identified here. Many of the proteins in Table 4 are known to be acute phase proteins (C-reactive protein, plasminogen, and serum amyloid P), involved in the complement pathway (Complement component C4), the coagulation pathway (antithrombin), or both (plasma protease C1 inhibitor), or lipid transport (apolipoproteins). Altered levels of several of these proteins have been reported to correlate with SIRS and sepsis,[8,33−36] as both the complement and coagulation pathways are known to be activated.[8−11]

Conclusions

Sepsis is a very complicated disease, common in the critically ill, that still has no truly effective early diagnosis strategy or treatment. It can strike rapidly, in a matter of days, and is associated with substantial morbidity and mortality. Plasma represents a proven resource in the quest for understanding

the complex interactions of the biochemical cascades that lead to disease and, further, in the identification of biomarkers for disease diagnosis.[32] Here we report the unique combination of immunodepletion, 3D LC separation and MS/MS analysis to offer some important insights into the interactions that surround the onset of sepsis and the potential identification of protein biomarkers in this event. This platform allowed for the removal of the highly abundant proteins and thus the detection of previously suppressed low-abundance proteins. Subsequent analysis using an in-house-developed high-resolution separation and tandem mass analysis enabled the detection of ~3000 lower abundance plasma proteins and the ultimate observation of 10 potential sepsis biomarkers, with the down-regulation of 7 proteins including those involved in lipid transport, as well as the up-regulation of 3 proteins observed in plasma from SIRS patients. The use of this platform has important implications in the field of plasma protein profiling and biomarker discovery and its applications could be far-reaching in disease diagnosis.

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