Intact-protein-based High-resolution Three-dimensional Quantitative Analysis System for Proteome Profiling of Biological Fluids*

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The substantial complexity and vast dynamic range of protein abundance in biological fluids, notably serum and plasma, present a formidable challenge for comprehensive protein analysis. Integration of multiple technologies is required to achieve high-resolution and high-sensitivity proteomics analysis of biological fluids. We have implemented an orthogonal three-dimensional intact-protein analysis system (IPAS), coupled with protein tagging and immunodepletion of abundant proteins, to quantitatively profile the human plasma proteome. Following immunodepletion, plasma proteins in each of paired samples are concentrated and labeled with a different Cy dye, before mixing. Proteins are subsequently separated in three dimensions according to their charge, hydrophobicity, and molecular mass. Differences in the abundance of resolved proteins are determined based on Cy dye ratios. We have applied this strategy to profile the plasma proteome for changes that occur with acute graft-versus-host disease (GVHD), following allogeneic bone marrow transplantation (BMT). Using capillary HPLC ESI Q-TOF MS, we identified 75 proteins in the micromolar to femtomolar range that exhibited quantitative differences between the pre- and post-GVHD samples. These proteins included serum amyloid A, apolipoproteins A-I/A-IV, and complement C3 that are well-known acute-phase reactants likely reflecting the post-BMT inflammatory state. In addition, we identified some potentially interesting immunologically relevant molecules including vitamin D-binding protein, fetuin, vitronectin, proline-rich protein 3 and 4, integrin-α, and leukocyte antigen CD97. IPAS provides a combination of comprehensive profiling and quantitative analysis, with a substantial dynamic range, for disease-related applications.

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Comprehensive profiling of the serum and plasma proteomes has substantial relevance to the identification of circulating protein biomarkers that may have predictive value and utility for disease diagnosis and management. As an orthogonal separation technique, two-dimensional (2-D)1 PAGE (1–3) is seminal to the field of proteomics, especially with respect to its parallel quantitative display of proteins. 2-D PAGE allows for the separation of complex protein mixtures, derived from cells, tissues, or biological fluids, on the basis of two different physicochemical properties: the isoelectric point (pI) of the proteins and their relative molecular mass (M₉). However, intrinsic limitations of 2-D PAGE do not allow for sufficient capacity for analysis of low-abundance proteins in such complex protein mixtures as the plasma proteome. Hence, many alternative strategies have been explored (4–7). For example, a shotgun technique designated multidimensional protein identification technology (MudPIT) coupled to an orthogonal 2-D HPLC system consisting of cation exchange and reverse-phase (RP) chromatography with ESI MS/MS for peptide analysis has been developed (8, 9). However, analysis of random peptides from complex digests may not be sensitive to protein post-translational modifications and other types of protein processing and may therefore miss many relevant protein isoforms associated with disease states.

The dynamic range of plasma protein abundance spans at least 12 orders of magnitude. Proteomic analysis of sufficient sample to detect low-abundance proteins invariably means excessive loading of albumin and several other high-abundance proteins that comprise greater than 90% of the total protein mass of plasma. Excessive loading of the high-abundance proteins may interfere with the analysis of lower-abundance proteins (10). We have combined immunodepletion of abundant plasma proteins and protein labeling with Cy dyes with an orthogonal, three-dimensional (3-D) intact-protein analysis system (IPAS) to quantitatively profile the human plasma proteome for disease-related changes, using paired samples. This quantitative analysis system separates mixtures of proteins labeled with Cy dyes in the first dimension

1 The abbreviations used are: 2-D, two-dimensional; RP, reverse-phase; IPAS, intact-protein analysis system; GVHD, graft-versus-host disease; BMT, bone marrow transplantation; ID, inner diameter; HGF, hepatocyte growth factor.
accompanying centrifugation in Centricon YM3 (3-kDa $M_c$ cutoff) concentrators according to manufacturer’s instructions (Millipore, Bedford, MA). Protein in the concentrated samples was quantitated and the volume adjusted to 1.5 ml in lysis buffer (30 mM Tris (pH 8.5), 8 M urea, 4% CHAPS), then placed on ice in a 15-ml centrifuge tube.

Cy5 and Cy3 dyes were used to label the samples obtained before and after GVHD, respectively. Briefly, the Cy dyes were each resuspended in $N,N$-dimethylformamide at a concentration of 1 mM, then allowed to react with the plasma proteins at a concentration of 4 nmol Cy dye/mg protein. A minimal protein labeling reaction on the ε-amino group of lysine residues was performed on ice in the dark for 30 min. The reaction was quenched by addition of 50-fold molar excess (relative to dye) of l-lysine for 10 min.

**Protein Separations**—The workflow of 3-D separations is shown in Fig. 1. Mixed paired plasma was first resolved by liquid-based IEF for 6 h at 10 °C. A total of 20 fractions were collected into separate vials using vacuum aspiration, then stored at −80 °C until use. Each of the 20 fractions was further resolved by RP HPLC in the second dimension. The separation column was a POROS R2/10 RP column: 4.6-mm ID × 100 mm, packed with 10-μm cross-linked polystyrene-divinylbenzene (Applied Biosystems, Foster City, CA). The trap-column was a MacroTrap column: 3.0-mm ID × 8 mm, packed with polymeric RP HPLC packing (Michrom BioResources, Inc.). Protein samples from the first-dimension separation were injected onto the trap-column and washed with water and buffer A, respectively. The desalted protein sample was passed through the separation column during a 60-min gradient elution run at a flow rate of 1.5 ml/min. The mobile phases used were A, 98% H$_2$O + 2% ACN + 0.1% TFA and B, 10% H$_2$O + 90% ACN + 0.1% TFA.

Eighty second-dimension fractions were collected from each first-dimension fraction, for a total of 1,600 fractions. Each second-dimension fraction was further resolved by SDS-PAGE. RP HPLC fractions were lyophilized and dissolved in 20 μl of sample buffer (120 mM Tris–HCl (pH 6.8), 10% SDS, 20% glycerol, 3% DTT, 0.03 μM bromophenol blue). After boiling for 5 min, the protein samples were loaded onto 18 × 16-cm (0.75-mm spacers) 7.5–15% gradient polyacrylamide gels. The gels were bonded to the inner (low fluorescence; Amersham) plate using bind-saline according to the manufacturer’s protocol. Proteins were electrophoresed at 12 mA until the dye front reached the bottom of the gel followed by fixation in 30% methanol, 7% acetic acid for 1 h. In all, 40 SDS-PAGE gels, two emanating from each first-dimension fraction, were obtained from the pooled patient samples, and 40 additional gels were obtained from each of the four individual patient samples.

**Protein Visualization and Image Analysis**—Gels were scanned...
directly using a 9200 Typhoon Scanner. Protein expression was quantitatively analyzed with the DeCyder Software Package (DeCyder version 3.5; Amersham). Thresholds for assigning differential expression between the two pools were set at a minimum 1.5-fold change.

Gel pieces from selected bands were excised for MS analysis using the Ettan Spot Picker (Amersham Pharmacia Biotech).

**MS Analysis—**

Gel pieces were washed successively in H2O, 25 mM NH4HCO3 (pH 8.0), and 100% ACN, then vacuum-dried and incubated in 10 l of trypsin solution (6.3 ng/lin 5mM NH4HCO3 pH 8.0) for 16 h at 37 °C. The resultant peptide mixtures were extracted with 25 l of 0.1 TFA/50% ACN. The peptide extracts were vacuum-dried, resuspended in 20 l of mobile phase A (98% H2O/2% CAN/0.1% FA) and then analyzed using nanoflow capillary HPLC ESI MS/MS in Q-TOF micro (Micromass, Manchester, United Kingdom). MS/MS spectra were acquired in the automated MS to MS/MS switching mode, with an m/z-dependent set of collision offset values. Doubly and triply charged ions were selected and fragmented, with argon as the collision gas. The acquired spectra were automatically processed and searched against the nonredundant Swiss-Prot protein sequence database (Version 42.3) using ProteinLynx Global Server 1.1 (Micromass), with the following parameters: precursor-ion mass accuracy, 0.1 Da; fragment-ion mass accuracy, 0.1 Da; no modification; one missed cleavage; trypsin was used as the proteolytic enzyme. Smoothing was applied using the Savitzky Golay mode, with the smooth window (channels) set at and a smooth number of 2; the minimum peak width at half height (channel) was set at 4, with a centroid top of 80%; charge states were calculated and peaks de-isotoped.

The column used in nanoflow capillary HPLC was packed with C18 (7-cm packing length 75-μm ID capillary tube). The flow rate was 350–500 nl/min with a 1-h gradient elution run for each sample.

**RESULTS**

**Quantitative Protein Differences Between Pre- and Post-GVHD Samples—** A total of 194 protein bands exhibited a 2.0-fold or greater reduced intensity after onset of GVHD. Some of the bands migrated to the same position in the third dimension from contiguous second-dimension fractions likely representing the same proteins that were collected into two or more fractions. However, 85 bands were considered to represent distinct protein forms based on their occurrence in non-neigh-
boring fractions in the first- or second-dimension separations or based on their occurrence as distinct \( M_r \) bands in the third-dimension separation. Protein was digested from corresponding gel pieces and analyzed by nanoflow HPLC ESI Q-TOF MS, yielding 48 distinct protein identifications with two or more peptides, based on protein sequence database analysis (Table I). Some of the protein bands that yielded identifications by MS yielded more than one protein identification, with a frequency of 5% for dual identifications from the same band. We have observed that when two proteins were identified in a digest from a band, frequently one of the two proteins also occurred in a neighboring band, with the two bands occurring in too close proximity to be cleanly cut out by the spot picker.

The abundance in human plasma of the identified proteins was evaluated (Table I) to assess the sensitivity and the dynamic range of IPAS. Some of the proteins that have been detected are relatively abundant proteins whose levels range from \( 10^{-3} \) to \( 10^{-6} \) M. These proteins include serum amyloid A and P, apolipoproteins A-I/IV/C-III, and complement C3/C4. Such proteins usually occurred in more than one fraction. Other proteins such as B-lymphocyte surface antigen, leukocyte antigen CD97, and T cell receptor \( \beta \) were generally found in far less abundance. These proteins usually occurred in fewer fractions than the relatively more-abundant proteins, and some occurred as a single band from a single fraction.

To assess the extent to which protein differences observed between pre- and post-GVHD pools are reflected in differences in paired, nonpooled pre- and post-GVHD samples from individual patients, plasma from four additional individual patients were examined (Fig. 2). We singled out nine protein bands that exhibited quantitative differences between pre-
and post-GVHD pools for which identifications were obtained by MS for their identification and quantitative analysis in non-pooled samples. We found concordant quantitative changes and identity between the pooled and the nonpooled samples (Fig. 2, Table II). MS/MS data from the pooled sample were corroborated by MS of corresponding protein bands in the individual samples yielding the same protein identity. Through this approach we were able to confirm several of the potentially immunologically relevant proteins detected in the pooled sample in one or more of the individual samples, including T cell receptor β, vitronectin, integrin α-L, and proline-rich protein 4.

**Detection of Protein Isoforms by IPAS**—In some cases, among the identified proteins that exhibited quantitative changes with GVHD, the same proteins occurred in different positions in the 3-D separations, indicative of differences in Mr and/or pl, suggestive of their occurrence as isoforms (Table I). Some of the differences between isoforms may represent chemical post-translational modifications. Others may represent cleavages, given the differences in observed molecular masses. Frequently one isoform exhibited up-regulation whereas another isoform exhibited down-regulation, as shown for apolipoprotein A-I (Fig. 3), a protein that is affected.

**Fig. 2.** Protein differences reflected in immunodepleted pre- and post-GVHD pooled samples as compared with identical fractions obtained from the nonimmunodepleted pre- and post-GVHD individual patient samples. Fraction 6A is shown for both samples. Identified proteins are as indicated.

**TABLE II**

Concordance of fold change among individual and pooled patient’s sera for some biologically relevant proteins

| Relevant upregulated potential biomarker proteins | Patient 1 ID | Patient 1 fold change | Patient 2 ID | Patient 2 fold change | Patient 3 ID | Patient 3 fold change | Patient 4 ID | Patient 4 fold change | Pooled patients ID | Pooled patients fold change | p value |
|--------------------------------------------------|--------------|-----------------------|--------------|-----------------------|--------------|-----------------------|--------------|-----------------------|-----------------|-----------------------------|---------|
| HLA Class 1                                       | MALDI-MS     | 1.5                   | MSMS         | 2.4                   | Visual       | 1.6                   | Visual       | 1.7                   | Visual          | 2.1                         | 0.002   |
| T Cell Receptor beta                              | MALDI-MS     | 1.6                   | MSMS         | 1.6                   | Visual       | 2.5                   | Visual       | 1.5                   | Visual          | 2.4                         | 0.005   |
| Integrin-alpha                                    | MALDI-MS     | 1.8                   | MSMS         | 1.7                   | Visual       | 4.3                   | MSMS         | 1.9                   | Visual          | 2.6                         | 0.010   |
| Annexin A2                                        | Visual       | ND                    | MSMS         | 2.0                   | Visual       | 2.3                   | Visual       | 2.1                   | Visual          | 5.9                         | 0.030   |
| Serum Spreading Factor (Vitronectin)              | Visual       | 1.9                   | Visual       | 1.6                   | Visual       | 2.1                   | MSMS         | 4.1                   | MSMS            | 5.3                         | 0.010   |

| Relevant downregulated potential biomarker proteins | Patient 1 ID | Patient 1 fold change | Patient 2 ID | Patient 2 fold change | Patient 3 ID | Patient 3 fold change | Patient 4 ID | Patient 4 fold change | Pooled patients ID | Pooled patients fold change | p value |
|-----------------------------------------------------|--------------|-----------------------|--------------|-----------------------|--------------|-----------------------|--------------|-----------------------|-----------------|-----------------------------|---------|
| B Lymphocyte Surface Antigen CD97                   | Visual       | -1.5                  | Visual       | -1.7                  | Visual       | -1.3                  | Visual       | -1.1                  | MSMS            | -5.4                         | 0.100   |
| Leukocyte Antigen CD97                             | Visual       | -1.4                  | Visual       | -2.0                  | ND           | Visual               | MSMS         | -1.6                  | MSMS            | -2.9                         | 0.030   |
| Proline rich Protein                               | Visual       | ND                    | MSMS         | -2.1                  | Visual       | -1.8                  | Visual       | -2.5                  | MSMS            | -2.8                         | 0.004   |
| Lysozyme C                                         | Visual       | ND                    | MSMS         | -2.0                  | Visual       | -1.8                  | Visual       | -2.6                  | MSMS            | -2.7                         | 0.004   |

The p value is from a two-sided T-test of no change, based on log-transformed data.
by GVHD (11) and also known to exhibit cleavage in part as a result of the systemic inflammation that occurs during acute GVHD (12, 13).

**Effect of Immunodepletion of Abundant Proteins**—To test the efficacy of the immunodepletion procedure, both the flow-through and the fractions bound to the immunodepletion column (Fig. 4) were collected, concentrated, and subsequently resolved by 2-D PAGE. Analysis of the flow-through fraction revealed almost complete immunodepletion of the targeted proteins. Comparison of lanes from nondepleted plasma, with corresponding lanes from immunodepleted plasma, indicated that several bands that exhibited 2.0 or greater-fold change following GVHD were obscured by abundant proteins in the nonimmunodepleted paired plasma (Fig. 2).

**Comparison of 2-D PAGE and IPAS**—An aliquot of the same sample of immunodepleted, labeled plasma was resolved by 2-D PAGE (Fig. 5), in addition to its analysis by IPAS. For optimal resolution, a total of 200 μg of immunodepleted plasma proteins was loaded onto each 2-D PAGE gel. The resolution of both the 2-D PAGE and the IPAS separations were compared by assessing the number of proteins that exhibited a 2.0-fold or greater quantitative change following GVHD. Only four such protein spots were detected by 2-D PAGE. The two neighboring spots located in the lower part of the 2-D gel in Fig. 5 were identified by MS/MS analysis as complement C3 whose plasma levels were known to be in-

**Flow-through fraction**

**Bound fraction**

**Fig. 3.** Protein bands identified as apolipoprotein A-I have differences in molecular mass and show discordant regulation with onset of GVHD. The full-length protein (fraction 6A, band 239 (left)) exhibits down-regulation with onset of GVHD, whereas the protein fragment (fraction 6A, band 240 (right)) exhibits up-regulation. Tryptic peptides identified by MS/MS are shown in *green* and *blue*, with each identified peptide shown in only one color (e.g. two colors are used to highlight contiguous peptides).
We have implemented a system to separate intact proteins in three dimensions and have examined the feasibility of this system to quantitatively profile the human plasma proteome before and after the onset of GVHD, leading to the identification of a large number of proteins that are affected by GVHD. We have compared the resolution achieved with this system relative to 2-D gels. Various schemes have been previously implemented for sample preparation prior to 2-D gel analysis to improve the yield of low-abundance proteins. We have compared the yield of protein differences related to GVHD with the 3-D system relative to DIGE involving tagging pairs of samples with different fluorescent dyes (Cy3 and Cy5) so that the sample preparation in this comparison is identical. Thus, using aliquots of the same samples for both DIGE and the 3-D system, we have demonstrated increased resolution of the 3-D system and increased sensitivity compared with 2-D gel analysis of the same tagged, paired samples.

It is clear that a greater number of proteins exhibited quantitative differences than the number of proteins that were identified. The extent of protein identification is related to the sensitivity of the methods utilized for protein identification. As such methods increase in sensitivity it is likely that additional proteins that occur at low abundance and that are detected using IPAS will be identified using MS. Of interest is the fact that several low-abundance proteins that exhibited quantitative changes by IPAS yielded identification by LC-MS/MS based on single peptides. Therefore their identification was not considered to be definitive. These include interleukin-22, tumor necrosis factor, and hepatocyte growth factor (HGF) (15–19), whose abundance is among some of the lowest for plasma proteins (<10^{-12} M). Tumor necrosis factor is known to be an important mediator of tissue damage in GVHD, and its role in alloreactive responses has been demonstrated (15–17). Interestingly, in an independent study based on immunoassays, an approximate 6-fold increase (from 555 ± 42 pg/ml to 3,098 ± 967 pg/ml) in the level of HGF with GVHD was reported (18). IL-22 is secreted by T cells and induces the production of acute-phase reactants in vitro and in vivo, suggesting its involvement in inflammation (19). Additional validation studies are needed to confirm the identity and quantitative changes in proteins identified based on single peptides.

We have identified in our study proteins previously known to be affected by GVHD as well as novel proteins not previously known to be affected by this disorder. Acute GVHD remains the major toxicity of allogeneic BMT. There is no definitive laboratory test that distinguishes GVHD from other toxicities and BMT complications such as veno-occlusive disease. Severe GVHD has an extremely poor prognosis (12). Thus the discovery of proteins in serum or plasma that are potential biomarkers for GVHD may lead to the development of a diagnostic test that is sensitive and specific for this disorder. The identification of 48 proteins that changed in their abundance with GVHD represents a first step toward such a test.

It should be noted that the identified protein bands that exhibited quantitative differences between the pre- and post-GVHD samples may represent particular isoforms of the proteins to which they are related or from which they are derived. Other isoforms may occur in plasma that were unaffected by GVHD. Thus, it is likely that some of the changes in plasma proteins associated with GVHD may be isoform specific as we have observed for some of the proteins including apoA1.

The ability to keep proteins intact during sample preparation and separation has important advantages compared with strategies that digest proteins at an earlier step. These advantages include the ability to quantify and recover proteins as well as to assess post-translational modifications and cleavage. Given the improved resolution achieved with the addition of a third dimension of separation of intact proteins, it would be reasonable to consider various alternatives in each of the three separation modes. For example, ion exchange-based separation may be substituted for ampholyte-based IEF in the first dimension, and size-based separation in the third dimension may be accomplished by capillary electrophoresis using polymers as a molecular sieve matrix that allows proteins to be resolved based on molecular mass (20–22). Capillary electrophoresis in the third-dimension separation could be multiplexed to increase throughput and would facilitate protein recovery by eliminating the need for gel cutting and protein extraction from gels (23–25). Automation of the separation system can also be envisaged.

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