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Transplantation proteomics

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Abstract

The field of proteomics is developing at a rapid pace in the post-genome era. Translational proteomics investigations aim to apply a combination of established methods and new technologies to learn about protein expression profiles predictive of clinical events, therapeutic response, and underlying mechanisms. However, in contrast to genetic studies and in parallel with gene expression studies, the dynamic nature of the proteome in conjunction with the challenges of accounting for post-translational modifications requires the translational proteomics investigator to understand the strengths and limitations of proteomics approaches. In this review, we provide an overview of proteomics approaches and techniques, and proteomics informatics for clinical transplantation investigators. We also review recent publications pertaining to transplantation proteomics, and discuss the implications and utility of urine proteomics for non-invasive investigation of transplant outcomes.

Keywords

transplantation; proteomics; surface-enhanced laser desorption ionization; matrix-assisted laser desorption ionization; mass spectrometry; biomarkers

Abbreviations

CAN, chronic allograft nephropathy; CID, collision-induced dissociation; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FT, Fourier transform; GBM, glomerular basement membrane; GVHD, graft vs. host disease; HPLC, high performance liquid chromatography; HSCT, hematopoietic stem cell transplantation; IMPDH, inosine monophosphate dehydrogenase; LC, liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; m/z , mass-to-charge ratio; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SELDI, surface-enhanced laser desorption ionization; SCT, stem cell transplantation; TOF, time of flight

The central dogma of biology is that DNA encodes RNA, which is translated to protein. The development of molecular biology technologies has paralleled this central dogma. Although the biochemistry of DNA, RNA and protein has been an active field of study for several decades, it is only in the past 10–15 yr that novel technologies have permitted high-throughput study of DNA, RNA and protein on a scale that is comparable with the size of the human genome, transcriptome and proteome, respectively. High-throughput proteomics is the most recent of these technologies to emerge, thereby rejuvenating interest in protein expression

among translational investigators who aim to leverage scientific discoveries for the betterment of patient care. The field of transplantation stands to benefit from the application of proteomics to discover biomarkers of outcomes and novel therapeutic targets.

In this review, we provide an overview of proteomics approaches and techniques, and proteomics informatics for clinical transplantation investigators. We also review recent publications pertaining to transplantation proteomics, and discuss the implications and utility of urine proteomics for non-invasive investigation of transplant outcomes.

Overview of proteomics

Proteomics analyses generally share several common steps (Fig. 1). Starting with a biological sample containing a complex mixture of a multitude of proteins, the main tasks are to (i) separate the proteins into smaller groups (fractions) of proteins [e.g. by polyacrylamide gel electrophoresis (PAGE), based on protein size], (ii) digest the proteins into peptide fragments, (e.g. with trypsin which cleaves proteins at specific and predictable sites), (iii) separate the peptide fragments (e.g. by liquid chromatography), (iv) ionize the peptide fragments [e.g. with electrospray ionization (ESI)], (v) measure the masses of the peptide fragments (e.g. with mass spectrometry, MS), and (vi) compare the peptide masses against extensive protein databases to determine the peptide sequences, and to piece together the likely protein(s) present in the original sample. The order of these steps may vary. For example trypsin digestion may precede or follow the ionization step, which in turn may precede or follow the fractionation step. As well, fractionation may first occur in one dimension (e.g. size), followed by ionization and MS, and then proceed to further separation of a given fraction in another dimension such as charge. The selection of specific methods for each step depends upon the nature of the protein sample, the goal of the studies, and the availability of the relevant technologies and expertise.

Proteomics technologies

The majority of reports in proteomics use MS-based technologies, although protein micro-arrays have also been utilized. Samples are ionized by an ion source and analyte ions of similar mass-to-charge (m/z) ratios are resolved by a mass analyzer. Ions then reach a detector that measures the intensity of ions at a given m/z . Spectra are coupled to databases for subsequent protein identification.

Biological samples must first be prepared by fractionation of protein mixtures and digestion of proteins to peptides. Because of the sheer number of proteins in any biological sample, this approach decreases the complexity of the mixture, making it more amenable to identification of individual proteins. Proteins are commonly fractionated using 2D gel electrophoresis. The first dimension separates by isoelectric point and is known as isoelectric focusing. For very simple mixtures, this 1D technique can be used alone. This is usually followed by sodium dodecyl sulfate (SDS) binding, which confers negative charge to the proteins in a fairly consistent manner such that, after application of a charge gradient in the second dimension, proteins are separated by mass as they travel through the PAGE. Gel spots of interest are then extracted and digested using a proteolytic enzyme such as trypsin. Peptide mixtures are further analyzed using one of the available MS technologies.

While this approach has been used for decades to separate protein mixtures, a number of limitations exist. Firstly, only proteins that are abundant enough to form visible spots on a gel can be further analyzed. Only a fraction of proteins known to be present in a mixture can be visualized using SDS-PAGE (1) and low abundance proteins can be missed by this method. Also, hydrophobic proteins are not well separated by charge-based methods and can aggregate in the gel without being well separated.

Liquid chromatography is an alternative method for fractionation. High-performance liquid chromatography (HPLC) can be used to overcome some of the limitations of SDS-PAGE. Samples can be separated based on a number of different properties, including anion exchange, cation exchange, hydrophobicity and others. Combinations of these properties can be leveraged to enhance separation, for example, by using first a cation exchange followed by separation based on hydrophobicity (reversed phase HPLC). The large number of fractions generated from the sample allows for less complexity in each fraction, increasing the likelihood of identification of peptides in the fraction. This modality can also handle smaller proteins and peptides, which makes digestion before separation an option. The advantage of this method sequence over the reverse can be seen when comparing HPLC with separation based on SDS-PAGE. While certain proteins may not be well separated by SDS-PAGE because of variations in charge, *a priori* digestion decreases this variation as smaller peptides have a narrower range of charge variability. Furthermore, high abundance proteins can make identification of lower abundance proteins difficult and technologies exist using specific affinity filter columns to remove these high abundance proteins, such as albumin, IgG, antitrypsin, IgA, transferrin and haptoglobin. However, filtering in this way risks losing proteins of interest as albumin avidly binds low molecular weight proteins and concentrates them over time by preventing renal excretion (2).

A mass spectrometer consists of three main components: an ionization source, a mass analyzer, and an ion detector (Fig. 1). The first two of these components encompass several technologies that can be combined in multiple conformations, depending on the resources, skills, and objectives of the operators.

Ionization sources

Peptides are ionized primarily using two techniques: matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI). Additionally, surface-enhanced laser desorption ionization (SELDI) has the capability of ionizing whole proteins and is similar in principle to MALDI (Fig. 1).

In MALDI, peptide mixtures are spotted onto a plate and an energy-absorbing molecule is applied. Once dried, the energy-absorbing molecule forms a matrix within which the peptides are embedded. A laser is then used to ionize the peptides, which then pass into the mass analyzer (Fig. 2).

In contrast, ESI ionizes samples directly from the liquid phase. Biological samples are fractionated by liquid chromatography and enter the ESI source at a continuous flow rate. The flow stream passes through a needle under high-voltage which ionizes the peptides into a mist of charged droplets. Ionized peptides then pass into the mass analyzer (Fig. 3).

SELDI is analogous to MALDI (Fig. 2), but rather than using fractionated and digested peptide mixtures SELDI is able to analyze whole proteins. Biological samples are spotted onto protein chips of varying substrates based on chemical properties (cationic, anionic, hydrophobic) or biochemical species (antibodies, enzymes, DNA, receptors). After application of the sample to the chip, the energy-absorbing matrix is applied and dried. A laser is used to ionize the proteins, which then pass through a time-of-flight (TOF) mass analyzer to a detector, thus generating m/z spectra. This technique is less labor intensive and less expensive than resolving peptides from biological samples using MALDI or ESI. However, the major limitation to SELDI is protein identification. That is, the spectra are whole-protein m/z ratios and therefore are not further subjected to sequence analysis. Given the complexities of the intact proteins, protein mass fingerprint identification is fraught with ambiguities and is not currently possible. However, SELDI has been used to generate informative predictive profiles of disease states

such as in the prognosis of certain cancers, as well as conditions related to transplantation, as we review below.

Mass analyzers

There are number of mass analyzers used in proteomic research. Commonly used mass analyzers include TOF, ion trap and quadrupole. Quadrupole and TOF can be used in combination. In addition, the Fourier transform (FT) ion cyclotron resonance technology is also gaining popularity because of the ultra-high resolution provided by FT. MALDI is generally coupled with a TOF analyzer while ESI is usually coupled with an ion trap, triple quadrupole, or a quadrupole TOF. However, there are a variety of alternative permutations in use in proteomic research.

An important concept to briefly mention is resolution. In this context, resolution refers to the ability of the mass analyzer to differentiate peptide species of slightly different m/z ratios. Visually, resolution differences can be seen in spectra of a conglomeration of many peptides as discrete peptide peaks vs. sloping humps. The mass analyzers discussed below have a range of resolutions. Combinations of these analyzers make use of the higher resolution of some analyzers with desirable features of others.

The TOF mass analyzer separates ions based on their flight time down a vacuum tube. As described above, MALDI samples consist of peptide mixtures in a dry, crystalline matrix that is pulsed with a laser to ionize the peptides. Ionized peptides travel through a TOF mass analyzer and reach the detector. Ions are separated by their size and speed with smaller ions traveling faster down the vacuum tube and reaching the detector first. Resolution in TOF analyzers is directly related to the time difference between ions passing through the analyzer and reaching the detector. As this technology was introduced in 1987, a number of modifications have improved the resolution of the device. The reflector was added to the TOF analyzer to compensate for differences in kinetic energy of ions. This allows ions of similar m/z ratios to reach the detector at the same time, improving the resolution. Another modification has been the use of tandem TOF analyzers, known as TOF–TOF. In this form, a collision cell is placed between the two TOF analyzers. This change allows for fragmentation of peptide ions ('precursor ions') into smaller peptides ('product ions') and generation of high-resolution spectra amenable to peptide sequencing algorithms.

The ion trap consists of two electrodes above and below a ring electrode (Fig. 4). Precursor ions enter the trap through the upper electrode. By adjusting the voltage of the electrodes, ions of a specific m/z ratio can be retained in the trap. Then the voltage is again adjusted to allow collision of the precursor ions with inert gas in the trap, after which the ions are released to the detector. This produces robust fragmentation of peptide ions to obtain peptide sequence data, although it has somewhat lower resolution than TOF.

Triple quadrupole mass analyzers were the original mass analyzers used for MS/MS in proteomics. Each quadrupole consists of four metal rods maintained under a current that generates an electric field. The frequency of the field can be adjusted to allow ions of a specific m/z ratio through at any given time. Three quadrupoles are placed in series with the middle one serving as a collision cell. Thus, ions pass through the first quadrupole (Q_1) and ions of a given m/z ratio are selected by adjusting the field. In the second quadrupole (q_2), ions collide with an inert gas and fragment ions are further separated in the third quadrupole (Q_3). Upon arriving at the detector, peptide sequence data are then obtained.

Combinations of these analyzers also exist, such as the quadrupole TOF analyzer that combines the Q_1 and q_2 of the triple quadrupole in series with a TOF analyzer in place of the Q_3 . The

collision cell within the analyzer allows for fragmentation of peptide ions and, thus, peptide sequence analysis.

The FT ion cyclotron is similar to the ion trap. In this technology, the FT uses a very powerful magnetic field and uses a FT algorithm to scan all ions in the trap at once. The ions resonate at very specific frequencies, which are in turn resolved by the FT. The resolution on these devices is very high, but cost and the additional technical expertise required to operate FT have limited their widespread use in proteomics.

Protein identification

One of the major goals of proteomics is the identification of proteins of interest. As described above, most MS or MS/MS studies give information about peptides that must be interpreted back to the protein level. Protein databases that contain peptide sequence information are essential to complete this task. The MS technologies outlined above give two general forms of data that can be leveraged to identify proteins. The first is known as peptide mass fingerprinting and the second is via direct peptide sequencing of some of the peptides for a given protein (i.e. partial protein sequencing).

Peptide mass fingerprinting is generally performed in concert with MALDI. Peptide m/z ratios are obtained with MALDI and these are usually singly protonated, such that a peptide with an m/z of 1135 Da can be assumed to have a mass of 1134 Da plus the mass of the single proton. Rather than giving true sequence data, the mass of the peptide species is compared with a mass in a peptide sequence database. This may seem somewhat unsatisfying on first glance. However, there are a number of features that enhance the data provided by this method. Firstly, MALDI-TOF MS generates high-resolution spectra to a number of decimal places. While a peptide of mass 1134 may have many matches in a database, the number of matches for 1134.6 becomes fewer, as does that for 1134.65 and so on. The resolution of MALDI-TOF MS allows for this degree of specificity. Secondly, there are many peptides generated by a MALDI experiment and using multiple peptide masses to search the database increases the probability of identifying one protein of interest. For example, while the sample peptide mass of 1134 Da may have 30 matches, including another peptide of 1296 Da may decrease the number of matches to 10. Thirdly, the proteolytic enzyme used for digestion provides additional information. Trypsin is most commonly used and this cleaves peptides in a very specific manner, specifically after lysine and arginine residues unless followed by a proline residue. Thus, peptide mass databases can generate *in silico* protein digestion information on proteins of known sequence. They are limited, however, by the information used in the database. An organism for which the entire protein sequence is not known or cataloged will limit the strength of the database.

True peptide sequence information is provided by MS/MS experiments. In studies that utilize ESI, fragmentation of peptide ions occurs within the mass analyzer within a collision cell. In addition, MALDI-TOF/TOF MS also utilizes a collision cell to generate peptide ions, as noted above. This phenomenon is known as collision-induced dissociation (CID) and generates ion fragment spectra that contain a range of ions based on the parent ion. For example, a peptide produced by trypsin digestion may contain 10 amino acid residues and will end with a lysine or arginine. Further fragmentation of the peptide ion leads to a group of product ions, which can then be compared with respect to their m/z values. The difference in m/z values between a series of product ions is compared with a table of the weights of amino acids, and peptide sequence data can be extrapolated. In reality, peptide mixtures are far more complex than this model suggests, and extrapolating sequence data in this manner would be unwieldy. Fractionation prior to MS/MS decreases the complexity of the sample.

Protein microarrays

Protein microarrays are not based on MS technology and have recently been reviewed (3). They are analogous in principle to DNA microarrays. Individual spots on slides are embedded with specific substrates. These substrates may be utilized in the forward or reverse phases. In a forward phase array, a substrate consisting of a known bait molecule, such as an antibody, is embedded onto a spot on the array. Biological samples are placed onto the array and binding occurs to spots of relevance. A second molecule is applied for detection of bound proteins or peptides. A reverse phase array consists of embedded proteins from a biological sample onto all spots on the array. Different antibodies are then applied to spots and binding is detected. For example, a tissue sample is embedded onto a microarray and antibodies are placed against known proteins in differing states of phosphorylation. This allows for insights into activated signal pathways within affected cell lines. The reverse phase protein microarray is now being used as part of clinical trials at the National Cancer Institute.

Applications of proteomics to transplantation

Human studies

Proteomics as a diagnostic tool has been used most extensively in the field of cancer research driven by the search for non-invasive biomarkers that would allow for early detection of cancers before patients become symptomatic or widespread metastasis makes outcomes unfavorable. The most well-known application has been in ovarian cancer. Petricoin et al. (4) studied SELDI-TOF MS as a screening tool in women with ovarian cancer. They were able to correctly discriminate patients with early stage ovarian cancer from non-malignant gynecologic disease and normal controls with a sensitivity of 100% and a specificity of 95%. The use of high resolution SELDI-TOF MS by Petricoin et al. continues to generate debates regarding issues of study design, technological consistency, and meaningful lower limits of protein mass (5–10). Other groups have used proteomics to identify diagnostic and prognostic proteomic patterns in patients with breast, cervical, and renal cancers (11–13). These technologies have been applied to clinical studies in transplantation, as well (Table 1).

Renal allograft rejection—Early diagnosis of acute rejection has obvious implications for graft survival. Urine proteomic profiling of acute renal allograft rejection has been investigated in three studies to date. Clarke et al. (14) performed SELDI-TOF MS on urine samples from 32 patients with renal allografts, of whom 17 had biopsy-confirmed acute rejection and the remainder had stable graft function. They demonstrated a group of protein peaks that differentiated acute rejection from stable graft function. Using the Ciphergen (Fremont, CA, USA) Biomarker Pattern Software package, two protein peaks (3.4 and 10.0 kDa) served as biomarkers that could separate the two groups with a sensitivity of 83% and specificity of 100%, although this group did not identify the proteins in question, nor did they validate their results with an independent dataset.

Schaub et al. (15) performed a more comprehensive comparison of urine proteomic profiles to renal allograft biopsy samples in patients with clinically defined stable graft function (n = 22), acute rejection (n = 18), acute tubular necrosis (n = 5), and recurrent glomerulopathies (n = 5). They also compared urine proteomic profiles of 28 healthy controls and five women with acute bacterial cystitis. Using SELDI-TOF MS, they identified a ‘rejection pattern’ that was evident on visual inspection of software-generated gel views of the spectra. The rejection pattern included three groups of peaks with m/z of 5270–5550, 7050–7360, and 10530–11100 Da. This pattern was seen in 17/18 patients with acute rejection and in 4/22 patients with stable graft function. None of the urine profiles from the patients with acute tubular necrosis, recurrent glomerulopathies, cystitis, or healthy controls had this pattern. Although this study did not have an independent validation group, they followed a subset of patients within the stable graft

function and acute rejection groups, and the majority of these patients had urine proteomic profiles that matched their clinical course. Another strength of this study is their use of allograft biopsies to categorize all of their transplant patients, including those with stable graft function, rather than inferring stability from normal serum creatinine levels. In addition, this group characterized the protein peaks found in their rejection patients, which were identified as cleavage products of β -2 microglobulin (16). In their follow-up paper, they sequenced the proteins in their rejection-pattern peaks and achieved coverage of almost the entire sequence of β -2 microglobulin. They also described the breakdown of β -2 microglobulin into its cleavage products and associated SELDI-TOF MS peaks.

O’Riordan et al. (17) performed SELDI-TOF MS on urine samples from a group of 23 renal allograft recipients with biopsy-proven acute rejection, 22 patients with stable graft function and 20 healthy controls. Using two separate informatics techniques, they selected a group of protein peaks that differentiated acute rejection from stable graft function with a sensitivity of approximately 90% and a specificity of approximately 80%. They also observed another protein peak that differentiated stable graft function from healthy controls with a sensitivity and specificity of 100%.

Cardiac allograft rejection—Borozdenkova et al. (18) studied potential markers of cardiac allograft rejection in a cohort of adults. Seventeen patients underwent cardiac transplantation and had protocol endomyocardial biopsies performed during the first 6 months post-transplant. A subset of four patients had a total of 33 biopsy samples incubated with radio-labeled methionine and 2D gel electrophoresis was performed on these samples. Newly synthesized proteins were assumed to have incorporated radiolabeled methionine and would identify proteins of interest relevant to the clinical state at the time of the biopsy. Thirteen proteins were identified as differentially expressed in acute rejection. MALDI-TOF MS was followed by peptide mass fingerprinting with identification of 11/13 differentially expressed proteins, including eight cardiac specific proteins and two stress proteins (heat shock protein 27 and $\alpha\beta$ crystallin). Three of these peptides were then measured in serum samples of the entire group drawn pretransplant and at the time of biopsies. One of these proteins, tropomyosin-1, was found to be significantly elevated in the serum of patients with acute rejection, while $\alpha\beta$ crystallin showed an increasing trend but did not reach significance.

Graft vs. host disease—Hematopoietic stem cell transplantation (HSCT) is curative of many cancers and non-oncologic diseases. However, graft vs. host disease (GVHD) remains one of the most important problems following HSCT. Kaiser et al. (19) collected urine on 35 patients following allogeneic stem cell transplantation (SCT), five patients after autologous SCT, and five ICU patients with sepsis. Urine proteomic profiling was performed and compared with a cohort of healthy individuals. They used a capillary electrophoresis technique for protein separation, which is similar to isoelectric focusing but with greater resolution. This was then coupled to ESI-TOF MS to generate differentiating spectra. This study found 21 polypeptides that distinguished between healthy and SCT patients with a sensitivity of 100% and specificity of 95%. Furthermore, they found a group of 16 polypeptides expressed in SCT patients with and without GVHD that differentiated these two groups with a sensitivity of 100% and specificity of 82%. The polypeptides expressed in GVHD were present 5–15 days prior to the clinical diagnosis of the disease. They also compared patterns of GVHD with sepsis, and although there was some overlap, they identified a pattern that discriminated between the two states with a sensitivity of 100% and specificity of 97%. Using MALDI-TOF/TOF MS, they identified two of the polypeptides whose expression was increased in GVHD as Leukotriene A4 Hydrolase and Albumin. Although this study did not have an independent validation group, cross-validation was performed. This study also demonstrates the utility of urine proteomics in non-renal disease states.

The studies described above reflect both the advantages and pitfalls of proteomic profiling. These studies had essentially the same goal of identifying non-invasive preclinical biomarkers. The informatics approaches used to derive their protein peaks of interest were different in all studies. Also, using the same technique, the three studies on renal allograft rejection identified different groups of protein peaks. None of these studies had independent subject groups to perform validation of their findings, although the GVHD study did perform cross validation. Thus, generalization to clinical application has not yet been demonstrated but the potential is intriguing.

Animal studies

Chronic allograft nephropathy—Joosten et al. (20) used proteomics to identify antigens of interest in a rat model of chronic allograft nephropathy (CAN). As the predominant glomerular finding in humans and rats with CAN is thickening and double contours of the glomerular basement membrane (GBM), they focused their attention on proteins of the GBM. Using the Fisher to Lewis rat model of transplantation and chronic rejection, they found IgG1 antibodies against GBM antigens seen only in those rats with CAN. Collagenase-digested GBM samples were subjected to proteomic studies to identify the antigens recognized by these anti-GBM antibodies. After 2D gel electrophoresis, these antibodies were incubated within the gel to identify which spots contained associated antigens. The two spots identified were removed from the gel, underwent trypsin digestion, and were then subjected to a nano-spray technique with a quadrupole TOF analyzer. Using peptide mass fingerprinting, they found that one spot consisted of peptides identified from the heparan sulfate proteoglycan perlecan, while the second spot contained peptides from the $\alpha 1$ chain of type VI collagen and the $\alpha 5$ chain of type IV collagen. Both of these proteins are localized to the endothelial side of the GBM, making them logical targets of these antibodies.

Cyclosporine nephrotoxicity—Calcineurin inhibitors revolutionized solid organ transplantation by decreasing rates of acute rejection at the price of long-term nephrotoxicity. Steiner et al. (21) studied the effect of cyclosporine on kidney homogenate protein expression in Wistar rats. Wistar rats were treated with either cyclosporine or placebo, sacrificed and their kidneys homogenized and subjected to 2D gel electrophoresis. After iso-electric focusing and SDS-PAGE, one spot was further evaluated that was found to be the most differentially expressed in the two groups. This spot was further fractionated and digested with trypsin. Peptide sequencing identified the down-regulated protein as calbindin-D, a 28 kDa Vitamin D-dependent calcium-binding protein. Using an ELISA against calbindin-D, they found that decreased levels of the protein correlated with the length of treatment with cyclosporine. Normally, calbindin-D transports calcium found in the distal tubule of the kidney from the tubular lumen to the bloodstream. Cyclosporine is known to interfere with the effects of calcineurin and other calcium-binding proteins, and microcalcifications are seen as a histological manifestation of cyclosporine nephrotoxicity. In a follow-up study, Aicher et al. (22) compared calbindin-D levels in the kidneys of rats, dogs, monkeys and humans treated with cyclosporine, as dogs and monkeys do not manifest nephrotoxicity from cyclosporine. They found that distal tubular staining for calbindin-D was decreased in humans and rats treated with cyclosporine compared with untreated controls. Additionally, dogs and monkeys did not manifest any difference in calbindin-D levels between those treated with cyclosporine and controls.

Tolerance in liver transplantation—Pan et al. (23) applied proteomics to study immune tolerance in a rat model of orthotopic liver transplantation (OLT). Serum was collected from rats that had undergone OLT and was compared with serum from naïve rats. A 2D electrophoresis was performed using isoelectric focusing and SDS-PAGE. Fifteen significantly differentially expressed spots were found on the gel and subjected to trypsin digestion followed

by MALDI–TOF MS. These 15 proteins were identified and included a number of acute phase reactants such as haptoglobin. Haptoglobin was further studied given its known inhibitory effect on T-cell proliferation. Using immunohistochemistry, the intracellular localization of haptoglobin within the hepatocyte was related to clinical manifestations of either rejection or tolerance. The inhibitory effects of haptoglobin were also demonstrated by its inhibition on a mixed lymphocyte reaction.

***In vitro* drug studies**

Immunosuppressive drugs are an essential part of post-transplant care. Newer agents have come on the scene over the past decades, such as mycophenolate mofetil (MMF) and sirolimus, while older drugs like cyclosporine and tacrolimus have found uses outside the transplant arena. Each of these medications has a described site of action (e.g. calcineurin inhibition) although there are many details regarding the mechanism of action of downstream effects that remain unknown. Proteomics has been used to better understand these functions.

Cyclosporine—The immunosuppressive effects of cyclosporine are due to its inhibition of calcineurin, which becomes unable to dephosphorylate and activate nuclear factor of activated T lymphocytes (NFAT). This prevents the transcription of IL-2 and related cytokines. The effect of cyclosporine on T lymphocyte protein synthesis was reported by Mascarell et al. (24). Isolated mouse T lymphocytes were stimulated with Concanavalin A in the presence or absence of cyclosporine. Radiolabeled methionine was added to culture to evaluate newly synthesized proteins. After cell lysis, 2D gel electrophoresis with SDS-PAGE was performed and spots were compared between the two conditions. Surprisingly, there were more protein spots in the cyclosporine-treated cells than in the untreated group. There were patterns unique to each group with some protein spots present in the cyclosporine-treated group while other spots were present in the untreated group. Certain spots developed in the untreated group over time correlating with known patterns of cytokine expression observed after T-cell stimulation. While this study did not identify any of the proteins in question, it demonstrated that cyclosporine not only inhibits cytokine expression, but also stimulates the synthesis of a unique set of proteins.

Mycophenolate mofetil—MMF was introduced in the early 1990s and has since become an integral part of transplant immunosuppression. The active metabolite mycophenolic acid (MPA) inhibits inosine mono-phosphate dehydrogenase (IMPDH) preventing the synthesis of guanosine monophosphate and DNA. Escobar-Henriques et al. (25) used a yeast model to evaluate protein expression in MPA-treated cells after treatment with radio-labeled methionine. MPA-treated yeast showed decreased growth that could be reversed by the addition of guanine. 2D gel electrophoresis and SDS-PAGE were performed and gels were compared with previously described yeast protein maps with identified spots (26). Three proteins were up-regulated and 27 proteins were down-regulated in MPA-treated yeast. The up-regulated proteins were identified as two stress-related proteins and the yeast analog of IMPDH. Four of the down-regulated proteins were further studied by over-expressing their genes to overcome the effect of MPA. Two proteins, *cdc37p* and *sup45p* were found to have increased resistance to MPA when over-expressed. These proteins are involved in cell cycle progression and translation termination, respectively. The effects were felt to be independent of guanosine depletion and the authors hypothesized a role in the toxicity seen with MPA. Interestingly, they also measured RNA levels of the *CDC37* transcript and found that they were unchanged with MPA treatment. This emphasizes a well-known, important concept in proteomics, namely that measuring RNA levels does not necessarily correlate with protein expression.

Rapamycin (sirolimus)—Rapamycin is currently the newest clinically available immunosuppressive agent in the transplant armamentarium. It binds to the mammalian target

of rapamycin (mTOR) and inhibits cell proliferation. Two studies have used proteomics to further evaluate the molecular effects of rapamycin.

Grolleau et al. (27) studied the effect of rapamycin on Jurkat T cells using DNA microarrays and proteomics. After assessing the changes in gene expression seen with rapamycin, 2D gel electrophoresis was performed with SDS-PAGE. The gel results were compared with protein maps previously described by this same group (28). They found changes in 111 spots of which 70 were up-regulated and 41 were down-regulated. Twenty-two spots were matched to their protein map for identification, which correlated with 16 genes of which 11 were found in the DNA microarray experiments. These proteins exhibited a range of functional categories and those that were down-regulated included proteins involved in signaling, growth, RNA metabolism, cellular structure and metabolism, while up-regulated proteins included one nuclear protein and one membrane protein. Again, RNA levels in microarray data correlated with only a fraction of proteins expressed, although this may be limited by the fact that the authors did not directly identify the proteins in this study but rather used a previously studied protein map.

Huang et al. (29) studied the effects of rapamycin in a yeast model. They used a previously described reverse phase protein microarray of the entire yeast proteome (30) to assess for changes related to treatment with rapamycin in a group of organic molecules they called small-molecule inhibitors of rapamycin (SMIRs). Six SMIRs were identified that inhibited the anti-proliferative effect of rapamycin. To evaluate the mechanism of actions of SMIRs, they began by using DNA microarrays to study the effect of gene expression in the presence of rapamycin with and without a subset of SMIRs. SMIR4 was found to completely reverse the changes in gene expression induced by rapamycin, while SMIR3 changed the expression of a subset of genes. They then used biotin-conjugated SMIR3 and SMIR4 to probe the yeast protein microarray with detection of binding by fluorescent-labeled streptavidin. SMIR3 bound to eight proteins and SMIR4 bound to 30. Only one protein spot bound strongly to SMIR3, a protein analogous to a mammalian tumor suppressor. SMIR4 bound strongly to five proteins out of a total of 30 spots. They further studied these proteins by evaluating the effect of rapamycin on yeast with deletions of the proteins, as their absence should confer sensitivity to rapamycin even in the presence of SMIRs. Only one protein was identified that fit this criterion, a protein of unknown function named ybr077p. The deletion of this protein led to hypersensitivity to rapamycin and showed no change after the addition of SMIR4, as well as SMIR3. The authors further expand on this effect evaluating its role in concert with other knockouts and suggest a role for ybr077p in the TOR pathway.

The urine proteome

Large-scale genomics and proteomics investigations that aim to distinguish disease from normal require clear and distinctly defined control groups. In the proteomics domain, the definition of the 'normal proteome' is elusive because of the dynamic nature of protein expression even in healthy individuals. However, the normal range is known for many serum proteins such as albumin, immunoglobulins, protein hormones, and binding proteins. The definition of the normal urine proteome is a bigger challenge. Urine proteins reflect the processes that maintain plasma protein homeostasis within very narrow ranges. Appropriate renal responses to changes in the circulation may require excretion and/or reabsorption of large amounts of a given set of proteins. Therefore, the urine proteome may be more appropriately defined as 'appropriate' vs. 'inappropriate' for a given set of normal states or variables such as volume status, blood pressure, body mass index, gender, physical activity, posture (upright vs. recumbent), and even the time of day. Complicating the construction of a benchmark dataset of control urine or serum samples is the inability to define the healthy state with certainty. An

individual who is clinically healthy at the time of sample collection may have early disease processes that are only observable at some molecular level.

Several groups have attempted to delineate the normal human urine proteome by characterizing the proteins present in urine from healthy individuals. In two related publications, Spahr et al. (31) and Davis et al. (32) studied an undisclosed number of samples of 'normal male urine from a commercial pooled source'. They optimized their methods in an attempt to overcome the limitations of complex mixture analyses and listed 124 urinary proteins present in the pooled samples. Thongboonkerd et al. (33) utilized 2D-PAGE and MALDI-TOF MS to identify 47 distinct proteins in urine samples obtained from an undisclosed number of healthy volunteers. While there is some overlap between the findings of the two groups, it is not clear to what extent the variables and health states listed above contribute to these findings. In an attempt to establish a normal 2D human urine proteomic map, Oh et al. (34) utilized 2D electrophoresis and MALDI-TOF MS to identify 113 proteins in pooled urine collected from 20 young, healthy male subjects and 20 young, healthy female subjects. The authors noted the difficulty in comparing their 2D maps, citing technical differences, gel preparation methods, and environmental and genetic factors as potential sources of intermap noise. Pieper et al. (35) utilized 2D electrophoresis, MALDI-TOF MS and LC-ESI MS/MS to characterize the proteins in multiple samples from a healthy male and a healthy female volunteer. They were able to identify 150 proteins within roughly 420 spots. They also utilized their method to compare the urine proteome of a single patient with renal cell carcinoma prior to and following nephrectomy, and found several differentially expressed proteins, but again no mention is made of correcting for important baseline physiologic, anthropomorphic and environmental variables that can affect urinary protein excretion.

Proteomics informatics

The evolution of high-throughput gene expression studies has spawned developments and innovations in bioinformatics approaches for large numbers of genes. Much of what bioinformaticists have learned from genomics about normalization, noise modeling, reproducibility, clustering, classification models, and differential expression can be extrapolated to proteomics data. The field of informatics continues to broaden. Informatics as applied to proteomics includes algorithms for *de novo* peptide sequencing from MS/MS spectra, inference of secondary and tertiary protein structure from MS/MS data, matching peptide and protein data to databases, and predictive modeling in correlation with clinical outcomes. Currently, the predominant additional challenge of proteomics is large-scale protein identification, for which the power of bioinformatics approaches is largely limited by the availability of extensive protein databases with sufficient detail to permit unambiguous identification of proteins. High-throughput identification of novel, previously unknown proteins or rare post-translational modifications is not possible with current databases and requires more innovative approaches. Proteomics informatics approaches will continue to be the focus of active development and innovation.

Summary

One can imagine the application of proteomic profiling to any disease state to characterize biomarkers of disease before clinical manifestations and end-organ damage becomes apparent. Mechanistic studies of disease could be enhanced by studying protein modifications and activation states, and by offering insight into signaling pathways. As alluded to above, both beneficial and toxic effects of drugs could be studied using proteomic profiling, both for evaluating changes in known proteins as well as investigating unforeseen changes in the proteome. Large, robust benchmark datasets of normal urine proteomic profiles corrected for important health states and variables will empower the discovery to disease-associated profiles.

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References

1. Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc Natl Acad Sci USA* 2000;97:9390–9395. [PubMed: 10920198]
2. Liotta LA, Ferrari M, Petricoin E. Clinical proteomics: written in blood. *Nature* 2003;425:905. [PubMed: 14586448]
3. Espina V, Mehta AI, Winters ME, et al. Protein microarrays: molecular profiling technologies for clinical specimens. *Proteomics* 2003;3:2091–2100. [PubMed: 14595807]
4. Petricoin EF, Ardekani AM, Hitt BA, et al. Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 2002;359:572–577. [PubMed: 11867112]
5. Conrads TP, Fusaro VA, Ross S, et al. High-resolution serum proteomic features for ovarian cancer detection. *Endocr Relat Cancer* 2004;11:163–178. [PubMed: 15163296]
6. Baggerly KA, Edmonson SR, Morris JS, Coombes KR. High-resolution serum proteomic patterns for ovarian cancer detection. *Author Reply* 2004;11:585–587.
7. Liotta LA, Petricoin EF, Veenstra TD, Conrads TP. High-resolution serum proteomic patterns for ovarian cancer detection. *Endocr Relat Cancer* 2004;11:585–587.
8. Baggerly KA, Morris JS, Edmonson SR, Coombes KR. Signal in noise: evaluating reported reproducibility of serum proteomic tests for ovarian cancer. *J Natl Cancer Inst* 2005;97:307–309. [PubMed: 15713966]
9. Liotta LA, Lowenthal M, Mehta A, et al. Importance of communication between producers and consumers of publicly available experimental data. *J Natl Cancer Inst* 2005;97:310–314. [PubMed: 15713967]
10. Ransohoff DF. Lessons from controversy: ovarian cancer screening and serum proteomics. *J Natl Cancer Inst* 2005;97:315–319. [PubMed: 15713968]
11. Becker S, Cazares LH, Watson P, et al. Surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) differentiation of serum protein profiles of BRCA-1 and sporadic breast cancer. *Ann Surg Oncol* 2004;11:907–914. [PubMed: 15383419]
12. Wong YF, Cheung TH, Lo KW, et al. Protein profiling of cervical cancer by protein-biochips: proteomic scoring to discriminate cervical cancer from normal cervix. *Cancer Lett* 2004;211:227–234. [PubMed: 15219946]
13. Won Y, Song HJ, Kang TW, Kim JJ, Han BD, Lee SW. Pattern analysis of serum proteome distinguishes renal cell carcinoma from other urologic diseases and healthy persons. *Proteomics* 2003;3:2310–2316. [PubMed: 14673781]
14. Clarke W, Silverman BC, Zhang Z, Chan DW, Klein AS, Molmenti EP. Characterization of renal allograft rejection by urinary proteomic analysis. *Ann Surg* 2003;237:660–664. [PubMed: 12724632] discussion 664–665.
15. Schaub S, Rush D, Wilkins J, et al. Proteomic-based detection of urine proteins associated with acute renal allograft rejection. *J Am Soc Nephrol* 2004;15:219–227. [PubMed: 14694176]
16. Schaub S, Wilkins JA, Antonovici M, et al. Proteomic-based identification of cleaved urinary beta2-microglobulin as a potential marker for acute tubular injury in renal allografts. *Am J Transplant* 2005;5:729–738. [PubMed: 15760396]
17. O’Riordan E, Orlova TN, Mei JJ, et al. Bioinformatic analysis of the urine proteome of acute allograft rejection. *J Am Soc Nephrol* 2004;15:3240–3248. [PubMed: 15579528]
18. Borozdenkova S, Westbrook JA, Patel V, et al. Use of proteomics to discover novel markers of cardiac allograft rejection. *J Proteome Res* 2004;3:282–288. [PubMed: 15113105]
19. Kaiser T, Kamal H, Rank A, et al. Proteomics applied to the clinical follow-up of patients after allogeneic hematopoietic stem cell transplantation. *Blood* 2004;104:340–349. [PubMed: 15054046]

20. Joosten SA, Van Dixhoorn MG, Borrias MC, et al. Antibody response against perlecan and collagen types IV and VI in chronic renal allograft rejection in the rat. *Am J Pathol* 2002;160:1301–1310. [PubMed: 11943715]
21. Steiner S, Aicher L, Raymackers J, et al. Cyclosporine A decreases the protein level of the calcium-binding protein calbindin-D 28 kDa in rat kidney. *Biochem Pharmacol* 1996;51:253–258. [PubMed: 8573191]
22. Aicher L, Wahl D, Arce A, Grenet O, Steiner S. New insights into cyclosporine A nephrotoxicity by proteome analysis. *Electrophoresis* 1998;19:1998–2003. [PubMed: 9740060]
23. Pan TL, Wang PW, Huang CC, Goto S, Chen CL. Expression, by functional proteomics, of spontaneous tolerance in rat orthotopic liver transplantation. *Immunology* 2004;113:57–64. [PubMed: 15312136]
24. Mascarell L, Frey JR, Michel F, Lefkovits I, Truffa-Bachi P. Increased protein synthesis after T cell activation in presence of cyclosporin A. *Transplantation* 2000;70:340–348. [PubMed: 10933161]
25. Escobar-Henriques M, Balguerie A, Monribot C, Boucherie H, Daignan-Fornier B. Proteome analysis and morphological studies reveal multiple effects of the immunosuppressive drug mycophenolic acid specifically resulting from guanylic nucleotide depletion. *J Biol Chem* 2001;276:46237–46242. [PubMed: 11535588]
26. Perrot M, Sagliocco F, Mini T, et al. Two-dimensional gel protein database of *Saccharomyces cerevisiae* (update 1999). *Electrophoresis* 1999;20:2280–2298. [PubMed: 10493132]
27. Grolleau A, Bowman J, Pradet-Balade B, et al. Global and specific translational control by rapamycin in T cells uncovered by microarrays and proteomics. *J Biol Chem* 2002;277:22175–22184. [PubMed: 11943782]
28. Hanash SM, Strahler JR, Chan Y, et al. Data base analysis of protein expression patterns during T-cell ontogeny and activation. *Proc Natl Acad Sci USA* 1993;90:3314–3318. [PubMed: 8475073]
29. Huang J, Zhu H, Haggarty SJ, et al. Finding new components of the target of rapamycin (TOR) signaling network through chemical genetics and proteome chips. *Proc Natl Acad Sci USA* 2004;101:16594–16599. [PubMed: 15539461]
30. Zhu H, Bilgin M, Bangham R, et al. Global analysis of protein activities using proteome chips. *Science* 2001;293:2101–2105. [PubMed: 11474067]
31. Spahr CS, Davis MT, McGinley MD, et al. Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. I. Profiling an unfractionated tryptic digest. *Proteomics* 2001;1:93–107. [PubMed: 11680902]
32. Davis MT, Spahr CS, McGinley MD, et al. Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. II. Limitations of complex mixture analyses. *Proteomics* 2001;1:108–117. [PubMed: 11680890]
33. Thongboonkerd V, McLeish KR, Arthur JM, Klein JB. Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. *Kidney Int* 2002;62:1461–1469. [PubMed: 12234320]
34. Oh J, Pyo JH, Jo EH, et al. Establishment of a near-standard two-dimensional human urine proteomic map. *Proteomics* 2004;4:3485–3497. [PubMed: 15529407]
35. Pieper, Gatlin CL, McGrath AM, et al. Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. *Proteomics* 2004;4:1159–1174. [PubMed: 15048996]

Fig. 1. Diagrammatic overview of proteomics methods and technologies. ESI, electrospray ionization; FTICR, Fourier transform ion cyclotron resonance; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; Q, quadrupole; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SELDI, surface-enhanced laser desorption/ionization; TOF, time of flight.

Fig. 2. Schematic of ion source for MALDI and SELDI. Analyte is embedded in the matrix and pulsed with a laser. This confers charge to the analyte and it enters the mass analyzer, which then generates a mass/charge vs. intensity spectrum.

Fig. 3. Schematic of ion source for ESI. Analyte from liquid phase enters through spray needle, is ionized, and then enters the mass analyzer.

Fig. 4. Schematic of Ion Trap mass analyzer. Ionized analytes enter the trap. Adjusting the electrode voltage retains ions of a specific m/z . Then ions collide with gas in the trap to produce product ions that generate tandem MS spectra.

Summary of transplant proteomics studies to date

Table 1

Authors	Technology	Samples	Target process
Clarke et al. (14)	SELDI-TOF MS	Human urine	RAR
Schaub et al. (15,16)	SELDI-TOF MS	Human urine	RAR, ATN
O'Riordan et al. (17)	MALDI-QTOF MS	Human urine	RAR
Borozdenkova et al. (18)	SELDI-TOF MS	Human endomyocardium and serum	CAR
Kaiser et al. (19)	2D GE MALDI-TOF MS CE	Human urine	GVHD
Joosten et al. (20)	ESI-TOF MS		
Steiner et al. (21)	MALDI TOF/TOF MS 2D GE nanospray quadrupole TOF	Rat GBM	CAN
Pan et al. (23)	2D GE SDS-PAGE ELISA	Rat kidney	CsA nephrotoxicity
Mascarell et al. (24)	SDS-PAGE MALDI-TOF MS	Rat serum	Immune tolerance
Escobar-Henriques et al. (25)	2D GE SDS-PAGE	Mouse T lymphocytes	CsA immunosuppression
Grolleau et al. (27)	2D GE SDS-PAGE	Yeast	MMF immunosuppression
Huang et al. (29)	2D GE SDS-PAGE reverse phase protein microarray	Rat T cells	Rapamycin immunosuppression
		Yeast	Rapamycin immunosuppression

2D GE, 2-dimensional gel electrophoresis; ATN, acute tubular necrosis; CAN, chronic allograft nephropathy; CAR, cardiac allograft rejection; CE, capillary electrophoresis; CsA, cyclosporine; ESI, electrospray ionization; GBM, glomerular basement membrane; GVHD, graft vs. host disease; MALDI, matrix assisted laser desorption ionization; MMF, mycophenolic acid; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; QTOF, quadrupole time of flight; RAR, renal allograft rejection; SDS, sodium dodecyl sulfate; SELDI, surface enhanced laser desorption ionization; TOF, time of flight.