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Application of Proteomics Technology to the Field of Neurotrauma

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ABSTRACT

Near-completion of the Human Genome Project has stimulated scientists to begin looking for the next step in unraveling normal and abnormal functions within biological systems. Consequently, there is new focus on the role of proteins in these processes. Proteomics is a burgeoning field that may provide a valuable approach to evaluate the post-traumatic central nervous system (CNS). Although we cannot provide a comprehensive assessment of all methods for protein analysis, this report summarizes some of the newer proteomic technologies that have propelled this field into the limelight and that are available to most researchers in neurotrauma. Three technical approaches (two-dimensional gel electrophoresis, direct analysis by mass spectrometry, including two-dimensional chromatography coupled to mass spectrometry and isotope coded affinity tags, and antibody technologies) are reviewed, and their advantages and disadvantages presented. A discussion of proteomic technology in the context of brain and spinal cord trauma follows, addressing current and future challenges. Proteomics will likely be very useful for developing diagnostic predictors after CNS injury and for mapping changes in proteins after injury in order to identify new therapeutic targets. Neurotrauma results in complex alterations to the biological systems within the nervous system, and these changes evolve over time. Exploration of the "new nervous system" that follows injury will require methods that can both fully assess and simplify this complexity.

Key words: brain trauma; mass spectroscopy; proteomics; spinal cord trauma

INTRODUCTION

THE MAPPING OF THE HUMAN GENOME has provided new technologies and theories necessary to tackle evaluation of very complex biological systems. Assessment of genetic information and the interactions of genes with environmental influences will advance understanding of basic neurobiology, pathophysiology of disease states, and potential therapies. However, genetics cannot completely answer the questions that arise in studying injury in the nervous system. Indeed in a variety of fields, scientists criticize the use of genomics as a tool, because DNA sequencing provides only a snapshot of the different ways a cell may use its genes. Any cell constantly reacts to its

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changing environment, creating a dynamic system (Persidis, 2000), and there seems to be a low correspondence ($R^2 = 0.61$) between changes at the transcription level and changes at the proteomic level (Ideker et al., 2001). Although proteomics is in some ways a "catch-all" term, it conceptualizes the many complex interactions that occur in the cellular repertoire for proteins. These events include genome-coded processes and posttranslational modifications, as well as interactions among proteins, nucleic acids, lipids, and carbohydrates (Persidis, 2000).

As of 2001, the Human Genome Project estimated that there are only about 30,000 genes (Claviere, 2001; Venter et al., 2001). Although the final figure is currently under debate (Shouse, 2002), this relatively low number of genes suggests greater roles for regulation of RNA translation and posttranslational modifications of resulting proteins in the cellular response to normal and pathological stimuli. Researchers have been investigating individual proteins or protein families in basic biology and disease for decades, but the field of proteomics offers a more global perspective. Because such analysis embraces the study of large numbers of inter-related proteins and their involvement in selected physiological or pathological states, a more robust appreciation of the injured nervous system may emerge.

This report serves as a beginning for the broad appreciation of the burgeoning field of proteomics. Here, we discuss the advantages and disadvantages of selected methodologies, and how they can be applied to study cellular events that occur after trauma to the brain or spinal cord. We will concentrate on the "gold standard" two-dimensional gel electrophoresis (2D GE) and a representation of emerging technologies for the field of neurotrauma that can be used in laboratories that do not have proteomics per se as a focus. The usefulness of proteomic investigation in neuroscience in general is currently under intense discussion (Grant, 2001; Grant and Blackstock, 2001), and the field of neurotrauma can position itself to embrace or reject findings as they emerge.

CURRENT AND FUTURE TECHNIQUES FOR PROTEOMIC ANALYSIS

Acknowledged challenges for proteomics research surround the analysis of complex mixtures of proteins to identify expression profiles after a physical or chemical stress, and these issues certainly apply in neurotrauma. It has been estimated that there are around 300,000 proteins in the human proteome and likely similar numbers in animal models. The large number of proteins relative to genes can result from alternative splicing of transcripts, direct protein modifications due to specific cleavages, or other post-translational events. Because so many potential proteins and multiple time points will be involved in any analysis of neural injury, approaches must be based on established biochemical principles and amenable to high throughput technologies. Techniques should allow sorting out of protein complexity, as well as analyzing and identifying proteins in low abundance or with atypical characteristics, such as basic or glycosylated proteins. Methods still need to be refined for studying protein–protein interactions, protein structure, and metabolic pathways. It is unlikely that one method of analysis can yield full information; most likely, a combination of different methods, each with its own specific advantages, will need to be employed. In addition, a major challenge will come in data storage and analysis, and biocomputing must be given extensive consideration whenever proteome-directed research is undertaken.

Two-Dimensional Gel Electrophoresis followed by Mass Spectrometric Identification of Proteins

This approach is the classic method for analyzing multiple proteins. Proteins are separated in the first dimension by isoelectric focusing (a property that depends on the relative amounts of acidic and basic amino acids in each protein) and in the second dimension by size. It is possible to see over 1,000 well-resolved proteins on a single gel. While the application of 2D GE is not new to neuroscience research (Amess and Tolkovsky, 1995; Buonocore et al., 1999; Charriaut-Marlangue et al., 1996), the new interest in this method is the ability to directly identify proteins that are differentially expressed by mass spectrometry (MS).

To identify proteins, each spot of interest is cut from the gel and digested with trypsin. The masses of the tryptic peptides are then obtained by MS (either MALDI TOF [Matrix-Assisted Laser Desorption Ionization Time-of-Flight] or LC-MS [Liquid Chromatography coupled to a Mass Spectrometer] and MS/MS) and are used to search databases for proteins that best match the experimental fragments obtained. With LC-MS/MS, it is possible to further subdivide the fragments and obtain amino acid sequence information which, when added to the database search, increases the chance of matching the protein.

Several improvements have made this method more robust and reproducible, including the discovery of better detergents and buffer combinations, new pH gradient strips, pre-cast SDS slab gels, more sensitive gel stains, and the development of difference gel electrophoresis (DIGE) (Unlu et al., 1997), which is now marketed freely as a kit by Amersham). The DIGE system involves a modification of the normal 2D gel method such that one is able to resolve both control and experimental samples in one gel. The two protein samples that are to be com-

pared, for example a control tissue and an injury tissue, are each pre-labeled with one of two cyanine dyes, Cy3 or Cy5. The labeled samples are mixed and co-migrated on the same gel in both dimensions, removing imperfections in the separation due to differences in the gel matrix, pH field, and other procedural effects. Because the samples are co-migrated, it is easy to quantify changes in protein expression and pick proteins that are altered by the treatment. The co-migration also reduces the number of gels that need to be performed for statistical purposes. The coupling of this method with mass spectrometry to identify proteins that are differentially expressed has recently been validated by Tonga et al. (2001) and is reviewed by Patton (2002).

These advances result in better resolution and visualization. Despite the improvements, 2D GE/MS remains technically complicated, and requires at least triplicate samples to be processed for statistical purposes. In addition, some classes of proteins are not detectable, including those that are rare (i.e., low abundance), small (i.e., under 2 kDa), glycosylated, of basic pH, or are integral membrane proteins. Table 1 lists the advantages and disadvantages to this method.

Overall Assessment of 2D GE

In general, 2D GE, especially DIGE, works well for protein discovery and is currently the most widely used technique for detecting proteins that have changed upon treatment. It is the only high-resolution method currently available to detect changes in post-translational modifications, including phosphorylation, which is critical to many proteins of significance to neuroscience. But 2D GE is still not able to detect the entire proteome, missing proteins that are present in low abundance and that have isoelectric points outside the normal range of pH 4–9. A substantial advancement would be the development of high-throughput affinity purification methods for low abundance proteins. Kits to affinity purify specific groups of proteins, for example phosphoproteins, are now available through some commercial vendors, and these have catapulted the study of differential protein expression. New techniques and improvements continually arise in this area; however, the need for specialized training of personnel is critical for 2D GE and MS.

Non–Gel-Based Separations of Proteins Coupled to Mass Spectrometry

This technical category is very broad and constantly evolving (Washburn et al., 2001). Methodologies include, but are not limited to, separations of protein digests by various chromatography procedures. Table 2 summarizes advantages and disadvantages of these methods. Several two-dimensional chromatography methods have been developed that separate proteins by orthogonal chromatography steps, for example, ion exchange followed by reverse phase high-performance liquid chromatography (HPLC) (Washburn et al., 2001). Complex fractions can be further resolved by mass spectrometry (MS and MS/MS). For instance, advances with this technology enabled the identification of as many as 1,484 yeast proteins in a single experiment. This technology is particularly useful for comprehensive proteome projects, and specifically can be applied to identify proteins, for example, integral membrane proteins, that are normally missed by 2D GE.

Another innovative technology in this general area has been the development of isotope coded affinity tags (ICAT) (Gygi et al., 1999; Patton, 2002). A new, im-

Table 1. Two-Dimensional Gel Electrophoresis with Mass Spectrometry

Advantages	*Disadvantages*
Simultaneous high resolution of multiple proteins	Technical expertise required
Rapid comparison of multiple gels	Sample-to-sample variability
Ability to fluorescently label proteins and co-migrate them in the same gel	Multiple controls/experiimental samples necessary
Improved reagents available	Many rare proteins not visible
Compatible with pre-fractionation of samples (enriches rarer proteins)	Some classes of proteins not detectable (e.g., <2 kDa, basic, glycosylated)
Mass spectrometry identifies lower amounts of protein	Integral membrane proteins are underrepresented in gels
Identify some post-translational modifications	
High-throughput capability	
Discovery of new, differentially expressed proteins	

Table 2. Non–Gel-Based Mass Spectrometry Methods

Advantages	*Disadvantages*
No two-dimensional gel electrophoresis for initial separation	Requires high-resolution separation methods
Direct comparison of complex protein samples	For ICAT method, proteins without cysteine residues missed (10–15%)
High-throughput capability	Unlikely to identify post-translational modifications
Analyze wider range of protein concentrations	Requires sophisticated mass spectrometers and skill in running them
Discover novel/differentially expressed proteins and peptides	Commercially available reagents are very expensive
Analyses of proteins that are missed by two-dimensional gel electrophoresis (basic, acidic, small, integral membrane proteins)	

proved version of these tags, named cleavable ICAT, is now available in kit form from Applied Biosystems. Briefly, ICAT refers to a pair of affinity directed reagents, which differ from each other by nine mass units (a difference of nine ^{13}C-groups in the linker portion of the tag), and that target cysteine residues in proteins. A pair of reagents is used to tag the full proteome from control and treated cells (or tissue) with one or the other ICAT reagent, respectively. Every protein containing a cysteine residue would then be tagged appropriately. The reaction is highly specific and occurs with high efficiency. After tagging, the two protein groups are mixed and digested with trypsin. Fragments tagged by the reagents are separated on an avidin column, and the mixture is then further separated and analyzed by LC-MS/MS. Fragments generated from proteins in equal abundance in the two tissues will be only nine mass units apart (or multiples of nine, if the fragment contains more than one Cys), and of equal height in the mass spectrometer. Fragments from proteins that are differentially expressed will appear to be higher (or lower) than their corresponding partners. These fragments can then be targeted for MS sequencing. Identities of the parent compounds can be obtained by comparing the sequences to databases. While this method is outstanding for determining the amount of protein in relation to a treatment or injury, it is not useful for the detection of changes due to post-translational modifications. Because the protein is identified solely on the basis of MS/MS fragmentation of a single peptide, ICAT analysis works best with mass spectrometers that have high resolution and mass accuracy.

Overall Assessment of Direct Mass Spectrometry Methods

Non–gel-based mass spectrometry methods for identifying differentially expressed proteins have great potential. These methods depend on high-resolution chromatography to separate complex mixtures of proteins prior to mass spectrometry, requiring capillary chromatography for sensitivity and high-resolution mass spectrometry for identification of proteins. In addition, new advances in bioinformatics are required for complex analyses of very large data files that are generated by these procedures. New developments in all these areas appear frequently in the literature, and as these methods become more robust and routine they may enable direct proteome analyses.

ICAT has the potential to provide excellent data in a short time frame and is worth considering, if the proper mass spectrometer is available. Even though post-translational modifications may not be amenable to this analysis, it can yield information for proteins that are newly synthesized in response to a stressor. This method may be the technique of choice for analyzing "pull downs" or "immunoprecipitates" without having to analyze the samples first by gel electrophoresis. Importantly, ICAT and other direct methods of global protein analysis complement the 2D GE/MS method.

Protein and Antibody Chips

The concept of evaluating thousands of proteins at once by a chip-based method is very appealing; however, this technology is still in early stages of development and will require considerable work to arrive at a point where it can be widely used to assess global differential protein expression. There are, however, several commercial companies that are currently offering protein arrays (Table 3) and others that have them under development (ProteoMonitor, 2002). Many approaches are used, but two main types of chips are being investigated: antibody-based and protein-based. In the case of antibody-based chips, antibodies to proteins of interest are fixed on an

Table 3. Companies Currently Offering Protein Microarrays

Company name and website	*Chip description*
Adaptive screening www.adaptive-screening.com	Recombinant protein array
BD Biosciences Clontech www.clontech.com	Antibody array; fluorescent technology
Discerna www.discerna.co.uk	*In situ* array; cell-free synthesis
Hypromatrix www.hypromatrix.com	Four array products and custom antibody array services
Jenni Array Technologies www.jenni.com	Peptide, small molecule, kinase substrate arrays
Luminex www.luminexcorp.com	Microsphere/bead; cell signaling and kinase activity
Molecular Staging www.molecularstaging.com	Antibody arrays; rolling circle amplification technology
Pepscan www.pepscan.nl	Peptide arrays anchored to glass slides
Zeptosens www.zeptosens.com	Six pre-spotted microarrays with recognition elements

Adapted from ProteoMonitor (www.proteomonitor.com). Total listing was 30 companies, many of which are still developing their products.

inert surface (i.e., glass slide, membrane, beads) and probed with the full complement of proteins from the tissue of interest. Antibody-based chips will be used primarily to measure differential expression. Protein-based chips rely on placing proteins directly on the surface, and would be used primarily to test for protein–protein or protein–drug interactions. There are advantages and disadvantages to chip technology. For antibody-based chips, advantages include the use of single-chain, Fv antibody phage libraries to rapidly find appropriate antibodies for a large number of antigens. For protein-based chips, a critical need will be the use of recombinant vectors (e.g., full length expression; LaBaer personal communication) for producing a full complement of proteins, including rare ones, for placement on a chip. Additional technical advantages are outlined in Table 4. However, disadvantages (Table 4) may likely include the need for prior knowledge of the protein (i.e., not ideal for protein discovery) as well as availability of high-affinity antibodies for the proteins to be examined.

Overall Assessment of the Chip and Antibody Method

This technology holds considerable promise for identifying differentially expressed proteins. Thus, it will be

Table 4. Chip Technology

Advantages	*Disadvantages*
Measurement of hundreds of proteins in parallel	Prior knowledge of protein required
Antibodies of differing affinities can be on one chip	Necessary to have the antibodies
Possible use of very small samples (microdissection)	Antibody specificity is critical
Potential to distinguish post-translational modifications	Limited use with post-translational modifications (antibody must distinguish modified site)
Methods for amplifying signals under development	Dependent on antibody affinity and protein concentration

important to begin to consider how to build chips that may be of specific interest to CNS trauma. One starting point is the application of antibodies to proteins that are already known to be involved in CNS injury and/or repair. In the last two decades, an extensive body of literature has developed with respect to biochemical and cellular changes following traumatic injury to the brain and spinal cord. This knowledge could be a valuable starting point for developing "injury chips." In addition, studies using DNA microarrays could provide valuable information about changes at the mRNA level that may suggest additional proteins to evaluate. Although the technology is not standardized, starting to work in the area now could ensure that chips are developed that will be useful to the neurotrauma field in the future.

Bioinformatics

In order to achieve the best value of any method, full and meaningful analysis will be critical, and any proteomics approach will generate extensive data. It will be important to develop methods, or to apply methods developed by others, to interpret and categorize information in a useful manner. Approaches to analyzing proteomic data are already being addressed in a variety of fields. The use of large-scale proteomics technologies yielding proteome-wide maps to study expression or interaction will depend heavily on information storage, representation, and analysis. It is possible to access proteomics databases and software through the World Wide Web; however, the evolution of resources is very rapid. Trends and probable changes are discussed in a paper by Wojcik and Schachter (2000). Other investigators have also recognized the need for fast, accurate computational analysis of protein function, and have begun development of large-scale computational systems for the analysis of sequence and structure of proteins (Weir et al., 2001). In addition, researchers have employed searching algorithms to study proteomics spectra generated by mass spectroscopy (Petricoin et al., 2002). Investigators in CNS injury can usefully appropriate existing bioinformatics strategies and sculpt them to meet their specific needs.

DISCUSSION

The gold standard for protein determination is still two-dimensional gel electrophoresis (2D GE) followed by mass spectrometry (MS). This two-stage method allows for both hypothesis-driven and discovery-driven research strategies. New methods on the horizon include separations of proteins by non–gel-based methods followed by MS, methods that rely exclusively on molecular biology, and protein/antibody chips. Although both antibody-based and protein-based chips are now available commercially, development is at a very early stage and interpretations of any studies will be cautious. All of these methods permit parallel processing of several proteins at once and have the potential for increasing the sensitivity of detection to allow the use of small amounts of material. With improvements in such methods, rare proteins and cell type–specific proteins may be evaluated.

Although the study of proteins is not new, the field of "proteomics" is. In the past, several studies of CNS injury have used the high-resolution power of 2D gel electrophoresis (Amess and Tolkovsky, 1995; Buonocore et al., 1999; Charriaut-Marlangue et al., 1996; Jenkins et al. 2002; Leski and Steward, 1996). The work of Kirschenbaum and Pulsinelli (1990) performed over a decade ago to analyze differences in phosphorylation patterns in ischemic rat hippocampus, striatum, and neocortex tissues was extremely interesting and forward thinking for its time. Now with the coupling of MS to aid in the identification of proteins that are regulated, this 2D GE approach becomes even more powerful. Many of the newer mass spectrometric techniques can identify proteins down in the attomole (10^{-18} moles) to low femtomole (10^{-15} moles) levels.

The study of CNS trauma is a dynamic and exciting area of neuroscience, and because there is a readily defined event, dissection of complex neurobiological consequences can be attempted in this context. Information gained will provide insight into processes of cell death, regeneration, and plasticity that will have relevance to many other developmental or degenerative neurological disorders. There are limitations to current proteomic technologies, including minimal rapid high-throughput screening and detection of post-translational modifications, as well as issues regarding appropriate and meaningful bioinformatics/data analysis and interpretation. In neurotrauma, protein modifications will be important: injury initiates a complicated sequence of cellular events that affects many aspects of cell signaling, genome-coded events, protein–protein interactions, and post-translational processes. It is clear that proteomic technologies could have a large impact on the study of CNS injury. Information obtained may be extremely valuable for several reasons: (1) use of high-throughput screening for proteins after injury may provide a timecourse of how large numbers of critical events are simultaneously altered after CNS injury; (2) use of proteomic techniques could help to develop reliable biomarkers for CNS trauma and post-injury outcome; and (3) proteomic techniques could be useful for screening potential therapeutic targets.

Both the field of proteomics and the application of proteomic technologies to CNS trauma are in early stages.

Proteomics as a field is growing and changing almost daily. At this point, there does not appear to be a "magic bullet" proteomic technology that will be applicable for every research question. Each investigator will need to carefully consider the goals of the proposed experiments in determining the appropriate proteomic approach to utilize. There will be many technical challenges that CNS trauma investigators will face as the field advances. As they begin to utilize proteomics more regularly, the potential "payoff" to be attained with this type of data far outweighs the current difficulties and limitations.

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