PROSPECT

Proteomics in Biomarker Discovery and Drug Development

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Abstract   Proteomics is a research field aiming to characterize molecular and cellular dynamics in protein expression and function on a global level. The introduction of proteomics has been greatly broadening our view and accelerating our path in various medical researches. The most significant advantage of proteomics is its ability to examine a whole proteome or sub-proteome in a single experiment so that the protein alterations corresponding to a pathological or biochemical condition at a given time can be considered in an integrated way. Proteomic technology has been extensively used to tackle a wide variety of medical subjects including biomarker discovery and drug development. By complement with other new technique advances in genomics and bioinformatics, proteomics has a great potential to make considerable contribution to biomarker identification and to revolutionize drug development process. This article provides a brief overview of the proteomic technologies and their application in biomarker discovery and drug development. J. Cell. Biochem. 89: 868–886, 2003.   © 2003 Wiley-Liss, Inc.

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PROTEOMICS

The rapid progresses in genomics and spectacular advances in mass spectroscopic technology in last several years have led us into a new field, proteomics. Today, proteomics has been growing to be a revolutionary tool in various studies in molecular medicine including biomarker discovery and drug development. Disease involves alterations in protein expression and thus offers a basis for detection of biomarkers and drug targets through examining the protein expression profiles with proteomics. By directly analyzing proteins in body fluids including serum, spinal fluid, urine, and exhaled breath, proteomics can globally identify and monitor biomarkers and thus change the way we diagnose, treat and prevent disease. By comprehensively inspecting the entire proteome in given tissues or cells treated with drug or drug candidates, proteomics provides a detailed map of protein interactions related to disease-associated pathways thus facilitate drug development. In this review article, we aim to briefly summarize the technology of proteomics and its application in biomarker discovery and drug development.

Definition of Proteomics

Proteome refers to the entire protein complement expressed by a genome and proteomics is the study of the proteome [Wasinger et al., 1995; Wilkins et al., 1996]. Since the proteome is dynamic, proteomics can be defined as a research field that involves the large-scale identification, characterization, and quantitation of proteins expressed in a cell line, tissue, or organism under given conditions. Proteomocs is a powerful approach that integrates recent technological advances in high-throughput protein separation, mass spectrometry (MS), genomic database, and bioinformatics to address important physiological and medical questions.

Protein Expression and Modification

Proteins, not genes, are functional molecules in cells and represent important targets for the therapeutic intervention. It is impossible to accurately predict genes and their structures by genomic data and bioinformatics. Only through the study of proteins can the existence of a particular gene be confirmed and the function of the protein expressed by the gene be characterized. Moreover, protein expression and the expression level are not the direct reflection of the mRNA levels in cells and protein expression also subjects to post-translational modification that exists in hundreds different types. As a result, a gene could express different types of proteins with different amounts under different conditions. These varied phenotypes are responsible for the actual biological functions of a gene in cells. Proteomics is established to address these problems. The applications of proteomics include the study of protein expression and modification under a given biological condition, the characterization of protein functions in a genome, the identification of protein localization and compartmentalization at a given time, and the determination of protein–protein interactions related to a biological process. In this regard, proteomics can be classified into three main types, expression proteomics, functional proteomics, and structural proteomics [Graves and Haystead, 2002].

Expression Proteomics

Expression proteomics looks at the different display of proteins expressed in a given tissue, body fluid, or cell and thus searches for bio-markers and/or drug targets. In this approach, entire protein extracts are separated to generate protein profiles. By comparing the protein profiles between a health or control sample and a diseased or drug-treated sample, proteins altered in their expression levels and patterns (e.g., modification) are identified. Further characterization can determine if these proteins are disease-specific or drug-associated targets.

Functional Proteomics

Functional proteomics focuses on a group of proteins assembling a specific biological function and studies their protein–protein or protein–DNA/RNA interactions and post-translational modifications. Specific types of proteins or sub-proteomes are isolated through affinity chromatography or other chemical method and then subjected to further proteomics analysis based on how these proteins in the biological system interact with each other, in pairs or in protein complexes, within complicated cellular pathways. This approach allows a selected group of proteins to be characterized in response to internal or external signals and thus provides information about protein signaling, disease pathogenesis, and protein–drug interactions.

Structural Proteomics

Structural proteomics aims at mapping out the structures of all the proteins or protein complexes in a specific cellular organelle and building up the relationships of these proteins in a global view. This type of approach attempts to characterize all the proteins in a genome and integrate their protein–protein interactions into a "proteomics map" with "functional, structural, and location annotation." One can also isolate or pre-fragment the proteins or protein complexes from specific organelles and then analyze their locations and their relations with other proteins, which make up the functional characteristics of these organelles.

TECHNOLOGY PLATFORMS OF PROTEOMICS

There are several proteomic platforms available at the moment. A typical workflow of a platform generally consists of, in subsequence, sample preparation or extraction, protein separation or pre-fractionation, comparative

profiling of protein expressions, proteolytic digestion and mass analysis, protein identification through database matching or protein sequencing, and finally protein validation and biochemical characterization. The commonly used proteomic techniques are briefly introduced as follows.

2-Dimensional (2-D) Gel Electrophoresis

The first and most commonly used technique in proteomics is 2-dimensional gel electrophoresis (2-DE). In 2-DE, proteins are extracted from cells or tissues and then separated according to their isoelectric points (pIs) in the first dimension and their molecular weights in the second dimension to generate protein profiles. 2-DE has a powerful resolution of separating thousands of proteins in a single gel, a capacity that no other method can overpass in protein separation up today. After 2-DE separation, proteins in gels can be visualized by Coomasie blue or silver staining and gel images are digitalized and analyzed with a computer-aid special software. Interesting (altered) protein spots are identified, cut off and subjected to in-gel tryptic digestion. The resulting peptide mixtures are analyzed with MS to obtain so-called peptide fingerprints, which determine protein identifications through matching to genomic and proteomic databases.

2-DE is primarily used in expression proteomics in which protein profiles are compared qualitatively and quantitatively between any given sample pair. The appearance and disappearance of protein spots tell the difference of protein expression while the varied intensity of the spots reflects the different protein expression levels under a given condition. 2-DE protein profiling is especially useful in biomarker discovery in which comparison can be made between normal and diseased samples including tumor tissues and bodily fluids. 2-DE also features a great strength of separating protein isoforms resulting from protein post-translational modifications, alternative mRNA splicing and proteolytic processing due to a disease condition or drug treatment. These kinds of protein modifications and cellular processing change the molecular mass and the pI (charge) of proteins, leading to the appearance of different spots in 2-D gels. This resolving ability of 2-DE has been extensively employed in the studies of functional and structural proteomics.

Mass Spectrometry

After protein separation by 2-DE, MS is applied to produce mass spectra for protein identification. MS is an analytic tool to obtain protein structural information such as peptide masses, amino acid sequences, type and location of protein modification. The recently tremendous improvement of MS in sensitivity, resolution, and mass accuracy made this analytic tool a core technology in proteomics. Before MS analysis, proteins are subjected to "in-gel" digestion with a protease and the proteolytic peptides are extracted from the gels. When applied to MS, protein peptides are ionized with a "soft" ion source, and the ionized peptide mixture is resolved in a mass analyzer based on the mass/charge (m/z) ratio of the peptides, which are then detected in sequence by a detector and are finally displayed from low to higher mass as a mass spectrum.

The most widely used MS is matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) [Yates, 1996, 1998]. With MALDI-TOF, digested peptides are mixed with a chemical matrix that can absorb energy from light, and the mixture is co-crystallized in spots on a sample plate. When a laser beam fires at the spots, the matrix absorbs energy and transfers it to the peptides which "flight" in gas phase by picking up a proton and thus being charged. These ionized species are then resolved by their different flight time in a high-vacuum tube to reach the detector. For most proteins, $\sim$10 ng (200 fmol for a 50-kDa protein) can be reliably detected by MALDI-TOF. MALDI-TOF is principally used in peptide mass fingerprinting and is a primary instrument in large-scale proteomics because it can be integrated into a high-throughput automatic workflow of proteomics.

Another frequently used MS in proteomics is nano electrospray ionization tandem MS (ESI MS/MS) [Yates et al., 1997, 1999]. In ESI MS/MS, a microcapillary tube containing 1 μl of peptide solution sprays a fine mist of charged droplets generated from a potential difference between the capillary and the inlet to the mass spectrometer. Desolvated peptide ions are formed as the solvent evaporated in a high vacuum chamber, and are resolved to produce the first MS scan. From the MS scan, a peptide ion (parent ion) is selectively transmitted into a collision chamber where the

peptide is fragmented by interactions with an inert gas. The fragments of the peptide ion are then resolved based on their *m/z* ratio to generate the second MS spectrum with a series of small peptides that differ by only a single amino acid. By combining these small peptides together, the amino acid sequence of the parent peptide can be constructed.

In routine proteomics, proteins in question are generally analyzed firstly with MALDI-TOF MS through peptide fingerprinting. If proteins cannot be identified by fingerprinting due to insufficient number of proteolytic peptides or lacking suitable DNA database for confident matching, ESI MS/MS can be used for the amino acid sequencing. These two types of MS operate in different ways and produce complementary information suitable for protein identification and thus stay in the mainstream of the technology of proteomics.

Surface Enhanced Laser Desorption Ionization (SELDI)-ProteinChip Separation and Profiling

SELDI-ProteinChip system is a newly developed proteomics platform that uses patented technology to separate, profile, and analyze proteins at the femtomole level [Jr et al., 1999; Yip and Lomas, 2002]. With this approach, the surface of a protein chip is modified chemically or biochemically (Surface Enhanced) to be able to bind a certain group of proteins based on a specific physical property such as hydrophobicity, charge, etc. A small amount of crude biological sample such as serum or protein extracts is directly applied to the surface, incubated, and washed. The specific proteins or functional class of proteins that match to the chemical or biochemical properties of the protein chip are retained on the surface and therefore, separated from the protein mixture. The protein chip is then subjected to mass analysis by a ProteinChip reader (a SELDI-TOF MS) which generates mass profiles of the bound proteins in spectral or gel views. SELDI-ProteinChip system allows comparison of protein mass profiles to be made between any sample pair or among a group of samples from different biological conditions, and thus biomarkers or disease-related protein targets are identified. The great advantages of this technology include its abilities to use a very small amount of crude sample and to detect proteins with molecular weights lower than

6-kDa, which may not be well observed in 2-DE in general.

Protein Pre-Fractionation and "Short-Gun" Approach

Differing from the 2-DE/MALDI-TOF and SELDI-ProteinChip techniques stated above mainly focusing on protein differential expression, a new approach called "short-gun" or LC-MS/MS aims at total identification of entire proteins [McCormack et al., 1997; Peng and Gygi, 2001]. This method uses reversed-phase liquid chromatography (LC) to separate the tryptic digests of entire proteins followed by on-line ESI tandem MS for peptide sequencing. In this approach, total protein extracts from cells or tissue are pre-fractioned with an anion exchange column to simplify the protein mixture. Selections of protein fractions from the column are subjected to proteolytic digestion, and the resulting digested mixture is applied to LC separation coupled online with MS/MS measurement. The generated MS spectra of whole cellular fraction are carefully analyzed and protein identification is performed through peptide assignment and database searching. This short-gun approach features less sample manipulation and total protein peptide mapping. A recent study integrating this technique with a "top-down" approach (intact protein mass analysis followed by molecular weight database search) substantially increased the dynamic range and confidence of protein identification [VerBerkmoes et al., 2002].

Isotope-Coded Affinity Tags (ICAT) Modification and Chemical Proteomics

As an alternative to 2-DE separation, a recently introduced method, known as ICAT [Gygi et al., 1999], can be used for protein expression profiling. This technique depends on the chemical labeling of any pair of protein samples with two identical reagents isotopically different in mass, allowing the relative amount of protein to be quantitatively compared in the subsequent mass spectral determination. With ICAT modification, the pair of protein samples are labeled on their cystine residues, respectively, with either light or heavy form of ICAT chemical reagents and then mixed together for proteolytic digestion. The digested peptide mixture is further purified through avidin affinity chromatography by means of the biotin tag on ICAT reagents to isolate the ICAT-labeled

peptides. MS analysis of these peptides produces the peak ratios for different protein expression and subsequent MS/MS peptide sequencing results in identification of proteins in altered expression levels.

Similar strategies using specific protein-modifying reagents (tags) in facilitating expression profiling and functional studies of distinct protein families within a complex proteome is now classified as chemical proteomics [Jeffery and Bogyo, 2003]. These modifying reagents are designed and synthesized to be able to selectively react with distinct protein residues or protein families mechanistically or functionally. The modified proteins can be purified or isolated based on their tag properties and then the targeted proteins can be identified and/or further characterized with MS. Chemical proteomics has great potential in accelerating the studies of target identification and validation and drug discovery.

APPLICATION OF PROTEOMICS IN BIOMARKER DISCOVERY

Biomarkers are usually disease-associated proteins that can be detected and quantitatively measured for disease diagnosis, staging, prognosis, and treatment monitoring. The development of a disease condition is a multi-step process involving different biological pathways. Many proteins are altered in expression levels and/or expression types such as modification during this process. These altered proteins can be detected in tissue, blood, urine, or other body fluids and thus provide indicators for the disease. An ideal biomarker should have high specificity for a certain disease condition; this kind of biomarkers is rare, however. Most of biomarkers are those proteins expressed by many different types of diseases but with variant expression levels from type to type. Combining several unspecific biomarkers together may lead to a specific index for a particular disease. In this regard, proteomics offers suitable and powerful technique platforms for the biomarker discovery, characterization, and evaluation because of its capacity of globally examining the protein expression profiles under given conditions. Actually, since they were introduced, proteomics approaches, especially 2-DE/MALDI-TOF and SELDI/ProteinChip, have been extensively used to identify biomarkers for various diseases.

Cancer

Cancer is a prime target to study by proteomics. Lung cancer is one of most comprehensively studied malignancies using proteomics techniques. Substantial effort was made by Hanash et al., together with other researchers, to identify tumor-associated proteins as novel biomarkers for the early diagnosis and/or as new targets for therapeutic treatment [Hanash, 2001; Hanash et al., 2001; Naour et al., 2002; Wang and Hanash, 2002]. A number of potential tumor markers have been found in tissue, sera or tumor cells by using comparative 2-DE technique coupled with MS and microarray analysis or autoantibodies blotting [Brichory et al., 2001a,b; Chang et al., 2001; Madoz-Gurpide et al., 2001; Chen et al., 2002a]. Especially, cytokeratin isoforms were demonstrated to correlate with patient survival in the cancer [Gharib et al., 2002] and the overexpression of oncoprotein 18 was found to reflect the poor differentiation status in lung carcinoma [Chen et al., 2003]. A database integrated protein expression with mRNA levels in lung cancer has also been constructed to facilitate biomarker discovery and tumor classification [Oh et al., 2001]. To study the arsenic-induced cell transformation of lung cancer, we have performed a proteomic analysis by using SELDI-TOF ProteinChip technology [He et al., 2003a]. Differential protein profiles between control and arsenic-induced transformed lung cells distinguished several prominent protein peaks, indicating the potential of SELDI proteomics for identifying biomarkers for lung cancer [He et al., 2003a].

Another cancer that received extensive proteomics analysis is bladder tumor, including transitional cell carcinoma (TCC), squamous cell carcinoma (SCC), and adenocarcinoma. Celis research group has long been involved in the searching for biomarkers that may form the basis for diagnosis, prognosis, and treatment of bladder cancer using proteome expression profiling [Celis et al., 1999d; Celis et al., 2000; Celis and Gromov, 2003]. They have systematically analyzed hundreds of fresh tumors, random biopsies, cystectomies, and urines using proteomics and immunohistochemical analysis to establish comprehensive 2-DE databases of bladder cancer for tumor marker and protein target discovery [Rasmussen et al., 1996; Celis et al., 1999c]. Their studies also led to the

identification of several protein markers associated with TCC progression [Celis et al., 1996; Gromova et al., 1998] and SCC differentiation [Ostergaard et al., 1997; Celis et al., 1999b] and invasion [Celis et al., 1999a; Ostergaard et al., 1999]. In addition, ProteinChip technology has also been utilized to screen urine samples from TCC patients and five potential novel TCC biomarkers have been found through the study [Vlahou et al., 2001].

Many investigators have also concentrated on the identification of biomarkers for breast cancer [Hondermarck et al., 2001; Wulfkuhle et al., 2001; Luftner and Possinger, 2002]. Again, 2-DE protein profiling was used as a major approach to search for tumor-related proteins in tumor tissues [Franzen et al., 1996; Wulfkuhle et al., 2002] and breast tumor cells [Le Naour et al., 2001; Adam et al., 2003], while SELDI-ProteinChip was a sensitive tool to detect new biomarkers in serum [Li et al., 2002] and nipple aspiration fluid [Paweletz et al., 2001b; Cho et al., 2002]. In the unique case of breast cancer, the biomarker discovery in nipple aspirate fluid features an even special significance as it may lead to a potential non-invasive method in the diagnosis of the disease. A recent elegant experiment used bilateral matched pair breast ductal fluids from women with unilateral invasive breast carcinoma as specimens to examine protein expressions distinct between the breast pair with 2-DE proteomics [Kuerer et al., 2002]. This study has identified several differently expressed proteins that are associated with breast carcinoma and may be potential biomarkers with a clinical role.

Since the current clinical biomarker, prostate-specific antigen (PSA), lacks the specificity and sensitivity in the diagnosis of prostate cancer, efforts were made to analyze multiple proteins as signature proteomic patterns for distinguishing cancer from noncancer and thus as an index of biomarkers for accurate detection of prostate cancer [Adam et al., 2002a; Petricoin et al., 2002b; Qu et al., 2002]. Researchers also looked for alternatives of PSA by examining the proteomic alternations of PSA–antichymotrypsin complex [Qian et al., 1997], low-MW PSA [Charrier et al., 2001], and other tumor-associated proteins such as nuclear matrix proteins [Lakshmanan et al., 1998; Alberti et al., 2000]. In particular, improvements in sensitivity and accuracy have been obtained by applying laser capture microdissection (LCM) to accumulate tumor cells from tissue samples prior to SELDI/ProteinChip [Jr et al., 1999; Wellmann et al., 2002] or 2-DE/MALDI [Ornstein et al., 2000; Paweletz et al., 2001a; Ahram et al., 2002] proteomic analysis. These studies have resulted in identification of a number of unique proteins that are associated with the actual molecular events in carcinogenesis and may be potential candidates for new specific biomarkers.

Lacking specific biomarkers in clinical assessment is also the problem for hepatocellular carcinoma (HCC). Attempts have been taken to discover novel biomarkers for HCC early detection by means of proteomics [Seow et al., 2001; Shalhoub et al., 2001; Steel et al., 2001]. These include the identification of two HCC specific nuclear matrix proteins [Chew et al., 1997], novel protein Hcc-1 [Choong et al., 2001], a distinct repertoire of autoantibodies in HCC [Le Naour et al., 2002], aldehyde dehydrogenase variants [Park et al., 2002], and sarcosine dehydrogenase, liver carboxylesterase, peptidyl-prolyl isomerase A, and lamin B1 [Lim et al., 2002]. In addition, proteome databases have been also established to aid in pathological staging, protein profiling, and biomarker discovering of HCC [Seow et al., 2000; Cho et al., 2002; Liang et al., 2002].

Gastric and colon cancers are two common malignant tumors in digestive tract. Both diseases are traditionally identified and classified by histological criteria due to no specific and suitable biomarkers currently available for clinical assessment. However, only a limited number of proteomic studies involved in the direct discovery of biomarkers for these cancers, although proteome databases of tissue [Cole et al., 2000; Ha et al., 2002] and cell lines [Simpson et al., 2000; Hoffmann et al., 2001; Tomlinson et al., 2002] have been constructed. So far, attentions have been attracted to identify specific proteins or antigens that reflect the chemo- and thermo-resistant properties of stomach cancer [Sinha et al., 1998, 2001] and that are associated with *Helicobacter pylori*, the human pathogen that causes an inflammatory process leading to gastric cancer [Chang et al., 1999; Enroth et al., 2000; Haas et al., 2002]. For colon cancer, a few candidate proteins have been found in tissue samples to be potential markers for correlating to malignant transformation and tumor metastasis [Keesee et al., 1994; Chaurand et al., 2001a,b; Stulik et al., 2001; Brunagel et al., 2002].

Other cancers that received proteomic analysis for biomarker or antigen identification include ovarian cancer [Ardekani et al., 2002; Bandera et al., 2003; Liotta et al., 2003], renal cell carcinoma [Sarto et al., 1999; Balabanov et al., 2001; Klade et al., 2001], head and neck cancer [Patel et al., 2001; Srisomsap et al., 2002; Zhou et al., 2002], neuroblastoma [Prasannan et al., 2000], and leukemia [Hanash et al., 2002]. One recent study using 2-DE to analyze LCM ovarian tumor specimens revealed several uniquely over-expressed proteins in invasive cancer, which could be important markers and/or therapeutic targets [Jones et al., 2002]. Another worth-emphasized project utilized a serological proteomic pattern generated through SELDI technology as a screening tool to classify 116 masked serum samples and yielded a sensitivity of 100% and specificity of 95% in discriminating ovarian cancer from non-cancer [Petricoin et al., 2002a]. A new emerging proteomic technology, differential in-gel electrophoresis (DIGE), in combination with LCM, has also been claimed to be a powerful procedure for the molecular characterization of tumor progression and for the identification of tumor-specific biomarkers in esophageal scans cell cancer [Zhou et al., 2002].

Heart Disease

Heart disease, including various cardiovascular dysfunctions, is a leading cause of death in the world. This is partially due to the lack of sensitive biomarkers for diagnosis and the limited option of therapeutic treatments. Although the pathogenic mechanisms of cardiovascular diseases remind largely unknown, it can be speculated that extensive alternations of myocardial proteins accompany the disease processes and determine the progression and prognosis. Researchers in this area promptly realized that proteomics is a robust tool to globally characterize these underlying protein changes for better understanding of the basis of heart disease and for identification of novel target proteins for diagnosis and therapy [Dunn, 2000; Arrell et al., 2001b; Jiang et al., 2001; Macri and Rapundalo, 2001; Van Eyk, 2001; Jager et al., 2002].

As the pioneering work, several proteomic databases of human, dog, and rat myocardial tissues were constructed by Jungblut [Muller et al., 1996; Otto et al., 1996; Li et al., 1999], Dunn [Dunn et al., 1997; Evans et al., 1997], and

Pleissner et al. [1996]. These databases contain information on several hundred identified cardiac proteins and thus build a basis for studying alternations in protein expression in models of heart disease. Recently, completed identification and characterization of bovine heart proteins was also achieved by using 2-DE separation coupled with a new high-resolution MS, MALDI-TOF/TOF, which displayed both protein identify and modification on tryptophan residues [Bienvenut et al., 2002]. In addition, an Oxford team has reported the generation of a high-resolution 2-DE proteomic map of platelets, a crucial factor correlated with thrombotic diseases such as stroke and myocardial infarction [O'Neill et al., 2002]. All these databases form an essential platform for cardiovascular proteomics in facilitating future identification of biomarkers and drug targets for new therapeutic strategies. Along with these database construction, to better separate the complex proteins from cardiac muscles for protein identification, various improved methods have been developed by enhancing the solubilization [Labugger et al., 2002], pre-fractioning sub-proteomes [McDonough et al., 2002; Neverova and Van Eyk, 2002] and complementing separation techniques with LC-MS/MS [Edmondson et al., 2002].

Based on these databases and later-on proteomic characterization, several animal models have been employed in proteomic studies for analyzing functional proteins differently expressed in various cardiovascular conditions [Heinke et al., 1998, 1999; Pleissner et al., 1998; Weekes et al., 1999, 2003; Arrell et al., 2001a; Sironi et al., 2001; Schwertz et al., 2002]. A number of significantly altered proteins have been identified in the ventricular tissue of bovine with delated cardiomyopathy [Weekes et al., 1999], myocardial issue of rabbits with myocardial ischemia and reperfusion injury [Schwertz et al., 2002] and in cardiac tissue of mice with cardiovascular complications of AIDS with chronic alcohol consumption [Weekes et al., 2003]. By compared with human expression pattern, these proteins can be further examined and determined as potential biomarkers for characterizing the heart diseases.

Similar approaches were applied to study human heart and revealed several characteristic protein markers for dilated cardiomyopathy [Pleissner et al., 1997; Corbett et al., 1998]. Researches into the discovery of autoantibodies

in the sera of patients with myocarditis [Pankuweit et al., 1997], idiopathic dilated cardiomyopathy [Pohlner et al., 1997], and rheumatic heart disease [Tontsch et al., 2000] were also performed and resulted in the identification of some particular antigens including creatine kinase, dihydrolipoamide dehydrogenase, nicotinamide-adenine dinucleotide dehydrogenase, and ubiquinol-cytochrome-C reductase. Other studies looked at the modification and diversification of heat shock protein 27 [Scheler et al., 1997a; Scheler et al., 1999] and apolipoproteins A-I and II [Asztalos et al., 2000; Dayal and Ertel, 2002], the well-known markers for cardiac diseases, in response to different myocardial conditions. Identification and quantification of the distributing profiles of these proteins may form characteristic patterns for classifying heart diseases.

Arthritis, Hepatitis, and Others

Proteomic technologies have also been used to study inflammatory and immune diseases. Rheumatoid arthritis (RA) is one of the autoimmune diseases that have been subjected to proteomic analysis for novel autoantigens or autoantibodies in sera as biomarkers of the disease [Hueber et al., 2002]. A sera autoantibody to a 68 kDa antigen detected in RA synovial membranes can be a positive marker as it demonstrated a high sensitivity of 64% and specificity of 99% in distinguishing RA from non-RA and other rheumatic diseases [Blass et al., 1995], while anti-ribonucleoproteins C1/C2 present in systemic sclerosis and psoriatic arthritis may serve as a negative marker for their absence in RA sera [Heegaard et al., 2000]. Another potential diagnostic autoantibody, anti-alpha-enolase, was identified through analyzing serum samples of 255 patients with very early arthritis, and the antibody has a specificity of 97% for RA [Saulot et al., 2002]. In addition, 2-DE technique has also been employed to detect the protein alterations in RA salivary [Beeley and Khoo, 1999] and synovial fluid [Smith et al., 2001] and the potential of quantitative analysis of protein expression in forming the basis for new diagnostic methods has been discussed in these studies.

Since chronic infection with hepatitis B or C virus (HBV, HCV) is a major risk factor for the development of HCC, most proteomic studies concerning hepatitis focused on the HBV- and HCV-related HCC [Seow et al., 2001; Steel et al., 2001; Le Naour et al., 2002]. We have initiated a project using proteomic technology to globally examine HBV infected serum samples aiming at searching for serological biomarkers for the diagnosis of HBV liver inflammation, which still relies on biopsy test [He et al., 2003b]. Compared with normal and HBV-negative samples, at least seven proteins in HBV-positive sera were found significantly changed in both expression quantity and patterns. Some of these alterations reflect the difference between low and higher stages of inflammation. We are evaluating if a combination index integrating the alterations of these proteins can be a useful biomarker for HBV diagnosis and therapy.

Biomarker discovery by proteomics for other diseases is relatively limited but promising. For example, a complementary antigen, Mtb81, has been identified and claimed to be a novel serological marker for the diagnosis of tuberculosis co-infected with human immunodeficiency virus [Hendrickson et al., 2000]. Cystatin C, detected in cerebrospinal fluid by SELDI technology, has been believed to be a biomarker for pain in humans [Mannes et al., 2003].

APPLICATION OF PROTEOMICS IN DRUG DEVELOPMENT

Drug-development process involves many steps, including target identification, lead selection, small-molecular screening and optimization, and clinical testing. Proteomics is a promising approach in identification of protein targets and biochemical pathways involved in disease process and thus plays an important role in drug development. By studying interrelationships between proteins that occur in health and disease, proteomics contributes insight into determination of the pathophysiological basis for target identification. By charactering the protein expression profiles following drug treatment, proteomics provides molecular information to study the mechanistic basis for drug action and toxicity. Specific biomarkers identified by proteomics may serve as protein signatures to screen drug for its efficacy, resistance, and optimization. In this regard, some sub-disciplines such as chemical proteomics [Jeffery and Bogyo, 2003], topological proteomics [Owens, 2001], clinical proteomics [Krieg et al., 2002], toxicoproteomics [Steiner and Anderson, 2000], and

pharmacoproteomics [Kennedy, 2002; Meister, 2002] have been created to recognize the special contribution of proteomics in drug discovery. It is anticipated that the increasing promise of proteomics, together with the vast genetic information provided by genomics, will rationally revolutionize the target validation and drug development process.

Identification of Drug Targets

Proteins constitute the majority of drug targets on which drug design processes are initiated. Proteomics is an effective means to detect the targets by globally examining the protein expression alternation and protein–protein interactions occur in disease process and/or after drug treatment. Several recent reviews summarized the application of proteomics in identification of novel drug targets for leukemias [Hanash et al., 2002], *Mycobacterium tuberculosis* [Betts, 2002], and chronic skeletal diseases [Cho and Nuttall, 2002]. Proteomic analyses on pathogen–host interactions in chlamydial disease [Coombes et al., 2002] and in HCV and HIV [Tang et al., 2002] were also carried out to reveal the cellular proteins that physically interact with viral proteins and thus represent potential drug targets.

A common strategy in target identification is to analyze the proteomic profiles of cells treated by a growth factor or a regulatory enzyme. By compared with untreated cells, the altered proteins in the gene network regulation or signaling pathway in response to the treatment will be identified. Examples using this method include the identification of cytokine regulated targets in human intestinal epithelial cells [Barcelo-Batllori et al., 2002], potential targets of transforming growth factor-beta in lung epithelial cells [Kanamoto et al., 2002], and cellular targets regulated by the 12-phorbol 13-myristate acetate-stimulated MKK/ERK cascade in human erytholeukemia K562 cells [Lewis et al., 2000]. A similar approach to discover the targets in signaling pathways is to focus on the phosphorylated sub-proteomes isolated by immunoprecipitation prior to 2-DE separation. With this approach, novel signaling proteins or potential drug targets have been detected in primary human lymphocyte treated with IFN-alpha or IL2 [Stancato and Petricoin, 2001] and in human platelets after thrombin activation [Maguire et al., 2002].

Analyzing the proteomic profiles after treatment by a known drug is a similar strategy for the identification of new target proteins and for getting new insight into the mechanism of action for better drug design. With this method, HSP27 has been found to be a potential modulated target for antitumor drugs in breast cancer [Chen et al., 2002b], mortalin an anti-apoptotic protein in vascular smooth muscle cells [Taurin et al., 2002], aldehyde dehydrogenase 1, and quinone reductase 2 selective targets of the quinolines in red blood cell purine binding proteome [Graves et al., 2002] and several cellular stress proteins to be new targets of lovastatin, a lipid lowering agent, in liver [Steiner et al., 2000].

Chemical proteomics is another primary approach for target identification. Chemical proteomics distills a specific group of proteins from a complex proteome by attaching chemical probes to the specific proteins, which can then be studied in detail [Adam et al., 2002b; Jeffery and Bogyo, 2003]. Chemical probes targeting cysteine protease have been used to identify protein targets in processes such as cellular apoptosis [Faleiro et al., 1997], cataract formation [Baruch et al., 2001], and infection with malaria parasite [Greenbaum et al., 2002]. Other activity-based chemical probes have also been used in protein profiling under a disease state or condition to detect proteins distinguishable based on their activity and affinity with the chemical tags [Adam et al., 2002c; Jessani et al., 2002; Lind et al., 2002]. Besides, new chemical probes that target serine hydrolase [Kidd et al., 2001] and phenyl sulfate [Adam et al., 2002d] and non-directed activity-based probes [Adam et al., 2001] have been synthesized. Recently, two new techniques of chemical proteomics called fluorophore-assisted light inactivation (FALI) and chromophore-assisted laser inactivation (CALI) were introduced for protein target identification and validation [Beck et al., 2002; Rubenwolf et al., 2002].

In a more systematic way, Patterson described a large-scale proteomics-based target discovery platform consisting of cell biology, chemical proteomics, and bioinformatics technology [Patterson, 2002]. By using isotope labeling for quantitatively analysis of protein expression and correlating with gene-specific mRNA, the differences in the expression levels of nucleic acid and proteins suggest drug targets that directly cause disease or reveal biochemical

pathways that could be modulated by bioactive molecules. This system has been evaluated by applying it to study continuous cell lines derived from human pancreatic adenocarcinomas [Patterson, 2002].

Other studies are based on the recognition between proteins and antibodies or specific peptides to identify the binding partners or sites with potential role in therapeutics. The examples include the determination of conformational immunogenic sites by proteomic mapping of patient sera with post-therapy HACA [Spencer et al., 2002] and the identification of interaction partners of a tumor suppressor peptide, p21cip1/waf1 [Gururaja et al., 2003].

Action Mechanism of Drugs

Numerous proteomic studies have been performed to investigate the drug mechanism of action, biochemical basis of drug activity, and cellular pathways that drugs act on. The resulting data provided novel insights into drug action modes and elucidated the new modulating factors for new drug design. Differential protein expression profiling is a commonly used approach, which examines the comprehensive protein alteration after drug treatment to elicit new drug-associated parameters missed by conventional method. By characterizing the altered proteins using this approach in human tumors, the drug effects and regulated pathways have been investigated in Burkitt lymphoma cells treated with drug 5′-azacytidine [Poirier et al., 2001], in A549 lung adenocarcinoma cells stimulated with a synthetic cyclin-dependent kinase inhibitor [Kovarova et al., 2002], in colon cancer cells treated with butyrate [Tan et al., 2002], in acute promyelocytic leukemia cells induced by retinoic acid [Wan et al., 2001], and in renal cell carcinoma cells treated with interferon-gamma [Lichtenfels et al., 2002]. Other researchers addressed the drug action using animal cells, including probing for iso-proterenol-stimulated signal transduction pathways in the rat C6 glioma cell [Storm and Khawaja, 1999] and studying the antitumor activities of Ganoderma lucidum extracts in mouse spleen cells [Wang et al., 2002]. Animal models have also been directly utilized to study the drug mechanisms such as the inflammatory effects stimulated by interleukin and interferon in rat trigeminal ganglia [Friso et al., 2001], insulin processing mediated by insulin sensitizer drug rosiglitazone in pancreatic islets of obese mice [Sanchez et al., 2002] and the actions of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats [Bruno et al., 2002].

Peroxisome proliferators (PPs) are a group of chemicals that induce hepatic peroxisome proliferation, suppression of apoptosis, liver tumor formation, and beta-oxidation of fatty acids. The biochemical response of PPs is mediated by PPs-activated receptor but the mechanisms underlying the mediation process and the molecular events are poorly understood. A number of investigations have been concentrated on the proteomic analysis of mouse or rat liver in response to the PPs treatment in order to delineate PPs mediated signaling pathways and their impacts on hepatocarcinogenesis [Edvardsson et al., 1999; Chevalier et al., 2000; Macdonald et al., 2001; Chu et al., 2002; White et al., 2003], and to explore the possible therapeutic value of PPs in diabetes [Edvardsson et al., 1999].

Apart from these animal studies, some bacteria have also be examined by proteomics for their response to antibiotic agents or chemical stress [Singh et al., 2001; Duche et al., 2002; Bandow et al., 2003; Bruneau et al., 2003]. 2-DE was used to profile the bacterial proteins after treatment with antibiotics and alternatives. By comparing and characterizing the protein expressions, researchers aimed at obtaining new information on the antibiotic mechanisms and classifying novel drug candidates with unknown mechanisms of action.

Drug Toxicity and Screening

Another topic of proteomics application in drug development is the measurement of drug toxicity, including studies on mechanistic toxicology for evaluating current drugs and on predictive toxicology for screening new drug candidates [Hellmold et al., 2002; Kennedy, 2002]. Drug side-effects are common problems but the action mechanisms of the drug toxicity on human organs are largely unknown. In a typical proteomic experiment, protein extracts from a targeted organ after overdose or over-time dosing of a drug are separated by means of 2-DE or ProteinChips and the differentially expressed proteins are identified and analyzed. Further characterization of these altered proteins helps us understand the mechanism of toxicity for drug re-design and improvement, and the elicited drug-associated proteins can be used as predictive markers of toxicity for

classifying compounds and screening large numbers of drug candidates.

Currently, most of studies have been focused on defining the mechanistic basis for hepato- and renal-toxicity of various drugs such as acetaminophen [Fountoulakis et al., 2000; Ruepp et al., 2002], pyrimidine derivatives [Newsholme et al., 2000], methapyrilene, cyproterone acetate, and dexamethasone [Man et al., 2002], and 4-aminophenol, D-serine, and cisplatin [Bandara et al., 2003]. In many cases, specific proteins were identified and shown to correlate with molecular pathways of cellular toxicity of the drugs. Cardiovascular system is another common site that suffers drug toxicity. As demonstrated by conventional studies, cardiotoxicity could occur through various cellular and molecular mechanisms. Proteomics study on drug cardiotoxicity may present us a more comprehensive picture depicting how drug effects occur in heart and vascular system. Preliminary studies in this regard have been performed [Scheler et al., 1997b; Herman et al., 2001; Bandara and Kennedy, 2002]. Attempt has also been made to utilize the specific proteins as molecular signatures for studying or evaluating other drugs [Man et al., 2002]. In addition, Randic and Basak [2002] developed a new mathematical method for characterization of effects of different toxic agents on the cellular proteome, aiming to graphically illustrate the differential protein expression between normal and drug-treated samples for understanding drug toxicity. In parallel to the drug toxicity study, the cellular mechanisms of drug resistance in some diseases such as pneumonia [Cash et al., 1999], lymphocytic leukemia [Voss et al., 2001], and myeloma [Mitsiades et al., 2003] have also been explored by employing proteomic technology.

Studies on drug toxicity may not only elucidate the mechanism of toxic damage but also detect toxin-associated proteins, which can be used as markers of toxicity for drug screening. A full drug-screening program involves the establishment of comprehensive databases integrated with techniques and data from genomics, proteomics, and bioinformatics. For the proteomics part, these data are a combination of protein expressions of cells or organisms specifically in response to drug treatment resulting in unique expression patterns, molecular fingerprints, indicatives of drug efficacy, and potential toxicity. These information will

obviously accelerate the lead identification and improve the optimization of drug efficacy and safety in pre-clinical and clinical studies. One attempt to set up a proteomic program for drug screening was taken by Gianazza et al. [2002], who annotated 2-DE maps of rat serum proteins under control and experimental conditions with emphasis on species-specific components and the effects of acute and chronic inflammation. Anti-inflammatory drugs were screened and tested for their efficacy and toxicity on adjuvant arthritis, the correlation between biochemical parameters and therapeutic findings from the screening proved the sensitivity of the procedure in revealing "side-effects" of the test drugs.

In a recent report, Toledo-Sherman and Chen [2002] introduced a high-throughput virtual screening platform for drug selection by integrating genomics and proteomics initiatives. This platform emphasized the techniques adaptable to high-throughput that can tackle multiple targets and therapeutic areas simultaneously. At the core of this program, virtual screening relies on a structure-based docking and ranking method to identify bioactive molecules in compound library database. In contrast, another new method using the free binding energy between a ligand and receptor as a scoring strategy to screen drug candidates has been proposed [Bock and Gough, 2002]. A validation experiment with 2,671 samples demonstrated the effectiveness and sensitivity of the new method. Additionally, a proteomic map and database of lymphoblastoid proteins has been constructed for analyzing drug effects and lymphocyte cell diseases [Caron et al., 2002].

CONCLUSIONS

Proteomics is established on the basis of the tremendous technique advances in genomics, protein separation, and analysis. Proteomics features a global examination of protein expressions and thus the corresponding molecular events and cellular pathway interruptions in response to a certain disease condition can be inspected in a comprehensive and integrated way. Numerous efforts and progress have been made in proteomics technology in the last few years, its application in biomarker discovery and drug development is still at its early age, however. Improvements are still needed in rising the capacity for analyzing large size of samples, refining the resolution for separating

low abundant proteins and increasing the sensitivity for identifying small amounts of proteins by MS. High-throughput proteomic screening platforms integrated with genomics and bioinformatics for identifying biomarkers, drug targets, and drug leads represented one of the developing directions. Explorations in pre-fractionation of sub-proteomes for better resolution and in chemical probes for higher sensitivity and specificity are also encouraging. It can be anticipated that, by complement with other new technologies, proteomics holds great potential for identifying many specific biomarkers for clinical diagnosis, large source of drug targets for therapeutic intervention, and monitoring markers of drug toxicity and efficacy for drug screening and design.

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