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Journal of Chromatography B, 815 (2005) 275–284
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Methods of comparative proteomic profiling for disease diagnostics

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Received 17 August 2004; accepted 22 October 2004
Available online 26 November 2004

Abstract

The recent development of numerous technologies for proteome analysis holds the promise of new and more precise methods for disease diagnosis. In this review, we provide an overview of some of these technologies including two-dimensional gel electrophoresis (2DE), historically the workhorse of proteomic analysis, as well as some newer approaches such as liquid phase separations combined with mass spectrometry, and protein microarrays. It is evident that each method has its own strengths and weaknesses and no single method will be optimal in all applications. However, the continuing development of innovative strategies for protein separation and analysis is providing a wealth of new tools for multi-dimensional protein profiling that will advance our capabilities in disease diagnostics and our understanding of disease pathology.

Keywords: Comparative proteomic profiling; Disease diagnostics; Proteins biomarkers

Contents

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doi:10.1016/j.jchromb.2004.10.072

1. Introduction

Technologies used for the parallel analysis of large numbers of proteins are advancing rapidly. The increasingly powerful tools for proteomic studies are providing new opportunities for the discovery of protein biomarkers that will be useful for diagnosing disease, monitoring disease progression or the efficacy of treatment, identifying new therapeutic targets, and understanding the underlying mechanisms of disease.

Nucleic acid based technologies have been widely used in studies of comparative gene expression profiling for biomarker discovery. However, it is essential that these studies also be carried out at the protein level. Proteins are the functional readout of genetic information and protein activity can be affected by many factors that are not reflected in the RNA transcript population (transcriptome). For instance, there can be a substantial discordance between mRNA abundance and protein expression levels [1,2]. Further, over 200 different post-translational modifications [3] can regulate protein function by altering properties such as interactions with other biomolecules or sub-cellular localization. In developing tools for disease diagnostics, it is also important to consider that many of the biological fluids that are relatively accessible for analysis, such as serum, urine, and saliva, are rich in protein but very poor sources of nucleic acids for assay.

An ideal proteome screening methodology would combine high throughput capabilities with detection of as many protein products as possible in a sensitive, reproducible, and quantifiable manner. The wide-ranging biochemical heterogeneity of proteins makes it unlikely that any single separation and analysis method will be suitable for profiling the full proteome of any cell type, tissue, or biological fluid. In the following sections, we describe several of the tools that have been used or that are being developed for protein biomarker discovery and disease diagnostics, each with its own strengths as well as limitations. Several reviews of proteomic investigations in disease diagnosis have been published [4–9]. Here, we will emphasize recent studies that are illustrative of proteomic approaches currently being used.

2. Two-dimensional gel electrophoresis for proteome analysis

2.1. The basic technology

Two-dimensional gel electrophoresis (2DE), developed in the mid-1970s [10,11], was the first method to allow the resolution and simultaneous display of hundreds of proteins. Recent improvements in the implementation of this basic technology, together with the explosion of protein sequence information resulting from genomic studies, and the development of techniques for peptide analysis by mass spectrometry, have fueled the emergence of proteomics as a powerful tool for comparative gene expression profiling.

The straightforward application of 2DE in disease proteomics is well-demonstrated by a large study aimed at the discovery of proteins that might serve as prognostic biomarkers for survival of lung cancer patients [12]. Proteins from lung tissue of 90 patients with lung adenocarcinoma were resolved by 2DE and 682 protein spots were quantified and statistically analyzed for correlations with patient survival. Using the top 20 proteins that showed a significant correlation with survival, it was possible to generate a risk index that was highly predictive of outcome for patients with early stage tumors. Of a total of 46 spots shown to correlate with survival, 33 were identified by mass spectrometry, providing information regarding biological changes associated with the tumor tissue. Importantly, one of the proteins identified, phosphoglycerate kinase 1 (PGK1), was detected in serum where it retained its strong correlation with patient survival, suggesting that it may prove useful in screens of disease progression. It is worth noting that for most of the proteins identified in this study, similar associations with survival were not found at the mRNA level when the same [13] or different [14] tumor sets were examined by microarray analysis [12]. This underscores the value of conducting studies at multiple levels of gene expression.

2DE has been used by many investigators to compare the protein complements of diseased and healthy tissue. Among the more recent studies is one in which proteins from peripheral blood mononuclear cells (PBMCs) were compared between healthy individuals and those with rheumatoid arthritis [15]. Twenty-nine differentially expressed protein spots were found in gels that could be used in hierarchical clustering for the accurate separation of healthy individuals from those with arthritis. Some of the proteins were identified by mass spectrometry and have known roles in inflammatory or autoimmune processes [15], showing that this approach will be of value both diagnostically and in helping to understand the disease pathology. Another recent study used 2DE and mass spectrometry to identify proteins whose expression is modulated in oral tongue squamous cell carcinoma [16]. Approximately 600 protein spots from ten pairs of matched tumor and surrounding non-tumor tissue were compared by 2DE and spots showing consistent differences were identified by peptide mass fingerprinting. Many of the observed changes could be explained in terms of tongue tumor pathology, the increased vascularization of the tumor tissue, or were proteins whose expression has been found to be modulated in other tumors as well [16].

While direct comparisons of tumor and non-tumor tissue are clearly informative, it is also true that interactions among the heterogeous cell types that comprise the tumor microenvironment are critical to disease progression [17]. In order to capture proteins engaged in this intercellular cross-talk, methods are being devised to sample fluids that contact diseased tissue. For instance, nipple aspirate fluid is being examined for markers of breast cancer [18] and urine is being studied for markers of urinary tract disease [19]. Proteins from fluid that directly perfuses breast tumor tissue were collected from

supernatants of short term cultures of freshly excised tumor tissue [20]. These tumor interstitial fluid (TIF) proteins were characterized by 2DE with subsequent identification of 267 proteins by mass spectrometry, immunoblotting, or comparison to existing databases. Proteins were found representing many aspects of cellular metabolism, cell-cell interactions, and angiogenesis, indicating that TIF protein profiles will likely be a rich source of information related to the interplay between healthy and diseased cells, as well as to the body's defense response to the diseased tissue.

A novel application of 2DE has been in the discovery of circulating autoantibodies in cancer patients. There is evidence of a humoral immune response against tumor antigens in some cancer patients that might be used in serum-based assays of disease progression or in the development of anticancer vaccines [21–23]. Proteins isolated from tumor tissue or cell lines are resolved by 2DE and then transferred to membranes for immunoblotting against patient sera. Immunoglobulins present in the sera that have reactivity against tumor proteins can be detected in these 2D blots and the antigenic protein can be identified by mass spectrometry after alignment of the blot with a stained gel. Sera from patients with lung cancer [21,24], hepatocellular carcinoma [22], renal cell carcinoma [23,25], and neuroblastoma [26] have been studied in this way, and in each case, autoantibodies specific to a limited number of tumor proteins were found. Although it is incompletely understood why some tumor proteins become antigenic in a subset of patients, the antitumor antibodies often correspond to proteins that are overexpressed, mislocalized, or mutant in the tumor. There is also evidence that increased cytokine activity contributes to the development of autoantibodies in some patients [21].

2.2. Limitations and improvements to 2DE technology

Despite successes with 2DE, the method has many, often-described limitations. For instance, solubility problems can lead to an under-representation of hydrophobic membrane proteins, highly basic proteins are difficult to resolve in first dimension focusing gels, and the dynamic range of detection possible in gels can be exceeded by the dynamic range of protein abundance in samples, making the detection of low abundance proteins difficult. Many of these limitations are being addressed, both through improvements to the technology and by using 2DE together with other technologies to take advantage of the complementary strengths of each. New detergents are being used to extend the utility of 2DE to more of the low solubility proteins [27,28] and work continues to improve the resolution of basic proteins [29–31]. Gels that focus proteins in a very narrow pH range in the first dimension, so-called zoom gels, can be used to increase the number of proteins resolved in the 2D system [32].

2.2.1. Removal of abundant proteins

The problems posed by widely different levels of proteins in a sample are particularly notable in serum or plasma where proteins vary by as much as 12 orders of magnitude in abundance and a small number of proteins, including albumin, immunoglobulins, transferrin, haptoglobins, α1-antitrypsin, acid-1-glycoprotein, constitute as much as 80% of the total protein [33]. Affinity based methods are available for the specific removal of many of the abundant proteins, making minor but possibly informative proteins more accessible to detection and analysis [19,34]. Improvements in the detection of some serum proteins that can be gained by removal of albumin are illustrated in Fig. 1. It is also becoming evident that proteins such as albumin and the immunoglobulins can serve as carrier proteins, able to bind potentially useful biomarkers [35]. It will undoubtedly be beneficial to examine proteins and peptides that co-elute with the abundant proteins, as well as those left in the unselected population, in any separation strategy.

2.2.2. Sample prefractionation and laser capture microdissection (LCM)

Additional methods of sample fractionation prior to 2DE analysis are being used, essentially adding a third dimension to protein separations. More low abundance proteins become detectable when gels are loaded with proteins from individual fractions, rather than the total cell or tissue lysate. Of course, disadvantages to this approach are that it multiplies both the total number of gels required and the total amount of sample necessary for a given analysis as well as introducing the potential for protein loss or degradation as the number of sample handling steps increases. Nevertheless, liquid chromatographic separations, including those based on ion exchange, hydrophobic interactions, differential affinity, and size exclusion, have all proven useful in increasing the number of proteins resolved by 2DE (reviewed in [36]). Reversed-phase high performance liquid chromatography (RP-HPLC) was used to fractionate proteins from cultured human breast epithelial cells (HBL-100), cultured B-cells (BL60-2), and rat lung tissue [37]. Subsequent 2DE showed a reproducible fractionation that allowed detection of proteins not clearly visible in gels of unfractionated cell lysates, including some that were experimentally induced by the apoptotic agent staurosporine [37]. Solution phase isoelectric focusing has also been applied as a prefractionation step. A procedure for microscale solution isoelectrofocusing (musol-IEF) has been developed that uses a series of small volume chambers to form discrete pH zones for the high resolution separation of proteins based on p*I* [38]. Experiments with mouse serum [38] and human breast cancer cell extracts [39] have demonstrated that prefractionation by this procedure increases the loading capacity and greatly enhances the resolution possible with narrow pH range first dimension IPG strips.

Another form of sample prefractionation can be achieved at the cellular level. Tumor specimens invariably contain mixed populations of cells, with variable proportions of diseased and normal cells, as well as mixed cell types naturally occuring in the tissue. Clearly, protein expression differences arising from the disease state could be masked by the heter-

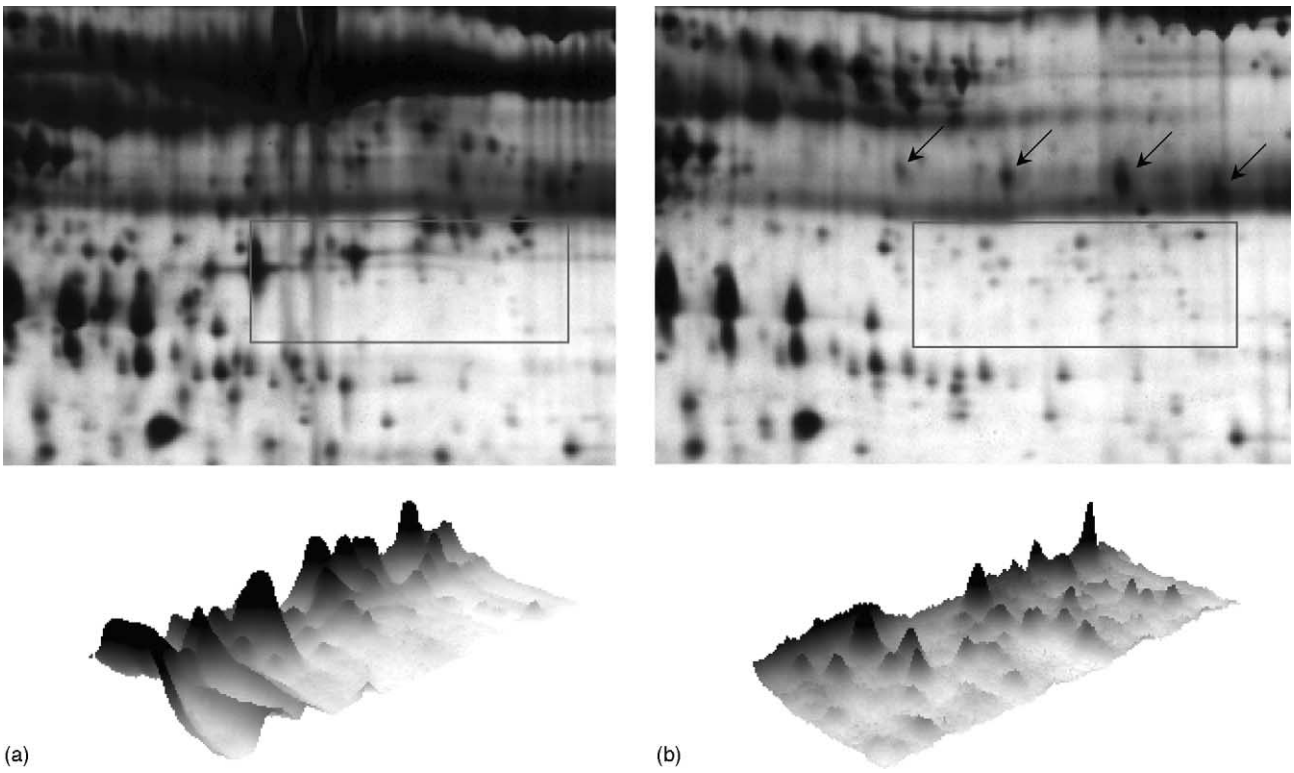

Fig. 1. Improved resolution and detection of serum proteins after human serum albumin (HSA) removal. A close-up view is shown of a 2D gel region including and just below the position of the major albumin spot: (a) gel with total serum proteins; (b) gel with HSA removed. A 3D image of the boxed region is shown below each gel. Note the numerous low abundance spots that can now be visualized for analysis. Arrows in panel (b) point to spots that are obscured in the gel shown in panel (a). Reprinted from [34] with permission.

geneity of the sample. Laser capture microdissection (LCM) allows precise dissection, so that malignant cells or their non-malignant counterparts can be cleanly separated from neighboring cells in biopsy material [40]. The value of LCM in proteomic profiling is illustrated in a recent study of pancreatic ductal adenocarcinoma (PDAC), where both normal and malignant ductal epithelial cells represent only a small percentage of the tumor mass [41]. When 2DE was used to compare proteins from non-malignant pancreatic tissue with those from normal ductal cells collected by LCM, there were numerous differences, presumably due to the small contribution made by ductal cells to the heterogeneous undissected tissue. LCM was then used to prepare populations enriched in normal or malignant ductal cells from pancreatic tumors. Nine differentially expressed proteins that varied consistently between the normal and malignant ductal cells could be detected by 2DE of the LCM collected samples [41]. In a second example, LCM was used to help in profiling proteins from matched normal ductal/lobular units and ductal carcinoma in situ (DCIS) of the breast [42]. In this study, too, distinct protein profiles were generated by 2DE of proteins isolated from frozen tissue sections or LCM collected epithelial cells, and the two methods of tissue sampling produced only partially overlapping lists of differentially expressed proteins [42]. LCM is a highly labor intensive procedure that yields limited amounts of material and so it is not suitable for screening large numbers of samples. Nevertheless, it sharply focuses comparisons of proteins found in a subset of cells from heterogenous tissue, and proteins identified in cell populations obtained by LCM can be pursued in larger sample sets by other analytical techniques, such as immunohistochemistry.

2.2.3. Two-dimensional difference gel electrophoresis (2D DIGE)

A serious bottleneck in the evaluation of 2D gels is the delineation of protein spot boundaries in the gel image, and the matching of spots in a series of gels so that quantitative comparisons can be made. Even with specialized imaging equipment and sophisticated software, the process requires time-consuming manual editing. This problem is exacerbated by gel-to-gel differences that arise from unavoidable minor variations in the efficiency of protein entry into the IPG strip, the transfer of proteins from the first to the second dimension gel, or in local areas of the gel composition itself. Two-dimensional difference gel electrophoresis (2D DIGE) is an analytical strategy designed to minimize these problems, making sample-to-sample comparisons easier and more accurate, as well as reducing the number of gels required to evaluate a series of samples [43,44], reviewed in [45].

In 2D DIGE, different size and charge matched fluorescent dyes, such as Cy3 and Cy5 derivatives, are used to covalently

label the proteins of two samples that are to be compared. The labeled protein samples are mixed together and then resolved in a single 2D gel. Fluorescent signal from the Cy3 and Cy5 dyes can be imaged separately, and the ratio of labeling can be determined for each spot, allowing quantitative comparisons between the samples to be made within individual spots in the image. Since the samples are run in a single gel, differences due to technical variations are avoided and the process of gel matching is eliminated. An internal standard can be added to the mix, comprised of a combination of equal amounts of each sample in the comparison series, labeled with a third fluorescent dye, such as Cy2 [46]. This refines the accuracy of quantitation and helps in making comparisons among multiple samples.

2D DIGE has been applied to a model system of breast cancer [47]. Protein expression patterns were compared between a cell line established from human breast luminal epithelium (HB4a) and a derivative cell line that overexpresses ErbB-2 (HBc3.6). Several proteins showing deregulation in the HBc3.6 cell line could be identified by mass spectrometry and are known to be associated with changes in cell morphology, proliferation, cell transformation, or metastasis [47]. In another study, proteins from colonic tumor tissue and nearby normal mucosa from six colorectal adenocarcinoma patients were compared by 2D DIGE [48]. Over 1500 spots were resolved and quantitatively analyzed by this method, yielding 52 discrete proteins, identified by mass spectrometry, that showed consistent differences between normal and cancerous tissue [48]. 2D DIGE has also been used to reveal differential protein expression in infiltrating ductal carcinoma of the breast (IDCA) [49].

The comparative power of 2D DIGE was combined with the specificity of LCM to discover potential markers of esophageal carcinoma [50]. Cancerous and normal squamous epithelial cells were dissected from frozen esophageal tissue sections and proteins were compared by DIGE. Numerous protein spots were found to vary more than three-fold in expression and are candidate markers of esophageal cancer. Two of the proteins were identified by mass spectrometry and their differential expression in normal and cancer cells was confirmed by immunoblotting, demonstrating the feasibilty of this approach [50].

3. Separation and analysis of proteins by liquid chromatography and mass spectrometry

There is a great deal of interest at the present time in developing gel-free systems for protein analysis because of their potential for multiplexing [51,52]. An analogy may be made to DNA sequencing, notably as utilized in the genome project which received a considerable boost when the switch from gel-based approaches to a gel-free technology took place. Multi-modular combinations of HPLC, liquid-phase isoelectric focusing (IEF), and capillary electrophoresis (CE) provide various options to develop high-resolution orthogonal 2D liquid phase-based strategies for the separation of complex mixtures of proteins. Such strategies include SEC–CE or SEC–RPLC as used by Jorgenson's group to fractionate protein mixtures in *Escherichia coli* lysates [53,54]. Le Coutre analyzed *E. coli* membrane proteins with affinity chromatography, followed by on-line RPLC–MS [55]. Feng reported the use of ion-exchange chromatography (IEC) followed by on-line eight-channel parallel RPLC–ESI-MS to purify recombinant proteins in a high-throughput fashion [56]. A major advantage of liquid separations is that proteins are maintained in solution that allows on-line intact protein characterization by MS as well as protein recovery. Our group developed a novel 2D IEF-RPLC system to fractionate or resolve large numbers of cellular proteins. These protein fractions were recovered and applied to protein biochips to determine their antigenicity in cancer [52,57,58]. The capacity of the 2D separation system in practice is limited to resolving no more than 10,000 protein forms according to Giddings' model, if each dimension has a capacity of 100; that capacity may not be sufficient to achieve complete resolution of a cell or tissue proteome. It is, therefore, beneficial to reduce sample complexity as much as possible.

With the emergence of soft ionization techniques such as fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), and electrospray ionization (ESI) more than a decade ago [59–61], biological mass spectrometry (Bio-MS) has become a standard tool for protein analysis [62]. Biological samples subjected to mass spectrometry consist of three major types: (1) tissues; (2) cell populations; and (3) biological fluids. Innovations in mass spectrometry continue to have a substantial impact on proteomics. Nano-electrospray techniques [63,64] combined with a hybrid quadrupole time-of-flight mass spectrometer tandem mass analyzer (ESI Q-TOF MS/MS) enable extensive fragmentations to produce collision-induced dissociation (CID) spectra that allow unambiguous protein identification by peptide sequence tags through protein sequence database searches. High-throughput proteomic analysis may also be performed with a MALDI Q-TOF MS/MS tandem instrument [65,66] and MALDI TOF-TOF MS/MS tandem mass spectrometry [67]. A new ion source for Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) enables quick changes between MALDI and ESI modes [68].

Mass spectrometry in conjunction with proteomics, has been utilized primarily for protein identification. However, it is possible to profile tissues and biological fluids directly using mass spectrometry. The potential of mass spectrometry to yield comprehensive profiles of peptides and proteins in biological fluids without the need to first carry out protein separations has attracted interest. In principle, such an approach would be highly suited for clinical applications because of reduced sample requirements and high throughput. This approach is currently popularized, particularly for serum analysis, by the technology referred to as surface-enhanced laser desorption ionization (SELDI) [7]. Proteins from a patient sample are captured by various types of surfaces with

different properties including adsorption, partition, electrostatic interaction, or affinity chromatography. Although such surfaces are referred to as "chips", they should not be confused with microarrays as they do not involve any type of arraying. Aside from the use of SELDI, the direct analysis of tissues or biological fluids may be simply accomplished using standard matrix-assisted laser desorption ionization without the use of proprietary surfaces. Some quite noteworthy findings have been reported using SELDI. They include the ability to accurately diagnose ovarian, prostate, breast, and other types of cancer with minimal sample requirement and with high throughput. A study of ovarian cancer that has attracted considerable attention demonstrated the ability of SELDI in combination with an algorithm, to correctly identify all cancer patients, including those with limited stage I disease [69].

MALDI mass spectrometry has been utilized in an innovative fashion to profile tissues in situ. A recent study utilized this approach to classify lung tumors based on their proteomic profile [70]. Proteomic spectra were obtained for 79 lung tumors and 14 normal lung tissues. More than 1600 protein peaks were detected from histologically selected 1 mm diameter regions of single frozen sections from each tissue. Class-prediction models based on differentially expressed peaks enabled the classification of lung cancer histologies, distinction between primary tumors and metastases to the lung from other sites, and classification of nodal involvement with 85% accuracy. The major drawbacks of direct analysis of tissues or biological fluids by MALDI or SELDI are the preferential detection of proteins with a lower molecular mass and the difficulty in determining the identity of proteins whose masses are measured because of lack of correspondence between the masses detected and those predicted for corresponding proteins, due to post-translational modifications.

There have been some concerns regarding the significance of the diagnostic patterns uncovered using SELDI because the molecules monitored in serum using this approach are likely to be present at concentrations many fold higher than traditional cancer biomarkers. Such markers, therefore, are unlikely to originate from the tumor and thus are considered to be epiphenomena of cancer produced by other organs in response either to the presence of cancer or to a generalized condition of the cancer patient such as debilitation or acute-phase reaction [71]. Thus, the role of MALDI and MALDI surfaces in profiling biological fluids remains to be determined.

4. Antibody and protein arrays

Antibody and protein arrays offer an attractive complement to separation and mass spectrometry methods for comparative proteomics research. Various technologies for probing binding interactions on arrays of immobilized antibodies, proteins or peptides are in development and use. Each technology has its own advantages, disadvantages, and optimal applications. These methods and their applications in comparative proteomics are reviewed here.

4.1. Antibody arrays

Antibody arrays are useful for measuring the abundance of multiple, specific proteins in low sample volumes. Antibody array methods are particularly well suited to profiling many candidate biomarkers in large sets of biological samples, such as serum, to identify individual proteins or groups of proteins that statistically associate with a particular condition. The multiplex capability of antibody arrays allows both the efficient testing of many individual candidate markers and also the evaluation of the use of multiple markers in combination. The use of multiple markers in combination may in some cases have higher diagnostic accuracy than individual markers. Since microarray experiments are generally rapid to run and easy to analyze, large clinical studies are possible, enabling the validation of multiple new or candidate markers.

Various technological implementations of antibody array experiments have been demonstrated. A variety of substrates and methods of antibody attachment have been used, such as passive adsorption of antibodies onto membranes [72–75], poly-L-lysine coated glass [76,77], or hydrogels [75,78], covalent linkage to amine-reactive coated glass [77,79,80], or linkage of biotinylated antibodies to streptavidin-coated glass [81]. The best choice of surface is not yet firmly established and may depend on the application or the detection method used. Factors to consider in evaluating surfaces are reproducibility and consistency in both the background and the signals, the signal levels relative to the background levels, and the ability of the surface to maintain the antibodies in their properly folded, reactive forms.

A variety of detection formats also have been employed. Sandwich assays, using a pair of antibodies specific for every target, have been developed in a chip format for the multiplexed detection of cytokines [80,82–85]. Sandwich assays have the potential for very high specificity and sensitivity of detection. Rolling-circle amplification (RCA) [82,84], tyramide signal amplification [80], and fluorescence [85] have been used as detection methods for multiplexed sandwich assays. RCA significantly enhances fluorescence signal and reduces detection limits in comparison to non-amplified fluorescence methods. Its advantages for microarray assays are that it is an isothermal process and that the amplification products are covalently attached to the spot of origin—a factor important for planar, multiplexed assays.

An alternative to sandwich assays are "label-based" assays, in which the proteins to be detected are labeled with tags that allow detection after capture by immobilized antibodies. A benefit of the label-based assays is that only one antibody per target is required (as opposed to two antibodies per target for a sandwich assay), making the development and testing of assays for new targets straightforward. This capability will be important for research in which multiple rare or newly discovered proteins are to be probed. Another

advantage of the label-based method is that competitive assays are possible, since two different samples, a test sample and a reference sample, can be co-incubated on an array. Competitive assays can lessen the requirement to match the concentrations of analytes to a particular linear range for each analyte. This feature may be important when a multiplexed assay measures different analytes in widely varying concentration ranges [86]. Competitive assays could be performed using a labeled reference sample and an unlabeled test sample [86], or both the test and reference samples could be labeled, each with its own distinguishable label [76].

Labeled proteins have been detected by fluorescence [74,78,87], RCA [75], or colorimetric methods [73]. RCA detection of labeled proteins was developed as a means to improve the detection sensitivity of the label-based antibody microarray assay [75]. Two pools of proteins were, respectively, labeled with biotin and digoxigenin and co-incubated on antibody microarrays. The biotin-labeled proteins were detected by RCA with green fluorescence and the digoxigenin-labeled proteins were detected by RCA with red fluorescence. The fluorescence was enhanced up to 30-fold relative to non-amplified fluorescence, and the reproducible detection of low-abundance proteins in serum samples was demonstrated.

Several reports have demonstrated the application of antibody microarrays to cancer proteomics research. Portions of frozen tumor specimens isolated by laser capture microdissection (LCM) were probed by antibody arrays to identify proteins both in the tumor tissue and in the surrounding stroma that had levels correlating with advancement of disease [73]. A similar study probed proteins in LCM-isolated tissue from hepatocellular carcinoma tumors and the surrounding environment, identifying proteins that may be associated with that disease [74]. Proteins in cultured colon carcinoma cells were profiled by antibody arrays to identify proteins that may be regulated in response to radiation exposure [77]. In a novel application, microarrays of antibodies spotted onto nitrocellulose specifically captured cells expressing specific membrane antigens [72]. Suspensions of leukocytes isolated from the blood of leukemia patients were incubated on microarrays of antibodies recognizing various CD antigens, and quantification of the bound cells by dark field microscopy identified antigens that accurately discriminated CLL lymphocytes from normal lymphocytes.

Another useful application for antibody arrays is to profile sets of proteins in blood serum or other readily sampled biological fluids to identify candidate markers for cancer diagnosis. Such an application was demonstrated in the reproducible and accurate measurement of multiple proteins in serum samples from prostate cancer patients and controls [78]. The clustering of antibody measurements and ELISA measurements from four replicate experimental sets measuring 53 different serum samples (Fig. 2) shows that replicate microarray measurements are highly reproducible and that the ELISA and microarray measurements substantially agree. A set of five candidate biomarkers was derived from the study with statistically different levels between cases and controls. This result established the feasibility and value of multiplexed serum biomarker detection. The further application and development of the above methods are sure to yield valuable results in cancer proteomics research.

4.2. Protein and peptide arrays

Protein and peptide arrays are complementary to antibody arrays. They are useful for probing the interactions of protein and peptides with other antibodies, proteins, or other molecules. The methods and applications of these technologies are discussed below.

"Reverse phase" protein arrays recently have proven useful for probing the abundance of specific proteins in sets of biological samples. Protein lysates from cell culture or tissue samples are spotted in microarrays onto nitrocellulose membranes. A labeled antibody specific for a particular protein is incubated on a microarray, and quantification of the bound antibody reveals the amount of that protein in each of the samples. Therefore, reverse phase array experiments measure a single protein in many samples, in contrast to antibody array experiments that measure many proteins in one sample. Several demonstrations of the use of the technology for profiling proteins in cancer have appeared. The technology was used to measure proteins relevant to apoptosis pathways in malignant and normal prostate tissue [88], to investigate defects in signaling in ovarian cancer tissues [89], and to profile multiple proteins in 60 cancer cell lines used by the National Cancer Institute to screen compounds for anticancer activity [90].

Protein arrays also have been made from purified or semi-purified proteins (as opposed to whole-cell lysates). High-throughput expression and purification methods were used to produce proteins, and the arrayed proteins were used to probe specific binding interactions. One study looked at the interactions of calmodulin- and phospholipid-interacting proteins with arrayed yeast proteins that had been expressed and purified from 5800 open reading frames [91]. An efficient method to produce arrays of proteins is to spot individual bacterial colonies of a cDNA library onto membranes, induce the colonies for protein expression, and lyse the cells on the membrane [92–94]. These arrays may be most useful for measuring protein–protein and protein–small molecule interactions, and may eventually find application in diagnostics and comparative proteomics.

Another method to produce proteins for arrays is to separate whole-cell lysates into the component protein fractions using multi-dimensional liquid chromatography. Multiple modes of separation in succession (for example, ion-exchange chromatography followed by reverse phase) have high resolving power, and liquid phase methods allow convenient fraction collection. As previously suggested [58], protein fractions separated by liquid chromatography and spotted onto microarrays could be used for the parallel interrogation of thousands of proteins. An advantage of using proteins taken from their native states is that modifications and al-

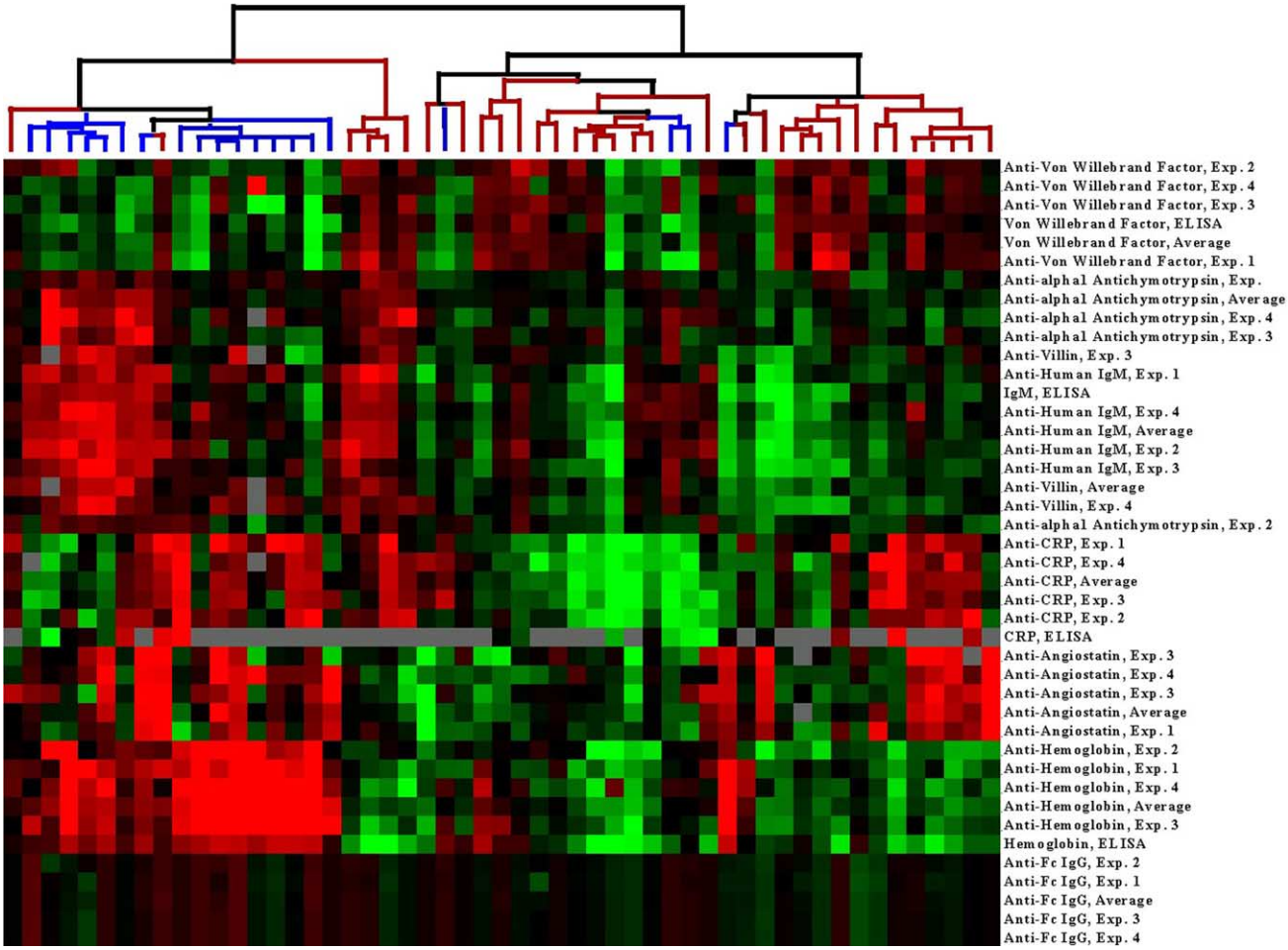

Fig. 2. Two-way hierarchical clustering of antibody microarray data. Serum samples from 33 prostate cancer patients and 20 healthy controls were measured with eight different antibodies in four independent experiment sets. The red-colored branches of the dendrogram indicate serum samples from the prostate cancer patients, and the blue-colored branches indicate serum samples from the controls. Each colored square represents one antibody measurement from one array. The color and intensity of each square represent the relative protein binding from the sample versus the reference, red representing higher from the sample and green representing higher from the reference. Black squares indicate relatively equal binding from the sample and reference, and gray squares indicate no data. Reprinted from [78] with permission.

terations to the proteins are present, in contrast to proteins expressed in foreign systems, such as in bacterial or insect cells, that may not have correct post-translational modifications. A valuable application of such microarrays is the study of immune responses in cancer patients [95–97]. Arrays of tumor-derived proteins were incubated with sera from cancer patients and controls, and the level of antibody binding to each protein fraction identified proteins that may commonly elicit immune responses in prostate cancer [95,96] and in lung cancer [97]. Circulating tumor-specific antibodies may be valuable for cancer diagnostics.

Peptide microarrays also have been powerfully used to study and characterize immune responses [98,99]. Sets of peptides from candidate targets of autoantibodies in various autoimmune diseases were collected and arrayed, and the arrays were incubated with sera from patients with autoimmune diseases such as autoimmune encephalomyelitis or multiple sclerosis. The detection of antibody binding at each peptide revealed the specificity of the autoimmune response in each patient. The mapping of immunoreactivity in the autoimmune patients could be used for diagnosis, prognosis, and tailoring of antigen-specific tolerizing therapy.

The above applications and technologies demonstrate the value of antibody, protein and peptide microarray methods. Further improvements to the technologies and dissemination of the methods should broaden their use and impact in biological research.

Acknowledgements

Work from the authors' laboratories is funded, in part, by the National Cancer Institute Early Detection Research Network. L.F.S. receives additional support from NIH grant AI055988 and Nucleonics Inc. B.B.H. acknowledges support from the Cancer Research and Prevention Foundation, the Department of Defense (DAMD17-03-1-0044) and the Van Andel Research Institute.

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