[Frontiers in Bioscience 14, 3292-3303, January 1, 2009]

Proteomics strategies for target identification and biomarker discovery in cancer

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. General considerations in sample selection for cancer proteomics
4. Two dimensional gel-based proteomics
5. LC-MS/MS-based proteomics
6. Quantitative MS and LC-MS
6.1. Sample labeling approaches
6.1.1. Stable isotope labeling of amino acids in cell culture (SILAC)
6.1.2. Heavy water labeling
6.1.3. Isotope-coded affinity tags (ICAT)
6.1.4 Isobaric Tags for Relative and Absolute Quantification of peptides (iTRAQ)
6.2. Label-free quantitation in LC MS-based proteomics
7. Biofluid peptide profiling by mass spectrometry after high-throughput single step peptide capture
8. Biomarker discovery in biofluids
9. Target identification for anti-angiogenic therapy
10. Concluding remarks
11. Acknowledgements
12. References

1. ABSTRACT

The revolution in genomics and proteomics has produced complex technologies enabling an insight into the functional effectors of cellular processes. In oncology these technologies lead to the identification of biological markers which may provide the starting point for the development and identification of diagnostic tests and therapeutic targets. To identify and validate reliable tumor markers within the proteome, it is necessary, prior to tandem mass spectrometry, to reduce sample complexity. This can be done by robust fractionation and separation techniques. This review addresses the discovery stage of onco-proteomics - the strategies for target identification and biomarker discovery in solid tumors and biofluids. The overview includes different proteomic methods, from gel-based to liquid chromatography (LC)-based separations of proteins/peptides, and the corresponding detection by mass spectrometry. The quantitative methods in mass spectrometry include techniques based on stable isotope labeling of proteins/peptides and label-free methods. A particular emphasis is given to proteomics-based biomarker discovery in biofluids (*e.g.* plasma, urine, secretome, cerebrospinal fluid) and target identification in tissue for anti-angiogenic therapies.

2. INTRODUCTION

Proteomics represents the large-scale analysis of protein expression, post-translational modifications and protein-protein interactions, thereby providing a link between gene and cellular function. Proteomics has been used for the analysis of proteins isolated from cells, tissues and biofluids. Proteomic data are validated and expanded by various technical disciplines including cell imaging by light and electron microscopy, protein, antibody and tissue arrays, DNA microarray/DNA chip and genetic readout experiments. Mass spectrometry (MS), in which biomolecules are ionized and their mass is measured following their specific trajectories in a vacuum system, has become the method of choice for the detection, identification and quantitation of proteins in complex biological samples. Large scale MS-based proteomics has become possible with the development of atmospheric pressure ionization methods in 1988 allowing for controlled ionization, fragmentation and subsequent analysis of large biomolecules (1, 2). The soft ionization techniques, electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) have revolutionized biological MS and introduced the grounds for disease-oriented proteomics. The availability of whole genome

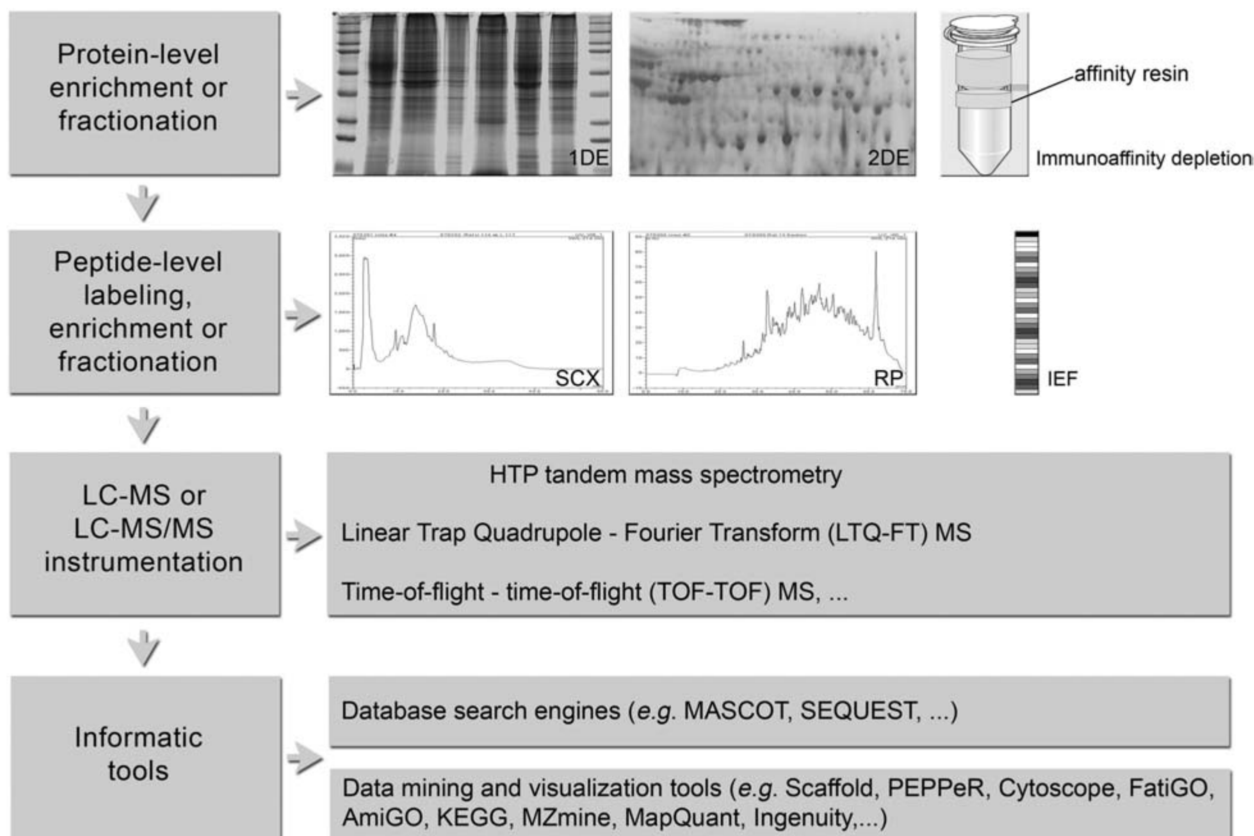

Figure 1. Schematic of possible proteomic workflows with regard to sample separation techniques at the protein or peptide level, mass spectrometry for protein identification and bioinformatic tools for data analysis. Common methods of protein enrichment include protein separation by size (one dimensional electrophoresis – 1DE), by size and isoelectric point (two dimensional electrophoresis - 2DE), and by affinity-based chromatography, where proteins are enriched based on preferential binding to a separation resin. At the peptide level, enrichment can be accomplished e.g. by liquid chromatography based on peptide charge (strong cation exchange - SCX) or hydrophobicity (reverse phase - RP), or based on the isoelectric point by isoelectric focusing (IEF) of peptides on an immobilized pH gradient gel. The enriched protein (or peptide) samples are detected and quantitated by high-throughput (HTP) tandem mass spectrometry, such as Linear Trap Quadrupole Fourier Transform LTQ-FT MS (*e.g.* in label free quantitation experiment) and TOF-TOF MS (*e.g.* in label based quantitation experiment). The resulting mass spectra are automatically searched in protein databases to retrieve the protein identity, using search engines such as MASCOT or SEQUEST. Large amounts of data obtained by tandem mass spectrometry and database searches can be further mined for functional correlations between the proteins and pathway visualization.

sequence databases and advances in bioinformatic tools has further contributed to MS-based proteomics. In recent years, proteomics has increasingly been applied to disease-related questions including 1. discovery of disease signatures and/or identification of disease-specific biomarkers, 2. uncovering molecular mechanisms of disease and 3. discovery of novel protein targets for therapeutic intervention (3). Biomarkers are molecular indicators reflecting disease-related physiological processes. They are of particular importance to indicate cancer risk, early detection, prognosis and to assess therapeutic response. The advantage of protein-based biomarkers include their proximity to (patho-)physiological processes, their structural diversity and a potential coupling to routine, antibody-based diagnostic assays.

The complexity *i.e.* the dynamic range of the human proteome is too large to be fully resolved with a single platform in a high-throughput manner. A strong experimental design including reduction of sample complexity prior to analysis is essential (Figure 1). Currently most proteomics strategies rely on a 'bottom up' approach meaning that at some point in the workflow, the (sub-) proteome of interest is digested and analyzed at the peptide level.

Excellent reviews on the different separation techniques and MS instruments for peptide analysis are available elsewhere (4). Here we will present the most common MS-based proteomics strategies (Figure 2) and highlight their use for biomarker discovery and target identification in cancer research.

3. GENERAL CONSIDERATIONS IN SAMPLE SELECTION FOR CANCER PROTEOMICS

For the discovery of reliable cancer biomarkers using patient material, standard operating procedures for

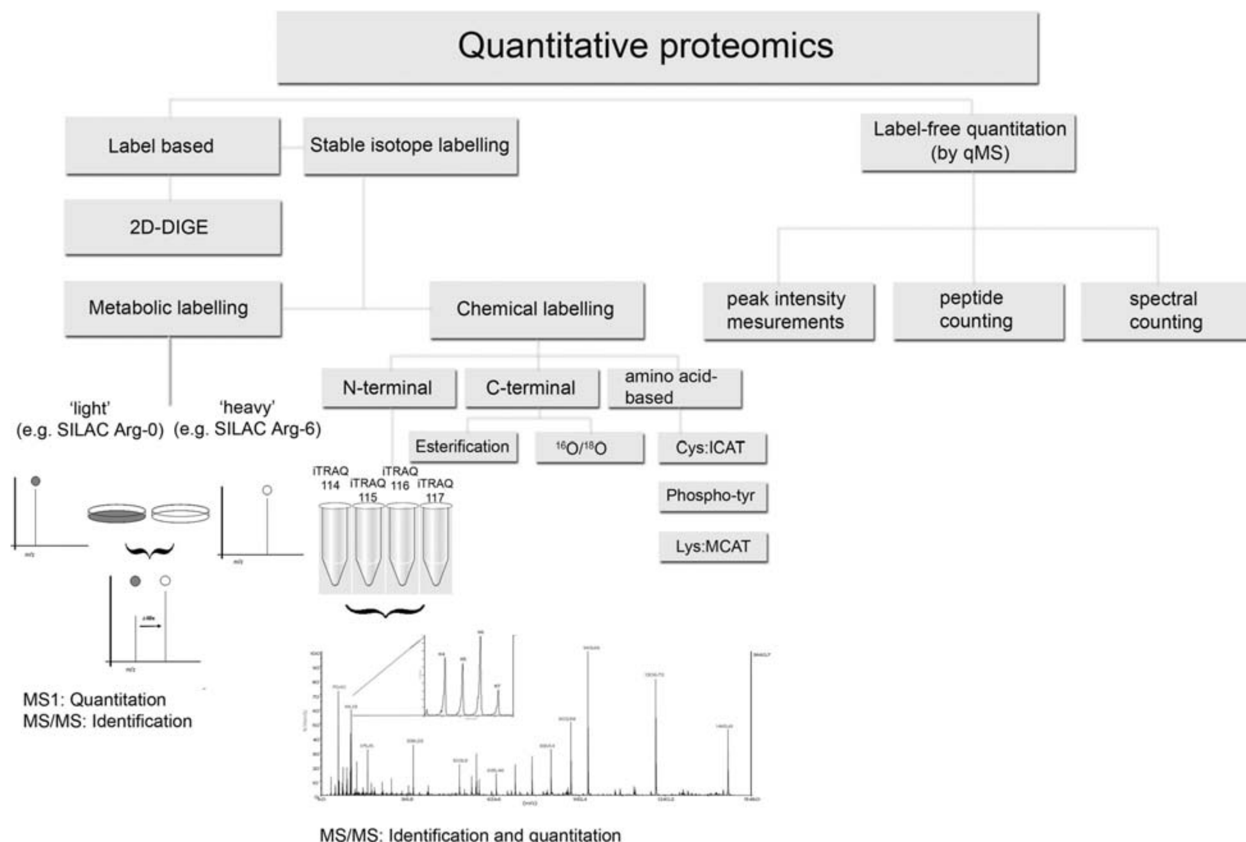

Figure 2. Diagram showing possible high throughput quantitative proteomic approaches. Quantitation of proteins/peptides is possible with label or label-free techniques, depending on the downstream applications and the technology available. A common labeling approach for gel-based proteomics is protein labeling with fluorescent CyDyes followed by 2D gel separation, where the quantitation of protein expression is assessed through the analysis of label intensity of gel images. Proteins are identified after quantitation. Stable isotope labeling includes metabolic and chemical labeling of the proteins or peptides. In metabolic labeling, cells are grown in media enriched in stable isotopes (*e.g.* Arg-0 and Arg-6 in SILAC experiment). Chemical labeling procedures involve chemical incorporation of different stable isotopes on a certain reactive terminus of the peptide/protein (N-terminal, C-terminal, amino acid based). The difference in the mass of the labeled peptides allows the quantitation of different samples and protein identification is usually achieved by MS/MS. Label free assessment of protein expression by mass spectrometry is based on the correlation between the number of the spectra/peptides or peak intensity and the abundance of the detected protein.

both clinical specimen handling and reduction of sample complexity is of utmost importance. Clinical tissue samples need to be well-defined in terms of pathology / morphology/ histology and body fluids need to be properly handled in a consistent manner. Factors that need to be considered before embarking on a cancer proteomics discovery experiment include: 1. sample selection (clinical or research model) appropriate for question/purpose of the research, 2. isolation of the relevant sub-proteome from the sample and 3. establishment of the optimal experimental conditions for each sample type. For example, a relevant proximal fluid (*e.g.* urine for bladder cancer, cerebrospinal fluid for glioma) may provide an enriched source for biomarker discovery as opposed to blood-plasma. Subsequently targeted detection techniques such as multi-reaction-monitoring-MS and antibody-based methods may be used in a second stage of analysis to validate the biomarker candidates in a larger series of samples and if needed in a convenient biofluid such as serum. If tissue is the sample of choice for discovery, tissue micro-dissection may be employed to reduce cellular heterogeneity and thereby enrich for relevant components (*e.g.* tumor cells). Furthermore, a careful pathological evaluation is important to ensure selection of well-defined clinical samples. With respect to sample processing, suitable buffer conditions, minimal lysis times and high yield protein isolation are recommended. For each sample type these conditions need to be evaluated and optimized and the reproducibility of the whole procedure assessed. Below we describe several commonly used strategies for downstream quantitative protein and peptide profiling. Table 1 provides an overview of these strategies along with a listing of their applications, depth of analysis, sensitivity and throughput.

4. TWO DIMENSIONAL GEL-BASED PROTEOMICS

In the two-dimensional gel electrophoresis (2DE) – based proteomics, proteins in a complex sample are separated on a polyacrylamide gel, first based on their iso-

Table 1. Overview of the most common methodologies used in cancer proteomics with regard to application, throughput, sensitivity, observed mass range, number of detected peptides/proteins and the possibility of protein identification

Method	Application	Throughput[1]	Sensitivity[2]	Observed mass range	Nr detected peptides/ proteins	Protein identity
High-throughput						
bead-MALDI-TOF-MS	pattern diagnostics	very high	low[3]	800-20'000 Da	200-400	optional
SELDI-TOF-MS	pattern diagnostics	high	low[3]	2'000-20'000 Da	50-150	no
In-depth						
2DGE	biomarker/ target discovery	low	medium	> 5 kDa	1'000-2'000	optional
1D-LC-MS/MS	biomarker/ target discovery	medium	medium (depl.)- high (enrichment)[4]	> 5 kDa	1'000-2'000	yes
2D-LC-MS/MS	biomarker/ target discovery	low	medium (depl.)- high (enrichment)[4]	> 5 kDa	>2'000	yes
GeLC-MS/MS	biomarker/ target discovery	low	medium (depl.)- high (enrichment)[4]	> 5 kDa	>2'000	yes

[1]Throughput; very high: > 100 samples per day; high: tenth of samples per day; medium: 1-2 hours per sample; low: > 10 hours-several days per sample. [2]Sensitivity; low: ug/ ml; medium: ug/ml- high ng/ml; high: ng/ml. [3]In combination with selective affinity surfaces (as in ImmunoMS) sensitivity may be greatly enhanced (medium). [4]In combination with abundant protein depletion (depl.) in body fluids and/or selective enrichment strategies (*e.g.,* sub-cellular fraction, glycoproteome) sensitivity is greatly enhanced.

electric point (pI) and in the second dimension based on their molecular weight. Protein samples can be pre-stained with fluorescent dyes (*e.g.* CyeDyes) which label the amino acid lysine. Quantitation of individual spots is based on staining intensity and is carried out on gel images using dedicated software. Spots of interest are excised, *in gel* digested with specific proteases (normally trypsin) and analyzed by MS. Matrix assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS) and electrospray ionization tandem MS (ESI MS/MS) are efficiently used for the identification of gel separated proteins. In MALDI-TOF MS spectra, the observed mass of a peptide is presented by its mass to charge ratio (*m/z*), and 'peptide mass fingerprints' are matched to the calculated peptide masses of a particular protein in the database. In ESI MS/MS the ionized peptides are sequenced and the sequences matched to the databases. 2D gel-based proteomics has a limited dynamic range often identifying the same high abundant proteins regardless of the system studied. This is especially apparent when analyzing unfractionated protein mixtures from whole cell/tissue lysates (5). Other analytical limitations of 2DE are the rather poor separation of high molecular weight and hydrophobic, membrane spanning proteins (6) and the co-migration of proteins complicating the quantitation of the spots (5). Several strategies are available to improve this technique including more sensitive staining methods (7, 8), larger gels (9), sample fractionation prior to separation, limited range IEF (gel zooming), 2DE compatible detergents and automation. A major advantage of the technique is its capacity to detect posttranslational changes by differential migration on the gel.

Using fluorescent labels, two-dimensional differential in-gel electrophoresis (2D-DIGE) has been successfully applied to study mechanisms of tumor (10), biomarkers in plasma and serum (11-13) and proteome differences in varying cancer types (14-16). In a search for biomarkers for routine screening in high-risk patient groups, Bengtsson and collaborators (14) screened more than 60 tissue samples from ovarian cancer. They identified statistically valid markers using non-supervised methods that distinguished between normal, benign, borderline, and malignant tissue. Nevertheless 2D-electrophoresis remains a labor-intensive approach that requires considerable skill and the throughput is limited. Therefore, for protein profiling of large series of clinical samples, gel free analysis coupled to liquid chromatography (LC-MS) or one dimensional gels coupled to LC (GeLC-MS) may be the methods of choice (Figure 1).

5. LC-MS/MS-BASED PROTEOMICS

Multi-dimensional separation-based approaches at the protein and peptide level are frequently coupled to liquid chromatography (LC)-MS which allows for automation (Figure 2). For biofluids, important up-front fractionation includes abundant protein depletion (17) and/or enrichment for information rich subproteomes such as the albuminome (18) or glycoproteome (19). For tissues, sub-cellular fractionation into *e.g.* organelles, (plasma-)membrane or nuclear fractions will enrich for subsets of proteins and increase sensitivity (20).

In a gel-free proteomics pipeline, mixtures of proteins are digested to peptides by specific proteases and separated - depending on the complexity of the sample - by one or more dimensions of LC, and coupled to automated MS/MS (21) (Figure 1). Microcapillary reverse phase (RP) LC (22-24) was initially used to separate the peptides on the basis of hydrophobicity. To be able to cope with the enormous dynamic range of the proteome it soon became clear, that more than one dimension of peptide separation is needed to provide enough separation power for the mass spectrometer to process hundreds of thousands of peptides present in these complex mixtures. Currently, mostly multidimensional peptide separations are used. The most common two-dimensional LC separation applied combines strong cation exchange (SCX) chromatography with RP chromatography coupled with automated MS/MS, first described as multidimensional protein identification technology (MudPit) (25-28). The peptide fractions separated by RP are injected to the mass spectrometer via online ESI or spotted to the MALDI target plates for subsequent offline analysis by MS/MS (29-31).

In an alternative combined gel- and chromatography-based bottom-up approach, proteins in a mixture are digested in-solution and separated on an immobilized pH gradient gel. 'Focused' peptides are extracted from gel pieces and subjected to LC-MS/MS. Using that approach 1820 distinct proteins were identified with capillary IEF from microdissected tissue samples from glioblastoma cancer patients (32-36). Another powerful method combines classical SDS polyacrylamide gel electrophoresis (SDS-PAGE) with LC in a so-called 'GeLC' approach. Complexity in the protein mixture is reduced by size separation of proteins using SDS-PAGE separation, proteins are in-gel digested and the resulting peptides are separated using single or multiple dimensions of LC (29, 37). The above described in-depth profiling methods are also amenable to quantitative MS, as described in the following sections.

6. QUANTITATIVE MS AND LC-MS

6.1. Sample labeling approaches

Quantifying changes in protein abundance between samples is a key requirement for profiling differences in cell state at the molecular level. Several methods for relative quantification have been developed either with stable isotope labels or chemical labels (reviewed in (38)) (Figure 2). In these methods proteins or peptides are differentially labeled by isotopically 'light' and 'heavy' labels. The samples (usually two or more) are pooled, separated, and peptides common to all the samples appear as peak pairs (triplets *etc*.), differing in mass determined by isotope label used. Molecular ion intensity as determined by peak heights or peak areas is compared to an estimated abundance of the peptides between the compared samples.

6.1.1. Stable isotope labeling of amino acids in cell culture (SILAC)

This metabolic labeling approach allows for multiplexing up to 3 labels. Stable isotope containing amino acids are added to cell culture media and incorporated in the proteins by metabolic incorporation (*in vivo*). Isotope labeled peptides are identified and quantified in the MS spectra as precursor ion pairs (or triplets) differing in mass by known amount and their relative abundance is measured by comparing peak intensities or areas. This approach has been successfully used to identify protein-protein interactions involved in EGFR signaling (39) and in insulin-dependent GLUT4 interactions (40).

6.1.2. Heavy water labeling

Tryptic proteolysis of proteins in the presence of light (H_2O^{16}) or heavy (H_2O^{18}) water results in enzymatic labeling of peptides with light (^{16}O) or heavy (^{18}O) oxygen by natural exchange of two oxygen atoms from the carboxy terminus of the proteolyzed peptides with two oxygen atoms from the surrounding water molecules (41). The heavy oxygen isotope labeled peptide differs from light oxygen isotope labeled peptide by 4Da and can be detected by a corresponding mass shift in the mass spectrum.

6.1.3. Isotope-coded affinity tags (ICAT)

The isotope-coded affinity tags (ICAT) (42) are biotin-tagged chemicals that are added onto the cysteine residues in proteins. Protein samples are labeled with isotopically distinct tags, combined and digested. The biotin tag allows isolation of ICAT labeled peptides by affinity chromatography which greatly reduces sample complexity, while keeping high protein coverage. Following enrichment of ICAT-labeled peptides, the sample is separated by (multidimensional) separation techniques and analyzed by either ESI MS/MS or MALDI MS/MS (30). Co-immunoprecipitation of proteins followed by analysis using ICAT LC-MS/MS technology has identified differences in the composition of subunits of 20S proteosomes (43), proteins interacting with transcription factors and large polymerase II pre-initiation complex (44, 45) and the mSin3 co-repressor complex (46). Moreover, organelle purification in conjunction with ICAT labeling and nanoLC-MS/MS provided a first comprehensive description of the protein constituents in the post-synaptic density of rat brain (47).

6.1.4. Isobaric tags for relative and absolute quantification of peptides (iTRAQ)

iTRAQ technology is a multiplexing protein quantitation strategy that provides relative and absolute measurements of proteins in complex mixtures (48). In this method, isobaric tags different for each sample are chemically linked to amine residues on peptides (N-terminal amine and lysine side chain amine). The differentially labeled intact peptide masses are indistinguishable, but produce diagnostic fragment peaks in MS/MS mode that provide relative quantitative information on proteins. iTRAQ reagent strategy currently contains up to eight isotopically distinct tags (an advantage over three in SILAC and two in ICAT) enabling time course and disease progression experiments in a multiplexed fashion. Straightforward labeling protocol, efficient and ubiquitous labeling of the peptides has rendered iTRAQ increasingly popular. Spiking the samples with an internal standard allows for absolute quantitation. This method has been applied to differential protein profiling in lung cancer where 51 differentially expressed proteins were found during epithelial-mesenchymal transition (49). In a search for biomarkers in endometrial cancer nine potential candidates have been discovered, using a combination of differentially labeled tags, iTRAQ and cleavable ICAT (cICAT) (50).

6.2. Label-free quantitation in LC MS-based proteomics

Recently, measurements of mass spectral peak intensities and spectral counts have emerged as promising methods for quantifying changes in protein abundance. Several studies have demonstrated a relationship between protein abundance and sampling statistics, such as sequence coverage, peptide count, and spectral count in label-free LC MS/MS shotgun proteomics (51, 52). Of these parameters, spectral counts, the number of MS/MS events observed for a protein in the mass spectrometer, shows the highest technical reproducibility (52). Spectral counting proved to be a more sensitive method for detecting proteins that undergo changes in abundance, as compared to peak area

intensity measurements that yield more accurate estimates of protein ratios (53). Quantification of absolute ion abundance may turn out to be a more sensitive method, as it does not rely on shotgun sequencing, so undersampling is not an issue. However, a sophisticated LC-MS data pre-processing pipeline is needed for this type of label-free analysis. Working towards this goal, there has been during the last 2 years an explosion in new bioinformatics tools that aid in automated data normalization, time alignment, peak detection, peak quantification, and peak matching, including MapQuant (54), MZmine (55), MSInspect (56), OpenMs (57) and PEPPeR (58). An inventory of pipe-line requirements and a comparison of open-source/freely available tools have recently been presented (59).

7. BIOFLUID PEPTIDE PROFILING BY MASS SPECTROMETRY AFTER HIGH-THROUGHPUT SINGLE STEP PEPTIDE CAPTURE

Biofluid peptide profiling approaches combining peptide capture with surface enhanced laser desorption ionization (SELDI)-TOF-MS or MALDI-TOF-MS, to measure intact peptides by their accurate molecular weight (mass) have proven to be a powerful tool for surveying endogenous small proteins and peptides comprising the serum peptidome or degradome (reviewed in (60)). These profiling methods are complementary to techniques such as electrophoresis and LC, which have a bias for detecting larger molecules. Using global capture chemistries such as C8 or C18 in serum, sensitivity is limited to high abundant peptides. However, when targeted affinity approaches are used, sensitivity is greatly enhanced while throughput is maintained. MALDI-TOF-MS-based serum profiling approaches are reproducible when careful study design and sample handling is combined with carefully controlled instrument calibration, automated sample preparation, and robust data preprocessing and analysis.

The clinical application of serum peptide profiling combines MS with bioinformatics to discover signatures of subsets of peptides and define a diagnostic classifier. Targeted tandem MS in a second stage of analysis may be optionally employed for identification of peptides underlying disease signatures. Over the last 5 years, many studies have established distinctive serum peptide patterns that correlate with clinically relevant parameters in cancer (see references in (61, 62)) (reviewed by Omenn *et al.* 2006). In particular, the pattern of processing by cancer-specific exoproteases may provide specific surrogate biomarkers (18, 63). In this study it was shown that a 68 peptide signature accurately distinguished 3 cancer types and controls. Recently, Lopez *et al.* (64, 65) applied MALDI-TOF-MS-based serum peptide profiling to albumin-bound peptides and proteins in an ovarian cancer study (65). Several biomarker panels enabled differentiation of stage I ovarian cancer from unaffected (age-matched) control women. Interestingly, targeted MS/MS of signature peptides isolated using the carrier protein-based approach identified protein fragments, many from low-abundance proteins or proteins not previously seen in serum. Together, the above findings suggest that serum low molecular weight proteome or peptidome contain fragments derived from 2 sources: (a) high-abundance endogenous circulating proteins and (b) cell and tissue proteins. Obviously, for a comprehensive analysis of cell and tissue-derived proteins in serum, more elaborate fractionation is needed combined with LC-MS/MS (see below).

8. BIOMARKER DISCOVERY IN BIOFLUIDS

Due to the complexity and large dynamic range of protein levels in biofluids, for large scale protein identification, multi-dimensional separation-based approaches at the protein and/or peptide level have emerged, most often coupled to LC-MS/MS. One promising approach to enrich for potential biomarkers while reducing sample complexity is the analysis of the glycoproteome of serum, cerebrospinal fluid and urine (66-68). Disease-specific glycosylation of proteins, secreted or stored, represent a rich source of potential disease markers in body fluids. Proof-of-principle of glycoproteomics was demonstrated in the analysis of plasma from mice with carcinogen-induced skin cancer where it was shown that normal and cancer sera can be unambiguously discriminated using unsupervised clustering of the resulting LC-MS peptide patterns (19). Targeted MS/MS of differential peptides in bladder cancer patients using concanavalin A affinity chromatography coupled to nanoLC-MS/MS revealed 186 proteins (including low abundant and 40 novel proteins) (69). Several proteins were associated with the presence of bladder cancer. These findings warrant further studies in large cohorts of bladder cancer patients.

Alternatively to the analysis of natural body fluids, proteins secreted or shed from cancer cells and tumor tissue (secretome) may provide a new avenue for discovery of blood-based biomarkers. During the last 2 years, several studies have shown differential analyses between the secretomes of cancer cell-types or –stages. Moreover, selected secretome candidates have been validated using antibody-based assays in serum or tissue microarrays (reviewed by Jimenez *et al.* (70)). In a pioneering study for breast cancer biomarkers in tumor derived medium (71), 267 proteins were identified including proteins involved in cell adhesion, motility and invasion. In the medium of prostate cancer cells, prostate-specific antigen (PSA) was detected in response to androgen stimulation (72) indicating the potential to identify low abundant proteins within the secretome. Two recent 2D-LC-MS/MS studies of considerable interest focused on breast cancer cell lines reflecting different stages of progression (73, 74). Analysis of conditioned media produced from 3 different breast cancer cell lines representing semi-normal, non-invasive and metastatic origins revealed about 1100 unique proteins in addition to known blood biomarkers such as

several kallikreins, various proteases, receptors, protease inhibitors, cytokines and growth factors. Moreover, of the 15 proteins previously reported to be altered in serum or tumor from women with breast cancer, 10 were found in the secretome of the tumor cell lines. These and other studies indicate that secretome analysis is a promising alternative approach for mining low abundant proteins. Many of the identified proteins may show a potential as circulating serum-based biomarkers.

9. TARGET IDENTIFICATION FOR ANTI-ANGIOGENIC THERAPY

An important turning point in solid tumor growth is the transition from the avascular to the vascular phase (1). Formation of blood vessels in the tumour bed allows unlimited tumor growth and facilitates metastasis, as tumour cells are in close contact with the vascular bed (2). Anti-angiogenesis therapy is a potentially powerful strategy to block and/or reduce tumor growth. The identification of novel angiogenesis-specific proteins is crucial for the development of new anti-angiogenic therapies and for the establishment of new biomarkers to follow treatment response.

Using a 2DE approach a set of proteins was identified that are potentially involved in the synergistic anti-angiogenic effects of vinblastine and rapamycin (75). Changes in the relative abundance of over 1000 proteins were detected in primary human endothelial cells treated with the angiogenesis-promoting steroid sokotrasterol sulfate using quantifiable cleavable ICAT labeling linked to microcapillary reversed-phase (microRP) LC-MS/MS (76). Among the up-regulated proteins, several were identified to be involved in cell communication and morphogenesis that were novel to endothelial cells. In another study aiming at identifying angiogenesis-related proteins in glioma, glioma blood vessels were dissected by laser microdissection and subjected to MALDI Fourier Transform mass spectrometry (MALDI FTMS) and nano-LC MALDI TOFTOF MS (77). 16 peptides were expressed in the glioma vessel group, compared to the normal brain, and four were confirmed by nano-LC MALDI TOFTOF MS. The expression of two proteins, colligin 2 and fibronectin was confirmed by immunohistochemistry.

In our laboratory we applied 4-plex iTRAQ technology linked to LC-MALDI on a human/rat xenograft glioblastoma model to identify differential protein expression in highly infiltrative, non-angiogenic brain tumors compared to fully angiogenic tumors (Figure 3). The animal model is based on the serial xenotransplantation of glioblastoma tumor spheroids in the brain of immunodeficient rodents (Figure 3A) (78). In this model the complex features of invasion and angiogenesis are partially separated into an invasive tumor in early generation rats and highly angiogenic and less invasive tumor in late generation rats. Invasive tumors show stem cell markers, such as nestin, vimentin, musashi (78). Although comparative genomic hybridization (CGH) showed the invasive and angiogenic tumors as well as the corresponding initial human GBM to have almost identical chromosomal profiles, DNA microarray analysis revealed specific gene expression patterns. This suggests that rather than clonal selection it is transcriptional regulation and interaction with the microenvironment that drives the phenotypic shift (78). Profiling the proteome in these two tumor phenotypes in our xenograft model is thus an important step forward in understanding the molecular mechanisms leading to invasion and angiogenesis. By iTRAQ we were able to identify over 7000 proteins (C.I. >95%) in membrane enriched fractions (Figure 3B), of which about 3000 were isoform and species specific (Rajcevic *et al.*, unpublished data). With specially developed software we were able to distinguish between the isoform-specific rat/stromal and human/tumor proteins and thus separate the host-tumor compartment of the model at the bioinformatics level. About 300 proteins (about 10%) were regulated at least 1.5 fold in angiogenic *vs*. invasive phenotypes ($p<0.05$). The majority of regulated proteins was of human origin (tumour-derived) and was upregulated in the angiogenic tumors. Based on protein profiling (Figure 3C) we assume that the host may be strongly involved in triggering the angiogenesis in the tumors, whereas the tumors themselves support the vascularisation, when triggered. The differential phenotypes of the xenograft model and our ability to distinguish between tumor and host proteins using advanced bioinformatics tools, provides a unique possibility to study the angiogenic switch in the context of tumor/host interactions.

10. CONCLUDING REMARKS

The quest for reliable, high quality cancer markers and specific therapeutic targets using various proteomic approaches requires ever increasing power of the proteomic analysis to yield proteins with the necessary sensitivity and specificity to discriminate between normal and cancer cells. Since the development of soft ionization techniques for bio-polymers including proteins, in mass spectrometry, huge progress has been made in MS-based proteomics with respect to sample processing, sensitivity, speed of data acquisition and data analysis. The challenge of the complexity of proteomes has been (partially) overcome by the combination of multidimensional fractionation and separation strategies. However, multi-dimensional strategies are typically low throughput. Therefore, the discovery phase of in-depth cancer proteomics studies to date, have generally yielded large numbers of promising biomarker/target candidates in relatively low numbers of biological samples. These candidates need to be followed up and validated using alternative high throughput methods. In addition to classical antibody-based techniques, targeted mass spectrometry employing multi-reaction monitoring is emerging as a promising high throughput, antibody-free validation approach to bridge the gap between discovery and clinical assay development. Only upon validation in independent cohorts, and with the right tools (high affinity reagents,

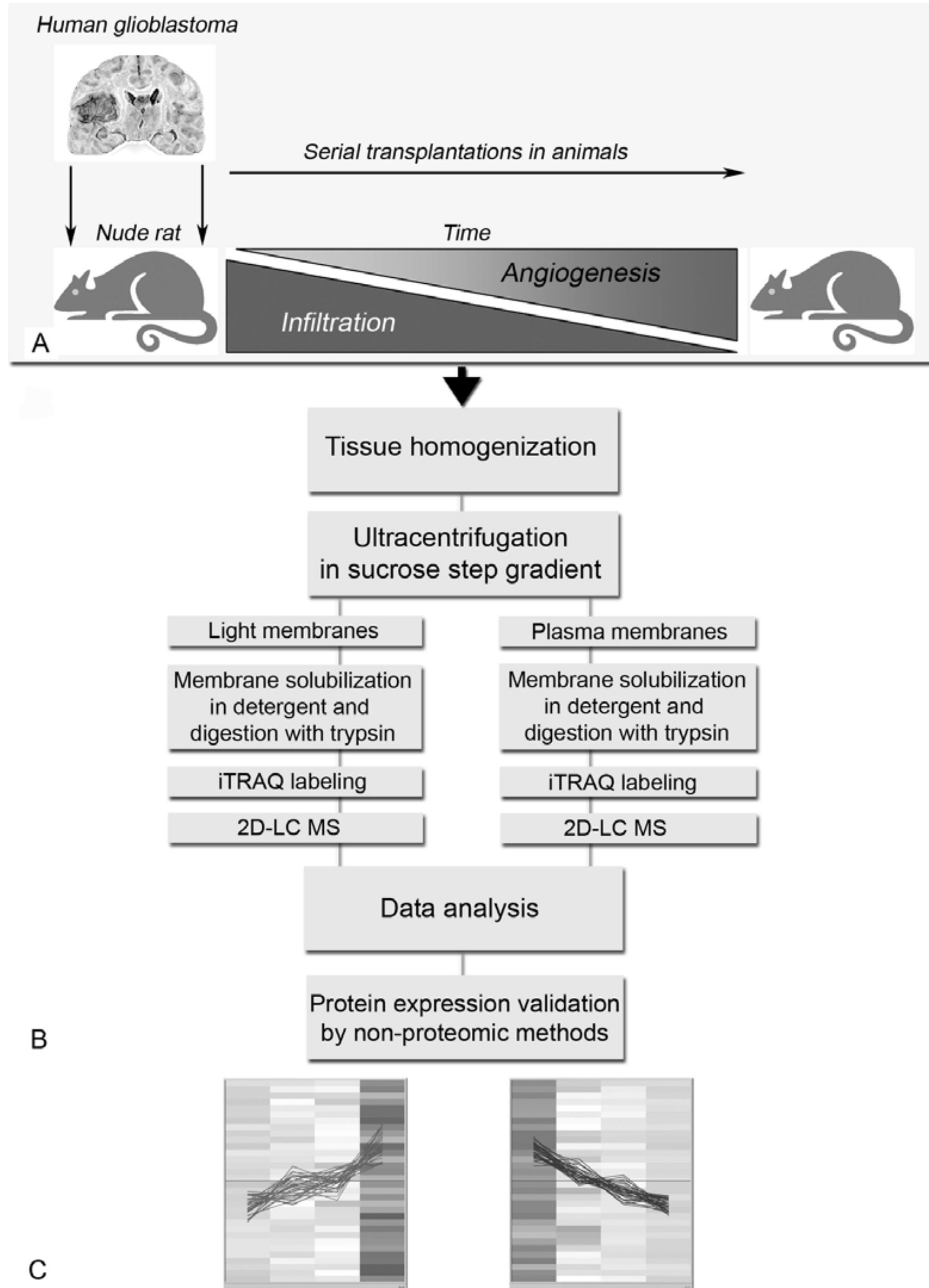

Figure 3. A workflow diagram of an iTRAQ 2D-LC MS approach applied to assess the protein expression differences in two distinct human Glioblastoma multiforme (GBM) xenografts growing in nude rats. (A) One phenotype displays a highly infiltrative angiogenesis independent growth, whereas by serial transplantation in animals, the tumour develops into a less infiltrative highly angiogenic phenotype (78). Thus, this model can be used to identify proteins involved in tumour cell invasion as well as angiogenesis. (B) Sample processing: tumors are homogenized in HEPES buffer, the membranes are separated by ultracentrifugation in a sucrose step gradient, solubilized in detergent (Rapigest, Waters, USA), digested by trypsin and labeled by 4 different iTRAQ labels (Applied Biosystems). Labeled samples are separated in two dimensions of LC and detected and quantitated by MS. The protein expression data are validated using non-proteomic techniques (*e.g.* Western blotting or immunohistochemistry). (C) Two representative protein profiles are shown, one representing proteins upregulated in the infiltrative phenotype and the 2nd one depicting proteins downregulated in the angiogenic phenotype.

typically antibodies), the translational medicine phase can be initiated and the promise of cancer proteomics exploited.

11. ACKNOWLEDGEMENTS

The authors acknowledge the support from the European Commission FP6 Integrated Project Angiotargeting (Contract No 504743) and from the Ministry of Culture, Higher Education and Research of Luxembourg.

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Key Words: Cancer Proteomics, Mass Spectrometry, Label-Free Proteomics, Quantitative Proteomics, Angiogenesis, Biomarkers, Biofluids, Glioma, Review

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