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Proteomic strategies in multiple sclerosis and its animal models

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Abstract

The early and precise diagnosis, the prognosis, and the clinical management of multiple sclerosis, remain a considerable challenge. In recent years, the development of novel and powerful proteomic techniques prompted the use of these approaches for the search of unique biomarkers in the cerebrospinal fluid of multiple sclerosis patients. A few studies have also utilized proteomics to delineate the profile of differentially expressed proteins in animal models of the human disease in order to gain global insights into affected pathways. The identification of differentially expressed proteins may be an initial step in the discovery of novel targets and mechanisms that play critical roles in the pathology of multiple sclerosis. Based on these findings, future investigations may elucidate the events leading to demyelination, axonal damage, and neurodegeneration, providing better insights into mechanisms governing the onset and progression of the disease. Although these proteomic studies provide valuable information, they are also faced with a number of challenges. The present review discusses some of the strengths and limitations of proteomic investigations as applied to multiple sclerosis.

Keywords

Autoimmune disease; Cerebrospinal fluid; Experimental autoimmune encephalomyelitis; Glia; Spinal cord inflammation

1 Introduction

Multiple sclerosis is an inflammatory disease of the CNS affecting myelin, oligodendrocytes, axons, and neurons. The disease is commonly observed in young adults between the ages of 20 and 40 and has a higher prevalence in women as compared to men. Presenting symptoms include diplopia, optic neuritis, paresthesias, paresis, poor motor coordination, fatigue, and cognitive dysfunction. Although the manifestation of clinical signs and the disease course are variable among affected individuals, the majority of multiple sclerosis patients experience a relapsing-remitting phase during which the sudden appearance of neurological symptoms is followed by complete or partial recovery. Subsequently, disability may progress over time and become irreversible, a disease form called secondary progressive. About 20% of patients are affected by the primary progressive form during which deficits augment without remissions.

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This review was envisioned as a concise overview of the multiple sclerosis and the relevant proteomic fields. Therefore, we apologize to those who published excellent work in both areas but were not cited here.

The diagnostic criteria of multiple sclerosis include clinical signs which are disseminated in time and space, presence of lesions in the brain and spinal cord detected by magnetic resonance imaging (MRI) and occurrence of oligoclonal Igs in the cerebrospinal fluid (CSF). As the exact diagnosis may require long-term observation, individuals who are definitively diagnosed with multiple sclerosis may already be experiencing the disease for a considerable period of time. The importance of early treatment has been emphasized [1] although this issue remains controversial [2]. Presently, there are no prognostic tools to predict whether subjects will develop the mild or more severe form of multiple sclerosis. Because of the aforementioned reasons, the discovery of biomarkers that can ascertain early diagnosis and accurate prognosis is of pivotal importance.

Historically, the neurological deficits observed in multiple sclerosis were attributed to the destruction of myelin and the consequent slowing of axonal conduction. Remissions were thought to be primarily the result of limited remyelination and resolution of inflammation. Although adult oligodendrocyte progenitor cells are present in the multiple sclerosis brain, and immature or even myelin-producing mature oligodendrocytes are found in white matter lesions, proper remyelination of axons is unsuccessful especially at later disease stages [3,4]. The reasons behind failure in remyelination are not well understood and remain controversial. Several studies have shown that pathological changes are not confined to the lesion site but also occur in normal appearing white matter [5–12].

Although originally described by Charcot in the 19th century, the contribution of axonal damage to multiple sclerosis pathology was long overlooked. There is now well-established evidence indicating that axonal injury and neurodegeneration are major factors contributing to neurological decline and permanent deficits [13–19]. Initially, it was suggested that axonal injury occurs at late disease stages and is the consequence of inflammatory demyelination. Recent investigations have demonstrated that neuronal and axonal anomalies are detectable at the earliest phases of multiple sclerosis and may not always be secondary to demyelination or inflammation [19–22]. In addition, diffuse gray matter pathology, which is already detectable at the start of the relapsing-remitting disease, is observed in all multiple sclerosis phenotypes [23–25]. Despite the consensus on the importance of neuronal and axonal damage in multiple sclerosis, the delineation of underlying mechanisms remains in its infancy.

Even though increasing reports describe the cellular, morphological, and functional changes that occur in the multiple sclerosis brain and spinal cord, limited information is available regarding the molecular mechanisms underlying neurological dysfunction. Yet, these studies are essential to the design of novel therapeutic interventions. Because postmortem CNS tissue offers only limited possibility of addressing mechanistic questions at the molecular level, these issues are being investigated by use of animal models of multiple sclerosis. The best-characterized models are experimental autoimmune encephalomyelitis (EAE) and Murine Theiler's virus-induced demyelinating disease. The collective term EAE encompasses various models which appear to mimic different aspects of the human disease. EAE can be induced in rodents by immunization with myelin components or transfer of encephalitogenic T cells and leads to different clinical courses depending on the disease-inducing agent and the animal species and strain. Myelin oligodendrocyte glycoprotein (MOG)-induced chronic EAE in C57Bl/6 mice, proteolipid protein (PLP)-induced relapsing-remitting EAE in the SJL mice, and myelin basic protein (MBP)-induced acute EAE in the Lewis rat are some of the frequently used models. Ascending paralysis is the most common symptom. CNS, and in particular spinal cord inflammation, demyelination, and axonal damage are some of the pathological hallmarks of EAE. One of the advantages of these animal models is that mechanisms of neurodegeneration, demyelination, remyelination, and inflammation as well as cell–cell interactions can be studied at well-defined stages, *e.g.*, before the onset of symptoms and throughout the course of the disease, under carefully controlled experimental conditions. These

models have been extremely helpful in the design and testing of therapeutic strategies and in the elucidation of molecular events underlying the pathology of the immune and CNS cells, albeit further corroboration of findings in multiple sclerosis is necessary to confirm their relevance to the human condition. The usefulness of EAE in multiple sclerosis research, its strengths, limitations, and pitfalls have been thoroughly discussed in a recent report [26].

In past years, the rapid development of proteomic approaches prompted interest in the application of these novel and powerful techniques to the discovery of potential multiple sclerosis-specific biomarkers in biological fluids and especially in the CSF. In addition, a number of studies delineated the differential protein expression profiles in tissue obtained from rodents afflicted with EAE, in order to provide initial insights into possible molecular mechanisms involved in deleterious or reparative processes. Whereas most proteomic approaches alone are not sufficient to unravel mechanisms and determine their role in the disease, they nevertheless are potent techniques providing global information about multiple events simultaneously. The knowledge acquired by proteomics can constitute the basis for additional investigations by use of other conventional biochemical, molecular, and *in vivo* techniques which will uncover the significance of the observed changes.

2 Strengths and limitations of global proteomic approaches used in the search of protein markers in multiple sclerosis

A number of studies investigating protein markers in biological fluids of multiple sclerosis patients or in tissues of animals affected by EAE have been published. These investigations used a variety of proteomic approaches including MS [27], 2-DE [28], and protein arrays [29]. Although some of these technologies have been in existence for nearly 50 years, only recent advances have enabled the proteomic analysis of cells and tissues at quantities obtained in a research or clinical laboratory environment. Since proteomic approaches have been thoroughly reviewed in many publications [30–34], we will discuss only the basic concepts to allow concise understanding of the strengths and limitations of these techniques in the context of multiple sclerosis research.

One of the aims of proteomics analysis is to identify changes in protein expression which may govern disease onset and progression in multiple sclerosis patients and related animal models. Therefore proteomic technologies are used to quantify and identify proteins. High throughput quantification of proteins has traditionally been accomplished by 2-DE and more recently by MS techniques. These approaches are usually employed to measure relative rather than absolute quantities. With 2-DE, a protein mixture is separated first by p*I* from acidic to basic properties followed by diagonal resolution according to size. Protein spot intensities can be compared with software assistance to determine changes in protein levels. Since many proteins that are present in the cells undergo PTM, a single protein may be represented as multiple spots on a 2-DE gel. Therefore an increase or decrease in the intensity of a spot reflects alterations in the levels of only this particular “version” of a given protein, rather than all the protein variants translated from the same gene. Therefore, it is essential to verify changes in protein expression by alternative means including Western blotting or assess functional alterations by use of activity assays. A typical 2-DE gel can display ~200–2000 protein feature spots depending on the chosen p*I* and size range as well as the geometric dimensions of the gel matrices [35]. The detected proteins tend to be the ones with high copy numbers and are usually involved in cellular functions important in the maintenance of steady state, including energy metabolism, structure integrity, stress response, and protein synthesis [35]. Since there are ~25 000 human genes that could give rise to over 1 million protein products as a result of RNA splicing and PTM, the current application of 2-DE is far from being comprehensive. Many regulatory proteins including signaling molecules, cytokines, and transcription factors are not routinely detected by typical 2-DE analysis due to their low abundance. However, it is possible

to expand 2-DE-based proteome coverage. First, the use of a narrower p*I* and molecular weight (MW) range, *e.g.*, p*I* of 5–7 and MW between 30 and 60 kDa to "zoom in" on display areas allows more proteins with similar p*I* and MWs to be resolved. Second, the utilization of biochemical techniques including subcellular organelle fractionation [36], phosphoprotein capture [37], and immunoprecipitation enables the "enrichment" of specific groups of proteins facilitating the display of target proteins on 2-DE gels. Third, the development of DIGE (GE Healthcare, Piscataway, NJ) technology enables proteins labeled with up to three different fluorescent dyes to be co-analyzed in the same gel matrices, facilitating more precise protein spot matching and accurate quantification statistics [38]. Although these technical fine tunings of 2-DE sample processing allow the analysis of a larger number of low abundance regulatory proteins, the information obtained is, in many ways, qualitative rather than quantitative. This is due to the numerous sample-handling steps that make comparative quantitative analysis impractical and error prone.

As alternatives to 2-DE, MS techniques have increasingly been used for protein quantification because of their ability to measure the abundance of more proteins with higher sensitivity. The most popular paradigm consists of digesting proteins into peptides by use of trypsin, followed by the quantification and identification of the generated peptides by MS. Thus, many proteins and especially membrane proteins, which are not amenable to 2-DE analysis due to limited solubility, can be quantified by MS because the peptides produced are likely to be more soluble. Auxiliary technologies supporting MS-based high throughput proteomic analysis are commercially available. These approaches enable the large-scale repetitive analysis of biological samples, providing data that can distinguish disease specific proteomic changes from random biological variations due to increased reliability in statistics.

Proteomic quantification by MS is rapidly evolving. Three methods have been commonly employed for tissue analyses with various degree of success: (i) label-free method [39]; (ii) isotopic coded affinity tags (ICAT) [40]; and (iii) isobaric tags for relative and absolute quantification (iTRAQ) [41]. In the "label-free" method, a peptide mixture derived from the tryptic digest of total protein extracts is first separated on an LC column and peptides are sequentially eluted based on their relative hydrophobicity. The LC eluent is subsequently introduced into a mass spectrometer and the signal for each peptide is measured and registered in the data system. The arbitrary quantity of the peptide is determined by measuring the integrated area of the peak corresponding to the retention time of the peptide in the LC column. The reliability and validity of each signal can be ascertained by repetitive runs of multiple specimens as well as replicates of each sample. The peptides showing statistically significant changes can then be selected for sequence analysis on the mass spectrometer. Finally, all peptides derived from the same protein can be collated. The success of this method is dependent on very high performance instruments including highly reproducible LC and mass spectrometers capable of generating high mass accuracy [42]. However, this method is not yet amenable to 2-D LC separation, limiting the proteome coverage. On the other hand, the ICAT method can analyze chemically derivatized peptides containing cysteine residues. Two versions of the elementally identical yet isotopically different chemical tags are available for binary comparison. Peptides from two sources can be mixed together for LC and MS analysis. The advantage of this method is the ability to compare peptide quantities within the same analytical step, without the possible errors resulting from the comparison of independent analyses. Moreover, since only cysteine containing peptides are analyzed, theoretically, more proteins can be quantitatively compared within a limited instrument time. One of the limitation of this technology is that most proteins are identified based on cysteine containing peptides, therefore, cysteine-free proteins cannot be quantified.

The recent development of the iTRAQ approach, enabled the simultaneous quantification of proteins obtained from the tissue of four distinct samples. A small molecule termed "iTRAQ

tag" is covalently added onto the peptides *via* primary amines, which include peptide N-termini and lysine side chains. When labeled peptides are analyzed in a tandem mass spectrometer, a "signature ion" derived from the iTRAQ tags can be observed in a mass spectrum for quantification purposes. There are currently four versions of iTRAQ tags that can be used to label peptides from four different sources. When the mixture of labeled peptides is analyzed by MS/MS, the relative ion intensities of the four signature ions with *m*/*z* 114, 115, 116, and 117 indicate relative changes in protein expression. The key feature of the iTRAQ tags is that they are isobaric, containing combinations of stable isotopes with identical total molecular mass. This ensures that iTRAQ-labeled peptides are treated as equal for chromatographic separation. Yet, they can be quantified by MS/MS analysis based on the signature ions.

3 Challenges in the proteomic analysis of the CSF from multiple sclerosis patients

Biofluids such as the CSF, plasma, and urine have long been used for the discovery of biomarkers as they can be obtained from patients relatively easily and they often reflect anomalies in protein secretion during pathological conditions [27]. The CSF can be obtained through lumbar puncture (spinal tap) which is used for the detection of oligoclonal bands reflecting abnormalities in Ig production, an indication of intrathecal production of antibodies. Lumbar puncture is sometimes used as one of the tools that help the diagnosis of multiple sclerosis.

The precise and early diagnosis of multiple sclerosis, the unpredictability of disease progression, the outcome of a particular therapeutic treatment in different individuals, and the correct clinical management of the disease are important issues that present challenges in patient care. Identification of unique markers that would ascertain the early and accurate diagnosis of the disease, distinguish multiple sclerosis phenotypes, predict the disease course, and provide insights into the pathophysiology of the CNS would be invaluable. To address these issues, a number of studies have applied a variety of novel proteomic approaches to the global analysis of biological fluids obtained from affected individuals including plasma and especially CSF. The overall goals of such studies were to create a databank of proteins found in the CSF of multiple sclerosis patients [43] and identify proteins that are uniquely detected in multiple sclerosis as compared to controls [28,44]. However, the search for unique disease markers by the analysis of the CSF presents multiple challenges.

3.1 The design of the studies: Choice of controls

One of the factors that can contribute to inconsistencies is the choice of controls. Due to bioethical reasons, most investigations cannot use CSF from healthy individuals as controls. Instead, multiple sclerosis samples are compared to those derived from subjects with other diseases which may or may not affect the CNS. This lack of uniformity in the design of different studies renders difficult the comparison of proteomic data between published investigations. Another factor is subject-to-subject variability. This is especially important when CSF of a small number of patients are pooled for proteome analysis. Even though investigators make an effort to pool samples from individuals with comparable multiple sclerosis phenotype, the manifestation and disease course vary considerably among affected individuals. Consequently, the protein content of the CSF can be fairly diverse among subjects. It is also important to keep in mind that even in the same patient, the levels of CSF proteins may fluctuate during the course of the disease. Because multiple sclerosis is a disorder affecting many cell types and pathways simultaneously or sequentially, it is likely that proteins that are found in the CSF of patients are also present in the CSF of healthy subjects or individuals affected by other diseases, albeit at different concentrations. Therefore, the search for proteins that are uniquely present in biofluids of subjects with multiple sclerosis may not be simple. Even if proteins found in the

CSF are not exclusive to multiple sclerosis, abnormal changes in their levels may still be relevant to the pathophysiology of the disease. In this case, the reliable discovery of disease markers may depend on the combined use of proteomic approaches with quantitative methods measuring the absolute concentrations of proteins of interest. In addition, it would be desirable to systematically compare the proteome profiles of subgroups of multiple sclerosis patients at a well-defined disease stage and in large cohorts in order to identify proteins which are consistently present in the CSF at a particular phase. However, given the circumstances under which samples are obtained and the labor intensive and costly nature of proteomic methods, this is not an easy task to accomplish.

3.2 Sample collection and conservation

Another challenge is the standardization of sample collection, conservation, and processing. Artifacts may result from contaminations especially from blood during sample collection. Because protein concentrations are more than 100-fold higher in blood than in CSF and many CSF proteins are also found in plasma, even a minor blood contamination may produce artifacts. The impact of blood contamination on CSF proteome analysis was recently studied [45]. These investigators used a combination of 2-DE and LC-MS/MS to examine CSF samples which were spiked with increasing amounts of blood. Four blood-related proteins, hemoglobin, catalase, peroxiredoxin, and carbonic anhydrase I were the most abundant contaminants. Even 0.001% blood contamination was within the detection limit of the 2-D LC-MS/MS. However, it is worth noting that in multiple sclerosis the blood–brain barrier (BBB) is breached and some of the blood-related proteins detected in the CSF may be due to the opening of the BBB rather than contamination. In another study, Dumont *et al.* [43] reported that out of 18 proteins which were found in multiple sclerosis but not in non-multiple sclerosis CSF, three were potential contaminants of skin origin.

The importance of sample conservation has been exemplified by a recent controversy with respect to cystatin C, a secreted cystein protease inhibitor [46–49]. Irani *et al* [47] analyzed the CSF proteome profile of patients with multiple sclerosis and those affected by other neurological diseases or transverse myelitis. Using SELDI-TOF MS analysis, these investigators found that the ratio of a 12.5 kDa cleavage product of Cystatin C to the 13.5 kDa full-length protein was significantly elevated in the CSF of multiple sclerosis patients as compared to the other groups. The authors concluded that this ratio could be used as a biomarker to identify a subgroup of multiple sclerosis patients. However, other investigations disputed this finding and indicated that the cleavage product is an artifact of sample conservation [46, 48,50]. Hansson *et al.* [48] used a proteomic approach similar to that employed by Irani and coworkers to analyze CSF obtained from 43 multiple sclerosis and 46 healthy controls. They found that both the full-length and the 12.5 kDa cleavage products were not different in multiple sclerosis patients as compared to controls concluding that the cleavage product is an artifact of sample conservation. These latter findings were also confirmed by Del Boccio *et al.* [46]. Yet, a fourth study by SELDI-TOF MS analysis indicated that 12.5 kDa cystatin C cleavage product is lower in subjects with multiple sclerosis, neuromyelitis optica, and inflammatory disease as compared to patients with noninflammatory disorders [49]. The aforementioned contradictory studies highlight the importance of sample handling in the analysis of the CSF by proteomic approaches.

3.3 Reproducibility of the proteomic technique

An additional issue of importance is the reproducibility and sensitivity of the proteomic technique employed. This is especially critical when differential expression profiles in control *versus* experimental samples are delineated by comparison of spot intensities in two different gels, as in the case of 2-DE. Terry and Desiderio [51] evaluated the between-gel reproducibility of 2-DE analysis of the human CSF, by running a single pooled CSF in ten different gels and

demonstrated that the technique is suitable for comparative proteomics. Similar studies have confirmed the reproducibility of the iTRAQ approach [52]. Other reports corroborated the consistency in results obtained by both 2-DE and iTRAQ [53].

4 Proteomic analysis of the CSF obtained from multiple sclerosis patients

A number of studies have used proteomic approaches in order to define the protein composition or identify differentially expressed proteins in the CSF of multiple sclerosis patients [28,43, 44]. One of the investigations by 2-DE and MS compared CSF obtained from three multiple sclerosis patients with that of three individuals affected by other inflammatory conditions, two with CNS sarcoid and one with viral meningitis [28]. Twenty-four proteins were present in the multiple sclerosis gel but not in controls. As 20 of these proteins were previously reported in normal human CSF, it was concluded that the four proteins, cartilage acidic protein, SPARC-like protein, tetranectin, and autotaxin t, were exclusively present in the CSF of multiple sclerosis patients. These four proteins have not been previously associated with multiple sclerosis or its animal models by use of other proteomic, genomic, or conventional biochemical and molecular approaches. Therefore, further studies on a larger cohort are necessary to determine whether they are indeed disease markers and what putative role they may play in the pathophysiology of multiple sclerosis.

A recent comprehensive study [44] analyzed the CSF proteome in multiple sclerosis by a combination of ultra-filtration and LC/MS. Centrifugal ultrafiltration allowed the separation of high molecular weight proteins from 5 to 50 kDa proteins which were further analyzed by LC/MS. The separation of high abundance proteins such as albumin and Igs enabled the more accurate analysis of lower-abundance proteins. It is worth mentioning that this procedure may also eliminate some putative biomarkers that associate with high abundance proteins leading to loss of information. Pooled CSF of eight relapsing-remitting multiple sclerosis patients in remission was compared to that obtained from six cancer patients (non-multiple sclerosis controls) who showed normal clinical CSF parameters and no cerebral or leptomeningial metastasis. These investigators identified 44 proteins, which were present in the CSF of multiple sclerosis patients but not in the non-multiple sclerosis controls. Several of these proteins including complement C3 and heat shock protein hsp90 have previously been implicated as putative multiple sclerosis markers [54]. Other proteins such as annexin I and II are increased in the brain of subjects with multiple sclerosis [55–57], whereas others including contactin 1, hepatocyte growth factor-like protein, neurexin-2 alpha precursor, caspase-14 were ascribed to multiple sclerosis for the first time.

In general, the profiling of CSF proteins to construct a reliable and comprehensive databank is on its own a formidable task because the proteins are highly diversified and in different abundance. The added variability resulting from sample handling and conservation, the use of diverse proteomic approaches with dissimilar sensitivity and the variety of programs available for data analysis, often lead to marginally overlapping results between different investigations. It is clear that the validation of results by comparison of different published report, will not always yield a clear-cut answer. Some of the challenges associated with the proteome analysis of the CSF have recently been extensively discussed [58].

Despite nonoverlapping results, taken together, these reports pinpoint a number of proteins which warrant further investigations. For example, the identification of annexin I and II, contactin-1, and hepatocyte growth factor-like protein in CSF of multiple sclerosis patients may be relevant to a number of processes including axonal function after demyelination, axon-oligodendrocyte interactions, remyelination, neurodegeneration, or neuroprotection.

Annexin I is localized to activated microglia/macrophages, lymphocytes and astrocytes found in multiple sclerosis and EAE lesions [55,56]. It plays a neuroprotective role against excitotoxicity in other pathological conditions [59] and may have a similar function in multiple sclerosis. In fact, excitotoxicity has been implicated in the pathogenesis of EAE [60,61]. Huitinga *et al*. [62] reported that administration of a peptide derived from annexin I attenuates the clinical severity of EAE. In contrast, annexin II mediates microglial activation induced by tissue plasminogen activator, a process that might have neurotoxic effects [63].

Contactin-1, a neuronal cell surface glycoprotein, associates with sodium channels and in particular, $Na_v1.2$ and $Na_v1.3$ and enhances their surface expression [64–66]. The levels of the sodium channel $Na_v1.2$ is increased in demyelinated but not injured CNS axons in MS and EAE [67,68]. Therefore, it has been suggested that the upregulation of $Na_v1.2$ may be a putative compensatory mechanism to restore axonal function. Thus, by modulating the surface expression of $Na_v1.2$, contactin-1 may contribute to this compensatory mechanism. Other studies have also shown that contactin plays a role in axogenesis, oligodendrocyte development, and myelination [69–71].

Finally, hepatocyte growth factor-like protein, also known as macrophage stimulating protein (MSP), has been shown to be a neurotrophic factor for motor neurons in different CNS regions by acting through its tyrosine kinase receptor RON (recepteur d'origine nantais) which is expressed on neurons [72–74]. It is worth noting that RON expression is decreased both in multiple sclerosis and EAE [75], and EAE symptoms, demyelination, axonal injury, and neuroinflammation are exacerbated in RON deficient mice. As MSP inhibits the production of proinflammatory cytokines in differentiated human monocytoid cells, the aggravation of EAE pathology in RON-deficient mice was attributed to an increase in macrophage-derived proinflammatory and neurotoxic substances in the absence of the suppressive action of MSP. However, the lack of a direct neurotrophic/neuroprotective effect of MSP on neurons might also have contributed to disease and injury severity in these mice.

In summary, despite many challenges, proteomic analysis of the CSF might not only lead to the identification of putative biomarkers but also provide some hints about novel directions to be pursued in multiple sclerosis research.

5 Generation of differential protein expression profiles in EAE

In the past several years, a number of studies delineated the gene expression profiles in the CNS of rodents affected by EAE. However, to date, only a very limited number of proteomics studies have been performed in this animal model [76–78]. Alt *et al.* [77] examined the extent of consistency between gene and protein profiles as it is well known that alterations in gene expression do not always reflect changes in protein expression. Genechip microarray analysis and 2-DE combined with MS were performed concurrently using a cerebral microvessel preparation derived from mice with EAE and controls. The goal was to identify genes and proteins that are involved in the opening of the BBB. The expression of 288 genes was modulated whereas only six differentially expressed proteins were detected. Although five of these proteins were represented in the genechips, the increase in the expression of only one of them, fibroleukin/fgl2, was confirmed by both microarray and proteomic analysis. This indicates a lack of significant correlation between results obtained by the two methods. Thus, findings obtained by genechip analysis need to be further confirmed by other methods such as Western blots, immunocytochemistry, or ELISA to ensure that changes in transcription are also reflected at the translational level. Reciprocally, proteomic methods may lack the sensitivity of gene microarrays and may fail to detect some of the differentially expressed proteins. Therefore, proteomic and genomic approaches may provide complementary information.

We have recently used the MBP-induced acute EAE model to elucidate the differential protein expression profiles in the lumbar spinal cord of affected rats at the peak of the disease as compared to adjuvant-treated controls [78]. Our first goal was to validate the usefulness of the iTRAQ approach to the study of the spinal cord proteome during pathological conditions. To achieve this goal we compared our data with results reported in the literature. We determined whether some of the differentially expressed proteins have previously been shown to be modulated in multiple sclerosis or EAE, and whether our findings are in agreement with earlier reports. Our second aim was to use this method in order to identify proteins not yet ascribed to EAE pathology. Such proteins could be potential targets for future investigations. iTRAQ analysis identified some proteins that have formerly been implicated in multiple sclerosis and/or EAE by use of conventional approaches, gene microarray analysis, or other proteomic techniques. In agreement with other published investigations, the changes in the levels of certain proteins were confirmed by iTRAQ analysis as well. These proteins are involved in neuroprotection, neuronal dysfunction, immune activation, and inflammation. In addition, we identified changes in PTM in several proteins previously implicated in multiple sclerosis and other inflammatory diseases [79]. Thus, iTRAQ can be used to study complex biological processes. However, our studies also provided insights into the limitations of this approach. We could not detect significant changes in a number of proteins which we have shown to be modulated in the spinal cord of rats and mice affected by acute or chronic EAE [80,81]. Among those was plasma membrane calcium ATPase 2 (PMCA2) whose protein and mRNA levels are reduced in EAE as assessed by Western blots and quantitative RT-PCR, respectively [81]. Although iTRAQ analysis showed some decrease in PMCA2 levels, the differences did not reach statistical significance. This was especially due to animal-to-animal variations and the small number of samples (two controls and two EAE) that can be labeled in a single iTRAQ experiment. In contrast, a larger number of samples can be analyzed simultaneously by Western blots, compensating for intersample variability. In the future, it will be necessary to determine whether pooled samples from a larger group of animals ensure better correlation between iTRAQ analysis and the formerly published results. We also identified over 20 proteins which are attributed to EAE pathology for the first time. We further confirmed the differential expression of some of these proteins by Western blots and initiated studies to unravel their cellular localization and distribution by use of immunocytochemistry. Further studies are necessary to elucidate their potential role in the disease.

Proteomic studies on EAE are still in their infancy. Future investigations should determine whether the profiles of differentially expressed proteins in one particular EAE model are validated in other EAE models in different species. It is also important to assess how the expression of identified proteins is modulated during the course of the disease. Most importantly, it will be necessary to establish their potential relevance to the human disease by analyzing their expression in postmortem multiple sclerosis brain and spinal cord. Finally, *in vivo* and *in vitro* investigations need to be performed to elucidate the role of these new targets in different pathological processes that occur in EAE and multiple sclerosis.

6 Profiling of the autoantibody response in multiple sclerosis and EAE

In recent years, interesting and promising proteomic approaches have been developed to define autoantibody responses in autoimmune diseases [82]. Extending the technique previously developed by Joos *et al*. [83], Mac-Beath and Schreiber [84], Haab *et al*. [85], Robinson *et al.* [29] established protein microarrays consisting of derivatized microscope slides on which putative peptides and autoantigens derived from target tissues are deposited. Using these arrays, small volumes of sera obtained from patients or experimental animals can be screened in order to delineate the diversity of autoantibody response at various phases of the disease and following therapeutic treatment. The detection of autoantigens can help the development of antigen-specific DNA tolerizing vaccines to treat multiple sclerosis and protein microarrays

can be useful to determine whether the profile of autoantibody production correlates with and predicts disease course. Thus, this approach may have diagnostic, prognostic, and therapeutic value.

To assess the usefulness of protein arrays, myelin arrays which included polypeptides of MBP, MOG, PLP, myelin-associated glycoprotein (MAG), cyclic nucleotide phospho-diesterase (CNPase), oligodendrocyte-specific protein, have been tested in acute and chronic EAE. These investigations indicated that enhanced autoantibody diversity may predict increased disease activity in acute EAE, and a rise in epitope spreading is associated with a more severe disease course in chronic EAE. The myelin arrays were further used to develop and select antigen-specific tolerizing DNA vaccines and to monitor their effects in EAE. The vaccines decreased the number of relapses in acute EAE and prevented epitope spreading. It is worth noting that some caution may be needed with respect to the application of tolerizing DNA vaccines to the treatment of multiple sclerosis as both beneficial and detrimental outcomes have been reported in EAE [29,86–91]. Similarly, a lipid microarray which included gangliosides, sulfatide cerebroside, and sphingomyelin was produced for the study of lipid-specific antibodies in the CSF of individuals with relapsing-remitting and secondary progressive multiple sclerosis and sera derived from acute EAE in the mouse [92]. Strong reactivity to sulfatide, oxidized phosphatidylcholine, and sphingomyelin and weaker reactivity to asialo-GM1 were observed in multiple sclerosis. Antibodies to sulfatide have previously been reported in the CSF of multiple sclerosis patients [93] and have been implicated in demyelination [94–96]. Serum obtained from mice with acute EAE contained autoantibodies against sulfatide, asialo-GM1, cerebroside, and other lipids. Coimmunization with sulfatide during induction of EAE and transfer of sulfatide-specific antibodies to mice with EAE lead to a more severe disease course.

In a recent study, Somers *et al* [97] validated the use of a powerful molecular approach, serological antigen selection [98], for the study of autoantibodies in the CSF or serum of multiple sclerosis patients. The authors used a normalized cDNA library derived from active chronic multiple sclerosis plaques and directionally cloned and displayed the cDNA repertoires on filamentous phage. The phages were then mixed with a pooled CSF sample obtained from ten patients with relapsing-remitting disease course. This step allows the binding of specific IgGs to their corresponding antigens displayed on the phage. The phage antigen-IgG complexes are then captured by use of immobilized antihuman IgG. The unbound, nonrelevant phages are washed and the bound phages are eluted, amplified by reinfection into bacteria and subject to three additional selection steps as described above. The clones obtained after the fourth round of selection were subjected to fingerprint analysis which indicated selective enrichment. To select cDNA products which bind to IgGs that are specific to multiple sclerosis, the authors established depletion strategies. The alternate use of normal and multiple sclerosis sera in the four selection steps, enabled the recovery of cDNA clones which might be specific for the disease. Although this approach appears promising for the detection of multiple sclerosis-related antigens and antibodies, further studies are required to identify the isolated clones and determine their specificity for multiple sclerosis as compared to other inflammatory or autoimmune diseases. To begin addressing this latter issue, the authors performed phage ELISA and assessed the immunoreactivity of ten CSF samples obtained from patients with noninflammatory or other inflammatory diseases. None of the samples were immunopositive. In the future, CSF of a larger cohort of nonmultiple sclerosis patients needs to be tested to validate these encouraging findings. It is worth noting that the success of this approach and its widespread applicability to large cohorts of multiple sclerosis patients may depend on a number of factors. The cDNA library must be representative, as much as possible, of the various types of lesions found in different multiple sclerosis patients. This issue is especially important in view of the studies of Lucchinetti *et al.* [99] who emphasized the profound heterogeneity of lesions between different multiple sclerosis patients. These investigators described four different patterns of lesions based on myelin loss, the location and extension of plaques, the

type of oligodendrocyte damage, and complement activation. In addition to the source of the cDNA library, the heterogeneity of the CSF between subjects may limit the meaningful interpretation of the results and the widespread applicability of the approach to different patients and multiple sclerosis phenotypes. The authors approached this issue by determining the frequency of the antibody response to two antigenic cDNAs in individual CSF obtained from ten patients as compared to the pooled CSF sample. They found that two out of ten samples were positive. In future studies, the use of a larger cohort may provide a better insight into the usefulness of this approach as a diagnostic or prognostic tool.

It is worth noting that earlier studies attempting to identify autoantibodies in the CSF and sera of multiple sclerosis patients also used the phage display technology [100]. However, these investigations employed random peptide libraries to bind IgGs. One limitation of this technique as compared to the newer approach used by Somers *et al.* [97] is that only antigen mimotopes are picked up and further investigations are necessary to identify the actual antigen. Subsequent studies by the same investigators [101,102] indicated that identical antibodies are present both in the serum and CSF albeit a number of them are particularly enriched in the CSF. However, many of the antibodies were also found in the serum of non-multiple sclerosis subjects. Most importantly, antibody profiles were patient-specific which can be an impediment for investigations focusing on the use of CSF antibodies as diagnostic or prognostic markers.

7 Other proteomic studies relevant to multiple sclerosis research

The causes of demyelination and the failure in remyelination are major topics of investigation in multiple sclerosis research. Many studies have focused on the function and pathology of oligodendrocytes, the myelin producing cells of the CNS, and oligodendrocyte–axon interactions. Over the course of many years, excellent work on this subject has been published by use of conventional approaches. Recent investigations have used proteomic methods to create a databank which may facilitate the study of oligodendrocytes and determine the changes that occur in these cells in response to specific stimuli [103–106].

An interesting study by Marta *et al.* [105] investigated how antibody crosslinking of MOG affects the expression and phosphorylation state of oligodendrocyte proteins, *in vitro*. The goal was to elucidate the molecular events that may lead to antibody-mediated demyelination with particular emphasis on MOG. Antibody-induced demyelination is an important component of multiple sclerosis pathology [99,107–110]. Antibodies to MOG are elevated in the CSF of multiple sclerosis patients [111–114] and have been implicated in demyelination in the human disease and in related animal models [114]. Total cellular protein obtained from MOG-crosslinked oligodendrocytes and controls were resolved on 2-DE and the immunoblots generated from the gels were probed with anti-phosphoamine acid antibodies. The phosphoproteins that showed changes in phosphorylation state were then identified by MS or by use of specific antibodies to probe immunoblots. Proteins involved in cellular stress responses, signal transduction and cytoskeletal stability were identified. Based on these findings and their previous work showing that crosslinking of anti-MOG antibody to oligodendrocytes induces repartitioning of MOG into lipid rafts leading to retraction of processes [115], a model of anti-MOG mediated demyelination was proposed.

A second study [104] described an approach to identify oligodendrocyte proteins, *in vivo*. This investigation compared the proteome profile of the rat optic nerve exposed to X-irradiation *versus* the contralateral control optic nerve containing a normal oligodendrocyte population. Previous studies had established that X-irradiation at birth selectively eliminates oligodendrocytes [116,117]. However, after post-natal day 14, oligodendrocyte progenitor cells repopulate the X-irradiated nerve, differentiate into myelin producing mature oligodendrocytes, and completely myelinate axons by postnatal day 28. The authors combined

this experimental model with 2-DE and MS, in order to identify proteins associated with oligodendrocytes, *in vivo*. Proteomics maps were simultaneously derived from the untreated P14 optic nerve as control, the X-irradiated P14 optic nerve which does not contain oligodendrocytes, and the X-irradiated P28 optic nerve which is myelinated. The rationale was that proteins which were absent from X-irradiated oligodendrocytes at P14 as compared to normal controls and were detectable at P28 in the X-irradiated optic nerve would be associated with oligodendrocytes. It is worth noting that the expression of axonal proteins may also be altered in the presence or absence of oligodendrocytes as glia-axon interactions play a role in the regulation of axonal proteins [118,119]. Nevertheless, the combined use of such a model in conjunction with proteomic techniques to identify proteins and conventional approaches that can localize the identified proteins to specific cells or axons, may provide insights into mechanisms of myelination.

Astrocytes are also important contributors to CNS inflammation [120] and are believed to play a role in multiple sclerosis and its animal models [121–124]. A number of studies have used proteomic approaches to create a databank of proteins expressed in astrocytes [125,126] or delineate their differential regulation upon activation of cells [127–129].

A comprehensive study using 2-DE coupled with MALDI-TOF and TOF/TOF analysis identified 191 proteins from primary mouse astrocytes after ascertaining that the cultures were not contaminated by neurons or microglia [126]. The identified proteins included those associated with antioxidant, chaperone and metabolic activity as well as cytoskeletal, signaling, proteosomal, and nucleic acid binding proteins. About 28% of proteins were represented on the gels by multiple spots indicating PTM. Three additional investigations used proteomic approaches to identify proteins which are modulated in activated astrocytes, *in vitro* [127–129]. In one of the studies [127], rat astrocytes were exposed to endothelin-1, a stimulus implicated in CNS injury and stroke. Ten proteins showed altered abundance in cultures exposed to endothelin-1 as compared to controls. Four of these proteins were associated with the actin cytoskeleton. In a second study, Falsig *et al.* [129] analyzed both protein and transcript levels of mouse astrocytes exposed to a mixture of inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and interferon-γ (IFN-γ). The abundance of proteins in controls *versus* activated astrocytes was assessed by silver staining of 2-D gels and by labeling of lysates obtained from controls and activated astocytes with two different isotopes. The two samples were then mixed and subject to 2-DE followed by MS. A total of 17 differentially expressed proteins were reported using these methods. Transcriptional analysis of similar samples by microarrays detected a higher number (182) of up- or down-regulated genes in activated astrocytes as compared to controls. A third report used 2-DE and MALDI-TOF MS to examine the conditioned medium of mouse astrocytes stimulated with IL-1β and TNF-α *versus* controls [128]. They identified secreted proteins which included proteases, protease inhibitors, antioxidant proteins, and cytosolic enzymes. In all three studies, most growth factors and cytokines known to be secreted or regulated by inflammatory stimuli or cellular activation, were not detected. This illustrates some of the limitations of the proteomic approaches in the study of isolated cells.

In sum, proteomic studies on select glial populations, especially when challenged with a stimulus that induces activation or causes pathology, can lead to a better understanding of their function. The insights provided by such studies can open new avenues of investigation elucidating the contribution of these cells to pathological conditions of the CNS including multiple sclerosis.

8 Conclusions

Proteomic approaches are potent tools to monitor global changes in protein expression in pathological conditions or to create databanks for the study of various cell types that play essential roles in diseases. One of the advantages is that the techniques do not depend on the availability of specific antibodies. Yet, it is worth noting that proteomic methods detect only some protein subgroups and may fail to identify others of critical importance. The abundance of the proteins, their solubility and the extent of the changes in expression may be some of the limiting factors. Thus, it would be prudent to use proteomic analysis in conjunction with other approaches such as gene microarray analysis and immunoblotting. Despite some limitations which might be resolved with the further development, refinement, and validation of the current techniques, invaluable insights into pathological processes can be obtained by use of proteomic methods.

Abbreviations

BBB
: blood–brain barrier

CSF
: cerebrospinal fluid

EAE
: experimental autoimmune encephalomyelitis

ICAT
: isotopic coded affinity tag

IL-1β
: interleukin 1β

iTRAQ
: isobaric tags for relative and absolute quantification

MBP
: myelin basic protein

MOG
: myelin oligodendrocyte glycoprotein

MSP
: macrophage stimulating protein

MW
: molecular Weight

PLP
: proteolipid protein

RON
: recepteur d’origine nantais

TNF-α
: tumor necrosis factor-α

9 References

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