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High-resolution peptide mapping of cerebrospinal fluid: a novel concept for diagnosis and research in central nervous system diseases

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Abstract

Peptides, such as many hormones, cytokines and growth factors play a central role in biological processes. Furthermore, as degradation products and processed forms of larger proteins they are part of the protein turnover. Thus, they can reflect disease-related changes in an organism's homeostasis in several ways. Since two-dimensional gel electrophoresis is restricted to analysis and display of proteins with relative molecular masses above 5000, we developed Differential Peptide Display (DPD), a new technology for analysis and visualization of peptides. Here we describe its application to cerebrospinal fluid of three subjects without a disease of the central nervous system (CNS) undergoing routine myelography and of two patients suffering from a primary CNS lymphoma. Peptides with a relative molecular mass below 20 000 were extracted and analysed by a combination of chromatography and mass spectrometry. The peptide pattern of a sample was depicted as a multi-dimensional peptide mass fingerprint with each peptide's position being characterized by its molecular mass and chromatographic behaviour. Such a fingerprint of a CNS sample consists of more than 6000 different signals. Data analysis of peptide patterns from patients with CNS lymphoma compared to controls revealed obvious differences regarding the peptide content of the samples. By analysing peptides within a mass range of 750–20 000, DPD extends 2D gel electrophoresis, thus offering the chance to investigate CNS diseases on the level of peptides. This represents a new approach for diagnosis and possible therapy.

Keywords: Peptide mapping; Neuropeptides

1. Introduction

Many pathogenic processes are reflected by characteristic changes in the content of extracellular body fluids like blood plasma, urine or cerebrospinal fluid (CSF); e.g. cystatin C from blood is used as a marker for glomerular filtration rate and chronic renal insufficiency [1]. Since peptides can be collected with those fluids in routine clinical settings, they are used in diagnosis and therapy. Nonetheless, amongst the proteinaceous products of the estimated

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30 000 human genes, many of still unknown identity may have diagnostic or even therapeutic relevance.

Different approaches are under way to gain access to this pool of possible biomarkers. Genomic technologies are expanded by proteomics which comprises technologies for the analysis of the protein content of cells and organisms [2]. Usually, proteome projects are based on two-dimensional gel electrophoresis as separation technology. This technique analyses proteins within a range of relative molecular masses between 5000 and ≥100 000 [3,4]. Though it has been successfully applied to CSF for the detection of proteins with diagnostic value for diseases of the nervous system [5], the group of peptides which comprehends many physiologically active or indicative components like hormones, cytokines and protein degradation products is neglected. Therefore, attempts were made to analyse the peptide content of different sample types, among them CSF [6–10]. In the meantime the field of Peptidomics™ was established, which is defined as *the technology for the comprehensive qualitative and quantitative description of peptides in biological samples* [11]. Within this field, we developed a new concept for the analysis of peptides, the Differential Peptide Display™ (DPD), which has been applied successfully to different body fluids [11].

CSF is ideally suited to reflect central nervous system (CNS) disease since it is in contact with all brain surfaces and all cerebral interstitial fluid drains into it. Since the introduction of lumbar puncture more than 100 years ago, the analysis of cells and proteins from CSF has been a valuable tool for diagnosis and classification of inflammatory, degenerative and neoplastic CNS diseases [12]. Nonetheless, simple and reliable diagnostic and prognostic markers are still lacking for a range of CNS diseases and sometimes biopsy or even open surgery are necessary for histological confirmation of diagnosis.

The diagnostic possibilities have markedly increased by improvement of sensitivity and precision of established methods of protein research, like radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) [13]. In addition, new mass-spectrometric techniques such as matrix-assisted laser-desorption/ionisation time-of-flight (MALDI–ToF) mass spectrometry (MS) and electrospray ionisation (ESI) have been developed [14–17]. DPD consists of a combination of peptide extraction, chromatographic separation and concentration with subsequent MALDI–ToF analysis. Already, mass spectrometry has been used for analysis of neuropeptides in CSF and human tissue [18,19]. Recently, a large variety of peptide fragments of neuroendocrine proteins were identified, e.g. chromogranin A and B, VGF and 7B2, leading to the assumption that they were products of prohormone convertase activity [10].

Here we present the application of MS as part of a new technology for the analysis of cerebrospinal fluid and the differential description of CSF peptide patterns. We compared patients without CNS disease, who were subjected to routine myelography due to spinal-nerve root compression syndrome, and patients suffering from primary CNS lymphoma, in order to demonstrate differences between them on the level of CSF peptides.

2. Experimental

After approval by the local ethics committee, written informed consent was obtained from all patients. Two were suspected to suffer from a primary CNS lymphoma (PCNSL) and three were punctured for routine myelography due to spinal-nerve root compression syndrome (Table 1). All CSF samples were prepared according to a common standard operating procedure. After lumbar puncture CSF was centrifuged for 10 min at 2000 *g* and the supernatant was stored at −80 °C before analysis. After thawing, peptides with a relative molecular mass of up to 20 000 were extracted from 0.5 ml CSF and separated using reversed-phase (RP) C_{18} chromatography. CSF was diluted 1:3.75 with water and pH adjusted to 2–3. This sample was loaded on an RP column (250×4 mm, Vydac, HP-ChemStation 1100 Agilent Technologies), the peptides were eluted with an acetonitrile gradient (4–80%) in 0.05% trifluoroacetic acid and separated into 96 fractions (Fig. 1a). Elution was monitored by UV detection and the retention time of major peptide peaks from repeatedly loaded extracts did not vary.

After lyophilization, each HPLC fraction was resuspended in a mixture of α-cyano-4-hydroxicinnamic acid (matrix) and L-fucose (co-matrix) in 0.1%

Table 1
Data for patients included in study

	Diagnosis	Duration of history	Sex	Age (years)	BMI	Concomitant disease	Medication
Control	Nerve root compression syndrome L5	2 years	Male	21	23.2	None	None
Control	Cervical nerve root avulsion	6 months	Male	26	23.4	None	None
Control	Failed back-surgery syndrome	1 year	Male	50	26.6	Coronary heart disease	Atenolol
PCNSL	Malignant non-Hodgkin B-cell lymphoma	3 weeks	Male	66	28.4	Arterial hypertension	Bisoprolol/hydrochlorothiazide, dexamethasone
PCNSL	Malignant non-Hodgkin B-cell lymphoma	4 weeks	Female	75	25.4	Arterial hypertension, coronary heart disease, depression	Losartan, verapamil, oxazepam, lorazepam

BMI, body mass index.

acetonitrile/trifluoroacetic acid (1:1) and applied to a matrix-assisted laser-desorption/ionisation (MALDI) target, followed by ambient temperature air drying. Sample ionisation was carried out by application of repeated single laser shots over a representative area of the sample spot. The accelerated ions were analysed in a matrix-assisted laser-desorption/ionisation time-of-flight (MALDI–ToF) mass spectrometer [20] (Voyager-DE STR mass spectrometer, Applied Biosystems, Framingham, MA, USA) in linear mode with delayed extraction.

Software, specifically designed by BioVisioN, was used for peak recognition, visualization and statistical analysis. The converted mass spectra (Fig. 1b) of all 96 chromatographic fractions generated from one sample were combined in one multi-dimensional diagram (peptide mass fingerprint) in sequence of their elution from the HPLC column. The *x*-, *y*- and *z*-axes are molecular mass, chromatographic fraction and mass-spectrometric intensity, respectively. In such a diagram every mass peak is depicted as a bar with the colour intensity increasing with the intensity of the mass-spectrometric intensity of that peak (Fig. 1b). The individual peptide mass fingerprints of each sample set were pooled to a joint peptide mass map (master map) representing the peptidomes of CSF from lymphoma and control patients (Fig. 1c).

Differences between patients and controls were detected by generating subtractive peptide maps.

The underlying peptides were identified using an ESI-qToF MS (Qstar, electrospray-ionisation quadrupole time-of-flight mass spectrometer, Applied Biosystems, Framingham, MA, USA). Peptide-fragment spectra were detected using nanospray in the full scan mode (voltage 800–1000 V, collision energy 20–40 eV) [21–23] and 200 scans per sample were accumulated. After charge-state deconvolution (Bayesian reconstruct tool of the BioAnalyst program package by Applied Biosystems) and de-isotoping (Voyager 5.1 software, Applied Biosystems), mass spectra were saved in a MASCOT generic file format, and were submitted to the MASCOT database search engine (Matrix Science, London, UK) [24]. Databases were searched with SwissProt (Version 39.6, www.expasy.ch) and MSDB (Version 010721, EBI, Europe). This procedure allows also identification of modified amino acids like, for example, phosphothyrosine as well as determination of its position.

3. Results

The DPD process, which comprises a combination of different technologies with analysis of peptides by mass spectrometry as the core technique (Fig. 1), was applied to the examination of CSF. The spectrometric data for each sample were processed to a multi-dimensional peptide mass fingerprint containing signals corresponding to relative molecular masses between 750 and 20 000. Components with a CSF concentration as low as the upper picomolar range were detected with a mass accuracy better than 500 ppm. The position of each peptide in such a fingerprint is precisely characterized by its relative molecular mass and its elution/retention time during the preceding chromatography with a shift of maximal one fraction between independent analyses. This

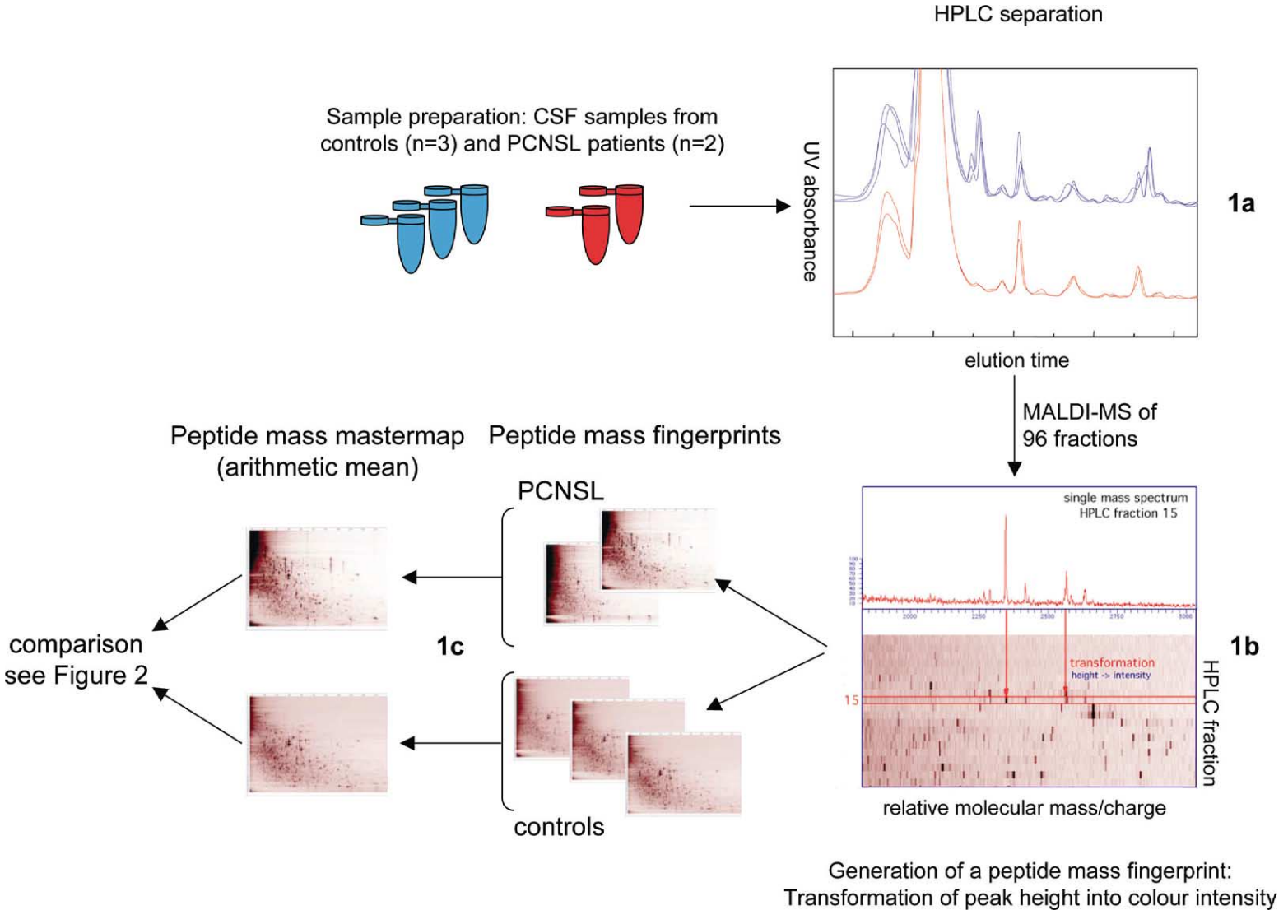

Fig. 1. Flow scheme of the Differential Peptide Display process. Peptides are extracted from 0.5-ml specimens of CSF and separated by RP-HPLC into 96 fractions using a C_{18} column. Each chromatographic fraction is analysed by MALDI–ToF-mass spectrometry and all 96 mass spectra generated from one sample are combined to a ''virtual 2-D gel picture'' (peptide mass fingerprint), i.e. transformation of MS spectra by rotation by 90° so that they are viewed from above. Thus, each peak is depicted as a bar with its colour intensity corresponding to the height of the relevant peak. The *x*-, *y*- and *z*-axis are molecular mass, chromatographic fraction and mass spectrometric intensity, respectively (m/z: molecular mass/charge).

precision allows the calculation of lymphoma- and control-specific master maps without extensive data processing.

The separation into 96 fractions increases sensitivity of the subsequent MS analysis resulting in the detection of more than 6000 different mass-spectrometric signals, which are in part redundant due to multiple charge states, oxidation products or mass spectrometric derivatives like sodium adducts.

Fig. 2 depicts the master maps for the PCNSL and the control patients. For each peptide the arithmetic mean of its intensity was calculated from the underlying individual datasets. Thus, a master map contains all peptides detected in CSF of the corresponding set of patients. It therefore represents the characteristic, group- or disease-specific CSF peptidome. Furthermore, the signal-to-noise ratio within such a master map is improved due to combination and averaging of the individual data sets.

Master maps of patients and controls are compared to identify qualitative as well as quantitative differences (Fig. 2). Signals with the same intensity in both datasets are depicted as white spots, whereas red or blue colour indicates stronger signals in CSF from lymphoma patients and healthy subjects, respectively. The extent of the difference between corresponding signals can be calculated as a measure of a component's suitability to discriminate between CSF from PCNSL patients and controls.

Several differences were found and one particularly distinct one was detected in fraction 60 with a mass-to-charge ratio (m/z) of 1378 (Fig. 3). It was identified as the double-charged signal of a 24 amino-acid fragment of serum albumin with the average relative mass of 2754 (amino acid residues 25–48, sequence DAHKSEVAHRFKDLGEENFKALVL).

Both signals showed a higher intensity in the samples from lymphoma patients than in controls. Here data from the double-charged component are presented since its signal intensity is in the linear part of the dynamic range of the MS detector. The single-charged albumin ion, however, occurs in concentrations too high for an optimal quantitation (Fig. 2). Fig. 3 depicts the section of the mass spectra generated from fraction 60 of all samples which contains the signal of the double-charged albumin fragment. The more intense colour of the spots in lymphoma samples correlate with a higher signal intensity and a larger amount of the corresponding peptide in the CSF sample.

4. Discussion

The basic concept of using CSF as a diagnostic medium is based on the assumption that CSF composition is a mirror of the physiological and pathophysiologic processes taking place within the brain tissue, the meninges, or the choroid plexus. The production and removal of CSF occur via different ways. The main quantity, i.e. two-thirds of CSF is generated by the choroid plexus, where blood plasma traverses the blood–CSF barrier. Many compounds are at least partially retained resulting in higher concentrations on the blood side. Another source of CSF is interstitial fluid that drains centrifugally from the cerebral interstitium to the subarachnoid space alongside the Virchow-Robin spaces [25]. It is derived from the blood–brain barrier, which has a much greater exchange and transfer surface than the blood–CSF barrier, but it is also more selective and the more commonly disrupted one [26].

About 80% of CSF proteins are thought to originate from plasma [12]. Due to their lipophilicity many proteins as well as peptides cross the blood–CSF barrier by simple diffusion. Furthermore, active transport occurs across the blood–brain barrier for example by saturable transport systems, which can be highly specific [27,28]. Peptides synthesized within the brain parenchyma reach CSF with the bulk flow of extracellular fluid or after secretion from ependyma or arachnoid membranes. In the choroid plexus some peptides are newly synthesized (e.g. cytokines and growth factors), others like leptin and interleukin enter the CSF space by active transport mechanisms and vice versa CSF peptides, e.g. soluble beta-amyloid protein are cleared actively into the blood [29].

The total number of peptides present in CSF is still not known. Pan et al. [30] described about 50 peptide families with central nervous activity crossing the blood–brain barrier. The DPD technology allows the simultaneous analysis of the variety of peptides extracted from CSF. An estimated number of more than 1000 different compounds were de-

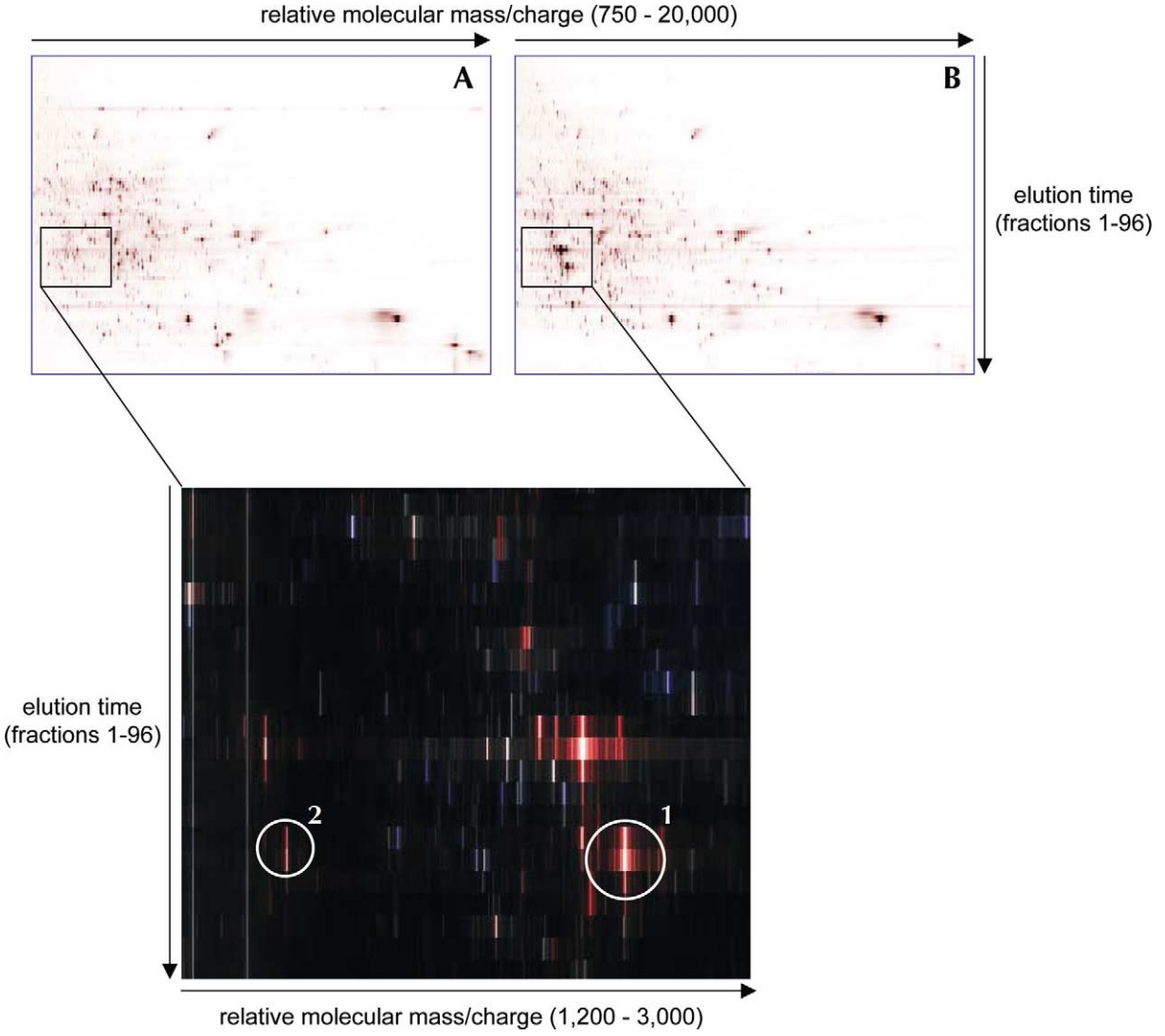

Fig. 2. Peptide mass maps (master maps) generated from CSF from healthy controls (A) and patients with PCNSL (B). Individual peptide mass fingerprints from three healthy subjects and two lymphoma patients were combined to an average map (master map) for each sample set: From the intensities which were measured in individual samples, the arithmetic mean was calculated for each signal whose position in the data matrix is precisely characterized by its elution time during chromatography and its molecular mass. Depicted is the pattern of peptides with relative molecular masses between 750 and 20 000. Every bar represents a signal from the mass-spectrometric analysis whose intensity correlates with the amount of a specific peptide in the sample. All samples from different individuals contain a common peptide pattern as well as interindividual differences. The part below depicts the comparison of peptide mass maps (master maps) from CSF from controls and lymphoma patients. Shown is a detail of the subtractive map with the range of relative molecular masses between 1200 and 3000. The average intensities from both sets were compared for every single signal position calculating the absolute difference. Signals with the same intensity in both sets are represented by white spots, whereas red and blue colour indicates stronger signals from lymphoma patients and controls, respectively. Labelled are the signals of the identified albumin fragment with $m/z = 2754$ (1) and the double-charged component at $m/z = 1378$ (2) (u: units, m/z: ratio of molecular mass and charge).

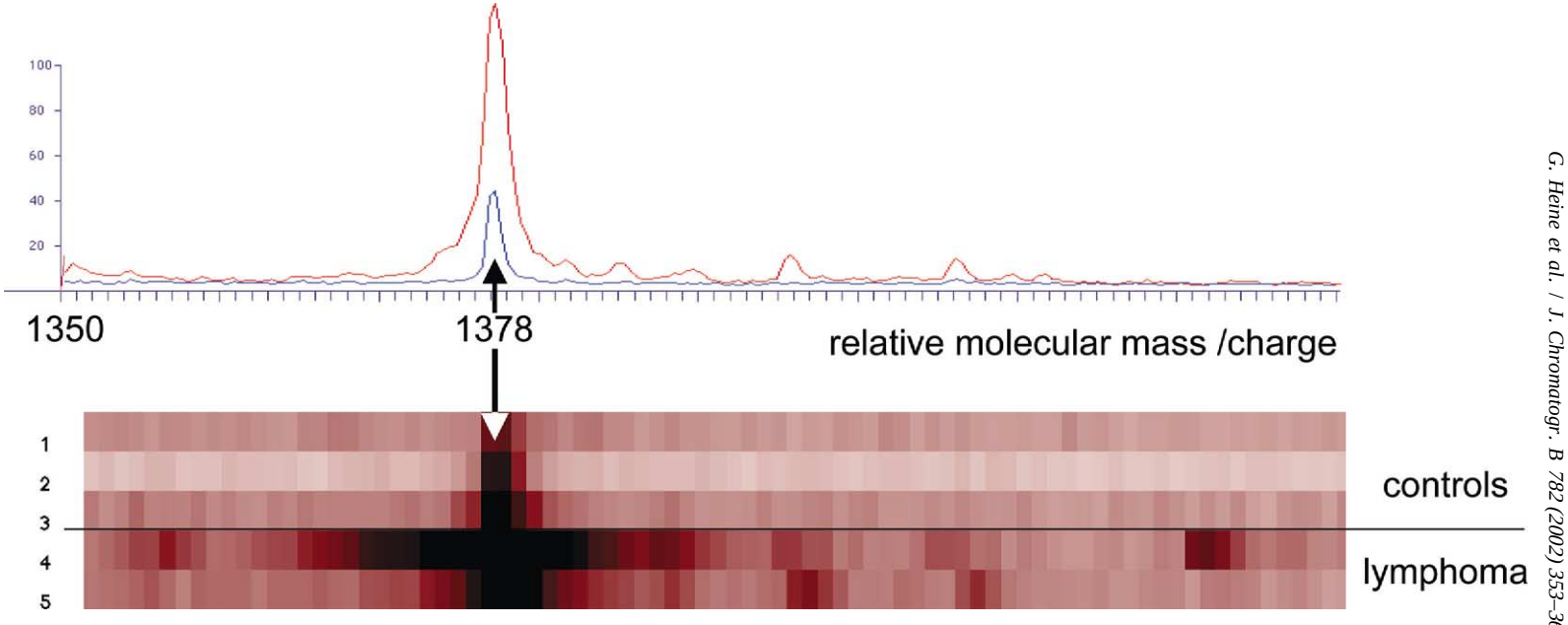

Fig. 3. Comparison of individual mass spectra generated from fraction 60 from controls and lymphoma patients. The data for every single CSF sample is shown, with controls depicted in lanes 1–3 and lymphoma samples in lanes 4 and 5. The signal intensity corresponds with the amount of peptide in the sample. The signals in lanes 4 and 5 are stronger, indicating that the corresponding peptide occurs in larger amounts in CSF from lymphoma patients. The curves on top show the average mass-spectrometric intensity of fraction 60 from the lymphoma (red) and the control samples (blue). Shown is the range of relative molecular masses between 1350 and 1450.

tected with relative molecular masses between 750 and 20 000. The central technique MALDI–ToF-MS is performed with a mass accuracy better than 500 ppm and is able to detect substances with a concentration of at least 500 pmol l^{-1} in the initial sample [31,32]. This corresponds to absolute amounts of femtomoles of peptides actually measured by MS. The elution profile varies by no more than one fraction and the similarity of peptide mass fingerprints obtained from different samples and measurements demonstrates the reproducibility of the quality and high resolution of the separation. This high accuracy allows the detection of a specific peptide in any peptide mass fingerprint, where its position is precisely defined by its molecular mass and its hydrophobicity, i.e. by its elution/retention time during the preceding HPLC separation.

So far we have analysed CSF samples from some hundred patients suffering from different diseases as well as from rats showing that there is a common, species-specific peptide pattern in CSF. Although the human CSF pattern is rather homogenous, we describe here that the DPD technology is able to detect differences in CSF which correlate with the disease status: As a clinical example patients with PCNSL were chosen, since this malignant disease, although rare, has its special diagnostic needs. PCNSL cannot be differentiated from a glioblastoma, the most common malignant glial tumour, solely by neuroimaging. However, the diagnostic and therapeutic features of the two entities are completely divergent. For example, a presurgical steroid therapy, as often applied for glioblastoma to reduce oedema volume, can cause a temporary regression of a lymphoma, which might prevent histologic diagnosis without being diagnostic itself. Since the therapy of choice for PCNSL is chemotherapy, sometimes combined with radiation, as opposed to surgical excision and radiotherapy in glioblastoma, a stereotactic biopsy is mandatory if a lymphoma is suspected [33]. A diagnostic CSF peptide pattern for differentiation between both entities with high sensitivity and specificity as well as a CSF marker for monitoring therapy and remission as well as for early detection of recurrence would be of great clinical value.

The peptide content of CSF from lymphoma patients and controls was characterized on the basis of five peptide mass fingerprints. Clear differences were spotted, but the number of subjects is too low for a profound statistical evaluation. The presented data have to be validated by analysis of a larger number of samples, including a comparison to CSF peptide master maps of glioblastoma patients. Nonetheless, this first approach demonstrates the principle of *Differential Peptide Display*, and its ability to compare the peptide patterns from different CSF samples in order to detect differences correlating with pathologic events.

For one of the signals, which showed a very clear difference in intensity between patients and controls, the corresponding peptide has been identified. The pathophysiologic relevance of the difference in the peptide patterns is supported by the results of the sequencing experiment: the increase in this albumin fragment is likely to be caused by a barrier disruption which typically occurs in tumour patients. The complete albumin molecule is traditionally used as a marker for the integrity of the brain barriers with the ratio of albumin concentration in CSF and blood correlating with the extent of the disruption [34,35]. It is questionable whether the occurrence of this distinct albumin fragment is specific to PCNSL or is a feature of a brain barrier disruption in general and therefore shared with other malignant brain tumours as well. Therefore, CSF from glioblastoma patients have to be included in further examinations.

This study demonstrates the course of a DPD analysis exemplarily. The work flow comprises four major steps: (1) extraction of peptides from biological sources and fractionation with reversed-phase HPLC, (2) analysis of all individual fractions using MALDI–ToF-mass spectrometry, (3) comparison of mass spectrometric data from distinct clinical populations and (4) identification of differences by sequencing of the corresponding peptides. DPD technology analyses the range of relative molecular masses below 20 000 and is therefore complementary to standard procedures of protein analysis using two-dimensional gel electrophoresis. It allows to compare different states of health on the level of extracellular peptides, as it is shown here for CSF from lymphoma patients in comparison with controls without CNS disease. DPD data are validated by identification of the underlying peptides, so their biological role in the clinical condition can be elucidated. Such an identification is performed by

complementing mass-spectrometric techniques like MS–MS, i.e. fragmentation by mass spectrometry and identification by database searches and, if necessary, by de novo sequencing, e.g. Edman sequencing. New insights may be obtained not only with regard to biomarkers, but also concerning degradation pathways of larger proteins and discovery of peptides with intrinsic biological activity.

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