

Cleavage of Cystatin C in the Cerebrospinal Fluid of Patients with Multiple Sclerosis

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Objective: The diagnosis of multiple sclerosis (MS) can be challenging because of the lack of a specific diagnostic test. Recent advances in proteomics, however, offer new opportunities for biomarker discovery and the study of disease pathogenesis. **Methods:** We analyzed cerebrospinal fluid (CSF) samples from 29 patients with MS or clinically isolated syndromes (CIS), 27 patients with transverse myelitis (TM), 50 patients with human immunodeficiency virus (HIV) infection, and 27 patients with other neurological diseases (ONDs) by surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy. **Results:** We found a unique protein of 12.5kDa that was 100% specific for MS/CIS compared with TM or OND. Low levels of this protein were found in some patients with HIV infection. Tandem mass spectroscopy of a tryptic digest of this 12.5kDa protein identified it as a cleavage product of full-length cystatin C (13.4kDa), an important inhibitor of cysteine proteases including the cathepsins. Although total cystatin C levels in the MS patients was not different compared with controls, the patients with the highest 12.5/13.4 peak ratios also had the greatest cathepsin B inhibitory activity. **Interpretation:** This suggests that cleavage of cystatin C may be an adaptive host response and may identify a subgroup of patients with MS.

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The accurate identification of patients with multiple sclerosis (MS) can be challenging at the time of disease onset. Even with magnetic resonance imaging (MRI), evoked potentials and cerebrospinal (CSF) studies, the diagnosis is still based on clinical criteria. Although reliable serological tests are available for most autoimmune diseases, no such assay is available for the diagnosis of MS in part because no single antigen has been specifically associated with the disease. Nevertheless, the availability of effective immunomodulatory therapy makes it important to identify biological markers that reliably distinguish MS from other neurological diseases.

The recent development of a protein chip platform based on surface-enhanced laser desorption/ionization (SELDI) time-of-flight mass spectroscopy allows for the high-throughput analysis of complex protein mixtures. This method requires microliter amounts of sample and has a sensitivity in the subfemtomole range. Using this technique, Petricoin and Liotta reported specific biomarkers for some types of cancer.¹ However, tumors are cell type specific and usually follow a predictable clinical course; hence, biomarker discovery

using cell extracts, serum, or other body fluids has progressed rapidly in this field. In contrast, multiple immune cells, neuroglia, and neurons have complex interactions with one another in MS, and these interactions can vary over time. Thus, the clinical course of MS is both variable and unpredictable and biomarker discovery for this disease poses unique challenges. In a recent attempt to identify disease-specific biomarkers for MS, CSF from five patients was analyzed by two-dimensional gel electrophoresis. Despite the small sample size, 15 proteins were found to be differentially expressed in the CSF of MS patients compared with controls.² In this study, we analyzed CSF samples by SELDI time-of-flight mass spectroscopy from a larger sample size of well-characterized patients and controls. Analysis of CSF has several advantages over serum for biomarker discovery in neurological disease. CSF better represents local events in the brain as compared with serum. Furthermore, high-abundance proteins in serum may mask the low abundant, low molecular weight proteins that are the likely candidates for biomarkers. We identified several proteins that were significantly dysregulated in patients

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Table 1. Demographics of Patients with MS/CIS

Sample No.	Age (yr)	Sex	Race	Diagnosis	Duration (mo)	No. of Prior Attacks	Last Attack (wk)	EDSS at LP	Steroid (last mo)	IMA at LP
31	23	M	Asian	RR	24	3	24	1.5	No	—
42	30	F	White	RR	18	3	8	2.5	No	—
45	39	M	White	RR	11	2	6	2.5	No	—
52	49	F	Black	RR	5	2	8	2	No	—
61	44	F	White	RR	11	2	10	3.5	No	—
78	35	F	White	RR	92	3	14	1	No	—
79	54	F	Black	RR	8	2	8	3	No	—
109	24	F	White	RR	31	2	52	0	No	—
151	39	F	White	CIS ^b	3	1	12	1.5	No	—
152	32	F	White	CIS	2	1	8	2.5	Yes	—
168	45	F	White	RR	42	2	6	2.5	No	—
169	42	F	White	RR	66	3	12	2.5	No	—
171	45	M	White	RR	7	2	8	3	No	—
174	63	F	Black	SP	168			6	No	+
185	37	F	White	SP	102			7	No	+
191	33	F	Black	RR	48	3	4	2.5	No	—
192	54	M	White	CIS ^b	4	1	3	2.0	Yes	—
247	26	F	White	RR	7	2	2	1.5	No	—
251	33	F	White	CIS ^b	3	1	4	3	Yes	—
252	24	F	Black	RR	10	3	12	2.5	No	—
256	53	F	White	RR	6	2	8	2	No	—
259	27	F	White	RR	5	2	4	2	No	—
267	40	M	White	RR	40	2	7	2	No	—
271	40	F	Black	RR	13	2	6	2	No	—
303	37	F	Black	CIS ^b	2	1	8	2.5	No	—
329	44	M	White	RR	24	2	8	1.5	No	—
330	54	M	White	RR	58	4	8	2.5	No	—
331	37	M	White	CIS	2	1	5	2	Yes	—
332	31	F	White	RR	30	2	8	2.5	Yes	—

with MS or CIS, one of which was a cleavage product of cystatin C. Our findings have important implications for the diagnosis of MS and for understanding disease pathogenesis.

Methods

Patient Selection

All CSF samples used in these studies were obtained from patients undergoing a lumbar puncture as part of their diagnostic evaluation being conducted through the Adult Neurology Clinic at the Johns Hopkins Hospital. A protocol approved by our INSTITUTIONAL REVIEW BOARD for Human Subjects Research allowed us to collect a small additional sample along with each diagnostic specimen. Written informed consent was obtained from each patient before these samples were obtained. Individuals with definite MS (n = 23) were diagnosed according to current criteria.^{3,4} Six patients had clinically isolated syndromes (CIS) and abnormal cranial MRI scans consistent with MS.⁴ Four of these patients have since had second clinical attacks and thus have confirmed MS. CSF samples from patients with various other neurological disorders (ONDs) (n = 27) were used as

controls. A diagnosis in each of these patients was defined according to individual disease criteria. These samples represented both inflammatory (n = 12) (n = 3 each with neurosarcoidosis and viral meningoencephalitis, n = 1 each with acute inflammatory demyelinating neuropathy, chronic inflammatory demyelinating neuropathy, primary central nervous system [CNS] lymphoma, human immunodeficiency virus (HIV) infection with progressive multifocal leukoencephalopathy, lumbosacral plexitis and CNS Lyme's disease) and noninflammatory neurological diseases (n = 15) (n = 3 each with normal pressure hydrocephalus and amyotrophic lateral sclerosis, n = 2 with pseudotumor cerebri, and n = 1 each with meningioma, drugs induced delirium, spinocerebellar degeneration, Alzheimer's disease, hereditary myelopathy and Parkinson's disease). For the purpose of this study, CSF was considered inflammatory in the control samples if one or more of the following abnormalities were present: white cell count more than five cells/mm³, detectable oligoclonal bands or IgG index greater than 0.8. CSF samples from another 27 patients with acute transverse myelitis (TM) and 50 patients with HIV infection (22 without dementia; HIV-ND and 28 with dementia; HIV-D) were used as other

Table 1. Continued

CSF wbc	CSF prot	OCB	IgG Index	T2 br Lesion	T2 Cord Lesion	Enhancing Brain Lesion	Enhancing Cord Lesion	T1 Holes	MRI Criteria ^a	12.5/13.4 Ratio
3	26	2	1.4	21		2		4	+	0.1262
8	42	1	1.1	2	1	1	0	0	-	0.0989
16	33	5	1.5	7		1		0	+	0.0879
7	57	6	1.2	7		2		0	+	0.1059
1	32	3	0.8	22		0		6	+	0.0776
6	27	3	1.3	2	1	0	0	0	-	0.0759
3	28	6	2.2	8		2		1	+	0.1262
4	21	8	2.8	4		0		0	-	0.0989
6	49	4	2	10		1		0	+	0.0879
18	62	6	1.7	8		2		0	+	0.1059
1	36	3	1.1	0	1	0	0	0	-	6.1187
1	17	2	0.9	2	0	0	0	0	-	3.9665
24	45	4	1.4	3	1	1	1	0	+	14.547
1	47	6	1.9	25	2	0	1	7	+	7.0584
2	32	2	1.4	16	2	0	0	6	+	9.0739
1	22	3	1.9	2	1	0	0	0	-	5.7216
2	55	1	0.8	1	2	1	1	0	+	10.146
8	18	1	1.5	1	2	0	1	0	-	4.3576
4	24	2	0.7	11		3		0	+	0.4099
9	33	7	3.4	6	3	1	0	0	+	0.779
6	45	5	2.2	4		0		0	-	0.296
2	34	0	0.5	2		0		0	-	0.5492
42	58	2	3.5	4		1		0	+	1.9112
1	23	1	2.1	14		0		1	+	3.5913
20	44	2	1.1	14	2	3	2	0	+	12.276
4	33	2	1.1	4		0		0	-	2.3348
2	37	7	1.4	17		6		3	+	6.3686
26	34	3	1.3	4		1		0	+	4.9795
5	48	3	1.4	4		1		0	+	2.5962

^aMeets criteria for MRI abnormality consistent with diagnosis of MS.

^bCIS patients that have since converted to clinically definite MS.

MS = multiple sclerosis; CIS = clinically isolated syndrome; EDSS = Expanded Disability Status Score; MRI = magnetic resonance imaging; IMA = immunomodulatory therapy; RR = relapsing-remitting; SP = secondary progressive MS; OCB = oligoclonal band; CSF = cerebrospinal fluid; LP = lumbar puncture.

controls. All patients except three with TM had an inflammatory CSF, but none had oligoclonal bands or an elevated IgG index. Samples from HIV infected patients were taken from the prospectively followed North Eastern AIDS dementia cohort.⁵ None of the patients had opportunistic infections.

Demographic and clinical data for the patients with MS/CIS was obtained by direct patient interview or from the relevant medical records (Table 1 provided online). With the exception of two patients with secondary progressive MS who were already on disease-modifying therapy, none of our patients had received any treatment other than corticosteroids before the time of CSF acquisition. An Expanded Disability Status Scale (EDSS) score was obtained at the time of CSF acquisition by an examiner who was blinded to the results of our analyses. Each patient also had an enhanced cranial MRI scan within 2 weeks of their lumbar puncture. The total number of T2 hyperintense lesions, T1 hypointense lesions, and gadolinium-enhancing T1 lesions meeting a greater than or equal to 3mm cutoff criteria was determined from each scan by a single blinded examiner. Each scan was also judged for whether it met the formal requirements for

an abnormality consistent with MS according to published criteria.^{3,4}

Protein Chip Assay

All CSF samples were handled equally and placed immediately on ice and centrifuged at 3,000 rpm for 10 minutes. The cell-free samples then were stored at -80°C in 0.5ml aliquots. For protein chip analysis, a single aliquot of CSF was thawed and immediately realiquoted into 50µl volumes and refrozen at -80°C. Each sample was thawed once more before analysis. CSF samples were initially analyzed using the weak cation exchange (CM10) and the hydrophobic chip (H50) protein chips (CIPHERGEN Biosystems, Fremont, CA). These chips bound proteins with specific physicochemical properties, which then were resolved by SELDI time-of-flight mass spectroscopy (CIPHERGEN Biosystems, Fremont, CA). Spectra derived from CM10 chips showed a greater number of peaks and a better resolution of low molecular mass species and were used in all subsequent assays. The protein chip arrays were assembled into a deep well type Bioprocessor assembly (CIPHERGEN Biosystems). Before sample loading, the

arrays were equilibrated with 150 μ l of binding buffer (50mM ammonium acetate buffer, pH = 4.0). Each spot on the array then was incubated with 15 μ l of CSF diluted in binding buffer to a final volume of 150 μ l with gentle agitation for 1 hour at room temperature. The spots were washed in the same buffer three times, after which 1 μ l of 50% saturated sinapinic acid (SPA) dissolved in 50% acetonitrile, 0.5% trifluoroacetic acid solution was added. The chips were air-dried and SPA was reapplied. The protein chips were analyzed in the ProteinChip biology systems reader (model PB-SIIc; CIPHERGEN Biosystems) using a laser intensity of 2.6 microJoules and a sensitivity setting of 5. Resulting spectra were noise-filtered, baseline subtracted, and calibrated with CIPHERGEN's "All-in-One Protein standard" consisting of cytochrome C (12,360.2Da), myoglobin (16,951.5Da), and GAPDH (35,688Da). Biochemical properties of the unique peaks identified in CSF samples were further characterized by changing the pH of the binding buffer (range, 4.0–9.0). The stability of these peaks was also determined by monitoring the effects of freeze/thaw cycles on the CSF, heating of samples to 50°C for 30 minutes or leaving them at room temperature for 16 hours. Each sample was analyzed in duplicate. All peaks obtained through the peak detection process were aligned using the Biomarker Wizard tool in the CIPHERGEN ProteinChip software (version 3.1). Peaks of similar (0.3%) mass/charge (m/z) ratio were clustered across all spectra. Each cluster then represented a particular protein.

Data Analysis

All data were internally normalized by total ion current. Spectra used for further analysis had normalization factors less than 2 standard deviations from the mean. The comparison of peak intensities and the ratios of the 12.5 and 13.4kDa peak among the patient groups was done by a one-way analysis of variance using a Tukey–Kramer comparison test. Linear regression curves were generated using Graph Pad Prism to determine if there was a correlation between cystatin C (Graph Pad Software Inc., San Diego, CA) levels and cathepsin B activity.

Enrichment of 12.5kDa Protein

A single CSF sample (MS267) that had a prominent 12.5kDa peak was selected for further study. One milliliter of CSF was semipurified in 100 μ l aliquots. CSF (100 μ l) was incubated with 50 μ l of equilibrated protein A beads for 5 minutes at room temperature to remove IgG. The supernatant was collected and diluted 1 to 5 with 50mM Tris, pH 9.0. Ten microliters of Q Hyper D strong anion exchange beads (CIPHERGEN) equilibrated with 50mM Tris, pH = 9.0, was incubated with each sample aliquot for 5 minutes at room temperature. The supernatant was collected and dialyzed in four changes of 1 liter ultrapure water overnight. Purification of the 12.5kDa peak was confirmed by SELDI time-of-flight mass spectrometry.

Tris-Tricine Gel Electrophoresis

All 10 aliquots processed in a manner described above were combined, lyophilized, and resuspended in 45 μ l ultrapure water to which 45 μ l of Tricine sample buffer (BioRad, Hercules, CA) with 2% β -mercaptoethanol was added. Proteins

were resolved using precast 16.5% Tris-Tricine SDS-PAGE gels (BioRad). The anode buffer consisted of 0.2M Tris-HCl, pH 8.9, and the cathode buffer consisted of 0.1M Tris-HCl, 0.1 M Tricine, 0.1% SDS, pH 8.3. Samples were diluted in 10ml of 50mM Tris-HCl, 4% wt/vol SDS, 12% wt/vol sucrose, 5% vol/vol β -mercaptoethanol, and a trace of bromophenol blue, pH 6.8. After denaturation at 97°C for 5 minutes, samples were loaded onto the gel with 30 μ l/lane. Gels were run at 200 mamps for 3 hours. After electrophoresis, gels were fixed, stained with a Silver Stain Plus Kit (BioRad, Hercules, CA), and dried between two pieces of cellophane.

Protein Digestion and Peptide Extraction

The 12.5kDa band was excised after silver staining of the gel. Tryptic digestion and peptide extraction were performed on the excised band.⁶ The gel band was destained in 15mM potassium ferricyanide/50mM sodium thiosulfate followed by washing with water and dehydration with acetonitrile. The isolated gel band was then incubated for 45 minutes at 55°C with 10mM dithiothreitol followed by incubation with 55mM iodoacetamide for 30 minutes at room temperature. The sample then was washed and dehydrated with alternating washes of 5mM ammonium bicarbonate followed by acetonitrile. After drying the extract in a speedvac for 15 minutes, tryptic digestion was performed with 12.5 μ g/ml trypsin in 5mM ammonium bicarbonate overnight at 37°C. Peptides were extracted with successive incubations of 25mM ammonium bicarbonate, followed by 5% formic acid and then acetonitrile. Samples were dried, cleaned, and concentrated using an OMIX C₁₈ pipette tip according to manufacturer's instructions (Varian, Palo Alto, CA).

Protein Identification by Tandem Mass Spectrometry

An Axima CFR MALDI-TOF mass spectrometer (Kratos, Manchester, UK) was used for protein identification and accurate mass measurements. Two microliters of the cleaned peptides along with 125fmol of a three-point calibrant mixture were spotted via the dried droplet method with 0.3 μ l saturated α -cyano-4-hydroxycinnamic acid (CHCA; Sigma, St. Louis, MO) in 50% ethanol/50% ddH₂O. Internal calibration was applied and the monoisotopic masses of the tryptic digest peaks were acquired. Tandem mass spectrometry (MS/MS) was performed on selected peaks. The monoisotopic masses of the tryptic digest peaks were combined with fragment data from the MS/MS into a single Mascot (www.matrixscience.com) search. To obtain an accurate mass of the peaks 12.5 and 13.4kDa, a CSF sample containing these peaks was processed as described above on a CM-10 chip. Before the addition of matrix, a three-point mass calibrant mixture was added directly to the sample spot to allow for internal calibration. Using a modified holder (with permission of CIPHERGEN Biosystems), we then analyzed these chips for accurate mass using an Axima CFR MALDI-TOF mass spectrometer.

Immunodepletion of Cystatin C

Twenty microliters of rabbit anti-human cystatin C or rabbit anti-fusin antisera (DakoCytomation, Carpinteria, CA) was bound to 10 μ l of protein A beads equilibrated in phosphate-

buffered saline, pH 7.4, by rocking at room temperature for 1 hour. Ten microliters of equilibrated protein A beads alone was used as another control. Each sample was washed three times with phosphate-buffered saline, pH = 7.4. A CSF sample was selected that contained both the 13.4kDa and the 12.5kDa peaks. Thirty microliters of this CSF was added to each of the above sample and rocked for 1 hour at room temperature. Fifteen microliters of the supernatant was applied to CM-10 arrays and analyzed as described above.

Cystatin C Levels

A sandwich enzyme-linked immunosorbent assay was used to measure cystatin C levels in the CSF samples according to the manufacturers instructions (Alexis Biochemicals, San Diego, CA). Each CSF sample and standard was analyzed in duplicate. Concentration of cystatin C in each CSF sample was determined using a standard curve and expressed as relative fluorescence units.

Cathepsin B Activity

Cathepsin B activity, a known substrate of cystatin C, was measured using an activity assay kit (Biovision Research Products, Mountain View, CA). This fluorescence-based assay utilizes the preferred cathepsin-B substrate sequence Arg-Arg labeled with amino-4-trifluoromethyl coumarin (AFP). Cathepsin-B cleaves the synthetic substrate RR-AFC to release free AFC. THP-1 cells (a monocytic cell line) were used as a source of cathepsin B. Cell lysates were prepared using a lysis buffer provided with the assay kit. Cell lysates from 1×10^6 cells were added to 50 μ l of CSF in a microtiter plate (quantity sufficient 100 μ l). Two microliters of substrate Ac-Arg-Arg-AFC was added to each well and incubated for 1 hour at 37°C. Absorbance was measured using a fluorescent plate reader with a 400nm excitation filter and 505nm emission filter. Controls included reaction buffer alone and a ca-

thepsin B inhibitor provided in the kit. All samples were analyzed in duplicate.

Results

A total of 217 peaks with a signal-to-noise ratio of 5 to 1 in the mass range of 3 to 100kDa were identified in the CSF samples. SELDI mass spectra for 12,000 to 13,500 m/z range from a representative control and MS patient is shown in Figure 1. Replicate samples were averaged and then analyzed by a Mann-Whitney *U* test, using a *p* value cutoff of 0.01. We found two peaks that were significantly elevated and another two peaks that were significantly diminished in the MS/CIS samples (Table 2 provided online). Interestingly, two of these peaks appeared to have a reciprocal arrangement, such that all MS/CIS patients in whom the 12.5kDa peak was elevated, the 13.4 peak was diminished. The 13.6kDa peak was a broad peak and may represent a complex mixture of proteins. A peak at 3.9kDa (see Table 2 provided on line was also significantly elevated in the patients with MS/CIS; however, the peak height was small and had only a twofold increase in the MS/CIS patients compared with controls. Hence, we have not pursued the identity of these proteins at this point. The 12.5kDa peak was present in 19 of 29 MS/CIS patients and in none of the patients with OND or TM. Its presence alone provided 100% specificity but only 66% sensitivity for diagnosis of MS when compared with these diseases. The 12.5kDa peak was found in some patients with HIV infection; the levels were small and significantly lower when compared with the MS/CIS patients. Because of a recip-

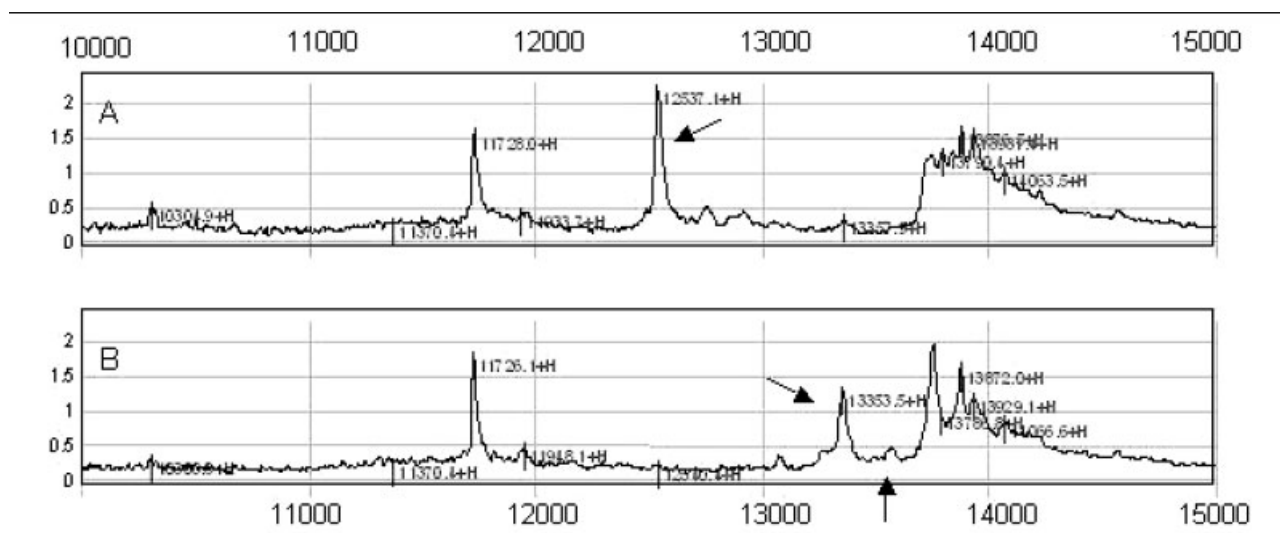


Fig 1. Representative cerebrospinal fluid spectra generated by surface-enhanced laser desorption/ionization analysis. (A) Patient with multiple sclerosis showing a prominent peak at 12.5kDa (arrow). The 13.4kDa peak is blunted. (B) The 12.5kDa peak is absent from the control patient. However, the 13.4kDa peak is prominent (slanted arrow). Another small peak at 13.6kDa is also noted (vertical arrow) which is absent from the spectra of the patient with multiple sclerosis.

Table 2. Peak Intensities Significantly Altered in Patients with MS

Peak	OND (mean intensity +SD)	MS (mean intensity +SD)	<i>p</i>
Protein peaks elevated in MS			
12.5kDa	0.04 + 0.03	0.46 + 0.42	<0.0001
3.9kDa	0.15 + 0.11	0.29 + 0.22	0.007
Protein peaks diminished in MS			
13.4kDa	0.73 + 0.26	0.41 + 0.32	0.0005
13.6kDa	0.20 + 0.06	0.13 + 0.08	0.0005

MS = multiple sclerosis; OND = other neurological disorder; SD = standard deviation.

cal relationship between the 12.5 and 13.4kDa peaks, we calculated a ratio of the 12.5kDa to 13.4kDa peak for comparison purposes. The ratios of the two peaks were significantly elevated in the MS/CIS group (mean \pm standard error [SE], 4.632 ± 0.909) compared with OND (mean \pm SE, 0.109 ± 0.011 ; $p < 0.001$), TM patients (mean \pm SE, 0.068 ± 0.006 ; $p < 0.001$), HIV ND (mean \pm SE, 1.646 ± 0.124 ; $p < 0.05$), and HIV-D (mean \pm SE, 1.815 ± 0.187 ; $p < 0.05$; Fig 2). To examine the stability of this protein in CSF, we reanalyzed three samples after leaving them at room temperature for 4 hours and overnight. We found that the 12.5kDa peak was stable with no change in CSF stored at room temperature for up to 4 hours and only a slight increase after overnight storage of CSF at room temperature. The peak was also not affected by heat treatment.

Despite the small samples sizes, we analyzed our data to determine if there was a correlation between the intensity of the 12.5kDa peak and the clinical pattern of MS (CIS, remitting relapsing, secondary progressive), measures of disease activity (duration since last attack, total lesion burden or contrast enhancement on MRI), or effect of treatment (Table 1). Although no correlation

could be found with any of these parameters, there were significantly higher levels in those patients whose last attack involved infratentorial regions (brainstem, cerebellum, and spinal cord) when compared with those individuals whose last attack involved supratentorial regions ($p = 0.02$; Fig 3). Interestingly, however, CSF from patients with acute transverse myelitis showed a prominent 13.4kDa peak in all samples, whereas the 12.5kDa peak was not visualized in any of them.

To identify the protein corresponding to the 12.5kDa peak, we studied its binding properties to CM-10 chips at different pH values. We found that the overall binding properties of the 12.5 and 13.4kDa peaks were similar, because decreased binding with increasing pH was observed (data not shown). Although maximal binding was seen at pH = 4.0, small amounts of this protein were still bound to the cation exchange chip even at pH 9.0, suggesting that the pI of this protein is greater than 9.0. For purification purposes, we chose a CSF that showed high levels of the 12.5kDa protein. This sample was first run through a protein A column to remove IgG, followed by treatment using a strong anion exchange spin column. Proteins that passed through these columns were collected and anal-

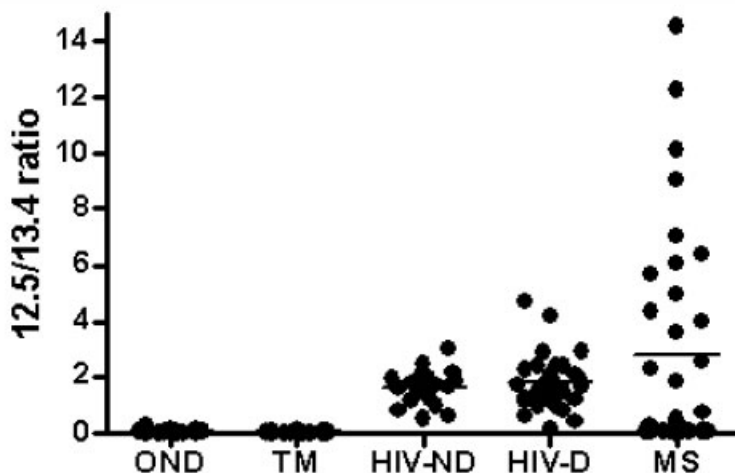


Fig 2. Comparison of the ratio of the 12.5 to 13.4kDa peak in cerebrospinal fluid from different disease states. The 12.5 to 13.4 peak ratio was significantly elevated in the multiple sclerosis (MS) group compared with other neurological diseases (ONDs) ($p < 0.001$), transverse myelitis (TM; $p < 0.001$), human immunodeficiency virus (HIV-ND; $p < 0.05$), and HIV-D ($p < 0.05$).

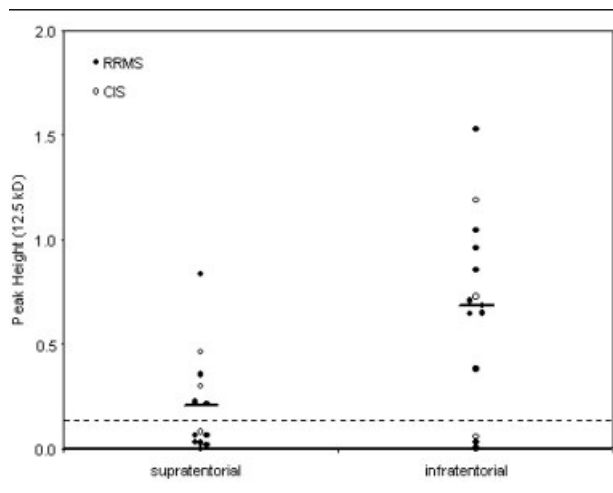


Fig 3. Effect of anatomical location of last clinical attack on 12.5kDa peak height in the cerebrospinal fluid of multiple sclerosis/clinically isolated syndromes (CIS) patients. The peak was significantly higher in patients with recent infratentorial disease activity compared with those with a supratentorial involvement ($p < 0.05$).

ysis by the CM10 chip showed the 12.5kDa peak had been enriched (Fig 4). This protein then was resolved by a tris-tricine gel and the corresponding band was sequenced by MALDI MS/MS. Combining the mo-

noisotopic masses of the tryptic peptides with the MS/MS fragment data yielded a Mascot score of 166 for human cystatin C (accession no. 14278690) with 51% sequence coverage. The MS/MS data from two peptides (1226.68Da, 2060.92Da) yielded Mascot ion scores greater than 40 (Table 3). This combination of sequence and mass fingerprint information allowed for an unambiguous identification of human cystatin C. Intact MW measurements of the 12.5kDa and 13.4kDa peaks obtained via the Axima CFR were 12,538Da and 13,361Da, respectively. The difference of 823Da between the two peaks corresponds to the mass of the last eight amino acids at the carboxy terminal of cystatin C (accession no. 14278690), consistent with the conclusion that 12.5kDa is a cleavage product of cystatin C.

The identity of this 12.5kDa protein was further confirmed by immunodepletion from CSF samples using antisera to cystatin C followed by SELDI time-of-flight mass spectroscopy analysis. We chose CSF known to have both the 12.5 and 13.4kDa peaks. As shown in Figure 5, exposure of the CSF to either protein A beads alone (Fig 5A) or to protein A beads bound to rabbit anti-fusin antisera used as a control antisera to an irrelevant antigen (Fig 5B) had no effect on the detection of these proteins. However, protein A beads bound to anti-

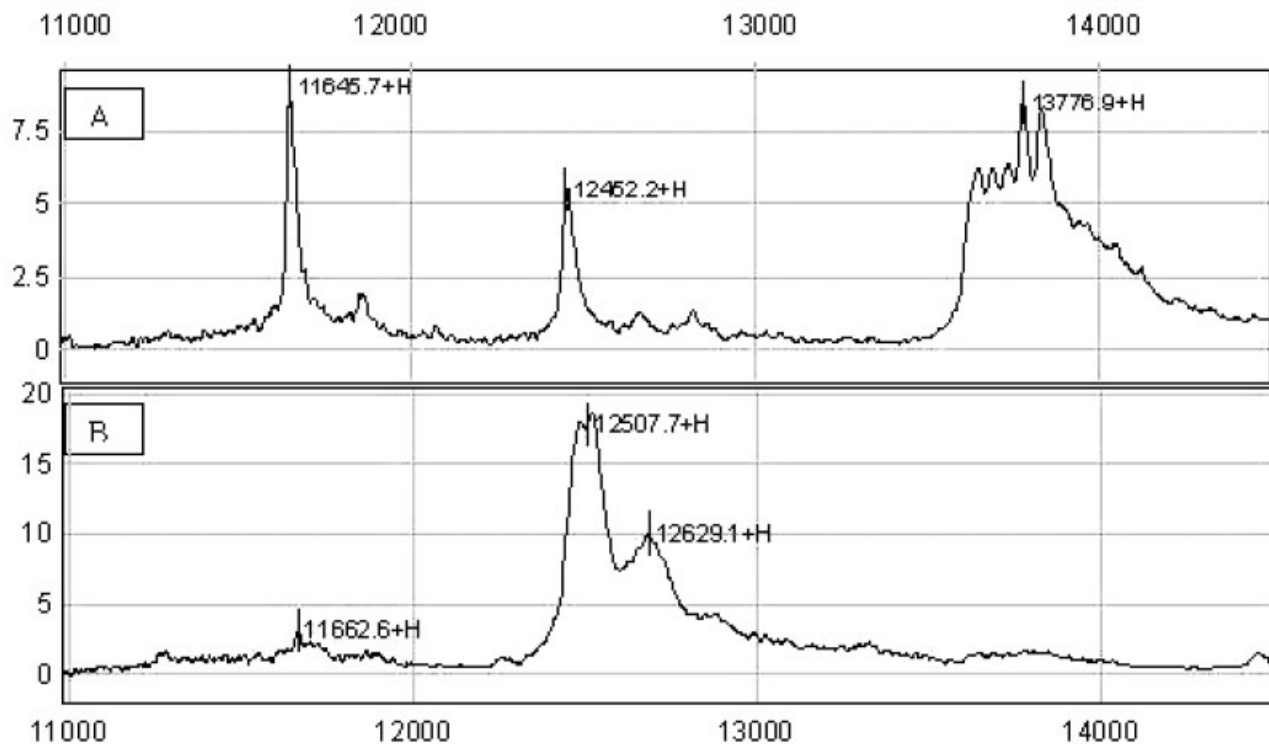


Fig 4. Partial purification of the 12.5kDa protein from cerebrospinal fluid (CSF). (A) CSF incubated with protein A beads to remove IgG and then analyzed by surface-enhanced laser desorption/ionization (SELDI) time-of-flight mass spectroscopy shows the presence of the 12.5kDa protein. (B) CSF was further exposed to strong anion exchange beads and reanalyzed by SELDI time-of-flight mass spectroscopy, which shows the removal of the 11.6 and 13.8kDa complexes and relative enrichment of the 12.5kDa protein.

Table 3. Peptides Recovered from Tryptic Digestion of the 12.5kDa Protein Band (amino acid residue, observed molecular weight, and sequence are shown)

Residues	Observed MW	Sequence
1–8	825.00	SSPGKPPR
9–25	1800.91	LVGGPMDASVEEEGVRR
9–25	1816.94	LVGGPMDASVEEEGVRR + oxidation (M)
25–36	1382.76	RALDFAVGEYNK
36–36	1226.68	ALDFAVGEYNK [ion score 60]
46–45	2303.93	ALDFAVGEYNKASNDMYHSR + oxidation (M)
37–45	1080.54	ASNDMYHSR
37–45	1096.55	ASNDMYHSR + oxidation (M)
46–53	912.62	ALQVVRAR
76–92	2060.92	TQPNLDNCPFHDQPHLK + (carbamidomethyl) [ion score 41]

cystatin C antisera (see Fig 5C) immunodepleted both the 12.5 and the 13.4kDa peaks confirming that both of them are cystatin C. A new peak at 12.1kDa was now seen likely representing a protein unmasked protein by the removal of cystatin C.

We next measured total cystatin C levels in the CSF of the patients with MS/CIS (mean \pm SEM = 9.3 \pm 0.3 units) and compared it with that of patients with OND (11.1 \pm 0.4 units). No significant differences were found between the two groups. Because cystatin C is a protease inhibitor that specifically blocks cathepsin B activity, we also measured cathepsin B activity in the CSF of patients with MS/CIS. A significant inverse correlation ($p < 0.05$) between the cystatin C levels and cathepsin B activity was found, suggesting that the cystatin C in the CSF of MS/CIS patients is bioactive (Fig 6A). To determine if cleavage of cystatin C alters

its ability to inhibit cathepsin B, we compared the 12.5/13.4kDa peak ratio with cathepsin B activity in the MS patients. MS patients with peak ratio greater than 0.1 the cathepsin B levels were 486 \pm 68.8 units (mean \pm SEM), and in MS patients with peak ratio less than 0.1 the levels were 697 \pm 52.8 (mean \pm SEM; $p = 0.06$). Further analysis of the MS group that showed a 12.5 to 13.4kDa peak ratio of greater than 0.1 shows that patients with the highest CSF 12.5 to 13.4 ratios also exhibited the greatest inhibition of cathepsin B activity (see Fig 6B), suggesting the possibility that cleavage at the C terminal region may actually enhance its inhibitory function.

Discussion

Identification of biomarkers for MS not only is of diagnostic importance but such markers could be used to

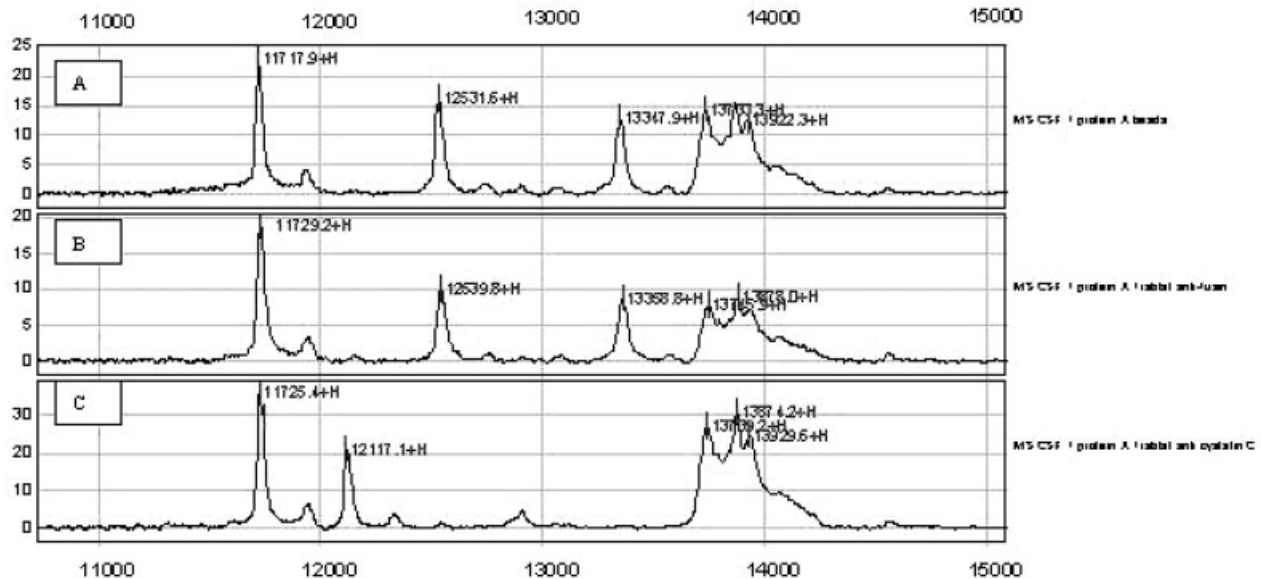


Fig 5. Immunodepletion of cystatin C from cerebrospinal fluid (CSF). CSF was analyzed by surface-enhanced laser desorption/ionization (SELDI) time-of-flight mass spectroscopy after incubation with either (A) protein A beads alone, (B) protein A beads bound to rabbit anti-fusin antisera, or (C) protein A beads bound to rabbit antisera to cystatin C. Both the 12.5kDa and the 13.4kDa proteins were selectively removed by the anticystatin antisera.

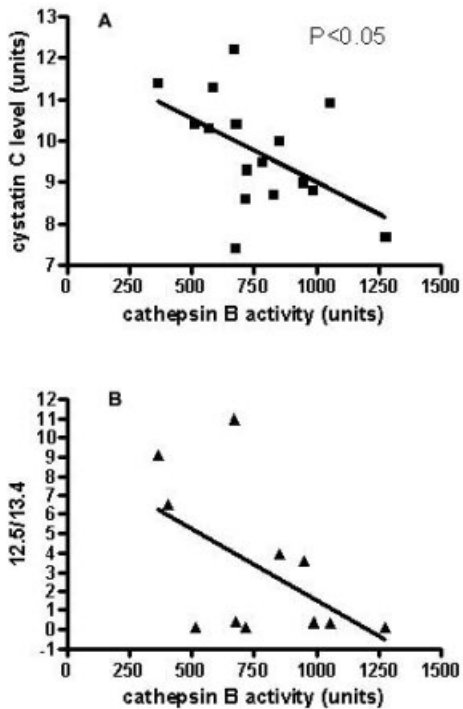


Fig 6. Correlation of cystatin C levels and cathepsin B activity in the cerebrospinal fluid (CSF) of multiple sclerosis (MS)/clinically isolated syndromes (CIS) patients. (A) Higher cystatin C levels were associated with lower cathepsin B activity, suggesting that cystatin C in the CSF of MS/CIS patients had not lost its cysteine protease inhibitory activity. (B) In some patients 12.5 to 13.4 peak ratios were associated with decreased cathepsin B activity.

predict future clinical events and may also be used for monitoring the effect of treatment. We demonstrate that CSF samples are a reliable biological specimen for SELDI analysis in search for biomarkers of MS. A distinct technical advantage of using CSF over serum is that it does not require preclearing of large and abundant proteins in serum that may mask the proteins of interest, which are usually present at much lower concentrations. The samples are also more likely to represent local events within the CNS compared with serum.

We used SELDI time-of-flight mass spectrometry to identify several novel protein peaks in the CSF of patients with MS/CIS compared with other controls. We focused in the mass range of 3 to 30kDa and compared only those proteins that bound to the weak cation chip. We identified a unique peak at 12.5kDa in the CSF of patients with MS/CIS. The identity of the 12.5kDa protein was established as a cleavage product of cystatin C formed by the removal of the last eight amino acids from the carboxy terminal of the protein. Because this 12.5kDa peak was found in two thirds of MS/CIS samples and not in any of the controls with

OND or TM, this may be a novel biomarker for MS and hence of diagnostic and pathogenic significance. Higher concentrations of this protein in patients with infratentorial lesions may be caused by the anatomical proximity of the lesions to the lumbar thecal sac from where the CSF was withdrawn or caused by unique features of MS lesions at these sites. However, the absence of the peak in patients with transverse myelitis may suggest that the pathophysiology of the lesions in the spinal cord of patients with TM and MS may be different. A previous study that included CSF samples from normal controls did not identify a 12.5kDa peak.⁷

Several lines of evidence suggest that the 12.5kDa peak is a breakdown product of the 13.4kDa peak. The intensity of the 12.5kDa peak and that of the 13.4kDa peak seem to be reciprocally related to each other and the sequence analysis of the 12.5kDa peak revealed that it corresponds to cystatin C, which is known to have a molecular mass of 13.4kDa.⁷ Heating the CSF had no effect on the levels of the 12.5 and 13.4kDa peaks, whereas repeated freeze-thaw cycles and overnight storage of CSF at room temperature resulted in a slight increase in the 12.5 peak intensity, which suggests that heat treatment may denature the protease that cleaves the 13.4kDa protein into the 12.5kDa form. These observations have important implications for future studies for biomarker discovery efforts in MS that will require the use of prospectively collected samples with strict adherence to uniform protocols for the collection, centrifugation, and storage of CSF samples.

Cystatin C is an inhibitor of cysteine proteases including cathepsins B, H, K, L, and S.⁸ It is present in high concentrations in CSF compared with serum and other body fluids.⁹ The protein is a nonglycosylated molecule of 120 amino acids formed after removal of a 26-amino acid signal peptide.¹⁰ Thus, any altered activity or levels of cystatin C would also result in dysregulation of cathepsin function which have been implicated in a variety of effects including degranulation of cytotoxic lymphocytes¹¹ and in processing of major histocompatibility class II antigen in monocytes.¹² A previous study that measured cystatin C levels in CSF of MS patients by enzyme-linked immunosorbent assay also found diminished levels in patients compared with healthy controls. Conversely, levels of cathepsin B were increased in CSF and brain of patients with MS.^{13,14} In contrast, although we did not have access to totally normal CSF, our studies did not show any significant difference between the cystatin C levels in the MS patients compared with patients with ONDs. Interestingly, other studies have shown that cystatin C levels are increased in the CSF of patients with Alzheimer's disease⁷ and Creutzfeldt-Jakob disease.¹⁵ In both these studies, CSF was analyzed by SELDI and the 13.4kDa

protein was further sequenced to identify it as cystatin C. In Icelandic patients with a hereditary form of amyloid angiopathy, a mutated form of cystatin C (Leu68Gln substitution) has been found. This protein accumulates in the amyloid deposits and is truncated by 10 amino acids at the amino terminal.¹⁶ This region is critical for the functional activity of cystatin C.¹⁰ Leukocyte elastase has been shown to cleave cystatin C at Val₁₀-Gly₁₁ resulting in loss of its ability to bind to cathepsins.¹⁷ In our experiments, one of the peptides from the tryptic digest of the 12.5kDa protein that matched to cystatin C contained an intact Leu₅-Val₁₀-Gly₁₁ and an intact amino-terminal region suggesting the presence of a novel cleavage site at the carboxy terminus in the MS patients. The mass differences between the 12.5 and 13.4kDa proteins suggest that the cleavage site is at eight amino acids from the carboxy terminal end of the protein.

The role of cystatin C in the pathogenesis of MS is not understood. Elevated serum cystatin C levels have recently been shown to be a strong predictor of death in patients with cardiovascular disease.¹⁸ We did not find any significant difference in the total cystatin levels in the MS/CIS patients compared with controls. Our data suggest that the total levels of cystatin C are inversely proportional to cathepsin B activity. Furthermore, it appears that cleavage of cystatin C did not lead to any augmentation of cathepsin B activity. In fact, the patients with the highest 12.5 to 13.4 ratios seemed to have the highest cathepsin B inhibition activity as well. This raises the possibility that cleavage of cystatin C at the carboxy terminus may lead to enhanced activity of this protein. This is in keeping with previous studies in which the protease inhibiting effects of the molecule have been ascribed to the amino terminal region of the molecule.¹⁰ Cleavage of the carboxy terminus of cystatin C thus may be an adaptive host response in MS. If confirmed, this raises the possibility that development of other inhibitors of cysteine proteases may have some therapeutic potential. Short synthetic peptidyl-diazomethyl ketones have been developed that mimic the activity of the amino terminal domain of cystatin C, and their in vitro use leads to inhibition of bone matrix degradation by cysteine proteases resulting in decreased bone resorption.¹⁹ E64 derived from *Aspergillus japonicus* is also a strong irreversible inhibitor of cysteine proteases.^{20,21} Several other compounds have been designed to inhibit the activity of cysteine proteases.⁸ Nonetheless, measurement of levels of cystatin C and its breakdown product in the CSF of MS patients may identify a subtype of MS. However, larger sample sizes from MS patients at different stages of disease are needed to further validate our observations. Still, the absence of the cystatin C cleavage product in the CSF of patients with TM and

other neuroinflammatory diseases suggests that inflammation alone is not sufficient for cleavage of this protein. Therefore, this cleavage product may not only identify a subgroup of patients with MS/CIS but it may also be able to separate these patients from other inflammatory diseases.

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