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Cerebrospinal Fluid Proteomic Profiling of HIV-1-Infected Patients with Cognitive Impairment

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Advanced HIV-1 infection is commonly associated with progressive immune suppression and the development of cognitive, motor, and behavior disturbances. In its most severe form, it is diagnosed as HIV-1 associated dementia (HAD) and can progress to profound functional disability and death. Despite prodigious efforts to uncover biomarkers of HAD, none can adequately reflect disease onset or progression. Thus, we developed a proteomics platform for HAD biomarker discovery and used it to perform a pilot study on cerebrospinal fluid (CSF) from HIV-1-infected people with or without HAD. A 2-dimensional electrophoresis (2-DE) map of a HAD CSF proteome was focused on differentially expressed proteins. 2-DE difference gel electrophoresis (2-D DIGE) analysis showed >90 differences in protein spots of which 20 proteins were identified. Differential expression of 6 proteins was validated by Western blot tests and included vitamin D binding protein, clusterin, gelsolin, complement C3, procollagen C−endopeptidase enhancer 1, and cystatin C. We posit that these proteins, alone or together, are potential HAD biomarkers.

Keywords: proteomics • 2-dimensional electrophoresis • DIGE • cerebrospinal fluid • HIV • cognitive impairment • HAD

Introduction

HIV-1 invades the brain soon after the initial seroconversion reaction.[1] Virus can enter the brain in free form or through infected CD4+ T cells and macrophages. Virus ingress through the blood-brain barrier (BBB) is facilitated by its functional breakdown.[2] Over periods measured in years and following long standing productive viral replication and immune compromise, HIV often affects the development of behavioral, motor, and cognitive deficits that, in their most severe form, are called HIV-1 associated dementia (HAD).[3] Disease mechanisms revolve around the induction of a metabolic encephalopathy caused by secretory products released from immune-competent and virus-infected brain mononuclear phagocytes (MP; perivascular macrophages and microglia).[4] This is commonly associated with a multinucleated giant cell encephalitis, whose pathological features include neuronal injury, myelin pallor, and astro- and micro-gliosis.[5] Although the mechanisms for disease are not fully understood, they are based, in measure, on the abilities of immunological products and viral proteins released from infected cells, to damage neurons. Despite the widespread use of anti-retroviral therapy, disease incidence and prevalence remains on the rise.[6]

Currently, HAD is a diagnosis of exclusion made primarily on clinical grounds when concurrent opportunistic infections, psychiatric disorders, and malignancies are eliminated.[7] Neuropsychiatric evaluations demonstrating significant cognitive dysfunction with or without behavioral and motor disease manifestations support diagnosis of HAD.[8−10] HAD usually but not always is associated with immune suppression and high viral loads. Thus, a number of studies have been initiated to seek biomarkers relevant for this disease diagnosis as well as use in therapeutic monitoring for anti-retroviral responses and for adjunctive medicines.[11−13] Such studies have uncovered potential biomarkers for HAD including arachidonic acid and its metabolites, platelet activating factor, matrix metalloproteinases, soluble Fas (sFas) and Fas ligand, amyloid beta 42 and tau, pro-inflammatory cytokines, quinolinic acid, glycolipids, activation markers, and oxidative stress markers.[14−20]

CSF surrounds the brain and spinal cord providing nutritive, protective, and environment support for the nervous system.[16] Its functions include buoyancy, buffering, and delivery of electrolytes and micronutrients to the brain parenchyma.[21] It is well accepted that CSF proteins and/or their fragments will

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reflect central nervous system (CNS) metabolic activity and can be reflective of ongoing brain pathological processes.[22,23] Approximately a quarter of cellular proteins secreted or shed within the CNS are present in the intracellular milieu.[24] Therefore, it is reasoned that the CSF proteome can provide valuable information about the CNS in disease.[25−29] Recent advances in proteomics combine multidimension analyses of the global protein composition with mass spectrometric identification. This opens up the possibilities towards gaining insights into ongoing pathological events in the CNS through examination of the CSF proteome. However, evaluation of the CSF proteome remains a challenge for a number of reasons. First, protein concentration in CSF ranges from 0.2 to 0.8 mg/mL, which is approximately 100 times less than what is present in serum.[31] Second, albumin, transferrin, and immunoglobulins constitute more than 70% of already low levels of proteins in CSF.[32,33] Third, biomarkers may be present at very low abundance and because of the high turnover and dynamic nature of the CSF there may be limited accumulation of disease-associated proteins.[34]

Thus, the focus of this study is to find changes in the CSF proteomes that differentiate HAD form HIV-1 infected subjects without cognitive impairment. To achieve this goal, we used two-dimensional difference gel electrophoresis (2-D DIGE) for protein profiling and electrospray ionization liquid chromatography tandem mass spectrometry (ESI−LC−MS/MS) peptide sequencing for protein identification. Western blot analysis was used to validate the data set. The proteins identified associated with HAD provide unique insights into the CSF proteome, which reflect the tempo and progression of neurological decline associated with advanced viral infection.

Materials and Methods

CSF Sample Acquisition. CSF was acquired from HIV-1 infected individuals with or without cognitive impairment by the National NeuroAIDS Tissue Consortium (NNTC, Request #R101) and the NeuroAIDS Research Program at the University of Puerto Rico Medical Sciences Campus (UPR-MSC). Patients were scored as HAD based on neuropsychological examinations.[35,36] Fluids were obtained as part of routine examinations in clinic and stored as 1 mL aliquots. CSF samples were shipped frozen to the University of Nebraska Medical Center (UNMC) on dry ice. Use of CSF samples in this study has been approved by UNMC Institutional Review Board (#196−05-EX).

CSF Sample Preparation. One milliliter of CSF was supplemented with 0.1% Triton X100 and protease inhibitors (BioVision, Mountain View, CA). The samples were filtered through 0.2 μm spin filters (Life Science Products, Frederick, CO) at 4000*g* for 2 min to remove particulate matter. The Multiple Affinity Removal System Hu-6 (Agilent Technologies Inc., Santa Clara, CA) was used for selective removal of the 6 most abundant proteins. The system consists of high-performance liquid chromatography (HPLC) column 4.6 × 50 mm^2 and optimized buffers for sample loading, washing, eluting, and regenerating. CSFs were diluted with 3 volumes of buffer A (ready to use phosphate buffer) and then applied to a column at flow rate 0.25 mL/min (EZStart HPLC Shimadzu, Columbia, MD). One milliliter of diluted sample was used per run. Flow-through fractions were collected from 3.5 to 10.5 min. Bound proteins were eluted with buffer B (ready to use elution buffer) at the flow rate of 1 mL/min from 15 to 18.5 min and the column was regenerated with buffer A for the next 13.5 min at 1 mL/min flow rate. Depleted CSFs were concentrated on spin filters Vivaspin 6 with 5 kDa cutoff membrane (Vivascience, Hannover, Germany) by centrifugation at 4000*g* at 4 °C. Sample volume was reduced to 0.1 mL.

Proteins were purified by precipitation using a 2D Clean-Up Kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. After precipitation, protein pellets were resuspended in 7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 1% Pharmalyte (pH 3−10) before minimal labeling or 0.1% Triton X100 before saturated labeling. Protein concentration was measured using a 2D Quant Kit (GE Healthcare Piscataway, NJ) and Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Minimal Labeling of CSF Proteins with CyDyes. Fifty micrograms of proteins from CSF from nondemented (ND) or HAD patients were labeled with Cy3 or Cy5 dyes respectively (CyDye Minimal Labeling Kit, GE Healthcare Piscataway, NJ). Four hundred picomols of dye per 50 μg of proteins was used. Twenty-five micrograms of protein from each CSF was mixed, labeled with Cy2 minimal dye, and used as an internal standard. Cy2-, Cy3-, and Cy5-labeled samples and 250 μg of nonlabeled pooled CSF were mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 1% Pharmalyte pH 3−10) and loaded on one Immobiline strip (24 cm, pH 3−10, GE Healthcare Piscataway, NJ) by overnight rehydratation. The experiment was repeated three times using three pairs of CSFs (ND and HAD).

Saturation Labeling for Analytical Gels. Five micrograms of six CSF samples were speed vacuum-dried and resuspended in lysis buffer (30 mM Tris-HCl pH 8, 7 M urea, 2 M thiourea, 4% CHAPS). Proteins were reduced by incubation with 2 nM TCEP [tris-(2- carboxyethyl) phosphine hydrochloride] at 37 °C for 1 h then labeled with 4 nM of Cy5 saturation dye for 30 min at 37 °C. One volume of sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% Pharmalyte, 130 mM DTT) was added to the samples.

An internal standard was prepared using 5 μg of each sample mixed together then speed vacuum-dried. Protein pellet was resuspended in lysis buffer (30 mM Tris-HCl pH 8, 7 M urea, 2 M thiourea, 4% CHAPS) and reduced with 2 nM TCEP per 5 μg protein for 1 h at 37 °C. The proteins were labeled with 4 nM of Cy3 saturation dye per 5 μg protein for 30 min at 37 °C. One volume of sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% Pharmalyte, 130 mM DTT) was added to samples. Each 5 μg of proteins of CSF were labeled with Cy5 were mixed with 5 μg of internal standard labeled with Cy3 and rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 60 mM DTT, 1% Pharmalyte, pH 3−10) was added to the final volume of 450 μL.

Saturation Labeling for Preparative Gels. Remaining CSF samples were pooled and used in the preparative gel. Total amount of protein used was 280 μg. Samples were mixed and speed vacuum-dried. Protein pellet was resuspended in 0.25 mL of lysis buffer (30 mM Tris-HCl pH 8, 7 M urea, 2 M thiourea, 4% CHAPS) and treated with TCEP (2 nM per 5 μg protein) for 1 h at 37 °C. Proteins were labeled with Cy3 saturation dye for preparative labeling (4 nM per 5 μg proteins) for 30 min at 37 °C. To stop the reaction, sample buffer (7 M urea, 2 M thiourea, 4% CHAPS) was added to the final volume of 445.5 μL. Pharmalytes, 4.5 μL, pH 3−10 (1% final concentration) and 4.5 mg of DTT was added (final DTT concentration of 130 mM).

2-D Electrophoresis (2-DE) and DeCyder Analysis. First dimension separation was carried out with an IPGphor II

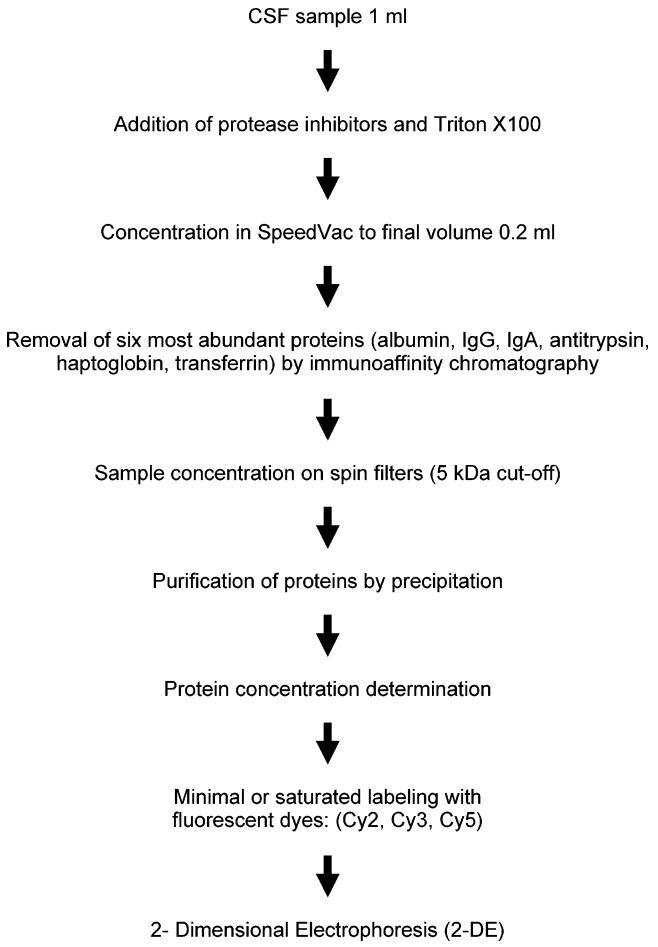

Figure 1. Flow chart presenting an outline of CSF sample preparation for 2-D DIGE. In minimal labeling HAD samples were labeled with Cy5, ND samples were labeled with Cy3 and controls were labeled with Cy2. In saturated labeling, HAD and ND samples were labeled with Cy5. Each sample was mixed with internal standard labeled with Cy3. For preparative gel, samples were labeled with Cy3 saturation dye.

apparatus (GE Healthcare Piscataway, NJ). Samples were loaded on Immobiline DryStrips gels (24 cm long) with linear immobilized pH gradient 3−10 by overnight rehydratation. Each gel contained the standard and one of the samples (analytical gels, after saturated labeling), pairs of samples (minimal labeling), or pooled material for the preparative run. Isoelectrofocusing step was carried out at a constant temperature of 20 °C with a total 45 kVh (500 V for 0.5 kVh, gradient to 1000 V for 0.8 kVh, gradient to 8000 V for 13.5 kVh, 8000 V for 30.2 kVh). For the preparative run after saturation labeling 65 kVh was applied (500 V for 0.5 kVh, gradient to 1000 V for 0.8 kVh, gradient to 8000 V for 13.5 kVh, 8000 V for 50.2 kVh). Before the second dimension, separation strips were incubated with an equilibration solution [50 mM Tris-HCL pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue] containing 100 mM DTT for 15 min. For protein alkylation, gel strips were incubated with 100 mM iodoacetamide in equilibration solution for next 15 min. Protein alkylation was applied only for samples labeled with minimal dyes. Then strips were loaded on the top of 12% polyacrylamide gels and fixed with 0.5% agarose. Second dimension was carried out with an Ettan Daltsix Electrophoresis System (GE Healthcare Piscataway, NJ) at 20 °C. Power was held constant to 5 W per gel for the initial 30 min and 17 W per gel until bromophenol blue reached the gel bottom. For visualization of protein spots, signals were collected at excitation wavelength for Cy2-, Cy3-, and Cy5-labeled samples at 488, 520, and 620 nm, respectively, using Typhoon 9410 Variable Mode Imager (GE Healthcare Piscataway, NJ). Gels were scanned at 100 μm resolution and analyzed using DeCyder 2-D 6.5 software (GE Healthcare Piscataway, NJ). For visualization of protein spots used for identification after minimal labeling, gels were stained with Sypro Ruby and scanned at 400 nm on Typhoon 9410. Spots selected for protein identification after DeCyder analysis were picked from gels by automatic Ettan Spot Picker (GE Healthcare, Piscataway, NJ) with 2 mm diameter tip.

In-Gel Digestion. Gel pieces excised from 2-DE gels were washed at room temperature with 200 μL 50% ACN/50 mM NH_4HCO_3 for 1 h. Gel pieces were dried, and 1 μg trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3 was added to each piece. Tryptic digestion was carried out overnight at 37 °C. Peptides were extracted with 60% acetonitrile and 0.1% trifluoroacetic acid (TFA), dried, and resuspended in 0.5% TFA. All samples were purified using ZipTip (Millipore, Billerica, MA) according to manufacturer's procedure and resuspended in 0.1% formic acid prior to mass spectrometric analysis.

Mass Spectrometry and Protein Identification. Peptides were fractionated on a microcapillary RP-C_{18} column (New-Objectives, Woburn, MA) followed by fragmentation using ESI−LC−MS/MS system (ProteomeX System with LCQDecaPlus, ThermoElectron, Inc., San Jose, CA) in a nanospray configuration. The spectra obtained from mass spectrometric analyses were searched using Sequest search engine (BioWorks 3.2 software from ThermoElectron Inc., San Jose, CA). In the TurboSEQUEST search parameters for .Dta Generation, we used a threshold of 50 000, precursor mass tolerance of 1.4 AMU, Group scan of 1, Minimum group count of 1, and minimum ion count of 15. In the TurboSEQUEST search parameters, we used peptide tolerance 2.0 AMU and fragment ions tolerance 1.0 AMU with charge state set on "Auto". An "in-house" indexed human.fasta.idx database with the following 5 key words: Homo, sapiens, human, man, primate was created from nr.fasta retrieved from ftp.ncbi.nih.gov. Keratins and cytokeratins were excluded from our human.fasta.idx database. Exclusion of these proteins was based on observation that identified keratins belonged to those characteristic for epidermis and thus could be a contaminant acquired during lumbar puncture. The database was updated every other week, and oxidated methionine was allowed in searches. For high-confidence protein identification, at least two unique peptides sequenced were required.

One-Dimensional Electrophoresis (1-DE) and Western Blot Analysis. 1-DE separations were performed using the NuPAGE gel system (Invitrogen Corp., Carlsbad, CA) in 4−12% gradient bis-tris gels under reducing conditions. For Western blot analyses, 2 μg of CSF protein after depletion were loaded per lane. Electrophoresis followed by transfer and immunodetection was performed as previously described.[37] The following primary antibodies were used: HYB 249−01 monoclonal anti-vitamin D binding protein (DBP; Abcam, Cambridge, MA), rabbit anti-cystatin C (U.S. Biological, Swampscott, MA), E5 monoclonal anti-clusterin (BD Biosciences, Lexinkton, KY), chicken IgY anti-complement C3 (GenWay, San Diego, CA), rabbit anti-procollagen C endopeptidase enhancer (PCPE-1;

Table 1. Protein Identification Summary

spot #	protein	NCBI	Swiss-Prot #	MW	p*I*	spots
B658	A Chain A, Human Serum Transferrin	4699632	Q06AH7	76959	6.97	17,18
A2409	A1BG_HUMAN Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein)	46577680	P04217	54272	5.58	19,20
A2590	alpha 2-plasmin inhibitor, alpha 2-PI {N-terminal, form A}	300028	P08697	54565	5.87	28
B824	Alpha-1-antichymotrypsin precursor	4165890	P01011	47651	5.33	32
A2271	Amyloid precursor-like protein 1 isoform 1 precursor	67782338	P51693	72247	5.54	16
C898	Angiotensinogen (serine (or cysteine) proteinase inhibitor	30584507	P01019	53154	5.87	9,33,35−37,39,40
C1380	Apolipoprotein A−I precursor (Apo-AI)	55637005	P02647	30777	5.56	80
A5783	Apolipoprotein AI, apo AI [human, spleen, Peptide Mutant, 88 aa]	253362	P02647	10161	4.76	82,83
B1095	Apolipoprotein A-IV precursor	71773110	P06727	45372	5.28	53−56
B1225	Apolipoprotein E	13097699	P02649	36154	5.65	63,66
B1208	Apolipoprotein E precursor	4557325	P02649	36154	5.65	54,57,60−71,75,83
A2590	ASPIC [*Homo sapiens*]	9368808	Q9NQ79	71420	4.95	28
B1831	B Chain B, Familial Als Mutant G37r Cuznsod	2982081	P00441	15904	5.87	85
A5678	beta-trace {N-terminal} [human, cerebrospinal fluid, Peptide Partial, 27 a	410564	P05067	2893	6.11	76−79
A1474	Ceruloplasmin	1620909	Q1L857	115471	5.43	2
C1221	CLU protein	18043615	P10909	52495	5.89	72
B1208	Clusterin	32891795	P10909	52495	5.89	57,58,60−62,65,69
B1204	Clusterin isoform 1	42716297	P10909	57832	6.25	59
C1080	CO3_HUMAN Complement C3 precursor	116594	P01024	187164	6.02	51,52
A2590	Complement 9	2258128	P02748	63173	5.43	28
B1351	Complement C4-B precursor	81175167	P0C0L5	192793	6.73	19,23,25,74
A2478	Complement component 3 [*Homo sapiens*]	40786791	P01024	187146	6.02	21,22
C1236	Complement component C4	2347136	P0C0L5	192793	6.73	73,74
C727	Complement factor B	2347133	P00751	85533	6.67	5,6,7,8
B930	Crystal Structure Of The Complex Of Actin With Vitamin D− Binding P	22219267	P02774	51216	5.17	38
B1565	D Chain D, Crystal Structure Of Human Apolipoprotein A−I	2914178	P02647	23403	5.55	80−83
B755	D Chain D, Human Complement Component C3c	78101272	P01024	71190	6.82	21−24,26
A4647	envelope glycoprotein gp120 [Human immunodeficiency virus 1]	85662893	A0A891	22754	5.08	71
C653	EPC-1	1732049	P36955	46342	5.97	1,6,42−49
B592	Gelsolin isoform b	38044288	P06396	80640	5.58	13−15
A1879	Growth-inhibiting protein 24 [*Homo sapiens*]	46981959	Q2TU88	47674	5.33	9
C880	Growth-inhibiting protein 25	46981961	Q2TU87	32821	5.71	32
B2156	H Chain H, N-Truncated Human Cystatin C	55669629	P01034	12328	7.86	89,90
C839	Hemopexin	11321561	P02790	51676	6.55	26
A2964	*Homo sapiens* angiotensinogen (serine (or cysteine) proteinase inhibitor	30584507	P01019	53227	5.87	33
B779	Human Serum Albumin	4389275	P02768	69367	5.92	30,31
A2518	Human Serum Albumin In A Complex With Myristic Acid and Tri-Iodobenzoic Acid	4389275	P02768	69367	5.92	31,53
C693	ITIH4_HUMAN	13432192	Q14624	103358	6.51	3
A2656	Kininogen	386853	P01042	71957	6.34	27,28
C730	MHC serum complement factor B	7145102	P00751	85533	6.67	6
C692	NrCAM protein	2511666	Q92823	143894	5.45	3,4
A2964	NUCB1_HUMAN Nucleobindin 1 precursor (CALNUC)	46397857	Q02818	53879	5.15	34
C1000	Pigment epithelium-derived factor	15217079	P36955	46342	5.97	42−47
A5782	PREDICTED: similar to Apolipoprotein A−I precursor (Apo-AI)	55637005	P02647	30778	5.56	76,82,83
A3401	Procollagen C-endopeptidase enhancer	51094573	Q15113	47972	7.41	50
A7176	Proprotein convertase subtilisin/kexin type 1 inhibitor	12804001	Q9UHG2	27372	6.22	84
B1474	Prostaglandin D2 synthase 21kDa (brain)	54696706	P41222	21029	7.66	76,79
C1181	Retinol Binding Protein	4558178	P02753	23010	5.76	67
B847	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin)	4502261	P01008	52602	6.32	33,35−37
C1000	Serine (or cysteine) proteinase inhibitor, clade F	12653501	P36955	46342	5.97	42,43,45−47
A4647	SHPRH protein [*Homo sapiens*]	109734410	Q149N8	193126	7.3	71
B592	Similar to gelsolin (amyloidosis, Finnish type)	17028367	P06396	85,698	5.9	10−15
B2029	The Structure Of Human Retinol Binding Protein	4558175	P09455	13406	5.5	68,87,88
C1221	Transthyretin	37483	P02766	15887	5.52	41,72,86,87
B782	Vimentin	5030431	P08670	53652	5.06	29,77
C956	Vitamin D-binding protein/group specific component	455970	P02774	52964	5.4	38

Sigma-Aldrich, St. Luois, MO), and monoclonal anti-gelsolin (BD Biosciences, Lexington, KY). Secondary antibodies included HRP-conjugated F(ab′)$_2$ fragment of goat anti-mouse, goat anti-rabbit, and donkey anti-chicken IgY (Jackson Immunoresearch, West Grove, PA). All antibodies were used at concentrations recommended by the manufacturer.

Results and Discussion

CSF Samples Processing for Proteomic Analysis. Thirty-eight CSF samples were collected and analyzed for this study. They contained proteins in a range of 0.16−0.9 μg/mL with the majority of samples (29 of 38) containing 0.16−0.60 μg/mL. CSF

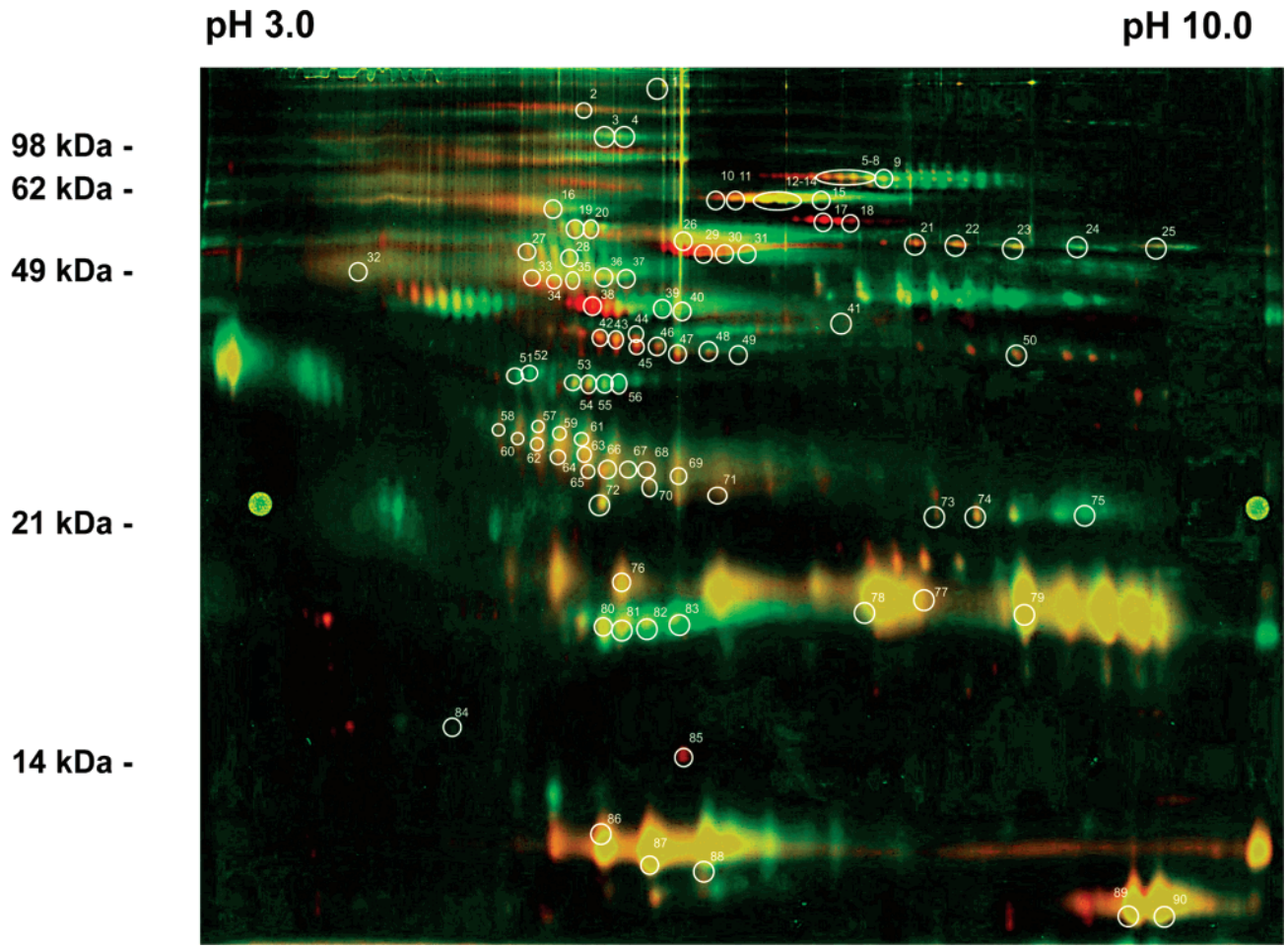

Figure 2. Representative gel from 2-D DIGE experiment, which included six CSF samples with Cy dye minimal labeling. Fifty micrograms of protein from ND (Cy3-labeled), 50 μg of protein from HAD (Cy5-labeled), and 50 μg of internal standard (Cy2-labeled) was loaded on one 2D gel. Gels for minimal labeling were stained with Sypro Ruby. After electrophoresis, gels were processed as described in Materials and Methods. White circles and numbers indicate identified proteins that correspond to Table 1.

samples were depleted of the six most abundant proteins. This included serum albumin, IgG, IgA, antitrypsin, haptoglobin, and transferrin. Details of sample processing are presented in Figure 1. The average yield after immunodepletion and 2D Clean-up processing was ~10−20% of original protein contents (data not shown). To secure sufficient amount of protein for mass spectrometry identifications, 75 μg of protein from one CSF sample was necessary for one large format 2-D DIGE gel with minimal labeling; we had only 6 samples (3 from HAD and 3 from ND group) in the cohort of samples obtained from NNTC that met these thresholds. This is a limitation in studying proteomes of CSF unless samples are pooled. Despite the high efficiency immunodepletion, traces of serum albumin and immunoglobulins were found (see Table 1).

2-D DIGE with Minimal Labeling of CSF from HAD and ND Patients. Using 2-D DIGE with minimal labeling proteomic platform, we compared protein patterns among the 6 CSF samples (3 HAD and 3 ND). All were from HIV-seropositive individuals and each sample contained at least 75 μg of protein after immunodepletion. In all instances, samples from patients with HAD (50 μg of protein) were labeled with Cy5 and those from ND (50 μg of protein) were labeled with Cy3. An equal mixture of samples (25 μg from each) were labeled with Cy2 and used as internal control. Pairing of samples for individual gels was based only on a diagnosis of HAD provided by NNTC. Figure 2 shows a representative gel of three 2-D DIGE experiments. Altogether, 425 protein spots were excised from all three gels and were further subjected to in-gel tryptic digestion and protein identification by ESI−LC−MS/MS. These yielded high-confidence identification in 221 spots and the combined results of high-confidence identifications are presented in Table 1. As expected, many proteins were identified in more than one spot. Broadly selected spots, those that indicated differential expression and those equally expressed in HAD and ND, were secured. Our goal was to find differentially expressed proteins as well as to create a map of proteins, which can be used as reference in our future experiments. Our criteria for selection of differentially expressed proteins were that a spot had similar DIGE Index in at least two out of three gels. In some instances, interpretation of data was difficult because one protein was identified in multiple spots, e.g., gelsolin, among which some spots were up- and some were down-regulated in HAD (data not shown). In other instances, up-regulation of vitamin D binding protein (DBP) was consistent in all gels and spots; however, the DIGE index varied from 1.1 to 60.5. Therefore, proteins which were identified with a DIGE Index > 2.0 and reproducible in all gels were selected for Western blot validation. Figure 4A−C presents results of such validation using samples from different cohort.

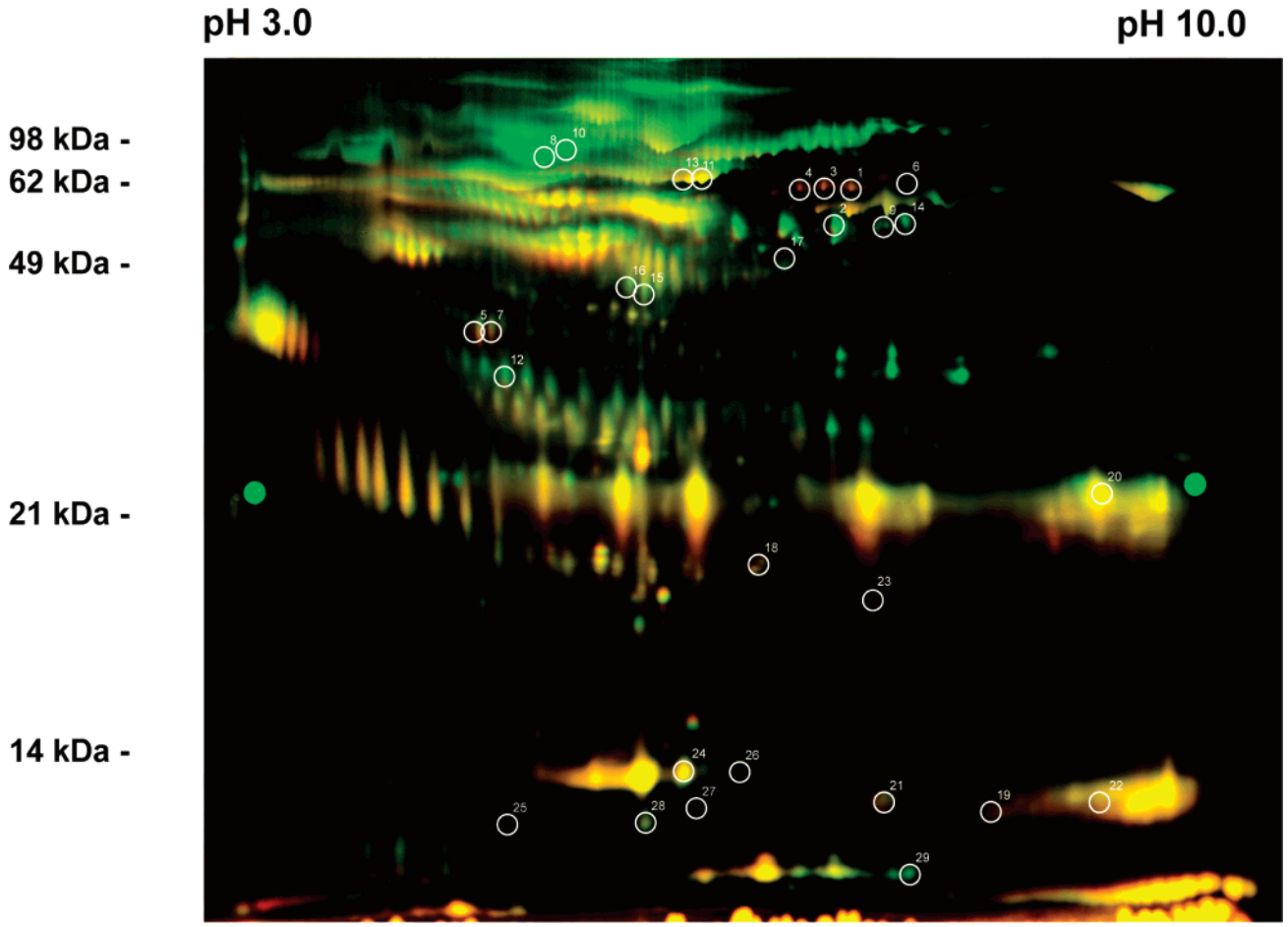

Figure 3. Representative gel from 2-D DIGE experiment, which included six CSF samples with Cy dye saturation labeling. Five micrograms of six CSF samples were labeled with Cy5 saturation dye. To prepare the internal standard, 5 μg of each sample were mixed. Next, proteins were labeled with Cy3 saturation dye. Five micrograms of each CSF labeled with Cy5 was mixed with 5 μg of internal standard labeled with Cy3 and run on one gel. White circles and numbers indicate identified proteins that correspond to Table 2.

Of 425 samples, we made 221 high-confidence protein identifications. Because of the inherent limitation of protein content among samples, 2-D DIGE with saturated labeling combined with preparative gel made of pooled samples was selected for the next experiment utilizing CSF samples with lower protein contents.

2-D DIGE with Saturated CSF Labeling from HAD and ND HIV-Infected Patients. This approach allowed us to use 10 μg of protein from each of 6 samples (3 HAD and 3 ND) from the NeuroAIDS Program at the UPR-MSC and remaining material was pooled to create one sample of 280 μg of proteins for one preparative gel. CSF samples were immunodepleted of the 6 most abundant proteins. For analytical runs, 5 μg of protein from each sample was labeled with Cy5, then an additional 5 μg of each sample were mixed together and labeled with Cy3. Five micrograms of this mixture was added to each sample labeled with Cy5. Samples labeled with Cy3 served as internal control. Pooled CSF designated for use in preparative gel were labeled with Cy3 (saturated labeling). All 7 gels (6 analytical and 1 preparative) were run concurrently, scanned, and the resulting images were analyzed using BVA module of DeCyder software. This module enables matching and comparison of multiple gels when the same internal standard is applied on every gel. The standardized abundance is derived from the normalized spot volume standardized against the intra-gel standard. We used Student's *t*-test of BVA module for automatic statistical analysis between two groups (ND and HAD). Figure 3 shows a representative gel with spot numbers corresponding to proteins listed in Table 2. In 6 analytical gels, 3878 spots were detected. Out of 3878 spots, 1350 met the following two criteria: (i) they were detected in at least two out of three gels in one group and (ii) their positions (coordinates) were matched on all gels where detected. Out of 1350 spots, 90 showed a statistical significant difference ($p < 0.05$) between groups and another 14 spots showed difference with significance between $0.05 < p < 0.06$. Ninety spots were matched with the preparative gel and evaluated for quality. Fifty-two were then subjected to in-gel trypsin digestion and LC−MS/MS identification. Results presented in Table 2 shows the summary of high-confidence protein identifications. Similar to the first series of experiments, some proteins were identified in multiple spots. Three proteins from this 2-D DIGE experiment were selected for further validation as potential biomarkers. They were: complement C3, procollagen C−endopeptidase enhancer 1 (PCPE-1), and gelsolin. Results of Western blot validation are presented in Figure 4D−F. Differential expression of these proteins was confirmed using samples from separate cohort that were used for 2-D DIGE. Proteins that were identified only in the UPR-MSC cohort are: gluthatione transferase, osteoglycin, paraxonase 1, UDP-GlcNAc:betaGalβ-1,3-

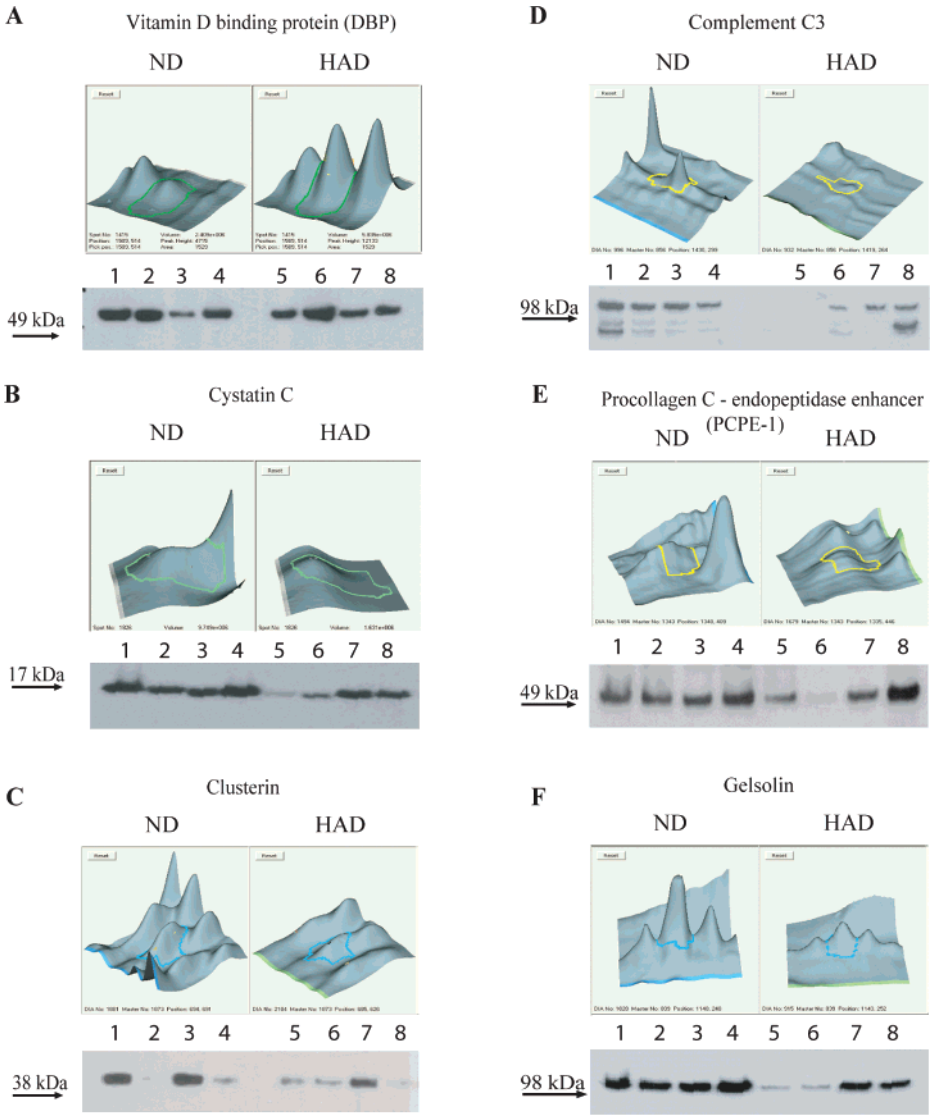

Figure 4. Western blot analyses of proteins selected base on 2-D DIGE experiments: (A) DBP, (B) cystatin C, (C) clusterin, (D) complement C3, (E) PCPE-1, (F) gelsolin. Western blot validation was performed using CSF immunodepleted from 6 most abundant proteins. (Top) DeCyder 3D views of representative spots, and (Bottom) Western blot analyses. Samples 1−4 are from ND patients, and samples 5−8 are from patients with HAD.

N-acetylglucosaminyltransferase 1, fibrinogen, and histocompability antigen HLA B*2705.

Changes in the biochemical composition of CSF could serve as a useful tool for investigations of CNS pathological events and as such a source for biomarker discovery. Despite more than two decades of research, there is lack of good laboratory tests (biomarkers) predicting and monitoring progression of HAD. A number of inflammatory or viral measures in CSF of diseased people have been proposed as being predictive markers of this disease.[11,15,38−40] Among them, cytokines were explored extensively by several groups. Macrophage chemotactic protein-1 (MCP-1) correlates with CSF viral loads and the degree of cognitive impairment.[41] In addition, a longitudinal study of 9 individuals with or at risk of HAD indicated that neurofilament protein was an indicator of CNS injury.[42]

Western Blot Validation of 2-D DIGE Results. Our approach for proteomic analysis of limited sample protein content was to perform a discovery phase and limit the sample numbers. This was followed by an expanded validation phase using a larger sample cohort. In our 2-D DIGE experiments presented here, we used altogether 12 CSF samples; 6 pairs each of HAD and ND. For Western blot analysis, we used another 8 CSF samples. All CSF samples used in biomarker discovery and validation were immunodepleted from the 6 most abundant proteins, and equal amounts of protein from each CSF sample (2 μg) were loaded onto 1-D SDS-PAGE gel. Figure 4 shows results of Western blot analysis of 6 proteins; 3 were selected from 2-D DIGE with minimal labeling (Figure 4A−C) and 3 were selected from 2-D DIGE with saturated labeling (Figure 4D−F).

Western blots presented in Figure 4 confirmed a trend of the differential expression of proteins seen in the 2-D DIGE experiments. However, the levels of individual proteins varied greatly from individual to individual. This was most profound for PCPE-1 where sample #8 showed higher level than in ND samples while the protein was almost absent in sample #6. Such pattern potentially might be indicative of advancement of HAD if confirmed using larger number of CSF samples. This would

Table 2. Selected Identification of Differentially Expressed Proteins in Six CSF Samples

#	DIGE index	*p* value	protein	p*I*	MW	NCBI	Swiss-Prot
1	−8.58	0.0025	Complement C3 precursor	6.02	187162	116594	P01024
2	−8.51	0.0027	Complement C3 precursor	6.02	187162	116594	P01024
3	−7.86	0.00064	Complement C3 precursor	6.02	187162	116594	P01024
4	−7.85	0.022	Complement C3 precursor	6.02	187162	116594	P01024
5	−6.65	0.0044	Complement C3 precursor	6.02	187162	116594	P01024
6	−4.45	0.0027	Glutathione transferase	8.92	23355	4504183	P08263
			Complement C3 precursor	6.02	187162	116594	P01024
			Complement C3 precursor	6.02	187162	116594	P01024
7	−3.86	0.032	Osteoglycin preproprotein	5.46	33922	7661704	P20774
			Paraoxonase 1	5.08	39731	19923106	P27169
8	−2.77	0.0029	Ceruloplasmin precursor	5.44	122204	4557485	P00450
9	−2.25	0.027	Complement C3 precursor	6.02	187162	116594	P01024
10	−1.87	0.035	Ceruloplasmin precursor	5.44	122204	4557485	P00450
11	−1.86	0.031	Gelsolin isoform a precursor	5.90	85696	4504165	P06396
12	−1.82	0.0084	Clusterin isoform 2	5.89	52494	42740907	P10909
13	−1.77	0.027	Gelsolin isoform, precursor	5.90	85696	4504165	P06396
14	−1.63	0.032	Complement C3 precursor	6.02	187162	116594	P01024
			Angiotensinogen preproprotein	5.87	53154	4557287	P01019
15	−1.53	0.035	UDP-GlcNAc:beta Galb−1,3-N-acetylglucosaminyltransferase 1	6.77	47119	5802984	O43505
			Cathepsin D [Precursor]	6.10	44552	4503143	P07339
			Apolipoprotein E precursor	5.65	36153	4557325	P02649
16	−1.51	0.037	C Chain C, Crystal Structure Of Fibrinogen Fragment D	5.87	36180	2781209	**
			Angiotensinogen preproprotein	5.87	53154	4557287	P01019
			Pigment epithelium-derived factor precursor (PEDF) (Serpin-F1)	5.97	46342	20178323	P36955
17	−1.35	0.025	Procollagen C-endopeptidase enhancer	7.41	47958	51094573	Q15113
			Alpha-2-macroglobulin precursor (Alpha-2-M)	6.00	163276	112911	P01023
18	−1.29	0.049	Family with sequence similarity 3, member C precursor	8.67	24680	7661714	Q5HY75
19	1.16	0.011	Cystatin C precursor	9.00	15799	4503107	P01034
			A Chain A, Prealbumin (Human Plasma)	5.52	13760	230651	P02766
20	1.25	0.042	Prostaglandin H2 D-isomerase	7.66	21028	32171249	P41222
			Prealbumin	5.52	15919	219978	P02766
21	1.25	0.016	Cystatin C precursor	9.00	15799	4503107	P01034
22	1.26	0.029	Cystatin C precursor	9.00	15799	4503107	P01034
23	1.57	0.047	A Chain A, Prealbumin (Human Plasma)	5.52	13760	230651	P02766
24	1.64	0.039	Prealbumin	5.52	15919	219978	P02766
			A Chain A, Prealbumin (Human Plasma)	5.52	13760	230651	P02766
25	1.81	0.0053	Prealbumin	5.52	15919	219978	P02766
26	1.84	0.043	A Chain A, Prealbumin (Human Plasma)	5.52	13760	230651	P02766
			Prealbumin	5.52	15919	219978	P02766
27	2.06	0.029	Prealbumin	5.52	15919	219978	P02766
28	2.09	0.032	Prealbumin	5.52	15919	219978	P02766
			A Chain A, Prealbumin (Human Plasma)	5.52	13760	230651	P02766
29	3.4	0.049	B Chain B, Human Class I Histocompatibility Antigen HLA-B(Asterisk)2705	6.07	11731	230014	**

need to be correlated more precisely with neuropsychological evaluations as well as measures of viral load and/or other parameters.

Currently, it is not clear whether differential protein expression patterns are signatures solely of HAD or of ongoing neuroinflammation.[43] The biological significance cystatin C down-regulation in HAD CSF remains undefined. Nonetheless, cystatins C and B likely play protective roles against endogenous and pathogen-derived proteases making their decreased expression during disease of some significance.[44] This may also be an indicator of ongoing viral infection in brain macrophages and microglia. Interestingly, in CSF of multiple sclerosis, patients' cystatin C levels are decreased,[45] whereas in CSF of patients with Alzheimer's or Creutzfeld-Jacobs diseases, they are increased.[46,47] Taking these findings all together, it is possible that the mechanism regulating cystatin C expression is modulated in a disease specific manner rather than being a general characteristic of brain macrophage inflammatory responses. Interestingly, we recently showed that cystatin B expression is increased in HIV-1-infected MDM secretome.[48] Nevertheless, we have not identified this protein in any of 12 CSF samples we examined, making its link to clinical disease unknown at the present time.

Differential expression of CSF's PCPE-1 was previously reported by Yuan and Desiderio.[28] The protein is down-regulated in HAD and its postulated function is inhibition of matrix metalloproteinases (MMP) and as such likely provides regulatory roles to slow disease progression. The fact that PCPE-1 was not found in the virus-infected macrophage secretome suggests that its origin in CSF is either from another cell type or serum leakage from a damaged BBB. Differential expression of gelsolin, which one isoform is secreted by phagocytic cells, is in accord with our previous observation of deregulation of cytoskeleton machinery in HIV-1-infected macrophages.[49] Providing functional links to these proteins for the development of HAD will certainly require more directed future studies.

Differential Levels of α-Chain of Complement C3. Consistently in all 2-D DIGE experiments, we observed down-regulation of protein(s) in two spots with molecular mass corresponding to 40 kDa and p*I* approximately 4.0 in which we identified complement C3. Further analysis of mass spectrometry data and sequence coverage correlated these two protein spots with the Complement C3c alpha′ chain fragment 2 (Swiss-Prot #P01027). Down-regulation of this fragment was consistent with the down-regulation of complement C3 pre-

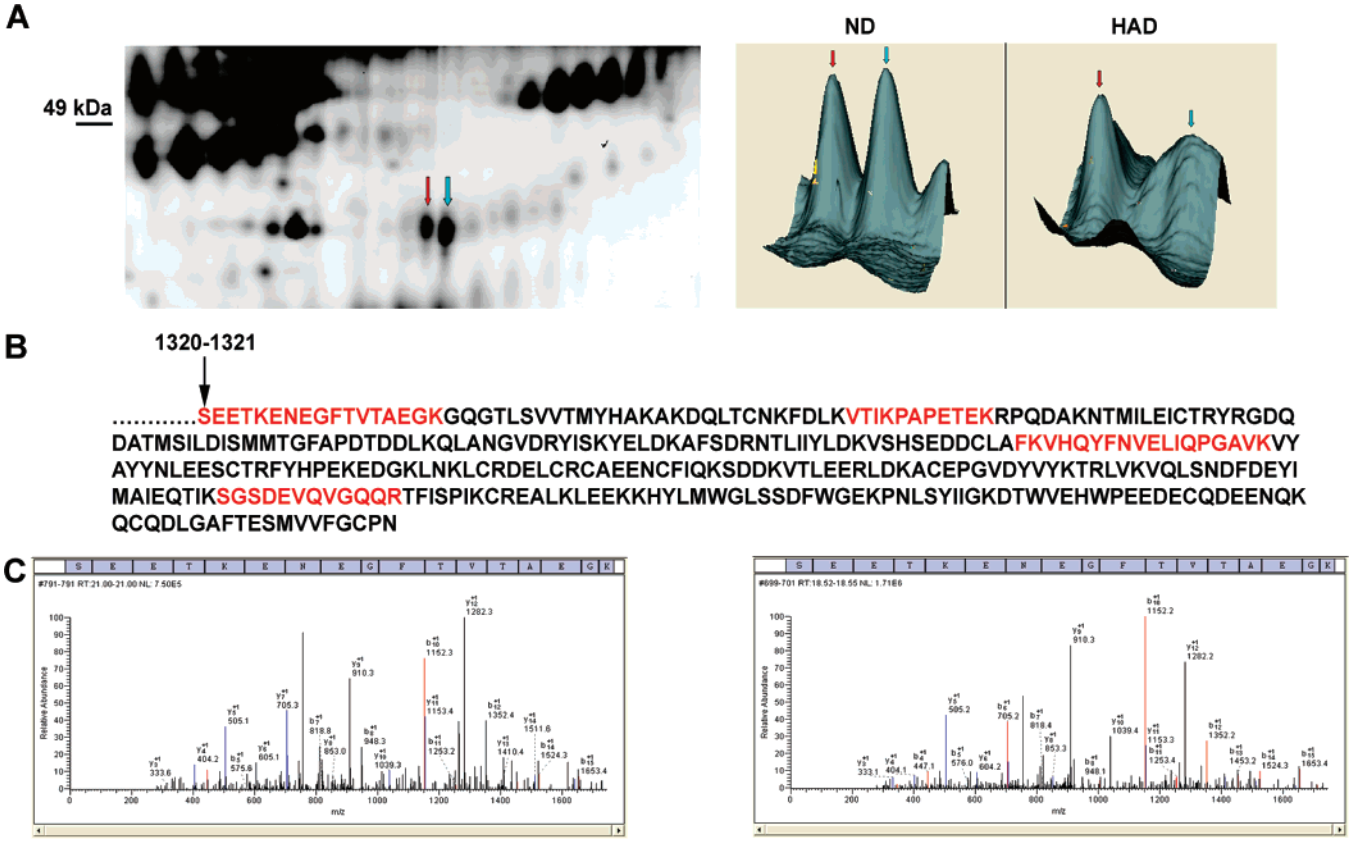

Figure 5. Analysis of α-chain of complement C3 protein detected after 2-D DIGE analysis in CSF. (A) (Left) Region of 2-DE gel with spots (indicated by arrows) where Complement C3c alpha′ chain fragment 2 (Swiss-Prot #P01027) was identified; (Right) 3-D views of indicated spots. (B) Amino acid sequence of complement C3 with polypeptides identified by mass spectrometry indicated by red color. (C) Two MS/MS spectra of N-terminal peptide (SEETKENEGFTVTAEGK) from α-chain of complement C3.

Table 3. HIV-1 Proteins Identified in 12 CSF Samples

MS #	protein	peptide sequence	NCBI	z	XCorr	ΔCn	ions
1735	envelope glycoprotein	R.IGPGQAFYATGAIIGDIRQAHCNISSDK.W	58220977	3	3.01	0.32	27/108
1781	envelope glycoprotein	K.GDMKNCSFNITTNIKGK.M	1845947	3	3.14	0.33	22/64
A6031	envelope glycoprotein	R.SENITNNVKNIIAQLTEPVK.I	62906476	3	3.04	0.41	26/76
A2964	envelope glycoprotein	R.SENITNNAKIIIAHLNESVEINCTR.-	72539346	3	2.97	0.3	14/96
A2389	envelope glycoprotein	K.SVRIGPGQTFYATGDVTGDIR.K	46948945	3	3.1	0.23	25/80
2259	envelope glycoprotein gp120	K.EALQEVVEQLR.Q	85662893	2	2.86	0.27	14/20
2247	envelope glycoprotein gp120	K.EALQEVVEQLR.Q	85662893	2	2.71	0.3	14/20
A4674	envelope glycoprotein gp120	K.EALQEVVEQLR.Q	85662893	2	3.09	0.24	14/20
A4658	envelope glycoprotein gp120	K.EALQEVVEQLR.Q	85662893	2	3.18	0.32	15/20
A4558	envelope glycoprotein gp120	K.EALQEVVEQLR.Q	85662893	3	3.13	–	19/40
4212	gag-pol fusion polyprotein	R.EFPSEQTRXNSPTR.A	37934098	3	3.01	0.23	25/52
A8489	gag-pol fusion polyprotein	R.EFPSEQTRXNSPTR.A	37934098	3	3.1	0.22	27/52
4253	gag protein	K.TSITMQRSNFKGPK.R	80975076	2	2.73	0.42	14/26
A4231	vpu protein	-.MLNLQARIDYR.L	13569324	2	2.54	0.35	14/20
2350	pol polyprotein	K.QFTEAVQK.I	13095233	1	2.08	–	10/14
A5787	vif protein	K.PPLPSVMKLTEDRWNK.P	2853437	3	3.15	0.31	26/60
1964	reverse transcriptase	K.LVDXRELNR.R	29650702	2	2.59	0.21	14/16

sented in Figure 4D. Figure 5A shows DeCyder analysis of the corresponding spots and region of 2-DE gel where the spots were found. Figure 5C shows two MS/MS spectra of N-terminal peptides, which indicate that serine (amino acid #1321) is the N-terminal. We have used several polyclonal antibodies against complement C3 to validate this result. All tested antibodies showed very weak immunoreactivity against the Complement C3c alpha′ chain fragment 2 (Swiss-Prot #P01027) in Western blot assays (data not shown).

Activation of complement has been postulated to play a major pathogenic role in a number of neurodegenerative disorders including Alzheimer's Disease, Parkinson's Disease, and multiple sclerosis.[50,51] It is possible that specific components and/or fragments of the complement system may be a common biomarker for more than one disease. Nevertheless, on the basis of our results, we propose that the Complement C3c alpha′ chain fragment 2 (Swiss-Prot #P01027) can be used as a potential biomarker of HAD. The advantage of using this fragment is that regardless of how the precursor of complement C3 is processed and the abundance of intermediate forms, levels of the α-chain will indicate the final step of processing.

Viral Proteins in CSF. Despite the fact that in most cases viral load in CSF samples was not detectable, we were able to identify several HIV-1 proteins, which are listed in Table 3. The

mass spectrometric identifications, however, were based on a single peptide, thus being classified as low-confidence identification. In two instances, polymerase polyprotein and envelope glycoprotein gp120, Sequest algorithm did not assign ΔCn values. Identification of a fragment of envelope glycoprotein gp120 (peptide sequence K.EALQEVVEQLR.Q, Table 3) in multiple spots and in multiple gels confirmed its conserved sequence, although BLAST search showed frequent mutations reported in this region. This peptide matched a database entry made in 2005 (Direct submission 20-Jan-2005 by Vidal, N. UMR 145, I.R.D, 911 av. Agropolis, 34394 Montpellier Cedex 5, FRANCE) of a subtype C that originated in Congo. None of the mutated peptides were found among samples from 12 individuals, who originated from four different U.S. geographical regions. Two other peptides belonging to envelope glycoprotein with partially overlapping sequences (R.SENITNNVKNIIAQLTEPVK.I and R.SENITNNAKIIIAHLNESVEINCTR.-) were identified by MS/MS sequencing (Table 3). Because C-terminal Arg residue in the latter peptide is linked with a Pro residue, we suspect that in this case the peptide was derived from already processed (degraded) protein.

Concluding Remarks

We used CSF proteomic profiling to identify putative biomarkers for HAD during advanced viral disease. Methods developed by our research for sample preparation, proteomic profiling, protein identification, and validation uncovered several "novel" biomarkers for disease. Based on the study results, we now conclude that 6 proteins, alone or in combination, are "putative" disease biomarkers. One of the limitations faced was the relatively small number of samples with sufficient protein contents to be investigated by 2-DE in combination with protein identification by LC−MS/MS. This is because collection of CSF from patients who must give informed consent remains a challenge. Nevertheless, after examination of our data based on proteomic analysis of 12 samples, we were able to select proteins differentially expressed in HAD and validated them by Western blot. The selection of these specific proteins as biomarkers in any event will certainly require a larger sample cohort. This work and another done in collaboration with colleagues at the UPR-MSC[43] provides a foundation for the role of differentially expressed proteins in HAD biomarker discovery. Another step will be to increase patients numbers as well as cross validation of these biomarkers with biological and immune parameters as a next step to protein validation. The accomplishment of such a goal will require a more systematic understanding of the CSF proteome for therapeutic monitoring together with neuroimaging and clinical evaluations.

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