Comparative proteomics of cerebrospinal fluid in neuropathologically-confirmed Alzheimer's disease and non-demented elderly subjects

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Objectives: *Diagnostic tests able to reveal Alzheimer's disease (AD) in living patients before cognitive ability is destroyed are urgently needed. Such tests must distinguish AD from other dementia causes, as well as differentiate subtle changes associated with normal aging from true pathology emergence. A single biomarker offering such diagnostic and prognostic capacities has eluded identification. Therefore, a valuable test for AD is likely to be based on a specific pattern of change in a set of proteins, rather than a single protein.*
Methods: *We examined pooled cerebrospinal fluid (CSF) samples obtained from neuropathologically-confirmed AD (*n*=43) and non-demented control subjects (*n*=43) using 2-dimensional gel electrophoresis (2DE) proteomic methodology to detect differentially expressed proteins. Proteins exhibiting expression level differences between the pools were recovered and identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.*
Results: *Five differentially-expressed proteins with potential roles in amyloid-β metabolism and vascular and brain physiology [apolipoprotein A-1 (Apo A-1), cathepsin D (CatD), hemopexin (HPX), transthyretin (TTR), and two pigment epithelium-derived factor (PEDF) isoforms] were identified. Apo A-1, CatD and TTR were significantly reduced in the AD pool sample, while HPX and the PEDF isoforms were increased in AD CSF.*
Discussion: *These results suggest that multi-factor proteomic pattern analysis of the CSF may provide a means to diagnose and assess AD.* [Neurol Res 2006; **28**: 155–163]

Keywords: Alzheimer's disease; biomarkers; cerebrospinal fluid; proteomics

INTRODUCTION

Alzheimer's disease (AD) affects millions worldwide and its incidence is projected to increase. There is a crucial need for a reliable diagnostic test that will detect AD antemortem before it causes disability. Therapies given very early in the disease course are more likely to be effective, as brain destruction in AD begins long before clinical signs and symptoms are recognized. Such a test would need to distinguish AD not only from normal elderly patients, but also from a number of other less common diseases that have nearly identical clinical syndromes (non-AD dementias). Additionally, the test would provide an accurate prediction for the eventual development of AD in clinically normal elderly individuals, as well as an index of disease severity, so that it would be useful in monitoring the efficacy of potential therapeutic agents. Despite many efforts, however, no single biomarker has met these expectations. This is not surprising, given the etiological heterogeneity of sporadic AD and its variability in clinical manifestations, range of neuropathological expression, senescence-associated morbidities, genetic background, and the wide variety of individual lifestyles and personality traits. It is likely, therefore, that a useful test for AD will be based on a specific pattern of change in a set of proteins, rather than a single protein. Proteomics technology is ideally suited to the discovery of such a diagnostic set. Cerebrospinal fluid (CSF) is produced mainly in the choroid plexus and to a lesser degree from the brain's interstitial fluid. It fills the ventricular cavities and encases the brain and spinal cord[1]. Because the CSF is in direct contact with the brain's extracellular spaces, the CSF chemistry reflects brain chemistry. Proteomic analysis of this fluid has the potential to reveal subtle pathological changes[2]. Neurodegenerative diseases such as AD are believed to originate in the brain and therefore testing of peripheral organs and blood is far less likely to provide diagnostic data early in the course of disease. Many diseases of the brain are already diagnosed through CSF analysis, including multiple sclerosis[3], Creutzfeldt-Jakob disease[4,5], as well as numerous infectious and

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neoplastic conditions[6]. There has long been interest in a CSF test for AD as well.

Cerebrospinal fluid is derived from the choroid plexus, blood and brain parenchyma. The choroid plexus is a major site of origin for CSF proteins[7] and several of these have recently been reported to be altered in AD, including cystatin C[8], transthyretin[9], retinol-binding protein and α-2 microglobulin[10]. Other evidence has also pointed to abnormal structure and function of the choroid plexus in AD[11,12]. The most abundant CSF proteins are derived from the blood, with their concentrations dictated by properties of the vascular blood-brain barrier (BBB). If there is BBB dysfunction in AD, as has been suggested[13–16], there should be increased concentrations of blood-derived CSF proteins in AD subjects. While some early studies found albumin to be increased in AD CSF, later studies have not confirmed this, but have rather suggested that albumin may be increased in vascular dementia but not AD[17]. A recent study using proteomics technology reported that albumin was present at increased concentration in the CSF from patients with frontotemporal dementia, but not in the CSF of AD cases[18].

Because AD is a disease of brain parenchyma, it is perhaps more likely that it will be brain-derived, rather than blood- or choroid plexus-derived proteins that will provide both a diagnostic signature and informative pathogenic data. The neurosecretory VGF precursor[19] and apolipoprotein E (Apo E)[10] are examples of CSF proteins mainly derived from the brain that have recently been found, using proteomic methods, to be altered in AD CSF. Many other brain-derived molecules have been reported to be altered in AD CSF such as: β-2-microglobulin, retinol binding protein, ubiquitin, α-2-haptoglobin, glial fibrillary acidic protein, apolipoprotein A-I, apolipoprotein J, beta-trace, kininogen, α-1 antitrypsin, cell cycle progression 8 protein, α-1 beta glycoprotein and F2-isoprostane[10,20–27]. The most accurate laboratory test for AD currently available is the CSF ratio of Aβ/tau or Aβ/phosphorylated tau protein. Both molecules are brain-derived and specifically related to the signature pathologic lesions of AD, but these still fall short of the required sensitivity (80–90%) and specificity (50–85%) for an unequivocal diagnostic test[27–32]. A more specific diagnostic panel of proteins might be constructed, however, from other elements of the AD pathogenic cascade.

Inflammation is thought to play a major role in AD by perpetuating or accelerating the primary destructive processes[33–35]. Altered concentrations of inflammation-associated cytokines, including tumor necrosis factor-α and tumor necrosis factor-β, have been reported in mild cognitive impairment and AD patients[36]. It is possible that a characteristic disease-specific profile of inflammatory proteins may be present in the CSF. Identification of the critical molecular pathways could indicate effective therapeutic targets. As with all putative CSF markers, however, their validity and usefulness for the clinical diagnosis of AD have still not yet been adequately assessed.

In this study, we have used a proteomic approach in which we examined CSF pools from large numbers of neuropathologically-diagnosed AD and non-demented (ND) subjects. We assessed potential AD biomarkers through 2-dimensional gel electrophoresis (2DE) and scanning densitometry to reveal differentially expressed molecules in the two patient group proteome cohorts and assigned protein identifications by mass spectrometry analysis. Our approach differs from most previous proteomic studies of AD CSF in that we have examined large numbers of cases and all cases have been neuropathologically diagnosed.

HUMAN SUBJECTS AND METHODOLOGY

Human subjects

Post-mortem CSF was obtained from the Brain Donation Program at Sun Health Research Institute. Sun Health Research Institute is an affiliate of Sun Health Corporation, a non-profit, community owned and operated health care provider in Sun City, Arizona. The Institute's Brain Donation Program has been in existence for >17 years and has enrolled over 1600 individuals over that time. Donors have volunteered specifically for the program. The CSF was taken from the lateral ventricles of deceased donors within 4 hours of death. The CSF was centrifuged and divided into 1 ml samples in polypropylene tubes and immediately frozen at −80°C. Standardized neuropathologic examinations were performed on every brain. Diagnostic criteria used are as follows: AD cases are rated according to the current consensus criteria established by the NIA-Reagan Institute[37]. All cases are also classified according to the Consortium to Establish a Registry for AD (CERAD) criteria[38] for the diagnosis of AD, and all cases are assigned a Braak stage[39]. Dementia with Lewy bodies (DLB) coexists with AD in ~30% cases and therefore is a source of variation within the AD category that must be considered. For the present study, cases with combined AD and DLB were excluded. Inclusion criteria for AD subjects included: (1) clinical diagnosis of dementia or AD; (2) neuropathologic diagnosis of AD with no other major neuropathologic diagnosis; (3) CERAD neuritic plaque density of moderate or frequent; (4) Braak stages V or VI; (5) post-mortem interval of 4 hours or less. Inclusion criteria for non-demented elderly control subjects included: (1) neuropsychologic assessment consistent with normal cognitive performance; (2) no major neuropathologic diagnosis; (3) CERAD neuritic plaque density zero or sparse; (4) Braak stages I or II. Forty-three control subjects with banked CSF available met these criteria. To match these, we selected the 43 most recent AD subjects that met the above-mentioned inclusion criteria. The characteristics of these study subjects are summarized in the results section below.

Two-dimensional mini-gel electrophoresis of CSF proteins (pH 4–7)

Cerebrospinal fluid aliquots, each containing 100 μg protein [bovine serum albumin protein assay kit, Pierce,

Rockford, IL, USA] were pooled from all AD and control subjects and combined in a manner to ensure that total protein amounts from all individuals were represented equally. Albumin and IgG were removed by incubating CSF with an affinity resin (Amersham Biosciences, Piscataway, NJ, USA) for 90 minutes at 4°C on a rocking platform. After separation from the resin through microspin columns at 6500 × g for 5 minutes, 0.5 ml CSF were mixed with 2 ml ice-cold acetone and incubated at −20°C overnight. Proteins were pelleted by centrifugation at 10,000 × g for 20 minutes at 4°C, air-dried for 5 minutes and mixed with 10 µl 2.5% sodium dodecyl sulphate (SDS) containing 150 mM dithiothreitol (DTT). The mixture was incubated at 80°C for 10 minutes, vortexed and diluted with 70 µl iso-buffer (9 M urea, 4% 3-[(cholamidopropyl)-dimethyl-ammonio]-propanosulfonate hydrate, 35 mM Tris-HCl, 65 mM DTT and 0.002% bromophenol blue) and 70 µl rehydration buffer containing 9 M urea, 4% immobilized pH gradient (IPG) buffer, 18 mM DTT and 0.002% bromophenol blue. Samples were incubated at room temperature (RT) for 30 minutes and applied to 7 cm IPG strips, pH 4–7 (Amersham Biosciences) by re-hydration in a 7–18 cm Immobiline DryStrip re-swelling tray (Amersham Biosciences) for 16 hours at RT. First dimension electrophoresis was performed with an Ettan IPGphor system (Amersham Biosciences). Pre-focusing steps were carried out to remove ionic components as follows: 1 hour at 50 V, 1 hour at 100 V, 1 hour at 200 V, 1 hour at 300 V, 2 hours at 400 V, 2 hours at 600 V and 2 hours at 800 V. Focusing was performed at 1200 V for 14 hours to complete 20,000 Vh. Strips were equilibrated in 5 ml buffer containing 50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 65 mM DTT for 15 minutes at RT followed by a second equilibration step in the same buffer with 200 mM iodoacetamide instead of DTT. The second dimension was performed with Nu-PAGE 4–12% Bis-Tris ZOOM mini-gels (Invitrogen, Carlsbad, CA, USA) combined with a 3-[N-morpholino]ethane sulfonic acid (MES) buffer containing 1 M MES, 1 M Tris-HCl, pH 7.3, 16 mM ethylenediamine tetraacetate (EDTA) and 69 mM SDS. After electrophoresis at 200 V for 35 minutes, the mini-gels were fixed for 10 minutes with 50% methanol and 10% acetic acid, stained for 3 hours with colloidal blue staining kit (Invitrogen) and destained with deionized water overnight.

Data analysis

Gels were scanned with a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA) and analysed with the PDQuest 2D-gel analysis software, v.7.2 (Bio-Rad). The protein spots were detected and matched between groups and a reference gel generated by merging spots from all the gels studied. Gel spots of interest were carefully removed using sterile techniques and stored in siliconized tubes at −20°C until trypsin digestion.

Protein recovery and identification

A solution of 50 mM ammonium bicarbonate, 50% MeOH was used to destain the gel plugs for 20 minutes at RT. The solution was aspirated and discarded and the destaining repeated a total of three times. The gel plugs were incubated with 100 µl 75% acetonitrile for 20 minutes. The acetonitrile was removed and the gel plugs dried in a SpeedVac for 15 minutes. Reduction-alkylation was completed with a solution of 40 µl 10 mM DTT, 20 mM ammonium bicarbonate that was added to the dried gel plugs, incubated in water bath at 56°C for 45 minutes. The samples were removed from the water bath and cooled to RT. The solution was discarded and 40 µl 55 mM iodoacetamide (Bio-Rad) in 20 mM ammonium bicarbonate was immediately added and incubated at RT for 30 minutes in the dark. The solution was discarded and the gel plugs were washed by adding 50 µl 50 mM ammonium bicarbonate, 50% MeOH, incubating for 10–15 minutes at RT, aspirating and discarding the solution. In-gel digestion was performed by adding 140 ng trypsin [20 µl 20 µg trypsin, 1 ml 20 mM ammonium bicarbonate from a 20 µg vial of porcine trypsin sequence grade (Promega, Madison WI, USA)] and incubated at 37°C for 2 hours. After trypsin digestion, the reaction was stopped with 60 µl extraction buffer (50% acetonitrile, 0.1% trifluoroacetic acid (TFA)) and allowed to stand at RT for 20 minutes. The supernatant was removed and placed in a new Eppendorf tube. The same procedure was repeated but with 40 µl extraction buffer being added to the previous extraction.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis

To each tube with extract (peptides), 5–10 µl 50% acetonitrile and 0.1% TFA was added and mixed by aspirating up and down with the same pipette tip. From this extract, 0.5 µl was deposited on the MALDI-TOF plate and allowed to air dry. After the sample was dried on the plate, 0.5 µl α-cyano-4-hydroxy-cinnaminic acid (Fluka, Buchs, Switzerland) composed of 50% saturated solution of matrix and 50% acetonitrile, 0.1% TFA was added to the sample. Mass spectrometry data were acquired using a MALDI-TOF instrument (4700 Proteomics Analyser, Applied Biosystems, Inc., Framingham, MA) using standard acquisition methods as established by the manufacturer. Mass spectra were calibrated with trypsin autolysis peaks (1045.5 and 2211.1 m/z). Data spectra were recorded in positive-ion, delayed extraction mode with a 20 KV accelerating voltage.

Peptide mass fingerprint

Database searching of peptide mass fingerprints was carried out using the GPS Explorer and the Mascot search engine. The database selected from Swiss-Prot was *Homo sapiens* for each of the proteins analysed.

RESULTS

Forty-three AD and control cases were selected under the strict criteria described in the Methods section

above. All 86 individuals were classified as Caucasian. The average age in both AD and ND individuals was 81 years. The AD group was composed of 25 men and 18 women and the ND cohort was represented by 19 men and 24 women. On neuropathologic measures, the AD and control subjects differed significantly (2-tailed Wilcoxan Rank Sum Tests). The CERAD neuritic plaque scores (range 0–3) for the ND and AD were 0.21 and 2.95, respectively ($p<0.0001$). The Braak stages (range 0–6) were 1.77 and 5.50 for the ND and AD, respectively ($p<0.0001$). The white matter rarefaction scores (range 0–5) for the ND and AD cohorts were 1.40 and 3.48, respectively ($p<0.04$). This latter measurement represents the degree of white matter pathology associated with AD and is due mainly to demyelination and cell loss[40,41] and corresponds to the white matter hyperintensities and lucencies observed on magnetic resonance imaging (MRI) and CT scans.

Apo E, a cholesterol-carrying protein that is expressed in three allelic forms ($\varepsilon2$, $\varepsilon3$ and $\varepsilon4$), represents the best known risk factor for AD because individuals with the allele $\varepsilon4$ are at risk of developing early onset AD[42,43]. In multiple studies, the allelic frequency of Apo E $\varepsilon4$ is increased from an average of 14% for ND European populations[44] to ~50% in AD patients. All individuals in the present study were Apo E genotyped following standard techniques[45]. Our results indicated that in the ND group the Apo E allelic frequencies were as follows: $\varepsilon2=0.036$, $\varepsilon3=0.793$ and $\varepsilon4=0.171$, while in the AD group were: $\varepsilon2=0.035$, $\varepsilon3=0.791$ and $\varepsilon4=0.174$. In our study, in which strict criteria were employed to select AD and ND groups, no differences in Apo E allelic frequencies between the two cohorts were evident which may be due to sampling random chance.

Several potential markers for AD were identified in the 2DE proteome analysis of pooled CSF from AD and ND cases. Automatic scanning densitometry using the Bio-Rad PDQuest software demonstrated six protein spots that exhibited a statistically significant difference between AD and ND: apolipoprotein A-I (Apo A-I), cathepsin D (CatD), hemopexin (HPX), transthyretin (TTR) and two isoforms of pigment epithelium-derived factor (PEDF) (*Figure 1*). To ensure that the technique of CSF preparation, amount of CSF loaded, the isoelectric and electrophoretic steps, as well as the pattern of separation were reproducible, the complete protocol was repeated five times on five different occasions. For normalization, the percent integrated optical density (% volume) was calculated for each spot that had been matched to the reference gel. The means of the % volume values of spots were used for statistical analysis. The AD patient group was compared with the ND control group by a Student's *t*-test (*Figure 2*). The significance level was set at 0.05 (5%) and adjustments were made for multiple comparisons using Bonferroni's method. The Coomassie blue stained gel areas containing the molecules of interest were excised, submitted to tryptic digestion and the molecular masses of the resulting peptides identified by MALDI-TOF mass spectrometry. Their molecular characteristics are as follows:

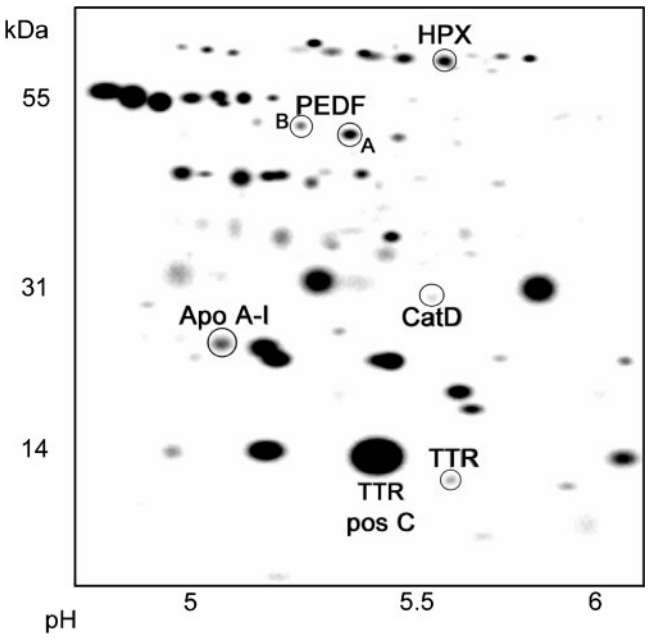

Figure 1: Two-dimensional master gel of postmortem human ventricular CSF pools. The protein spots were matched between AD and ND groups and a master gel generated from the spot data of all gels. The protein spots that were found to be statistically different between AD and ND pools by the Bio-Rad PDQuest software analysis are circled and designated by their acronyms. A full description of the gel characteristics and technical implementation is given in the methodology section. The heavy TTR spot was chosen as a positive control to test the quality of the scanning densitometry, trypsin digestion and MALDI-TOF mass spectrometry analysis

Apo A-I is a major component of the heavy density lipoproteins (HDL). Our study found significantly decreased Apo A-I levels in the AD pool compared with ND controls. Apolipoprotein A-I: accession number (ACC) P02647; molecular weight (MW) 30,758.9; protein score (PS) 174; number of tryptic peptides matched (NTPM) 20; protein confidence interval (PCI) 100%; intensity matched (IM) 37.71. Sequences of identified Tp: 37–47, 48–64, 52–64, 70–83, 84–101, 119–130, 121–130, 121–131, 131–140 ox., 132–140, 132–140 ox., 132–142, 143–155, 165–173 ox., 165–175 ox., 185–195, 202–212, 231–239, 240–250 and 251–262.

CatD is a lysosomal aspartic endopeptidase that we observed to be down-regulated in the AD CSF pool compared with ND controls. CatD: enzyme catalog 3.4.23.5; ACC P07339; MW 44,523.6; PS 60; NTPM 12; PCI 98.95%; IM 36.35. Sequences of identified Tp: 34–49 ox., 177–194, 195–205, 195–205 ox., 206–222 ox., 223–235, 267–287 c (c=carbamidomethyl), 288–309 c, 288–309 c, ox., 314–331 c, ox., 393–399, 393–403.

Hemopexin is a heme binding glycoprotein that was elevated in pooled AD patient CSF when compared with ND controls. Hemopexin: ACC P02790; MW 51,643.3; PS 131; NTPM 22; PCI 100%; IM 73.83. Sequences of identified Tp: 90–102, 92–102, 103–112, 116–124, 131–151 c, 131–151 a (a=acrylamide light),

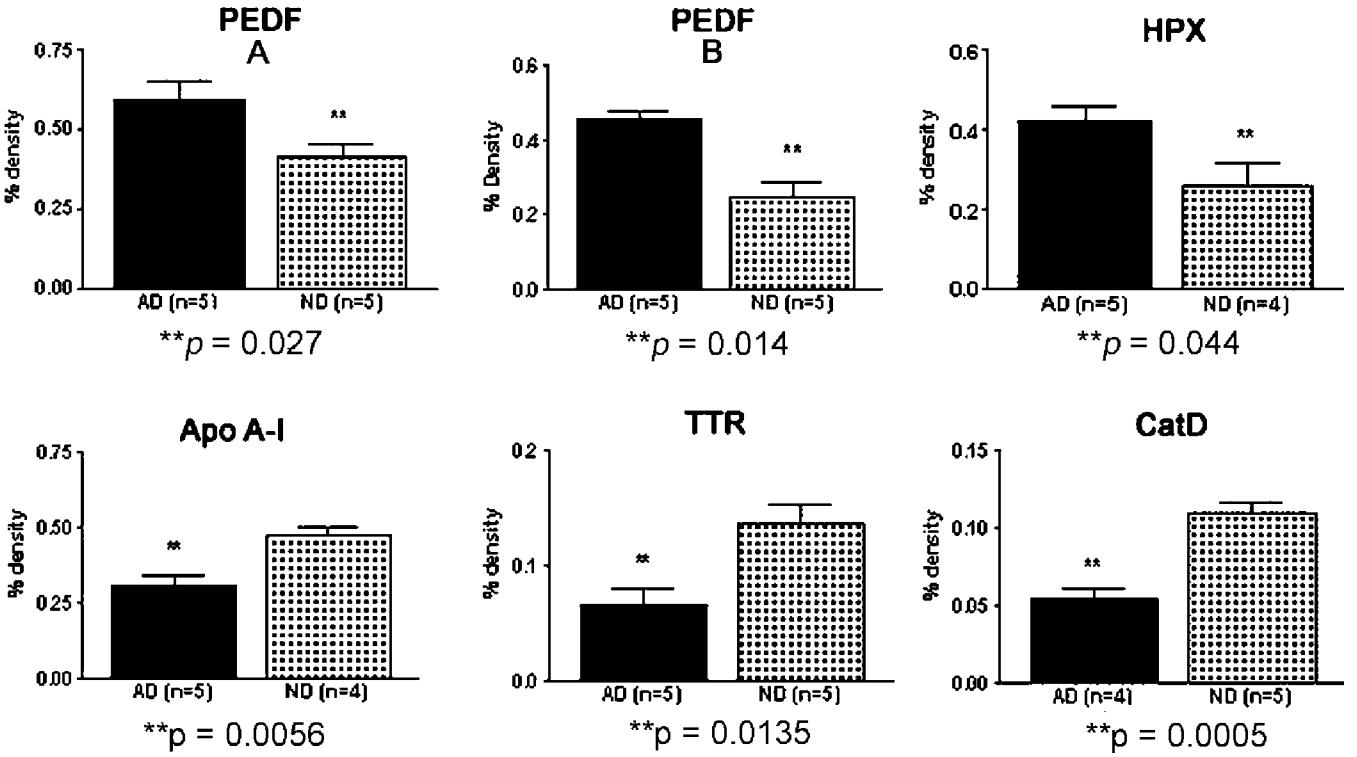

Figure 2: Histograms depicting the percentage of density of those protein spots that statistically differed between the CSF of AD and ND control pools. The bars represent standard error of the mean

152–166 c, 181–193 c, 198–208, 198–208 c, 209–219, 214–222, 223–234 c, ox., 226–234 c, 226–234 c, ox., 240–256 ox., 257–284 c, 318–332, 348–371 c, 379–386, 387–402, 412–441 c (2x).

Two isoforms of PEDF were increased in AD compared with ND controls. Pigment epithelium-derived factor isoform A: ACC P36955; MW 46,313.3; PS 68; NTPM 9; PCI 99.85%; IM 56.42. Sequences of identified Tp: 54–67, 70–99 ox., 107–123, 152–160, 175–189 ox., 198–214, 225–237, 226–237, 263–281 ox. Pigment epithelium-derived factor isoform B: ACC P36955; MW 46,613.3; PS 46; NTPM 6; PCI 76.40%; IM 11.46. Sequences of identified Tp: 54–67, 107–123, 152–160, 175–189 ox., 226–237, 400–411.

The level of TTR was found to be significantly decreased in the AD CSF pool versus the ND controls. Transthyretin: ACC P02766; MW 15,877; PS 59; NTPM 7; PCI 98.82; IM 22.90. Sequences of identified Tp: 30–41a, ox., 42–54, 55–68, 56–68, 101–123, 125–146, 125–147.

DISCUSSION

There is an immense range of individual variability among humans and the protein composition of the CSF is not an exception to this rule. Owing to this individual variability, large numbers of cases must be studied to identify a reliable panel of markers with the necessary sensitivity and specificity. Proteomic methods are extremely laborious, and perhaps because of this, previous studies have been severely limited by their small numbers of subjects. In order to circumvent and reduce the scope of these limitations, we combined CSF from large numbers of subjects into two pools, one for each diagnostic category. Our rationale was to use this approach to identify a subset of proteins that could be considered as preliminary candidates for a diagnostic panel. Subsequent tests on individual subjects would further refine and validate the panel.

Important in assessing the CSF as a potential source of biomarkers for AD is to consider the advantages and disadvantages in using either postmortem or antemortem CSF. Post-mortem CSF allows the accurate neuropathological confirmation of AD while the clinical diagnosis of AD is only 75–80% accurate, according to a recent analysis of data from the National Alzheimer's Coordinating Center (manuscript in preparation). The use of post-mortem subjects allows the direct correlation of candidate markers with the specific histopathologic lesions of AD in both AD and control subjects. Therefore, it is possible to identify not only markers associated with AD, but also markers that are associated with preclinical AD and thus might be used to predict the development of clinical disease. The use of post-mortem CSF also permits the collection of large volumes of CSF (~30 ml) which are unattainable from living individuals, thus allowing for a larger number of experiments. Other parameters to be considered are that the concentration of CSF proteins that originate from neurons and glial cells is higher in ventricular than lumbar CSF[46]. Therefore, the chances of finding good markers of AD might be better in ventricular CSF. We recognize, however, that post-mortem degradation may produce artefacts in post-mortem CSF and therefore we

intend to ultimately test our diagnostic panel on CSF drawn pre-mortem from cases that subsequently went on to autopsy and neuropathologic diagnosis.

Several reports concur that Apo A-I is decreased in the plasma or serum of patients with AD[47–50]. Apo A-I is produced in the small intestine and liver then secreted into plasma. It is responsible for reverse cholesterol transport, a process where Apo A-I attaches to and transports lipids in the plasma and increases cholesterol removal from peripheral tissues[51]. Apo A-I also plays a major role in regeneration of the central nervous system (CNS) and re-myelination[52]. Apo A-I has been found in association with Aβ deposits in the brains of AD patients and has been suggested to modulate amyloid toxic effects at several levels, including a capacity to physically inhibit aggregation and prevent β-sheet organization[53–55]. It also may protect the brain from amyloid precursor protein (APP) C-terminal toxicity through a synergistic interaction with alpha-tocopherol[56]. It is well established that Apo A-I, as a principal component of HDL, protects blood vessels against atheromatosis by removing cholesterol and shunting it to the liver[57]. The discovery that the Apo A-I Milano variant can reduce atheroma volume has generated interest in using Apo A-I and its mimetic peptides as therapeutic agents[58,59].

A CatD deficiency may cause massive neurodegeneration by activating microglia and eliciting inflammation[60] and it has been reported that CatD increases with normal aging[61,62], but not in patients with AD or critical coronary artery disease[63]. CatD may be directly involved in the generation of pathologic Aβ peptides[64–66] and increased endocytosis and cathepsin activity may increase amyloidogenesis in AD[67,68]. Alterations in lysosomal activity and permeability promote senile plaque and Aβ deposition that appear to be an early event in AD[69–71]. In addition, several cathepsins are abnormally distributed and are up-regulated in the AD brain[72,73]. CatD can degrade tau and may have a part in the generation of paired helical filaments (PHF)[74]. Inhibitors of CatD blocked the hyperphosphorylation of tau in the hippocampus[75].

Hemopexin is an acute phase reactant molecule, induced by pro-inflammatory cytokines IL-1 and IL-6, capable of binding metal ions, thus playing an important role in the maintenance of metal ion homeostasis[76]. In addition, HPX has the important function of protecting cells by scavenging and transporting heme to the liver, thus inhibiting potential oxidative damage from heme catalysis and heme-bound loss of iron[77,78]. Relevant to our studies, HPX was found to be 6.5-fold elevated in the plasma of pooled AD (n=10) patients compared with ND (n=9) controls[79].

The detection of two isoforms of the PEDF could be due to phosphorylation by protein kinase CK2 at positions Ser 24 and Ser 114 and by protein kinase A (PKA) at position Ser 227. These post-translational modifications are of functional importance because phosphorylation by CK2 eliminated PEDF neurotrophic activity and improved anti-angiogenic activity. Phosphorylation by PKA diminished PEDF anti-angiogenic activity[80]. In addition, the molecule contains an N-terminal pyroglutamate and is glycosylated at Asn 226 (Ref. 81). This molecule is a powerful inhibitor of angiogenesis and acts by inducing apoptosis in proliferating endothelial cells[82–85]. Paradoxically, it may also act as a neuroprotective factor, because it can reduce apoptosis induced by free radicals and ultraviolet (UV) damage[83,86,87]. Pigment epithelium-derived factor may thus protect neurons against damage caused by vascular diseases that affect the CNS. An imbalance of PEDF and vascular endothelium growth factor (VEGF) levels is reported in certain diseases and conditions, such as ocular neovascular disorders, macular degeneration, pterygia, idiopathic pulmonary fibrosis and hypoxic stress[88–94]. Capillary permeability is increased in the presence of VEGF, an activity that is inhibited by PEDF[95]. There is an age-related decrease in PEDF production[96,97], but this molecule has been reported to be elevated in the brains of AD patients and may protect against oxidative stress or glutamate-induced injury[98]. A significant decrease in the levels of CSF PEDF has been reported in frontotemporal dementia[18].

Transthyretin is synthesized in the liver and the choroid plexus and constitutes 25% of the total CSF protein concentration[7]. The molecule serves as a transporter for thyroxin and retinol and over 60 TTR mutants have been characterized that result in familial amyloid polyneuropathy[99]. Apparently, the TTR amyloid does not cause apoptosis, suggesting that the amyloid fibrils may represent a scavenger mechanism that captures toxins[100]. Transthyretin concentrations increase with age but have been reported to be significantly reduced in the CSF of AD patients[9,26,101–104]. Decreases of TTR are also observed in AD serum[102,105]. In some elderly individuals wild-type TTR causes a senile systemic amyloidosis[106]. Some of the TTR variants exhibit altered Aβ peptide affinities making them important targets for the study of AD[107]. Overexpression of APP in transgenic mice results in high levels of sAPPβ and TTR. These mice do not develop tau phosphorylation and neuronal death, but when the mice were injected with an antibody against TTR, increases in Aβ, tau phosphorylation and neuronal loss were observed[108,109]. Transthyretin is capable of sequestering both Aβ 40 and Aβ 42, thus preventing Aβ aggregation and it is negatively correlated with the number of senile plaques in AD[101,110–115]. Transthyretin, in combination with the receptor for advanced glycation end products, has the ability to activate nuclear transcription factor NF-kB, which promotes neuroinflammation[116].

In summary, the six CSF proteome molecular markers we identified appear to be directly relevant to AD pathology, Aβ metabolism and vascular and brain physiology. Owing to the complexity and heterogeneity of AD, the finding of a single diagnostic marker in the biological fluids seems a remote possibility. Rather, the diagnosis of AD may be more reliably accomplished by the statistical consensus of an array of relevant protein markers. The crucial first effort to identify candidate AD biochemistry status markers will now be followed by an

assessment of the capability of these molecules to reveal dementia reliably from analyses of individual CSF samples. In principle, the development of AD pathology should generate a characteristic alteration of protein expression and this pattern should be detectable in the CSF. Elucidating this pattern will not only provide a clinically useful diagnostic and predictive test, but contribute to our understanding of the molecular pathogenesis of AD and potentially identify and assess the effectiveness of therapeutic agents.

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