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Cerebrospinal Fluid Profile of Amyloid β Peptides in Patients with Alzheimer's Disease Determined by Protein Biochip Technology

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Key Words
Alzheimer's disease · β-Amyloid · Cerebrospinal fluid · Protein biochip · ELISA

Abstract
Amyloid-β peptides (Aβ) are major components of amyloid plaques in the Alzheimer's disease (AD) brain and have been proposed as diagnostic markers in cerebrospinal fluid (CSF). Aβ derived from brain may be processed into fragments before emerging in CSF. Therefore, we determined mass profiles of Aβ peptides in CSF of patients with AD and age-matched healthy control subjects (CTR) by using protein biochip technology. Aβ peptides were captured on the chip surfaces (spots) by the specific monoclonal antibody 6E10 and were then analyzed by integrated surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF-MS). We found Aβ species with mean molecular masses at 1,583.3 Da (corresponding to $A\beta_{2-14}$), 2,068.5 Da ($A\beta_{1-17}$), 2,166.4 Da ($A\beta_{1-18}$), 3,676.6 Da ($A\beta_{1-33}$), 3,789.4 Da ($A\beta_{1-34}$), 4,076.9 Da ($A\beta_{1-37}$), 4,134.0 Da ($A\beta_{1-38}$), 4,233.3 Da ($A\beta_{1-39}$), 4,332.4 Da ($A\beta_{1-40}$) and 4,516.8 Da ($A\beta_{1-42}$) in both AD (n = 24) and CTR (n = 24) subjects. $A\beta_{1-38}$ appeared to be a major Aβ species in human CSF along with $A\beta_{1-40}$. Quantitation revealed that CSF levels of $A\beta_{1-38}$ were significantly decreased in AD as compared to CTR subjects. The CSF profile of Aβ peptides may be used for diagnostic and therapeutic purposes in clinical studies.

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Introduction

β-Amyloid (Aβ) deposits, along with neurofibrillary tangles, are the major histopathological hallmarks of Alzheimer's disease (AD). The predominant protein component of Aβ plaques are strongly aggregating peptides with an approximate molecular mass of 4 kDa [1–3]. Whereas $A\beta_{42}$ is the major component of Aβ plaques, $A\beta_{40}$ is mostly found in vascular amyloid of brain blood vessels [4]. Cerebrospinal fluid (CSF) levels of $A\beta_{42}$ measured by ELISA were shown to be lower in AD patients as compared to controls [for a review, see 5], while conflicting results were reported from $A\beta_{40}$ measurements [6–12].

A number of reasons explaining the conflicting data were discussed, including heterogeneity of the patient sample and limitations of the ELISA method used, which do not display the various fragments of peptides captured by the monoclonal antibodies.

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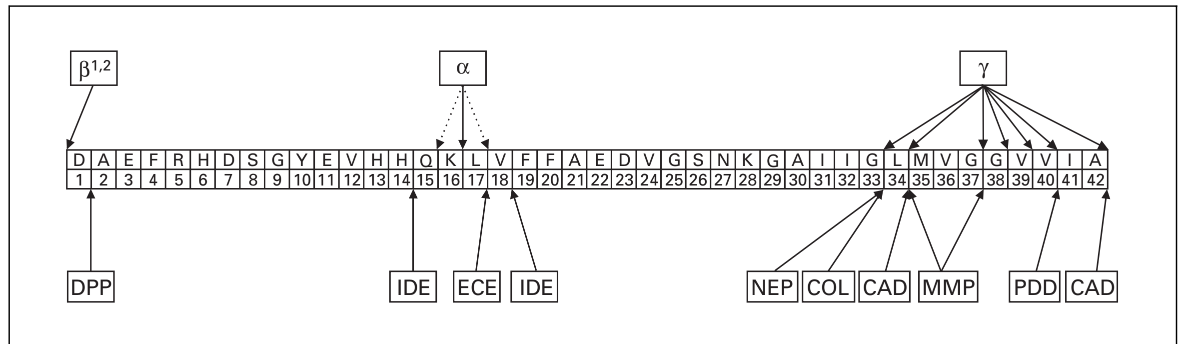

Fig. 1. Potential cleavage sites of Aβ peptide: secretases and putative proteases. α = Alpha secretase; β = beta secretase (BACE 1 and 2); γ = gamma secretase; DPP = dipeptidyl peptidase; IDE = insulin-degrading enzyme; ECE = endothelin-converting enzyme 1 (ECE-1); NEP = neprilysin; COL = collagenase; MMP = matrix metalloproteinase 9 (MMP-9); CAD = cathepsin D; PDD = peptidyl dipeptidase.

To clarify the patterns of Aβ peptides present in CSF we determined mass profiles of Aβ in patients with AD, as well as in age-matched healthy control subjects (CTR) by using a protein biochip technology (SELDI, Ciphergen) [13–17].

Subjects and Methods

Subjects

A diagnosis of probable AD was made according to NINCDS-ADRDA criteria [18] as well as ICD10 [19]. The clinical workup included neuropsychological testing, MRI scans and routine CSF investigations. The mean Mini-Mental State Examination (MMSE) scores were 21 ± 5.8 (SD) in the AD group (n = 24). The mean age (SD) was 71.2 ± 9 years; there were 10 females and 14 males. Healthy CTR (n = 24) were recruited among cognitively intact patients undergoing spinal anesthesia before surgical intervention. The mean age was 65.7 ± 12 years; there were 8 females and 16 males. The study was approved by the local ethics committee and subjects gave written informed consent prior to the investigation. CSF was obtained by lumbar puncture, aliquoted, immediately frozen at the bedside (–80 °C) and freshly thawed prior to the analyses.

Protein Biochip Analysis

Capturing Aβ peptides from CSF was done on SELDI Protein G-coated (PG20) ProteinChip® Arrays (Ciphergen Biosystems, Palo Alto, Calif., USA). Like PS20 ProteinChips, PG20 ProteinChip Arrays contain an epoxy surface which covalently reacts with amine and thiol groups and allows precoupling with recombinant Protein G. The specific monoclonal antibody 6E10 against Aβ epitope 1–17 (Signet Pathology Systems, Dedham, Mass., USA) was first purified on Protein G columns [ImmunoPure Protein (G) IgG Purification Kit, Pierce Biotechnology, Rockford, Ill., USA] and then added (2 µl of 0.2 mg/ml) on the microspots followed by incubation in a humidity chamber at room temperature for 1 h. Antibody solution was removed and the chip array was washed once in bulk with 8 ml phosphate buffer saline (PBS, pH 7.4) containing 0.5% (v/v) Triton X-100 for 10 min, then twice in bulk with 8 ml PBS for 5 min. The spots were coated with 5 µl crude CSF and incubated in a humidity chamber at 4 °C overnight. CSF was removed and spots were washed; first in bulk with 8 ml PBS (pH 7.4) containing 0.5% (v/v) Triton X-100 for 10 min, then washed in bulk twice with 8 ml PBS for 5 min, and finally rinsed with 1 m*M* HEPES buffer. α-Cyano-4-hydroxy cinnamic acid (CHCA) was dissolved in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. After drying, a 1:5 diluted solution of CHCA was added to the spots (0.5 µl) and mass identification was made by 100 averaged shots in a Ciphergen SELDI Protein Biology System II (PBS II). All data were normalized against a common matrix peak. Signal to noise (S/N) ratios above 2.5 were considered to be sufficient to submit the data to statistical analysis.

Statistical Analysis

Group comparisons were done by nonparametric Mann-Whitney U tests. Statistical significance was assumed at $p < 0.05$.

Results

We found CSF species of Aβ peptides with mean molecular masses at 1,583.3 Da (corresponding to $A\beta_{2-14}$), 2,068.5 Da ($A\beta_{1-17}$), 2,166.4 Da ($A\beta_{1-18}$), 3,676.6 Da ($A\beta_{1-33}$), 3,789.4 Da ($A\beta_{1-34}$), 4,076.9 Da ($A\beta_{1-37}$), 4,134.0 Da ($A\beta_{1-38}$), 4,233.3 Da ($A\beta_{1-39}$), 4,332.4 Da ($A\beta_{1-40}$) and 4,516.8 Da ($A\beta_{1-42}$) in both AD (n = 24) and CTR (n = 24) subjects (fig. 1a). Mass detection showed high accuracy with minimal intersample deviation and corresponded well to calculated peptide masses (table 1, fig. 1).

$A\beta_{1-38}$ and $A\beta_{1-40}$ were the most prominent Aβ species in CSF and fulfilled the requirement of an S/N ratio over 2.5 for quantitation (fig. 2a, b).

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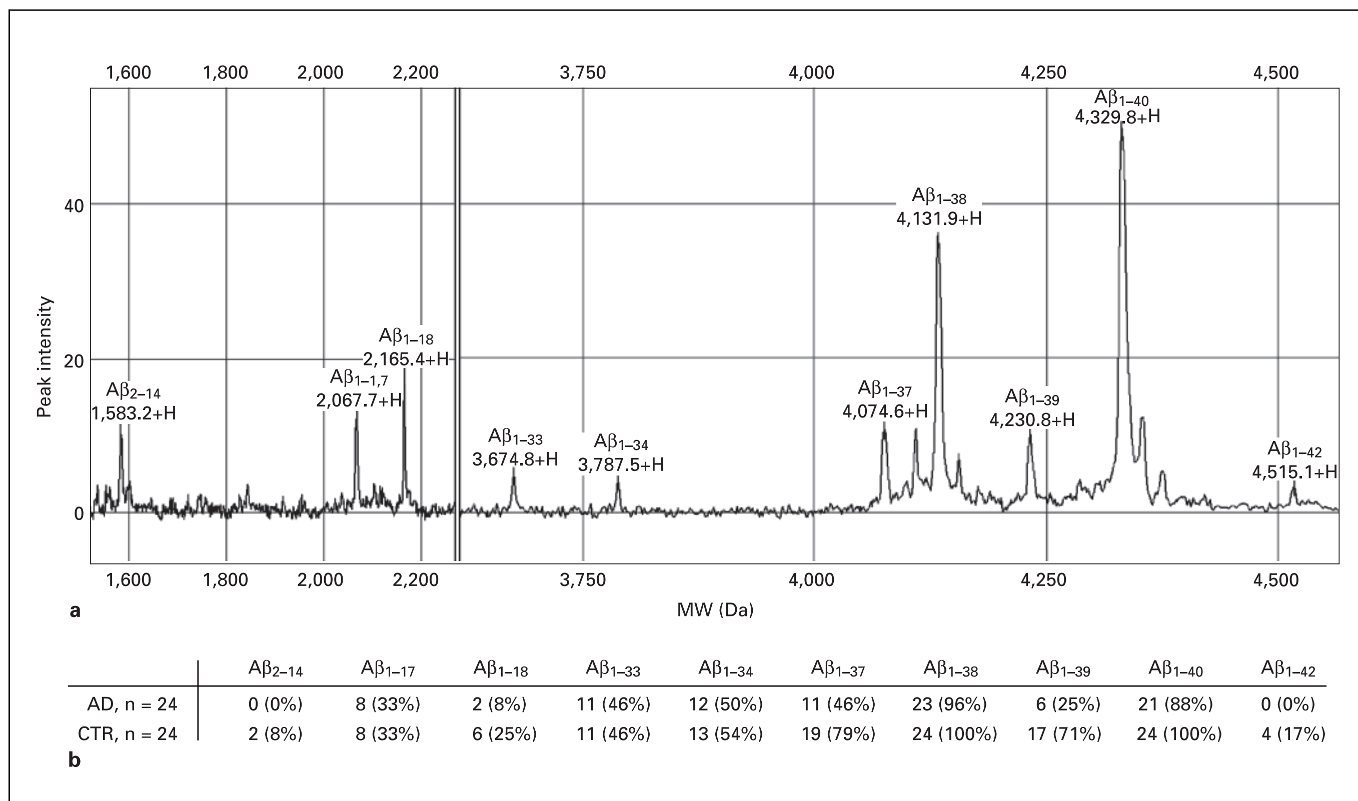

	Aβ2–14	Aβ1–17	Aβ1–18	Aβ1–33	Aβ1–34	Aβ1–37	Aβ1–38	Aβ1–39	Aβ1–40	Aβ1–42
AD, n = 24	0 (0%)	8 (33%)	2 (8%)	11 (46%)	12 (50%)	11 (46%)	23 (96%)	6 (25%)	21 (88%)	0 (0%)
CTR, n = 24	2 (8%)	8 (33%)	6 (25%)	11 (46%)	13 (54%)	19 (79%)	24 (100%)	17 (71%)	24 (100%)	4 (17%)

Fig. 2. a CSF profile of Aβ peptides (1 subject) captured by monoclonal antibody 6E10 on a protein biochip. **b** Percentage of Aβ peptide peaks with an S/N ratio higher than 2.5.

Table 1. CSF Aβ peptides captured by monoclonal 6E10 antibody

Aβ peptides	Mean MW observed, Da	SD Da	MW calculated Da	Amino acid sequence
2–14	1,583.3	0.08	1,583.6	AEFRHDSGYEVHH
17	2,068.5	0.69	2,068.2	DAEFRHDSGYEVHHQKL
18	2,166.4	0.65	2,167.3	DAEFRHDSGYEVHHQKLV
33	3,676.6	0.89	3,674.0	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIG
34	3,789.4	0.85	3,787.2	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL
37	4,076.9	0.98	4,074.5	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVG
38	4,134.0	1.00	4,131.6	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG
39	4,233.3	1.10	4,230.7	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV
40	4,332.4	1.00	4,329.9	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
42	4,516.8	1.63	4,514.1	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

Mean MW with standard deviation (SD) in daltons, calculated peptide MW in daltons and amino acid sequence. Only peaks with an S/N ratio higher than 2.5 were included in the analyses.

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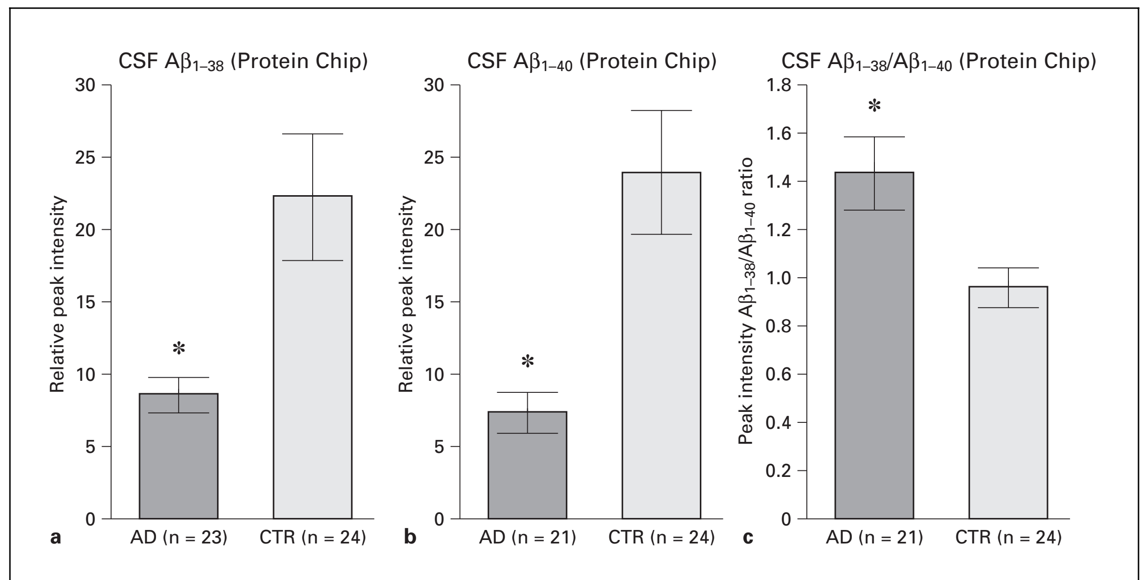

Fig. 3. CSF $A\beta_{1-38}$ (**a**) and $A\beta_{1-40}$ (**b**) peak intensity measured on the protein biochip after normalization to a common matrix peak in patients with AD and CTR. * p = 0.001 (**a**) and * p = 0.000 (**b**), Mann-Whitney test. Only peaks with an S/N ratio higher than 2.5 were included in calculations. **c** Mean peak intensity $A\beta_{1-38}/A\beta_{1-40}$ ratio in CSF of patients with AD and CTR. * $p < 0.01$, Mann-Whitney U test.

The mean peak intensity corresponding to $A\beta_{1-38}$ [mean molecular weight (MW) 4,134 Da] was lower in CSF of AD patients (mean 8.65 ± 1.12 SE, n = 23), as compared to CTR (22.28 ± 4.25, n = 24; $p < 0.01$, Mann-Whitney U test) (fig. 3a). Similarly, the mean peak intensity corresponding to $A\beta_{1-40}$ (mean MW 4,332.4 Da) was lower in CSF of AD patients (mean 7.55 ± 1.18 SE, n = 21) as compared to CTR (23.98 ± 4.25, n = 24; $p < 0.01$, Mann-Whitney U test) (fig. 3b).

The mean peak intensity of the $A\beta_{1-38}/A\beta_{1-40}$ ratio was significantly higher in CSF of the AD patients (1.43 ± 0.15 SE, n = 21), as compared to CTR (0.95 ± 0.08, n = 24; p = 0.01, Mann-Whitney U test) (fig. 3c).

Discussion

We have determined mass profiles of Aβ peptides in human CSF by using protein biochip technology. We found Aβ species corresponding to $A\beta_{1-40}$ and $A\beta_{1-42}$ (4,333.5 and 4,517.7 Da, respectively), and additionally detected Aβ species corresponding to $A\beta_{2-14}$, $A\beta_{1-17}$, $A\beta_{1-18}$, $A\beta_{1-33}$, $A\beta_{1-34}$, $A\beta_{1-37}$, $A\beta_{1-38}$ and $A\beta_{1-39}$ in both AD patients and CTR subjects. Presence of Aβ species with similar mass profiles was recently reported in cell culture media, measured by immunoprecipitation-MALDI-TOF mass-spectrometric analysis (IP/MS) [20, 21], immunoprecipitation-HPLC-mass spectrometry [22], as well as by SELDI technology [13–16]. In human CSF, several of the mass peaks found in the present analyses, including $A\beta_{1-38}$, were also identified by MALDI-TOF [6] and SELDI [17]. Thus, protein biochip technology may allow for detection of a heterogeneous population of truncated Aβ peptides in human CSF. The exact origin of the various Aβ species is currently not known; in addition to the action of the APP secretases and endopeptidases, such as IDE and neprelysin (see fig. 1), other yet unidentified proteases may be involved. Interestingly $A\beta_{1-38}$ turned out to be one of the most prominent Aβ peptide in CSF, particularly in AD patients, where it showed an even higher peak intensity than $A\beta_{1-40}$. These results are in line with a recent report of increased $A\beta_{1-38}$ (in percent, relative to total Aβ) in CSF of patients with AD, as well as chronic neuroinflammation [6].

The generation of $A\beta_{1-38}$ peptide is still a matter of discussion. Beher et al. [16] showed that such C-terminally truncated peptides are generated by action of γ-secretase and not by a separate enzyme entity. In a cell culture model, Weggen et al. [23] and De Strooper and Konig [24] also demonstrated an increase of $A\beta_{1-38}$ peptides and a de-

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crease of $A\beta_{1-42}$ upon administration of nonsteroidal anti-inflammatory drugs with potential γ-secretase activities.

In conclusion, protein biochip technology may allow for both mass profiling and relative quantitation of Aβ species in human CSF, and may thus expand the methodological repertoire of diagnostic and therapeutic biomarker research in AD.

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