Brief communication

Alterations in the Balance of Amyloid-β Protein Precursor Species in the Cerebrospinal Fluid of Alzheimer's Disease Patients

Lopez-Font I, Boix CP, Zetterberg H, Blennow K, Sáez-Valero J

***To whom correspondence should be addressed**: Instituto de Neurociencias de Alicante, Universidad Miguel Hernández-CSIC, Av. Ramón y Cajal s/n, Sant Joan d'Alacant, E-03550, Spain. j.saez@umh.es. Tel.:+34 965919580, Fax:+34 965919561.

Abstract

This study assesses whether C-terminal fragments (CTF) of the amyloid precursor protein (APP) are present in cerebrospinal fluid (CSF) and their potential as biomarkers for Alzheimer's disease (AD). We demonstrate that APP-CTFs are detectable in human CSF, the most abundant being a 25-kDa fragment, probably resulting from proteolytic processing by η-secretase. The CSF level of this 25-kDa CTF is higher in subjects with autosomal dominant AD patients linked to *PSEN1* mutations, in demented Down syndrome individuals and in sporadic AD subjects compared to age-matched controls. Our data suggest that APP-CTF could be a potential diagnostic biomarker for AD.

Background

Accumulation of the β-amyloid peptide (Aβ) in the brain is an early and specific phenomenon associated with the pathogenesis of Alzheimer's disease (AD) (Scheltens et al., 2016). Many reports support that the determination of Aβ42 in cerebrospinal fluid (CSF) is a core biomarker for AD (Blennow et al., 2015). While the amount of the pathological species of Aβ is increased in the AD brain, their levels in CSF are decreased, probably due to increased brain deposition. To enable monitoring early disturbance in amyloid precursor protein (APP) and Aβ mis-metabolism additional biomarkers are needed.

Other plausible biomarkers for AD are additional fragments resulting from the processing of the amyloid precursor protein (APP), which may contribute to Aβ deposition in the AD brain. APP is a large type I transmembrane spanning protein consisting of a large N-terminal extracellular domain, a hydrophobic transmembrane domain, and a short intracellular C-terminal domain. APP is usually cleaved by α-secretase (ADAM10; leading to non-amyloidogenic pathway), or by β-secretase (BACE1; leading to amyloidogenic pathway), which causes the secretion of large sAPPα and sAPPβ N-terminal fragments (NTFs). CSF levels of sAPPα and sAPPβ show no change in AD (Olsson et al., 2016). The membrane remaining C-terminal fragments (CTFs) are always processed by γ-secretase generating shortest intracellular domain (AICD) peptides (for a review see Haass et al., 2012).

APP can also undergo alternative proteolytic processing pathways (see recent review Andrew et al., 2016). Thereby, concerted cleavage of β-secretase and α-secretase result in the secretion of sAPPβ and shorter Aβ peptides (Portelius et al., 2011). The presence in CSF of short NTF derivatives of APP, which generation does not involve α-secretase or BACE, has been also demonstrated (Portelius et al., 2010). More

interestingly in the context of this report, a recently discovered alternative physiological APP processing pathway driven by an asparagine endopeptidase named δ-secretase (Zang et al., 2015) or by a metalloproteinase named η-secretase (Willen et al., 2015) will generate alternative proteolytic metabolites, including NTFs and CTFs, of different molecular mass.

In addition to the different species of Aβ, all the large extracellular APP-NTFs have been studied in human CSF (Palmert et al., 1989; Ghiso et al., 1989; Willen et al., 2015); but the presence of APP-CTFs in CSF has not been reported to date. In this study we investigated if APP-CTFs are detectable in CSF, characterized the major APP-CTF immunoreactive band, and determined whether the levels of this peptide fragment are altered in autosomal dominant AD (ADAD), Down syndrome subjects with Alzheimer's type dementia (dDS), and sporadic AD subjects (sAD).

Material and methods

Patients

Lumbar CSF samples were obtained from ADAD subjects that were all carriers of *PSEN1* mutations and who were part of the Genetic Counseling Program for familiar dementia (PICOGEN) at the Hospital Clínic (Barcelona, Spain). This group included 7 subjects carrying *PSEN1* mutations, and 7 age-matched non-mutation carriers from the same families (non-disease controls: NC). We also included lumbar CSF samples from 7 dDS subjects, along with 7 age-matched NC obtained from the Hospital Sant Pau (Barcelona, Spain). In addition, 20 patients with sAD defined as patients with cognitive symptoms and a CSF biomarker profile indicating AD (high total tau and phosphorylated tau together with low Aβ42 levels; see Supplemental Table 1) and 20 age-matched controls defined as (patients with non-specific symptoms without neurochemical evidence of AD) were also obtained from the Clinical Neurochemistry Laboratory (Mölndal, Sweden). All AD patients fulfilled the 2011 NIA-AA criteria for dementia (McKann et al., 2011).

Western blotting and immunoprecipitation

Samples of CSF (30 µL) were denatured at 98ºC for 5 min and were resolved by electrophoresis on 16.5% Tris-Tricine gels. Following electrophoresis, proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell Bioscience, GmbH). CTFs of APP were detected using the following anti-APP C-terminal antibodies: C1/6.1 (mouse monoclonal; Covance), A8717 (rabbit polyclonal; Sigma), and Y188 (rabbit monoclonal; Abcam), as well as with the rat antibodies 2D8 and 2E9 (Willem et al., 2015). To assess the specificity of the immunoreactive bands, samples were resolved by simultaneous detection of immunoreactivity to two antibodies. Blots were then probed

with the appropriate conjugated secondary antibodies and imaged on an Odyssey Clx Infrared Imaging System (LI-COR). Band intensities were analyzed using LI-COR software (Image Studio Lite). A control CSF sample was used to normalize the immunoreactive signal between blots.

For immunoprecipitation, samples were precleared for 2 h at 4 ºC by incubation with protein A-Sepharose (Sigma-Aldrich). Immunoprecipitations were performed at 4ºC by incubating 150 µL of CSF, overnight with the indicated anti-APP antibody previously coupled to protein A-Sepharose using dimethyl pimelimidate dihydrochloride (Sigma-Aldrich Co). Precipitated proteins were washed with PBS and eluted with 0.1M glycine buffer at pH 2.5. After pH neutralization, supernatants were denatured in Laemmli sample buffer at 98ºC for 5 min and subjected to SDS-PAGE. The membranes were then probed with an alternative anti-APP antibody.

Measurement of T-tau, P-tau and Aβ42 by ELISA

Total tau (T-tau), phosphorylated tau (P-tau) and Aβ1-42 (Aβ42) concentrations in CSF were measured using INNOTEST ELISA methods (Fujirebio Europe).

Statistical analysis

All data were analyzed using SigmaStat (Version 3.5; Systac Software Inc.) by Student's *t* test (two-tailed) for determination of exact *p* values. Correlation was assessed by linear regression. Results are presented as means ±SEM.

Results

APP-CTFs are present in human CSF

To determine the presence of APP-CTFs in human CSF, we first examined human CSF samples by Western blotting using three different anti-CTF antibodies (a schematic representation of APP and epitopes for antibodies is represented in Fig. 1A). Immunoblotting revealed a similar pattern of immunoreactive bands, with a predominant band of ~25 kDa (Fig. 1B). This band was also observed with the 2D8 and 2E9 antibodies, both against an extracellular domain close to the transmembrane domain, thus recognizing a η-secretase-generated CTF-APP (CTFη) (Willem et al., 2015). Simultaneous assay of combined fluorescence with the A8717 and 2E9 antibodies indicated that the major 25 kDa band is compatible with the CTFη; similar co-labelling was revealed with antibodies C1/6.1 and 2D8 (Supplemental Fig. 1). To further examine the identity of CTF bands in human CSF, we performed immunoprecipitation/Western blot analysis (Fig. 1C). CSF samples were immunoprecipitated using the A8717 antibody and blotted with the Y1887 antibody, confirming the identity of the 25-kDa band as an APP-CTF. Immunoprecipitating with 2D8 antibody and blotting with A8717 antibody confirmed the 25-kDa band as a CTFη.

The 25-kDa CTFη is increased in AD CSF

To assess whether APP-CTF levels are altered in AD, we first analyzed CSF samples from genetically determined AD subjects. The 25-kDa band was detected with A8717 antibody in all CSF analyzed (Fig. 2A). The level of the 25-kDa APP-CTF band in the CSF from ADAD subjects increased (95±27%; p= 0.01) compared to those in age-matched NC composed by non-mutation carriers from the same families (Fig. 2A). DS is also considered a pre-symptomatic AD (Dubois et al., 2014). Once more, an increase

(68±18%; p= 0.01) in the level of the 25-kDa APP-CTF band was determined in CSF samples from dDS patients, comparing these to age-matched NC (Fig. 2B). Finally, we found that the immunoreactivity for this band increases (35±9%; p= 0.01) in sAD compared to age-matched NC subjects (Fig. 2C). CSF samples from sAD subjects also showed similar increases for the 25-kDa band when analyzed with the 2D8 antibody (35±9%; p= 0.01), correlating tightly the estimation of the levels calculated with the A8717 antibody (Supplemental Fig. 2).

Discussion

Here, we demonstrate that APP-CTFs are detectable in human CSF. Particularly, the most abundant soluble APP-CTF is attributable to 25-kDa CTF recently characterize as the result of proteolytic processing by η-secretase (Willem et al., 2015). Canonical APP proteolysis occurs via α- and β-secretases, resulting in CTFα and CTFβ with a molecular mass lower than 15 kDa; but novel APP-CTF that migrate ~25 kDa have been described (Wang et al., 2015). As an intermediate of proteolytic process, the presence of any APP-CTF in CSF was unexpected. Anyhow, the presence in CSF of soluble forms of full-length membrane proteins containing the transmembrane and intracellular domains is not an unusual finding (Lopez-Font et al., 2015). The mechanisms by which these membrane-bound proteins reached the CSF are unknown, but neuronal death may be a major contributing factor.

Different APP C-terminal antibodies confirmed the 25 kDa CTFη as the predominant band in human CSF. As stated, these APP proteolytic fragments are derived from a constitutive processing step driven by a η-secretase that is partially in dynamic equilibrium with α- and β-secretase-mediated proteolysis (Haass et al., 1992; Willem et al., 2015; Lauritzen et al., 2016). The current canonical views about APP-related cleavage events are likely a partial understanding of the overall process. How

secretases compete for the APP substrate and whether subcellular compartmentalization of APP and secretases is responsible of the dynamic equilibrium between secretases is under discussion (Gandhi et al., 2004). Thus, one may speculate that the particular abundance of the 25-kDa CTFη in CSF is related with compartmentalization of secretases, resulting in prolonged time of residence of these CTFη in comparison with canonical CTFα and CTFβ. Anyhow, the other fragments derived of the further processing of CTFη by α- and β-secretases, the Aη-α and Aη-β fragments, have been also identified in human CSF with levels similar than those for Aβ (Willem et al., 2015).

Our determination of the CTFη-CTF levels by Western blotting displayed large overlap for sAD, whereas superior discrimination was obtained for ADAD and dDS subjects. Interestingly, APP CTFβ levels are also in frontal cortex brain homogenates obtained from patients (Pera et al., 2013). Further studies will indicate if distinct pattern of APP processing in ADAD and sAD affect the generation of different APP-CTFs. In our study all the pathological groups were compared with age-matched controls obtained from the same center of sample collection. The possibility that APP-CTF levels varies with age should be considered and deserve a specific study with larger number of samples. In any case, it will be valuable to replicate these finding using techniques such as ELISA, to evaluate their true potential as biomarkers. However, this desirable outcome will be challenging. We have described that the soluble full-length APP, containing the C-terminal domain, also exists in CSF as heteromeric complexes compromising other sAPP species (Cuchillo-Ibañez et al., 2015), and transmembrane domain, as well intracellular C-terminal domain could participate in APP dimerization (discussed in Isbert et al., 2012). Thus, future studies should develop custom pan-specific antibodies targeting the predicted N-terminal sequence of the APP-CTFs present in CSF for preventing co-precipitation of other APP fragment. Anyhow, even

the disadvantages of Western blotting for quantitative analysis, we considered that based on the data present in this study, there are sufficient evidence to justify further studies on the determination of APP-CTFs as a potential diagnostic biomarker of AD.

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Competing interests

The authors have no competing interests to disclose in connection with this article.

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Figure Legends

***Figure 1*. Soluble CTFs of APP are present in human CSF**. (**A**) Schematic representation of full-length APP processing by secretases. The epitopes for the antibodies used in this study are indicated. (**B**) Western blotting of three human CSF samples from non-demented controls subjects, resolved with the indicated antibody. 2D8 and 2E9 also detect other soluble fragments around ~15 kDa, termed Aη, generated after concerted cleavage by η-secretase and α/β-secretases (see Willem et al., 2015). 2D8 antibody also detects Aβ monomers. (**C**) CSF aliquots (Total, T) were immunoprecipitated with the indicated antibody and precipitated proteins (bound fraction, IP) were immunoblotted with the indicated alternative antibody. In the absence of capture-antibody (IPc), no bands were observed.

***Figure 2*. Higher levels of the 25-kDa APP-CTF band in the CSF of AD subjects.** (**A**) Representative blot of the APP-CTF in the CSF samples from 7 symptomatic ADAD and 7 age-matched NC, which were from the same families that ADAD subjects but that did not carry mutations (black symbol; see also Table 1). Densitometric quantification of the immunoreactivity from the 25-kDa band is also shown. (**B**) Representative blot and densitometric quantification in CSF from 7 DS subjects with dementia of the Alzheimer's type (dDS) and 7 age-matched NC. (**C**) Representative blot and densitometric quantification of the immunoreactivity from the 25-KDa APP-CTF in CSF samples from 20 sAD and 20 age-matched NC subjects. Immunodetection was performed with the A8717 antibody. *p* values are displayed.

***Supplemental Figure 1*. SDS-PAGE analysis and fluorescence detection of soluble CTFs of APP in human CSF**. To probe that the 25-kDa specie resolved with different antibodies is the same, three aliquots of CSF were analyzed by SDS-PAGE and resolved with (**A**) A8717 and 2E9 antibodies, or (**B**) C1/6.1 and 2D8 antibodies, simultaneously. The fluorescence of the secondary antibodies was detected with the Odyssey CLx Infrared Imaging system (LI-COR).

***Supplemental Figure 2*. Corroboration of the increase in the 25-kDa APP-CTF in the CSF of sAD subjects.** Representative blot and densitometric quantification of the 25-kDa APP-CTF in the CSF samples from 20 probable sAD 20 age-matched NC subjects blotted with the A8717 antibody (same data that in Fig. 2) and with the 2D8 antibody. A positive correlation was obtained when immunoreactive levels estimated with both antibodies from the same subjects were confronted. Regression linear coefficient (R) and *p* values are displayed.

Table 1: **Clinical, demographic data and classic CSF biomarker levels.**

Group	Age (years)	Gender	CSF Aβ42 (pg/mL)	CSF T-tau (pg/mL)	CSF P-tau (pg/mL)
sAD	71±2 [55-86]	15F/5M	412±19**	665±52**	84±6**
NC	72±2 [57-88]	6F/14M	739±32	233±14	36±2
ADAD	43±2 [31-49]	5F/2M	266±49**	883±204**	168±69*
NC	39±3 [25-47]	5F/2M	809±94	245±29	46±4
dDS	53±2 [43-57]	4F/3M	422±17*	767±170*	108±19*
NC	48±2 [47-53]	5F/2M	751±84*	160±26	33±5

The *PSEN1* mutations included in this study from ADAD cases (autosomal dominant AD subjects) corresponded to 3 carriers of L286P, and one S169P, L173F, L235R and L282R. The data represent the means ±SEM, and for age the range of values is also indicated. All the pathological groups were compared with age-matched NC obtained from the same Hospital. Significantly different $**p < 0.005$; $*p < 0.05$ from the NC group. This study was approved by the ethics committee at the Miguel Hernandez University and it was carried out in accordance with the Declaration of Helsinki.