Apolipoprotein E, transthyretin and actin in the CSF of Alzheimer's patients: relation with the senile plaques and cytoskeleton biochemistry

Aksam Merched[a], Jean-Marie Serot[b], Sophie Visvikis[a], Dominique Aguillon[a], Gilbert Faure[c], Gérard Siest[a,*]

[a] *Centre de Médecine Préventive, UPRES, 2, rue du Doyen Jacques Parisot BP 7, F-54501 Vandoeuvre-lès-Nancy, France*
[b] *Hôpital Hotel Dieu, 54350 Mont Saint-Martin, France*
[c] *CRIP, Centre de Recherche en Immuno-Pathologie, Faculté de Médecine de Nancy, BP 184, F-54500 Vandoeuvre-lès-Nancy, France*

Received 28 November 1997; revised version received 20 February 1998

Abstract We measured the levels of two β-amyloid (Aβ)-sequestering proteins, apolipoprotein (Apo) E and transthyretin (TTR), in ventricular human cerebrospinal fluid (CSF) of Alzheimer's disease (AD) patients and controls in relation to brain histological findings. We also studied actin levels in CSF as a marker of the biochemical role of these two proteins in the cytoskeleton. We show that TTR levels in CSF were significantly decreased in AD patients compared to controls and negatively correlated with the senile plaque (SP) abundance. Moreover, actin levels were positively linked to TTR levels and increased in CSF samples of patients homozygous for the ApoE ε4-allele. We propose that TTR and ApoE4 may have competition in the aggregation of Aβ and its deposition in the SP of AD brain. The relationships between ApoE, TTR and actin could suggest a metabolic implication of ApoE genetics and TTR levels in cytoskeletal biochemistry which may be relevant to the pathogenesis of AD.

Key words: Transthyretin; Apolipoprotein E; Actin; Cerebrospinal fluid; Senile plaque; Alzheimer's disease

1. Introduction

Amyloid β (Aβ) is the major component of senile plaques found in Alzheimer's disease (AD) brain. Aβ also exists in a soluble form in physiological fluids such as cerebrospinal fluid (CSF) and plasma as well as in the conditioned media of different cultured cell lines [1–3]. The mechanism by which Aβ forms amyloid fibrils is unknown. But emerging evidence suggests that amyloid fibril formation is a critical step in the pathogenesis of AD. CSF contains several factors that promote the solubility, transport and clearance of Aβ, mainly two important proteins apolipoprotein E (ApoE) and transthyretin (TTR).

ApoE has three common isoforms, E2, E3 and E4, encoded by alleles ε2, ε3 and ε4. The ε4 allele is over-represented in late-onset and sporadic AD; and its importance as a risk factor for its development is now well established [4–6]. TTR is a homotetrameric protein each subunit of which has a molecular weight of 15 kDa. Within the central nervous system (CNS), TTR is the only known CSF protein synthesized solely by the choroid plexus [7]. In CSF, TTR is the principal thyroxine-binding protein [8], which suggests its possible implication in the active transport of thyroid hormone from the blood stream into CSF [7]. Several lines of evidence point to an active role of ApoE and TTR in fibril formation in the brain. ApoE binds to β-peptide with high affinity in vitro [9], promotes Aβ fibrillogenesis in vitro and induces accumulation of the β-amyloid peptide in cultured vascular smooth muscle [10–12], while TTR inhibits and prevents this fibril formation [12,13]. TTR in lumbar CSF was shown to be correlated negatively with the degree of dementia in patients with dementia of Alzheimer type [14]. Recently, we have shown that ApoE levels increased in lumbar CSF in cases with normal aging and more extensively with AD [15,16], which has now been confirmed by others [17,18]. We also reported that TTR levels increased with aging but decreased with AD [19].

The aim of this work was to investigate how the two Aβ amyloid-sequestering proteins ApoE and TTR may contribute to the balance of amyloid formation in AD. We took into account senile plaque (SP) abundance in the brain of the same patients. We also verified the potential action of these proteins in the cytoskeletal biochemistry by estimating, for the first time, actin levels in the CSF, the major component of the microfilament. We studied these relationships because the intracellular organization of actin filament seemed to be regulated by thyroxine, mainly transported by TTR in the CSF [20]. We hypothesized that ApoE may also be involved in actin regulation via its avidity for this protein [21]. We investigated the ApoE polymorphism effects on these protein levels in relation to brain histological findings.

2. Materials and methods

2.1. Patients and samples

This study was performed with 30 patients. Twenty patients were confirmed by neuropathology examination at autopsy to have AD without other complicating neurological or neuropathological features (84.40 ± 1.79 years, five males and 15 females). Ten sex- and age-matched controls (83.10 ± 3.49 years, three males and seven females) were free of AD or other known neurological diseases (five with cerebrovascular disease without dementia, three multi-infarct dementia, two with degenerative disc disease). The NINCDS-ADRDA work group histological criteria for the diagnosis of 'definite Alzheimer's disease' were used. Autopsies were performed within 24 h in the nursing home of the Hotel Dieu Hospital in Mont Saint-Martin (France). Informed consent for brain autopsy had been obtained from the families of all patients. The CSF samples were obtained following opening of the cranial cavity and withdrawal of the CSF syringe. Brain sections including middle temporal gyrus, middle frontal gyrus, and inferior parietal lobule as well as hippocampus CA1 sections were ob-

*Corresponding author. Fax: (33) 383 44 87 21.
E-mail: gerard.siest@cmp.u-nancy.fr

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; Aβ, β-amyloid peptide; AD, Alzheimer's disease; Apo, apolipoprotein; CNS, central nervous system; CSF, cerebrospinal fluid; SP, senile plaques; TTR, transthyretin

PII S0014-5793(98)00234-8

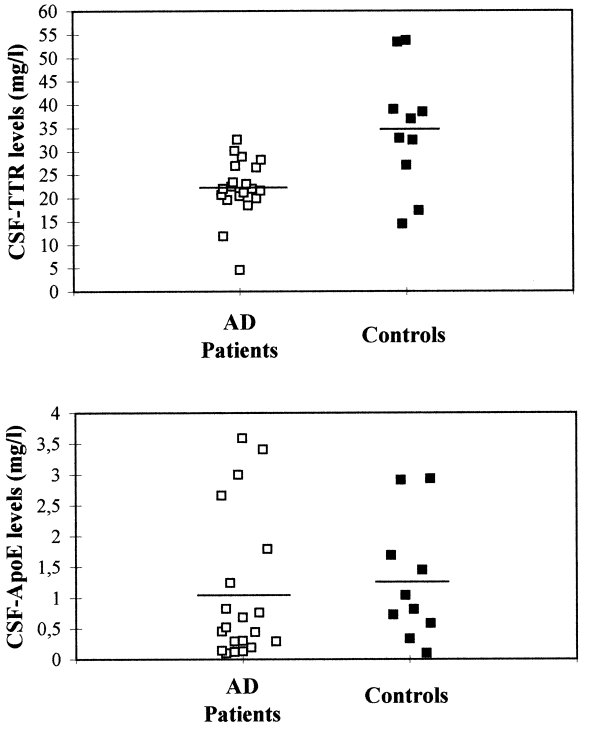

Fig. 1. Comparison of ApoE and TTR levels in CSF of AD patients and control patients. Each value is the mean of triple assays. Bars represent the mean values.

tained from all patients. Samples were frozen in liquid nitrogen and stored until analyzed.

2.2. Histological analyses

Semiquantitative grading of senile plaques and neurofibrillary tangles in the tissue section were performed by immunofluorescence using monoclonal antibodies: antihuman β4 amyloid (Dako, Glostrup, Denmark) and antihuman Tau1 (Boehringer Mannheim, Mannheim, Germany) with FITC anti-mouse Ig (Institut Pasteur Productions, Marnes, France) as a second-step reagent previously described [22].

For AD patients, immunofluorescence revealed many SP with homogeneous frequency in the three neocortical regions. Control patients presented a few SP and threads. SP were classified as follows: +, some; ++, moderate; and +++, abundant.

2.3. Protein measurements

The measurement of CSF total protein was made using a dye binding colorimetric method based on pyrogallol red-molybdate complex (Biotrol, Paris, France) with a Cobas Mira analyzer (Roche Diagnostics, Neuilly-sur-Seine, France). ApoE measurements in CSF were performed by a sandwich enzyme-linked immunosorbent assay (ELISA) as described elsewhere [16]. TTR was measured automatically using an Array protein system (Beckman, Brea, CA, USA) as described by the manufacturer with anti-TTR antibody (Beckman Instruments, Galway, Ireland). The coefficients of analytical variation were 4.9% and 3.7% for inter-assay and intra-assay, respectively. Actin levels were estimated by quantitative study of samples separated using high resolution, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) performed with the ISO-DALT system [23]. The staining of the 2D gels was performed with a neutral method using silver nitrate [24] with sensitivity in the nanogram range. Alternatively, proteins separated by 2D-PAGE were transferred to sheets of nitrocellulose (Schleicher&Schuell, Inc., Dassel, Germany). Primary antibodies to human TTR and actin were obtained from Sigma Biochemicals. Alkaline phosphatase conjugated anti-goat and anti-mouse was used as a secondary antibody. The 2D gels (18×16×0.15 cm) were digitized using an Agfa 800 laser scanner with 1024 gray levels, an optical density range of 0–3.2 and a spatial resolution of 250 μm. The image data were analyzed on a SUN workstation by the HERMeS program [25]. Calibration and normalization for staining and detection variation were done using the TTR content of samples measured by nephelometry. To avoid variations which may be due mainly to the silver staining of actin between samples, we studied actin and TTR values from the same gel. In this way, the standardization was essentially internal for each gel. Minor variations in load volumes, for instance, would not alter the results. Values for CSF samples in this study were represented by averages of two or more replicates.

2.4. ApoE polymorphism study

First, PCR amplification of the sequences spanning the ApoE polymorphic sites was performed. Then, ApoE genotyping was performed on DNA isolated from nucleated blood cells [26] and digested with *Hha*I as described elsewhere [27]. When blood was not available, polymorphism was studied by phenotyping ApoE in CSF samples using two-dimensional electrophoresis.

2.5. Statistical analysis

All data were analyzed with the BMDP statistical software (Los Angeles, CA). The mean values were compared using the non-parametric Kruskal-Wallis test. We used the Spearman rank test to evaluate the relationship between ApoE, TTR and actin levels. $P \leq 0.05$ was considered significant.

3. Results

The analysis showed no significant difference in the levels of ApoE (±S.E.M.) between AD and control groups (1.05±0.26 mg/l, $n=20$ vs. 1.26±0.31 mg/l, $n=10$ respectively, $P=0.32$) (Fig. 1), but the mean CSF level of TTR in patients with AD was significantly lower than that observed in controls (22.21±1.41 mg/l vs. 34.52±4.12 mg/l respectively, $P=0.0094$). Neither ApoE nor TTR levels were significantly correlated with those of albumin (not shown).

A semiquantitative estimation of senile plaques showed a negative correlation ($P=0.035$) between the SP abundance and the mean levels of TTR in CSF of the same patients (Fig. 2). The TTR levels were significantly higher in CSF of patients with a few SP compared to those of patients with abundant SP ($P<0.05$). Correlation was lacking between ApoE levels and SP abundance ($P=0.74$). We showed a positive correlation between ApoE ε4-allele frequency and the SP abundance ($P<0.05$, by χ^2 test). Age did not affect the SP abundance ($P=0.77$). We also investigated the effect of age on ApoE and TTR levels in CSF. Neither ApoE nor TTR changed significantly with age (data not shown).

In the CSF, analysis of TTR and acting using 2D-PAGE

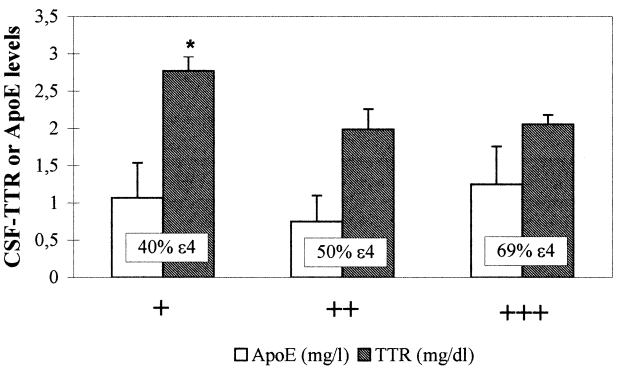

Fig. 2. ApoE and TTR concentrations in CSF of AD patients as a function of the SP abundance in the brain of the same patients. SP ranged as follows: +, some ($n=7$ patients); ++, moderate ($n=7$); +++, abundant ($n=7$). The frequency of ε4 in each group is shown. *$P<0.05$. Bars are S.E.M.

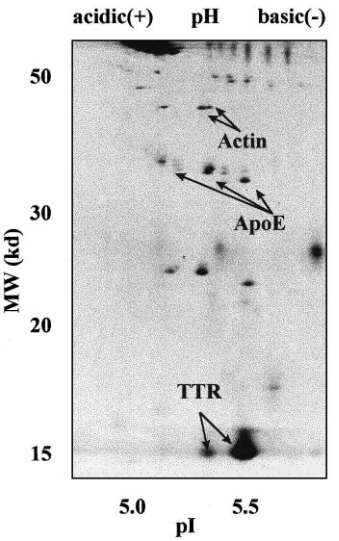

Fig. 3. High resolution 2D-PAGE of silver-stained human CSF proteins. Actin (p*I*: 5.24 and 5.28; MW: 43 kDa) and TTR spots (p*I*: 5.55 and 5.3; MW: 14 kDa) are indicated by the arrows.

(Fig. 3) showed two spots for actin with molecular masses (MM) of about 43 kDa and different isoelectric points (p*I*) of 5.24 and 5.28 and two or three spots for TTR (MM = 14 kDa and p*I* 5.04–5.55). The spots of actin and TTR were identified either by immunostaining of the 2D blot or by comparison with published maps. The correlation between actin spot intensities and those of TTR showed a positive relationship for all patients including AD and controls ($r = 0.54$, $P = 0.0088$, $n = 20$, Fig. 4). The correlation remained significant without the control patients ($r = 0.60$, $P = 0.019$, $n = 14$). While that with the control group was not significant ($r = 0.46$, $P = 0.25$, $n = 7$), no difference was found in the actin levels between AD and controls group (1.41 ± 0.22 mg/l ($n = 15$) vs. 1.41 ± 0.28 mg/l ($n = 7$), $P = 0.94$).

When we took into account the effect of the ApoE alleles relative to actin levels variability (Fig. 5), we found an association with the dose of the ε4 allele ($P < 0.05$). The levels

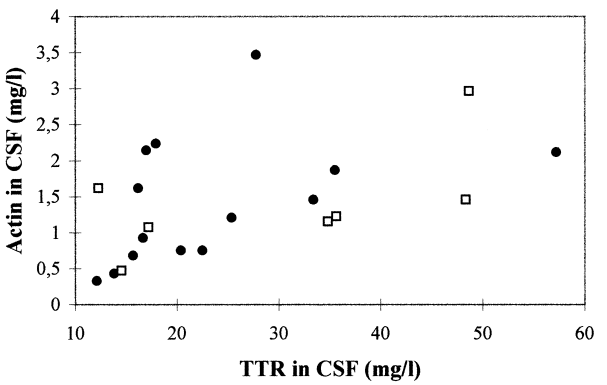

Fig. 4. Relationship between actin and TTR levels in CSF of patients with AD ($n = 14$) and with other neurological diseases ($n = 7$). The levels are reflected by the total intensities of actin and TTR proteins separated by 2D-PAGE, digitized using an Agfa 500 laser scanner and analyzed by HERMeS image analysis software. Spearman correlation coefficient with all patients was $r = 0.54$, $P = 0.0088$. The closed circles and the open squares correspond to AD and the neurological diseases groups, with $r = 0.60$ ($P = 0.019$, $n = 14$) and $r = 0.46$ ($n = 7$, $P = 0.25$), respectively.

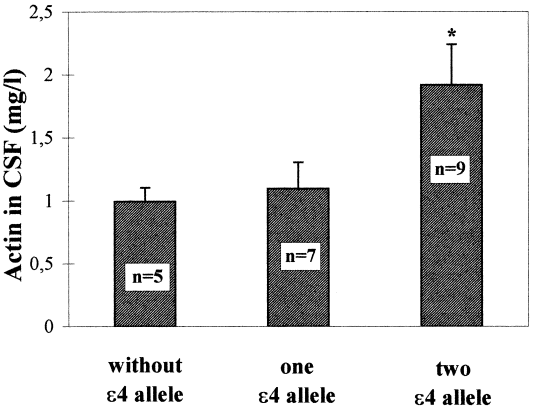

Fig. 5. Analysis of actin levels in CSF of all patients (14 with AD and seven with other neurological diseases) according to the ε4 allele dose of ApoE. Statistical differences between allelic groups were calculated using Kruskal-Wallis test, $*P < 0.05$. Bars are S.E.M.

were: 1.01 ± 0.09 mg/l ($\pm$S.E.M.) for no ε4 allele carriers ($n = 6$); 1.09 ± 0.21 mg/l for one ε4 allele carriers ($n = 7$) and 1.92 mg/l ± 0.32 for two ε4 allele carriers ($n = 9$). Actin levels were not statistically correlated with those of albumin ($r = 0.30$, $P = 0.20$, $n = 19$) nor were they linked to the SP abundance.

4. Discussion

This is the first report investigating transthyretin and actin concentrations in ventricular human cerebrospinal fluid of patients clinically and pathologically confirmed to have had AD. A few biochemical studies of Alzheimer's patients have involved the analysis of CSF for transthyretin. A previous study showed a negative association of TTR with degree of dementia in lumbar CSF of patients with AD [14]. Now, we report a negative correlation between TTR and one major hallmark in the AD brain, the senile plaques. We also show that TTR levels in AD CSF were significantly lower compared to those of patients suffering from other neurological diseases and we confirmed our recent findings in lumbar CSF [19]. We note that ventricular TTR values in the present study are higher than the lumbar ones [14,19] and this is due to the fact that TTR, in contrast to most CSF proteins, decreases from the ventricles to the lumbar space [7].

Meanwhile, we showed that the CSF mean levels of ApoE in AD patients did not differ significantly from those in the neurological patients. The results of studies regarding lumbar CSF ApoE in AD are apparently controversial (see [15]). However, the level of ApoE seems to be dependent on the clinical state, genetics and other factors; but it is likely to be linked to the death and damage caused by disease or injury. Here, no specific association of CSF ApoE levels with the pathology of Alzheimer could be determined, probably because the control group seemed to have had high levels of ApoE [15]. Singly, variation of ApoE levels in CSF had no evident implication in AD.

The precise mechanism by which TTR may be involved in AD is unknown. Our findings provide strong support for a role of TTR in Alzheimer's pathogenesis by sequestering Aβ and preventing fibril formation, as suggested by several in vitro assays [10,11,27]. The decreased CSF mean levels of TTR observed in patients with AD may reflect a decreased

synthesis of this protein by the choroid plexus in the brain. This is probably linked to the alterations in the structure of the latter which we have recently found with aging and more extensively with Alzheimer's pathology [28]. However, we cannot exclude an accelerated uptake of TTR from CSF via a specific receptor [29].

Another side of TTR implication poorly investigated in AD may be at the levels of hormonal regulation and delivery. In CSF, where TTR is the principal thyroxine binding protein, the TTR-thyroxine complex seems to have a distributive function, assuring availability of thyroxine to cells through the CSF circulation [30]. To better estimate this role, we studied the actin levels as potential target of the hormonal distributive action of TTR in the brain cytoskeleton. Thyroxine was shown to regulate the actin polymerization in the microfilament structure in astrocytes [20], e.g. the equilibrium between G-actin (soluble) and F-actin (insoluble) and consequently, the soluble form levels of actin in the extracellular fluid. The positive correlation that we reported here between actin and TTR levels in the CSF and the decrease of TTR levels with AD may have such biological relevance. Further investigations are needed to confirm this hypothesis.

The high levels of actin in CSF obtained with the ε4 allele carrier patients may be due to the extensive damage in the brain cells of these patients in relation to the ε4 allele [31]. Interestingly, ApoE seems to have allele-dependent affinity for some cytoskeleton components such as tau, MAP2 and actin [21], probably controlling the stability of the cytoskeleton and the normal neuronal function. The ε4 allele is thought to have a negative contribution to this process. The correlation reported here between actin in CSF and the ε4 allele may reflect one cytoskeletal abnormality characterized by a destabilization of the actin filaments, a process that might be enhanced by the ApoE4 isoform. Alternatively, TTR and ApoE4 could have different effects on actin regulation, indirectly by interacting with APP which was shown to be associated with the cytoskeleton [32] and seems to be directly implicated in the actin expression [33].

As a whole, TTR levels in CSF appear to be specifically decreased in AD and negatively correlated with SP abundance. These results reinforce the designation of TTR as a potential factor implicated in fibrillogenesis prevention and point to the utility of TTR determination in CSF of AD. Actin in the CSF may serve as a marker of the brain cytoskeletal integrity and with a lesser extent of particular TTR and/or ApoE functions in the brain.

Acknowledgements: We wish to thank the patients' families for their generous cooperation and understanding. We thank W. Ruff for improving the language of this paper and M. Starck and M.P. Recouvreur for their technical assistance. This work was partially supported by the 'Alzheimer 54' Institution and the Region de La Lorraine.

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