Disease Markers 22 (2006) 119–125
IOS Press

Altered β-amyloid precursor protein isoforms in Mexican Alzheimer's Disease patients

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Abstract. *Objective*: To determine the β-amyloid precursor protein (βAPP) isoforms ratio as a risk factor for Alzheimer's Disease and to assess its relationship with demographic and genetic variables of the disease.
Methods: Blood samples from 26 patients fulfilling NINCDS-ADRDA diagnostic criteria for AD and 46 healthy control subjects were collected for Western blotting for βAPP. A ratio of βAPP isoforms, in optical densities, between the upper band (130 Kd) and the lower bands (106–110 Kd) was obtained. Odds ratios were obtained to determine risk factor of this component.
Results: βAPP ratio on AD subjects was lower than that of control subjects: 0.3662 ± 0.1891 vs. 0.6769 ± 0.1021 (mean $\pm$ SD, $p < 0.05$). A low βAPP ratio (< 0.6) showed an OR of 4.63 (95% CI 1.45–15.33). When onset of disease was taken into account, a βAPP ratio on EOAD subjects of 0.3965 ± 0.1916 was found vs. 0.3445 ± 0.1965 on LOAD subjects ($p > 0.05$).
Conclusions: Altered βAPP isoforms is a high risk factor for Alzheimer's disease, although it has no influence on the time of onset of the disease.

Keywords: β-Amyloid precursor protein, Alzheimer's Disease, risk factor

1. Introduction

Alzheimer's Disease (AD) is the most common type of dementia [3,12] and is characterized by mental loss and progressive neurodegeneration leading to severe mental impairment and death [23]. β-amyloid deposits in the parenchymal senile plaques and in cerebral blood vessel walls as well as neurofibrillary tangles within neurons are the hallmarks of the disease [6, 21]. The gene locus for β-amyloid precursor protein (βAPP) is located on chromosome 21 and codes for a 770 aminoacid, transmembrane protein. This protein, when cleaved by a set of proteases -termed secretases- produces an $A\beta_{1-40}$ peptide and an $A\beta_{1-42}$ self-aggregating variant clearly implicated in the clinical syndrome of AD [17,23].

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ISSN 0278-0240/06/$17.00

Although the etiological role of this component has already been put on more cautious grounds [23], the hypothesis that AD might be a systemic disease has led researchers to look for peripheral cells that may harbour changes related to this disease [11]. On one such cell, the platelet, proteolytic processing of an amyloidogenic species of βAPP [13] and further secretion of AD Aβ amyloid peptide has been found [14]. The aim of the present work was to determine whether an altered β-amyloid precursor protein (βAPP) isoforms ratio is a risk factor of the disease. We also assessed the relationship of βAPP isoforms ratio with demographic and genetic data (*ApoE* polymorphisms). Western-blotting for βAPP on platelets and *ApoE* genotyping was performed in a comparative study on 26 AD patients and 46 control subjects.

2. Methods

2.1. Subjects

Blood samples were obtained from a total of 26 Alzheimer's Disease patients with clinical suspicion of Alzheimer's Disease, and from 46 normal, healthy subjects who attended the Clínica de Trastornos Cognitivos y Demencias at the Hospital de Especialidades del Centro Médico Nacional de Occidente in Guadalajara, Jalisco. Informed consent was obtained from the patient and caregiver (when indicated) by a staff physician. The study was approved by the Institutional Review Board of the Centro Médico Nacional de Occidente. In the AD group, there were 9 (34.6%) women and 17 (65.4%) men, whereas 31 (67.4%) women and 15 (32.6%) men were control subjects. The mean age of the AD population was 60.96 ± 12.85 years, whereas that of the control subjects was 67.73 ± 9.46 years.

Diagnosis of AD was made by: 1) A Mini Mental State Evaluation (MMSE) [10] performed during the first visit, with a score less than 25 for alphabetized subjects and 20 for non-alphabetized subjects, 2) DSM-IV criteria for dementia [9] and 3) NINCDS-ADRDA criteria for diagnosis of probable Dementia of Alzheimer Type [19]. Control subjects were recruited according to the following: 1) A MMSE score of $\geqslant 25$ for alphabetized subjects and of $\geqslant 20$ for non-alphabetized subjects, 2) No fulfilment of DSM-IV criteria and 3) No family history of AD or any other dementia (family data were always confirmed by a second informant who was a first degree relative of the patient, mainly a sibling). The following demographical data was obtained from all subjects: Date of birth, age, gender, age at diagnosis, education, and family history of AD or any other dementia (defined as a first-degree relative with such a disease).

All subjects underwent somatic and neurological examination, including laboratory and imagenologic studies (computed tomography and magnetic resonance imaging).

2.2. Blood collection

Fasting blood samples from patients and control subjects were obtained between 9:00 and 11:00 AM. Blood was drawn from a vein in the antecubital fossa by a 19-gauge needle. The tourniquet was carefully released after its application and blood was collected in 6 tubes containing potassium EDTA (for a total of 30 ml of blood), by a trained physician. Each sample was mixed gently and cooled to 4°C while transportation and platelet isolation was made.

2.3. Platelet isolation

Between 10 minutes and one hour after blood extraction, one 5 ml blood sample was centrifuged 10', at 200 g to separate platelet-rich plasma. Platelet-rich plasma was separated by means of a plastic pipette, avoiding aspiration of both the buffy coat and the blood pellet. Platelets were then pelleted by a second centrifugation (15' at 700 g), washed with cold buffer and stored at -80°C until assayed.

2.4. Western-blot analysis

Western-blotting was performed by means of platelet resuspension in TRIS buffer (50 mM TRIS, 120 mM NaCl, 0.5 % Noridet 40, pH of 7.4) with a specific set of protease inhibitors. Protein concentration was determined using the Lowry method [15]. Electrophoresis in 10% polyacrylamide gel was performed for 25 micrograms of each sample and then transferred to nitrocellulose membranes. Membranes were then pretreated in 5% whole milk in PBST medium for at least one hour, followed by overnight incubation with rabbit anti-APP primary antibody capable of detecting several isoforms of both mature and immature amyloid β(A4) precursor protein, including APP 695, APP 770 and APP 751 (2452 APP antibody, detects APP independent of phosphorylation state; Cell Signalling Technology Inc., Beverly, MA., USA [22]). After 3 times washout of membranes with 5% whole milk in

PBST medium, peroxidase-conjugated goat anti-mouse IgG was added for 2 hours at room temperature. After membranes were washed as previously described, antigen-antibody complexes were revealed by X-ray radiation. Quantitative Western blot analysis was performed by means of computerized assisted imaging (Electrophoresis Documentation and Analysis System, Kodak Digital Science, ID 3.0.2). Results are expressed as the ratio, in optical densities (OD), between the upper band (130 Kd, corresponding to the full-length mature APP) and the lower bands (106–110 Kd corresponding to the APP isoforms) of the APP [8].

2.5. *ApoE genotyping*

ApoE genotypes were determined according to a standard protocol in which DNA was obtained according to the Miller technique [20]. DNA amplification of exon 4 (244 bp) of *ApoE* was further amplified in a total Polymerase Chain Reaction (PCR) volume of 15 μl containing 200 mM dNTPs, 10 pmol of primers, 1.5 mM $MgCl_2$, and 2.5 U *Taq* polymerase (Invitrogen, Life Technologies). The following primers were employed: 5'-TCGCGGGCCCCGGCCTGGTACA-3' upstream and 5'-GAACAACTGAGCCCGGTGGCGG-3' downstream. PCR conditions consisted of an initial melting temperature of 94°C (5 min), followed by 30 cycles of melting (95°C, 30 sec), annealing (60°C, 30 sec) and extension (70°C, 1 min). The amplified product distinguished *ApoE* (ε_2, ε_3, ε_4) polymorphisms. Samples were separated using 6% polyacrylamide gel electrophoresis (29:1) followed by silver staining. Digestion by the *HhaI* enzyme was made to reveal the ε_2 (91 and 83 bp), ε_3 (91 and 48 bp) and ε_4 (72, 48, 35 bp) fragments. Electrophoresis in 12% polyacrylamide gel (19:1) was realized to detect such fragments.

2.6. *Statistical analysis*

Data were analysed using SPSS v. 10 software system. Data are expressed as mean $\pm$ SD. Statistical evaluation was performed by a two-tailed t test, regression analysis and chi-square test with Yates correction as appropriate. A correlation test was made to compare age vs. βAPP isosoforms ratio. Severity of the disease according to βAPP isoforms ratio was also performed. Odds ratios and 95% confidence intervals (CI) were also calculated. A $p < 0.05$ was taken as statistically significant.

Table 1
Demographic data in an AD Mexican population and control subjects

	AD subjects	Control subjects
Age	60.96 ± 12.85*	67.73 ± 9.46
Male/Female	17/9	15/31
MMSE	14.64 ± 5.31*	28.33 ± 1.96
Ages of education	8 ± 5.19*	11.71 ± 3.81
ApoE ε4 allele frequency&	50**	10

*$p < 0.001$.
& Frequency is given as percentages.
** OR (95% CI): 9.40 (2.88–32.66) $p < 0.001$.

3. Results

As already mentioned in Methods, there were a total of 26 Alzheimer's Disease patients who attended the Clínica de Trastornos Cognitivos y Demencias. We recruited 46 healthy subjects with no familiar history of dementia as control subjects.

In the AD group, there were 9 (34.6%) women and 17 (65.4%) men, whereas 31 (67.4%) women and 15 (32.6%) men where control subjects. The mean age of the AD population was 60.96 ± 12.85 years, whereas that of the control subjects was 67.73 ± 9.46 years. *ApoE* ε4 allele frequency was significantly higher in AD patients than in controls (0.5 vs. 0.1, OR 9.40, 95% CI 2.88–32.66; $p = 0.00002$) (Table 1).

3.1. *β-Amyloid*

At Western blotting, two main bands with molecular weights of 106 to 110 Kd and 130 Kd were observed. The upper, 130 Kd, band corresponded to the full-length, mature APP. The lower, 106–110 Kd, band corresponded to the APP immature isoforms (Fig. 1). A relation of the upper and lower bands yielded a ratio in which a high ratio ($\geqslant 0.6$) was related to a normal APP mature/immature isoform relation, while a lower ratio (< 0.6) was related to an altered APP mature/immature isoform relation [8]. The APP ratio on AD subjects was lower than that of control subjects: 0.3662 ± 0.1891 vs. 0.6769 ± 0.1021 (mean $\pm$ SD, $p < 0.05$), with an OR of 4.63 (95% CI 1.45–15.33) (Fig. 2). No statistical significance was found for gender in AD patients: 0.4190 ± 0.2870 in males vs. 0.3638 ± 0.3252 in females (mean $\pm$ SD, $p > 0.05$) (Table 2). Due to the abnormal proportion of Early-Onset Alzheimer's Disease patients in our sample, we performed an analysis between EOAD an LOAD patients, founding a βAPP ratio on EOAD subjects of 0.3965 ± 0.1916 vs. 0.3445 ± 0.1965 on LOAD subjects ($p > 0.05$).

Table 2
βAPP distribution in an AD Mexican population and control subjects

	AD subjects (N)	Control subjects (N)
Age		
40–49 yrs.	0.5038 ± 0.3275 (7)	0.4993 ± 0.2071 (6)
50–59 yrs.	0.3986 ± 0.2440 (6)	0.3716 ± 0.2135 (6)
60–69 yrs.	0.3997 ± 0.3654 (4)	0.6461 ± 0.1872 (17)
70–79 yrs.	0.3492 ± 0.3033 (8)	0.4309 ± 0.2596 (10)
80–89 yrs.	0.08547 (1)	0.6948 ± 0.1700 (7)
Total	0.3662 ± 0.1891 (26)*	0.6769 ± 0.1021 (46)
Severity of disease		
Mild	0.1944 ± 0.1485 (3)	
Moderate	0.5046 ± 0.5358 (19)**	
Severe	0.40 ± 0.29 (4)	

*$p < 0.05$, among groups.
**$p < 0.05$, as compared to other severities of disease.

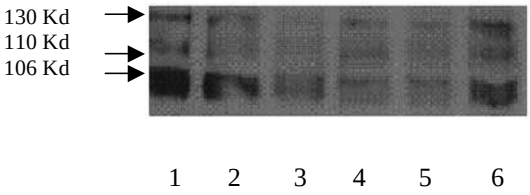

Fig. 1. Western blot analysis of βAPP on Alzaheimer's Disease patients. Mature (130 Kd) and immature (110, 106 Kd) βAPP isoforms are shown. Lanes 1, 2 and 6 show greater mature βAPPs, while lanes 3–5 show lesser mature isoforms.

3.2. Analysis of βAPP isoforms with Age, Severity of disease, and ApoE alleles

A regression analysis showed no correlation of βAPP isoforms with age ($r = -0.24, p > 0.05$, Fig. 3). When severity of disease was taken into account, we found a significant relation of βAPP with Moderate-AD as compared to Mild an Severe-AD, although with a high variability (0.1944 ± 0.1485 vs. 0.5046 ± 0.5358 and 0.40 ± 0.29, respectively, $p < 0.05$ for Moderate-AD, Table 2). There was no association between βAPP ratios and any specific *ApoE* allele. When taken together, both a low βAPP ratio and ε3/ε4 genotype, a stratified OR of 5.15 (95% CI 1.92–14.30, $p < 0.001$) was obtained, whereas a low βAPP ratio and ε4/ε4 genotype showed a stratified OR of 4.14 (95% CI 2.12–31.03). Other *ApoE* genotypes/βAPP ratios were not significant.

4. Discussion

β Amyloid is a major component of the pathogenesis of AD. Its presence has been demonstrated in platelets and has been thought of a peripheral marker of the disease. In this study we attempted to demonstrate whether increased immature βAPP isoforms would be observed in plasma of AD patients and if it would have some effect on certain demographic and genetic factors. In concordance to previous studies, we observed a significant decreased APP ratio on AD subjects as compared to that of control subjects [5,30]. This means that an increased degradation of this precursor protein is being performed in peripheral tissues. Whether or not this phenomenon reflects a similar process occurring in the brain of AD subjects is still a mater of controversy. However, the existence of this phenomenon can not be minimized, for processing and secretion of this precursor protein has been already demonstrated and its importance as a predictor for conversion to dementia of Alzheimer type in subjects with Mild Cognitive Impairment has recently been proved [5].

The precise mechanism of amelioration of APP concentration is not deciphered yet. Modifications in the splicing mechanism, in the stability of messenger RNA encoding for APP 751/770 or in the regulation of translation processes are some hypothesis [8] that need further investigation. Studies on platelet membrane fluidity are being conducted in our laboratory and tend to give promising information which could be combined with data from the hypothesis mentioned above.

Our study is consistent with the results of Strittmatter *et al.* concerning an increased *ApoE* ε4 allele frequency in AD patients [29]. A 4.5-times risk of presenting AD as related to the *ApoE* ε3/ε4 genotype and a 9.4-times risk related to the ε4 allele was also found. This is in accordance to studies published before showing an increased risk of presenting AD as related to either ε4 heterozygocity or homozygocity [1,2,4,7,16].

Our study also shows an AD population close to being an Early Onset AD (EOAD) population due to

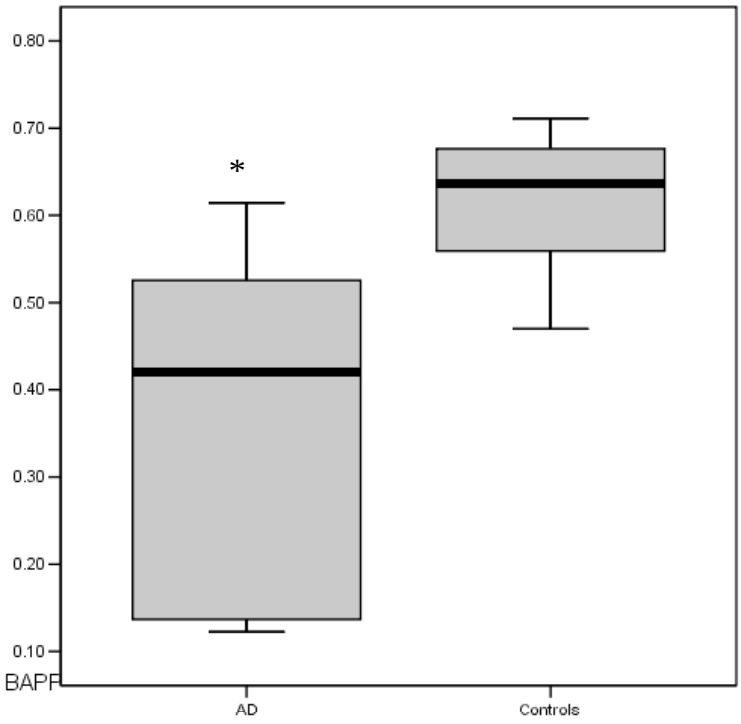

Fig. 2. Distribution of βAPP ratios in an AD population vs. control subjects. $^{*}p < 0.05$.

a high proportion of patients younger than 60 years of age (13 patients, 56.5%). Because of this, statistical analysis between EOAD patients and Late Onset AD (LOAD) was made. There were no statistical differences among both groups in neither the APP isoforms nor any of the *ApoE* genotypes. This disagrees with previous reports stating that increased heterozygotic/homozygotic *ApoE* ε4 genotypes are related in cases of LOAD [27]. This may also explain the statistical data relating an anticipation of the age at onset of this disease in patients expressing this *ApoE* allele. We can not exclude a different epidemiological proportion of *ApoE* ε4 alleles on any of the two groups (EOAD and LOAD), though, for a small population was studied and the demographic variations expected by the Hardy-Weinberg distribution call for such a bias if more subjects are to be taken into account. The fact that this study was undertaken in a reference hospital recruiting people from a main capital city and its surroundings may have contributed to a certain degree of selectivity of the population.

It is known that *ApoE* is related to increased cardiovascular disease and an altered lipid profile [30]. We believe that the strict adherence to the NINCS-ADRDA criteria we employed on selecting these patients may have helped to eliminate both mixed dementia (AD and vascular dementia) and those cases in which a suspicion of vascular disorder was held, reducing thus the frequency of *ApoE* ε4 allele on LOAD patients (and thus explains the disagreement with other author's data [24, 28]). We suggest that taking this into account may reduce the *ApoE* ε4 bias on LOAD on further studies and look for other items that may influence the incidence of the disease when an age-related factor is sought. However, we do not deny the influence of this variant on the pathologic process of the disease.

Previous studies have revealed that *ApoE* genotyping does not provide sufficient sensitivity or specificity to be used alone as a diagnostic test for AD [18] so we suggest that further scrutiny is to be done and that this data must be employed as an assistant in the diagnosis of AD. A post-mortem pathologic study of the brain of these patients is already considered and we believe these data will be useful for comparison of these findings with the "standard gold" diagnostic test. Up to this moment, few brains from this population are available and such a low number is not yet enough if significant data are to be obtained.

The coexistence of β-amyloid with the *ApoE* ε4 allele in the pathogenesis of AD has long been

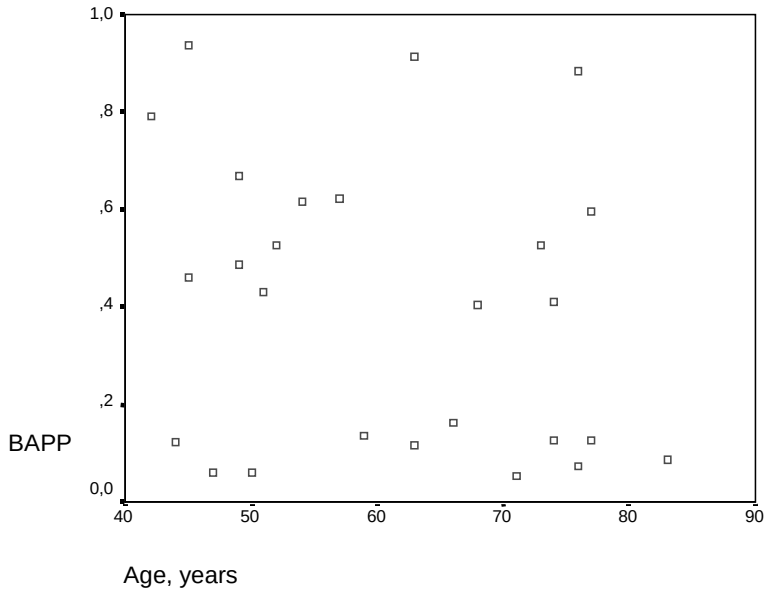

Fig. 3. βAPP isoform ratio in relation to age in an AD population. Age had no significant relation ($r = -0.24$, $p > 0.05$).

thought [26,31]. Increased risk of presenting AD in *ApoE* ε4 allele porters as well as augmented βAPP degradation in platelets led us to consider the probability of relating both factors to determine a combined model for risk factor evaluation. We observed a 5-times risk of presenting the disease when both ε3/ε4 genotype and a low APP ratio (taken as a ratio below (0.6) were stratified, and a 4-times risk with both the ε4/ε4 genotype and a low APP ratio. These data confirms the relevance of peripheral APP altered isoforms as an assessment of risk factor of the disease [24]. The stratified analysis helped us to exclude the effect of *ApoE* genotypes on the low βAPP ratio. We observed no significant OR amelioration or increase of the low βAPP ratio, thus strengthening the importance of a low βAPP ratio in peripheral blood as an indicator of the disease. Although we do not support its employment as a predictor or a unique tool for diagnosis, we believe both elements may be good indicators of the disease when used in conjunction with clinical and laboratory data.

Acknowledgements

This work was made possible by a grant from the Consejo Nacional de Ciencia y Tecnología (CONACYT) and the Instituto Mexicano del Seguro Social (IMSS).

We thank Dr. Alfredo Celis de la Rosa for his statistical support and Dr. Conchita and Isabel Villegas, M. Sc., for their technical assistance.

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