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Human Platelet Tau: A Potential Peripheral Marker for Alzheimer's Disease

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Abstract. Platelets are a major peripheral reservoir of the amyloid-β protein precursor, so they have been considered as a potential biological marker of Alzheimer's disease (AD). Here, it is demonstrated that tau protein is also present in platelets and that the levels of oligomeric species of this protein could serve as a novel and reliable biological marker for AD. Blood samples were obtained from 15 AD patients and 10 paired-age controls and platelets were separated via differential centrifugation. The purity of platelets was determined by flow cytometry and microscopy and the presence of tau was determined by immunofluorescence and immunoblots with tau specific antibodies. Immunofluorescence and immunoblot patterns of platelets were positive for tau. Immunoblots also showed the presence of high molecular weight (HMW) variants of tau that appeared to correspond to oligomeric forms of the protein. The ratio of HMW tau with respect to tau monomeric species was significantly higher in AD patients than controls. The present study is the first description of the presence of tau in platelets. The analysis of different tau fractions in platelets could serve as a new biological marker for AD.

Keywords: Alzheimer's disease, cognitive impairment, human platelets, molecular biomarkers, protein self aggregation, tau proteins

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent cause of dementia and an important public health problem. Major clinical features are a progressive decline of cognitive functions, memory, language, and visuospatial orientation. Neuropathological features of AD include a gradual and widespread neuronal loss, senile plaques presence, and neurofibrillary tangles. Senile plaques are mainly made of amyloid-β (Aβ), while the major constituent of neurofibrillary tangles is the microtubule-associated protein tau, which is extensively hyperphosphorylated in AD brains [1].

At present, the diagnosis of AD is based on clinical, neuroimaging, and neuropsychological examination. The necessity for biological markers of AD has been raised. These instruments could allow an early and correct diagnosis of AD to clinicians and also can be used for monitoring drug response in clinical trials [2]. New AD criteria [3] propose the use of biomarkers for the diagnosis of AD, such as volumetric and functional neuroimaging tools and studies of biochemical markers in bodily fluids, including measuring key proteins of the neuropathology (Aβ, tau, and hyperphosphorylated tau isoforms) in cerebrospinal fluid (CSF) [4, 5].

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Nevertheless the diagnosis based on examination of the CSF remains unsatisfactory, mainly because it requires the use of invasive techniques such as lumbar puncture [6].

As a way to face those problems, new and non-invasive biomarkers available in blood, saliva, and urine are currently under investigation [7]. Platelets have been postulated as a good peripheral model of the processes developing in central nervous system in AD. They are the major peripheral reserve of the amyloid-β precursor protein (AβPP), and the analysis of different fractions of AβPP in platelets has been suggested as a marker in the diagnosis and follow up of patients with AD [8–12]. So far no reports have been published on the presence and molecular nature of tau protein in platelets. Considering the role tau protein plays in the physiopathology of AD, an integrated set of experiments was carried out to demonstrate the presence of tau in platelets and its potential role as a novel biomarker.

MATERIALS AND METHODS

Antibodies

Monoclonal antibody Tau-5 (generous gift from Dr. Lester Binder, Northwestern University, Chicago, IL) that reacts with residues 210–230 in the proline-rich domain of human tau protein was used to identify tau variants. For detecting phosphorylated tau, PHF-1 antibody, which recognizes phosphorylated residues Ser396 and Ser404, was used (generous gift from Dr. Peter Davies, Albert Einstein College of Medicine, NY, USA). Alexa fluor 555 Goat anti-mouse conjugated (Sigma) was used as secondary antibody for immunofluorescence.

Subjects

The sample was divided in two groups (see Table 1): patients with probable AD (15) and control subjects (10). Patients were recruited from the Cognitive Neurology & Dementia Unit (Unidad de Neurología Cognitiva y Demencias – UNCD) of the Neurology Service, Hospital del Salvador in Santiago, Chile. Diagnosis of probable AD was established by a neurologist (AS) based on NINCDS/ADRDA criteria [13]. All patient underwent regular neurological, neuropsychological, and neuroimaging evaluation (brain scan and/or nuclear magnetic resonance imaging) in order to support the diagnosis. Controls were recruited at community centers in the metropolitan area of Santiago and among relatives of AD participants in the study. All controls underwent a comprehensive medical and neurological examination to ascertain that they were free of any other significant medical, neurological, or psychiatric illness and a mental examination in order to exclude subjects with cognitive impairment.

Table 1
AD and control subjects features. Values are presented as percentage or means (95% confidence interval in parenthesis). In the left column, *p* values of the differences between AD and control subjects are presented. Student's *t* test and Fisher's exact test were used for comparison between means and frequencies respectively

	AD	Control	*p*-value
Male–female (%)	46.7–53.3	30.0–70.0	0.678
Age	80.9 (65–105)	68.2 (52–79)	0.005
GDS	4.9 (3–6)	1.2 (1–2)	0.000
MMSE	15 (2–28)	28.4 (28–30)	0.001
Education (years)	7.4 (2–17)	9.6 (4–13)	0.293

All subjects resided in the metropolitan area of Santiago (around 41% of Amerindian and 59% Caucasian ancestors). Mental examination was performed with the Chilean's version of the Minimental test of Folstein (MMSE) [14] for both control and AD subjects. Staging of dementia severity was established with the Global Deteriotion Scale (GDS) [15].

The entire study was approved by the Committee on Ethical Issues of Hospital Salvador (Santiago, Chile) and the Medical Ethics Committee of the ICC, and all subjects provided informed consent prior to the initiation of the study. In the case of demented subjects, the informed consent was obtained from caregivers and/or legal guardians.

Blood samples

Five ml of peripheral venous blood samples were obtained by antecubital blood extraction with a vacutainer system in a Plus Blood Collection tube K2 EDTA (BD Vacutainer K2E, 10.8 mg), the sample was maintained and transported at room temperature until processing the same day (2 h as a maximum time before processing). Hemolyzed samples were excluded from this study.

Platelets purification

Platelets were isolated by differential centrifugation [16]. Centrifugation steps were executed at room temperature in a centrifuge Rotina 35 R Hettich Zentrifugen. Platelet-rich plasma (PRP) was obtained by a centrifugation step at $200 \times g$ for 10 min. PRP was

subjected to the second centrifugation step at 200×*g* for 10 min to remove contamination with red and white blood cells. A third centrifugation step was carried out at 1600×*g* for 10 min and a platelet pellet was obtained. Platelet-poor plasma was removed and the platelets were carefully resuspended in NH_4Cl 0.83% at room temperature for 5 min in order to lyse remainding red cells and then centrifugated at 1500×*g* for 10 min. The supernatant was discarded and the platelet pellet was washed in phosphate buffered saline (PBS; 1.4 mM NaCl, 0.02 mM KCl, 0.1 mM Na_2HPO_4, 0.017 mM KH_2PO_4)–1 mM EDTA and pelleted by a centrifugation step at 1500×*g* for 10 min. This last washing step was repeated once. The resulting pellet of platelets was maintained in ice to avoid proteolysis until continuation of the corresponding protocol.

Sample preparation for platelets proteomic

Platelets proteins were extracted by adding 150 μl cold RIPA lysis buffer (5.0 mM Tris–HCl pH 7.5, 1.5 mM NaCl, 10% NP-40: 10% deoxycholate: 20 mM EDTA pH 8.0, 500 mM NaF, 1% SDS) and 2 μl of a protease inhibitor cocktail (Sigma). After a centrifugation step at 1500×*g* for 10 min at 4°C the supernatant protein concentration was determined by Bradford method (BioRad) according to the manufacturer instructions. Proteins were stored at –80°C until proteomic study.

Proteomic study

Platelet proteins were separated by one-dimensional (1D) polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) in minigels [17] in a Mini-Protean III System (BioRad). A total of 50 μg of all platelets protein were loaded per well in 10% acrylamide gels. Proteins were transferred in a mini tank transfer system (BioRad) to a nitrocellulose membrane at 80 mV for 90 min. The membrane was blocked with 5% non fat dry milk for 1 h at 25°C. Immobilized proteins were probed with specific primary antibodies: Tau-5 (2 μg/ml) or PHF-1 (1 : 100) at 4°C overnight and anti-human β-actin 1 : 4000 (Sigma) for 1 h at 25°C. The membrane was then incubated with the secondary antibody HRP conjugated 1 : 5000 (Santa Cruz biotechnology) for 1 h at 25°C. Immunoreactive bands were detected using Super Signal West Pico chemioluminescent substrate (Pierce, Rockford, IL) in Fuji X-ray films. Films were scanned and band intensities quantified using the ImageJ 1.40 software (National Institutes of Health, USA).

Platelet immunofluorescence

Platelets were suspended and fixed in 50 μl 2% formaldehyde for 10 min at 25°C, then pelleted at 1200×*g* for 5 min at 25°C, washed twice with PBS and pelleted again at 1200×*g* for 5 min at 25°C. Then were resuspended in 50 μl of 0.1% Triton X-100 for 10 min at 25°C followed by incubation in 50 μl of PBS−1% BSA for 30 min at 25°C, centrifuged at 1200×*g* for 5 min at 25°C, resuspended in 50 μl of PBS−1% BSA with the primary antibody Tau-5 (50 μg/ml) and maintained at 4°C overnight. Platelets were pelleted by a centrifugation step at 1200×*g* for 5 min at 4°C, washed three times with PBS and pelleted again by a centrifugation at 1200×*g* for 5 min at 4°C, then resuspended in 50 μl of PBS−1% BSA with the secondary antibody conjugated with Alexa 555 1 : 300 (Sigma) and maintained at 4°C for 60 min. Platelets were washed, resuspended in 25 μl of PBS. Platelets were mounted on a slide and visualized in a fluorescence microscope (Axiovert 200 M Zeiss).

Flow cytometry analysis

Fixed platelets were suspended in 200 μl of PBS and counted in a Hemocytometer Counting Chamber (Neubauer). One thousand platelets/ml were analyzed in FACScan using a FACSDiva 6.1.1 software (Becton Dickinson, San José, CA, USA).

Statistics

Statistical analyses were conducted with SPSS 13.0 for WINDOWS. Means were compared with independent samples *t*-test. Frequencies were compared with Fisher's exact test. The critical *p*-value for statistical significance was set at $p = 0.05$.

RESULTS

The main clinical and demographic features of AD patients and controls are presented in Table 1. Gender and educational level were similar in both groups, but some minor differences in ages were found. Platelets sample purity was assessed by flow cytometry for ensuring the efficiency of purification procedure by differential centrifugation. Platelets correspond to 89.4% of the sample (Fig. 1). The presence and patterns of tau signal in purified platelets (total and phosphorylated forms of tau) was determined by immunofluorescence with Tau-5 and PHF-1 antibodies and also by immunoblot. Immunofluorescence experiments

demonstrated the presence of tau protein in platelets, but no differences in the pattern or signal intensity between control and AD subjects were found (Fig. 2, panels B and E). Platelets actin cytoskeleton was visualized with Alexa-conjugated phalloidin (Fig. 2 A and D). The same was found with the PHF-1 antibody (not shown). Immunoblots of total platelets proteins with tau specific antibodies showed the presence of several immunoreactive fractions with molecular weights ranging from approximately 60 kDa (the expected molecular weight for tau) to about 240 kDa (Fig. 3). It is remarkable that high molecular weight fractions of tau (HMWtau) correspond to the most significant fraction of tau immunoreactivity in AD subjects as compared with the controls. In fact, some protein bands at high molecular weight are appreciable only in AD samples.

When the ratio of the different fractions of platelets tau was analyzed, a significant difference in the ratio of HMWtau (tau bands of molecular weight over 80 kDa) to low molecular weight fractions LMWtau < 80 kDa was found in AD subjects when compared to paired age controls. For AD samples the mean ratio HMWtau/(LMWtau) was 3.893 (SD 2.506), versus a ratio of

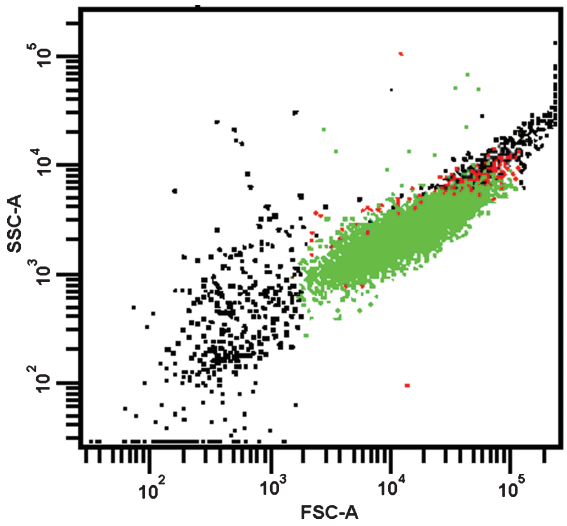

Fig. 1. Platelets purity assessed by flow cytometry. Light-scatter profile of platelets isolated from whole blood and fixed. Data are collected and displayed using logarithmic-orthogonal and logarithmic-forward light scattering. Single platelet events are identified by their characteristic light-scatter properties. In green, singles platelets count (84.1%); in red all platelets – singles plus aggregated (89.8%).

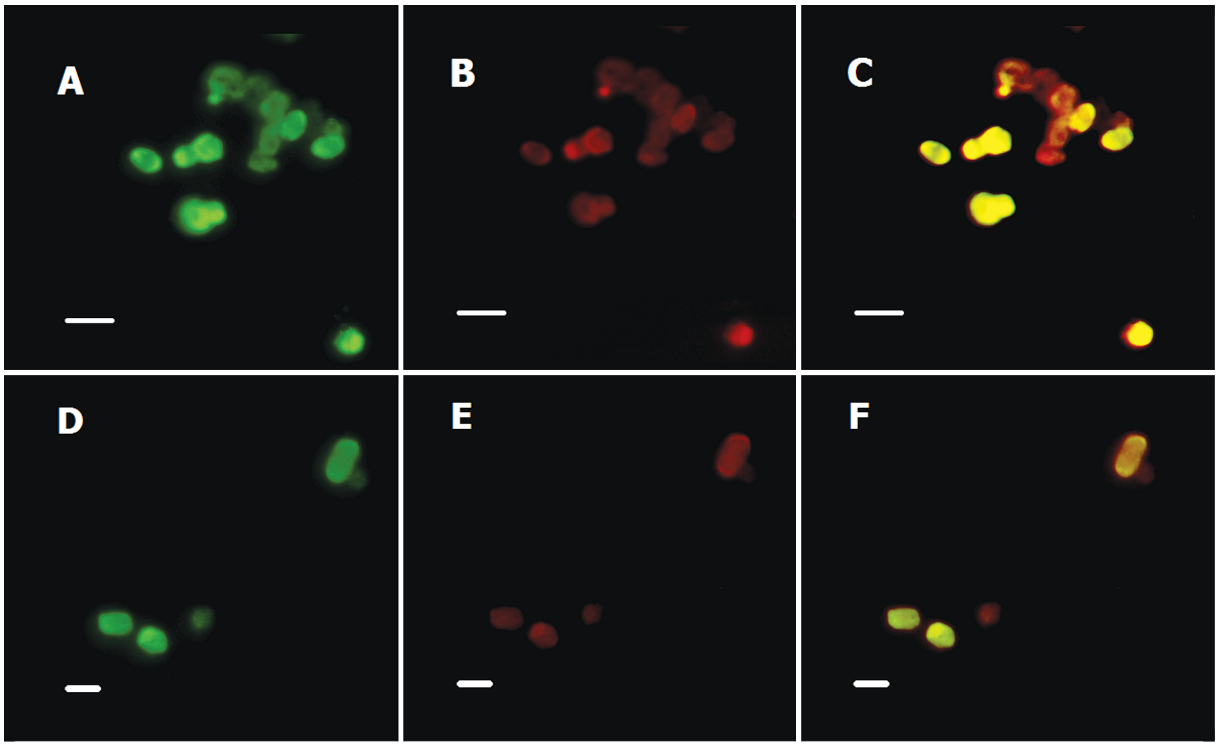

Fig. 2. Fluorescence microscopy of platelets isolated from control subjects (A–C) and AD patients (D–F). A and D: F-actin (Alexa conjugated phalloidin); B and E: tau immunofluorescence (Tau-5 antibody); C and F: merge. Bar represents 5 μm

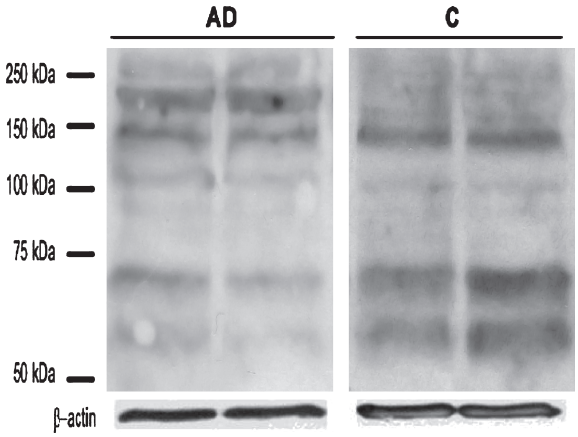

Fig. 3. Immunoblot of platelet tau forms with Tau-5 antibody. HMW tau bands can be seen at weights over the 80 kDa in AD subjects (AD) and control subjects (C), but a more important fraction of tau migrates at high molecular weight in AD samples when compared to control subjects. β-actin as load control at the bottom.

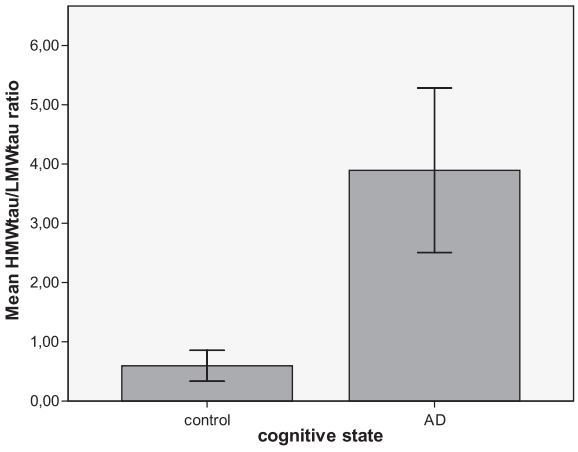

Fig. 4. Differences between tau species ratio in AD patients as compared with control subjects. Ratio is represented in arbitrary units of densitometric quantization of tau species (HMWtau/LMWtau) detected with the Tau-5 antibody in control and AD subjects. Bars: 95% confidence interval ($p = 0.000$).

0.594 (SD 0.364) for neurologically normal controls (t test $p = 0.0001$), as seen in Fig. 4.

DISCUSSION

Definitive diagnosis of AD requires demonstration of specific, microscopic lesions in the brain by histopathological postmortem analyses. At present the diagnosis of AD is mainly based on clinical features of the disease and could be established only in patients with mild to severe dementia. Therefore, it was evident the need of non-invasive biomarkers that not only help to improve accuracy of diagnosis, but also allow an early detection of AD so disease modifying therapies can be used. An ideal marker should identify the molecular abnormalities associated with both the neuropathological lesions that characterize the disease and its correlation with cognitive impairment [18]. In this context, measurements of tau and mainly hyperphosphorylated tau are promising biomarkers in patients with AD but also in subjects with mild cognitive impairment, considered in many cases to be an early or preclinical stage of the disease [4, 5].

However a limitation for the already established CSF markers is the requirement of a relatively invasive methodology, namely lumbar puncture [6]. Thus, blood, saliva, or urine samples are considered to be more accessible and less invasive, thus minimizing the practical challenges of sampling a reliable marker for the condition.

Tau, HMWtau, and tau aggregates

The microtubule-associated protein tau is the major component of the microtubule-associated proteins in axons and plays critical physiological roles in stabilizing microtubules and inducing its own assembly [19]. Under pathological conditions, tau self-aggregates into paired helical filaments, which turn into the neurofibrillary tangles during AD, one of the neuropathological hallmarks of AD and tauopathies [1].

Our results showed that tau is also present in platelets from peripheral blood. Notably, several molecular species of tau with molecular weights ranging from around 80 kDa to 240 kDa can be detected in immunoblot studies and the proportion of the different forms of tau differs in control and AD patients. The difference observed in AD patients and controls could not only be explained by difference in age. Indeed, we have some preliminary data showing that in cognitively normal subjects platelets tau ratio does not increase with age (Farías et al., unpublished data).

The nature of the high molecular weight species of tau remains elusive. A higher molecular weight human tau variant has been described mainly in the peripheral nervous system and in cell lines derived from the neural crest [20], which has been named to as "heavy tau", because its molecular weight of around 110–130 kDa as compared with the 66–68 kDa of normal adult human tau or light tau [21–25]. Both the heavy and the light human tau variants are differentially expressed from a single gene by an alternative splicing mechanism. Anyway, none of tau isoforms have been detected so far in platelets. Therefore, ours

is a novel finding of tau variants in platelets. Alternatively, high molecular weight forms of tau may correspond to aggregates of the nearly 60 kDa forms of tau, and this seems to be the case in this study on the basis of electrophoretic experiments under different conditions. These types of multimers of tau that can be visualized in immunoblot analyzes, even in reducing and denaturing conditions, have been described previously in brains of the mouse model of tauopathy and also in brains of patients with AD and frontotemporal dementia and parkinsonism linked to chromosome 17 [26]. The accumulation of such multimers could correlate to the pathophysiology of the disease in both CNS and in the peripheral blood cells [27]. As a matter of fact, is noteworthy that, in AD samples, as HMWtau increases LMWtau tends to decrease, a finding that is compatible with tau monomers consumption as higher order oligomers are being formed. Structural aspects, as well as the precise mechanisms of oligomerization, and correlations of these platelets tau aggregates with the levels of cognitive impairment will be a matter of future investigation avenues.

Therefore, our preliminary studies support the hypothesis that HMWtau fractions correspond to oligomeric aggregates of this protein. So we postulate that the relative amount of those HMWtau fractions may serve as a novel biochemical marker of AD. A more complete analysis and biochemical characterization of tau fractions and their relative amount in larger populations is under way.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=749).

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