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Presenilin 2 Mutation Does Not Influence Expression and Concentration of APP Forms in Human Platelets

Lucia Pastorino,[1] Francesca Colciaghi,[1] Gabriella Marcon,[2] Barbara Borroni,[3] Elisabetta Cottini,[3] Flaminio Cattabeni,[1] Alessandro Padovani[3] and Monica Di Luca[1]

[1]Institute of Pharmacological Sciences, University of Milano, Milano, Italy
[2]Department of Neurology, University of Udine, Udine, Italy
[3]Department of Medical Science, University of Brescia, Brescia, Italy

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Abstract

Background: The pattern of platelet amyloid precursor protein (APP) forms is altered in sporadic Alzheimer's disease patients, compared with both control subjects and non-Alzheimer's disease-demented patients. The aims of this study were to evaluate in platelets of symptomatic and presymptomatic patients carrying the mutation Met239Val in presenilin 2 (PS2) whether: i) PS2 and presenilin 3 (PS1) were expressed in platelets; ii) an altered expression of different APP isoforms mRNAs could be related to the presence of the mutation; and iii) an abnormal pattern of APP forms was associated to the mutation.

Materials and Methods: Reverse transcription-polymerase chain reaction (RT-PCR) of APP isoforms, PS1 and PS2 was performed on RNA extracted from platelets of three PS2 Met239Val mutated subjects, seven sporadic Alzheimer's patients and nine control subjects. The pattern of platelet APP forms at protein level was evaluated in the same population of subjects by means of Western blots analysis with specific antibody.

Results: We found that PS1 and PS2 were expressed correctly in human platelets. When the relative amount of expression of mRNA coding for APP 771/751–695 was measured, a similar ratio of expression was found in PS2-mutated subjects, compared with both sporadic Alzheimer's patients and to control subjects. Furthermore, when APP forms were evaluated in platelet homogenates by means of Western blots analysis with appropriate antibody, no difference was found in the pattern of APP forms in presence of PS2 mutation in platelets, compared with control subjects.

Conclusions: These results indicated that PS2 was expressed in human platelets and that PS2 mutation did not affect APP forms pattern, thus, suggesting that in this peripheral cell the pathological effect of PS2 mutation might occur upstream of the amyloid cascade.

Introduction

Accumulation of the amyloid-β peptide (Aβ) in the brain parenchyma and vasculature is an invariant event in the pathogenesis of both sporadic and familial Alzheimer's disease (AD). Aβ originates by proteolytic processing from a larger precursor protein (the amyloid precursor protein; APP), which is an integral transmembrane cell-surface protein present as numerous alternatively spliced isoforms derived from a single gene localized on chromosome 21 (1,2). APP can be processed by the action of different secretases: α, β and γ. For α-secretase, two different candidates have been proposed: ADAM 10 (*a d*isintegrin *a*nd *m*etalloprotease 10) and TNF-α converting enzyme (TACE) (3,4), members of disintegrin/metalloproteases family (3,4). On

Address correspondence and reprint requests to:
M. Di Luca, Inst. of Pharmacological Sciences, University of Milano, via Balzaretti, 9, 20133 Milano — Italy.
Phone: 0039-02-20488374; Fax: 0039-02-29404961;
E-mail: Monica.Diluca@unimi.it

the other hand, β-secretase is a transmembrane aspartic peptidase recently cloned (5–8). The α-secretase activity prevents the formation of Aβ; whereas, β-secretase cleavage, occurring most likely in the intracellular organelles (9,10), is mandatory for the formation of Aβ, in conjunction with γ-secretase.

Since APP isoforms are expressed in neuronal, as well as in several non-neuronal, tissues and cell lines, there has been an increasing interest aimed to evaluate whether abnormalities in neuronal APP processing may be reflected in a peripheral cell. Along this line, three major APP isoforms (MW ranging from 130 to 106 kDa) were found in the membranes of resting platelets, and both platelets and megakaryocytes express three APP transcripts encoding for APP 695, APP 751, and APP 770 (11,12). In addition, it has been reported that, upon stimulation, platelets are capable of releasing a protein functionally identical to the platelet coagulation factor XIa inhibitor, which represents a truncated form of APP containing the Kunitz domain (13). More recently, Li and colleagues (14) showed that platelets could release, when activated by thrombin, both the α-secretase fragment, sAPP, and Aβ, suggesting that these cells possess the whole repertoire of enzymes necessary for APP processing. Finally, there is evidence that modifications in the concentration/processing of APP in platelets are present in advanced stages of AD (15–19). In particular, an alteration of APP forms in the platelets of sporadic AD patients was reported recently (20), being the ratio between the 130 kDa APP form and the lower (106–110 kDa) forms specifically decreased in AD patients group, compared with values of both control subjects and patients affected by other neurodegenerative disorders associated with dementia. Furthermore, the alteration of platelet APP forms ratio was different among subsets of AD patients grouped according to clinical dementia rating (CDR) scores. Moreover, there was a positive correlation with individual mini-mental state examination (MMSE) scores, suggesting that this value varied according to the severity of clinical symptoms (19–21). Notwithstanding, the pattern of platelet APP forms in familial AD has been unexplored so far.

A common cause of familial AD is mutation of the genes encoding for presenilin 1 and 2 (22–26). In the present study, we analyzed platelet APP forms in subjects characterized by the presenilin 2 familial Alzheimer's disease (FAD) mutation Met239Val PS2 (Flo10) (27). PS2 gene, located at chromosome 1, encodes for a potential seven-transmembrane domain protein largely localized in the endoplasmic reticulum. At present, two different missense mutations in the PS2 gene have been linked to FAD. These two mutations most likely may cause AD by altering the metabolism of APP and/or Aβ or by binding to new proteins like calsenilin (28) and β catenin (29,30), thereby, accelerating the process of amyloidogenesis and/or cell death.

Aims of the present work were to investigate the platelets of patients carrying the mutation PS2 Met239Val (Flo10) for whether: i) PS2 and PS1 were expressed in platelets; ii) the presence of PS2 mutation could be related to an altered expression of different APP isoforms mRNAs; and iii) PS2 mutation carriers showed an alteration of the pattern of APP forms. Answering these questions allowed us to further investigate the role of PS2 mutation in APP processing in a peripheral cell.

Materials and Methods

Selection of Subjects

The study was undertaken on seven sporadic AD patients [age, mean $\pm$ standard error of the mean (SEM) in years: 65 $\pm$ 7], on nine age-matched control subjects (age, mean $\pm$ SEM in years: 68 $\pm$ 5) and on three subjects carrying the PS2 Met239Val mutation. Among the three mutated subjects, two were symptomatic and one pre-symptomatic; their age ranged between 35 and 80 years. These subjects belonged to a large family of Italian origin, in which the majority of its members live in Friuli Venezia Giulia, with small branches of kindred in France and the U.K. In the affected members, neurological examination and medical records documented the clinical evidence. Genetic analysis of PS2 and Apolipoprotein E (ApoE) were carried out on family members that were affected and non-affected.

The diagnosis of probable Alzheimer's disease was made according to National Institute of Neurological Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCS-ADRDA) criteria (31). Exclusion criteria for all groups were the following: head trauma; metabolic dysfunction; hematological diseases; alcohol abuse; drug abuse; delirium; mood disorders; actual treat-

ment with acetylcholinesterase inhibitors or with medications affecting platelet functions, such as anticoagulants, antiplatelet drugs, serotoninergic agonists-antagonists, and corticosteroids. All subjects included had a standardized clinical workup based on neurological examinations, laboratory blood and urine analysis, neuropsychological assessment, and neuroimaging study (Head CT and/or MRI). Before enrollment in the study, subjects or their legal caregiver completed an informed consent, after the nature and possible consequence of the study was explained. Personnel who were blind for diagnosis carried out platelet preparation and subsequent analysis.

Platelet Preparation and Protein Detection

Platelets were obtained from 30 ml of fresh blood by centrifugation as previously described (18,20). Briefly, 27 ml of blood were collected into one volume of 3.8% sodium citrate (in presence of 136 mM glucose), mixed gently, and centrifuged at 200X g for 10 min at room temperature. Platelet-rich plasma was separated from the blood pellet using a plastic pipette, avoiding the aspiration of the buffy coat. Platelets were then collected by further centrifugation. The platelet pellet was washed twice, resuspended at a concentration of 5 μg/μl in ice-cold Tris-HCl 10 mM, pH 7.4, containing Ethylen-glycol-bis (betaaminoethylethen)-N,N,N^1,N^1-tetraacetic acid (EGTA) 1 mM, phenyl-methyl-sulfonyl fluoride 0.1 mM, and a complete set of protease inhibitors (Complete™, Boehringer-Mannheim Corp., Indianapolis, IN).

Platelet proteins were separated in 6% SDS-PAGE and electroblotted to nitrocellulose membrane. Immunostaining reactions were performed with 22C11 (Boehringer-Mannheim; dilution 1:250), to recognize all APP forms, and with monoclonal antibody anti-presenilin 1 (Chemicon, Temecula, CA, USA; dilution 1:1000), to recognize human presenilin 1. Peroxidase conjugated antibodies (Kierkegard, Gaithersburg, Maryland, USA; dilution 1:10000) were utilized to perform enhanced-chemiluminescence reactions (Reinessance™, NEN Dupont, Boston, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted from platelets by acid-phenol method using an RNA extraction kit (Bio/RNA-X Cell™, Bio/Gene, Greensbury Farm, Bolnhurst, U.K.). 200 ng of total RNA were used to perform the first strand cDNA synthesis using oligo (dt) and Murine Moloney Leukemia virus (Mu-MLV/RT, Perkin-Elmer, Branchburg, New Jersey, USA; 40U per sample). Following RT reaction, PCR amplification for different mRNAs was performed by using specific primers as follows: APP+ 958, APP-1213, according to Golde et al. (32); and PS1/PS2 degenerated primers, according to Lee et al. (33), to amplify a cDNA fragment encoding amino acids 83–278 of human PS1 and the homologous region of human PS2. PS1 and PS2 were further amplified by means of nested-PCR using for PS1: sense ATA CCC CAT TCA CAG AAG AT, antisense: ATG AAC TAT TTG TCC GG, and for PS2 sense: GTA GCC ACC ATC AAG TCT GT, and antisense GCG GAC CAC TCT GGG AGG TA. All PCR reactions were carried out by using Taq polymerase 2.5 U per sample (Perkin-Elmer) in a programmable heating block (Perkin-Elmer, Gene Amp PCR System 2400) using the following scheme cycles: denaturation 94°C, 30 sec; annealing 55°C, 30 sec; extension 72°C, 1 min, for number of cycles specified in the text. PCR products were electrophoresed in 3% agarose gel; gels were stained with ethidium bromide (EtBr) and photographed under ultraviolet (UV) light.

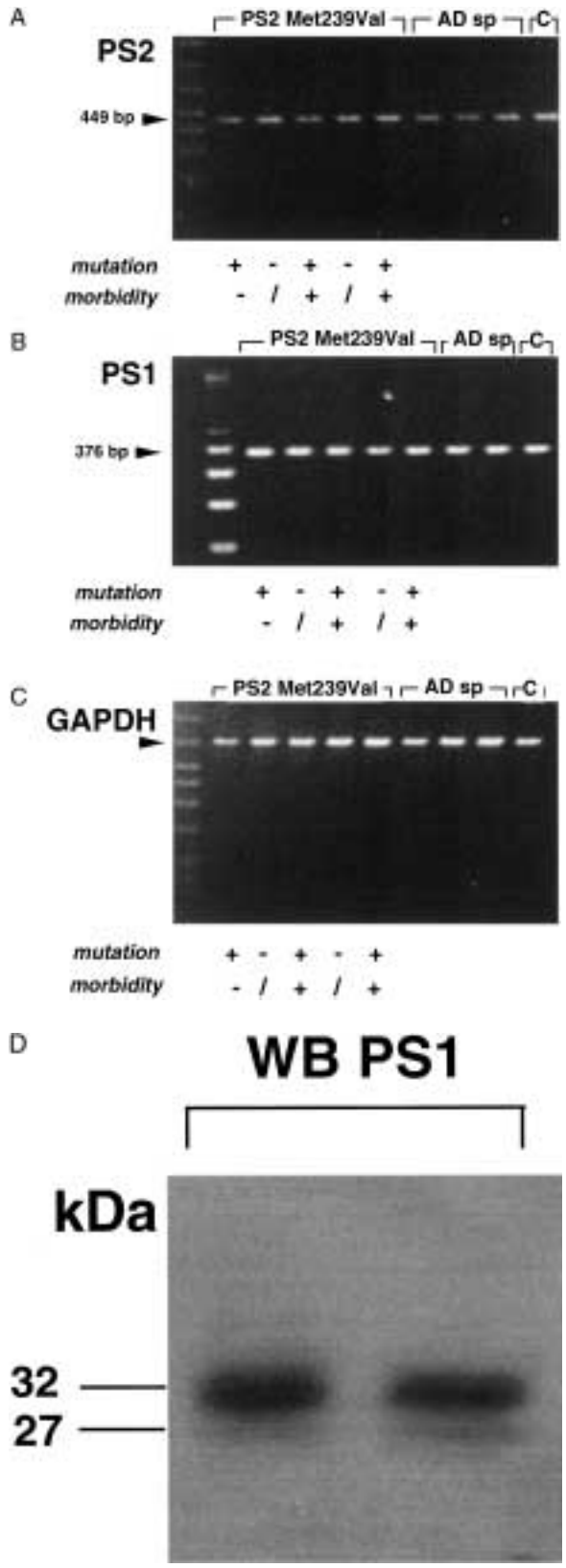
Data Analysis

Quantitative analysis of Western blots and EtBr-stained cDNAs were performed by means of computer-assisted imaging (Image, developed by Dr. Rasband, National Institutes of Health, Bethesda, MD). Statistical evaluations were performed according to one-way analysis of variance followed by Scheffé as post hoc comparison test.

Results

PS1/PS2 Are Expressed in Human Platelets

We first addressed the question concerning whether PS1 and PS2 were expressed in human platelets. Previous studies demonstrated that both PS1 and PS2 were expressed in a variety of human tissues; however, the expression of PS in platelets still needs further study.



To assess the presence of mRNA coding for PS1 and PS2 in platelets, RT-PCR analysis was performed in platelets obtained from patients carrying the PS2 mutation Met239Val (n = 3), sporadic AD patients (n = 7), and control subjects (n = 7 matched for age, sex, and ethnicity with sporadic AD patients and two subjects of the Flo10 family not carrying the mutation). The expression of other proteins relevant for the pathogenesis of AD, i.e. APP isoforms, was analyzed as well, and the expression of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

To evaluate the expression of PS1/PS2, the cDNA obtained after RT of platelet mRNA obtained from individual subjects was PCR-amplified with degenerate primers pairs that hybridized to highly conserved sequences within PS1 and PS2 cDNA, as previously reported (33). Subsequently, DNA sequences specific for the two proteins were further amplified with specific primers in a more restricted region.

As a result of this procedure, we obtained a PCR product of 449 bp for PS2 and 376 bp for

Fig. 1. Presenilin 2 (PS2) and presenilin 1 (PS1) expression in human platelets from Met239Val-mutated subject, sporadic Alzheimer's disease (AD) patients and from control subjects. Representative ethidium bromide (EtBr)-stained gels from reverse transcription-polymerase chain reaction (RT-PCR) analysis of (B) PS1 and (A) PS2 expression performed on mRNA extracted from platelet lysates using oligo (dt) for reverse transcription and degenerated primers, encoding the region between aa 83- and 278 of PS1 and the homologous region for PS2, for the first PCR amplification. The resulting PCR products were further amplified in a restricted region to separately amplify PS1 and PS2. DNA fragments of 449 bp and 376 bp were obtained for PS2 and PS1, respectively. The expression of PS1 and PS2 was normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression used as internal control for each sample (see Fig. 1C). The expression of either PS1 or PS2 was similar in all experimental groups. The presence of the Met239Val mutation is reported for each family member. (D) representative Western blots analysis performed with monoclonal antibody anti-presenilin1 on platelet homogenate obtained from that represented in Fig. 2C. ADsp = sporadic AD patients; C = control subjects.

PS1 as shown in Figures 1A (PS2) and 1B (PS1). When such a sequence of PCR reactions was performed on individual mRNA extracted from platelets of nine controls, seven sporadic, and three familial AD patients, no difference in the relative expression of PS1 and PS2 was found. Figure 1D shows a representative Western blots analysis performed on platelet homogenate of two control subjects with monoclonal antibody anti-PS1.

Expression of APP 770/751/695 in Platelets of Mutated and Non-mutated Subjects

To evaluate possible differences in the relative expression of platelet APP isoforms in the presence or absence of a PS2 mutation, the expression of the three major isoforms of APP 770/751/695 was tested in the same samples.

To estimate the relative levels of alternatively spliced APP transcripts, we used a semi-quantitative RT-PCR strategy. The ratio of APP 770/751 mRNA in individual subject platelets was determined by direct comparison of the level of cDNA generated at a given cycle within the linear range of amplification. Figure 2 shows a cycle-dependent RT-PCR amplification of 50 ng of platelet mRNA using primers flanking the alternative splice site and, therefore, amplifying the three APP isoforms, 770/751/695, in the same sample. A linear range of amplification was obtained between 24 and 32 cycles. When such experimental conditions were applied (Fig. 3A), no major differences were found for the expression of platelet APP isoforms in PS2-mutated subjects, compared with either sporadic AD patients or control subjects. Furthermore, when the ratio between the levels of APP 770 cDNA and APP 751 cDNA was calculated, no differences were found among the three experimental groups (Fig. 3B, cumulative data expressed as mean ± SEM of nine control subjects, seven sporadic AD patients, and three PS2 Met239Val subjects). In all experimental samples, data were normalized for the expression of the housekeeping gene GAPDH.

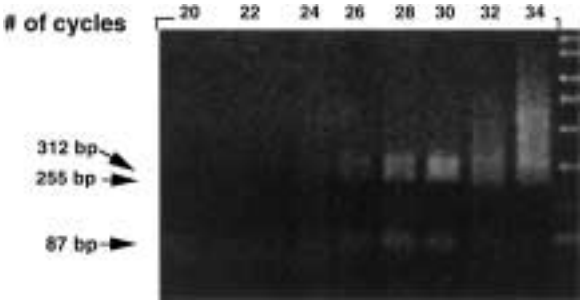


Fig. 2. Polymerase chain reaction (PCR) amplification of amyloid precursor protein (APP) 770/751/695 from control human platelets. Ethidium bromide (EtBr)-stained gel of PCR products after 22, 24, 26, 28, 30, 32, and 34 cycles of amplification. Markers of DNA fragments are in the far-right lane. APP 770, 751, and 695 were amplified as 321, 218, and 86 bp fragments, respectively.

Concentration of APP Forms in Platelets

We then tested the concentration of APP forms at the protein level in platelets of PS2-mutated subjects. An altered ratio between the 130 kDa APP form and the 106–110 kDa was previously reported in sporadic AD patients, compared with control subjects (18). Figure 4 shows a representative Western blots analysis performed with 22C11, a monoclonal antibody raised against a N-terminal domain of APP common to all APP forms. Three immunoreactive bands in the range of 130–110/106 kDa were found in platelet homogenates prepared from sporadic AD, control subjects, and PS2 missense-mutated patients, as shown in Figure 4. The density ratio between the upper form (130 kDa) and the lower forms (106–110 kDa) was measured by means of computer-assisted imaging (see "Materials and Methods" session), and the value normalized for the immunostaining of a platelet constitutive protein, i.e. actin (data not shown). The value of the ratio between 130 kDa and 110–106 kDa forms in PS2-mutated subjects was similar to the ratio measured in control subjects (PS2 mutated: mean ± SEM: 0.973 ± 0.2; controls: 0.87 ± 0.15; mutated vs. controls: $p = 0.78$). On the other hand, 130 kDa/110–106 kDa ratio measured in sporadic AD patients was significantly different, compared with both control and familial AD subjects (sporadic AD: 0.35 ± 0.15; $p > 0.001$ vs. controls and vs. PS2-mutated). The values reported in this study for control subjects and sporadic AD patients were consistent with previous data (18).

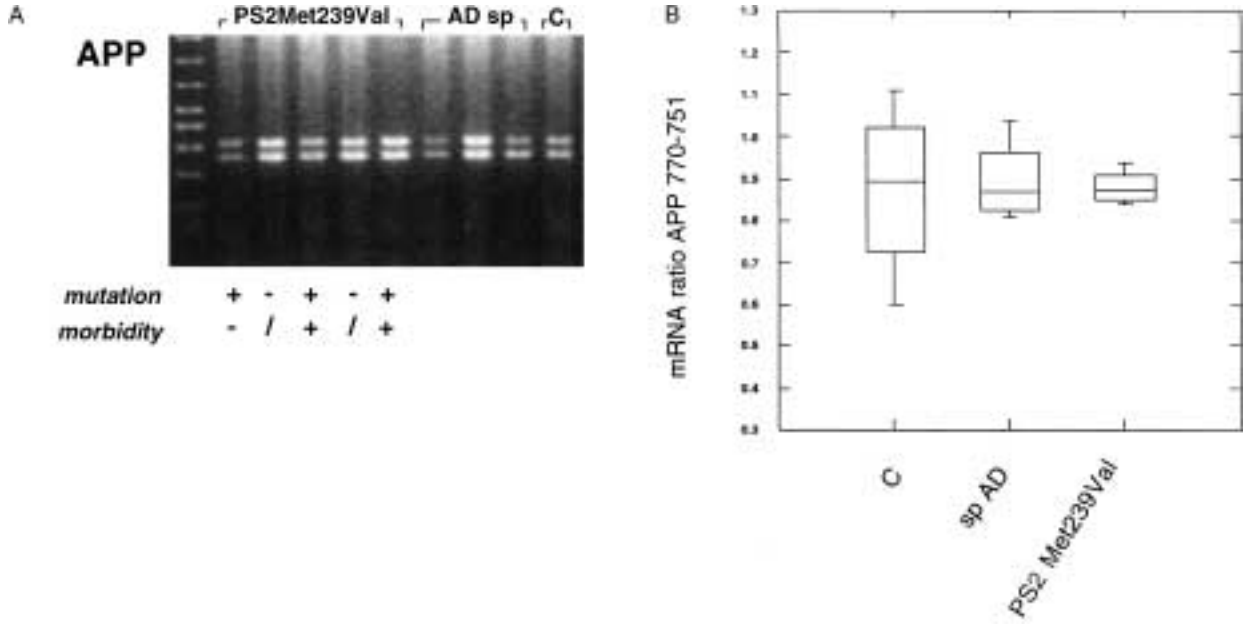


Fig. 3. Expression of APP isoforms in platelets of sporadic AD patients, Met239Val mutated subjects and controls. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of amyloid precursor protein (APP) 770/751/695 in platelets obtained from presenilin 2 (PS2)-mutated subjects (PS2 Met239Val), sporadic Alzheimer's disease patients (ADsp) and control subjects (C). APP 777/751/695 were amplified in 28 PCR cycles. No difference in the relative expression of APP forms was observed in Met239Val-mutated subjects, compared with both control and sporadic AD patients. The presence or absence of the mutation is indicated in each family member. Markers of DNA fragments are in the left lane. (B) Box plot of the density ratio of APP 770 and APP 751 in platelets of Met239Val-mutated patients, sporadic AD patients (ADsp) and control subjects (C). The optical density of APP 777 and 751 DNA fragments was quantified from EtBr-stained gels after 28 cycles of PCR amplification by means of computer-assisted imaging. Data were normalized for GAPDH expression. No difference in the relative expression of APP770/APP751 was observed between the experimental groups analyzed.

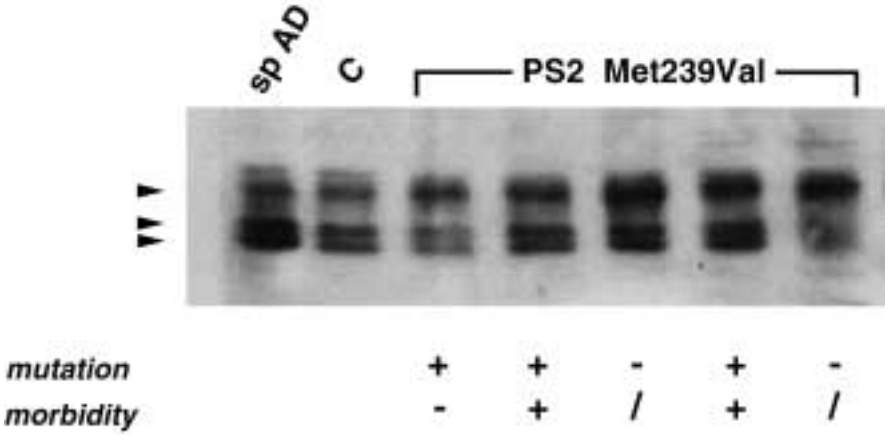


Fig. 4. Representative Western blots analysis of amyloid precursor protein (APP) forms in platelets obtained from Met239Val-mutated subjects, sporadic AD patients (spAD) and control (C). Proteins transferred into nitrocellulose paper were stained with 22C11, a monoclonal antibody raised against a N-terminal region common to all APP forms. A 130 kDa form and a doublet at 106–110 kDa are present (arrowheads). Immu-noreactive bands were measured by means of computer-assisted imaging. No difference in the ratio between 130- and 106–110 kDa forms was observed in presenilin 2 (PS2)-mutated subjects [mean ± standard deviation (SD): 0.973 ± 0.2, Optical Density (OD)], compared with controls (0.87 ± 0.15; mutation carriers vs. controls: $p = 0.78$), but it was significantly different from sporadic AD patients (0.35 ± 0.15; $p > 0.001$ vs. controls and vs. PS2 mutated). The presence of the mutation in the family members is reported.

Discussion

Our study shows that human platelets express presenilins transcripts, with the level of PS2 expression lower relative to PS1 mRNA. These results are in agreement with data previously reported on other tissues and cells (33,34). The expression of presenilins transcripts in platelets is similar in control subjects and in sporadic and familial AD patients (PS2 Met239Val mutation). Furthermore, our results show that in platelets the mutation Met239Val of PS2 gene does not affect either the relative expression of APP isoforms 770/751–695 or the relative concentration of APP forms in platelets. It has been previously demonstrated that the pattern of platelet APP forms is altered specifically in sporadic AD, compared with control subjects and non-Alzheimer's-demented patients matched for demographic characteristics (18–20), although the mechanism related to these abnormalities is still a matter of investigation. However, since we previously observed that the expression of APP isoforms was not altered in AD patients, compared with control subjects, one might speculate that the observed protein alteration was due to a modification of APP processing/metabolism. Our finding might suggest that platelets, acting as a reservoir for APP, may represent an easily accessible source of human material available to study the biochemistry of APP processing. In this respect, investigating whether APP forms can be altered in platelets of familial AD could shed light on the role of the periphery as a "delegate" for APP processing and Aβ deposition. With the present study, carried out on a limited number of patients belonging to a PS2 Met239Val-mutated family, we observed that both symptomatic and presymptomatic individuals did not show any alteration in the distribution of platelet APP forms, compared with control subjects, either at mRNA or at protein level. How does this observation correlate with the presence of PS2 mutation? One possible explanation for this apparent lack of correlation in platelets, could reside on the subcellular organization of these circulating cells that is dramatically different from that of a cell deriving from neuronal lineage. In fact, the subcellular localization of presenilins in platelet organelles is, at present, unknown and it might represent a key limiting factor for the biological function of this protein. Furthermore, to date, the physiopathological role played by both wild type and missense-mutated presenilins remains to be established, although several results have provoked hypotheses about the protein functions in both physiological and pathological states.

In particular, many authors, by analyzing different tissues and cell lines, claim that the pathogenic mechanism of AD through presenilins mutations might involve the amyloid cascade. This was suggested by means of different approaches, including in vitro studies and genetically modified animals (35). This hypothesis was further supported by the observation that a generalized effect of PS1 and PS2 mutations is to increase the concentration of Aβ42 in plasma, an effect that, however, was not present in sporadic AD patients (26).

On the other hand, some evidence suggests that the mechanism by which the pathogenic PS mutation causes AD might be different from Aβ42 production, but that it can occur upstream of Aβ formation/deposition. This mechanism might involve perturbed calcium regulation, which in turn, determines an enhanced elevation of intracellular calcium and a higher vulnerability to excitotoxicity (36,37). Furthermore, several lines of evidence suggest that presenilins may be involved in apoptosis. In one report, a truncated form of PS2 was shown to act as a dominant negative inhibitor of T-cell receptor-induced apoptosis in a mouse T-cell hybridoma (38). In another report, overexpression of PS2 was found to increase apoptosis induced by trophic factors withdrawal in PC12 cells and a FAD-associated PS2 mutation could induce apoptosis even in the absence of trophic factor withdrawal (39). Furthermore, PS1 mutations recently were shown to potentiate neuronal apoptosis through destabilization of β-catenin (29,30), and patients carrying a FAD-associated PS1 mutation (PS1Δ9) were characterized by absence of senile plaques (40). These observations, taken together, suggest that presenilin FAD-associated mutations increase the susceptibility of cells to death, with a mechanism that can precede Aβ42 formation. This further suggests that presenilins may act with a mechanism/mechanisms other than increased Aβ production in cells (37).

Our data showing that APP forms concentration in platelets of FAD-patients is not changed, if compared with control subjects, is in line with this hypothesis. Furthermore, we show that the relative amount of APP transcripts is not altered in platelets of FAD patients. Li et al. (41) proposed that, due to their localization at a nuclear level, missense-mutated presenilins

could induce chromosomal misaggregation, causing formation of aneuploid cells carrying trysomy for chromosome 21 that carries the gene coding for APP. Since the relative ratio measured for platelet APP forms could be dependent on the level of mRNAs coding for the two major APP isoforms in platelets, APP770 and APP751, we explored the possibility that the presence of a missense mutation on PS2 could determine alteration of any of mRNAs coding for APP770 and APP751. Since the ratio measured between APP770 mRNA and APP751 mRNA did not show any statistically significant difference, compared with the ratio measured for both control subjects and sporadic AD patients, one might speculate that, at least in this peripheral compartment, the normal pattern of APP forms (130kDa vs. 110–106 kDa bands) in PS2 mutated patients is not due to a compensatory mechanism, such as overexpression of one of the APP isoforms.

In conclusion, this study demonstrates that human platelets express PS2, PS1, and APP forms, and that sporadic AD patients, but not PS2 mutation carriers display an abnormal pattern of APP forms in platelets. Although the normal functions and the pathogenic mechanisms of presenilins remain to be established, the data described above suggest that, in this peripheral cell, the pathological effect of PS2 mutation might occur upstream of the amyloid cascade and does not involve alteration in APP processing.

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