**α-Secretase ADAM10 as Well as αAPPs Is Reduced in Platelets and CSF of Alzheimer Disease Patients**

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**Abstract**

**Background:** Members of membrane-bound disintegrin metalloproteinases (ADAMs) were shown to be capable of cleaving amyloid precursor protein (APP) at the α-cleavage site in different cell systems. One of the candidates α-secretases identified in this family is ADAM10. The present study addresses the following major questions: 1) Are the levels of an α-secretase candidate (i.e., ADAM10) reduced in accessible cells of Alzheimer Disease (AD) patients? 2) Are ADAM10 levels in the peripheral cells of AD patients related to a concomitant decrease in αAPPs?

**Materials and Methods:** Western Blot analysis of ADAM10 is performed on platelet homogenates from 33 sporadic AD patients and on 26 age-matched control subjects. Moreover, the levels of α-secretase metabolite (αAPPs) are tested both in platelets and cerebrospinal fluid (CSF) of the same pool of subjects by means of Western blot with a specific antibody.

**Results:** A significant decrease of platelet ADAM10 levels is observed in patients affected by probable AD when compared to control subjects and this is paralleled by a reduced level of αAPPs released from platelets. Moreover, in the same pool of AD patients, αAPPs levels were reduced concomitantly in CSF.

**Conclusions:** ADAM10 is expressed in platelets. A reduced level of ADAM10 is observed in platelets obtained from AD patients compared to age-matched controls. Further, in the same pool of AD patients, a qualitatively and quantitatively similar decrease in αAPPs is present both in thrombin-activated platelets and CSF, thus suggesting that alterations of APP processing might occur both in the neuronal compartment and peripheral cells.

**Introduction**

The amyloid precursor protein (APP) is a crucial element in the amyloid cascade and in the pathogenesis of Alzheimer Disease (AD). It can be processed by different pathways involving either α, β, or γ cleavage. The major physiologic route involves cleavage by α-secretase along the secretory pathway from the Golgi to the plasma membrane (1–5). The α-secretase cleavage occurs within the amyloid (Aβ) domain of APP and results in the secretion of the large and soluble extracellular domain of APP, the so-called αAPPs. Although numerous cells possess a basal level of α-secretase activity, proteolysis of APP by this enzyme is enhanced by diverse intracellular pathways such as activation of protein kinase C (PKC) (6). Accordingly, activation of membrane receptors coupled to PKC has been shown to increase APP α-secretase cleavage (7). In addition, experimental evidence suggests that αAPPs may exert a neuroprotective effect and may enhance learning and memory processes. Thus, the regulation of APP metabolism through α-secretase pathway appears to be relevant for the pathogenesis of AD.

Members of membrane-bound disintegrin metalloproteinases (ADAMs) were shown to be capable of cleaving APP at the α-cleavage site in different cell systems. The candidate α-secretases identified so far are ADAM 9 (8,9), ADAM10 (10,11), and ADAM17 (also known as TACE) (6,12). Although all these proteases share common structural homologies and are sensitive to peptide hydroxamates (13), there is evidence that ADAM17 does not possess inducible α-secretase activity (6), and that only ADAM10 shows basal and PKC-stimulated α-secretase activity, as well as other properties expected for the proteolytic processing of APP (10).

Because the α-secretase and the amyloidogenic β-secretase pathways were shown to be mutually exclusive (7), evaluating α-secretase activity in patients is crucial to understanding its role in the pathogenesis of AD.

To this end, we focused our attention on the platelet, a peripheral cell that has been shown to be relevant in studying the physiologic and pathologic processing of APP ex vivo (14–22).
In fact, platelets express the three major APP isoforms and their metabolites such as αAPPs and Aβ (23–25).

The present study addresses the following major questions:

1. Is there any detectable α-secretase candidate in platelets?
2. Is there any difference in α-secretase between patients with AD and controls?
3. Is there any relationship between APP metabolites measured in platelets and in cerebrospinal fluid (CSF) of the same AD patient?

These results will help in understanding, in a more comprehensive way, ex vivo, one aspect of the complex events determining AD pathogenesis, eventually providing new means for targeted pharmacologic intervention.

Materials and Methods

Characteristics of the Subjects

This study was undertaken on 33 patients with probable AD and 26 age-matched neurologic control subjects recruited at the Neurological Department of University of Brescia. The diagnosis of probable AD was made according to National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINDS-ADRDA) criteria. Exclusion criteria for all groups were head trauma, metabolic dysfunctions, hematologic diseases, alcohol abuse, delirium, mood disorders, treatment with acetylcholinesterase inhibitors (21), and with medications affecting platelet functions (antiplatelet drugs, anticoagulants, antiplatelet drugs, serotoninergic agonists-antagonists, and corticosteroids). All subjects included had a standardized clinical workup based on neurologic examinations, laboratory blood and urine analysis, neuropsychological assessment, and neuroimaging study (head CT and/or MRI). Before enrolment in the study, subjects or their legal caregivers completed an informed consent form after the nature and possible consequences of the study was explained.

Personnel who were blind for diagnosis carried out platelet preparation and subsequent analysis.

Cerebrospinal Fluid

CSF was obtained from 12 of 26 control subjects and 15 of 33 AD patients by lumbar puncture with the patient sitting in the upright position. The CSF was immediately aliquoted in presence of a complete set of protease inhibitors (Complete Roche, Mannheim, Germany) and frozen at −80°C until assayed. Protein concentration was measured by Bradford method and 4 μg of total proteins were loaded on the gel (running gel: acrylamide 6%) and electroblotted. The same amount of CSF proteins was processed in parallel in each sample for control subjects and AD patients. αAPPs staining and quantitative analysis were performed as reported below (SDS-PAGE and Western blot analysis).

Platelet Preparation

Twenty-seven milliliters of blood were collected into 3 ml 3.8% sodium citrate (in presence of 136 mM glucose), mixed gently, and centrifuged at 200 × g for 10 min. The interval between blood drawing and the first centrifugation was 20–25 min at maximum. Platelet-rich plasma was separated from the blood cells using a plastic pipette, carefully avoiding the aspiration of the buffy coat. Platelets were collected by centrifugation at 1200 × g for 20 min. Platelet pellets were washed twice with Tris-HCl 10 mM pH 7.4 and resuspended in ice-cold lysis buffer containing Tris-HCl 10 mM, pH 7.4; EGTA 1 mM; phenylmethyl-sulfonyl fluoride 0.1 mM; and a complete set of protease inhibitors (Complete, Roche). Homogenates were then subjected to three rounds of freeze thawing and 15 sec of sonication at 0°C.

Platelet Activation

Platelet pellets were washed and resuspended in Tyrod's buffer according to Bush (26) and incubated with thrombin (1 U/ml) in the presence of MgCl2 and CaCl2 (activation condition) and in absence of both MgCl2 and CaCl2 (basal condition), stirring for 15 min at room temperature.

After activation, platelets were centrifuged at 1200 × g for 15 min and resuspended in lysis buffer (Tris-HCl 10 mM, pH 7.4; EGTA 1 mM; phenylmethyl-sulfonyl fluoride 0.1 mM; and a complete set of protease inhibitors [Complete, Roche]). The supernatants were desalted by using DG10 columns (Biorad, City, CA, USA) and the total proteins were separated on 6% SDS-PAGE and electroblotted to nitrocellulose membranes. αAPPs staining and quantitative analysis were carried out as described below (SDS-PAGE and Western blot analysis).

Platelet Fractionation

Seventy microliters of platelet homogenates, prepared as reported, were centrifuged at 100,000 × g for 1 hr at 4°C to separate membrane (pellet) and soluble (supernatant) fractions. The centrifugation step was repeated both for the soluble and the membrane fractions after resuspension in lysis buffer.

RNA Extraction and RT-PCR Experiment

Total cellular RNA was extracted from human platelets and from human cortex (kindly supplied by Dr. G. Battaglia, Besta Institute of Neurology, Milan, Italy) by acid-phenol method using an RNA extraction kit (Bio/RNA-X Cell, Bio/Gen).

One microgram of total RNA was used to perform the first strand cDNA synthesis using oligo(dt), M-MLV-RT, and RNase H Minus 200 U/1 μg RNA (Promega, Madison, WI, USA). Following reverse transcriptase reaction, PCR amplification was...
performed by using specific primers for ADAM10 (284 bp): sense 5’ AACACGAGAACGTGGATTTGCA 3’, antisense 5’ CAAGCCAGACAAATACGCATC 3’. Commercial primers were used to amplify human GAPDH as housekeeping gene: sense 5’ ATG ACC CCT TCA TTG ACC 3’, antisense 5’ TGTTTCACCACCTCTTTG 3’. All PCR reactions were carried out by using Taq polymerase, 2.5 U per sample (PE Biosystem, Faster City, CA, USA) in a programmable heating block (Gene Amp PCR System 9600, PE Biosystem, Faster City, CA, USA) with the following scheme cycles: denaturation 94°C for 1 min; annealing 56°C for 1 min; and extension 72°C for 1 min for 30 cycles. PCR products were electrophoresed on 2% agarose gel; gels were stained with ethidium bromide (EtBr) and photographed under UV light.

Immunoprecipitation
Fifty micrograms of human cortex proteins were incubated in RIA buffer containing NaCl 200 mM, ethylenediamine tetraacetic acid (EDTA) 10 mM, Na2HPO4 200 mM, ethyl-

Results
Identification and Characterization of ADAM10 in Human Platelets
A representative RT-PCR analysis performed using specific primers for ADAM10 on RNA extracted from control platelets and from human cortex is shown in Figure 1. Amplification of a housekeeping gene, such as GAPDH, was also used. The presence of a specific transcript for ADAM10 at 284 bp is shown both in platelets (Plt) and human cortex, transcribed into cDNA, and amplified by PCR with ADAM10-specific oligonucleotide primers. Single transcript of the expected size was obtained both in platelets (Plt) and human cortex (hCx). GAPDH expression was estimated as internal control for each sample. Bp markers are reported in the right-most lane.
cortex (hCx). A representative immunoblot analysis of platelet ADAM10 in a control subject is shown in Figure 2A. Staining with an ADAM10 specific antibody revealed a major band at 68 kDa. Immunoreactivity of the 68 kDa band increases with the platelet protein concentration (Fig. 2A). No unspecific signal was obtained reacting nitrocellulose with the secondary antibody alone, thus confirming the specificity of the 68-kDa band (rightmost lane). WB analysis performed in total human cortex homogenates, using the same polyclonal antibody, revealed several immunoreactive bands (Fig. 2B, left panel). Therefore, human cortical samples were immunoprecipitated to better detect specific ADAM10 immunoreactive proteins (Fig. 2B, right panel). Under these conditions (Fig. 2B, right panel, lane +), two major bands are visible: one corresponds to the enzyme with the prodomain (85 kDa; upper arrow) and the second one, at 68 kDa, corresponds to the major band observed in platelets. As expected, a major signal, corresponding to IgG, is present at about 50 kDa. An aliquot of the sample was precipitated with protein A in absence of the primary antibody to test for unspecific precipitation (Fig. 2B, right panel, lane −).

The glycosylation state of platelet ADAM10 immunoreactive band was tested. Figure 3 shows a representative immunoblot analysis performed with anti-ADAM10 on platelet homogenates before and after treatment with N-glycosidase F, and after incubation with neuraminidase and O-glycanase. ADAM10 undergoes a downward bandshift of roughly 6 kDa in its apparent MW when treated with N-glycosidase F. Neuraminidase and O-glycanase experiments failed to produce a shift of ADAM10 immunoreactive band. Molecular weight markers are reported on the right.

![Fig. 2](image-url) **Fig. 2.** WB analysis of ADAM10 in human cortex and human platelets. (A) Representative WB analysis of ADAM10 in whole platelet homogenate of a control subject. Increasing protein concentrations were loaded on gel (8% SDS-PAGE). The arrow shows the migration of the 68-kDa ADAM10 immunoreactive band. No other bands are present at higher MW. No unspecific signal was obtained by incubating nitrocellulose with the secondary antibody alone (rightmost lane). Molecular weight markers are reported on the right. (B) Left panel: Representative WB analysis of ADAM10 in human cortical homogenate of a control subject. Fifty micrograms of proteins were loaded on 8% acrylamide gel. Molecular weight markers are reported on the right. Right panel: Representative WB analysis of ADAM10 in human cortex after immunoprecipitation: 50 µg of human cortex proteins were incubated in presence (+) and in absence (−) of pAb anti-ADAM10 in RIA buffer and then precipitated with protein A-agarose beads. The immunoprecipitated samples were then loaded into 8% SDS-PAGE and recognized with pAb anti-ADAM10. Two forms are present (arrowheads), enriched in the immunocomplex in comparison with WB analysis (left panel). A major band is present at 85 kDa, corresponding to the precursor protein, and a second band at 68 kDa, corresponding to the mature form of the protein. They represent two specific forms because they are not detected when the total protein was incubated in the absence of antibody (−). Molecular weight markers are reported on the right.

![Fig. 3](image-url) **Fig. 3.** Biochemical characterization of ADAM10 in human platelets. (A) Analysis of ADAM10 N-glycosylation in control platelets: 100 µg of total platelet proteins were incubated with (+) and without (−) N-glycosidase F, loaded on 8% acrylamide gels and ADAM10 identified by immunoblotting. N-glycosidase F treatment causes a downward bandshift of 6 kDa of the ADAM10 immunoreactive band. Molecular weight markers are reported on the right. (B) Analysis of ADAM10 O-glycosylation in control platelets: 100 µg of total platelet were incubated with (+) or without (−) neuraminidase. 50 µg were removed and further incubated with (+) or without (−) O-glycanase. Twenty micrograms of total proteins were loaded on 8% acrylamide gels and ADAM10 identified by immunoblotting. O-deglycosylation experiments failed to produce a shift of ADAM10 immunoreactive band. Molecular weight markers are reported on the right. (C) Western blot analysis performed with an antibody specific for ADAM10 in platelet subcellular fractions. Immunoreactivity is present in platelet homogenate (H) and in particulate (P), but not in the soluble fraction (S).
O-glycanase treatment (Fig. 3B) failed to produce any downward shift of the ADAM10 band, suggesting the presence of N- but not O-glycosylation sites in protein, as previously described in transfected cell systems (10). When different subcellular compartments (Fig. 3C) were probed with the same antibody, the ADAM10 immunoreactive band was enriched in the membrane fraction (P).

Expression of ADAM10 in Platelets of Controls and AD Patients

We then quantified, by means of Western blot analysis, the level of ADAM10 in platelets obtained from control subjects and AD patients. Table 1 shows the demographic characteristics of patients included in the study.

Figure 4A shows the results of a representative double immunostaining for ADAM10 and β-actin in whole platelet homogenate of two control subjects and two AD patients.

Fifteen micrograms of total proteins was loaded on gel. The signal for β-actin was linear in a protein range of 5–50 μg and with a range of antibody dilution from 1:1000 to 1:10000. An anti-ADAM10 dilution of 1:2000 and anti-β actin of 1:3000 was used.

The densitometric analysis of immunoblots revealed no significant differences in the actin immunoreactivity among the two experimental groups, whereas a decrease in immunostaining for ADAM10 was found in AD subjects. The ratio between ADAM10 and β-actin immunoreactivity was determined and the relative scattergram shown in Figure 4B. A statistically significant reduction in the ratio of ADAM10/β-actin immunoreactivity was present in AD subjects (controls, n = 26; AD, n = 33; p < 0.0001). To further confirm clinical probable AD diagnosis, for all subjects included, whole platelet homogenates were processed by means of Western blot analysis with mAb 22C11, recognizing all APP forms: APP form ratio (APPr) given as the ratio between APP immunoreactive band at 130 kDa and APP form at 106–110 kDa was calculated (14,17,19). As previously reported, a highly significant decrease in APPr was found in AD patients when compared to control subjects (Table 1; p < 0.001).

APP Metabolism in Platelets of Controls and AD Patients

It has been shown that αAPPs is stored in α-granules and it can be released from platelets during degranulation (23,24).

The release of αAPPs was measured from platelets of a subsample of controls (n = 12) and AD subjects (n = 15), probing media of isolated platelets exposed to human thrombin (1 U/ml), which activates platelet aggregation and α-granule degranulation.

Platelets were resuspended in Tyrode’s buffer in absence or presence of thrombin. Media were loaded into gels and probed with mAb 6E10 to measure αAPPs.

Figure 5A shows αAPPs immunoreactivity in media of non-activated (−) and thrombin-activated (+) platelets from a representative control subject and AD patient. Although the spontaneous basal release of αAPPs was not significantly different, the

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**Table 1. Demographic and clinical variables of the sample**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls</th>
<th>Alzheimer Patients</th>
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</thead>
<tbody>
<tr>
<td>Cases (N)</td>
<td>26</td>
<td>33</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>63.19 ± 6.10</td>
<td>68.15 ± 6.18</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>9/17</td>
<td>13/20</td>
</tr>
<tr>
<td>MMSE (mean ± SD)</td>
<td>29.28 ± 1.1</td>
<td>18.0 ± 3.7</td>
</tr>
<tr>
<td>APP ratio (mean ± SD)</td>
<td>0.90 ± 0.29</td>
<td>0.31 ± 0.19*</td>
</tr>
</tbody>
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*p < 0.001
thrombin-induced release of αAPPs was markedly lower in AD patients when compared to controls. The immunoreactivity of αAPPs bands in thrombin-activated platelets was quantified by image analysis. As shown in Figure 5B, a highly significant difference was found between AD and control samples (p < 0.001).

It has been previously shown that both familial and sporadic AD patients show reduced levels of αAPPs in CSF (27–29). Therefore, we addressed the questions whether the reduction of thrombin-induced release of αAPPs observed in AD platelets is associated with CSF changes in αAPPs, thus reflecting central abnormalities of APP metabolism.

To answer this question, we measured αAPPs levels in CSF of the same pool of AD patients and control subjects who were tested for platelet αAPPs. CSF samples (4 μg of total proteins in a volume ranging from 10–15 μl) were probed with mAb 6E10. Figure 6A shows a representative Western blot analysis performed with mAb 6E10 in two control subjects and two AD patients. The immunoreactivity of the 110-kDa band, corresponding to αAPPs form in neuronal cells, was significantly reduced in CSF of AD patients when compared to control subjects, as confirmed by quantitative analysis reported in Figure 6B (p < 0.05).

**Discussion**

The main finding reported herein is that platelets show the expression of the α-secretase candidate ADAM10 and that levels of ADAM10 are significantly decreased in platelets of AD patients when compared to control subjects. In addition, because ADAM10 has been shown in several in vitro systems to be capable of metabolizing APP we measured, in platelets of control and AD patients, levels of the major α-secretase metabolite, αAPPs.

We show that a significant decrease in thrombin-stimulated release of αAPPs occurs in platelets of AD patients, compared to controls, and this is paralleled by qualitatively and quantitatively similar decreases of αAPPs levels measured in the CSF of the same patients.

The identification of ADAM10 in platelets, although expected, has never been reported before. ADAM10 has been reported to be the most likely physiologic adult brain α-secretase (30). Moreover, ADAM10 appears to colocalize, in brain tissue, best with β-secretase (BACE) and APP, thus suggesting that it represents an important-amyloidogenic secretase (30). Our results showed that platelets express the same transcript for ADAM10
as the human cortex. In platelets, however, a major band at 68 kDa is found, whereas in cortex there are two forms at apparent molecular weights of 85 and 68 kDa.

Previous studies performed in transfected cell systems (10) identified two ADAM10 forms at apparent MWs of 85 and 68 kDa, and proposed that the 85-kDa form represents the proenzymatic form. The finding that in platelets only the 68 kDa, N-glycosylated form was identified might depend on the fact that platelets are pseudocells derived from maturation of megakaryocytes. It may be hypothesized, however, that megakaryocytes contain both forms, whereas platelets may contain only the mature 68 kDa form of the enzyme.

The observation that ADAM10 levels are markedly reduced in AD patients has several implications; ADAM10 is involved in basal nonstimulated processing of APP (10). Because α- and β-secretase pathways appear to be mutually exclusive, it is likely that reduction of ADAM10 allows for β-secretase amyloidogenic cleavage of the protein. This hypothesis was recently supported by the finding that increased α-secretase activity and αAPPs release after cholesterol reduction in neural cell lines results in a decreased secretion of both Aβ and βAPPs (11). This effect is probably due to reduced substrate availability for β-secretase cleavage. Accordingly, our study shows that thrombin-induced αAPPs release from platelets of AD patients is reduced when compared to controls.

The biochemical mechanisms responsible for αAPPs and Aβ formation in platelets are not fully understood, although several groups have reported abnormalities in these parameters in AD subjects (15–17). APP metabolites’ production and release from platelets has been linked to two important intracellular pathways—cytochrome oxidase (COX) and PKC activation (31)—suggesting that multiple pathways can be ascribed to the observed decreased of thrombin-induced αAPPs release in AD patients. It may be proposed that decreased levels of ADAM10 as well as a modification of the intracellular cascade might modulate APP trafficking and processing (31). Whether these different mechanisms converge or act in parallel to affect APP metabolism remains to be established.

All data presented here were obtained ex vivo in AD patients using an easily accessible peripheral cell (platelets).

The use of a peripheral cell to study a molecular aspect of neuropathology may raise some concerns, primarily with the feasibility of a peripheral cell mirroring biochemical pathways occurring in the central nervous system. However, the appropriate-ness of using platelets in the multiple similarities between platelets and neuronal cells: platelets store and release neurotransmitters and bear appropriate transporters and receptors normally expressed by neuronal cells (18,32). These similarities, although provocative, underscore the use of platelets for the search of clinical markers for either neuropsychiatric or neurologic disorders. Our findings that a qualitative and quantitative similar alteration of αAPPs levels is present both in platelets and CSF of the same patient are consistent with this hypothesis. In fact, although a reduction of CSF αAPPs levels in AD patients has been previously reported (27–29), our results show a possible correlation between peripheral and central compartment in the same subject.

In conclusion, our findings show a reduction of ADAM10 levels in vivo, indicating that ADAM10 is one of the major players in the molecular pathogenesis of AD and supports the hypothesis that increasing its activity/expression might prove beneficial for AD.

Acknowledgments

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