Soluble Amyloid Precursor Protein 770 Is Released from Inflamed Endothelial Cells and Activated Platelets

A NOVEL BIOMARKER FOR ACUTE CORONARY SYNDROME*

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Background: Separate monitoring of the cleavage products of different amyloid β precursor protein (APP) variants may provide useful information.

Results: We found that soluble APP770 (sAPP770) is released from inflamed endothelial cells and activated platelets as judged by ELISA.

Conclusion: sAPP770 is an indicator for endothelial and platelet dysfunctions.

Significance: How sAPP770 is released in vivo has been shown.

Most Alzheimer disease (AD) patients show deposition of amyloid β (Aβ) peptide in blood vessels as well as the brain parenchyma. We previously found that vascular endothelial cells express amyloid β precursor protein (APP) 770, a different APP isofrom from neuronal APP695, and produce Aβ. Since the soluble APP cleavage product, sAPP, is considered to be a possible marker for AD diagnosis, sAPP has been widely measured as a mixture of these variants. We hypothesized that measurement of the endothelial APP770 cleavage product in patients separately from that of neuronal APP695 would enable discrimination between endothelial and neurological dysfunctions. Using our newly developed ELISA system for sAPP770, we observed that inflammatory cytokines significantly enhanced sAPP770 secretion by endothelial cells. Furthermore, we unexpectedly found that sAPP770 was rapidly released from activated platelets. We also found that cerebrospinal fluid mainly contained sAPP695, while serum mostly contained sAPP770. Finally, to test our hypothesis that sAPP770 could be an indicator for endothelial dysfunction, we applied our APP770 ELISA to patients with acute coronary syndrome (ACS), in which endothelial injury and platelet activation lead to fibrous plaque disruption and thrombus formation. Development of a biomarker is essential to facilitate ACS diagnosis in clinical practice. The results revealed that ACS patients had significantly higher plasma sAPP770 levels. Furthermore, in myocardial infarction model rats, an increase in plasma sAPP preceded the release of cardiac enzymes, currently used markers for acute myocardial infarction. These findings raise the possibility that sAPP770 can be a useful biomarker for ACS.

Alzheimer disease (AD) is characterized by intracellular accumulation of neurofibrillary tangles and extracellular deposits of amyloid β (Aβ) peptides in the brain (1, 2). Neurotoxic Aβ is generated from amyloid precursor protein (APP) by sequential proteolytic cleavage by β-site amyloid precursor protein cleaving enzyme (BACE1) and the γ-secretase complex (3). Alternatively, APP can be cleaved at the α-site within the Aβ sequence by ADAM family proteases. Cleavage of APP at the α-site and β-site produces N-terminal parts of APP referred to as sAPPα and sAPPβ, respectively. APP has three kinds of alternatively spliced mRNA isoforms (4, 5), APP695, APP751, and APP770 (6), of which APP695 is predominantly expressed in neurons (7). We previously found that vascular endothelial cells express APP770 and produce Aβ40/42 (8). Increasing evidence suggests that AD patients have cerebrovascular brain lesions at an early stage (9–14). Another important finding is

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3 The abbreviations used are: AD, Alzheimer disease; ACS, acute coronary syndrome; Aβ, amyloid β-peptide; AMI, acute myocardial infarction; AP, angina pectoris; APP, amyloid precursor protein; BMEC, brain microvascular endothelial cell; CSF, cerebrospinal fluid; KPI, Kunitz-type protease inhibitor; MI, myocardial infarction; nPMCI, non progressive mild cognitive impairment; pMCI, mild cognitive impairment; PRP, platelet-rich plasma.
that Aβ deposition within the walls of small arteries in the brain, called cerebral amyloid angiopathy, is observed in most AD patients (15–18). Although the origin of the vascular Aβ deposits remains an issue under discussion, a recent Aβ-immunotherapy study indicated that Aβ depositions in the brain parenchyma and blood vessels occur independently (19). We anticipate that measurement of sAPP770α/β in in vivo samples will enable us to judge whether the increased sAPP secretion is caused by increased processing of endothelial APP770 or neuronal APP695. In fact, both sAPPα and sAPPβ have been extensively analyzed for their α- and β-cleavage activities, respectively (20, 21). However, the currently available APP ELISA systems detect APP695, APP751, and APP770 mixed together.

In this study, we first established an ELISA system to specifically detect sAPP770 in human samples. Using this ELISA, in addition to endothelial sAPP770, we unexpectedly found that platelets store and release large amounts of sAPP770 upon activation. Therefore, we applied this ELISA to acute coronary syndrome (ACS) patients, including those with unstable angina and acute myocardial infarction (AMI), since endothelial injury, platelet activation, and thrombus formation are key events in the origin and progression of atherosclerosis and in the pathogenesis of ACS (22). We found that the plasma sAPP770 levels are significantly higher in AMI patients. Furthermore, myocardial infarction (MI) model rats showed a significant and rapid increase in plasma sAPPα. These findings raise the possibility that sAPP770 could be a useful marker for optimal management of ACS.

**EXPERIMENTAL PROCEDURES**

**Subjects**—The clinical study was approved by the Ethical Committees of RIKEN, Tohoku University, and Fukushima Medical University. The study population of AD-related diseases consisted of 56 patients aged 72.4 ± 1.0 years who underwent evaluations for memory disturbance at the Tohoku University Hospital Outpatient Clinic on Dementia. Clinical assessments by geriatricians and neuropsychological examinations, including the Mini-Mental State Examination (MMSE), were carried out as described previously (23). According to our established criteria (23), 5, 13, and 25 patients were diagnosed as having non-progressive mild cognitive impairment (nMCI), progressive MCI (pMCI), and AD, respectively. Briefly, for a 2-year follow-up period, patients who showed unchanged or improved cognitive functions were categorized as nMCI, while patients with amnestic MCI who progressed to AD were classified into pMCI. Overall, 13 of 56 patients were found to be cognitively normal at the baseline investigation. A study population of ACS patients admitted to Fukushima Medical University Hospital was enrolled. The clinical diagnosis of stable angina pectoris (AP), unstable AP, and AMI was made by physicians according to the Guidelines for Treatment of Acute Coronary Syndrome (JCS 2007) and finally determined by coronary angiography. Plasma samples were drawn from the aorta of patients with stable AP, unstable AP, and AMI (n = 20 each) during coronary angiography. Peripheral blood was taken from the AMI patients at the time of hospital admission. Fifteen AMI patients in the first analysis and 26 additional AMI patients in the confirmatory analysis were evaluated. As a control, peripheral blood was taken from 19 subjects without any cardiovascular risk factors.

**Materials**—The materials used in this study were sourced as follows: tissue culture media and reagents including DMEM from Invitrogen; protein molecular weight standards from Bio-Rad; recombinant human IL-1β, TNFα, and IL-6 from R&D Systems; TAPI-0 from Peptide Institute Inc.; collagen from Nycoderm; all other chemicals from Sigma or Wako Chemicals. The anti-APP(C) antibody recognizes the C-terminal part of APP. The anti-OX2 antibody was raised against the synthetic oligopeptide KTTQEPLARDPVKL after conjugation with bovine thyroglobulin. The commercially available antibodies used were mouse monoclonal anti-APP 22C11 (Chemicon) and anti-Kunitz-type protease inhibitory domain (KPI) (Chemicon). Cardiac Troponin-I and Creatine Kinase ELISA Kits (Life Diagnostics Inc.) were used to monitor rat serum troponin-I and serum creatine kinase levels, respectively.

**Expression Plasmids and Cell Culture**—Human APP695-pcDNA3.1, APP751-pcDNA3.1, and APP770-pcDNA3.1 were constructed in a previous study (8). Human brain microvascular endothelial cells (BMECs) (Applied Cell Biology Research Institute) were cultured in CS-C Complete Medium supplemented with 10% FBS and used within four passages.

**Platelet Preparation**—Blood (16 ml) was collected from healthy volunteers on the day of the experiment using vacuum blood collection tubes (Nipro) containing 3.8% sodium citrate. Platelet-rich plasma (PRP; 2.3 × 10⁸–2.7 × 10⁸ platelets/ml) was collected by centrifugation at 200 × g for 20 min. Platelets were collected from the PRP by centrifugation at 900 × g for 10 min in the presence of 4 mmol/liter citrate. The platelet pellet was resuspended in modified HEPES-Tyrode buffer (134 mmol/liter NaCl, 12 mmol/liter NaHCO₃, 2.9 mmol/liter KCl, 0.34 mmol/liter NaH₂PO₄, 1 mmol/liter CaCl₂, 5 mmol/liter HEPES, 5 mmol/liter glucose, pH 7.4) to a density of 2.5 × 10⁹ platelets/ml. Aliquots (200 µl) of PRP or platelets were used for platelet aggregation assays, which were performed in siliconized glass cuvettes at 37 °C with constant stirring at 1000 rpm in a TPA-4C aggregometer (Tokyo Photoelectric Co.). Platelet aggregation was initiated by adding collagen (final concentration, 3 µg/ml). At each time point, the PRP or platelet suspensions were centrifuged, and the platelet pellets were solubilized with 200 µl of T-PER buffer (Thermo Fisher Scientific Inc.) containing a Complete protease inhibitor mixture (Roche). The resulting cell lysates were evaluated by ELISA and Western blot analyses.

**Rat MI Model**—Male Sprague-Dawley rats weighing 290–310 g were purchased from Clea Japan Inc. For the MI rats, permanent coronary occlusion was performed at the proximal portion of the left coronary artery as previously described (24). The same surgical procedure was performed for a group of sham rats, except that the suture around the coronary artery was not tied. Plasma and serum (10.5 ml) were collected from each rat at 0, 1, 2, and 3 h after surgery, and the levels of plasma sAPPα, serum cardiac troponin-I, and serum creatine kinase were measured.

**Quantification of sAPP Using ELISA Systems**—A Human sAPP Total Assay Kit and Mouse/Rat sAPPα Assay Kit (IBL-
Japan) were used for measurements of human sAPP695, APP751, and APP770, and rat sAPPα, respectively, according to the established protocols. The APP770 ELISA system was recently introduced at IBL-Japan as a commercialized product. Briefly, a 96-well plate was coated with the anti-OX2 antibody, and an HRP-labeled anti-APP R101A4 antibody was used as the detection antibody. Human cerebrospinal fluid (CSF; 1:16 dilution), plasma, and serum (1:75 dilution) were evaluated by both the APPtotal and APP770 ELISAs. Measurement of sAPP in human samples was performed by an operator blinded to the diagnosis.

APP Detection—COS cells expressing human APP695, APP751, or APP770 or platelet lysates were solubilized in T-PER buffer containing a Complete protease inhibitor mixture. sAPP in the platelet releasates was pulled down with heparin-agarose (Thermo Fisher Scientific Inc.). The COS cell lysates (5 μg of protein), and platelet-derived samples (corresponding to ~5 × 10^6 platelets) were subjected to SDS-PAGE (5–20% gradient gel), and transferred to nitrocellulose membranes. For Western blot analyses, the membranes were incubated with anti-APP 22C11 (1:1000 dilution), anti-OX2 (1:100 dilution), anti-APP(C) (1:1000 dilution), and anti-sAPPα (6E10) (1:1000 dilution) antibodies. Appropriate HRP-conjugated donkey anti-goat IgG (Jackson Immuno-Research Laboratories), anti-mouse IgG, and anti-rabbit IgG (GE Healthcare) antibodies were used as the secondary antibodies (1:1000 dilution). The ECL Prime Blocking Agent and ECL Advanced Chemiluminescent Substrate (GE Healthcare) were used for detection of the bound antibodies. The detected signals were quantified with a Luminimage Analyzer LAS-1000 PLUS (Fuji Film).

RESULTS

Quantification of sAPP770 in in Vivo Samples—Our previous finding of APP770 expression in endothelial cells (8) prompted us to develop a sandwich ELISA system that specifically detects APP770, and not APP695 or APP751 (Fig. 1A). In this study, we also used a conventional APPtotal ELISA for comparison. In the APPtotal ELISA, both immobilized and detection antibodies detect APP695, APP751, and APP770. For the APP770 ELISA, we used the same detection antibody as the APPtotal ELISA, and developed an immobilized antibody against the OX2 domain, as a unique domain of APP770. As shown in Fig. 1B, an anti-N-terminal APP antibody (22C11) detected APP695, APP751, and APP770, while the anti-OX2 antibody specifically detected APP770 only. Our newly developed APP770 ELISA had a linear range of 50 pg/ml to 9 ng/ml. As shown in Fig. 1C, the APPtotal ELISA detected sAPP695, sAPP751, and sAPP770 secreted from COS cells transiently overexpressing APP695, APP751, and APP770, respectively, while the APP770 ELISA exclusively detected sAPP770. Since different standard samples were used in the APPtotal and APP770 ELISAs, direct comparisons of the measured APP770 levels are difficult.

The endogenous level of sAPP770 secreted from human BMECs was detected by the new ELISA (Fig. 2A). IL-1β is known to enhance sAPP secretion from endothelial cells (25) and neurons (26). Indeed, addition of IL-1β and TNFα, but not IL-6, slightly but significantly increased the levels of sAPP770 secreted from BMECs (Fig. 2A). Since a selective TACE inhibitor, TAPI-0, partially blocked the secretion of sAPP770 from BMECs (Fig. 2B), TACE is at least partially involved in the production of sAPP770 in endothelial cells, similar to the case for sAPP695 production (20). Next, to clarify the extent to which sAPP770 accounts for the total sAPP in in vivo samples, we measured the sAPPtotal and sAPP770 levels in human serum and CSF samples. Even though the total sAPP levels were 700–900 ng/ml in both human serum and CSF samples, their sAPP770 levels showed a marked difference. The ratio of sAPP770 to sAPPtotal in CSF was ~7.4%, and markedly lower than that in serum (~46%) (Fig. 2C), possibly because the blood–brain barrier prevents the influx of serum sAPP770 into the brain. Therefore, it is considered that most of the CSF sAPPtotal is derived from neuronal sAPP695, while the serum sAPPtotal is mostly derived from sAPP770. Owing to the technical limitation of the standard samples, direct comparisons of
increased (Fig. 3A). When platelets were isolated and stimulated with collagen, release of sAPP770 was observed (Fig. 3C), similar to the case for PRP. Taken together, we concluded that platelets express APP770 and release APP770 upon activation. Western blot analyses of the resting and activated platelets using a series of anti-APP antibodies (Fig. 3D) showed that the sAPP (~120 kDa, gray arrowheads) detected with 22C11, but not proportionally detected with the anti-APP(C) antibody, was already present in the resting platelets (Fig. 3E). Since this sAPP signal was also detected with the anti-OX2 and anti-KPI antibodies, we confirmed that sAPP770 was actually present in the platelets. In accord with the decrease in sAPP770 observed in the platelets upon activation, sAPP770 was markedly increased in platelet releasates. Western blot analyses using anti-sAPPα antibodies showed the release of sAPP770α from activated platelets. In addition to sAPP770, full-length APP770 (~140 kDa, black arrowheads), which was detected by both the anti-APP(C) and anti-OX2 antibodies, was also present at much lower levels. These results indicate that shedding of APP770 does not make a significant contribution to the release of APP770 upon platelet activation.

**sAPP770 Levels Are Unchanged during the Disease Process of AD**—Even though our APP770 ELISA system is unable to differentiate sAPP770β from sAPP770α, we expected that simultaneous analyses by the APP770 and APPtotal ELISAs in clinical samples would provide useful information about endothelial sAPP770 and neuronal sAPP695 in vivo. First, we wanted to clarify whether the serum and CSF sAPP770 levels show any unique correlations with the disease process of AD. As shown in Fig. 4A, the levels of CSF sAPP770 were quite similar between control subjects, and npMCI, pMCI, and AD patients, although the CSF sAPPtotal levels tended to be higher in the npMCI, MCI, and AD patients than in the control subjects (Fig. 4B). Indeed, there was a significant difference in the CSF sAPPtotal levels between the control subjects and AD patients. Taken together, it is conceivable that CSF sAPP695 tends to increase in the early stages of AD pathogenesis. Again, serum sAPP770 was not apparently changed in the AD-related diseases (Fig. 4C). We did not find a positive correlation for the serum sAPPtotal levels between the AD patients and control subjects (Fig. 4D), probably because sAPP695 is not a major component of serum sAPP.

**Plasma sAPP770 Is Significantly Higher in AMI Patients**—Owing to the lack of correlation between CSF/serum sAPP770 and the AD-related disease status, we moved to analyses of the serum and plasma sAPP770 levels in ACS patients. It is well known that initial endothelial injury and subsequent platelet activation are closely related to the pathological cascade of ACS (30). First, we analyzed APP770 in plasma samples taken from the aorta during catheterization of the heart in stable AP, unstable AP, and AMI patients (n = 20 each). We found that the plasma APP770 levels were sequentially increased in stable AP, unstable AP, and AMI patients (Fig. 5A), suggesting that the grade of endothelial injury and platelet activation could be monitored by the plasma APP770 level. Next, to evaluate the practical application, we analyzed sAPP770 in plasma and serum samples from peripheral blood of normal subjects (n =
19) and AMI patients (n = 15). Compared with the normal subjects, the AMI patients had significantly higher levels of plasma APP770 (Fig. 5B) and unexpectedly lower levels of serum APP770 (Fig. 5C). As a result, the ratio of plasma sAPP770 to serum sAPP in the AMI patients was \( \approx 1.06 \pm 0.16 \), and remarkably higher than that in the normal subjects (\( \approx 0.26 \pm 0.03 \)) (Fig. 5D). We set the cut-off value for the plasma sAPP770/serum sAPP770 ratio at 0.48, which accounted for the mean \( \pm 2 \) S.D. for the normal subjects. As a result, we found that 94.4% of the normal subjects were under the cut-off value, while 100% of the AMI patients showed higher values. The results showed that the sensitivity and specificity of the plasma sAPP770/serum sAPP770 ratio were 100 and 94%, respectively, thereby highlighting the usefulness of this ELISA for the diagnosis and management of AMI. Furthermore, we performed a confirmatory analysis using another set of peripheral blood samples from AMI patients, and observed reproducible results, in which the AMI patients had higher plasma sAPP770, lower serum sAPP770, and higher ratio of plasma sAPP770 to serum sAPP770 (supplemental Fig. S1). Finally, comparative analyses between the plasma sAPP770/serum sAPP770 ratio and current standard biomarkers such as serum troponin-T (Fig. 5E) and creatine kinase (Fig. 5F) were performed in the AMI patients. When the cut-off values for the troponin-T and creatine kinase levels were set at 0.1 and 5 ng/ml, the sensitivities of these assays were 73 and 53%, respectively, emphasizing the high sensitivity of the plasma sAPP770/serum sAPP770 ratio. We did not find any correlations between the plasma sAPP770/ serum sAPP770 ratio and the levels of these cardiac enzymes, probably reflecting different underlying mechanisms for eleva-
Analysis of Plasma Amyloid Precursor Protein 770

DISCUSSION

Our newly established APP770 ELISA system enables us to measure APP770 in vivo samples, and thereby allows the levels of another type of sAPP, most likely sAPP695, to be estimated. Even though the sAPP total levels were similar between CSF and serum samples, we found that CSF sAPP770 was markedly lower than serum sAPP770, strongly suggesting that CSF mostly contains sAPP695. Meanwhile, most of the serum sAPP is considered to be sAPP770, ~75% of which is derived from platelets upon activation. We observed that the sAPP total levels in CSF were higher in MCI and AD patients than in normal subjects, while CSF sAPP770 showed similar levels between control subjects, and npMCI, MCI, and AD patients. Taken together, it is most likely that MCI and AD patients have increased levels of CSF sAPP695. Indeed, a previous report showed that the elevated CSF BACE1 activity in AD patients is positively correlated with both CSF sAPPα and sAPPβ (32), although there are also conflicting observations (33). The current limitation of our APP770 ELISA system is that sAPP770 and sAPP770β are detected together. We are now on the way to developing an sAPP770β sandwich ELISA, which would potentially be useful for assessing vascular Aβ formation.

Our finding that the inflammatory cytokine TNFα enhanced sAPP770 secretion from endothelial cells indicates that sAPP770 could be a marker for endothelial inflammation. Furthermore, we found that platelets released sAPP770 upon activation, which is considered to be the well-characterized PN2/APP (27, 28), a platelet α-granule protein. PN2/APP has recently been shown to exhibit anticoagulant activity in vivo (34, 35), possibly because of its inhibitory activity toward several prothrombotic enzymes (36). Our Western blot analyses showed that a much larger amount of sAPP770 (PN2/APP) compared with full-length APP770 was already present in resting platelets and immediately released upon activation. Therefore, plasma sAPP770 is potentially a promising marker for indicating platelet degranulation. Indeed, we found that AMI patients have significantly higher levels of plasma sAPP770. The reason why serum sAPP770 was reduced in AMI patients remains unclear. However, this could arise through either consumption of platelet α-granule proteins upon persistent platelet activation or endothelial dysfunction. As illustrated in Fig 7, the pathological cascade of ACS consists of multiple steps, such as endothelial injury, activation of platelets, formation and rupture of plaque that includes deposition of macrophages and T lymphocytes, and myocardial damage and cell death. Currently used AMI biomarkers, such as cardiac troponins, heart-type fatty acid-binding protein, aspartate aminotransferase, creatine

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phosphokinase, and lactate dehydrogenase, indicate myocardial damage. On the other hand, it is considered that plasma sAPP770 is increased by endothelial inflammation and platelet activation, which are placed in the early phase of ACS. Indeed, our experimental MI rat model showed that the increase in sAPP770 precedes the release of cardiac enzymes. In addition, we observed that unstable AP patients had higher levels of plasma sAPP770 than stable AP patients. Taken together, our findings

FIGURE 5. Plasma sAPP770 is significantly higher and serum sAPP770 is lower in AMI patients. A, plasma sAPP770 increases sequentially in stable AP, unstable AP, and AMI patients. Plasma samples drawn from the aorta of patients with stable AP, unstable AP, and AMI during coronary angiography were evaluated by the APP770 ELISA. The data shown are means ± S.E. (n = 20). *, p < 0.05; **, p < 0.005. B and C, plasma sAPP770 (B) and serum sAPP770 (C) from peripheral blood samples were measured in normal subjects (n = 19) and AMI patients (n = 15). The horizontal lines represent the mean value in each group. **, p < 0.005; ***, p < 1 × 10^{-5}. D, ratios of plasma sAPP770 to serum sAPP770 in AMI patients and normal subjects. The horizontal lines represent the mean value in each group. ***, p < 1 × 10^{-5}. E and F, comparative analyses between the plasma sAPP770/serum sAPP770 ratio and serum troponin-T (TnT; E) and creatine kinase (CK-MB; F) were performed in the AMI patients. The cut off-value in each assay is shown by the dashed line.

FIGURE 6. Increase in plasma sAPP770 precedes myocardial injury. A–C, MI model rats were produced by ligating the left coronary artery. At 0, 1, 2, and 3 h after surgery, both plasma and serum samples were taken from the MI and sham rats for ELISA analyses. The levels of plasma sAPPα (A), serum cardiac troponin-I (cTn-I) (B), and serum creatine kinase (CK) (C) are shown as means ± S.E. (n = 8). *, p < 0.0076.
highlight the possibility of plasma sAPP770 as a promising biomarker for judging the early stage of ACS.

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