EFFLUX TRANSPORT OF SERUM AMYLOID P COMPONENT AT THE BLOOD–BRAIN BARRIER

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Serum amyloid P component (SAP), a member of the innate immune system, does not penetrate the brain in physiological conditions; however, SAP is a stabilizing component of the amyloid plaques in neurodegenerative diseases. We investigated the cerebrovascular transport of human SAP in animal experiments and in culture blood–brain barrier (BBB) models. After intravenous injection, no SAP could be detected by immunohistochemistry or ELISA in healthy rat brains. *Salmonella typhimurium* lipopolysaccharide injection increased BBB permeability for SAP and the number of cerebral vessels labeled with fluorescein isothiocyanate (FITC)–SAP in mice. Furthermore, when SAP was injected to the rat hippocampus, a time-dependent decrease in brain concentration was seen demonstrating a rapid SAP efflux transport *in vivo*. A temperature-dependent bidirectional transport of FITC–SAP was observed in rat brain endothelial monolayers. The permeability coefficient for FITC–SAP was significantly higher in abluminal to luminal (brain to blood) than in the opposite direction. The luminal release of FITC–SAP from loaded endothelial cells was also significantly higher than the abluminal one. Our data indicate the presence of BBB efflux transport mechanisms protecting the brain from SAP penetration. Damaged BBB integrity due to pathological insults may increase brain SAP concentration contributing to development of neurodegenerative diseases.

Keywords: blood–brain barrier, brain efflux transport, brain endothelial cells, lipopolysaccharide, neurodegenerative diseases, neuroimmunology, permeability, serum amyloid P component

Introduction

Serum amyloid P component (SAP), a member of highly conserved lectin fold superfamily and pentraxin serum protein family, consists of five noncovalently associated identical subunits [1, 2]. SAP has diverse and important protective functions in mammals [3]. Native SAP binds to DNA, chromatin, glycosaminoglycans, amyloid fibrils, bacteria, bacterial lipopolysaccharide (LPS), complement components, and polymorphonuclear neutrophil cells [2]. The physiological roles of SAP include among others the clearance of cellular debris at the sites of inflammation and protection against chromatin-induced autoimmunity [2, 3]. SAP contributes to innate immunity against bacterial infections; however, the binding of SAP to some bacteria may protect the pathogen and enhance its virulence [3, 4]. Knockout mice lacking SAP were shown to be more susceptible than wild-type mice to lethal infection with *Escherichia coli* O111:B4 that SAP does not bind, but they survived potentially lethal infections with *Strep-*

tococcus pyogenes or *E. coli* J5, pathogens to which SAP binds [3]. However, SAP protected mice against Shiga toxin 2-caused hemolytic–uremic syndrome, a fatal complication of enterohemorrhagic *E. coli* O157:H7 infection [5]. SAP could also bind to *Streptococcus pneumoniae*, increased the complement deposition, improved phagocytosis of the pathogen, and protected against pneumonia [6]. SAP was shown to bind to influenza virus and increase antiviral activities including inhibition of hemagglutination, neutralization of viral infectivity, and inhibition of neuraminidase [3, 7]. Moreover, SAP production induced by experimental malaria infection in mice inhibited intraerythrocytic malaria parasites both *in vitro* and *in vivo* [8]. SAP also binds fungal cell wall and interacts with *Candida albicans* resulting in the lack of host neutrophil response in invasive candidiasis in the gastrointestinal tract [9]. SAP proved to be a granulocyte adhesion inhibitor, and it could prevent the accumulation of granulocytes in the lungs in a murine model of acute respiratory distress syndrome (ARDS) [10].

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SAP binds to all types of amyloid fibrils, protects them from proteolysis, and contributes to systemic amyloidosis [11, 12]. Accumulation of amyloid fibrils in connective tissues, viscera, and in the walls of blood vessels can result in organ damages, and targeted pharmacological depletion of intravascular SAP overload may have therapeutic effects [12, 13]. Administration of a novel synthetic bis-D-proline compound could produce sustained depletion of SAP and clinical improvement in patients with systemic amyloidosis in an open label clinical trial [14]. In a mouse model of systemic AA amyloidosis, the amount of circulating SAP could be decreased by 90% using this novel compound and intravenous administration of anti-SAP antibody significantly reduced splenic and hepatic AA amyloid deposition [12].

SAP is always present in pathognomonic lesions of Alzheimer's disease (AD), one of the major types of senile dementia associated with memory loss, impairment of cognition, and changes in behavior, and SAP depletion provided promising results in a recent pilot clinical study on patients with mild to moderate AD [15]. AD is characterized by the presence of senile amyloid plaques and intraneuronal neurofibrillary tangles [16–18]. Accumulated pathological data confirm the binding of SAP to amyloid- β (A β) fibrils in AD lesions including cerebrovascular and intracerebral plaques, neurofibrillary tangles, and deposits of amyloid angiopathy [19–23]. Moreover, local SAP synthesis is markedly accelerated in the regions of brain affected by AD [17, 24]. Deposits containing SAP are also seen in other neurodegenerative diseases including Creutzfeldt–Jakob disease, Pick's disease, Parkinson's disease, and Lewy body disease [20–22, 25]. SAP also has direct neurotoxic effects; we have previously demonstrated that SAP induces apoptosis in the cerebrocortical culture of rat brain *in vitro* [26–28] and in rat brain after intrahippocampal administration *in vivo* [29]. SAP is finally localized in the nuclei of dying neurons and induced an increased production of A β *in vitro* [27].

Human SAP is produced mainly in the liver and secreted into the blood circulation. The synthesis rate and also the secretion of SAP into the blood are increased under different pathological conditions including malignancy, rheumatoid arthritis, pregnancy, and neurodegenerative diseases, such as AD [1, 30]. The excess of secreted SAP is precipitated in amyloid deposits resulted by different chronic inflammatory processes [31]. SAP is also synthesized in brain, and increased mRNA and protein levels were detected in brain of AD patients compared to controls [17, 24, 32]. However, no significant increase in SAP production could be detected in postmortem samples from hippocampus and frontal cortex of nondemented subjects with AD neuropathology [32]. Available data about SAP levels in cerebrospinal fluid (CSF) of AD patients are controversial: a study showed elevated SAP concentration [33], whereas the others [34–37] did not detect significant differences between AD and age-matched control patients. However, CSF SAP level correlated with cognitive function measured by Mini-Mental State Examination [34],

and a low value was associated with two-fold increased risk of progression to AD in case of mild cognitive impairment patients [36]. Interestingly, increased local SAP synthesis in the brain of AD patients did not substantially contribute to the CSF levels, probably because of deposition in amyloid plaques [37]. Mulder et al. [37] determined the CSF index values for pentraxins SAP and C-reactive protein (CRP) and found that SAP index is six to eight times lower, which indicates limited diffusion of SAP from blood to brain. This difference between the transport of SAP and CRP could not be explained by differences in molecular weights, iso-electric points, or negative charges of the two pentraxins. We supposed that specific characteristics of SAP transport through the blood–brain barrier (BBB) might be partly responsible for this phenomenon.

Mammalian BBB actively controls cellular and molecular trafficking between the blood and the brain thereby feeding and protecting the central nervous system (CNS) [38]. The continuous layer of cerebral endothelial cells attached to each other by tight intercellular junctions constitutes the morphological basis of the BBB. Brain capillary endothelial cells have a dynamic interaction with other neighboring cells including astroglia, pericytes, perivascular microglia, and neurons. Receptor-mediated transport is responsible for the brain penetration of different compounds including peptides and proteins, as well as for their clearance. BBB transport can be unidirectional or bidirectional operating in both blood-to-brain and brain-to-blood directions [38]. In the present study, we investigated the influx and efflux transports of SAP through the BBB *in vitro* and *in vivo* and further interpretations are also discussed.

Materials and methods

Materials

All reagents were purchased from Sigma, unless otherwise indicated. Fluorescein isothiocyanate (FITC)-labeled human SAP was prepared by a reaction with 500 μ g SAP and 2.4 mg FITC in 1.2 ml 500 mM NaHCO₃ (pH = 9.5) buffer for 1 h at 4 °C. FITC-labeled SAP was then purified on a Hypersil-Keystone GFS-300 gel chromatographic column in 25 mM NH₄HCO₃ (pH = 7.5) buffer as eluent and lyophilized.

Animals

For the experiments, 3-month-old CBA $\times$ BL6 male mice and adult male Lati–Wistar rats (Lati, Budapest, Hungary) were used. The animals were kept in standard conditions and received tap water and rat chow *ad libitum*. The experiments conform to the European Communities "Council directive for the care and use of laboratory animals" and were approved by local authorities (XVI./03835/001/2006).

LPS, SAP, and FITC–SAP treatment in vivo

For the visualization of SAP penetration from blood to brain, the experimental treatment groups were the following: vehicle-, LPS-, SAP-, and LPS + SAP-treated animals ($n = 5$ mice in each). The mice received three intraperitoneal (i.p.) injections of *Salmonella typhimurium* LPS, 100 μg dissolved in phosphate-buffered saline (PBS, pH 7.4) in a single dose ($t = 0$ h, 6 h, 24 h), as it was described [39, 40]. Human SAP (Calbiochem) was reconstituted from lyophilized powder in sterile distilled water, resulting in a solution with physiological ion concentration and a protein content of 1.25 mg/ml. Both LPS and vehicle (PBS) treated mice received 250 μg SAP in 200 μl total volume, i.v., 1 h before the end of experiment. In a separate set of study, FITC-labeled SAP was dissolved in sterile PBS (1 mg/ml), and 250 $\mu\text{g}/\text{mouse}$ was given i.v. to the tail vein of vehicle or LPS injected mice ($n = 5$). Brain penetration changes were investigated after 1 h distribution time. At the end of the experiments, animals in deep anesthesia were perfused, and brains were dissected and processed for fluorescent or immunodetection of SAP.

To study the brain efflux of SAP, adult male rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and positioned in a Kopf stereotaxic apparatus. Ten micrograms of SAP dissolved in 1 μl of PBS was injected into the hippocampus during a period of 5 min via a 1- μl Hamilton microsyringe fixed on a Kopf microinjection unit. The coordinates of the injection site were: from bregma, AP: -5.3 , L: 1.7 , H: -2.9 according to Paxinos and Watson [41]. The needle was left in place for 5 min following infusion to minimize dragging of injected liquid along to injection track.

Immunohistochemistry for SAP

Following transcardial perfusion with 20 ml PBS for 5 min and with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4; 30 ml/mouse for 10 min), brains were removed and left for a 24-h postfixation period in 4% paraformaldehyde–PB at 4 °C. For cryoprotection, tissues were immersed in 30% sucrose in 0.1 M PB. Samples were embedded in Tissue Tek medium (Sakura Finetechnical Co., Ltd., Japan), frozen, and sectioned in a Leica cryostat (Germany). Sections of 15 μm thickness were melted on silane-coated glass slides and dried at room temperature for min 1–2 h before staining. Sections were rehydrated in PBS, three times for 5 min, then digested in 0.5 mg/ml trypsin in Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl, pH 7.4), containing 0.1% (w/v) CaCl_2 , at room temperature for 40 min. Enzymic digestion was stopped by washing the slides in ice-cold distilled water for 10 min, followed by PBS wash 3 $\times$ 5 min at room temperature. Nonspecific binding of the antibodies was reduced by a 1-h preincubation in blocking buffer containing 10% (v/v) normal goat serum (NGS) in PBS at room temperature. Sections were incubated with rabbit polyclonal antihuman

SAP antibody (DAKO; dilution 1:100) at 4 °C overnight, then with CY3 conjugated anti-rabbit IgG (dilution 1:100) in the dark at room temperature for 1 h, with both reagents diluted in 10% (v/v) NGS in TBS. Extensive washing in TBS (3 $\times$ 5 min each) was repeated between and after these steps. Finally, sections were dipped in distilled water and mounted in GelMount (Biomedex, USA), left to dry in the dark, then examined by Nikon Eclipse TE2000 fluorescent microscope (Nikon, Japan) using conventional rhodamine filter. Pictures were taken by a Spot RT digital camera (Diagnostic Instruments, USA).

Imaging FITC–SAP in mouse brain

Following perfusion, postfixation, cryoprotection, and cryosectioning, coronal mouse brain sections (30 μm) were mounted in GelMount and examined by fluorescent microscopy and by confocal microscopy (Leica, Germany).

Cell culture

Primary cultures of cerebral endothelial cells were prepared from 2-week-old rats [42–44]. Forebrains were collected in ice cold sterile PBS; meninges were removed with the help of sterile Whatman 3 M filter paper, then gray matter was minced by scalpels to 1 mm³ pieces and digested with 1 mg/ml collagenase CLS2 (Worthington) in Dulbecco's modified Eagle medium (DME) for 1.5 h at 37 °C. Microvessels were separated by centrifugation in 20% bovine serum albumin (BSA)–DME (1000 $\times$ g, 20 min) from myelin containing elements, and further digested with 1 mg/ml collagenase–dispase (Roche) in DME for 1 h. Microvascular endothelial cell clusters were separated on a 33% continuous Percoll gradient from red blood and other cells (1000 $\times$ g, 10 min), collected, and washed twice in DME before plating on collagen type IV and fibronectin-coated dishes, multiwell plates (Costar), or cell culture inserts (Transwell clear, 1 cm²; pore size 0.4 μm , Costar). For immunofluorescent staining, brain endothelial cells were cultured on glass coverslips coated with a biological matrix derived from corneal endothelial cells [42]. Cultures were maintained in DME supplemented with 5 $\mu\text{g}/\text{ml}$ gentamicin, 20% plasma-derived bovine serum (First Link, UK), 1 ng/ml basic fibroblast growth factor (Roche), and 100 $\mu\text{g}/\text{ml}$ heparin. In the first 2 days, culture medium contained puromycin (4 $\mu\text{g}/\text{ml}$) to selectively remove P-glycoprotein negative contaminating cells [42–44]. Cultures reached confluency within a week and were used for experiments.

To induce BBB characteristics, brain endothelial cells were cocultured with rat cerebral glial cells [42, 43]. Primary cultures of glial cells were prepared from newborn Wistar rats. Meninges were removed, and cortical pieces were mechanically dissociated in DME containing 5 $\mu\text{g}/\text{ml}$ gentamicin and 10% fetal bovine serum and

plated in poly-L-lysine-coated 12-well dishes and kept for minimum 3 weeks before use. In confluent glial cultures, 90% of cells were immunopositive for the astroglia cell marker glial fibrillary acidic protein, while the remaining 10% was immunopositive for CD11b, a marker of microglia. For coculture brain, endothelial cells in cell culture inserts were placed into multiwells containing astroglia at the bottom of the wells with endothelial culture medium in both compartments. When brain endothelial cells became confluent, 550 nM hydrocortisone was added to the culture medium [42–44]. Before experiments, cells were treated with CPT-cAMP (250 μ M) and RO 201724 (17.5 μ M; Roche) for 24 h to tighten junctions and elevate resistance [42–44].

Permeability assays for FITC–SAP

To measure the permeability of rat brain endothelial cell monolayers to FITC–SAP, cell culture inserts were transferred to 12-well plates containing 1.5 ml Ringer–Hepes solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 5.5 mM D-glucose, 20 mM HEPES, pH 7.4) in the lower, abluminal compartments. In upper, luminal chambers culture, medium was replaced by 500 μ l Ringer–Hepes containing 25 μ g/ml FITC–SAP. The multiwell plates were placed on a horizontal shaker (100 rpm) at 37 °C or at 4 °C, and inserts were transferred at 30, 60, 90, 120, 180, and 240 min to a new well containing Ringer–Hepes solution. The concentrations of the FITC–SAP in samples from the compartments were determined by a fluorescent plate reader (Fluoroskan, Labsystems; emission: 525 nm, excitation: 440 nm). Flux across cell-free inserts was also measured. Transport was expressed as nanogram of tracer diffusing from luminal to abluminal compartments or as permeability coefficients (P_e ; in 10^{-6} cm/s). Calculations are described in detail in previous publications [42–44].

To measure the transendothelial permeability for FITC–SAP from abluminal to luminal direction, cell culture inserts were transferred to 12-well plates containing 1.5 ml solution of 25 μ g/ml FITC–SAP in Ringer–Hepes in the basolateral compartments and kept on a horizontal shaker (100 rpm) at 37 °C or at 4 °C. In apical chambers, culture medium was replaced by 500 μ l Ringer–Hepes solution and changed to fresh Ringer–Hepes solution at 30, 60, 90, 120, 180, and 240 min. The concentrations of the FITC–SAP molecules in samples were determined by fluorescent plate reader.

Measurement of FITC–SAP release from brain endothelial cells

To load brain endothelial cells with FITC–SAP, cells cultured on filters were incubated with 25 μ g/ml FITC–SAP in Ringer–Hepes for 1 h at 37 °C. The cells were then washed with PBS, and the solutions in the upper and lower

compartments were changed to fresh Ringer–Hepes at 0, 60, 120, and 180 min. The concentrations of FITC–SAP in samples were determined by fluorescent plate reader. FITC–SAP release from brain endothelial cells was expressed as nanogram of tracer diffusing to either luminal or to abluminal direction during a given time on a given surface (1 cm^2).

Visualization of FITC–SAP uptake in brain endothelial cells

Rat brain endothelial cell monolayers cultured on biological matrix-coated glass coverslips were incubated with 25 μ g/ml FITC–SAP dissolved in Ringer–Hepes for 3 h at 37 °C or at 4 °C. After several washes in PBS, the cells were stained for tight junction protein ZO-1, and cell nuclei were stained with bis-benzimide. Briefly, cultures were washed in PBS and fixed with ethanol (95%) – acetic acid (5%) for 10 min at –20 °C. Nonspecific binding sites were blocked with 3% BSA in PBS, then cells were incubated with anti-ZO-1 polyclonal antibody (Zymed, USA) for 1 h 30 min. Incubation with secondary antibody Cy3-labeled anti-rabbit IgG lasted for 1 h. Between incubations, cells were washed three times with PBS. Coverslips were mounted in GelMount, examined by fluorescent microscope, and photographed by a Spot RT digital camera.

Determination of SAP content of rat brain hemispheres

Right hemispheres of rat brains were exposed to 1 μ g human SAP while left hemispheres were used as unexposed control. Rats were decapitated after 30 min, 1, 3, 5, and 7 days. Rat hemispheres were homogenized in Dounce homogenizer in PBS supplemented with protease inhibitors (Protease Inhibitor Cocktail Set I, Calbiochem) and centrifuged at 10,000 g for 15 min. Nunc Maxisorp plates were covered by antihuman SAP (Calbiochem) in 1:100 dilution and washed with PBS. Supernatants of tissue homogenate were diluted to 1:10 in Tris-buffered saline (TBS) supplemented with 0.1% bovine serum albumin (BSA) and 0.05% Tween-20, and 100 μ l sample per well was loaded. Anti-human SAP in 1:800, anti-mouse IgG conjugated to biotin in 1:20,000, and extravidin peroxidase in 1:200 dilution were used. Plates were developed by 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate and read at 450 nm wavelength in a multiwell plate reader.

Statistical analysis

All data presented are means $\pm$ S.E.M. The values were compared using the analysis of variance followed by Newman–Keuls test. Changes were considered statistically significant at $P < 0.05$.

Results

Extravasation of SAP to brain in LPS-treated and nontreated mice

No specific immunostaining could be detected in brain sections of mice injected with vehicle, LPS or SAP alone (Fig. 1A–C). In the LPS-treated group, which received also SAP for 1 h, SAP could be visualized, that became associated to brain microvessels, or diffused to the parenchyma (Fig. 1D).

The LPS-induced SAP extravasation to brain was restricted to a portion of brain vessels, mainly in midbrain. Most vessels with positive SAP staining were found in thalamus, where perivascular brain mastocytes are located in the highest number.

The specificity of the SAP immunostaining was verified on human kidney sections with known amyloid deposits. The SAP immunohistochemistry gave similar pattern to Congo red staining, while no labeling was seen if the primary antibody was omitted (not shown).

In a separate experiment, intravenously injected FITC-labeled human SAP was detected in brain tissue of mice. No FITC–SAP leakage to brain was seen in vehicle-treated mice (Fig. 1E). However, LPS treatment increased

the number of cerebral vessels labeled with FITC–SAP (Fig. 1F). Localization of the labeled SAP was vascular; association to brain endothelial cells could be demonstrated by confocal microscopy (Fig. 1F). Leakage of FITC–SAP could also be observed. These results are in accordance with LPS-induced increase in BBB permeability to SAP demonstrated by immunohistochemistry.

Demonstration of efflux transport of human SAP in rat brain

To study the fate of human SAP in brain, SAP was microinjected to the left hemispheres of rat brains and its level was determined by ELISA 30 min, 1, 3, 5, and 7 days following injection. No SAP was detected in control left hemispheres of rats during the whole period of experiments. The total SAP content in the treated left hemispheres was 600 ng at the first time point after 30 min. The total amount of SAP decreased to 200 ng in 1 day and reached near base level of right hemisphere in 3 days demonstrating a rapid SAP efflux transport *in vivo* (Fig. 2).

Permeability of cultured rat brain endothelial monolayers to human SAP

Transport of FITC–SAP was observed in rat brain endothelial cells from both luminal to abluminal (blood to brain)

Fig. 1. Immunohistochemistry of SAP on brain cortex sections in control (A), LPS- (B), SAP- (C), and LPS + SAP- (D) treated animals. Extravasation of human SAP can be seen around the brain vessels only in LPS-treated mice (D). Detection of i.v. injected FITC–SAP in brain tissue of mice by confocal microscopy (E). LPS treatment increased the FITC–SAP leakage to brain parenchyma in the frontal cortex (F). Bar = 50 μ m

Fig. 2. Time-dependent decrease of SAP content in rat brain after microinjection of SAP to the left hippocampus. SAP content of the left brain hemisphere decreased rapidly after SAP exposition and reached the base level of unexposed right hemisphere in three days ($n = 5$). Statistics: ANOVA followed by Newman–Keuls multiple comparison test. Significant changes: *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ compared to the values measured in the same time in the right hemisphere; in the samples from the left hemisphere: ^a $P < 0.001$ compared to the value measured at 0.02 day (i.e., 30 min), ^b $P < 0.05$ compared to the value measured at Day 1, ^c $P < 0.05$ compared to the value measured at Day 3, and ^d $P < 0.05$ compared to the value measured at Day 5. No time-dependent significant difference was found in the samples from the right hemispheres

Fig. 3. Transendothelial permeability for FITC-SAP in rat brain endothelial cell monolayers at 37 °C and 4 °C from the luminal to abluminal and abluminal to luminal directions (A). All values presented are means $\pm$ S.E.M. ($n = 5$). Statistically significant differences ($P < 0.001$) are indicated between values measured. Fluorescent microscopy images show FITC-SAP uptake (green) to primary RBE cells *in vitro* at 37 °C (B). Cell nuclei are stained by bis-benzimide (blue), intercellular junctions are delineated by ZO-1 immunostaining (red). No labeled protein uptake to the cells can be observed at 4 °C (C). Bar = 10 μ m

Fig. 4. Measurement of FITC-SAP release into the luminal and abluminal compartments after loading RBE cells 25 μ M FITC-SAP for 1 h. All values presented are means $\pm$ S.E.M. ($n = 5$). Statistically significant differences are indicated between values measured (* $P < 0.01$, ** $P < 0.001$)

and abluminal to luminal (brain to blood) directions at physiological temperature (Fig. 3). This process was temperature sensitive; FITC-SAP transport was strongly decreased at 4 °C (Fig. 3), indicating energy-dependent, active process typical for transcytosis. FITC-SAP uptake could be also visualized in the cytoplasm of brain endothelial cells at 37 °C, but not at 4 °C (Fig. 3B and C). The permeability coefficient for FITC-SAP in the abluminal to luminal direction in primary rat brain endothelial cells was significantly higher than in the other direction (Fig. 3), indicating a possible net efflux of SAP across the BBB.

To test further the direction of SAP flux from endothelial cells, they were loaded with FITC-SAP, then the release was examined in both luminal (blood) and abluminal (brain) directions (Fig. 4). The release of FITC-SAP on the luminal side was significantly higher than at the other side (Fig. 4), suggesting an efflux transport for SAP.

Discussion

This study was initiated by the observation that SAP, a protein involved in innate immunity, does not penetrate the brain in physiological conditions, but it is present in the CNS in pathologies and it is unknown how SAP crosses the BBB. Morphological and functional abnormalities in the cerebral microvasculature, such as profound irregularities in the course of vessels and the vascular basement membrane, changes in brain endothelial proteins like transporters, and increases in perivascular infiltrates, are likely to play important role in AD [45, 46]. The integrity of the BBB, estimated by use of the ratio of CSF

to serum albumin concentrations, was well preserved in mild dementia and the disturbance is aggravated during the progression of dementia in AD [47]. Microvascular segments directly surrounded by amyloid plaques or representing cerebral amyloid angiopathy show increased permeability [21]. Proteomic analysis from a preliminary study shows strong upregulation of SAP in capillary cerebral amyloid angiopathy [48], and this finding is supported by immunochemistry indicating significantly more SAP deposits in microvascular A β deposits [23, 49].

Previous studies suggested that SAP content of the AD brain can be originated from local synthesis in CNS [24, 32] or SAP synthesized by the liver can be transported through BBB by an unknown mechanism [21]. Our *in vitro* studies on the brain endothelial monolayers demonstrated that SAP can cross BBB in both directions. The transport was reduced at lower temperature showing the presence of an active transport mechanism for SAP. *In vitro* experiments also revealed that the release of SAP from endothelial cell to the luminal side is significantly higher than that to the abluminal side. This observation suggests that

efflux of SAP is preferred at the BBB which is in good accordance with the *in vivo* data indicating rapid clearance of SAP from the CNS after intrahippocampal administration. SAP was not detected in the CNS after intravenous injection indicating that it does not cross intact BBB in mice. However, bacterial LPS administration made the diffusion of SAP across the injured BBB possible. It should be noted that SAP has a biological effect on its own; it attenuated the LPS-induced increase on BBB permeability by interacting with LPS [40].

We have recently confirmed that SAP significantly potentiates the barrier-weakening effect of A β 1–42 peptide on an *in vitro* reconstituted model of the BBB [43]. In combination with A β , SAP aggravated A β -induced decrease in transendothelial electrical resistance and increases in BBB permeability for paracellular marker sodium fluorescein and transendothelial marker albumin through primary brain endothelial monolayers, although SAP treatment alone did not result in significant change in these permeability parameters [43]. This *in vitro* BBB permeability observation is in agreement with data demonstrating that SAP stabilizes A β fibrils in cortical amyloid plaques and cerebrovascular amyloid deposits.

In recent years, intravenous SAP overload may derive from exogenous sources during diagnostic procedures [50, 51] or therapeutic attempts based on versatile protective effects of SAP in pulmonary fibrosis [52] or renal fibrosis [53]. In order to meet the clinical need, pharmaceutical grade human SAP component has been recently isolated and characterized from normal human donor plasma for clinical use [4]. Although *in vivo* diagnostic imaging studies with ¹²³I-labeled SAP given intravenously [50, 51] could not detect binding to brain in AD patients, one cannot exclude that repeated high intravascular SAP doses can induce or exacerbate atherosclerosis or cerebral amyloid angiopathy. Accumulation of SAP is a well-known finding in human atherosclerotic lesions [54]. One may also speculate that SAP can also extravasate to brain and binds to A β in case the integrity of the BBB is not preserved due to neuroinflammation, brain tumor, or trauma. Our experiments revealed that although SAP can pass through the intact BBB from blood to brain, the gross transport to this direction must be zero because of the fast and efficient efflux transport. This efflux mechanism can explain the very low CSF index values for SAP observed by Mulder et al. [37]. However, the reduction of CSF SAP levels found in demented AD brains may also suggest that SAP located in brain interstitial fluid is quickly deposited to amyloid plaques [34, 36, 37].

According to the amyloid hypothesis of AD, accumulation of A β in the brain is the primary pathogenic event in AD. Recent evidence indicates that A β within the intravascular space is linked to A β deposited in the brain suggesting that transport of A β between the brain, blood and CSF, mainly across the BBB, regulates brain A β [55, 56]. A β can be cleared from the brain by receptor-mediated mechanisms mediated by low-density lipoprotein receptor and the receptor for advanced glycation end products [55, 56].

It should be noted that bacterial LPS increases brain levels of A β by increased influx and decreased efflux mechanisms, as well as increased neuronal production of A β [57, 58]. A recent study has confirmed that small variations in human serum albumin concentration in the CSF can control A β fibril formation in brain interstitium by bounding A β and trapping it in a nonfibrillar form [59]. SAP can also be cleared by an unknown manner from the CNS after intranasal administration in wild-type control mice. Intranasal injection of SAP to amyloid precursor protein transgenic mice revealed that it binds to amyloid deposits preventing the clearance of both SAP and A β [60]. According to these data, the inhibition of A β –SAP interaction [13] and the clearance of the components by the BBB offer new promising routes of AD therapy [56].

Because SAP plays significant role in the development and stabilization of both amyloid plaques and neurofibrillary tangles [61, 62], the inhibition of SAP–A β binding was proposed as a novel target in AD [13, 28, 29, 61]. Our present results suggest that SAP in the brain may originate sources from the circulation at the early stage of AD in addition to the neurons. The efflux of SAP seems to be dominant in healthy brain, but it is inhibited by the association of SAP to amyloid deposits in AD [60]. This interaction inhibits the proteolytic decomposition of the main plaque forming peptide A β as well [13, 28, 29, 61]. This mutually disadvantageous interaction holds the amyloid components in a trap and strengthens the amyloid generating and stabilizing process which eventually leads to widespread neuronal loss. The inhibition of the bond between SAP and A β results in free SAP and A β can be proteolytically cleaved and cleared from the brain [28, 29, 63]. Free SAP can be removed from the CNS by the active efflux transport demonstrated here. These observations strengthen the hypothesis that effective inhibitors of the SAP–A β interaction may result in significant advance in AD therapy. Our present data contribute to the knowledge on SAP penetration to the CNS during AD and to development of anti-amyloidotic drug research.

In conclusion, the results of our study support a role for BBB efflux transport of SAP, an immune molecule with versatile effects in various physiological and pathological conditions including autoimmunity, infections, systemic amyloidosis, fibrosis, and neurodegenerative diseases.

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