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Brain Serum amyloid P levels are reduced in individuals that lack dementia while having Alzheimer's disease neuropathology

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Abstract

The neuropathological signs of Alzheimer's disease (AD) include beta amyloid plaques and neurofibrillary tangles. There is a significant population of individuals that have these key hallmarks but show no signs of cognitive impairment, termed non-demented with AD neuropathology (NDAN). The protective mechanism allowing these individuals to escape dementia is unknown. Serum amyloid P (SAP) is a serum protein associated with wound repair that is elevated in the brains of Alzheimer's patients and binds to amyloid plaques. Using immunoblotting and immunohistochemistry, we evaluated SAP levels in post mortem samples of hippocampus and frontal cortex in age-matched controls, AD, and NDAN individuals. AD individuals had significantly increased SAP levels compared to normal controls, while NDAN samples had no significant difference in SAP levels compared to normal controls. Our results suggest that low levels of SAP in plaques marks the brains of individuals that escape dementia despite the presence of beta amyloid plaques and tangles.

Keywords

Alzheimer's; serum amyloid P; beta amyloid plaque; neurofibrillary tangle; hippocampus

Introduction

Alzheimer's disease (AD) is a terminal neurodegenerative disorder characterized by cognitive loss and neuronal death for which there is currently no cure [1]. Two key neuropathological hallmarks of AD are amyloid plaques, formed by extracellular aggregates of beta amyloid (A β) peptide, and intracellular neurofibrillary tangles, composed of hyperphosphorylated tau protein. Mild cognitive impairment (MCI) is an intermediate state on the path to AD, where 10–15% progress to full stage Alzheimer's, and MCI individuals have a limited number of plaques and tangles [2]. Interestingly, some people with high levels of beta amyloid plaques and neurofibrillary tangles, referred to as non-demented with AD neuropathology (NDAN), do not develop dementia [3–5]. Understanding the mechanism involved in such resistance would be important for the future development of novel, effective treatments for AD.

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Serum amyloid P (SAP) is a glycoprotein secreted by the liver into the blood, and is a member of the highly conserved pentraxin family, characterized by a planar disc arrangement of five, non-covalently associated monomers [6,7]. SAP does not readily cross the blood brain barrier; however, neurons in the brain can produce SAP, and this production is upregulated in AD brains [8] although there is no difference in the cerebrospinal fluid (CSF) levels of SAP between AD patients and age-matched normal controls [9–11]. Among AD patients, one study using 70 patients found that higher CSF levels of SAP tended to correlate with less dementia (9), while a smaller study using 20 patients did not observe any trend (10). SAP is a pattern recognition protein that binds components of pathogens as well as apoptotic and autoimmune material [12,13]. SAP is involved in wound repair and the innate immune response [14–16], and is a potent regulator of monocyte responses controlling fibrosis [17–21]; however, SAP also potentially contributes to the pathogenesis of Alzheimer's disease through the binding of amyloid plaques and neurofibrillary tangles (NFTs) [22–24]. SAP has been found to enter neurons, and at elevated levels can induce apoptosis *in vitro* and *in vivo* [25,26]. Additionally, SAP was described to stabilize amyloid plaques by preventing the proteolysis of A β fibrils [27]; however, other results suggest that addition of SAP actually inhibits initial fibril formation and dose-dependently increases the solubility of A β peptide [28]. SAP binds to fibrils in all types of amyloid deposits, but SAP is not essential for this deposition [29]. However, amyloid deposition throughout the body in reactive systemic amyloidosis is significantly delayed in SAP knockout mice, suggesting that SAP plays an important role in the pathogenesis of amyloid diseases [30,31].

In this report, we show that SAP levels are reduced in the brains of NDAN individuals compared to the levels in the brains of AD patients. This reduction can be detected in both the hippocampus and frontal cortex. SAP staining is colocalized to the amyloid plaques and is reduced in NDAN hippocampal tissue compared to AD. This suggests that a reduced presence of SAP associated with senile plaques characterizes the amyloid deposits in the brain of individuals who do not develop dementia despite the presence of amyloid plaques and neurofibrillary tangles consistent with fully symptomatic AD.

Experimental Procedures

Cases

Frozen brain tissue samples were obtained from the Oregon Brain Bank at Oregon Health and Science University (OHSU) in Portland, OR. Donor subjects were enrolled and clinically evaluated in studies at the NIH-sponsored Layton Aging and AD Center (ADC) at OHSU. Control subjects were participants in brain aging studies at the ADC. Subjects received annual neurological and neuropsychological evaluation, with CDR assigned by an experienced clinician. Autopsies were carried out post mortem from 2 to 24 hours, with most cases under 10 hours (Table 1a). All cases (n=4) classified as Alzheimer's fulfilled the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria for definitive Alzheimer's with histopathologic evidence of amyloid plaques and neurofibrillary tangles in the hippocampus and neo-cortex, as well as cognitive impairment with a mini-mental state exam (MMSE) score below 10. Control cases did not display cognitive impairment (MMSE of 28–30) and had either no or a very limited number of amyloid plaques and neurofibrillary tangles. Mild cognitive impairment (MCI) cases had some cognitive impairment with MMSE scores in the mid 20s, and intermediate levels of amyloid plaques and neurofibrillary tangles. Non-demented with AD neuropathology (NDAN) cases displayed little to no cognitive impairment (MMSE 26 to 30), while having extensive amyloid plaques and neurofibrillary tangles in the hippocampus (Figure 1) and neo-cortex (data not shown), comparable to full on-set Alzheimer's (Table 1b).

Western Blots

0.5 g of hippocampus or frontal cortex was homogenized in 1.5 ml of 0.32 M sucrose in 0.1 mM CaCl_2 with 1% protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and 1x phosphatase inhibitor cocktail (Thermo, Waltham, MA). Samples were diluted 1:20 in PBS/0.2% SDS/Triton-X 100, and protein concentration was determined by Bradford (Sigma). Samples were normalized to 2 mg/ml in 50 μl SDS-PAGE sample buffer, run on 4–20% SDS-PAGE gels (Bio-Rad, Hercules, CA) and visualized by Coomassie stain to further confirm protein concentration. SAP concentration was determined by Western blotting as described previously [32] with the following modifications. Commercially available SAP (EMD Biosciences, Rockland, MA) was used as loading controls. SAP was detected using a rabbit anti-SAP antibody (Epitomics, Burlingame, CA) at a 1:10,000 dilution. Quantitation of band intensity was performed via ImageJ (Rasband, WS, NIH, Bethesda, MD) and statistical analysis was done with GraphPad Prism (Graphpad Software, San Diego, CA).

Immunohistochemistry

Five mm sections of the mid hippocampus were brought out of storage at -80°C and equilibrated to -20°C before embedding in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA). 10 μm sections were cut and affixed to Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA), for storage at -80°C . After briefly equilibrating to room temperature, sections were rinsed in 0.1 M PBS and then fixed in ice-cold 4% paraformaldehyde for 15 minutes. After two brief washes in 0.1 M PBS, the sections were blocked and permeabilized for 1 hour in 0.1 M PBS containing 10% goat serum, 0.03% Triton-X, and 0.1% phosphatase inhibitor (Thermo Fisher). Incubation with primary antibodies in 0.1 M PBS containing 10% serum and 0.1% phosphatase inhibitor was carried out overnight at room temperature. Antibodies used were 4G8 anti-beta amyloid (mouse, 1:800) and pTau (Ser202; mouse, 1:1000) from Pierce (Rockford, IL). Following two PBS washes, the slides were incubated for 1 hour with Alexa Fluor 488 and 594 secondaries (1:600; Invitrogen, Carlsbad, CA) in 0.1 M PBS containing 10% serum and 0.1% phosphatase inhibitor. Slides were again rinsed twice in PBS and once in distilled water before a 10 minute incubation with 0.3% Sudan Black B (EMD Chemicals, Gibbstown, NJ) in 70% ethanol to block lipofuscin autofluorescence [33]. After two more rinses in distilled deionized water, Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) was applied (Vector Laboratories, Burlingame, CA), and coverslips were mounted. Nail polish was applied to seal the edges.

For beta-amyloid and SAP staining, the same procedure was followed except that the sections were blocked and permeabilized for 1 hour in PBS/4% BSA/0.03% Triton X-100. Sections were incubated overnight at room temperature with 1:800 4G8 in PBS-4% BSA. The next day, the slides were briefly washed twice in PBS, and the sections were incubated with 1:500 rabbit anti-SAP antibody (Epitomics) for 1 hour at room temperature, along with negative control antibodies. After four 5-minute washes, the slides were incubated with donkey anti-mouse Dylight 488 and donkey anti-rabbit rhodamine red X (Jackson ImmunoResearch) in PBS-4% BSA for 30 minutes.

Image Analysis

Images were acquired using a confocal laser-scanning module (Bio-Rad Radiance 2000 with LaserSharp software; Hercules, CA) mounted on a Nikon E800 microscope with a 20x/0.75NA objective (Nikon USA; Melville, NY). Images were acquired with 488 nm and 568 nm excitation. Images for comparisons were acquired with constant settings for laser power, detector gain, amplification gain, and offset. For the beta-amyloid and SAP staining, DIC and fluorescence images were acquired using an Olympus FV1000 confocal microscope with a 20x/0.75NA objective and Micromanager software [34].

Results

SAP levels in the hippocampus and frontal cortex of NDAN individuals are significantly decreased compared to AD

SAP levels were previously found to be elevated in the brains of AD patients [8]. We also found that SAP levels were elevated in the hippocampus and frontal cortex of AD patients compared to controls with a four-fold increase to 110 ng of SAP/mg of protein in AD (Figure 2A, B). Our results differ from the 1 µg of SAP/mg protein previously reported [8], because we looked at total homogenate rather than protein concentrations of supernatants after centrifugation of homogenates. Interestingly, SAP levels were significantly elevated in both the hippocampus and frontal cortex for AD patients compared to NDAN samples, even though both sets of cases had comparable number of amyloid plaques and neurofibrillary tangles that SAP associates with (Figure 2A, B). In the NDAN samples, the level of SAP in the hippocampus and frontal cortex were comparable to the controls. Levels of SAP in MCI patients were intermediate in the hippocampus, while at normal levels in the frontal cortex, which is to be expected due to the significantly lower number of plaques and neurofibrillary tangles found in MCI compared to AD [2].

SAP staining is reduced at Aβ plaques for NDAN hippocampal sections compared to AD

SAP co-localizes with amyloid plaques in the brains of AD patients [35]. On stained hippocampal tissue sections, no Aβ plaques or SAP staining was visible in the normal-age-matched controls (Figure 3). NDAN sections had numerous plaques with extremely light staining of SAP, also localized to the plaque (Figure 3). The number of plaques in AD patients was similar to NDAN, but AD tissue sections had relatively strong SAP staining co-localized to the plaques (Figure 3).

Discussion

SAP levels are elevated in the brains, but not the CSF, of patients with Alzheimer's disease. These patients have cognitive impairment and a significant number of amyloid plaques and neurofibrillary tangles in the memory and cognitive function area of the brain [36,8]. Mild cognitive impairment (MCI) patients only have slight cognitive impairment, fewer amyloid plaques, and significantly less neurofibrillary tangles [2]. Non-demented with AD neuropathology (NDAN) individuals have extensive amyloid plaques and neurofibrillary tangles like AD, while having little to no cognitive impairment [4,5]. Here, we report that NDAN individuals have significantly lower levels of SAP compared to AD with levels approaching those of normal age matched controls. However, it is not possible at this point to mechanistically link the decreased levels of SAP to cognitive rescue.

SAP is expressed by neurons in the brain, and normal elderly will develop low levels of amyloid plaques and neurofibrillary tangles [8,37]. Therefore, at least low levels of SAP are expected to be, and were, found. The MCI samples showed intermediate levels (between AD and control) of SAP in the hippocampus, and in the frontal cortex the levels were indistinguishable from normal-age matched controls. This is also expected, since MCI individuals have significantly less infiltration of neurofibrillary tangles at that stage of the disease and possibly reduced plaque numbers. However, the low levels of SAP in NDAN individuals was not expected as high levels of plaques and tangles are located in both sections of the brains and SAP readily binds to both. This lack of SAP could therefore reflect biochemical changes leading to reduced SAP deposition. Further studies are needed to clarify the exact mechanisms involved.

The lack of dementia and low SAP levels in NDAN individuals could potentially be due to a reduced inflammatory response. Typically in Alzheimer's, the presence of amyloid plaques

and neurofibrillary tangles activate inflammatory cells, including the microglia and macrophages, resulting in the release of cytokines and other inflammatory agents [36]. The microglia could upregulate SAP production, which can in turn bind more A β and result in additional plaques. For NDAN individuals, however, SAP could be acting predominantly in its role as an anti-inflammatory agent through opsonization and clearance of apoptotic neuronal cells, as well as downregulating the activation of infiltrating monocytes and macrophages with local IL-10 production [18]. There would be a balance between SAP acting in a positive manner as an anti-inflammatory agent and SAP binding to amyloid plaques detrimentally as a pattern recognition protein. We speculate that in the case of AD, the balance is shifted to SAP predominantly binding to fibrils and plaques, while in NDAN individuals SAP is predominantly acting to reduce inflammation.

The drug R-1-[6-[R-2-carboxy-pyrrolidin-1-yl]-6-oxohexanoyl]pyrrolidine-2-carboxylic acid (CPHPC) cross-links SAP pentamers to increase the clearance of SAP from the body, resulting in decreased SAP levels in the serum and CSF of Alzheimer's patients [38,39]. An intriguing possibility is that CPHPC could be used to prevent the onset of dementia by removal of SAP from the brain. It remains to be seen how CPHPC will affect amyloid clearance in the long term, and if it will help restore cognitive function.

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Fig. 1. Pathological signatures of AD occur in cognitively intact individuals (NDAN). Reactivity for A β plaques (green, top row) and phosphorylated tau (green, bottom row) in the dentate gyrus of individuals classified as control (Braak 1, Plaque 3, MMSE 30), AD (Braak 6, Plaque 1, MMSE 2), or NDAN (Braak 6, Plaque 1, MMSE 27). DAPI mounting media was used to visualize nuclei (blue). Bar 100 μ m

Fig. 2.

SAP levels in the hippocampus and frontal cortex of NDAN individuals are significantly decreased compared to Alzheimer's. SAP levels in homogenized brain tissue were measured by Western blot and compared to normal brain controls. Statistical significance was determined by 1-way ANOVA, Tukey's test. *, $p < 0.05$; **, $p < 0.01$. Results are mean $\pm$ SEM (n=6 for control hippocampus, n=5 for MCI and AD hippocampus, n=4 for NDAN hippocampus; n=3 for frontal cortex)

Fig. 3. SAP staining is reduced at A β plaques for NDAN compared to Alzheimer's. Dentate gyrus sections were stained for A β (green) and SAP (red). Bar is 50 μ m. Representative of n=3

Table 1a

Case studies

Diagnosis	Age	Sex	PMI	Braak Stage ^a	Plaque Score ^a	MMSE
Control ^b	82.7	M	14	1	4	N/A
Control ^b	83.1	M	2	1	1	N/A
Control ^b	77.4	F	12	0	4	N/A
Control	>89	F	18	0	4	28
Control	80	M	2	1	3	30
Control	28	F	15.5	0	4	N/A
Control	43	F	10	0	4	N/A
Control	51	M	9.5	0	4	N/A
Control	60	F	40.5	0	4	N/A
MCI ^c	>89	M	23	3	2	26
MCI ^c	89	F	20	3	2	22
MCI ^c	>89	F	12	3	2	27
MCI	>89	F	4	2	3	25
MCI	79	F	12	2	4	28
AD ^b	>89	M	3.25	6	1	2
AD ^b	61	M	4	6	1	2
AD ^b	79	N/A	6	6	1	N/A
AD	82	F	19	6	2	9
AD	60	N/A	4.5	6	2	N/A
AD	64	M	9.25	6	1	1
AD	62	F	6.5	6	2	7
AD	87	M	8.75	6	2	N/A
NDAN ^b	87.8	M	3	4	1	29
NDAN ^b	>89	F	4.5	6	1	27
NDAN ^c	87	F	2.5	2	3	29
NDAN	87.6	F	10.5	5	2	29

Diagnosis	Age	Sex	PMI	Braak Stage ^a	Plaque Score ^a	MMSE
NDAN	89	F	2.5	5	2	27
NDAN	>89	F	18	6	4	30

Abbreviations are as follows: MCI - mild cognitive impairment, AD - Alzheimer's disease, NDAN, non-demented with Alzheimer's disease neuropathology, M - male, F - female, PMI - postmortem interval in hours, MMSE - Mini-Mental State Examination, N/A - not available.

^aScoring based on Braak and Braak [39].

Tissue sample from hippocampus unless otherwise noted.

^bFrontal cortex tissue only

^cHippocampus and frontal cortex tissue

Table 1b

Case studies comparison

	<u>Ages</u>	<u>Sex</u>	<u>PMI</u>	<u>Braak</u>	<u>Plaque</u>	<u>MMSE</u>
Control	28 – >89	4M/5F	13.7 ±3.8	1 ± 1	4 ± 1	29 ± 1
MCI	79 – >89	1M/4F	14.2 ±3.4	3 ± 1	3 ± 1	26 ± 1
AD	60 – 87	2M/2F	9.6 ±2.5	6 ± 0 *	2 ± 1 *	6 ± 2 *
NDAN	87 – >89	1M/5M	6.8 ±2.6	5 ± 1 *	2 ± 1 *	29 ± 1

* denotes values significantly different than control values (p < 0.05; 1-way ANOVA, Dunnett's test)