Original Paper

The Increase of D-Amino Acids in Alzheimer's Disease-Related Proteins Isolated from the Brains of SAMP8 Mice

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Abstract: Recently, the presence and function of D-amino acids in mammals, including humans, have been reported. In particular, a relationship between an increase in the D-amino acid content of specific proteins and aging and senescence-related diseases has been proposed. However, this hypothesis remains unproven. Here, we focused on Alzheimer's disease, a well known senescence-related disease, and used high performance liquid chromatography (HPLC) to analyze the degree of change in the D-amino acid content in the Alzheimer's disease-related proteins PP1C, GLRX1 and NF-L. Cerebrums of SAM (senescence-accelerated mice), an animal model of Alzheimer's disease, were used. Increases in D-serine and D-aspartic acid contents with aging in PP1C were found. PP1C is a catalytic subunit of PP1 (serine/threonine phosphatase 1). Tau, one of the most important molecules involved in Alzheimer's disease, is a target of PP1C. Hyperphosphorylation of Tau is thought to be a trigger of Alzheimer's disease. Moreover, the involvement of PP1C in cognitive function and memory, independently of dephosphorylation of Tau, has been suggested. The results of this study suggest that substitution of L-amino acids with D-amino acids alters the physical properties of PP1C, and this, in turn, causes its malfunction, which may lead to the onset of Alzheimer's disease.

Keywords: *Alzheimer's disease, D-amino acid, SAMP8, proteinphosphatase 1, aging*

Introduction

Proteins synthesized in humans are composed mainly of L-amino acids. However, in recent years, it has been reported that the content of D-amino acid increases with age in structures or tissues such as the eye (Fujii et al. 1994), brain (Fisher et al. 1986, Kaneko et al. 1995), skin (Fujii et al. 2002), and bone (Ohtani et al. 1998, 2007). These D-amino acids are present in proteins (Kasai et al. 2010, Powell et al. 2003, Ritz-Timme et al. 2003).

When D-amino acids are substituted for L-amino acids in a protein, the protein undergoes a change in structure. This change in structure is likely to alter the features and functions of the protein. Moreover, in some proteins

associated with aging associated-diseases, the original L-amino acids have been replaced by D-amino acids (Fisher et al. 1986, Fujii et al. 1994, Kaneko et al. 1995, Powell et al. 2003, Kasai et al. 2010). The crystallin associated with cataracts, as well as myelin basic protein, which is thought to be involved in multiple sclerosis, contain D-amino acids (Fisher et al. 1986, Fujii et al. 1994). A relationship between amino acid isomerization and protein aging has been hypothesized from the findings of such studies, but specific proof has not yet been found.

Alzheimer's disease is a neurodegenerative condition in which the risk of development increases with aging (Grill and Cummings 2010). The senile plaque that is characteristic of this disease is composed of tangles

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of amyloid beta (Joachim and Selkoe 1989). It is thought that this abnormally structured amyloid beta has a cytotoxic effect, causing insufficiency and apoptosis of nerve cells (Liu and Hong 2005, Simakova and Arispe 2007). In the amyloid beta obtained from the brains of Alzheimer's patients, the first Asp (aspartate), seventh Asp, and 23rd Asp have been changed to D-Asp (Shapira et al. 1988, Roher et al. 1993, Shimizu T et al. 2002).

Moreover, in recent years, the presence of hyperphosphorylated Tau protein in the brains of Alzheimer's patients has been confirmed (Spillantini et al. 1997, Alonso et al. 2010). Hyperphosphorylation of Tau protein, which functions to maintain the cytoskeleton, is considered to participate in the decline of neuronal cells (Schindowski et al. 2008). Amyloid beta and hyperphosphorylated Tau protein are now considered to be important, independent causative molecules in Alzheimer's disease (Huang and Jiang 2009, Zempel et al. 2010). However, despite the accumulation of data, we still do not have enough to clarify the mechanism of the cytotoxic effects of amyloid beta.

In this study, I hypothesized that obstruction of cell survival by amyloid beta and hyperphosphorylated Tau is part of the expression of malfunction that occurs frequently in the aged brain. I then postulated that the increase in D-amino acid content of the aged brain affects the physical properties of proteins and thus give rise to senescence-related neurodegenerative disease.

To clarify these relationships, I detected the D-amino acid ratios in three Alzheimer's disease-related proteins: 1, Glutaredoxin (GRX) is involved in many cellular functions, including DNA synthesis, signal transduction, and defense against oxidative stress (Holmgren 1989); 2, Neurofilament light chain (NF-L) is one of three major neurofilaments. Neurofilaments are members of the intermediate filament protein family and are neuron-specific. Accumulation of neurofilaments is observed during the progression of numerous neurological diseases (Liu et al. 2004); and 3, Type 1 serine/threonine protein phosphatase (PP1C) is a general protein phosphatase (Kimura et al. 2007). An increase in PP1C mRNA expression in senile plaques has been reported (Ginsberg et al. 2006).

For these experiments, senescence-accelerated mice (SAM) were used as Alzheimer's disease model animals, and HPLC was used for D-amino acid analysis.

Materials and Methods

Animals

The SAM is an accelerated aging model that was established from a common genetic pool of the AKR/J strain of mice. The SAM model was established in 1981 by Takeda et al. (1981). SAM includes nine major SAMP (senescence-accelerated mouse prone) substrains and three major SAMR (senescence-accelerated mouse resistant) substrains, each of which exhibits characteristic disorders. Because the SAMP8 strain shows learning and memory deficits (Butterfield and Poon 2005), SAMP8 is regarded as an Alzheimer's disease model animal. Here, I used 20-week-old SAMR1 for comparison with 20-week-old SAMP8. The progress of aging in SAMP8 is more rapid than normal animal and more over the genetic background of SAMP8 does not become clear yet. Therefore, 8 week-old SAMR1 was used as the non-senescence animal. Animals were obtained from a commercial source (Japan SLC, Shizuoka, Japan).

Sample extraction

Cerebral cortices were removed from 8-week-old and 20-week-old SAMR1 and 20-week-old SAMP8 immediately after decapitation. Tissues were cut into pieces and homogenized in lysis buffer [20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Tween20, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM glycerol-2-phosphate disodium salt hydrate]. Before the lysis buffer was used, 10 µl protease inhibitor cocktail (Sigma, MO, USA) and 10 µl 100 mM phenylmethanesulfonylfluoride were added to each 1 ml of lysis buffer. Incubation of tissues for 15 min at 4 °C was followed by centrifugation at 15,000 rpm, 5 min at 4 °C. The protein concentrations in the supernatant were measured by Bradford method and the supernatant was used for Western blotting and immunoprecipitation followed by HPLC analysis.

Western blotting

Western blotting analyses were performed according to established procedures. Equal amounts of protein (20 µg) were separated on Supersep gel (Wako, Osaka, Japan) and transferred to PVDF Immobilon-P™ membranes (Millipore, MA, USA). Blocking One™ (Nacalai Tesque, Kyoto, Japan) was used for blocking. The following primary antibodies were used: anti-GLRX (R&D Systems, MN,

USA); anti-NF-L (EnCor Biotechnology, FL, USA); and anti-PP1C (Exalpa Biologicals, MA, USA). Digital images of protein bands on the membranes were captured by a LAS-3000 imager (FUJIFILM, Tokyo, Japan).

Immunoprecipitation for HPLC sample preparation

Each protein was obtained by using a Protein G Immunoprecipitation Kit (Thermo, IL, USA) in accordance with the manufacturer's instructions. Each of the antibodies mentioned above was used at 50 to 100 ng. To improve the precision of the HPLC analysis, protein extracts were filtered and desalted by using ZipTipC4™ (Millipore). Final solutions from the ZipTipC4™ were expelled from the ZipTip by using 75% acetonitrile in 0.1% trifluoroacetic acid. The purified samples were HCl-hydrolyzed in a Pico-Tag™ work station (Waters, Tokyo, Japan) at 108 °C for 7 h. Each hydrolyzed sample was dissolved in 15 µl of 200 mM borate buffer, and 10 µl of the 15-µl sample was mixed with 20 µl of NBD-F (4 - fluoro-7-nitrobenzofurazan). Samples were then heated at 60 °C for 5 min for the amine-labeling reaction with NBD-F. The sample solutions were cooled, and the reaction was stopped by addition of 70 µl 1% acetic acid in methanol. Then 50 µl of each sample solution was used for HPLC fractionation. Throughout the NBD-F labeling reaction the samples were protected from light.

HPLC system

Chromatographic analysis was performed on an LC-VP system (Shimadzu, Kyoto, Japan) consisting of the following components: SIL-10AD, LC-10AT_{VP}, FCV-10AL_{VP}, DGU-12A, CTO-10AS_{VP}, RF-10AXL, and SCL-10A_{VP}. The LC solution software controlled both the HPLC system and the data analysis. To quantify serine (Ser) and Asp, mobile phase A (1% tetrahydrofuran, 0.02% TFA, 10% ACN in MilliQ) and mobile phase B (1% THF, 0.02% TFA in ACN) were used for the elution of D/L-amino acid mixtures; a gradient from 0% B to 25% B over 50 min and 25% B to 75% B in 40 min was used through a TSKgel ODS-80TsQA reverse-phase column (Tosoh, Tokyo, Japan). After substitution of the mobile buffer with methanol, each of the amino acid fractions was separated into D- and L- with an OA2500-S chiral column, (SCAS, Osaka, Japan). The mobile phase of chiral-HPLC was 5 mM citric acid buffer (5 mM citric acid in methanol). The concentrations of D/L-Ser and D/L-Asp were calculated as follows: the enantiomeric ratio of

NBD-D-Ser or NBD-L-Ser was quantified as a percentage of the total (D+L)-Ser concentration, which was determined by the peak area ratio of the Ser derivative on the chromatogram recorded by LC solution (Shimadzu). The NBD-D-Asp and NBD-L-Asp ratios were calculated in the same way.

Immunohistochemistry

Cryosections (10 µm) of brains obtained from 8-week-old SAMR1, 20-week-old SAMR1, and 20-week-old SAMP8 were air-dried and washed in PBS. After being blocked with 5% skim milk, sections were probed with anti-PP1C (Exalpa Biologicals, MA, USA) overnight at 4 °C. The second antibody incubation, with Alexa488 conjugated goat anti-rabbit IgG (Invitrogen, CA, USA) was performed over 1.5 h at room temperature while shielded from light.

After a second blocking with 5% skim milk, sections were probed with anti-Tau (Cell Signaling Technology, MA, USA). The second antibody incubation, with anti-mouse IgG Dylight 649 (Jackson Immuno Research Europe, Suffolk, UK) was performed over 1.5 h at room temperature. Samples were mounted in Vectashield™ Mounting Medium (Vector Laboratories, CA, USA) and examined under an FV1000 confocal microscope (Olympus, Tokyo, Japan).

Results

Protein expression

Western blotting was used to detect the presence of Alzheimer's disease-related proteins in cerebral homogenates obtained from 8-week-old SAMR1, 20-week-old SAMR1, and 20-week-old SAMP8 (Fig. 1). The GLRX antibody detected one band (~12 kDa) that exhibited equal intensity in each experimental group. Similar data was obtained for PP1C (36 to 38 kDa). The NF-L antibody detected two bands, one at approximately 68 kDa and one smaller. I regarded the smaller band as insufficiently denatured NF-L.

Overall, no substantial differences were observed in the expression of these Alzheimer's disease-related proteins among the three types of cerebral homogenate.

Western blotting images of immunoprecipitates of Alzheimer's disease-related proteins are shown in Figure 2.

HPLC detection of the ratio of D-amino acids to L+D amino acids in Alzheimer's disease-related proteins

Many reports have described the biological functions of D-Ser and D-Asp and the related diseases (Fujii et al.

Figure 1 Western blotting analysis of Alzheimer's disease-related proteins obtained from cerebral cortices of SAMP8 and SAMR1 at the indicated age. Alzheimer's disease-related-proteins in SAM cerebral cortices 1 extracts were detected on PVDF membranes. GLRX, 12 kDa; NF-L, 68 kDa; PP1C, 36 to 38 kDa. Each cerebral cortices extract was loaded at a concentration of 20 µg/lane.

1994, Kaneko et al.1995, Ritz-Timme et al. 2003). The molecular characteristics of Ser and Asp cause them to

Figure 2 Western blotting analysis of Alzheimer's disease-related proteins extracted by immunoprecipitation.

Figure 3 Ratios of D-Ser and D-Asp to total Ser + Asp in Alzheimer's disease-related proteins in cerebrum of SAMR1 and SAMP8 at the indicated age. * $P < 0.05$, Student's t-test.

tend to isomerize from L-amino acid to D-amino acid more easily than other amino acids. This is why I focused on the isomerization of Ser and Asp.

I used a reverse-phase column to separate the Ser and Asp amino acid fractions from protein solutions of GLRX, NF-L, and PP1C obtained from the cerebral cortex of 8-week-old SAMP8, 20-week-old SAMP8, and 20-week-old SAMR1. The D/L-mixtures of the Ser fraction and Asp fraction were then separated into D- and L- amino acid fractions with a chiral column, and the ratio of each D-amino acid to the D+L-mixture was calculated (Table1, Fig. 3).

The statistical difference was determined by Student's t test. Difference with $P < 0.05$ was considered significant. The D-Ser ratios in GLRX did not indicate a significant difference (Fig. 3). In the D-Asp ratios, there was no significant difference between 8-week-old SAMR1 and 20-week-old SAMR1 ($P = 0.059$). The D-Asp ratio in 20-week-old SAMR1 was higher than that of 20-week-old SAMP8.

In NF-L, high D-Ser ratios were detected in 8-week-old SAMR1 and 20-week-old SAMR1 (Fig. 3). The cause that is not senescence may influence this change.

The ratio of D-Ser obtained from PP1C was significantly greater in 20-week-old SAMP8 than in 20-week-old SAMR1 ($P = 0.0034$). A non-significant increase was found between 8-week-old SAMR1 and 20-week-old SAMR1 (Table 1, Fig. 3). The D-Asp ratio was significantly greater in 20-week-old SAMR1 than in 8-week-old SAMR1 ($P = 0.0245$), and significantly greater in 20-week-old SAMP8 than in 8-week-old SAMR1 ($P = 0.0299$) (Fig. 3).

Localization of PP1C with Tau in SAM brains

Because changes in D-amino acid content in SAM with aging were detected in PP1C, immunohistochemical analysis was performed to confirm PP1C expression in SAM brains. Expression of PP1C, molecules of which are ubiquitous in animal cells, was observed in the cerebral cortex (Fig. 4). Particularly obvious localization of PP1C in the cell body was apparent. Tau is a collection of microtubule-associated proteins that is involved in microtubule assembly and stabilization in the neuron. Expression of Tau was somewhat localized to the neurites. Distinct co-expression of PP1C and Tau was observed (Fig. 4).

Figure 4 Immunohistochemical and confocal microscopic analysis of Tau and PP1C in SAM cerebral cortices. Upper panels (A, B, C) show Tau localization. Middle panels (D, E, F) show PP1C localization. Lower panels (G, H, I) show merged images of PP1C and Tau. There were no obvious differences in the expression of PP1C and Tau among the SAM of various ages. Scale bars are 300 μm .

Table 1 Ratios of D-Ser and D-Asp to total Ser and Asp in Alzheimer's disease-related proteins from SAM mice of two strains and ages.

Proportions of D-amino acid, D/(D+L) $\pm$ SEM		SAM		
Alzheimer's disease-related protein		SAM		
D-amino acid		SAMR1	SAMR1	SAMP8
		8 weeks	20 weeks	20 weeks
GLRX	D-Ser (n = 2)	0.46% $\pm$ 0.02%	0.33% $\pm$ 0%	0.46% $\pm$ 0%
	D-Asp (n = 3)	2.83% $\pm$ 0.26%	4.14% $\pm$ 0.34%	3.41% $\pm$ 0.09%
NF-L	D-Ser (n = 3)	0.77% $\pm$ 0.51%	0.95% $\pm$ 0.76%	0.43% $\pm$ 0.08%
	D-Asp (n = 3)	2.63% $\pm$ 0.93%	3.00% $\pm$ 0.97%	2.78% $\pm$ 0.29%
PP1C	D-Ser (n = 3)	0.26% $\pm$ 0.02%	0.35% $\pm$ 0.09%	0.40% $\pm$ 0.08%
	D-Asp (n = 3)	2.29% $\pm$ 0.38%	2.83% $\pm$ 0.38%	3.03% $\pm$ 0.65%

Discussion

The results of this study show that the content of D-amino acids in PP1C, an Alzheimer's disease-related protein, increased in aging progressing animal. Replacement of L-amino acids with D-amino acids can be considered to influence the bioactivity of the protein, because it changes physical properties such like structure. PP1 is a serine/threonine-specific protein phosphatase involved in the regulation of a variety of cellular processes, such as cell division, glycogen metabolism, muscle contractility, and protein synthesis (Cohen 2002, Hurley et al. 2007). PP1C is its catalytic subunit. Mouse studies suggest that PP1 functions as a suppressor of learning and memory (Genoux et al. 2002). Because defects in learning and memory are among the signs of Alzheimer's disease (Calley et al. 2010), a relationship between PP1 and Alzheimer's disease can be assumed (Knobloch et al. 2007). Furthermore, Tau, which is regarded as an important molecule in Alzheimer's disease, is a substrate for PP1C (Liu et al. 2005).

Tau is a microtubule-binding protein that serves to stabilize microtubules in growing axons. Tau is hyperphosphorylated in paired helical filaments, the major fibrous components of the neurofibrillary lesions associated with Alzheimer's disease (Gache et al. 1990). Production of pseudophosphorylated Tau, which mimics the disease-associated hyperphosphorylated Tau, causes apoptosis in neuronal cells. Six Tau protein isoforms have been identified, all of which are phosphorylated by glycogen synthase kinase 3 (GSK 3), in vitro (Mandelkow et al. 1992).

SAMP8 is an Alzheimer's disease model animal in which learning and memory defects have been reported (Flood and Morley 1998); hyperphosphorylation of Tau has been detected in SAMP8 brains (Canudas AM et al. 2005). However, expression analysis of GSK 3 isoforms showed no differences in SAMP8 in comparison with age-matched SAMR1 (Canudas AM et al. 2005). This indicates that the phosphorylation state of Tau is regulated not by GSK 3, but in another way.

In this study, the increase of D-amino acids in PP1C with aging, particularly in Alzheimer's disease model animal, SAMP8, was clarified. Replacement of L-Ser and L-Asp with the D-amino acids D-Ser and D-Asp in PP1C will change the physical properties of PP1C. The structural change induced by substitution D-amino acid might cause a low yield of PP1C obtained from 20-week-old SAMP8

in immunoprecipitation, through inhibition of antigen-antibody reaction.

Such change in PP1C may inhibit its Tau-dephosphorylating function. The hyperphosphorylated Tau, which plays a casual role in Alzheimer's disease, seems to be closely associated with PP1C that includes D-amino acids. Hopefully the bioactivity of PP1C containing D-amino acids will become apparent in future studies.

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SAMP8 脳に発現するアルツハイマー病関連タンパク質において 検出された D- アミノ酸量の増加

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要旨：これまで、生物を構成するアミノ酸は L- 体が主体であると考えられてきた。しかし近年、L- アミノ酸の光学異性体である D- アミノ酸の生体内での機能が明らかになりつつある。本研究では、加齢とともに発症リスクの高まるアルツハイマー病に注目し、アルツハイマー病関連タンパク質に含有される D- アミノ酸の加齢に伴う量の変化を解析した。実験にはアルツハイマー病のモデル動物である老化促進モデル動物 SAMP 8 系統 (SAMP8) を用いた。加齢の進んだ SAMP8 脳から抽出したアルツハイマー病関連タンパク質のうち、タンパク質脱リン酸化酵素である PP1C において D- アスパラギン酸と D- セリンの量が増加していることが明らかになった。PP1C はアルツハイマー病の原因分子のひとつである Tau の脱リン酸化作用を持つことから、D- アミノ酸含有 PP1C の生理活性低下と、アルツハイマー病との間に何らかの関連性があることが示唆された。

キーワード：アルツハイマー病、D- アミノ酸、SAMP8、PP1C、加齢