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Protein L-isoaspartyl *O*-methyltransferase inhibits amyloid β fibrillogenesis *in vitro*

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Fibrillar aggregates of β-amyloid peptide (Aβ) are major constituents of the senile plaques found in the brains of patients suffering from Alzheimer's disease (AD). Previous studies have shown that spontaneous isomerization or racemization of aspartyl residues in Aβ peptides leads to conformational changes in the secondary structure and increased aggregative ability of the peptides. Protein L-isoaspartyl *O*-methyltransferase (PIMT, EC 2.1.1.77) is a repairing enzyme converting L-isoaspartyl/D-aspartyl residues in damaged proteins to normal L-aspartyl residues. In this study it was investigated, whether PIMT is able to modulate Aβ fibrillogenesis *in vitro* by methylation of isoaspartyl residue using purified 5Aβ and PIMT. A Thioflavin-T (Th-T) binding assay conducted after aging Aβ *in vitro* (37 °C, pH 7.4 in PBS) revealed that PIMT inhibited the increase of fluorescence caused by amyloid fibrillogenesis. Western blot analysis revealed that high molecular Aβ aggregates (>200 kDa) only occurred during Aβ incubation, while they were reduced in response to incubation with PIMT and AdoMet. Additionally, circular dichroism (CD) showed that the β-sheet structure was increased in Aβ peptides in a time-dependent fashion, while PIMT suppressed the β-sheet transition after 24 h. Finally, transmission electron microscopy (TEM) revealed that PIMT reduced the size of the Aβ aggregates and induced a different pathway, leading to the formation of amorphous structures. Taken together, these findings indicate that isoaspartyl methylation leads to partial blockade of fibrillogenesis of Aβ by inhibiting the β transition in the Aβ peptide.

1. Introduction

Alzheimer's disease (AD) was first described by the Germen physician Alois Alzheimer (1864–1875) in 1906 in a neuropathological study of the brain of a 51-year-old woman who had exhibited progressive cognitive dysfunction and psychosis (Treusch et al. 2009). AD is characterized by accumulation of extracellular amyloid plaque and intracellular neurofibrillar tangles in human brain tissue (Walsh et al. 1999). AD is currently widespread, and it is expected that the number of patients will radically increase as society ages unless defensive or remedial medications are discovered (Sanders et al. 2009).

Amyloid fibrils and misfolded protein aggregates are pathological characteristics of age-related neurodegenerative diseases such as AD and are believed to be a general cause of dementia (Tsvetkov et al. 2008; Yan and Wang 2006). The senile plaques that accumulate are predominantly composed of aggregated amyloid β (Aβ) peptides (Fukuda et al. 1999). Aβ peptides are 40 or 42 amino acid residues in length and are derived from proteolytic cleavage of amyloid precursor protein (APP), which is a transmembrane protein that functions in cell migration and cell-cell interaction (Haass and Selkoe 2007; Selkoe 1999). Aβ 42 is much more prone to aggregation and more toxic to neurons than Aβ 40 (Yan and Wang 2006). Amyloid β monomer is converted into amyloid fibrils through a sequence of steps that involve the formation of various intermediate assemblages such as oligomers (Hardy and Allsop 1991) and protofibrils (Fukuda et al. 1999; Walsh et al. 1997). However, the characteristics of the pathogenic species of Aβ and the mechanism by which aggregation leads to cell damage are currently the topics of strong debate. In fact, it has been proposed that intermediate species of the Aβ aggregation process, and not fibrils, are the pathogenic species (Johnson and Aswad 1991).

Protein L-isoaspartyl *O*-methyltransferase (PIMT, EC 2.1.1.77) is a protein repair enzyme that converts L-isoaspartyl or D-aspartyl residues in damaged proteins to normal L-aspartyl residues by transferring a methyl group to an α-carboxyl group at an isoaspartate from the *S*-adenosyl methionine (AdoMet) (Roher et al. 1993). This repair enzyme is highly conserved in many species and highly expressed in the brain or testis (Murakami et al. 2008). PIMT-deficient mice manifest neurodegenerative changes that are concomitant with the accumulation of L-isoaspartate in the brain (Selkoe 1999).

Aβ peptides that have been posttranslationally modified by isomerization, racemization, pyroglutamylation and truncation are found in AD brains (Roher et al. 1993; Shin et al. 2003). Three aspartyl residues positioned at 1, 7 and 23 in the Aβ peptide are able to undergo isomerization or racemization. The Asp7 of Aβ from senile plaques has been shown to be modified to L-isoAsp at a rate of 55.0% (Sakai-Kato et al. 2007). Additionally, isomerized Aβ peptide at Asp1, 7 was found to have an increased propensity to form β-pleated sheets *in vitro* (Finder and Glockshuber 2007). Isoaspartate formation of position 23 of Aβ increased fibril formation and deposition of senile plaques (Shin et al. 2003). Moreover, racemization at position

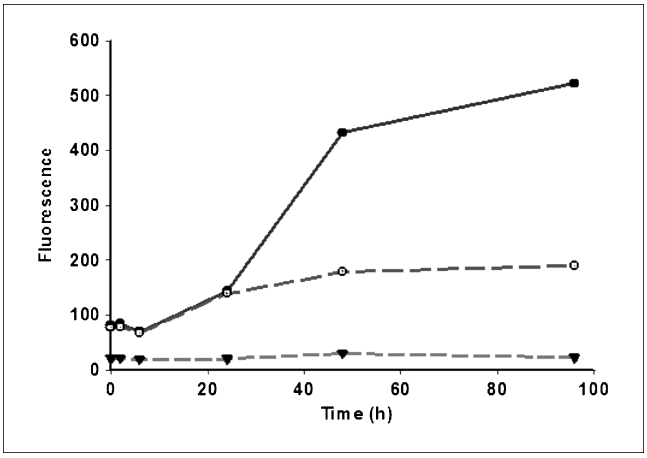

Fig. 1: The effect of PIMT on the fibril formation of Aβ as determined by Th-T assay. ●, Aβ((100 μM); ○, Aβ (100 μM) co-incubated with PIMT (10 μM) and AdoMet (5 μM); ▼, PIMT (10 μM) co-incubated with AdoMet (5 μM). All samples were incubated in PBS (pH 7.4) at 37 °C for 4 days. Each solution (40 μl) was added to 0.1 M Gly-NaOH buffer (pH 8.5) with 5 μM ThT. The fluorescence intensity was measured at an excitation of 450-nm and an emission of 482-nm using a Spectramax M2 fluorescence spectrometer

23 in Aβ peptide accelerated the aggregation and fibril formation (Sanders et al. 2009). Furthermore, Aβ species including isoAsp23 were detected by immunohistochemical analysis in lowa-type familial AD using anti-isoAsp23 antibody (Treusch et al. 2009). Isomerization of protein causes structural changes and abnormal function or enhances the aggregative ability in modified proteins. Although isoAsp is a substrate of PIMT, it has not been determined if it is able to convert isoAsp residues in Aβ.

In this study, we examined the effect of PIMT on the aggregation of Aβ *in vitro*. Pentamers of Aβ peptides that were expressed and purified from *E. coli* were aged with purified PIMT. We used thioflavin-T (ThT) as an indicator of fibril formation, circular dichroism (CD) to monitor the change of secondary structure, while Western blot and transmission electron microscopy were used to observe the effects of PIMT on the morphology of aggregates.

2. Investigations and results

To evaluate the ability of PIMT to interfere with Aβ fibril formation, we used a thioflavin-T fluorescence assay to quantify amyloid fibril formation. As shown in Fig. 1, the ThT fluorescence curve showed a sigmoid shape representing three phases: the nucleation phase (lag phase), the polymerization phase and the equilibrium phase (stationary phase). The fluorescence curves of Aβ were similar regardless of whether the samples were co-incubated with or without PIMT and AdoMet for 24 h. Additionally, treatment of the Aβ sample with PIMT and AdoMet had no effect on the nucleation phase, whereas Aβ that was incubated alone showed a significant increase in fluorescence between 24 and 48 h during the polymerization phase. Finally, Aβ that was incubated with PIMT and AdoMet exhibited only a slight increase in fluorescence, suggesting a high inhibitory effect against amyloid fibrillogenesis.

To estimate the potential β-sheet structure of each sample, we conducted CD spectral analysis. The Aβ peptide exhibited a negative maximal value at 218 nm, where a β sheet structure is typically detected. In addition, the curves of the CD spectra showed a time-dependant increase in β transition (Fig. 2A). The β sheet content in the Aβ peptide that was co-incubated with PIMT and AdoMet remained at the initial value during incubation for 24 h (Fig. 2B-D). Additionally, the curves of Aβ incubated with or without PIMT and AdoMet showed the same content of β sheet structure based on the absorbance at 218 nm (Fig. 2B). However, as the incubation time increased, the gap between the two curves at 218 nm gradually increased, with the content of β sheet in the Aβ peptide incubated alone increasing and the level in the Aβ peptide incubated with PIMT remaining unchanged. Taken together, these CD data indicate that PIMT showed an inhibitory tendency against the time-dependant increase in the β-pleat sheet in the Aβ peptide.

To determine if PIMT inhibits the formation of Aβ high molecular aggregates, Western blot analysis using SDS-PAGE was conducted. As shown in Fig. 3, high molecular aggregates (>200 kDa) of Aβ appeared rapidly and the band intensity of the monomer decreased after 24 h when Aβ was incubated alone (Fig. 3 lane 1, 3, 5 and 7). However, when Aβ was co-incubated with PIMT and AdoMet, the intensity was reduced, indicating that the aggregates were smaller (Fig. 3 lane 5 and 7). These findings demonstrate that PIMT partially inhibited the formation of large aggregates of Aβ.

Finally, we observed the morphology of Aβ aggregates by TEM. As shown in Fig. 4A, there were no distinct structures observed in micrographs of fresh Aβ. When the samples were incubated for more than 48 h, we observed a large amount of aggregates and a high content of amyloid fibrils (Fig. 4B). However, the apparent difference between the Aβ aggregates with or without PIMT was confirmed. Inspection of a large number of TEM fields failed to reveal fibrillar aggregates (Fig. 4C). The shape of the aggregates in the micrographs of Aβ incubated with PIMT were unusual and fibrils were not present (Fig. 4D). These findings indicate that PIMT inhibited the formation of fibrils and converted the aggregation pathway to induce the formation of an amorphous structure.

3. Discussion

Although Aβ is produced in normal individuals, amyloid fibril formation is only observed in the brains of patients with AD. Therefore, unusual factors must enhance the aggregation of Aβ protein in AD. We focused on the effects of isomerization/racemization of aspartyl residues on Aβ aggregation because, in addition to being found in AD, isomerized/racemized Asp is associated with other aged proteins and diseases. However, it is debatable whether these modifications in Aβ are the reason for or a consequence of the aggregation of Aβ because isomerization/racemization of Asp 23, which had been believed to play a principal role in Aβ aggregation, but recently has not effects on that (Murakami et al. 2008). Nevertheless, many studies conducted to evaluate modifications of Asp 1 and 7 had been accumulated excepting isoAsp 23. The substitution of Asp 1 and Asp 7 with isoaspartyl residues on the Aβ peptide is known to increase the content of β-pleat sheets (Fabian et al. 1994; Fukuda et al. 1999). In addition, racemization of the Aβ peptide positioned at Asp 7 and 23 is known to accelerate fibrillogenesis by increasing their β-pleat sheet content (Fabian et al. 1994; Roher et al. 1993). Particularly, isomerization of the Asp 7 residue results in zinc-induced oligomerization of Alzheimer's disease amyloid β (1–16) peptide (Tsvetkov et al. 2008). As a result, it is possible that isomerization or racemization of Asp in Aβ peptide is able to cause amyloid fibrillogenesis.

In most studies that have been conducted to date, aggregation experiments have employed monomer, truncated or modified Aβ peptide to observe the aggregation stages from monomers to fibrils or aggregates (Cohen et al. 2006; Sakai-Kato et al. 2007; Sanders et al. 2009). These experiments have examined the effects of isomerization or racemization in Aβ aggregation using synthesized monomeric peptides containing L-isoAsp

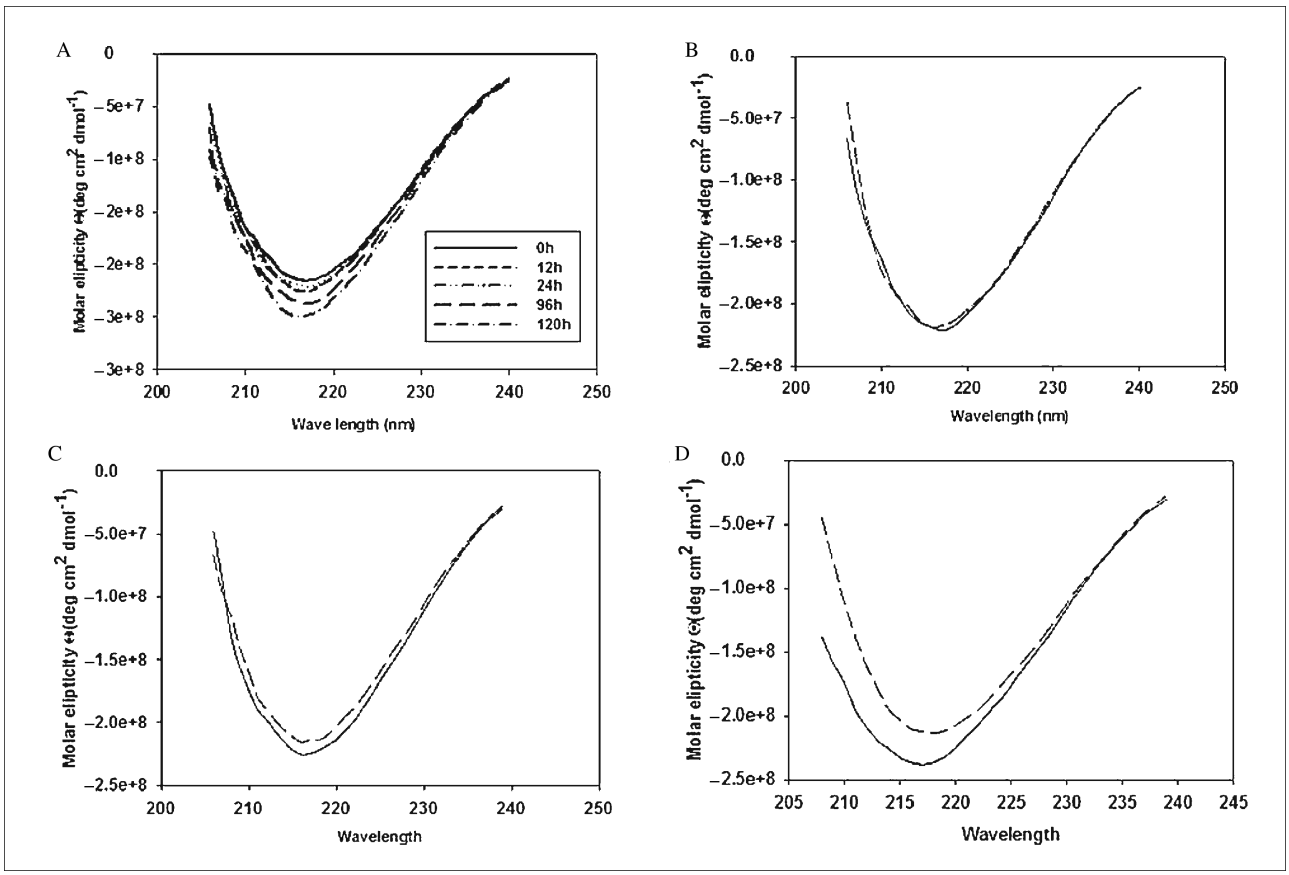

Fig. 2: Secondary structural changes as indicated by the CD spectra. (A) Time-dependent change in the Aβ secondary structure. Aβ (100 μM) incubated in PBS alone at 37 °C for 5 days (B-D) Effect of PIMT on (sheet transition of Aβ. All samples were incubated in PBS (pH 7.4) at 37 °C. Aβ: solid line, Aβ (100 μM) co-incubated with PIMT (10 μM) and AdoMet (5 μM): dotted line (B) 0 h, (C) 12 h, (D) 24 h incubation. All samples were placed in a quartz glass cuvette with a 1-mm path length and immediately transferred to a Jasco J-810 CD spectrometer and the spectra were then recorded at 0.1-nm intervals (50 nm/min, 37 °C). The obtained data were reported in molar ellipticities (deg *cm^3/dmol)

or D-isoAsp. However, in the present study, we used purified pentameric Aβ without modification. The results of this study demonstrated that 5Aβ as an oligomer was conformationally molten and could form amorphous aggregates or be converted to an amyloidogenic nucleus to initiate amyloid fibril formation. Amyloid fibrils that consisted of 5Aβ formed a highly organized structure due to repeating β-pleated sheets and were insoluble.

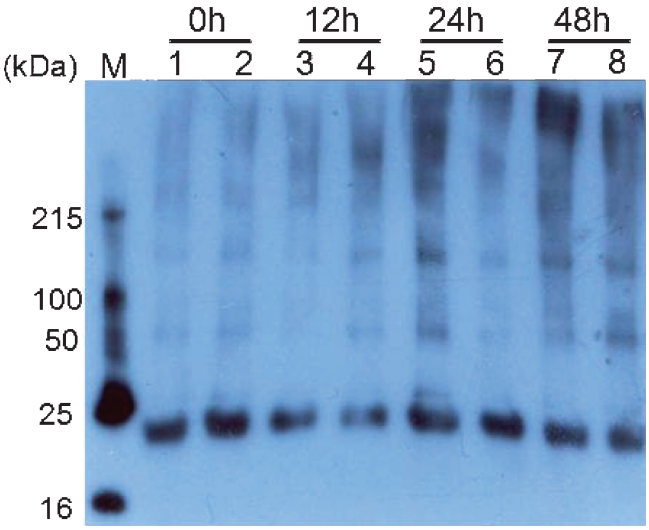

Fig. 3: Western blotting of Aβ incubated with or without PIMT and AdoMet. Aβ (100 μM) was incubated in PBS at 37 °C with or without PIMT (10 μM) and AdoMet (5 μM) for 2 days. Lanes 1, 3, 5 and 7 lanes: Aβ incubation; lanes 2, 4, 6 and 8: Aβ(co-incubated with PIMT and AdoMet. Immunoblotting was performed with monoclonal antibody 6E10. Molecular weight standards are indicated (in kDa) at the left

As shown in Fig. 1, the fluorescence of Aβ increased significantly after 24 h. However, this time for initiation of the polymerization phase was slower than the time required for synthesizing Aβ containing iso/D Asp and Aβ1–42 monomer, for which this phase is typically initiated within 10 h (Fabian et al. 1994; Shin et al. 2003). Generation of spontaneous isomeration or racemization requires proper aging time in the aspartyl residues (Johnson and Aswad 1991). Therefore, it is likely that the delayed initiation of the polymerization phase was caused by spontaneous isomerization or racemization in Aβ peptide positioned at Asp 1, 7 or 23. In addition, the fluorescence level of the stationary phase in Aβ that was incubated alone was lower than that of Aβ incubated with PIMT and AdoMet. This was likely due to normal Aβ peptide being recovered from isomerization or racemization by PIMT (Fig. 1). The effect of PIMT on the tendency of Aβ to form β-sheet structures was clear in the CD spectra. Specifically, the Th-T binding assay showed that the formation of fibrils increased after 24 h, while the β-sheet contents of Aβ increased remarkably from 24 h to 120 h (Fig. 2A). This increase was due to the spontaneous accumulation of iso/D-Asp, succinimide or isoaspartyl methyl ester, which likely increased the propensity of a polypeptide to undergo β-sheet formation (Orpiszewski and Benson 1999). PIMT inhibited the increase in β-sheet content in the Aβ peptide through methylation of isoAsp (Fig. 2B-D). Western blot analysis revealed the formation of Aβ aggregates as a band location and intensity on the gel. The 5Aβ monomer, which was under 25 kDa, was predominant until 12 h, whereas high molecular aggregates above 200 kDa were observed at 24 and 48 h. Similar to the ThT assay, which showed that 5Aβ required a nucleation time of approximately 12 h, peptides underwent spontaneous modifica-

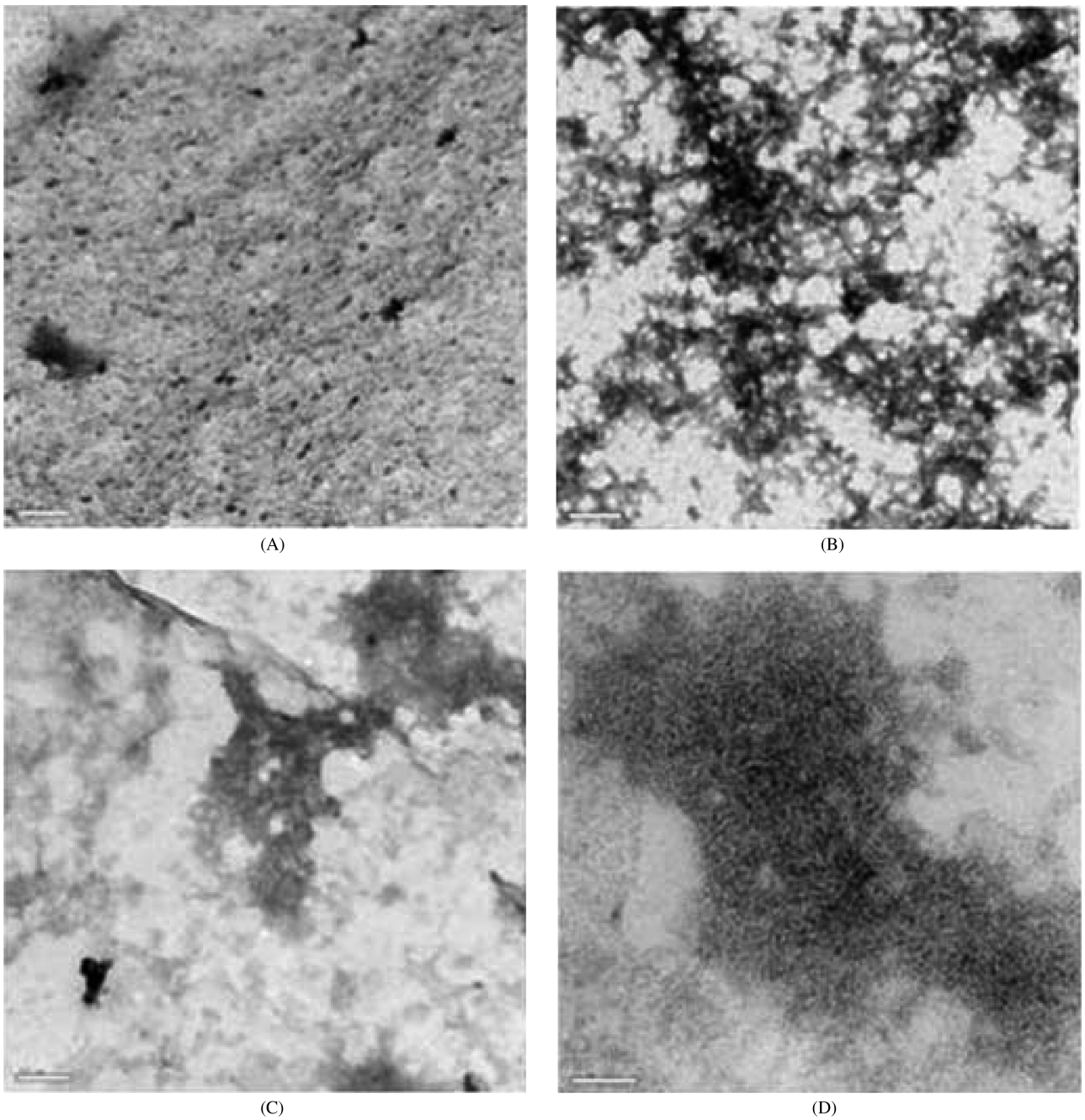

Fig. 4: Transmission electron microscopy (TEM) images of Aβ aggregates. Aβ (100 μM) was incubated with or without PIMT (5 μM) and AdoMet (5 μM) in PBS (pH 7.4) at 37 °C. The protein samples were then applied to carbon-coated Formvar grids and allowed to air-dry. After staining for 2 min with 2% uranyl acetate, the samples were observed under a JEOL JEM-3011 high resolution transmission electron microscope. (A) Fresh Aβ, (B) Aβ incubation for 48 h, (C, D) Aβ co-incubation with PIMT and AdoMet for 48 h. The magnification of (A-C) was × 20,000 while that of (D) was × 50,000

tion for 12 h rapidly and then formed high molecular weight aggregates after 12 h. However, these aggregates were reduced by co-incubation with PIMT and AdoMet, indicating that PIMT partially prevented the formation of 5Aβ aggregates during the polymerization phase.

Longer expending fibers, fiber complexes and characteristics of Aβ1–42 were observed in the TEM images of 5Aβ incubated alone for 48 h than in the other samples. These aggregates exhibited thicker forms than those formed by Aβ monomers, which was caused by the high 5Aβ concentration (100 μM) used in our experiment. When Aβ was co-incubated with PIMT and AdoMet, amorphous structures were detected without the fibril complexes that were observed when Aβ was incubated alone. These amorphous structures pass through the aggregation pathway which is differ to form the fibrils (Treusch et al. 2009).

In conclusion, this study demonstrated that PIMT inhibited the formation of fibrils by preventing an increase of β-sheet tendency in Aβ peptides, which induced the formation of amorphous structures. Our results indicate that spontaneous isomerization or racemization is one of the causes of fibrillogenesis and that PIMT may be a potent inhibitor of amyloid fibril formation.

4. Experimental

4.1. Materials

KSI-$(A\beta_{1-42})_5$-His_6 transformed *E. coli* strain BL21(DE3)pLysS was kindly provided by Professor Mook-Jung (Seoul National University, Korea). Recombinant porcine brain protein isoaspartyl methyltransferase (r-pbPIMT-His_6) transformed *E. coli* strain BL21 and anti-Aβ1–17 antibody (6E10) were purchased from Signet Laboratories Inc. (Dedham, MA, USA).

S-adenosylmethionine (AdoMet) and thioflavin T (ThT) were acquired from Sigma (Saint Louis, MO, USA).

4.2. Expression of KSI-($A\beta_{1-42}$)$_5$-His$_6$(5Aβ) and r-pbPIMT-His$_6$

The expression procedures were based on the method described by Kuliopulos et al. (1994) with some modifications. KSI-($A\beta_{1-42}$)$_5$-His$_6$ (5Aβ) was expressed in *E. coli* strain BL21(DE3)pLysS. A starter culture was grown overnight in 5 ml of LB medium supplemented with 100 μg/ml ampicillin. The starter culture was diluted 100 fold into 500 ml of fresh LB medium, and the cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the absorbance at 595 nm reached an OD of 0.3∼0.5. The expression culture was grown at 28 °C for 6 h, after which the cells were collected by centrifugation (4,000 x g, 10 min, 4 °C) and stored at -20 °C until use. The expression of pETPIMT was induced using a previously described protocol (Kuliopulos et al. 1994).

4.3. Purification of 5Aβ and PIMT

Purification of 5Aβ was conducted according to the method described by Kuliopulos et al. (1994), with some modifications. Briefly, the cell pellets were resuspended in 50 ml of binding buffer (5 mM imidazole, 40 mM Tris-HCl, pH 7.9, 500 mM NaCl), after which they were disrupted by sonication in binding buffer and then centrifuged at 12, 000 × g for 10 min at 4 °C to obtain the insoluble pellet. The insoluble fraction was then dissolved in 50 ml of binding buffer containing 8 M urea. The dissolved pellet was then centrifuged at 12, 000 × g for 10 min at 4 °C, after which the supernatant was loaded at a flow rate of 1 ml/min onto a HisTrap™ column (Amersham Pharmacia Biotechnology, Upsala, Sweden) that had previously been equilibrated with binding buffer containing 8 M urea. The 5Aβ fractions were then eluted with equilibration buffer containing 300 mM imidazole. Next, the elution fractions were dialyzed against PBS containing 8 M, 4 M and 2 M urea, and PBS alone. The cell pellet containing rPIMT was then re-suspended in binding buffer (pH 8.0, 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) at 4 ml/g wet weight. The resuspended cells were then disrupted by sonication and centrifuged for 30 min at 12, 000 × g to remove the intact cells and cell debris. Next, the supernatant was loaded onto a HisTrap™ column that had previously been equilibrated with binding buffer at a flow rate of 1 ml/min. The rPIMT fractions were subsequently eluted with binding buffer containing 200 mM imidazole. The enzyme fractions were concentrated by ultra-filtration using an Amicon apparatus (Amicon Co., Beverly, CA, USA) and membrane (Millipore Co, Billerica, MA, USA), after which they were digested with r-enterokinase (Novagen Co., Madison, WI, USA) to remove the his-tag from the PIMT. Finally, 10 units of r-enterokinase were incubated with 30 mg of PIMT in buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl and 2 mM $CaCl_2$ for 18 h at 20 °C.

4.4. Protein aging

Purified 5Aβ was diluted with PBS to a concentration of 100 μM and then incubated at 37 °C for 1, 12, 24 and 48 h in the presence or absence of 10 μM purified PIMT and 5 μM AdoMet, which is a methyl-group donor.

4.5. Thioflavin-T binding assay

Each Aβ solution was diluted to 10 μM in 5 μM thioflavin T and 0.1 M glycine-NaOH buffer (pH 8.5, 37 °C). After 3 min of binding time in the dark, each sample was transferred to a 96 well plate and the fluorescence intensity was measured at an excitation of 450 nm and an emission of 482 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) controlled by the SoftMax Pro v5 software (Molecular Devices).

4.6. Circular dichroism

Spectral data were obtained using a J-810 CD spectrometer (Jasco, Tokyo, Japan) at 37 °C with a 50 nm/min scan rate and a 1 nm bandwidth from 200 to 250 nm. Samples were placed in a quartz glass cuvette with a 1 mm path length and then immediately transferred to a spectrometer. The baseline was acquired in PBS buffer that did not contain Aβ, PIMT or AdoMet. The obtained data were expressed as ellipticities.

4.7. Western blot

Concentrations of Aβ were quantified using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The concentrations of the samples were then adjusted using loading buffer that contained β-mercaptoethanol as a reducing agent, boiled for 5 min and then separated by 5% SDS-PAGE. Next, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bilerica, MA, USA, as reported previously (Mollah et al. 2011)). The membranes were then blocked for 2 h in TBS containing 0.5% Tween-20 and 5% (w/v) dry skim milk powder, after which they were incubated overnight with anti-Aβ 1–17(6E10) antibody as the primary antibody. Next, the membranes were washed with TBS-T and incubated for 2 h with an anti-mouse lgG, HRT-linked secondary antibody (Millipore, Bedford, MA, USA). For chemiluminescence detection, the probed blots were incubated for 5 min with Immobilon Western HRT substrate (Millipore, Bedford, MA, USA as reported previously (Kim et al. 2010)).

4.8. Transmission electron microscopy

Aβ (100 μM) samples were incubated alone or with PIMT (10 μM) and AdoMet (5 μM) for 0 and 48 h, after which they were absorbed onto a carbon coated Formvar film mounted on 200 mesh copper grids (Hyun, Seoul, Korea) and blotted with filter paper. The grids were then allowed to air dry, after which they were stained for 10 sec with 2% uranyl acetate and viewed using a JEOL JEM-3011 high resolution transmission electron microscope.

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