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Non-toxic conformer of amyloid β may suppress amyloid β-induced toxicity in rat primary neurons: Implications for a novel therapeutic strategy for Alzheimer's disease

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ABSTRACT

The 42-mer amyloid β-protein (Aβ42) oligomers cause neurotoxicity and cognitive impairment in Alzheimer's disease (AD). We previously identified the toxic conformer of Aβ42 with a turn at positions 22–23 ("toxic" turn) to form oligomers and to induce toxicity in rat primary neurons, along with the non-toxic conformer with a turn at positions 25–26. G25P-Aβ42 and E22V-Aβ42 are non-toxic mutants that disfavor the "toxic" turn. Here we hypothesize that these non-toxic mutants of Aβ42 could suppress Aβ42-induced neurotoxicity, and examined their effects on the neurotoxicity, aggregation, and levels of the toxic conformer, which was evaluated by dot blotting using a monoclonal antibody (11A1) against the toxic conformer. G25P-Aβ42 and E22V-Aβ42 suppressed the neurotoxicity and aggregation of Aβ42 as well as the formation of the toxic conformer. The neurotoxicity induced by Aβ42 was also significantly reduced by the treatment of 11A1, but not of Aβ-sequence specific antibodies (6E10 and 4G8). Since recent studies indicate that Aβ oligomers contain parallel β-sheet, the present results suggest that the non-toxic mutants of Aβ42 without the "toxic" turn could prevent the propagation process of the toxic conformer of Aβ42 resulting in suppression of the formation of the toxic oligomers. This could be a promising strategy for AD therapeutics.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by the deposition of amyloid fibrils [1]. The deposits are mainly composed of 40- and 42-mer amyloid β-proteins (Aβ40, Aβ42), which are produced from amyloid β-protein precursor by two proteases, β- and γ-secretase, respectively [2]. Aβ42 is considered to play a pivotal role in the pathogenesis of AD because it aggregates faster and induces more potent neurotoxicity than Aβ40 [3]. There are accumulated studies that the intermediate assemblies (oligomers) of Aβ contribute to the neurotoxicity, synaptic loss, and behavioral disorder [4,5]. In spite of intensive researches targeting Aβ42 oligomers in the world, the ideal therapeutic medicines have not yet been developed. One of the reasons might be scarce information on the structure related to the neurotoxicity of Aβ42.

Our group previously identified the toxic conformer of Aβ42 with a turn at positions 22 and 23 ("toxic" turn) and the non-toxic conformer with a turn at positions 25 and 26 [6]. We also developed a monoclonal antibody (11A1) targeting the "toxic" turn in Aβ42 [7], which detected the intracellular Aβ as well as extracellular senile plaques in the brain sections of sporadic AD patients [7,8] and the induced pluripotent stem cells (iPSCs)-derived neurons from familial and sporadic AD patients [9]. These studies suggest that the toxic conformer could be involved in the pathogenesis of AD. It is therefore indispensable to develop a strategy to remove the toxic conformer of Aβ42 for AD therapeutics without adverse effects.

Abbreviations: AD, Alzheimer's disease; Aβ40, 40-mer amyloid β-protein; Aβ42, 42-mer amyloid β-protein; DIV, days in vitro; IgG, immunoglobulin G; iPSCs, induced pluripotent stem cells; MTT, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; Th-T, thioflavin T; Veh, vehicle; Wt, wild-type.

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In previous solid-state nuclear magnetic resonance (NMR) analyses [6], the ratio of the non-toxic conformer in the fibrils of wild-type (Wt) Aβ42 was higher than that in the fibrils of E22K-Aβ42 (Italian-type mutant), which induce more potent neurotoxicity than Wt-Aβ42 [10]. The mutants G25P-Aβ42 and E22V-Aβ42 are non-toxic and are unlikely to form "toxic" turn structure [11]. We hypothesized that these non-toxic mutants of Aβ42 hinder the formation of the "toxic" turn in Aβ42. This study validates this hypothesis by demonstrating the toxic effects of these mutants in rat primary neurons by 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and aggregation by thioflavin T (Th-T) test. The levels of the toxic conformer were also evaluated by dot blot using 11A1 antibody. Furthermore, the neutralizing effects of 11A1 as well as other Aβ-sequence specific antibodies (6E10 and 4G8) on Aβ42-induced neurotoxicity were tested.

2. Material and methods

2.1. Materials

Neurobasal medium and B-27 supplement were purchased from Life Technologies (NY, US). Sodium glutamate, L-glutamine, 1 mol/L ammonium solution, MTT, 2-propanol were purchased from Nacalai Tesque (Kyoto, Japan). The antibodies against Aβ1–17 (6E10) and Aβ17–24 (4G8) were purchased from Covance (CA, US). Antibody against the toxic turn of Aβ42 (11A1) was provided by Immuno-Biological Laboratories (IBL: Gunma, Japan). Immunoglobulin G (IgG) was purchased from Vector Laboratories (CA, US).

2.2. Neuronal cultures

Animals were treated in accordance with the guidelines of the Kyoto University Animal Experimentation Committee and the guidelines of The Japanese Pharmacological Society. Neuronal cultures were obtained from cerebral cortices of fetal Wistar rats (Nihon SLC, Shizuoka, Japan) at 17–19 days of gestation as described previously [12,13]. Cultures were maintained in Neurobasal medium with 2% B-27 supplement, 25 μM sodium glutamate, and 0.5 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2. After 4 days in culture, the medium was replaced with sodium glutamate-free Neurobasal medium. Only mature cultures (8–12 days in vitro (DIV) were used for the experiments. In all experiments, B-27 supplement without antioxidants was utilized during the treatment of Aβ42 as described previously [14].

2.3. MTT assay

Neurotoxicity was assessed by MTT assay according to the previously reported protocol [10] with slight modifications. Aβ42, synthesized as previously reported [10], was dissolved in 0.02% NH_4OH to 200 μM. After 30 min of pre-incubation on ice, Aβ42 solution diluted by 0.02% NH_4OH to the appropriate concentrations was added to the culture medium (1 or 10 μM) for dissolution. Final concentration of NH_4OH was 0.002% in the culture medium. After treatment of Aβ42 (1 μM from 8 to 12 DIV or 10 μM from 10 to 12 DIV), MTT assay was performed. After incubation of Aβ42 at 37 °C for 2 or 4 days, the culture medium was replaced with medium containing 0.5 mg/mL MTT, and cells were incubated for 30 min at 37 °C. 2-Propanol was added to lyse the cells, and absorbance was measured at 595 nm with an absorption spectrometer (microplate reader model 680, Bio-Rad Laboratories, CA, US). The absorbance obtained by the addition of vehicle was taken as 100%. The medium of vehicle treatment of each experiment contained 0.002% NH_4OH.

2.4. Dot blot

Aβ42 was dissolved in 0.02% NH_4OH at 200 μM. After 30 min of pre-incubation on ice, Aβ42 solution was diluted to 20 μM by 50 mM phosphate-buffered saline (PBS), and was incubated at 37 °C. At each time point, Aβ42 solution was gently mixed, and 2 μL of the solution was applied to a methanol-hydrophilized PVDF membrane as previously described [14]. After 10 min, the membrane was blocked by 2.5% non-fat milk in 10 mM tris-buffered saline containing 0.1% Tween-20. After blocking, the membrane was incubatedwith 11A1 antibody (1 μg/mL) [7] overnight at 4 °C, followed by the incubation with the secondary antibody for 1 h at room temperature. Spots were visualized with an enhanced chemiluminescence detection system using image reader (RAS4000, Fujifilm, Tokyo, Japan).

2.5. Thioflavin T (Th-T) assay

Aggregation of Aβ42 was evaluated by Th-T assay according to the previously reported protocol [10] with slight modifications. Each Aβ derivative was dissolved in 0.02% NH_4OH at 200 μM. The peptide solution (20 μM) diluted with 50 mM PBS was incubated at 37 °C for 0–8 h. Two microliters of each Aβ solution was added to 198 μL of 5 mM Th-T in 50 mM Gly-NaOH, pH 8.5. Fluorescence intensity was measured at 420 nm-excitation and 485 nm-emission with spectroscopic microplate reader (FLEX STATION II, Molecular Devices, CA, US).

2.6. Statistics

The statistical significance of differences was analyzed by one-way analysis of variance and post hoc multiple comparisons using Tukey's test. Statistical significance was defined as $P < 0.05$. All data were expressed as the mean ± SEM.

3. Results

3.1. Effects of G25P-Aβ42 and E22V-Aβ42 on Aβ42-induced neurotoxicity

Proline is known as an inducer of turn structure, while it hardly exists in β-sheet. On the other hand, valine is used as a turn breaker [15]. We investigated the effect of G25P-Aβ42, a mutant that induces a "non-toxic" turn at positions 25–26, on the Wt-Aβ42-induced toxicity by MTT assay using rat primary neuronal cultures. Wt-Aβ42 decreased the cell viability almost to 60% at the concentration of 10 μM for 2 days, whereas G25P-Aβ42 (10 μM) significantly rescued Wt-Aβ42-induced neurotoxicity (up to approximately 80%) (Fig. 1). E22 V-Aβ42, a mutant that disfavors a "toxic" turn at positions 22–23, also prevented Wt-Aβ42-induced neurotoxicity, and the viability was slightly higher than that of G25P-Aβ42. Indeed, G25P-Aβ42 and E22V-Aβ42 showed no neurotoxicity (Fig. 1). Similarly, the neurotoxicity induced by Wt-Aβ42 (1 μM for 4 days) was decreased by the addition of G25P-Aβ42 (1 μM) or E22V-Aβ42 (1 μM) (data not shown).

3.2. Effects of G25P-Aβ42 and E22V-Aβ42 on the formation of toxic conformer of Aβ42

We previously showed that the toxic conformer of Aβ42 could readily form oligomers [6], and that the increase of the toxic conformer preceded neurotoxicity in primary neurons [14]. To examine the effect of G25P-Aβ42 and E22V-Aβ42 on the formation of 11A1-reactive Aβ42 aggregates, we performed dot blot using 11A1 antibody for shorter incubation time (4 h) than the incubation time (48 h) in MTT test. In the Wt-Aβ42 solution, the

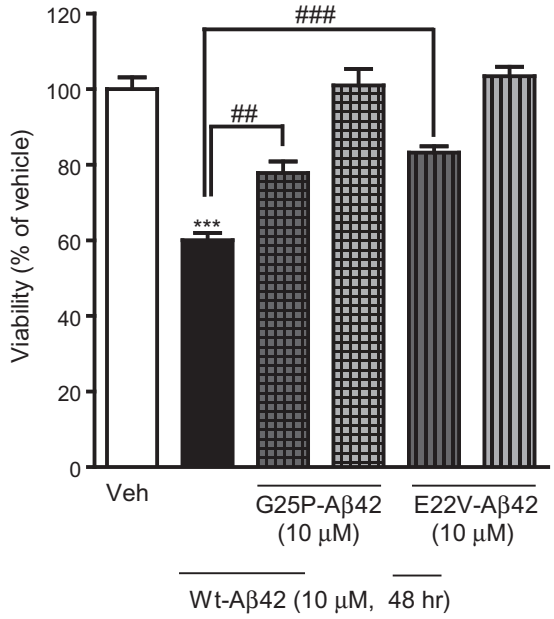

Fig. 1. Effects of G25P-Aβ42 and E22V-Aβ42 on Aβ42-induced neurotoxicity by MTT assay. Rat primary neurons were exposed to Wt-Aβ42 (10 μM) for 2 days in the presence or absence of G25P-Aβ42 (10 μM) or E22V-Aβ42 (10 μM) at 37 °C. Veh, vehicle. $^{***}P < 0.001$ vs. vehicle, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

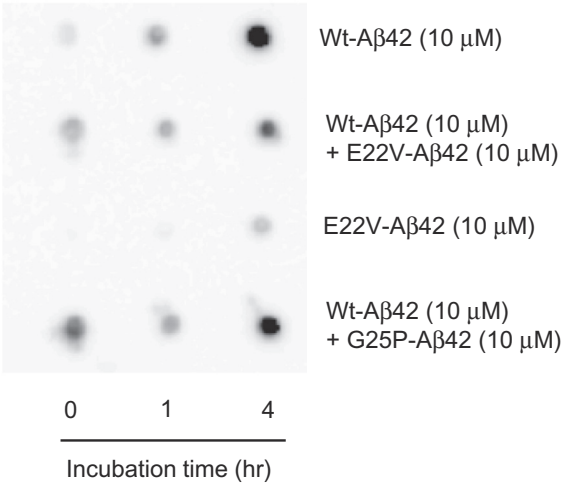

Fig. 2. Effects of G25P-Aβ42 and E22V-Aβ42 on the formation of toxic conformer of Aβ42 by dot blot. Dot blots were performed using 11A1 after incubation for 0–4 h in the Wt-Aβ42 solution (10 μM) in the presence or absence of G25P-Aβ42 (10 μM) or E22V-Aβ42 (10 μM) at 37 °C.

immunoreactivity of 11A1 increased in a time-dependent manner, and these results are in good agreement with the previous ones [14]. In a similar ratio (1:1) to the toxicity test, when Wt-Aβ42 (10 μM) was co-incubated with G25P-Aβ42 or E22V-Aβ42 (10 μM), E22V-Aβ42 suppressed the levels of the toxic conformer of Wt-Aβ42 (Fig. 2). We confirmed that E22V-Aβ42 showed almost no signals. G25P-Aβ42 also showed suppressive effect against the formation of the toxic conformer. However, the suppressive effect of G25P-Aβ42 was not so potent as that of E22V-Aβ42, but G25P-Aβ42 did not exacerbate the formation of toxic conformer of Wt-Aβ42 (Fig. 2). Given the recent structural studies that Aβ oligomers contained parallel β-sheet [16], these results imply that the non-toxic conformer might suppress the levels of toxic conformer by direct interaction with the toxic conformer of Aβ42.

3.3. Effects of G25P-Aβ42 and E22V-Aβ42 on the aggregation of Aβ42

Next, we carried out Th-T tests to evaluate the effect of G25P-Aβ42 and E22V-Aβ42 on the aggregation of Wt-Aβ42. In a similar ratio (1:1) to the toxicity test, G25P-Aβ42 (10 μM) or E22V-Aβ42 (10 μM) completely suppressed the increase in Th-T fluorescence of Wt-Aβ42 (10 μM), respectively (Fig. 3A and C). On the other hand, Wt-Aβ42 (20 μM) showed the strong fluorescence, whereas G25P-Aβ42 (Fig. 3A) and E22V-Aβ42 (Fig. 3B) did not. Interestingly, in another ratio (1:3) of non-toxic Aβ42 (5 μM) to Wt-Aβ42 (15 μM), E22V-Aβ42 inhibited the aggregation of Wt-Aβ42 more strongly than G25P-Aβ42 (Fig. 3B and D). Neither G25P-Aβ42 (5 μM) nor E22V-Aβ42 (5 μM) aggregated (Fig. 3B and D). The larger suppressive effect of E22V-Aβ42 on the aggregation than G25P-Aβ42 is consistent with that on the levels of toxic conformer (Fig. 2). These indicate that the E22V-Aβ42 containing a turn-breakable sequence might inhibit the process of transformation into β-sheet of Aβ42 required for its aggregation.

3.4. Effects of anti-Aβ42 antibodies on Aβ42-induced neurotoxicity

Neutralization using Aβ antibody is also one of the approaches to remove the toxic conformer of Aβ42 for AD therapeutics. We carried out the neutralization in the MTT test using conformer-specific 11A1 together with sequence-specific antibodies, 6E10 (Aβ1–17) and 4G8 (Aβ17–24), as a controls. We treated cells exposed to Wt-Aβ42 (1 μM) with 0.1 mg/mL (=*ca.* 0.67 μM) antibodies for 4 days because the toxicity of Wt-Aβ42 (1 μM) after a 2-day incubation was not evident (data not shown). The co-treatment of 11A1 significantly rescued the cell viability, whereas cotreatment with 6E10, 4G8, or IgG did not (Fig. 4A). In the case of E22P-Aβ42, a more potent mutant of Aβ42 [11] the similar results were obtained (Fig. 4B). We confirmed that these antibodies alone were not neurotoxic (Fig. 4C).

Kayed et al. [17] reported that only the conformation-specific antibody (A11) inhibited the Aβ42-induced toxicity, but not 6E10, like our experiment (Fig. 4). It is not surprising when it is considered that the epitope sequence of 6E10 could not be involved in the neurotoxicity of Aβ42. On the other hand, the other anti-Aβ42 antibody 4G8 showed also no suppressive effect against Aβ42-induced neurotoxicity in this study. Taking it into account that 4G8 is not reactive to the toxic conformer [14], 4G8 failed to remove the toxic conformer and showed no neuroprotection.

4. Discussion

Collectively, we demonstrated the significant suppression of Aβ42-induced neurotoxicity and aggregation by non-toxic Aβ42 mutants (G25P-Aβ42 and E22V-Aβ42) that disfavor "toxic" turn at positions 22–23, and that these suppression could be triggered by decreasing the formation of toxic conformer (Figs. 1–3). Anti-toxic Aβ42 antibody (11A1), but not 6E10 and 4G8, significantly neutralized the neurotoxicity of Aβ42 (Fig. 4). Thesefindings suggest that the non-toxic Aβ42 suppress β-sheet formation for aggregation and inhibit the toxic conformer formation of Aβ42, leading to neurotoxicity. Because both the formation of β-sheet and the toxic conformer are associated with Aβ oligomerization, these Aβ42 mutants might be a novel drug candidate for the battle with AD.

Given the previous study of systematic replacement with proline that Aβ42 aggregates contain intermolecular β-sheet at positions 15–21 and 24–32 [11], G25P-Aβ42 and E22V-Aβ42 may

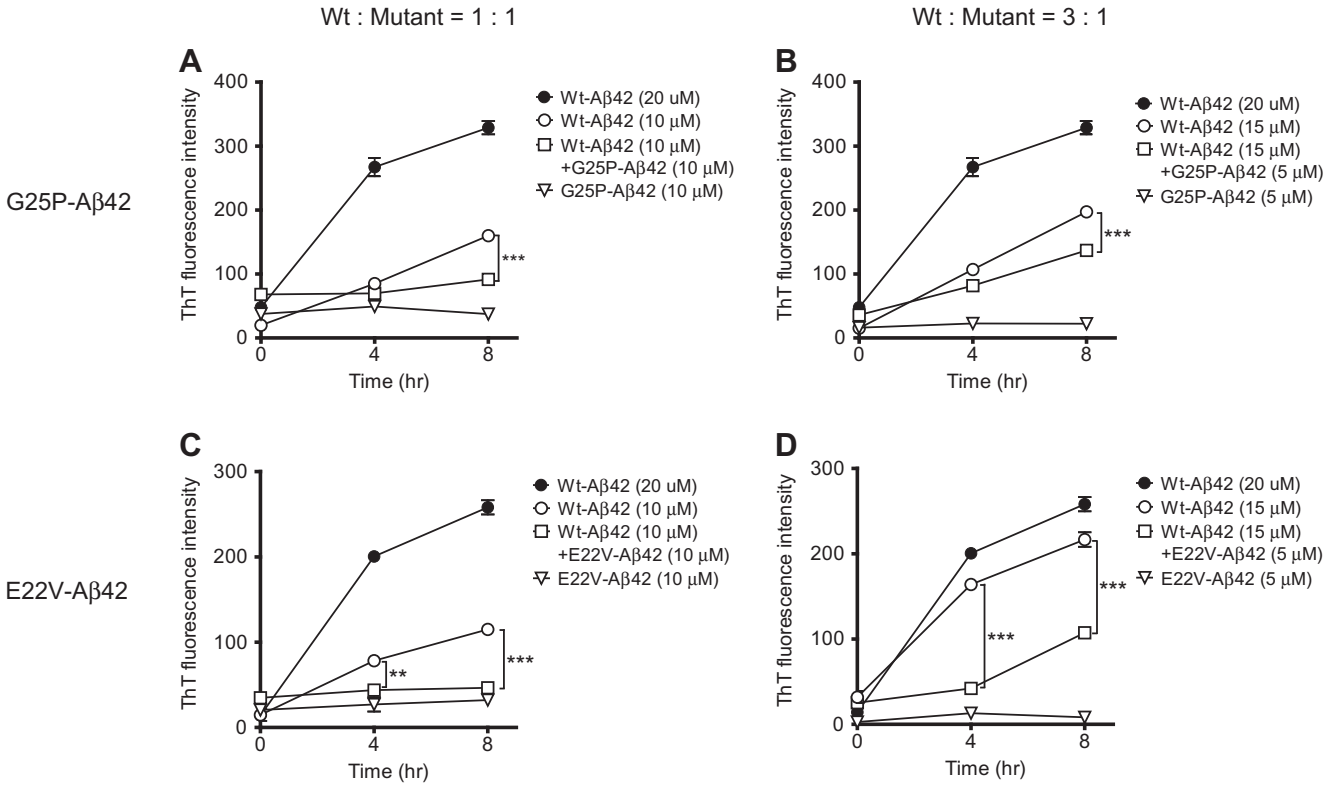

Fig. 3. Effects of G25P-Aβ42 and E22V-Aβ42 on the aggregation of Aβ42 by Th-T test. Wt-Aβ42 in the presence or absence of (A, B) G25P-Aβ42 and (C and D) E22V-Aβ42 at the indicated concentrations was assessed using ThT tests at 37 °C. $^{**}P < 0.01$, $^{***}P < 0.001$.

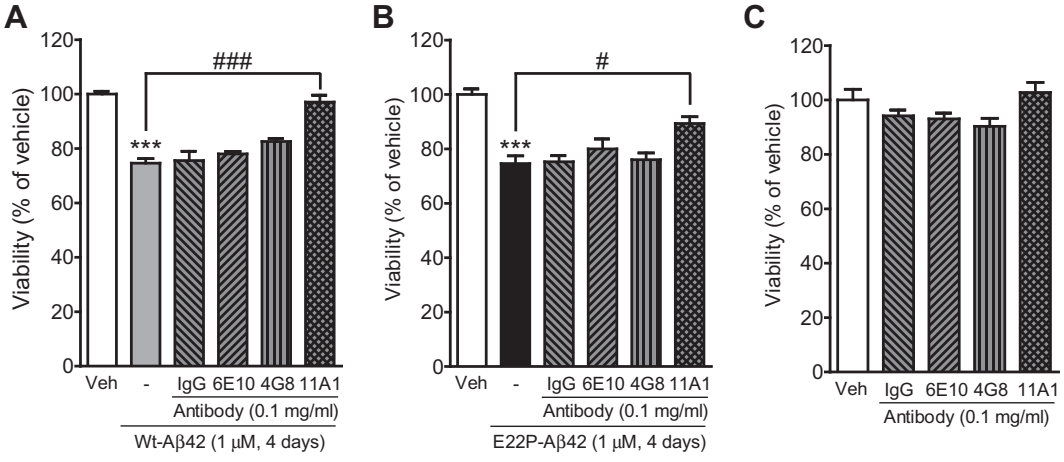

Fig. 4. Effects of anti-Aβ42 antibodies on Aβ42-induced neurotoxicity by MTT assay. Rat primary neurons were exposed to (A) Wt-Aβ42 (1 μM) or (B) E22P-Aβ42 (1 μM) for 4 days in the presence or absence of anti-Aβ antibodies (0.1 mg/mL = *ca.* 0.67 μM). (C) Neuronal cells were treated with antibodies alone. 11A1, anti-toxic turn of Aβ42 at positions 22–23; 4G8, anti-Aβ17–24; 6E10, anti-Aβ1–17; IgG, immunoglobulin G. Veh, vehicle. $^{***}P < 0.001$ vs. vehicle, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$.

intercalate the toxic conformer through parallel β-sheet with high affinity. Recent reports show the propagation of amyloidogenic proteins such as α-synuclein [18] and prion [19] as well as Aβ [20]. These proteins can aggregate to form seed in a rate-limiting step as a template, followed by the swift elongation into oligomers or fibrils. In our recent research, E22P-Aβ42 showed rapid formation of trimer [6], which could be the seed of the Aβ aggregation. This evidence suggests that the toxic conformer works as a template leading to the conformational change of the non-toxic conformer into the toxic conformer followed by propagation. This concept is supported by the result that Wt-Aβ42 exhibited the time-dependent formation of the toxic conformer [14]. Notably, 11A1 antibody protected against the toxicity of Aβ42 (1 μM) even at low concentration (0.1 mg/mL approximately equivalent to 0.67 μM) (Fig. 4). Considering that 11A1 is strongly reactive to the trimer of E22P-Aβ42 [7], the potent neuroprotective effect of 11A1 could be derived from the removal of the seed of the toxic conformer and the prevention of the oligomer formation. G25P-Aβ42 and E22V-Aβ42 may inhibit propagation process of the toxic conformer mediated by high affinity to β-sheet, suppressing the formation of the toxic oligomers and β-sheet. The non-toxic Aβ42 might have a physiological role for suppression of the propagation

of the toxic conformer and the up-regulation of the non-toxic conformer might lead to the onset of AD.

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