

SCIENTIFIC REPORTS



OPEN

Monoclonal antibody with conformational specificity for a toxic conformer of amyloid β 42 and its application toward the Alzheimer's disease diagnosis

Received: 29 February 2016

Accepted: 14 June 2016

Published: 04 July 2016

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Amyloid β -protein (A β 42) oligomerization is an early event in Alzheimer's disease (AD). Current diagnostic methods using sequence-specific antibodies against less toxic fibrillar and monomeric A β 42 run the risk of overdiagnosis. Hence, conformation-specific antibodies against neurotoxic A β 42 oligomers have garnered much attention for developing more accurate diagnostics. Antibody 24B3, highly specific for the toxic A β 42 conformer that has a turn at Glu22 and Asp23, recognizes a putative A β 42 dimer, which forms stable and neurotoxic oligomers more potently than the monomer. 24B3 significantly rescues A β 42-induced neurotoxicity, whereas sequence-specific antibodies such as 4G8 and 82E1, which recognizes the N-terminus, do not. The ratio of toxic to total A β 42 in the cerebrospinal fluid of AD patients is significantly higher than in control subjects as measured by sandwich ELISA using antibodies 24B3 and 82E1. Thus, 24B3 may be useful for AD diagnosis and therapy.

A hallmark of Alzheimer's disease (AD) is amyloid deposition in senile plaques that consist mainly of 40- and 42-residue amyloid β -proteins (A β 40 and A β 42)¹. These proteins are generated from A β -protein precursor by two proteases, β - and γ -secretases. A β aggregates (oligomerizes) through intermolecular β -sheet formation to exhibit neurotoxicity. The term "aggregation" in this context is defined as the change from A β monomers into amyloid fibrils *via* oligomers or protofibrils. A β 42 plays a more important role in AD pathogenesis than A β 40 because of its stronger ability to aggregate and show neurotoxicity². Multiple lines of evidence have proposed that the soluble oligomeric assembly of A β is more exclusively involved in neuronal death and cognitive impairment than its insoluble fibrils and protofibrils³. The minimal unit of these oligomers, which have been divided into low molecular-weight oligomers (2–12-mer) and high molecular-weight oligomers (24–100-mer), is thought to be either a dimer⁴ or trimer⁵ (2 or 3 \times *n*-mer). A β oligomer accumulation is among the earliest phenomena during the progression of AD pathology compared with other AD-related events such as hyperphosphorylation of tau protein, decreased hippocampal volume, and lowered glucose metabolism⁶. These highlight the significance of early and accurate diagnosis by targeting A β oligomer.

We have previously reported using solid-state NMR⁷ and systematic proline replacement⁸ that A β 42 is held in equilibrium of the toxic conformer with a turn at Glu22 and Asp23, and the non-toxic one with a turn at Gly25 and Ser26 (Fig. 1a). In particular, the proportion of A β 42 toxic conformer, in which residues Gln15~Ala21 and Val24~Ile32 are involved in forming an intermolecular parallel β -sheet of A β 42 aggregates, may contribute to AD

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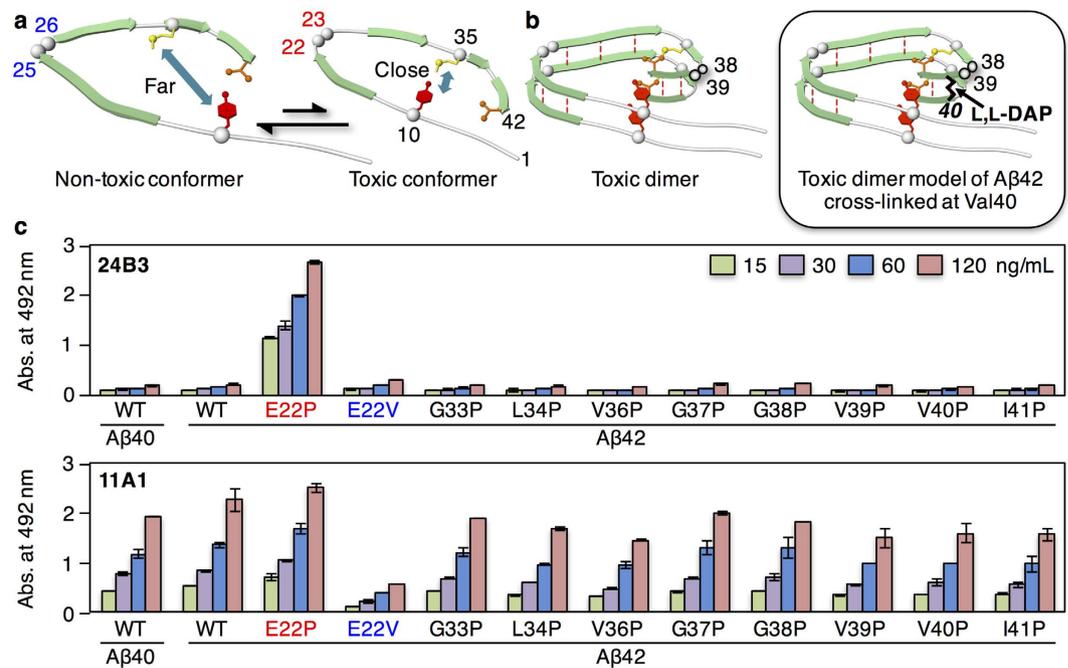


Figure 1. Development of antibody 24B3 that specifically recognizes a toxic conformer of A β 42 with a turn at Glu22 and Asp23. (a) A β 42 is held in equilibrium of the toxic conformer (right) with turn at positions 22 and 23, and the non-toxic one (left) with turn at positions 25 and 26. The proportion of toxic conformer of A β 42 could be enhanced by E22P or E22K mutations⁷. (b) Dimerization of the toxic A β 42 conformer that has a turn at Glu22 and Asp23 to induce close interaction between Tyr10 and Met35 for formation of the S-oxidized radical cation of Met35 and a C-terminal hydrophobic core for stabilization of radicals for long-lasting oxidative stress. Based on this hypothesis, a toxic oligomer model of A β 42 covalently cross-linked using L,L-DAP at Val40 was designed. (c) Enzyme immunoassay using 24B3 and 11A1 (15, 30, 60, 120 ng/mL) to detect A β 42 mutants (2.5 μ g/well) substituted with proline at Glu22, Gly33, Leu34, Val36, Gly37, Gly38, Val39, Val40, and Ile41 together with A β 40, A β 42, and E22V-A β 42, coated on plates.

pathologies as well as cerebral amyloid angiopathy (CAA) such as Italian mutation⁷. Moreover, the C-terminal hydrophobic core derived from another turn at Gly38 and Val39, in addition to the intramolecular anti-parallel β -sheet (Met35~Gly37 and Val40~Ala42), accelerates the aggregation (oligomerization) of A β 42⁹. The contribution of the C-terminal turn structure to the superior ability of A β 42 to form oligomers has also been reported by other groups^{10–12}. Based on this knowledge, we have proposed a dimer model of A β 42 with the “toxic turn” at Glu22 and Asp23 and the C-terminal core, which is supposed to be a partial structure of toxic oligomers of A β 42 (Fig. 1b)⁹.

We previously reported a conformation-targeted monoclonal antibody (11A1)¹³ against the toxic turn of A β 42 generated by immunization with E22P-A β 10–35, a minimum moiety for neurotoxicity including the toxic turn as a Pro-X corner (X: variable amino acid residue). 11A1 reacted with intracellular as well as extracellular A β depositions. In particular, the intracellular accumulation of A β could originate preferably from A β oligomers in AD brains^{13,14}, AD-model mouse brains^{14,15}, and neurons differentiated from induced pluripotent stem cells of AD patients¹⁶. However, the obvious reactivity of 11A1 with senile plaques (formed by extracellular A β accumulation) composed of fibrillar A β as well as monomeric A β hampers its application towards the diagnosis of AD. Indeed, because present diagnoses using sequence-specific antibodies against less-toxic fibrillar and A β 42 monomer run a risk of false positives (approximately ~30%) of AD^{17,18}, conformation-specific antibodies targeting neurotoxic A β 42 oligomers have garnered much attention for developing more accurate diagnostic methods. Several conformation-specific antibodies such as anti-prefibrillar A β oligomers (OC)¹⁹, and anti-A β 42-derived diffusible ligands (ADDLs) antibodies have been applied to AD diagnosis using cerebrospinal fluid (CSF). Although these attempts were likely successful in the limited cohort, almost all the target molecules of these antibodies could be A β oligomers that move into the fibrillar stage (on-pathway)²⁰, so the possibility of false positives in the future trials using these antibodies cannot be excluded. Therefore, the development of monoclonal antibodies that specifically recognize toxic A β oligomers that exist as stable soluble intermediates (off-pathway) is an important challenge for selectively removing neurotoxic A β oligomers.

In this paper, we report a novel monoclonal antibody (named as 24B3) with greatly enhanced selectivity for the toxic A β 42 conformer compared with 11A1, the first generation conformation-targeted antibody. Here, we describe the characterization of 24B3 by physicochemical and biochemical analyses. Notably, 24B3 recognizes the toxic oligomer model of A β 42 (Fig. 1b) more preferentially than the corresponding monomer. Furthermore, 24B3 was applied to the diagnosis of AD using human CSF, as determined by a novel sandwich enzyme-linked immunosorbent assay (ELISA) that uses a combination of 24B3 and an anti-N-terminus-specific antibody, 82E1.

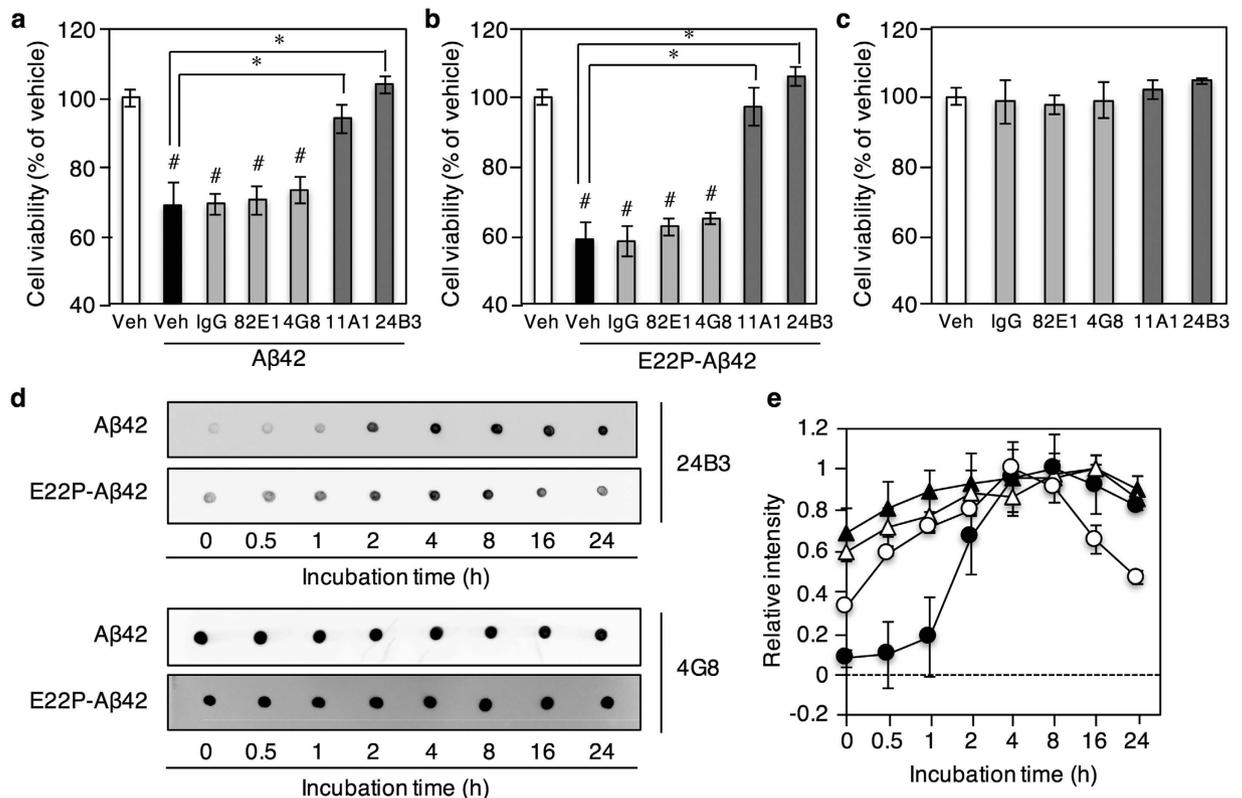


Figure 2. Prevention of A β 42-induced neurotoxicity by sequestering A β 42 oligomer using 24B3. (a,b) Neurotoxicity of (a) A β 42 and (b) E22P-A β 42 (1 μ M) on SH-SY5Y cells, and effects of IgG, 82E1, 4G8, 11A1, and 24B3 (0.1 mg/mL) on A β -induced neurotoxicity after 48 h incubation, determined by MTT assay. (c) Effects of antibodies alone on the cell viability. * p < 0.05 vs vehicle alone, * p < 0.05. Data are expressed as mean \pm s.e.m. (n = 3). (d,e) Dot blotting for A β 42 and E22P-A β 42 using 24B3 and 4G8 for detection. A β solution (25 μ M) was incubated for the indicated duration at 37 $^{\circ}$ C, and 1 μ L of solution was spotted on the membrane at each time point. (d) Representative blots and (e) densitometric quantifications of immunoblot signals from triplicate samples are shown in (d), respectively. ●, 24B3 for A β 42; ○, 24B3 for E22P-A β 42; ▲, 4G8 for A β 42; △, 4G8 for E22P-A β 42.

Results

Development of antibody 24B3 that specifically recognizes the toxic conformer of A β 42. To generate the antibody that specifically recognizes the “toxic turn” at Glu22 and Asp23 of A β 42, the seven monoclones previously selected using E22P-A β 10–35¹³ containing the toxic turn were re-evaluated in detail based on their ability to react with various A β 42 mutants with a proline replacement mainly at C-terminal region⁸. In order to precisely evaluate the selectivity for toxic conformer of A β 42, the concentration of clones used in this re-evaluation (15–120 ng/mL) was lower than that of the previous study¹³ (125–1,000 ng/mL). Enzyme immunoassay (EIA) identified a unique clone, named 24B3, as showing the strongest immunoreactivity with E22P-A β 42 among the other mutants in a dose-dependent manner (Fig. 1c). The specificity of 24B3 for E22P-A β 42 was much higher than that of 11A1. Moreover, 24B3 barely bound to E22V-A β 42, in which valine is known as a turn breaker, while 11A1 reacted weakly with E22V-A β 42 (Fig. 1c). These results suggest that 24B3 is more conformation-specific for the toxic A β 42 conformer compared with 11A1 since the proportion of toxic conformer of A β 42 could be enhanced by E22P mutation.

Prevention of A β 42-induced neurotoxicity by 24B3 occurs through sequestration of A β 42 oligomers.

To determine the effects of 24B3 on A β 42-induced neurotoxicity, we performed the MTT assay on SH-SY5Y human neuroblastoma cells, a neuronal cell culture model. Not only 24B3 but also 11A1 significantly rescued the neurotoxicity induced by both A β 42 and E22P-A β 42 as a toxic conformer surrogate⁸, whereas neither 4G8 nor 82E1 (anti-N-terminus of A β 42) could rescue (Fig. 2a,b). Notably, the protective effects of 24B3 were slightly higher than that of 11A1. All of these antibodies did not affect cell viability (Fig. 2c). Because we previously reported the neuroprotective potential of 11A1 on rat primary neurons²¹, the additional experiments were carried out. We then obtained similar results using primary neuronal cultures originated from rat (Supplementary Fig. 1). The slight inhibition of cytotoxicity by 11A1 was also observed with PC12 cells¹³. These observations are consistent with previous findings that A11 rescues A β 42-induced toxicity in SH-SY5Y cells, whereas 6E10 does not²², raising the possibility that 24B3 may target the oligomeric species of A β responsible for neurotoxicity in a manner similar to A11 although the haptens of these antibodies are different from each other. These results suggest that conformation-specific antibodies may be more beneficial in treating AD pathology than

sequence-specific antibodies, and that A β aggregates sequestered by 24B3 may contain oligomers formed by the toxic A β 42 conformer.

Since both A β 42 and E22P-A β 42 induced neurotoxicity in a time-dependent manner²¹, we determined by dot blotting how 24B3 detects the toxic conformer of A β 42. Regarding the immunoreactivity of 24B3, the relative intensity of E22P-A β 42 gradually increased to a maximum after 4 h of incubation, whereas A β 42 reacted more slowly than E22P-A β 42 (Fig. 2d,e). It should be noted that the relative quantification of reactivity of 24B3 against A β 42 was carried out under the longer exposure of blots due to its moderate affinity. It is noteworthy that the potent immunosignal of E22P-A β 42 against 24B3 immediately after dissolution means more rapid formation of the toxic conformer by E22P-A β 42 compared with A β 42. In contrast, the relative intensity by 4G8 against A β 42 was potent even after 0 h of incubation, and it remained almost constant up to 24 h (Fig. 2d,e). Under the longer exposure in the case of E22P-A β 42 treated with 4G8 because of its weak affinity, the similar results to A β 42 treated with 4G8 were obtained (Fig. 2d,e). These results suggest that the A β 42 oligomers responsible for neurotoxicity can be attributed to those detected by 24B3, but not those detected by 4G8.

Synthesis and characterization of the E22P-A β 42 dimer as a toxic oligomer surrogate. We recently validated optically active L,L-diaminopimelic acid (DAP)²³ as a useful linker near the intermolecular β -sheet region (Ala30) of E22P-A β 40²⁴. This strategy was applied to the synthesis of the E22P-A β 42 dimer as a toxic oligomer model for A β 42. The E22P mutation not only enhanced the neurotoxicity of A β 42 ~10-fold²⁵, but also increased the ratio of toxic conformation of A β 42⁷. According to our proposed dimer model (Fig. 1b), Val40 in the C-terminal hydrophobic core was replaced with the linker. Solid-phase synthesis using Fmoc-L,L-DAP as a substitute for Val40 provided a sufficient amount of E22P-A β 42 dimer (Fig. 1b) with high purity (6.0% yield, >98% purity, Supplementary Fig. 2).

The aggregative ability of the E22P-A β 42 dimer with a covalent linker at Val40 was evaluated using thioflavin-T (Th-T), a reagent that fluoresces when bound to A β aggregates, and transmission electron microscopy (TEM). E22P-A β 42 aggregated with a lag time of ~4 h and a maximum fluorescence value after being incubated for 96 h (Fig. 3a), and its velocity for aggregation was higher than that of A β 42, as previously reported²⁵. In contrast, the fluorescence of the E22P-A β 42 dimer remained almost unchanged even after a 96 h incubation (Fig. 3a). Given the moderate increase in the fluorescence after incubation for 168 and 336 h, these time-point samples were subjected to TEM analysis. As shown in Fig. 3b, we observed short and globular aggregates (oligomers) predominantly in the E22P-A β 42 dimer, unlike the typical fibrils found in E22P-A β 42.

Moreover, circular dichroism (CD) spectrometry was measured to analyze the secondary structure of the E22P-A β 42 dimer. A positive peak at ~190 nm and a negative peak at ~210 nm began to increase gradually after dissolution, suggesting that a random structure transformed into a β -sheet architecture in the E22P-A β 42 dimer in a manner similar to E22P-A β 42 (Fig. 3c).

The ability of the E22P-A β 42 dimer to form oligomers was further studied using size exclusion chromatography. As a control reference, the peaks corresponding to the monomer of E22P-A β 42 and its 6~8-mer were observed immediately after dissolution, but these peaks disappeared almost completely after 1 h incubation (Fig. 3d). This observation suggests the formation of insoluble fibrillar aggregates of E22P-A β 42, as observed in Fig. 3b. In contrast, the E22P-A β 42 dimer formed stable oligomers (comprising 6~8-mers) during incubation for ~24 h even after dissolution (Fig. 3d). It should be noted that the peak of E22P-A β 42 dimer did not appear to be observed even at the initial time point (Fig. 3d). Actually, we confirmed the presence of E22P-A β 42 dimer in reverse-phase HPLC on ODS column (Supplementary Fig. 2b). Once dissolved in the PBS solution, the E22P-A β 42 dimer could intrinsically form stable oligomers during elution in size exclusion chromatography. Alternatively, in the size exclusion chromatography using Superdex75 10/300GL column, E22P-A β 42 dimer itself might not be able to give a sharp peak because of adopting various conformations. Similar phenomena were also observed in A β 42 monomer using reverse-phase HPLC on ODS column under the acidic condition; A β 42 was observed only as a broad peak²⁶.

The neurotoxicity of the E22P-A β 42 dimer was measured using the MTT assay on human neuroblastoma SH-SY5Y cells. After being incubated for 16 h when the stable oligomer of the E22P-A β 42 dimer is suspected to be predominant, the viability of cells treated with the E22P-A β 42 dimer was lower than that of E22P-A β 42 at 1~10 μ M, indicating that the E22P-A β 42 dimer is more neurotoxic than E22P-A β 42 (Fig. 3e). Overall, the E22P-A β 42 dimer can form stable oligomers with a β -sheet structure, suggesting that the E22P-A β 42 dimer is a suitable model for studying toxic A β 42 oligomers. However, additional oligomer models such as trimer or tetramer should be examined to conclude which oligomeric species are toxic.

Binding affinity of 24B3 for the E22P-A β 42 dimer. To investigate the potential of 24B3 to recognize toxic A β 42 oligomers, we performed an EIA test. 24B3 showed stronger immunoreactivity against the E22P-A β 42 dimer than E22P-A β 42; its specificity for the E22P-A β 42 dimer was much higher than that of 11A1 (Fig. 4a,b). In contrast, the relative affinity of 4G8 for the E22P-A β 42 dimer was weak (Fig. 4c).

Surface plasmon resonance (SPR) was employed to evaluate the binding constant of 24B3 for A β , in which A β was immobilized on a sensor chip based on a biotin-streptavidin interaction. The biotinylation of A β 42, E22P-A β 42, and the E22P-A β 42 dimer did not affect their aggregation profiles and neurotoxicity, respectively (Fig. 3a, Supplementary Fig. 3). 24B3 bound 6.5-fold more strongly to the E22P-A β 42 dimer ($K_D = 1.8$ nM) than 11A1 ($K_D = 10$ nM) (Table 1, Supplementary Fig. 4). In addition, consistent with the EIA test (Fig. 4a,b), the binding affinity of 24B3 for E22P-A β 42 ($K_D = 3.1$ nM) was ~8-fold higher than that of 11A1 for E22P-A β 42 ($K_D = 24$ nM). Taken together with the lower binding affinity of 24B3 than 11A1 for A β 42 (24B3: $K_D > 100$ nM, 11A1: K_D 15 nM), these results strongly suggest that 24B3 specifically recognizes toxic oligomers of A β rather than monomeric A β .

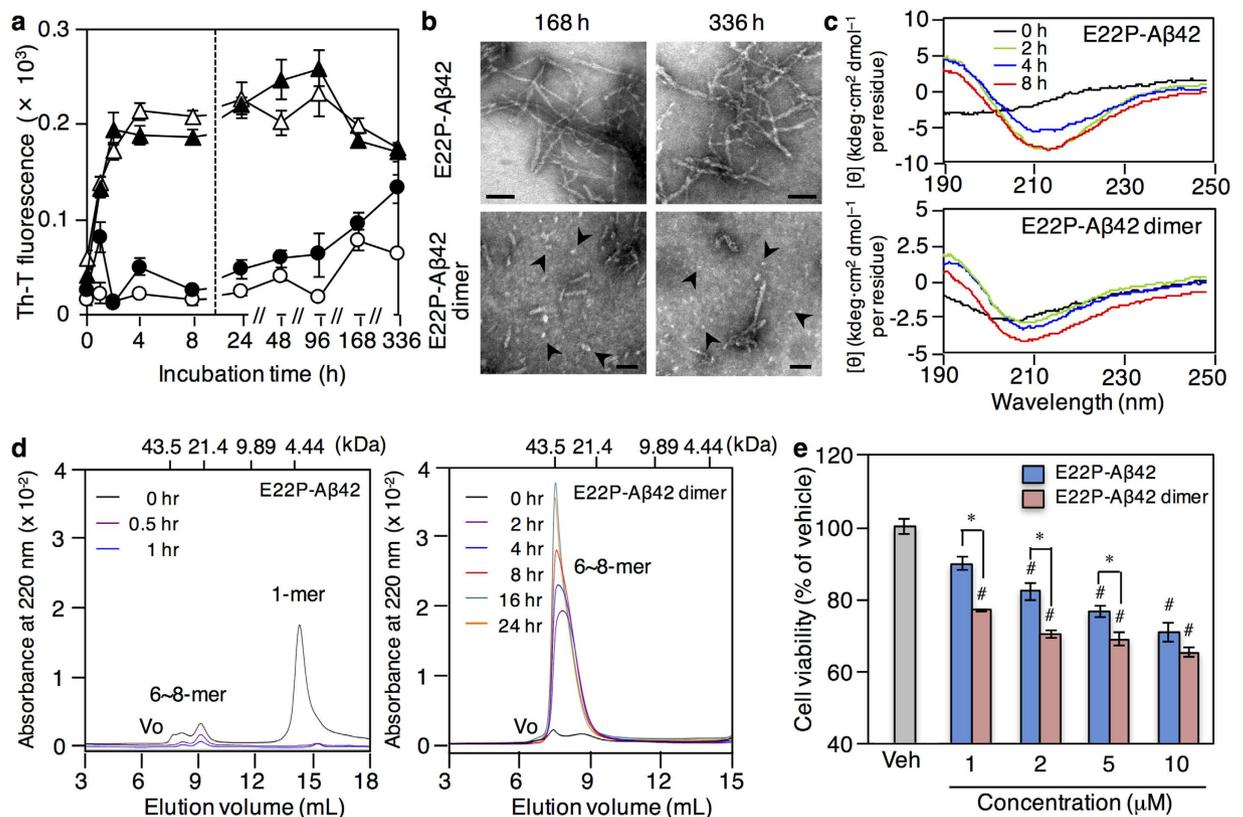


Figure 3. The E22P-A β 42 dimer forms stable 6~8-mer associated with neurotoxicity. (a) Th-T assay result for each A β (25 μ M) after incubation for the indicated duration at 37 $^{\circ}$ C. \bullet , the E22P-A β 42 dimer; \circ , the biotinylated E22P-A β 42 dimer; \blacktriangle , E22P-A β 42; \triangle , biotinylated E22P-A β 42. Data are expressed as mean \pm s.e.m. ($n = 8$). (b) TEM analysis of A β aggregates formed from E22P-A β 42 and E22P-A β 42 dimer after incubation for the indicated duration at 37 $^{\circ}$ C. Arrowheads indicate oligomers. Scale bar = 50 nm. Upper, E22P-A β 42; lower, the E22P-A β 42 dimer. (c) Analysis of secondary structure of E22P-A β 42 and the E22P-A β 42 dimer (25 μ M) after incubation for the indicated duration at 37 $^{\circ}$ C using CD spectrometry. (d) The ability of E22P-A β 42 and E22P-A β 42 dimer (25 μ M) to form soluble oligomers was evaluated by size exclusion chromatography after incubation for the indicated duration at 37 $^{\circ}$ C. The peptide was detected by absorbance at 220 nm. Molecular marker sizes are shown in kDa. V₀: void volume. (e) The viability of SH-SY5Y cells treated with either E22P-A β 42 or E22P-A β 42 dimer at the indicated concentration at 37 $^{\circ}$ C for 16 h. Data are expressed as the mean \pm s.e.m. ($n = 3$). Absorbance obtained after adding vehicle (Veh: 0.1% NH₄OH) was taken as 100%. $\#p < 0.05$ vs vehicle alone, $*p < 0.05$.

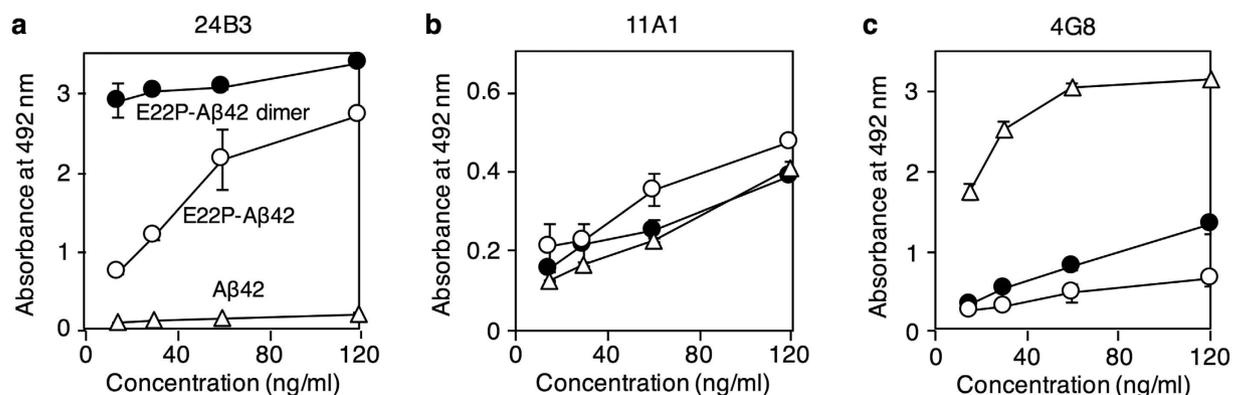


Figure 4. Specific recognition of E22P-A β 42 dimer by 24B3. Enzyme immunoassay using (a) 24B3, (b) 11A1, and (c) 4G8 (15~120 ng/mL) to detect A β 42, E22P-A β 42, and the E22P-A β 42 dimer (2.5 μ g/well) coated on plates.

| Antibody | Immobilized A β | k_a ($M^{-1} s^{-1}$) | k_d (s^{-1}) | K_D (nM) |
|----------|-------------------------|-----------------------------|-----------------------------|--------------------|
| 24B3 | A β 42 | $>5.0 \times 10^2$ | 5.0×10^{-5} | >100 |
| | E22P-A β 42 | $2.3 (0.015)^* \times 10^5$ | $7.2 (0.12) \times 10^{-4}$ | $3.1^{**} (0.072)$ |
| | E22P-A β 42 dimer | $2.3 (0.017) \times 10^5$ | $4.2 (0.25) \times 10^{-4}$ | $1.8^b (0.092)$ |
| 11A1 | A β 42 | $3.3 (0.12) \times 10^4$ | $4.8 (0.23) \times 10^{-4}$ | $15^c (0.93)$ |
| | E22P-A β 42 | $4.0 (0.040) \times 10^4$ | $9.5 (0.27) \times 10^{-4}$ | $24^d (0.81)$ |
| | E22P-A β 42 dimer | $9.2 (0.98) \times 10^2$ | $9.4 (1.6) \times 10^{-6}$ | $10^e (0.94)$ |

Table 1. Dissociation constant (K_D) together with association (k_a) and dissociation (k_d) rate constants of 24B3 and 11A1 for A β derivatives. *The values in the parentheses indicate standard deviation from triplicate experiments. **The different letters show significant differences.

| Case | Age | Sex | MMSE | Clinical diagnosis |
|------|-----|-----|------|--------------------|
| 1 | 68 | M | 24 | AD |
| 2 | 69 | F | 18 | AD |
| 3 | 77 | F | 21 | AD |
| 4 | 82 | F | 18 | AD |
| 5 | 75 | F | 18 | MCI |
| 6 | 84 | F | 21 | MCI |
| 7 | 78 | M | 13 | AD |
| 8 | 81 | M | 23 | MCI |
| 9 | 75 | M | N/A | AD |
| 10 | 82 | F | 23 | MCI |
| 11 | 84 | M | 22 | AD |
| 12 | 68 | M | 20 | AD |
| 13 | 79 | F | 28 | MCI |
| 14 | 58 | M | | Control |
| 15 | 73 | F | | Control |
| 16 | 70 | F | | Control |
| 17 | 71 | F | | Control |
| 18 | 68 | F | | Control |
| 19 | 81 | F | | Control |
| 20 | 77 | F | | Control |
| 21 | 69 | F | | Control |
| 22 | 71 | F | | Control |
| 23 | 77 | M | | Control |
| 24 | 78 | M | | Control |
| 25 | 74 | M | | Control |

Table 2. Summary of clinical diagnosis and mental state *. *MMSE, mini-mental state examination; AD, Alzheimer's disease; MCI, mild cognitive impairment; N/A, not available.

Ratio of toxic conformer to total A β 42 as a potential biomarker for AD pathology in CSF. It is becoming important to identify and validate biomarkers in biological fluids for AD diagnosis, especially for prediction of patients with mild cognitive impairment (MCI) who will convert to AD. Given the potential of 24B3 as a specific probe for toxic A β 42 oligomers, we developed a novel sandwich ELISA using a combination of anti-N-terminus antibody (82E1) for capture and 24B3 conjugated with horseradish peroxidase for detection. To determine if the ratio of toxic conformer to total A β 42 is correlated with the AD pathology in the brain⁷, we performed ELISA on human CSF samples from 13 patients with AD/MCI and 12 age-matched controls (Table 2); we combined the patients with MCI and those with AD to compare with the age-matched controls, since all the MCI patients in this study were those with "MCI due to AD"²⁷. Accordingly, the ratio of toxic conformer to total A β 42 in AD/MCI patients was significantly higher than that in the age-matched controls ($p = 0.0135$, Fig. 5a). In contrast, the difference in total A β 42 as one of the conventional biomarkers²⁸ was not significant ($p = 0.0863$, Fig. 5c). Intriguingly, the absence of a significant difference in the amount of toxic A β 42 conformer between AD/MCI and control groups ($p = 0.4620$, Fig. 5b) might be due to the disappearance of toxic oligomers by moving onto fibrillization of A β 42 monomer. Indeed, the reduction of A β 42 levels in CSF are originated from plaque formation by enhanced aggregation in the cerebral parenchyma as well as the disturbed clearance of A β from the cerebral parenchyma into CSF²⁸. These results signify the proportion of toxic conformer of A β 42 rather than its amounts. Considering its potential inaccuracy when to judge the diagnosis based on only the amount of total A β 42^{17,18}, the ratio of toxic conformer of A β 42 to total A β 42 could be a better substitute for precise diagnosis of AD.

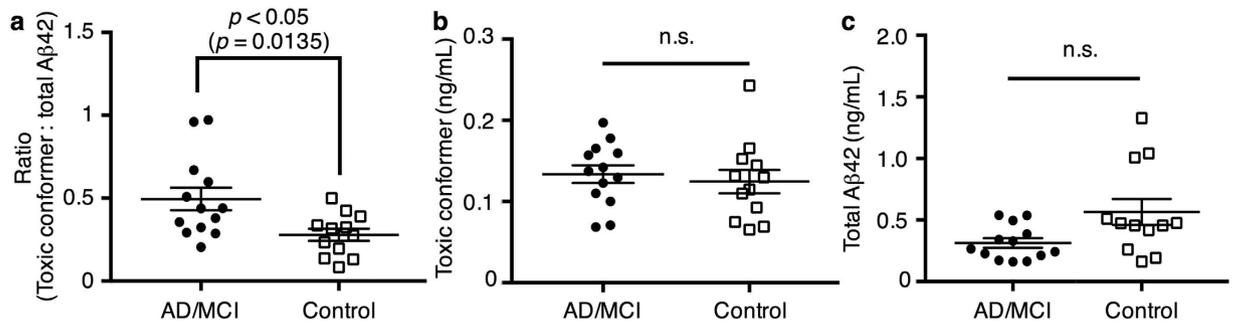


Figure 5. Comparing the ratio of toxic A β 42 conformer to total A β 42 in CSF between AD/MCI patients and age-matched individuals by sandwich ELISA. ELISA detection results: (a) ratio of toxic conformer of A β 42 to total A β 42, (b) toxic conformer of A β 42, and (c) total A β 42 in CSF of AD/MCI patients ($n = 13$) and age-matched controls ($n = 12$).

Discussion

Immunotherapy-mediated removal of A β aggregates is one of the first disease-modifying therapies for AD. However, several anti-A β antibodies recently tested in clinical trials failed²⁹, possibly because they were evaluated in later stage AD patients, when neuronal cells have been irreversibly damaged. In this stage, the elimination of A β 42 oligomers is no longer beneficial. Several ongoing immunotherapy trials involving patients diagnosed with preclinical AD or who carry a genetic mutation that induces AD symptoms may be more successful.

Our ideas are based on the concept that selective removal of toxic A β oligomers is important in immunotherapy since non-toxic A β plays a physiologically important role in synapse communication³⁰ and regulation of glucose metabolism³¹. We developed the conformation-specific monoclonal antibody 24B3, which specifically recognizes the toxic conformer of A β 42 in toxic oligomers (Fig. 4), and demonstrated a correlation between the ratio of toxic conformer to total A β 42 and the pathology of AD (Fig. 5). Moreover, 24B3 suppressed significantly the A β -induced cytotoxicity *in vitro*, while sequence-specific antibodies such as 4G8 with weaker affinity for the E22P-A β 42 dimer model did not (Figs 2 and 4). Several failures of passive immunization in clinical trials might be due to the insufficient affinity of sequence-specific antibodies³² for toxic A β 42 conformers. Recent unexpected findings on the neuronal hyperactivity induced from immunotherapy using a sequence-specific antibody (3D6) reaffirm the significance of conformation-specific antibody³³. Otherwise, they might be ascribed to the unintended elimination of physiologically necessary non-toxic conformers of A β 42^{7,34,35}, though other reasons such as inadequate timing and period to treat with anti-A β antibodies have already been proposed³⁶.

E22P-A β 42 as a toxic conformer surrogate readily forms toxic oligomers to inhibit long term potentiation (LTP)³⁷. Provided that A β 42 oligomerization involves the formation of various transient or intransient assemblies, which are on- or off-pathway aggregation products, it is indispensable to develop an “off-pathway” A β 42 oligomer model for developing anti-A β drugs with few side effects. To the best of our knowledge, this is the first report on the generation and characterization of an A β 42 dimer model, which is connected at the C-terminal hydrophobic region. Practical synthesis of A β dimers has thus far been limited to A β 40 dimers^{4,23,38}, partly due to the intrinsic and potent ability of A β 42 to aggregate during synthesis and preparation. Cross-linkage within this region has never been achieved in spite of its significance in oligomerization^{10–12}. Although one study has reported the synthesis of a dityrosine cross-linked A β 42 dimer at Tyr-10³⁸, its biological activity was not tested due to insufficient yield. Other oligomer models with high molecular weight such as ADDLs (~24-mer)³⁹ are also considered off-pathway aggregates. Dimers, trimers, and tetramers of A β 42, as prepared by photo-induced cross-linking of unmodified protein (PICUP) technology using 2,2'-bipyridyl-dichlororuthenium(II) hexahydrate as a catalyst, were mainly bound covalently at Tyr10⁴⁰, although it seems difficult to isolate these oligomers in a pure form. Recently, unique synthesis of N-terminal-tethered triple A β fragment (A β 25–35) has been reported⁴¹ as a trimeric model, which may be another minimal unit of toxic oligomers (2 or 3 \times n -mer) of A β 42. When we take into account these models as candidates of toxic oligomers, further investigation will be required to clarify the significance *in vivo*, though the pathology of the genetically modified mice to produce A β dimer cross-linked at Ser8 in N-terminal region was recently reported⁴².

We identified 24B3 based on the ability to bind the conformer possessing the turn structure at residues 22 and 23 using some proline-substituted mainly at C-terminal core region mutants of A β 42 (Fig. 1). Our findings indicate that 24B3 could be more favorable to the toxic conformer of A β 42 with the turn at residues 22 and 23 than to 11A1. It should be noted that 11A1 may bind various conformers with turns at other residues, even though 11A1 is not proline-specific like 24B3. The enhanced reactivity of 24B3 with the E22P-A β 42 dimer compared with E22P-A β 42 (Fig. 4) suggests the stabilization of the toxic A β 42 oligomer by formation of the C-terminal core and intermolecular β -sheet. Furthermore, the time-dependent reactivity of 24B3 towards A β 42 in solution (Fig. 2) reflects the amount of toxic A β 42 oligomers formed during incubation.

In previously reported sandwich ELISAs for A β oligomers⁴³, the same anti-A β antibodies were used for both capture and detection based on the idea that oligomers have multiple reactive sites. However, it is likely to be difficult to discriminate toxic A β oligomers from less-toxic fibrillar A β aggregates using such ELISAs, and their validity has thus been questioned⁴⁴. It is reasonable to use the conformation-specific antibody for detection after capturing total A β using 82E1 in sandwich ELISA. Decreased A β 42, increased total tau, and increased phosphorylation of tau, are currently the most accepted biomarkers for diagnosing AD (probable, possible, or definite AD)²⁸.

There have also been intensive studies on other biomarkers such as A β -related or tau-related molecules in order to increase diagnostic validity by modifying sensitivity and specificity. However, outliers largely affect the validity in these conventional biomarkers, and there have been no studies on the qualitative difference between various A β conformers in spite of accumulating structural studies of A β . Our group proposed the significance of the ratio of the toxic conformer to total A β 42 in AD pathogenesis⁷ using solid-state NMR analysis, which demonstrated that a ~2-fold increase in the ratio of toxic conformer of E22K-A β 42 to wild-type A β 42 can in part explain the extensive aggregative ability and neurotoxicity of E22K-A β 42²⁵. Although the ratio of A β 42 to A β 40 has been suggested as another biomarker⁴⁵, such a biomarker might correlate more preferably with the amount of senile plaque containing less-toxic fibrils rather than with the amount of toxic oligomers.

In summary, the target of a novel conformation-specific monoclonal antibody 24B3 could be oligomers that include the toxic conformer of A β 42, and the ratio of toxic conformer to total A β 42 could be an alternative evaluation criterion toward accurate diagnosis of AD. Although the analysis with a larger number of CSF samples is indispensable for further validation, the development of a less invasive test using plasma is an attractive goal for the future. 24B3 could be promising as a diagnostic tool of AD because of its superior affinity for toxic oligomers consisting of toxic conformation of A β 42, which is likely to be formed at an earlier stage before AD symptoms. Moreover, it could be applied to therapeutics during the early stages of AD.

Methods

Synthesis of biotinylated E22P-A β 42. Biotin-E22P-A β 42 was synthesized in a stepwise fashion on 0.1 mmol of preloaded Fmoc-L-Ala-PEG-PS resin (Applied Biosystems) by PioneerTM using the Fmoc method, as previously described²⁵. Biotin was added as the final residue. Briefly, after the completion of chain elongation and cleavage from the resin, the crude peptide was precipitated using diethylether, followed by purification using HPLC on Develosil ODS UG-5 column (20 mm i.d. \times 150 mm; YMC) with elution at 8.0 mL/min by an 80 min linear gradient of 10–50% CH₃CN containing 0.1% NH₄OH. Lyophilization yielded a pure peptide, the purity of which was confirmed by HPLC (>98%, 9.9% yield). The molecular weight of biotin-E22P-A β 42 was confirmed by liquid chromatography-mass spectrometry, followed by deconvolution (LC-MS; Acquity UPLC system H-class with Xevo G2-S, Waters); *m/z*, calculated: 4709.44; observed: 4708.95 [MH]⁺ (Supplementary Fig. 2a).

Synthesis of the E22P-A β 42 dimer and its biotinylated derivative. Fmoc-L,L-DAP was either synthesized as described previously²⁴ or purchased from Sigma. The E22P-A β 42 dimer (Fig. 1b) or the biotin-E22P-A β 42 dimer was synthesized as basically described above, except that a pseudoproline dipeptide, Fmoc-Gly-L-Ser(ψ Me,Mepro)-OH (Novabiochem), was utilized at the positions Gly25 and Ser26. Val40 was replaced with L,L-DAP as a cross linker. Regarding the E22P-A β 42 dimer, the purification using HPLC on YMC-Pack ODS-A column (20 mm i.d. \times 150 mm; YMC) with elution at 8.0 mL/min by a 70 min linear gradient of 20–60% CH₃CN containing 0.1% trifluoroacetic acid was carried out. Subsequent purification was performed using a YMC-Pack PROTEIN RP column (20 mm i.d. \times 150 mm; YMC) with elution at 8.0 mL/min by an 80 min linear gradient of 20–60% CH₃CN containing 0.1% trifluoroacetic acid. Lyophilization gave a pure peptide, the purity of which was confirmed by HPLC (>98%, 6.0% yield). The molecular weight of the E22P-A β 42 dimer was confirmed by LC-MS, followed by deconvolution; *m/z*, calculated: 8920.16; observed: 8920.29 [M]⁺ (Supplementary Fig. 2b).

Regarding the biotin-E22P-A β 42 dimer, the purification using HPLC on YMC-Pack ODS-A column (20 mm i.d. \times 150 mm; YMC) with elution at 8.0 mL/min by an 80 min curve gradient (curve 7 in Waters M600 system) of 30–60% CH₃CN containing 0.1% trifluoroacetic acid was carried out. Subsequent purification was performed using an XBridge BEH C18 Prep column (19 mm i.d. \times 150 mm; Waters) with elution at 8.0 mL/min by an 80 min linear gradient of 10–50% CH₃CN containing 0.1% NH₄OH. Lyophilization gave a pure peptide, the purity of which was confirmed by HPLC (>98%, 3.4% yield). The molecular weight of the biotin-E22P-A β 42 dimer was confirmed by LC-MS, followed by deconvolution; *m/z*, calculated: 9372.78; observed: 9372.92 [M]⁺ (Supplementary Fig. 2c).

HFIP treatment of A β . For treatment with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Wako), each A β was dissolved in HFIP at 1 mg/mL. After incubation at room temperature for 30 min, the solution was sonicated for 5 min, and dried *in vacuo*. The resultant film of A β was stored at –80 °C until use.

Thioflavin-T (Th-T) assay. The aggregative ability of each A β was evaluated with a previously described thioflavin-T (Th-T; Sigma) fluorescence assay²⁵. A β was dissolved in 0.1% NH₄OH at 250 μ M, followed by 10-fold dilution with phosphate buffered saline (PBS; 50 mM sodium phosphate, and 100 mM NaCl, pH 7.4) to a final concentration of 25 μ M. After incubating at 37 °C for the desired period, 2.5 μ L of the reaction solution was added to 250 μ L of 5.0 μ M Th-T in 5.0 mM Gly-NaOH (pH 8.5), followed by the measurement of fluorescence at 430 nm excitation and 485 nm emission using a microplate reader (Fluoroskan Ascent; Thermo Scientific).

Transmission electron microscopy (TEM). The A β aggregates after incubation for the desired period in the Th-T assay were examined under a H-7650 electron microscope (Hitachi). The experimental procedure has been described elsewhere²⁵. After the supernatant was removed from the pellets, the resultant aggregates were then suspended in water (100 μ L) by gentle vortex mixing, and centrifuged at 6,600 rpm for 1 min. These suspensions were applied to a 200 mesh Formvar-coated copper grid (Nissin EM), and allowed to dry in air for 5 min after being negatively stained for several seconds with 2% uranyl acetate and subsequently subjected to microscopy.

Circular dichroism (CD) spectrometry. The secondary structure of the A β dimer was estimated by CD spectrometry (J-805; JASCO) using a 0.1 mm quartz cell, as described elsewhere³⁷. The A β solution (25 μ M)

prepared above was incubated at 37 °C. An aliquot was loaded into the quartz cell, and CD spectra were recorded at 190–260 nm. The spectra of A β are shown after subtraction of the spectrum for the vehicle alone.

Size exclusion chromatography. The A β solution (25 μ M) was incubated at 37 °C. After the solution was collected periodically and centrifuged at $17,860 \times g$ at 4 °C for 10 min, the supernatant was analyzed by size exclusion chromatography on the Superdex75 10/300GL column (10 mm i.d. \times 300 mm; GE Healthcare) with elution at 0.6 mL/min by filtered- and degassed-PBS (pH 7.4), attached to a Waters LC system with a 2489 UV/Visible detector and 1525 binary HPLC pump controlled by EmpowerTM3 software (Waters), as described elsewhere²⁴. The peptide was detected by absorbance at 220 nm. Calibration curves of size exclusion columns were constructed using dextran standards (Mp: mean peak molecular weight, 43500, 21400, 9890, 4440) (Sigma) together with Blur dextran 2000 (GE Healthcare) as an indicator of the void volume (V_0).

MTT assay on SH-SY5Y cells. Human neuroblastoma, SH-SY5Y cells, maintained in a 1:1 mixture of Eagle's minimum essential medium (Wako) and Ham's F12 medium (Wako) containing 10% fetal bovine serum (Biological Industries), were used as a neuronal cell model to estimate the neurotoxicity of each A β with a slight modification to the previously described method²⁴. In brief, each A β was dissolved in 0.1% NH₄OH to generate a 10X stock solution. The resultant peptide solution (10 μ L) was diluted with 0.1% NH₄OH to appropriate final concentrations in medium before being added to 100 μ L of the culture medium of near-confluent cells (10⁴ cells/well) after one or two overnight incubation. In the case to test the effect of antibodies on the cells, the culture medium was replaced with fresh medium containing pre-incubated (30 min) A β solution with antibodies. After being treated at 37 °C for 16 or 48 h, 10 μ L of 5 mg/mL MTT (Sigma) was added to cells, followed by incubation for 4 h at 37 °C. After removing the medium, 100 μ L cell lysis buffer (10% SDS, 0.01 M NH₄Cl) was subsequently added to the cells. The resulting cell lysate was subsequently incubated overnight in the dark at room temperature before absorbance measurements were made at 595 nm with a microplate reader (MultiScan JX; Thermo Scientific). Absorbance obtained by the addition of vehicle (0.1% NH₄OH) was taken as 100%.

MTT assay on rat primary neurons. Animals were treated in accordance with guidelines by the Kyoto University Animal Experimentation Committee and guidelines by The Japanese Pharmacological Society. This study was approved by Kyoto University Animal Experimentation Committee. Neuronal cultures were obtained from the cerebral cortices of fetal Wistar rats (Nihon SLC) at 17–19 days of gestation as described previously²¹. Cultures were maintained in Neurobasal medium with 2% B-27 supplement, 25 μ M sodium glutamate, and 0.5 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO₂. After 4 days in culture, medium was replaced with sodium glutamate-free Neurobasal medium. Only mature cultures (8–12 days *in vitro*) were used for the experiments. In all experiments, B-27 supplement without antioxidants was utilized during the treatment of A β 42 as described previously²¹.

Neurotoxicity was assessed by MTT assay according to the previously reported protocol²¹. After 30 min of pre-incubation on ice for A β 42 solution (10 μ M) in 0.1% NH₄OH, followed by 10-fold dilution with Neurobasal medium, the medium containing A β was added to the cell culture for replacement. After incubation of A β 42 at 37 °C for 96 h, 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). The medium of vehicle treatment for each experiment contained 0.01% NH₄OH. The absorbance obtained by the addition of vehicle (0.1% NH₄OH) was taken as 100%.

Enzyme immunoassay (EIA). A 96-well Maxisorp plate (Nunc) was incubated with each A β (2.5 μ g/well) dissolved in 50 mM sodium carbonate for 2 h at room temperature, followed by treatment for blocking with 5% bovine serum albumin at 4 °C overnight, as described previously¹³. Briefly, after incubation with each clone obtained in the previous work¹³ for 1 h at room temperature, the plate was treated with a horseradish peroxidase-coupled anti-mouse IgG antibody (IBL), and quantified using *o*-phenylenediamine dihydrochloride substrate (Sigma) before measurements at 492 nm with a microplate reader (MultiScan JX; Thermo Scientific).

Surface plasmon resonance (SPR). Binding affinity tests were performed using a BIAcore X100 biosensor (GE Healthcare), as previously described¹³. In brief, sensor chip SA, to which streptavidin was anchored, was pre-conditioned by running HBS-EP buffer (GE Healthcare). Each HFIP-treated biotinylated A β dissolved in HBS-EP buffer (1 nM) was immobilized on the chip according to the manufacturer's protocol. The antibody was dissolved in HBS-EP buffer and injected over the chip-immobilized A β at a flow rate of 5 μ L/min. Either 20 mM Gly-HCl buffer (pH 2.0) or 5 M guanidine hydrochloride was used as the regeneration buffer. The values of response units (RU) obtained from a sample cell minus the RU obtained from a reference cell were used for analysis. Association and dissociation data were collected with flowing running buffer for 180 s and 360 s, respectively. The association and dissociation rate constants, and dissociation constant (k_a , k_d , and K_D) were calculated using a serial dilution series of antibody concentrations according to BIAevaluation 3.1 software (GE Healthcare).

Dot blotting. One microliter of each A β solution (25 μ M) was applied to a nitrocellulose membrane after incubation at 37 °C (0.2 μ m pore size; Bio-rad) as previously described²¹. After blocking in 5% non-fat milk dissolved in Tris-buffered saline containing 0.1% Tween-20 overnight at 4 °C, the membrane was treated with 24B3 or 4G8 (0.5 μ g/mL) for 1 h at room temperature before being incubated with secondary antibody. Development was performed with enhanced chemiluminescence and quantified using LAS-4000 (Fujifilm). ImageJ 1.42 (NIH) software was used to quantify the blots.

Subjects and collection of CSF samples. This study was conducted in accordance with the principles of Helsinki Declaration. The study was approved by the University Ethics Committee of Kyoto Prefectural University of Medicine. All subjects provided written informed consent to participate in the study. We collected CSF samples from 13 patients [aged 68–84 (mean \pm SD, 77.1 \pm 5.6) yr] with clinically diagnosed AD ($n = 8$) or MCI ($n = 5$), and 12 age-matched control subjects [aged 61–84 (mean \pm SD, 72.3 \pm 5.8) yr; see Table 2 for characteristics of study participants]. At the time of diagnosis, a full clinical history was taken, and physical and neurological examinations, Mini-Mental State Examination (MMSE), routine blood analyses, and magnetic resonance imaging (MRI) of the brain were performed for all subjects. The patients with AD met the criteria for probable AD defined in the diagnostic and research criteria established by the National Institute on Aging (NIA) and the Alzheimer's Association (AA)⁴⁶. All MCI patients were non-demented, not fulfilling the criteria for probable AD dementia⁴⁶, but met the criteria of "MCI due to AD" defined by NIA and AA²⁷. There were no significant differences in age between the AD/MCI group and the control group. None of the control subjects had memory complaints or any other cognitive symptoms.

Fresh CSF samples were obtained from the enrolled subjects and then immediately stored at -80°C until used for immunoassays. All lumbar punctures were performed in the early morning to exclude the effects of daily fluctuation in the levels of A β in CSF⁴⁷.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates (96 wells) were coated with 100 μL /well of 50 mM sodium carbonate containing 82E1 (IBL) and allowed to adhere overnight at 4°C . Plates were washed with PBS and blocked for overnight at 4°C with 200 μL /well of 1% (w/v) bovine serum albumin in PBS containing 0.05% NaN_3 . After two washes with PBS containing 0.02% Tween-20 (PBS-T), 100 μL of CSF was serially diluted in 1% bovine serum albumin in PBS-T before being added in triplicate to wells before incubation overnight at 4°C . After four washes with PBS-T, each well was treated with 100 μL of horseradish peroxidase-conjugated 24B3 for 1 h at 4°C . ELISA signals were detected by chemiluminescence using an enhanced chemiluminescent substrate (SuperSignal ELISA Femto Maximum Sensitivity, Thermo Scientific), and then measured with a microplate luminometer (SpectraMax Pro, Molecular Devices).

Synthetic E22P-A β 40 dimer²⁴ was used as a standard protein because the E22P-A β 40 dimer also containing the toxic turn at Glu22 and Asp23 was more stable than the E22P-A β 42 dimer. The concentration of the E22P-A β 40 dimer was determined using the Bradford assay (Bio-Rad). The amount of total A β 42 in CSF was determined by sandwich ELISA with a human β Amyloid ELISA Kit of A β 42 [Cat# 27711, human amyloid β (1–42) including (X-42)] (IBL) according to the manufacturer's protocols.

Statistical analysis. All data are presented as mean \pm s.e.m. The differences were analyzed with one-way analysis of variance (ANOVA) followed by Bonferroni's test or unpaired Student's *t*-test. These tests were implemented within GraphPad Prism software (version 5.0d). *p* values < 0.05 were considered significant.

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Acknowledgements

This study was supported by JSPS KAKENHI Grant Number 26221202 to K.I., K.M., T.S., T.K., and T.T., and by funds for life science research 2013 from Takeda Science Foundation to K.I. We thank Dr. Shunsuke Izumi at the Graduate School of Sciences, Hiroshima University for MALDI-TOF-MS measurements; Mr. Harukuni Tokuda at the Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University for assistance with cell culture; Dr. Kazumitsu Ueda, Dr. Noriyuki Kioka, and Dr. Yasuhisa Kimura at the Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University for use of the SPR biosensor; and Ms. Keiko Uchida and Tomoyo Takai of the National Institute of Biomedical Innovation, Health and Nutrition, for assistance with TEM.

Author Contributions

K.M., M.T., T.S., Y.I., M.H. and K.I. prepared the A β 42 dimer; K.M., M.T. and Y.I. performed aggregation and neurotoxicity experiments; T.K. and N.I. contributed to MTT assay using primary neurons; Y.M. and K.A. contributed to TEM; K.M. performed dot blotting, EIA and SPR experiments; R.I., H.T. and T.T. contributed to ELISA using CSF; M.M. contributed to preparation of 11A1 and 24B3 antibodies; and K.M., T.Sh., and K.I. designed the study and wrote the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Murakami, K. *et al.* Monoclonal antibody with conformational specificity for a toxic conformer of amyloid β 42 and its application toward the Alzheimer's disease diagnosis. *Sci. Rep.* **6**, 29038; doi: 10.1038/srep29038 (2016).



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