Monoclonal Antibody Against the Turn of the 42-Residue Amyloid β-Protein at Positions 22 and 23

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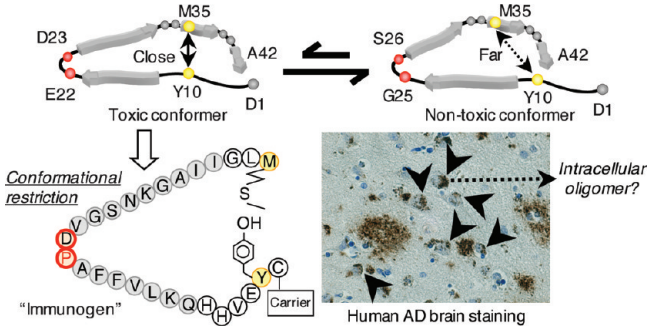
Abstract



Aggregation of the 42-mer amyloid β-protein (Aβ42) plays a critical role in the pathogenesis of Alzheimer's disease (AD). We have proposed a toxic conformer with a turn at positions 22 and 23, as well as a nontoxic conformer with a turn at positions 25 and 26, in Aβ42 aggregates from systematic proline scanning and solid-state NMR studies. Although recent clinical trials of immunization targeting Aβ42 aggregates have proved useful, some adverse effects were reported. One of the reasons was hypothesized to be excessive immunoreactions derived from the unintended removal of nontoxic Aβ42, which plays an important role in the physiological function. To develop a monoclonal antibody for toxic Aβ42, E22P-Aβ10-35, a minimum moiety for neurotoxicity containing the turn at positions 22 and 23, was used for the generation of antibodies, following the selection of clones using Aβ42 mutants of E22P (turn-inducing) and E22V (turn-preventing). The obtained clone (11A1) showed a high binding affinity (K_D = 10.3 nM) for Aβ42 using surface plasmon resonance. 11A1 also inhibited the neurotoxicity of Aβ42 in PC12 cells. Immunohistochemical studies showed that not only extracellular but intracellular amyloid was stained in human AD brains. In Western blotting analyses using human brains, low-molecular weight-oligomers rather than the monomer of Aβ were readily recognized by 11A1. These results imply that 11A1 could detect toxic Aβ42 oligomers with the turn at positions 22 and 23 and that 11A1 could be applicable for the therapeutic targeting of toxic Aβ42 in AD.

Keywords: amyloid, Alzheimer's disease, neurotoxicity, turn, human brain, transgenic mice

Alzheimer's disease (AD) is generally characterized by amyloid deposition in senile plaques that are mainly composed of 40- and 42-mer amyloid β-proteins (Aβ40 and Aβ42) (*1*, *2*). These proteins are produced from amyloid precursor protein (APP) by two proteases, β- and γ-secretases. Aβ42 plays a more important role in the pathogenesis of AD than Aβ40 because of its stronger aggregative ability and neurotoxicity (*3*). Oxidative stress is suggested to contribute to neurodegeneration associated with AD (*4*−*6*). One of the proposed mechanisms of the neurotoxicity of Aβ42 is related to radicalization at both Tyr10 and Met35 accompanied by the generation of hydrogen peroxide (*7*). On the other hand, there is substantial evidence that the oligomeric assembly of Aβ42 could induce AD via synaptotoxicity (*8*, *9*).

Immunization against Aβ is considered to be a promising approach for AD therapy because vaccination of transgenic mouse models of AD with Aβ42 aggregates resulted in a reduction of Aβ deposition and the prevention of cognitive impairment (*10*, *11*). However, clinical trials (AN1792) of immunization of AD patients against Aβ42 were interrupted because of severe adverse effects of excessive immune activation (*12*). Recently, a follow-up study has shown that immunization against Aβ42 suppressed Aβ depositions in AD patients but not the progressive cognitive impairment (*13*). One of the reasons for these problems might be the unintended elimination of both toxic and nontoxic forms of Aβ42,

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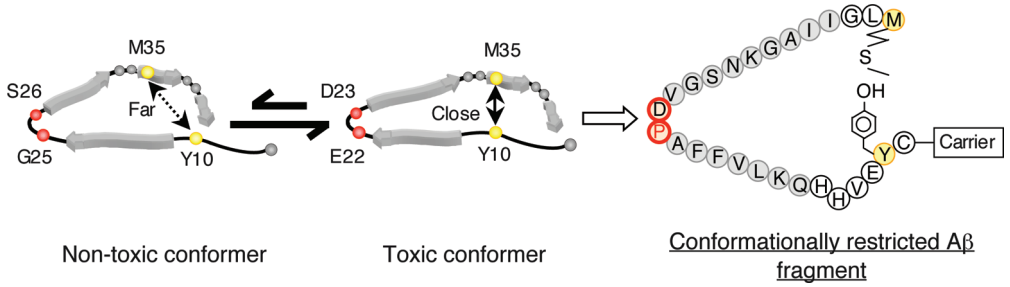

Figure 1. Nontoxic and toxic conformations of Aβ42 identified by our previous studies using solid-state NMR (*15*). The turn positions are different from each other. On the basis of the toxic conformation with a turn at positions 22 and 23, a conformationally restricted Aβ fragment was optimized as an immunogen to develop antibodies targeting toxic Aβ42.

whose role in physiological function is controversial at present. Quite recently, Tanzi and colleagues proposed the relevance of Aβ42 to the innate immune system as an antimicrobial protein (*14*). Aβ42 may have one conformer with the normal function required for brain regulation. Therefore, the discrimination of nontoxic from toxic Aβ42 is indispensable to block the progression of AD pathology and cognitive dysfunction effectively.

A recent investigation using solid-state NMR together with systematic proline replacement differentiated the toxic conformer with a turn at positions 22 and 23 in Aβ42 aggregates from the nontoxic one with a turn at positions 25 and 26; the former showed potent aggregative ability and neurotoxicity (*15*) (Figure 1). At least two conformers can exist in an equilibration of Aβ42. To generate a monoclonal antibody for toxic Aβ42, E22P-Aβ10−35, which contains both Tyr10 and Met35 required for neurotoxicity (*7*) and the turn at positions 22 and 23 as a Pro-X corner (X = variable amino acid residue) (*16*), was used as the immunogen (Figure 1). This paper describes the development and characterization of the 11A1 monoclonal antibody that was designed to target the toxic conformer of Aβ42. The 11A1 antibody demonstrated a high binding affinity for Aβ42 in surface plasmon resonance (SPR) analyses, inhibited Aβ42-induced neurotoxicity in PC12 cells, detected intracellular as well as extracellular Aβ in AD brain sections, and recognized low-molecular weight-oligomers rather than monomers of Aβ in AD brain extracts.

Results and Discussion

Development and Characterization of Monoclonal Antibody 11A1 against the Toxic Conformer of Aβ42

Clones were selected based on the ability to react with Aβ42 mutants that have a propensity to form a β-turn at positions 22 and 23 (*17*) (e.g., E22Q-Aβ42, E22G-Aβ42, E22K-Aβ42, E22P-Aβ42, and D23N-Aβ42), to obtain a unique clone named 11A1. As shown in Figure 2A (left), 11A1 showed stronger immunoreactivity with E22P-Aβ42 than Aβ42 and Aβ40, but weak affinity to E22

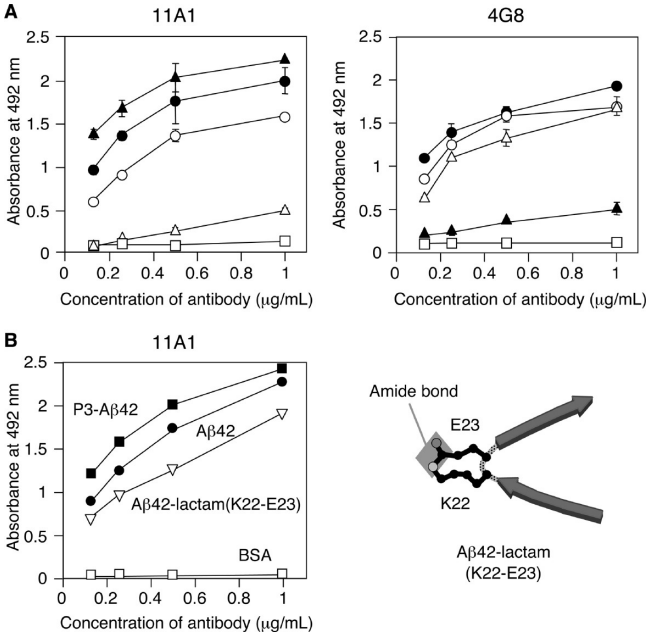

Figure 2. (A) Enzyme immunoassay of 11A1 (left) and 4G8 (right) monoclonal antibodies at concentrations of 0.125, 0.25, 0.5, and 1.0 μg/mL on Aβ42 mutants coated on to 96-well plates: ●, Aβ42; ○, Aβ40; ▲, E22P-Aβ42; △, E22 V-Aβ42; □, 0.1% (w/v) BSA. (B) Enzyme immunoassay of 11A1 antibody at concentrations of 0.125, 0.25, 0.5, and 1.0 μg/mL with Aβ42 mutants: ●, Aβ42; ■, P3-Aβ42; ▽, Aβ42-lactam(22K-23E); □, 0.1% (w/v) BSA and the structure of Aβ42-lactam(K22-E23).

V-Aβ42, in which valine was used as a turn breaker (*16*). 11A1 appeared not be proline residue specific since 11A1 bound to E22P-Aβ42, as well as Aβ42 to a similar extent. Our recent reports have examined conformationally restricted analogues of Aβ42 with a turn at positions 22 and 23: Aβ42-lactam (K22-E23, Figure 2B, right), in which the side chains at positions 22 and 23 were linked covalently (*15*), and the triple Aβ42 mutant (P3-Aβ42) with proline residues substituted at the three possible turn positions (22, 34, and 38) (*18*). 11A1 also bound efficiently to these mutants in a dose-dependent manner (Figure 2B, left). Therefore, 11A1 was thought to be sensitive to a turn at positions 22 and 23 of Aβ. The highly similar immunoreactivity of 11A1 for Aβ40 and

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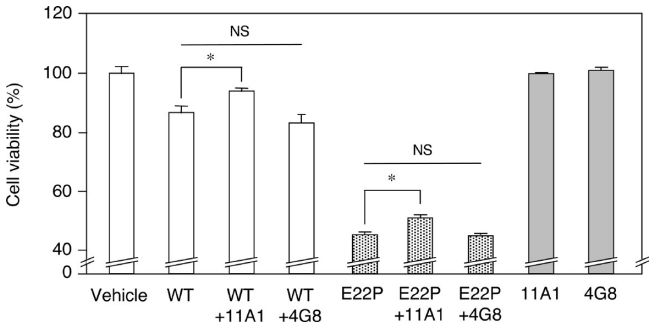

Figure 3. Neurotoxicity of Aβ42 (WT) and E22P-Aβ42 (0.1 μM) in PC12 cells, and their inhibition by 11A1 or 4G8 antibodies (0.36 μM, 0.054 mg/mL) estimated by MTT assay. Antibodies alone were used as the controls. *$p < 0.05$. Data are expressed as mean ± sem. NS: not significant.

Aβ42 (Figure 2A, left) might indicate the common presence of the turn at positions 22 and 23 of both Aβ42 and Aβ40. The existence of a turn at positions 22 and 23 in Aβ42 and Aβ40 was deduced from systematic proline replacement by us (*18*) and Wetzel's group (*19*), respectively.

On the other hand, a conventional anti-Aβ17-24 antibody, 4G8 (*20*), showed weak reactivity with E22P-Aβ42, while it bound effectively to E22V-Aβ42, as well as Aβ42 and Aβ40 (Figure 2A, right). These results suggested that 4G8 could not recognize the turn structure at positions 22 and 23 because E22V-Aβ42 could not readily form a turn structure at this position (*17*). The opposite reactivity of 4G8 from 11A1 for the Aβ42 mutants (Figure 2A) was reasonable because 4G8 could be residue specific; its epitope lies within the amino acids at positions 17−24 (*20*).

The dissociation constant ($K_D = k_d/k_a$) of 11A1 for Aβ42 calculated from the association (k_a) and dissociation (k_d) rate constants was determined from experiments using SPR. 11A1 showed a high binding affinity for Aβ42 (K_D = 10.3 nM, $k_a = 50.5 \times 10^3$ M^{-1} s^{-1}, $k_d = 0.522 \times 10^{-3}$ s^{-1}).

Inhibition of Aβ42-Induced Neurotoxicity in PC12 Cells by 11A1

Aβ42-induced neurotoxicity in PC12 cells in the presence or absence of 11A1 was determined using the MTT method (Figure 3). PC12 cells treated with Aβ42 at 0.1 μM showed lower viability than the control cells, and this toxicity was blocked by 11A1 at 0.36 μM (0.054 mg/mL) in a statistically significant manner. On the other hand, 4G8 antibody failed to inhibit Aβ42-induced cytotoxicity. Moreover, the neurotoxicity by E22P-Aβ42 (0.1 μM), which can more readily form the toxic conformer of Aβ42 than wild-type Aβ42 (*15*), was also inhibited by 11A1, but not by 4G8. These antibodies at 0.36 μM did not affect cell viability (Figure 3).

Although the inhibition of Aβ42 or E22P-Aβ42-induced toxicity by 11A1 is significant, the effect is not large, in contrast to the results in Figure 2. One of the reasons is presumably due to the difference of the ratio for Aβ doses to those of antibodies in the two independent experiments (Figures 2 and 3). Alternatively, the difference of experimental conditions will be assumed; the Aβ-immobilized plate in the enzyme immuno assay was used (Figure 2), while the culture in the cell test was utilized (Figure 3). Another possibility might be the lower effective concentration of 11A1 induced from the limitation of 11A1 solubility than 4G8; the concentrations of 11A1 and 4G8 after incubation for 48 h at 37 °C were 0.38 and 0.93 mg/mL (before incubation; 0.54 and 1.0 mg/mL), respectively. These results imply that 11A1 might be less stable, resulting in lower inhibition of Aβ42-induced cytotoxicity by 11A1 than expected. This problem of its solubility and stability should be solved before its application for therapy by improving the purification method (column, cell culture condition, and so on).

Detection of Intracellular as well as Extracellular Aβ in Human AD Brains by 11A1

Immunohistochemical studies of 11A1 and 4G8 antibodies were carried out using the frontal lobe and hippocampus regions of autopsied brains from 17 AD and 18 non-AD individuals (Table 1). As shown in Figure 4A (left), both antibodies reacted with typical amyloid plaques in the frontal lobe of AD patients, whereas interestingly some intracellular staining was detected only by treatment with 11A1 (Figure 4A, arrowheads). 11A1 showed mild intracellular staining even in non-AD individuals (Figure 4A, right). A similar pattern of intracellular as well as extracellular staining was observed in 11A1-treated hippocampus section of the same brains (Figure 4B). The evaluation of staining for senile plaques and intracellular amyloid in all 35 subjects is summarized in Table 1. To eliminate the possibility that this intracellular staining was nonspecific for amyloid, inhibition of the staining by the immunogen of 11A1 was investigated (Figure 5). Preincubation of 11A1 with its immunogen (200-fold molar excess) resulted in no staining in AD brain sections, indicating that 11A1 could detect Aβ within the cells as well as senile plaques (Figure 5, upper). In a control experiment, 4G8 did not react at all with the immunogen of 11A1 (Figure 5, lower).

Moreover, immunohistochemical studies of 11A1 were performed using two representative APP transgenic mice, Tg2576 (28 months old) and J20 (12 months old). However, the intracellular staining of amyloid in humans was weakly observed in mice, although senile plaques were detected significantly (Figure 6). It is notable that the intracellular amyloid detected by 11A1 was stained only in human brain sections but weakly in those of APP transgenic mice (Tg2576 and J20). This was similar to

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Table 1. Summary of Neuropathological Diagnosis and Immunohistochemical Data[a]

					11A1		
case	age	sex	CDR	Braak	SP	IA	NP diagnosis
1	96	M	3	6	+++	++	AD
2	83	F	3	5	+++	+++	AD
3	84	M	3	5	++	++	AD
4	86	F	1	5	+++	++	AD
5	91	F	1	5	++	+++	AD
6	82	F	3	5	+++	+++	AD
7	86	F	3	6	++	++	AD
8	84	M	2	6	+++	++	AD
9	76	M	3	6	++	++	AD
10	84	F	3	5	++	+	AD
11	84	F	2	4.5	++	++	AD
12	86	F	3	5	++	++	AD
13	87	F	3	5	++	+++	AD
14	74	M	0	5	+++	++	AD
15	82	F	1	5	++	+	AD
16	81	M	2	6	++	+	AD
17	87	M	3	6	+++	++	AD
18	79	M	0.5	2	—	+	non-AD
19	80	F	0	2	—	++	non-AD
20	82	F	N/A	2	—	+	non-AD
21	80	M	0	2	+	+	non-AD
22	75	M	0	1	—	+	non-AD
23	69	M	N/A	1	—	—	non-AD
24	70	M	0	1	—	+	non-AD
25	68	M	0	1	—	+	non-AD
26	67	M	N/A	1	+	+	non-AD
27	67	M	0	2	+	—	non-AD
28	80	M	0.5	2	+	—	non-AD
29	72	M	0	1	+	+	non-AD
30	81	M	0	1	—	+	non-AD
31	82	M	0	1	—	—	non-AD
32	78	F	N/A	1	—	—	non-AD
33	83	M	N/A	1	—	—	non-AD
34	77	F	0	1	—	+	non-AD
35	79	F	N/A	1	—	++	non-AD

[a] CDR, clinical dementia rating; Braak, Braak staging; SP, senile plaque; IA, intracellular amyloid; NP diagnosis, neuropathological diagnosis; AD, Alzheimer's disease. +, mild reactivity; ++, moderate reactivity; +++, strong reactivity; —, not detected; N/A, not available.

the antisoluble amyloid oligomer antibody (A11) developed by Glabe and colleagues; A11-positive staining existed only in human AD brains but not in Tg2576 mice brains (*21*). Although the production of conformation-sensitive antibodies targeting oligomers has been attempted by direct in vivo intracellular selection by Meli et al., almost no intracellular staining was observed in human brains (*22*). Hoshi and colleagues showed that monoclonal antibodies against amylospheroid, one of toxic Aβ aggregates associated with AD pathology,

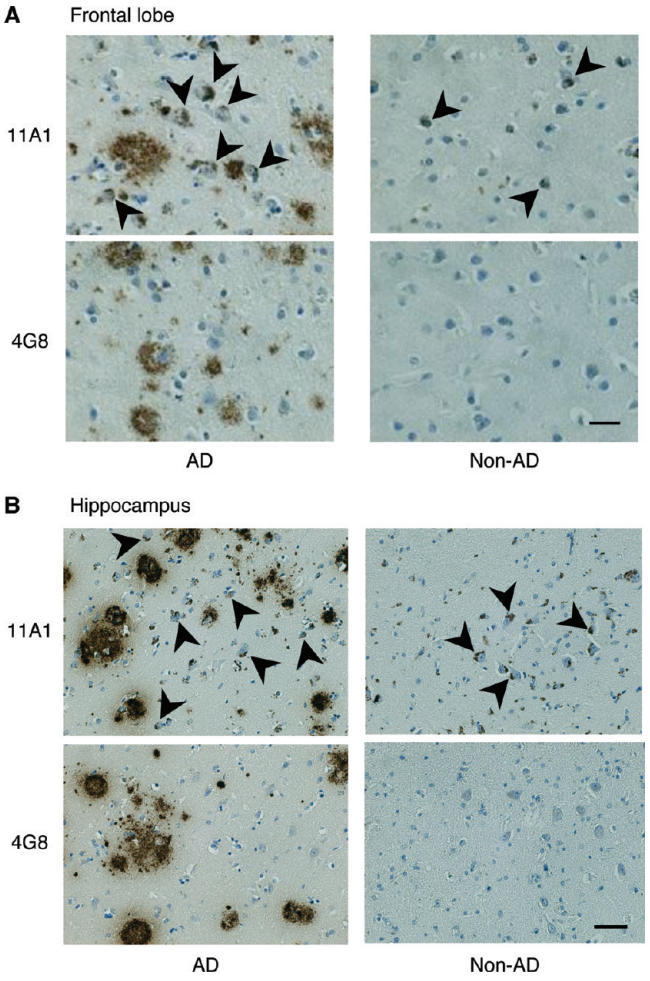

Figure 4. Immunohistochemistry in (A) the frontal lobe of human AD (case 1) and non-AD (case 18) patients and the hippocampus of human AD patient (case 1) and non-AD individual (case 18) using 11A1 (upper) and 4G8 (lower) antibodies, respectively. The scale bar in (A) and (B) represents 50 and 100 μm, respectively. Arrowheads indicate the staining of intracellular Aβ within the cells detected only in the 11A1-treated sections.

bound to only extracellular amyloid plaques in human brain sections (*23*). 11A1 is thus a unique antibody that preferably recognizes intracellular amyloid in human brain along with senile plaques.

As summarized in Table 1, moderate or strong reactivity for intracellular amyloid was frequently observed in almost all AD patients examined, while moderate staining was seen even in some of non-AD individuals (case 19 and 35), suggesting that intracellular amyloid could be involved in the progression of AD. Intracellular Aβ may be more important than extracellular Aβ because intraneuronal Aβ deposition frequently precedes extracellular Aβ accumulation in the human brain (*24*−*29*). Recently, mitochondrial toxicity, proteasome impairment, and synaptic damage due to intracellular Aβ have been suggested (*28*).

Ohyagi and colleagues (*30*) reported the enhancement of immunoreactivity for intraneuronal Aβ42 by autoclaving

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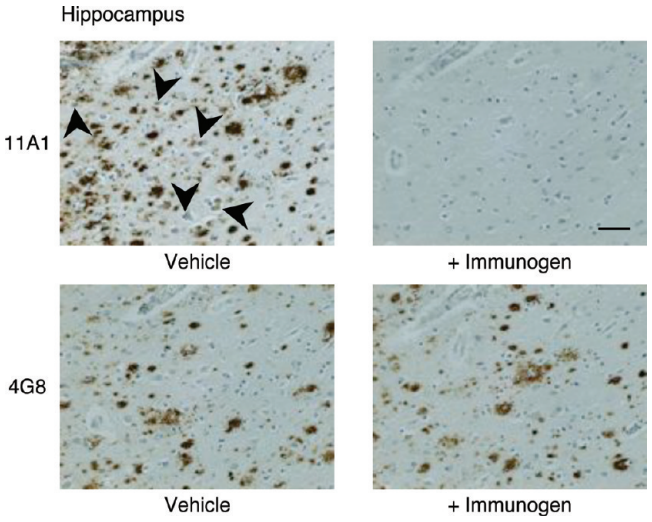

Figure 5. Inhibition assay in AD patient (case 2) by 11A1, 4G8 alone, or with the immunogen of 11A1 (200-fold molar excess) as indicated. Arrowheads indicate the staining of intracellular Aβ within the cells. The scale bar represents 100 μm.

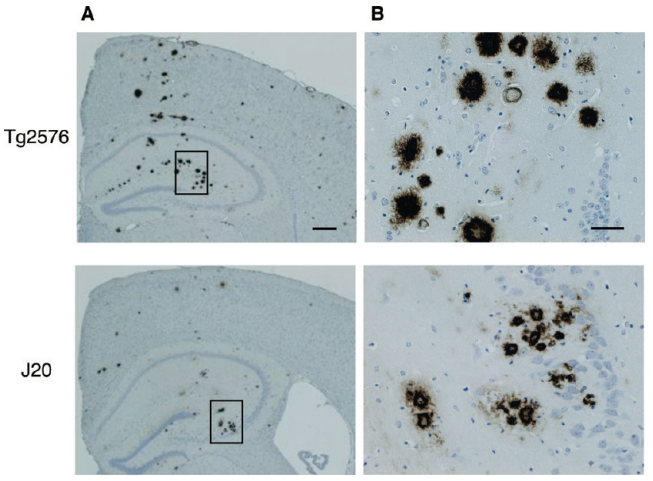

Figure 6. (A) Immunohistochemistry of the 11A1 antibody in the hippocampus and cortex of two representative APP transgenic mice (upper, female Tg2576 at 28 months old; lower, female J20 at 12 months old). The scale bar represents 500 μm. (B) High magnification of the area in box as shown in (A). The scale bar represents 50 μm.

and the reduction by conventional protocols just using formic acid in human sections. If 11A1 is conformation-, but not sequence-sensitive, formic acid treatment may somewhat alter the conformation of depositing Aβ. Recently, Christensen et al. revealed that formic acid treatment may be essential for staining highly aggregated Aβ in neurons (*31*). 11A1 may detect highly aggregated Aβ, which has a stable conformation against FA treatment in AD patients but not in the AD mouse model, although there are already other AD mouse models, such as the 3xTg mouse reported by Oddo et al. (*32*), which shows preferably abundant intraneuronal Aβ. Alternatively, the possibility cannot be completely ruled out that 11A1 could bind to other intracellular proteins with a sequence similar to Aβ with the turn at positions 22 and 23.

Recognition of Aβ Low-Molecular Weight-Oligomers rather than Monomer in Human AD Brains by 11A1

To address the question whether 11A1 can recognize Aβ oligomers in human brain, Western blotting was carried out using the Tris-buffered saline (TBS)-soluble fraction of frontal lobe tissues. Notably, in the low-molecular weight region of AD tissues, 11A1 potently reacted with low-molecular weight-oligomers (supposed to be mainly trimer) band (Figure 7A), while 4G8 and 82E1, whose epitope is the N-terminus of Aβ (*33*), strongly reacted only with the monomer of Aβ (Figure 7A, arrowhead). These data are consistent with our previous reports (*15*) describing the turn at positions 22 and 23 can induce the oligomerization of Aβ42.

On the other hand, in the high-molecular weight region, 11A1 exhibited a slightly different pattern from 4G8 and 82E1 (Figure 7A). The band pattern against anti-C-terminal APP antibody was different from that of 11A1 in the TBS-soluble fractions of AD (Figure 7B). Anti-C-terminal APP antibody reacts with three isoforms of APP (APP695, APP751, and APP770), but not with secreted APPα and APPβ after the α- and β-cut of APP, respectively (*34*). The 82E1 antibody does not show any cross-reactivity with APP (*33*). Furthermore, 11A1 failed to detect recombinant human APP, while anti-N-terminal APP antibody surely bound APP, whose presence was confirmed by Coomassie brilliant blue (CBB) staining (Figure 7C). These suggest that high-molecular weight bands might have originated from Aβ aggregates, but not APP. To check the authenticity of the antigenicity of 11A1, an absorption test using the immunogen was carried out (Figure 7D) because Western blotting generally has the problem that protein conformation is basically denatured during SDS-PAGE. As shown in Figure 7D, almost no bands for the low-molecular weight-oligomers were obtained after preincubation of 11A1 with its immunogen (200-fold molar excess), whereas weak bands in the high-molecular weight region were found. These results support the more preferable immunoreactivity of 11A1 for the low-molecular weight-oligomers; however, the denatured condition under SDS-PAGE might affect the amount of soluble Aβ oligomer detected in AD brains because 11A1 is supposed to be conformation-sensitive.

In the TBS-insoluble fraction of brain tissue, 11A1 reacted with the monomer band (Figure 7E, arrowhead) along with lots of mixed high-molecular weight-aggregates, whose pattern was almost compatible with those of other

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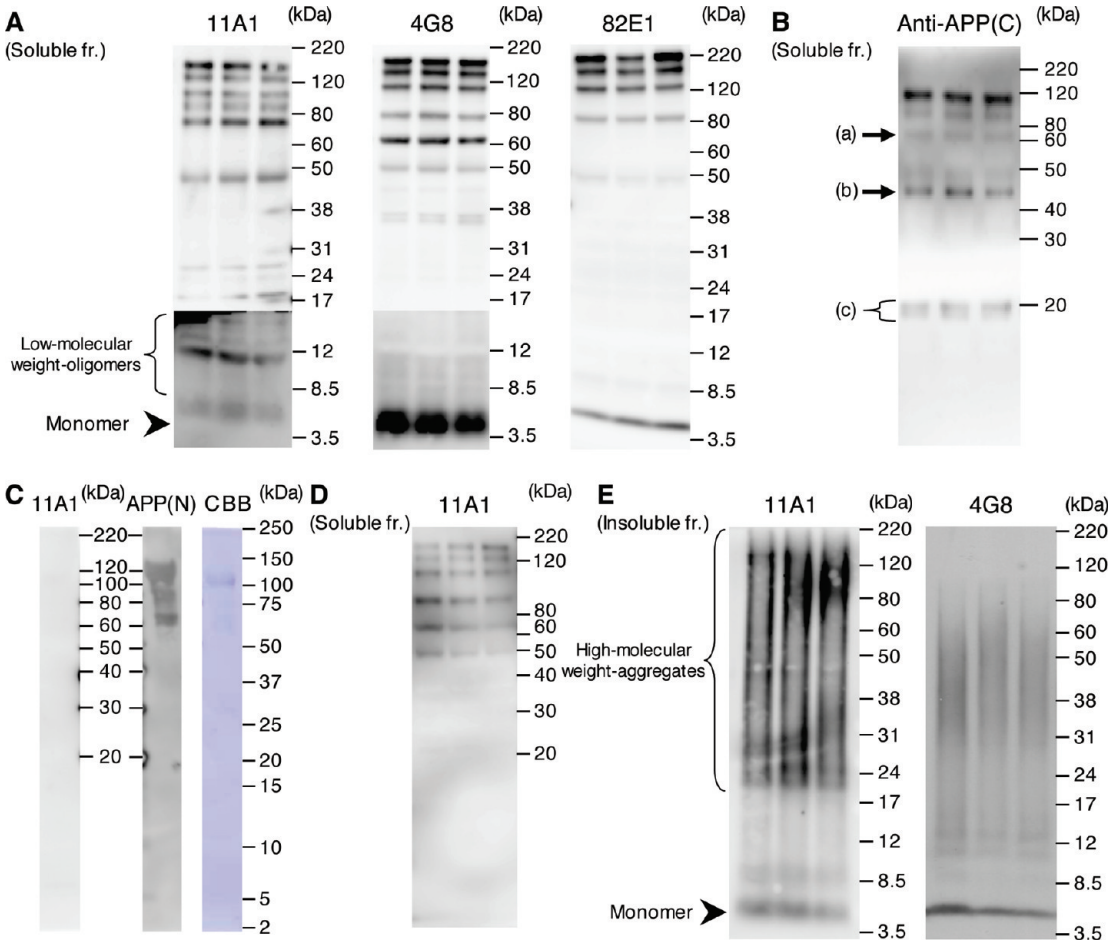

Figure 7. Western blotting of (A, B, D) TBS-soluble or (E) TBS-insoluble fractions of AD patients (case 3−5 in Table 1) and (C) recombinant human APP using (A, C, D, E) the indicated anti-Aβ antibodies; 11A1, 4G8, and 82E1 or anti-N-terminal APP antibody, (B) anti-C-terminal APP antibody. In the absorption test of D, the immunogen of 11A1 (200-fold molar excess) was used in AD patients (case 3−5). Arrowheads indicate Aβ monomers. The molecular markers of the kDa unit are shown together with blots. In B, a−c represent the lamin (70 kDa), β-actin (42 kDa), and C-terminal fragments (CTFs; 12−14 kDa), respectively, in which the lamin and β-actin were used as an internal standard. CBB: Coomassie brilliant blue.

conventional antibodies described in Saido et al. (*35*). In contrast, 4G8 detected the Aβ monomer more clearly than the high-molecular weight-aggregates in the insoluble fraction (Figure 7E); this supports that 11A1 can be conformation-sensitive because the Aβ monomer derived from the insoluble fraction is highly denatured by formic acid.

Conclusions

In this work, we developed a unique antibody, 11A1, against the toxic conformer of Aβ42, and 11A1 significantly detected intracellular as well as extracellular Aβ in human brain sections, and low-molecular weight-oligomers of Aβ in human brain extracts. A low but significant level of intracellular Aβ even in non-AD individuals suggests that 11A1 might recognize low-molecular weight-oligomers of Aβ with the potential for AD within the cells in human brains, although we should take into account that multiple manipulations and wash steps in the experiments could induce soluble Aβ oligomers in the brain extracts, which are known to be metastable. Further research will be needed to elucidate the cell types (e.g., neuron, microglia, and astrocyte) stained by 11A1 and whether intracellular Aβ stained in the human brains is an oligomer. To clarify whether toxic oligomers of Aβ42 with a turn at positions 22 and 23 exist in the brain of AD patients is our next goal. 11A1 recognizes the turn structure in the middle portion of Aβ sequence as an epitope, which is quite different from conventional anti-Aβ middle sequence-specific antibodies, leading to a difference in intracellular immunoreactivity in human brains. 11A1 might become a new tool to investigate the role of intracellular amyloid in the pathogenesis of AD and be applicable to anti-Aβ therapeutic approaches.

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Methods

Development of Monoclonal Antibody (11A1) for the Toxic Conformer of Aβ42 and the Enzyme Immunoassay

G9C, E22P-Aβ9-35 (CYEVHHQKLVFFAPDVGSNK-GAIIGLM) as an immunogen for the toxic conformer of Aβ42, was synthesized by the method described previously (*36*). The N-terminal glycine residue was replaced with a cysteine residue to bind to a carrier protein, bovine thyroglobulin, following the standard method (*33*). Mice (BALB/c, Charles River, Japan) were immunized weekly for a month with the conjugated G9C, E22P-Aβ9-35 (50 μg/mouse) mixed with complete Freund's adjuvant once followed by booster injections with the antigen in incomplete Freund's adjuvant three times. A 96-well Maxisorp plate (Nunc, Denmark) coated with various Aβ42 mutants (50 μg/mL) was incubated with the obtained clones for one hour at room temperature, followed by treatment with a horseradish peroxidase-coupled antimouse IgG antibody [Immuno-Biological Laboratories (IBL), Gunma, Japan], and quantified using 3,3′,5,5′-tetramethylbenzidine (Pierce, Rockford, IL) or *o*-phenylenediamine dihydrochloride substrate (Sigma, St. Louis, MO). A total of 45 clones were obtained and screened by the ability to bind Aβ42 mutants (E22P, E22Q, E22K, E22G, D23N) with a propensity to form a β-turn at positions 22 and 23 (*17*), and then subcloned to obtain seven monoclones. The screening was repeated to exclude weak or false positives. They were further screened by the inability to bind E22 V-Aβ42 without a propensity to form a turn at positions 22 and 23 to yield 11A1, whose isotype is IgG1. Mice used for the development of antibodies were maintained and studied according to the protocols approved by the Animal Care Committee of IBL Co., Ltd. The molecular weight of G9C, E22P-Aβ9−35 as an immunogen and E22P-Aβ9−35 for an absorption experiment were confirmed using matrix assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) (Supporting Information, Figure S1) as reported previously (*37*).

Binding Kinetics by Surface Plasmon Resonance (SPR)

Binding affinity tests were performed using a BIAcore X100 biosensor (BIAcore, Inc., Uppsala, Sweden). A CM5 sensor chip was activated as recommended by the manufacturer using an equimolar mix of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC), coupled with 5 μM Aβ42 in sodium acetate buffer (pH 4.8), and then blocked with ethanolamine. The antibody was dissolved in phosphate buffered saline (PBS) containing 0.1% Tween-20 running buffer (pH 7.4) and injected over the chip-immobilized Aβ42 at a flow rate of 10 μL/min. Guanidine hydrochloride (5 M) was utilized as a regeneration buffer. The association and dissociation rate constants (k_a and k_d) were determined using different antibody concentrations of 225, 450, 900, 1800, and 3600 nM. Samples of 90 μL were injected. Dissociation data were collected with flowing running buffer for 120 s. The values of the observed response units (RU) obtained in the sample cells minus the RU obtained from a reference cell were used for analysis. Kinetic parameters were evaluated using BIAevaluation 3.1 software (BIAcore).

Cytotoxicity Test Using PC12 Cells

To evaluate the cytotoxicity of Aβ using the MTT assay, we used PC12 cells, which have the potential to differentiate into neural cells. Since they are sensitive to Aβ proteins, they are generally used for estimating cytotoxicity as a neurotoxicity model (*38*). PC12 cells (RCB0009) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Aβ42 or E22P-Aβ42 alone or with antibody (11A1 or 4G8) at the concentration of 0.36 μM (0.054 mg/mL) was added to the undifferentiated cells, and incubated at 37 °C for 48 h; initially 3.6 μM (0.54 mg/mL) was the maximum solubility in buffer. The protein concentrations of antibodies after the MTT assay were determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA) according to the protocol of the manufacturer with bovine serum albumin used as the standard. The concentration of each Aβ used was 0.1 μM, which was close to the IC_{50} value of E22P-Aβ42. The details of the experimental procedure have been described previously (*17*).

Immunohistochemical Analysis

The frontal lobe and hippocampus in brain sections of AD (7 male, 10 female) and non-AD control (13 male, 5 female) individuals (Table 1) were used in the experiment with written informed consent obtained from the patients' families, and the consent was approved by the Ethical Committee of Tokyo Metropolitan Institute of Gerontology and Tokyo Metropolitan Geriatric Hospital. The National Institute on Aging−Reagan criteria modified were adopted for diagnosis of AD (*39*). The normal controls were defined as clinical documentation of unimpaired cognition as well as minimal senile changes, consisting of Braak's neurofibrillary tangle stage equal to or less than II, senile plaque stage equal to or less than A (*40*), and lacking any vascular, inflammatory or traumatic changes or tumors.

In two strains of APP transgenic mice [Tg2576 (*41*) at 28 months old and J20 (*42*) at 12 months old], brains were dissected, fixed in a 4% paraformaldehyde (Wako, Osaka, Japan) for 3−5 days, embedded in paraffin, and sectioned using a microtome at 5 μm thicknesses by standard techniques. The animals were housed in a 12-h light/dark cycle and were fed ad libitum. The mice used in the immunohistochemical study were maintained and studied according to protocols approved by the Animal Care Committee of the Tokyo Metropolitan Institute of Gerontology.

The human or mice brain sections were deparaffinized, rehydrated, and washed in PBS, followed by a brief treatment with formic acid. After incubation in 3% hydrogen peroxide in methanol to prevent endogenous peroxidation, the sections were blocked with 10% normal goat serum in PBS, followed by incubation with the primary antibody [11A1; 5 μg/mL, 4G8; 1 μg/mL (Signet, Dedham, MA)] overnight at 4 °C. Then, the sections were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Immunoreactivity was visualized using an ABC Elite kit (ABC Elite, Vector Laboratories, Burlingame, CA) in accordance with the manufacturer's protocol. 3,3′-Diaminobenzidine (Sigma) was used as a chromogen. The sections were counterstained with hematoxylin.

Preparation of Human Brain Extracts and Western Blotting Analysis

The frontal lobe in the brain of AD patients and non-AD control individuals was used for biochemical experiments.

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The definite diagnosis of AD was based on the presence of neurofibrillary tangles and neuritic plaques in the hippocampal formation and neocortical area. The neuropathological diagnosis of individuals used is summarized in Table 1.

Tissue in frontal lobe was homogenized in 10 volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.6) containing 150 mM NaCl (TBS), a mixture of protease inhibitors (Complete EDTA-free, Roche Diagnostics, Indianapolis, IN, USA), and a mixture of phosphatase inhibitors (Phos STOP, Roche Diagnostics) supplemented with 0.7 μg/mL pepstatin A (Peptide Institute, Osaka, Japan) and 1 mM phenylmethylsulfonyl fluoride (Sigma). The homogenates were centrifuged at 186,000 $\times$ g for 30 min at 4 °C using an Optima TL ultracentrifuge and a TLA100.4 rotor (Beckman, Palo Alto, CA, USA) to give supernatant (soluble) and pellet (insoluble) fractions. The pellet was dissolved by sonication in 70% formic acid containing a mixture of protease inhibitors based on the protocol of Saido et al. (*35*). The solubilized pellet was centrifuged at 186,000 $\times$ g for 30 min at 4 °C for 30 min, after which the supernatant was neutralized with 1 M Tris base of pH 11 (1:20, v:v) as an insoluble fraction.

The fractions (2 μg/μL) were subjected to Western blotting using 10−20% Tricine gels (Invitrogen, Gaithersburg, MD, USA) and transferred to PVDF membrane (0.2 μm pore size, Bio-Rad, Hercules, CA, USA). Recombinant human APP with protease nexin II was used to evaluate the cross-reactivity of 11A1 with APP (R&D, Minneapolis, MN, USA), and Coomassie brilliant blue was utilized to confirm the existence of proteins. Membranes were heated in PBS (1 min, microwave), blocked in TBS-T (TBS containing 0.01% Tween-20, 2.5% skim milk), and incubated with the primary antibody overnight at 4 °C [11A1; 5 μg/mL, 4G8; 1 μg/mL, 82E1 (IBL); 1 μg/mL, N-terminal or C-terminal APP (IBL); 1 μg/mL, actin (Sigma); 1 μg/mL, lamin (ImmuQuest); 1 μg/mL], followed by washing with TBS-T and treatment with the secondary antibody (1 h, room temperature). The development was performed with enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, England) and quantified using LAS-3000 (Fujifilm, Tokyo, Japan).

Supporting Information Available

Additional data including extended figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

K.M., N.K., T.Shir., T.Shim., and K.I. designed research; K.M., Y.H-S., N.M., and Y.N. performed research; Y.M., H.H., and S.M. contributed new reagents/analytic tools; K.M., N.K., T.Shir., T.Shim., and K.I. analyzed data; and K.M., T.Shim., and K.I. wrote the paper.

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Abbreviations

AD, Alzheimer's disease; Aβ, amyloid β; APP, amyloid precursor protein; CBB, Coomassie brilliant blue; MALDI-TOF-MS, matrix assisted laser desorption ionization-time-of-flight-mass spectrometry; PBS, phosphate buffered saline; SPR, surface plasmon resonance; TBS, Tris-buffered saline.

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