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RESEARCH ARTICLE

Accumulation of cellular prion protein within β-amyloid oligomer plaques in aged human brains

Reisuke H. Takahashi[1,2] | **Mayumi Yokotsuka**[1] | **Minoru Tobiume**[2] | **Yuko Sato**[2] | **Hideki Hasegawa**[2] | **Toshitaka Nagao**[1] | **Gunnar K. Gouras**[3]

[1]Department of Anatomic Pathology, Tokyo Medical University, Tokyo, Japan

[2]Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan

[3]Experimental Dementia Research Unit, Department of Experimental Medical Science, Lund University, Lund, Sweden

Correspondence
Reisuke H. Takahashi, Department of Anatomic Pathology, Tokyo Medical University, Nishi-Shinjuku 6-7-1, Shinjuku, Tokyo, 160-0023, Japan.
Email: takaharh@tokyo-med.ac.jp

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Abstract

Alzheimer's disease (AD) is the main cause of dementia, and β-amyloid (Aβ) is a central factor in the initiation and progression of the disease. Different forms of Aβ have been identified as monomers, oligomers, and amyloid fibrils. Many proteins have been implicated as putative receptors of respective forms of Aβ. Distinct forms of Aβ oligomers are considered to be neurotoxic species that trigger the pathophysiology of AD. It was reported that cellular prion protein (PrP^C) is one of the most selective and high-affinity binding partners of Aβ oligomers. The interaction of Aβ oligomers with PrP^C is important to synaptic dysfunction and loss. The binding of Aβ oligomers to PrP^C has mostly been studied with synthetic peptides, cell culture, and murine models of AD by biochemical and biological methods. However, the molecular mechanisms underlying the relationship between Aβ oligomers and PrP^C remain unclear, especially in the human brain. We immunohistochemically investigated the relationship between Aβ oligomers and PrP^C in human brain tissue with and without amyloid pathology. We histologically demonstrate that PrP^C accumulates with aging in human brain tissue even prior to AD mainly within diffuse-type amyloid plaques, which are composed of more soluble Aβ oligomers without stacked β-sheet fibril structures. Our results suggest that PrP^C accumulating plaques are associated with more soluble Aβ oligomers, and appear even prior to AD. The investigation of PrP^C accumulating plaques may provide new insights into AD.

KEYWORDS
amyloid plaque, Aβ oligomer, human brain, neuropathology, PrP^C

1 | INTRODUCTION

A characteristic hallmark of Alzheimer's disease (AD) is the deposition of amyloid plaques, which consist of β-amyloid peptides (Aβ), in the brain. Aβ is produced from the amyloid precursor protein (APP) by β- and γ-secretases. Aβ can exist in multiple forms as full length and various N- and C-truncated monomers, low and high molecular weight soluble oligomers, protofibrils, and fibrils, which form from the self-associated assembly. Soluble Aβ oligomers, precursors of amyloid fibrils, are proposed to be the main neurotoxic species in AD,

rather than amyloid fibrils with stacked β-sheet structures stained by thioflavin S (1–4). Aβ oligomers induce and accelerate Aβ seeding and play an important role in the early initiation of Aβ aggregation (5). Aβ oligomers are associated with early neuritic degeneration, especially in synaptic compartments (6,7), leading to cognitive impairment (8). Experimentally, mouse brains, brain slices, and primary neurons have been treated with synthetic Aβ oligomers or natural Aβ oligomer enriched extracts isolated from AD brains (9–11). Memory impairment, inhibition of long-term potentiation (LTP), synaptic dysfunction, and loss of dendritic spines were observed in such experiments (2,11,12).

The cellular prion protein (PrP^C) is a membrane-bound cell surface glycoprotein that is C-terminally bound to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor and positioned in lipid rafts (13,14). PrP^C is highly expressed on neurons, and it was recently reported that PrP^C is also highly expressed on neuronal exosomes (15). PrP^C can be the substrate for the production of the pathological isoform of PrP (PrP^{SC}), which plays an important role in prion diseases such as Creutzfeldt-Jakob disease (16). PrP^C and the prion paradigm also play key roles in other neurodegenerative diseases, including AD (17–19). It was identified that PrP^C is a high-affinity binding partner of Aβ oligomers (20–22). The N-terminus of PrP^C contains the binding site for Aβ oligomers (23), and this interaction is involved in Aβ neurotoxicity. The binding of Aβ oligomers to PrP^C with a co-receptor, metabotropic glutamate receptor 5 (mGluR5), in dendrites appears to cause neuronal degeneration via the activation of the Src kinase Fyn (24), leading to a loss of surface N-methyl-D-aspartate receptors (NMDARs) (25–27). Aβ oligomers affect the trafficking and endocytosis of PrP^C (28). The interaction between PrP^C and Aβ oligomers has been reported to impair synaptic plasticity (29) and LTP (9). Furthermore, dendritic spine loss (27), spatial memory deficits (29), and tau pathology spreading (30) were observed in AD mouse models due to this interaction. In an AD model mouse, the deletion of PrP^C rescued mice from the loss of synaptic markers, neuritic degeneration, early death, and AD pathology (29,31,32).

The mechanism by which Aβ oligomers cause neurotoxicity mediated by PrP^C is not well understood. It is presumed that the neurotoxic effects start with the binding of Aβ oligomers to PrP^C on the surface of neurons. Thus, Aβ oligomers and PrP^C might be expected to co-localize and accumulate together. The co-localization of PrP^C and Aβ42 oligomers was shown in hippocampal mouse primary neurons (21). It was also demonstrated that PrP^C was especially distributed to dendritic spines in neuronal cells and co-localized with Aβ in AD human brain tissues (25). However, it is possible that only specific Aβ oligomeric species are involved in these protein-protein interactions, and the endogenous types of Aβ molecules that are relevant to these interactions have not been precisely elucidated.

In this study, we immunohistochemically demonstrate the co-localization of endogenous Aβ and PrP^C in amyloid plaques of human brain tissue and characterize the amyloid plaques within which PrP^C accumulates. Accumulation of PrP^C within primitive or cored neuritic plaques in AD and AD mouse model brains was previously reported by several groups (30,33–35). However, PrP^C accumulating plaques are even seen in the aged brain without cognitive impairment, while they are rare in the advanced AD brain and not present in juvenile brains without amyloid plaques. Specifically, here we show that PrP^C accumulates within a subset of diffuse-type plaques, composed of more soluble and oligomeric Aβ in aging human brains. Since PrP^C accumulating plaques appear in aged, but not in juvenile, human brains, and are more likely to present with dementia, these plaques could result in the deterioration of cognition in early AD. More precise characterization of these PrP accumulating diffuse-type plaques might also facilitate early diagnosis of AD.

2 | MATERIALS AND METHODS

2.1 | Human brain tissue

We examined 30 cases (age, 29–95 years), 19 with biopsy specimens and 11 with specimens obtained at autopsy, for this study (Table 1). The information on plaque labeling in Table 1 reflects labeling with PrP antibody 3F4. Among the cases with biopsy specimens, two patients were clinically diagnosed with AD (Patient nos. 1, 2; Table 1). Four were diagnosed with or suspected of having dementia without AD (Patient nos. 3–6; Table 1), and eight were not diagnosed with either AD or dementia (Patient nos. 7–14; Table 1). Five biopsy specimens were obtained from young patients with glioblastoma at 29–39 years of age, and regions of brain tissue without tumor were used as control subjects (Patient nos. 15–19; Table 1). Among the autopsy specimens, one patient was both clinically and pathologically diagnosed with advanced AD (Patient no. 25; Table 1). One was only clinically diagnosed with AD (Patient no. 20; Table 1), and one was diagnosed with dementia without AD (Patient no. 21; Table 1). Five patients were not diagnosed with either AD or dementia (Patient nos. 22–24, 26, 27; Table 1). Three autopsy specimens were from young individuals at 28–49 years of age (Patient nos. 28–30; Table 1). The use of brain samples was approved by the ethics committee of the Tokyo Medical University (approval number: T2020-0230).

2.2 | Antibodies

The following well-characterized antibodies were used at the appropriate concentrations for immunohistochemical

TABLE 1 Characteristics of patients and pathological data

Biopsy

No.	Age	Gender	AD	Dementia	Amyloid plaque	PrP^C Plaque*	Diagnosis	BA
1	84	F	+	+	+	+	CAA, AD, involuntary movement	TPL
2	77	F	+	+	+	+	CAA, AD	FL
3	83	F	−	+	+	+	CAA, subdural hematoma, dementia, HT	OL
4	78	F	−	+	+	+	Cerebral hemorrhage, dementia	TL
5	86	M	−	+	+	+	Cerebral hemorrhage, dementia	PL
6	79	F	−	+	+	+	Glioblastoma, disorientation, hemiplegia	FL
7	74	F	−	−	+	+	Glioblastoma, hemiplegia	PL
8	78	M	−	−	+	+	Subcortical hemorrhage, diabetes mellitus type II	
9	75	M	−	−	+	−	CAA	
10	81	F	−	−	+	−	CAA	OL
11	71	F	−	−	−	−	Subcortical hemorrhage, hemiplegia	FL
12	81	M	−	−	−	−	Glioblastoma	FL
13	69	M	−	−	−	−	Metastatic carcinoma	FPL
14	71	M	−	−	−	−	Glioblastoma, aphasia, cerebral edema	TL
15	39	M	−	−	−	−	Oligoastrocytoma	TL
16	39	M	−	−	−	−	Anaplastic oligodendroglioma epilepsy	FL
17	38	F	−	−	−	−	Glioblastoma	FL
18	32	M	−	−	−	−	Metastatic sarcoma	
19	29	M	−	−	−	−	Germ cell tumor, headache, double vision	CBR

Autopsy

No.	Age	Gender	AD	Dementia	Amyloid plaque	PrP^C Plaque	Diagnosis	BA	PMT
20	95	M	+	+	+	+	Aspiration pneumonia, AD	FTL	9:42
21	83	M	−	+	+	+	Ischemic enteritis, cognitive impairment	TL	2:08
22	78	M	−	−	+	+	Malignant lymphoma, multiple Infarction	TL	14:35
23	70	M	−	−	+	+	Lung cancer, brain metastasis	PL	3:58
24	71	M	−	−	+	+	OMI, multiple infarction	FOL	3:08
25	75	M	+	+	+	−	Dissecting aneurysm, AD	TL	2:40
26	85	M	−	−	+	−	Intestinal necrosis, old cerebral infarction	FL	13:42
27	85	M	−	−	−	−	Bronchitis, congestive pulmonary edema	TL	6:00
28	49	F	−	−	−	−	Multiple organ failure	TL	15:11
29	37	M	−	−	−	−	Stomach cancer, carcinomatous meningitis	FL	11:14
30	28	M	−	−	−	−	CPE, cerebral edema	FL	2:21

Abbreviations: AD, Alzheimer's disease; BA, brain area; CAA, cerebral amyloid angiopathy; CBR, cerebral basal region; CPE, congestive pulmonary edema; F, frontal; HT, hypertension; L, lobe; O, occipital; OMI, old myocardial infarction; P, parietal; PMT, post mortem time; T, temporal.

*All results are evaluated by 3F4 antibody.

and immunofluorescent staining. The widely used and well-established antibody 6E10 is directed at amino acid residues 3–8 within the N-terminus of human Aβ and detects also Aβ-containing APP full-length and fragments (1:1000, BioLegend, previously COVANCE, SIG-39320, NJ, USA). 11A1 antibody (1:1000, Immuno-Biological Laboratories, 10379, Gunma, Japan) was generated against a toxic conformer with a turn at amino acid residues 22 and 23 in Aβ42, and recognizes oligomers rather than the monomer of Aβ (36). Well-characterized antibodies 3F4 (1:1000, BioLegend, previously COVANCE, 800301, CA, USA), 6H4 (1:1000, Prionics, 01-010, Schlieren-Zurich, Switzerland), and 12F10 (1:1000, Cayman CHEMICAL 189170, MI, USA), recognize amino acids 109–112, 144–152, and 142–160 of PrP, respectively. Anti-prion antibody 3H2 (1:500) (37) recognizes the N-terminus of PrP, amino acids 35–53. The PrP antibody T4 (1:1000 or 1:250) (38) is raised against the C-terminus of bovine-PrP-peptide corresponding to amino acids 221–239 equivalent to amino

acids 210–228 of human PrP. T4 is a rabbit polyclonal antibody, while all other antibodies used in this study are mouse monoclonals. Epitopes and predictive epitopes detected by the respective prion antibodies are depicted in Figure S1. Additionally, Aβ oligomer binding sites are also depicted (21,23).

2.3 | Immunohistochemistry

Brain sections were deparaffinized and autoclaved for 30 min at 121°C in antigen retrieval buffer (Nichirei Biosciences Inc. #415211, Tokyo, Japan) or incubated with 90% formic acid for 15 min at room temperature. Endogenous peroxidase was blocked, and sections were treated with 10% normal goat serum, and then incubated with the above-described primary antibodies.

For the preabsorption experiment, the 3F4 antibody (1 mg/ml) was incubated with glutathione S-transferase (GST) protein (0.5 mg/ml) or GST-PrP^C recombinant protein (0.5 mg/ml) in 1% bovine serum albumin (BSA). Each protein was adjusted in 1% BSA to a concentration 10 times higher than that of the antibody (3F4, 1 μg/ml; GST-PrP^C recombinant protein, 10 μg/ml; GST protein, 10 μg/ml). After the preincubation of the 3F4 antibody with each protein overnight at 4°C, the brain sections were treated with the mixture of the 3F4 antibody and proteins. The sections were then incubated with secondary antibodies followed by Envision+ (DakoCytomation, CA, USA) and labeled peroxidase was detected using diaminobenzidine.

2.4 | Immunofluorescence

For dual immunofluorescent labeling brain sections were incubated with the 6E10 and T4 antibodies followed by fluorescence-labeled secondary antibodies (1:200; Molecular Probes, OR, USA). For dual label Thioflavin-S (ThS) and 1-fluoro-2,5-bis (3-carboxy-4-hydroxystyryl) benzene (FSB) staining, the sections were incubated with filtered 0.01% ThS or FSB in 70% ethanol (ETOH) for 20 min, and then rinsed sequentially with 70% and 100% ETOH twice and embedded after immunofluorescent labeling by the 3F4 antibody.

2.5 | Congo red and Direct Fast Scarlet (DFS) staining

Briefly, after incubating the deparaffinized brain sections in 0.5% Congo red solution diluted by 100% ETOH or DFS stain solution (MUTO Pure Chemicals Co., LTD, Tokyo, Japan) for 20 min, the sections were rinsed in 0.2% KOH/80% ETOH solution or distilled water.

3 | RESULTS

3.1 | Specific accumulation of PrP^C within diffuse-type amyloid plaques in aged non-AD brain tissue

Amyloid plaques detected after autoclave pretreatment by the 6E10 antibody which detects Aβ and Aβ-containing APP products were observed in aged patients without dementia as evident in a representative case (Figure 1A, left). An adjacent brain section with the same pretreatment was subjected to PrP staining with an anti-PrP antibody, 3F4 and showed the accumulation of PrP^C within diffuse-type amyloid plaques that had been labeled by 6E10 in the adjacent section (Figure 1A, right). Since all human brain samples employed in this analysis were from patients without prion diseases, only PrP^C, and not PrP^{SC}, was detected in the brain sections by 3F4 staining. However, PrP^C is not accumulated within all of the 6E10-positive plaques, and the number of PrP^C accumulating plaques is much lower in comparison to antibody 6E10 labeling of plaques. Aβ-labeled plaques are shown in Figure 1 from a representative case from whom brain tissue specimens were obtained both by biopsy and at autopsy (see Table 1 for characteristics of the cases). Moreover, PrP-positive plaques were not always observed in brain tissue with 6E10-positive amyloid plaques (as indicated in Table 1). Results on PrP labeling of plaques in Table 1 were obtained using PrP antibody 3F4, which is one of the most established antibodies against prion protein and recognizes the epitope overlapping with the putative Aβ oligomer binding site. The specificity of the PrP^C accumulation within the plaques detected by the PrP antibody 3F4 was confirmed by preabsorption experiments employing GST protein and GST-PrP^C recombinant protein. The PrP^C accumulating plaques were still observed with treatment with the 3F4 antibody and just the GST protein, even though the concentration of the GST was 10 times higher than that of the 3F4 antibody (Figure 1B, left). However, none of the plaques were detected when treated with a combination of the 3F4 antibody and GST-PrP^C recombinant protein (Figure 1B, right), supporting the specificity of 3F4 labeling. For this, the concentration of the recombinant protein was 10 times higher than that of the antibody. Furthermore, the immunoreactivity detected by the 3F4 antibody was also observed with the other anti-PrP antibodies, 3H2, 6H4, 12F10, and T4 (Figure S2A-D) in the adjacent section confirmed by the 6E10 antibody (Figure S3). All the PrP antibodies revealed similar diffuse-type plaques and plaque distributions.

Accumulation of PrP^C within diffuse-type amyloid plaques was also confirmed in the same section by double immunofluorescent staining with Aβ antibody 6E10 and PrP antibody T4 (Figure 2A, upper row). Diffuse plaques stained by 6E10 antibody were co-localized with PrP^C stained by T4 antibody in biopsy brain tissue not

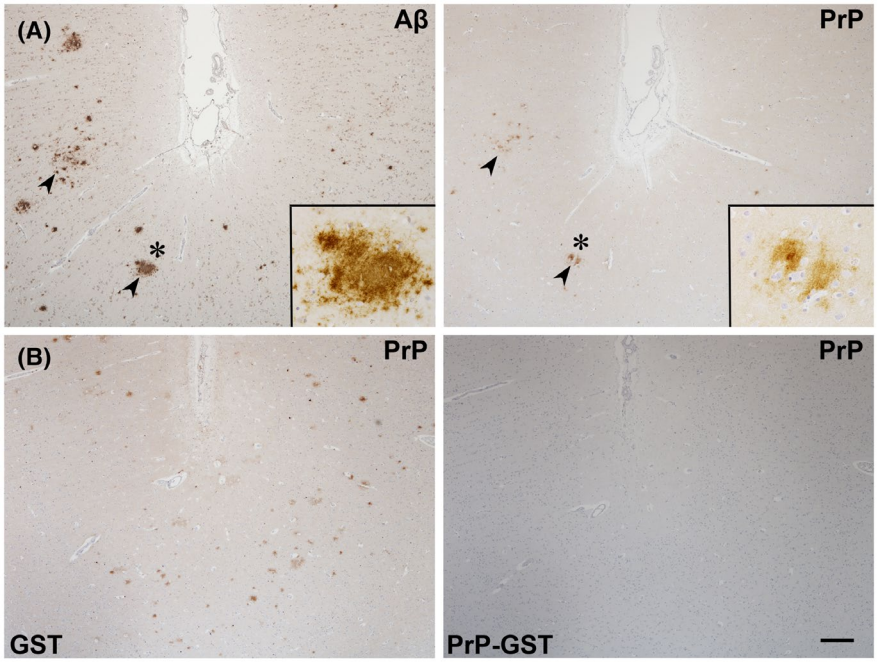

FIGURE 1 PrP^C is present in diffuse amyloid plaques without AD. Immunoreactivity of Aβ and PrP^C in brain tissue obtained at autopsy from a representative 71-year-old patient without dementia, and absorption of the 3F4 antibody by GST-PrP^C recombinant protein. PrP^C is depicted as PrP in the figure. (A) Aβ accumulation was detected by antibody 6E10 after autoclave pretreatment as diffuse-type amyloid plaques. Neuritic-type amyloid plaques were not seen by antibody 6E10 in this particular brain section (left). The accumulation of PrP^C, reminiscent of findings of diffuse-type amyloid plaque staining, was also detected in adjacent brain sections by PrP antibody 3F4 staining. A subset of the PrP^C accumulations was clearly co-localized within the plaques detected by the 6E10 antibody (arrowheads) (right). Focal accumulation of PrP^C in the same diffuse-type plaques stained by the 6E10 antibody (asterisks) was observed with higher magnification (insets). (B) Immunoreactivity of the 3F4 antibody preincubated with GST protein was preserved, and accumulation of PrP^C within the plaques was observed in a section from the same brain (left). 3F4 immunoreactivity was completely abolished in serial brain sections by preincubation with GST-PrP^C recombinant protein (right). Bar: 250 μm

diagnosed with dementia. To confirm the specificity of the staining an adjacent section was stained without primary 6E10 and T4 antibodies, respectively. The plaques were not observed at all even with longer exposure (Figure 2A, lower row). A representative diffuse-type plaque can be seen stained by both 6E10 and T4 antibodies, while a plaque with an amyloid core was only stained by the 6E10 and not with the T4 antibody in the non-AD case with dementia (Figure 2B). Thus, PrP^C accumulation can be observed within diffuse-type amyloid plaques in non-AD aged brain tissue.

3.2 | PrP^C accumulating plaques composed of more soluble Aβ oligomers without stacked β-sheet structures

To better characterize the PrP^C accumulating plaques in non-AD cases, we performed pretreatment with formic acid in addition to autoclave retrieval of brain tissue. We focused on one independent plaque to facilitate precise observation of the effect of formic acid pretreatment with serial sections. We saw that the 6E10 staining of this plaque was extremely diminished by formic acid treatment (Figure 3A and inset), while several small plaques now appeared following retrieval treatment after formic acid (Figure 3B and inset). The same large plaque in an adjacent section without formic acid did; however, show 3F4 antibody staining (Figure 3C and inset), and this 3F4 immunoreactivity was completely abolished by the pretreatment with formic acid (Figure 3D). These results suggest that PrP^C accumulating plaques are composed of more soluble Aβ, and/or Aβ-containing APP and/or APP products. Next, we tried to stain the same plaque in an adjacent section with antibody 11A1 (36) specific for neurotoxic Aβ42 oligomers. 11A1 immunoreactivity was observed in the same plaque (Figure 3E and inset) that was mostly removed by formic acid treatment (Figure 3F and inset). At higher magnification, some amyloid deposition seemed to be present after the treatment (Figure 3F inset), which might represent some labelings of insoluble Aβ or alternatively may represent soluble Aβ oligomers generated by the formic acid treatment. Intraneuronal Aβ42 detected by antibody 11A1 was also removed by formic acid (Figure 3E inset, asterisk, and 3F inset). These results suggest that most of the PrP antibody 3F4-positive plaques are composed of more soluble Aβ42 oligomers.

We next immunofluorescently investigated whether 3F4-positive plaques in the non-AD with dementia

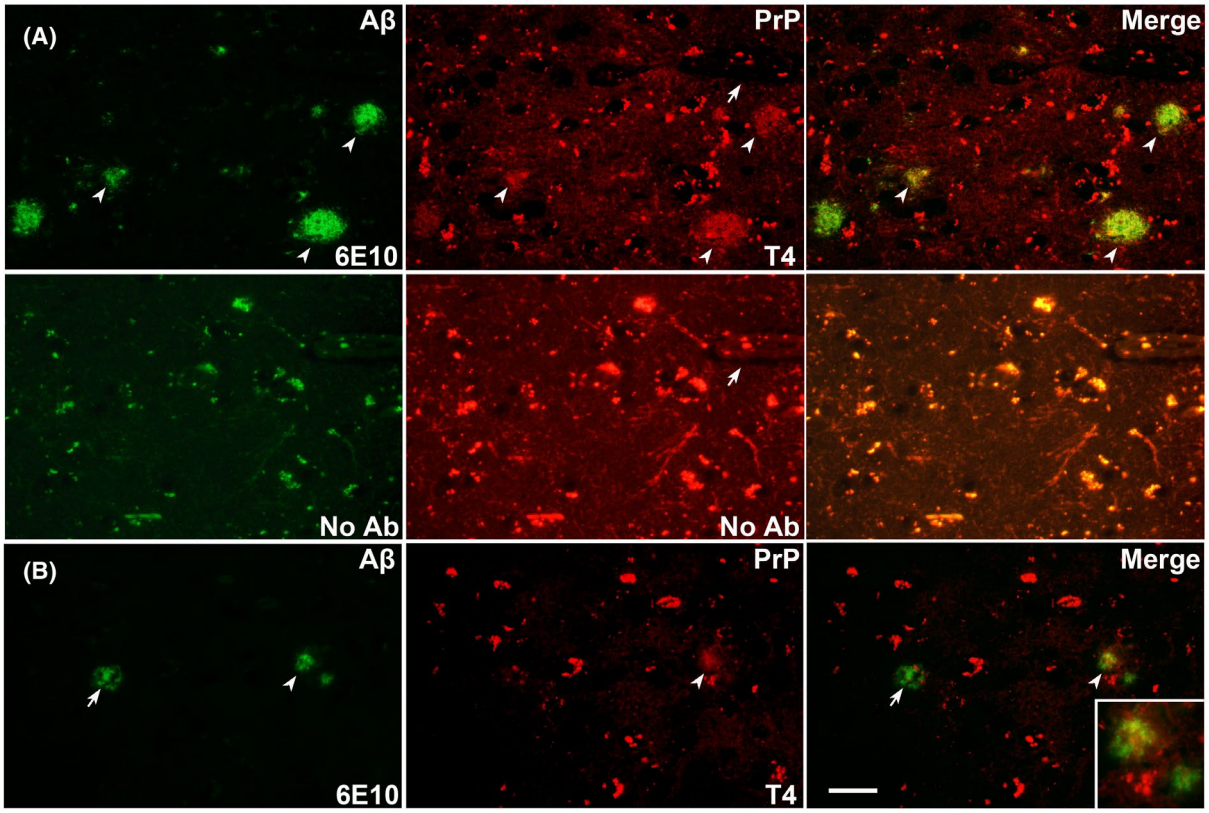

FIGURE 2 Co-localization of Aβ and PrP in diffuse-type amyloid plaques but not in cored plaques. (A) Diffuse plaques (arrowheads, upper row) stained by Aβ antibody 6E10 (green) revealed co-localization with PrP stained by antibody T4 (red) in biopsy brain tissue from a 74-year-old female case not diagnosed with dementia. To show that the marked red dots are autofluorescence rather than from primary antibody labeling, an adjacent section of the same area presented in the upper panel was stained without primary antibodies (lower row). To confirm the close vicinity of the sections, the same blood vessel is evident in the upper right corner (arrows). Even with longer exposure, the plaques were not observed without primary antibodies. In contrast, the green and red dot-like labeling showing marked intensities were completely co-localized and thus represent autofluorescence. (B) A diffuse-type plaque was stained by both 6E10 (green) and T4 antibodies (red) in a section from a biopsy brain tissue of an 86-year-old male case diagnosed with dementia (arrowhead), while a plaque with an amyloid core was only stained by the Aβ antibody 6E10 (arrow). A higher magnification view of the diffuse plaque (at arrowhead in the lower power image) is shown in the inset. Bar: 100 μm

cases ever labeled with thioflavin S (ThS), which detects stacked β-pleated amyloid fibrils. In a representative section, an amyloid core of a neuritic amyloid plaque was stained by ThS, but not by 3F4 antibody (Figure 4A and arrowheads). There was no evidence of the co-localization of ThS and antibody 3F4 in the merged image, even at higher magnification (Figure 4A inset). In contrast, there was no labeling of ThS within 3F4-labeled plaques (Figure 4B and arrowheads) that would, therefore, be classified as diffuse amyloid plaques. Co-localization of ThS and 3F4 labeling was also not observed with higher magnification (Figure 4B arrow and inset). In addition, we used the dye 1-fluoro-2,5-bis (3-carboxy-4-hydroxystyryl) benzene (FSB), which also detects β-pleated amyloid fibrils, instead of ThS (Figure S4). Similarly, in another case, a diffuse plaque was not labeled by ThS and labeled by 3F4 antibody (Figure S4 arrows), while a neuritic plaque with a very small amyloid core was labeled by both ThS and 3F4 antibody (Figure S4 arrowheads). While a neuritic plaque with a very small amyloid core was labeled by both ThS and 3F4 antibodies, obvious labeling of ThS was also not observed in PrP accumulating diffuse-type plaques in another non-AD case with dementia (Figure S5).

3.3 | PrP^C accumulation within various types of plaques in brain tissue clinically diagnosed with AD

In addition to the diffuse plaques, antibody 6E10 Aβ staining typically also reveals neuritic or classical plaques with amyloid cores in brain tissue, such as is seen in a case obtained by biopsy from a patient clinically diagnosed with AD (Patient no. 1; Table 1) (Figure 5A). Similar to Figure 1A of aged brain tissue without AD, only a subset of the 6E10-positive plaques in this AD brain was stained by the PrP antibody 3F4 (Figure 5B). However, in the neuritic plaques detected by antibody 3F4 staining, most of the amyloid cores

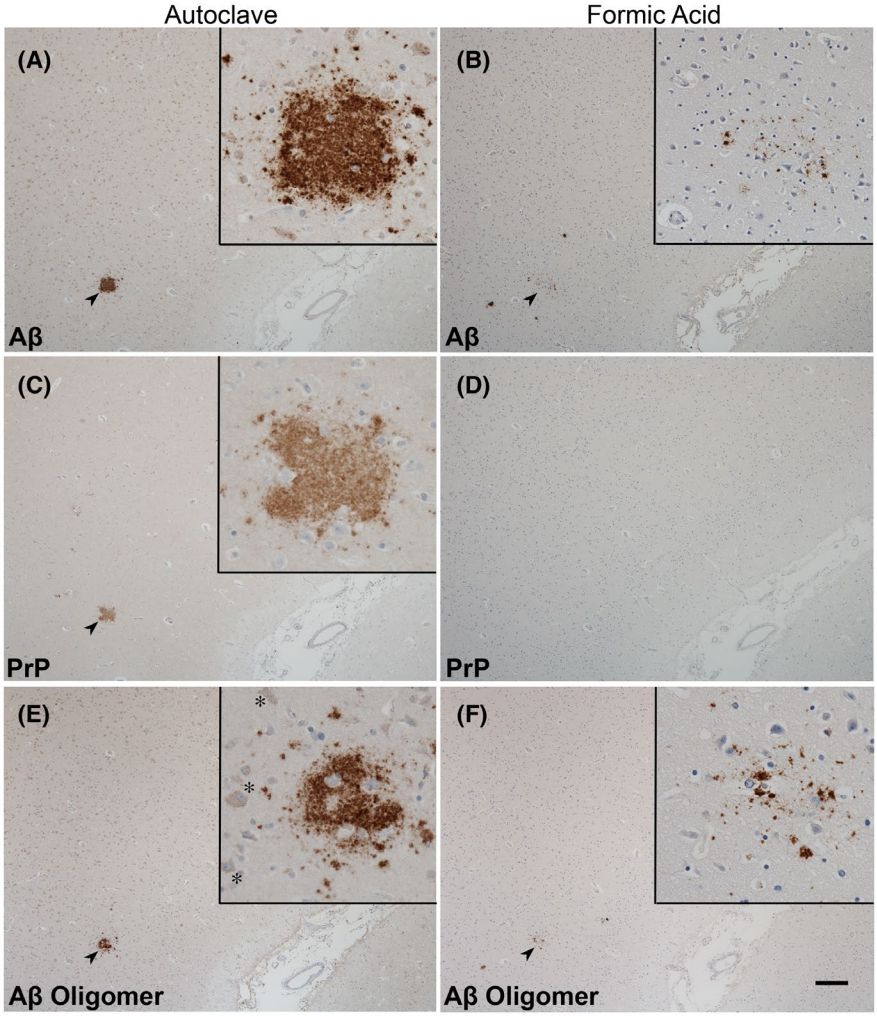

FIGURE 3 Abolition of PrP immunoreactivity following pretreatment with formic acid and labeling with Aβ oligomer antibody in a diffuse-type plaque. (A) Here we focused on one amyloid plaque to observe the alteration of the 6E10-immunoreactivity by formic acid pretreatment (arrowhead) and higher magnification of the markedly diminished plaque labeling is also depicted (inset). The brain tissue was obtained at autopsy from a 71-year-old patient without dementia. (B) The Aβ immunoreactivity was markedly diminished by formic acid (inset). (C) The same plaque was also immunolabeled by the PrP antibody 3F4 in serial brain sections (arrowhead) and was observed in a higher magnification view (inset). (D) PrP immunoreactivity was completely abolished by formic acid pretreatment. (E) Moreover, this plaque was also immunolabeled with the Aβ oligomer antibody 11A1 (arrowhead) and is also shown in a higher magnification view (inset). Intraneuronal Aβ was detected by the 11A1 Aβ oligomer antibody (asterisk). (F) Similarly to 6E10-immunoreactivity, the immunoreactivity of 11A1 was markedly diminished by formic acid pretreatment (arrowhead) and amyloid deposition newly appeared after the treatment (inset). Intraneuronal Aβ was also abolished by formic acid treatment (inset). Bar: 250μm

were not stained by antibody 3F4 in this case of AD, while the surrounding dystrophic neurites were stained (Figure 5B, left inset). The amyloid core that was not stained by the 3F4 antibody was instead stained by the Congo red dye (Figure 5B, right inset), denoting fibrillar amyloid. The 3F4-positive plaques in AD were, therefore, mainly of the diffuse type (Figure 5C), as detected in the other aged brain tissues. Moreover, in brain tissues from cases with clinically diagnosed dementia but not diagnosed AD (Patient no. 3; Table 1), the amyloid cores were stained by the 3F4 antibody, in addition to the dystrophic neurites (Figure 5D). Thus, some plaque cores have PrP labeling, although overall there is more labeling of diffuse plaques and of dystrophic neurites.

3.4 | Much less PrP immunoreactivity in amyloid plaques in brain tissue clinically and pathologically diagnosed with advanced AD

While numerous and remarkable 6E10-positive plaques were observed, faint staining by the 3F4 antibody was observed in plaques in a brain tissue specimen from a patient who was both clinically and pathologically diagnosed with advanced AD (Patient no. 25; Table 1) (Figure 6A,B). In addition, 3F4-positive diffuse-type plaques were not observed. At higher magnification, faint granular staining was only detected by the 3F4 antibody, especially in dystrophic neurites around amyloid cores in contrast to amyloid plaques markedly stained by the 6E10 antibody in the adjacent brain section (Figure 6B,C, asterisks).

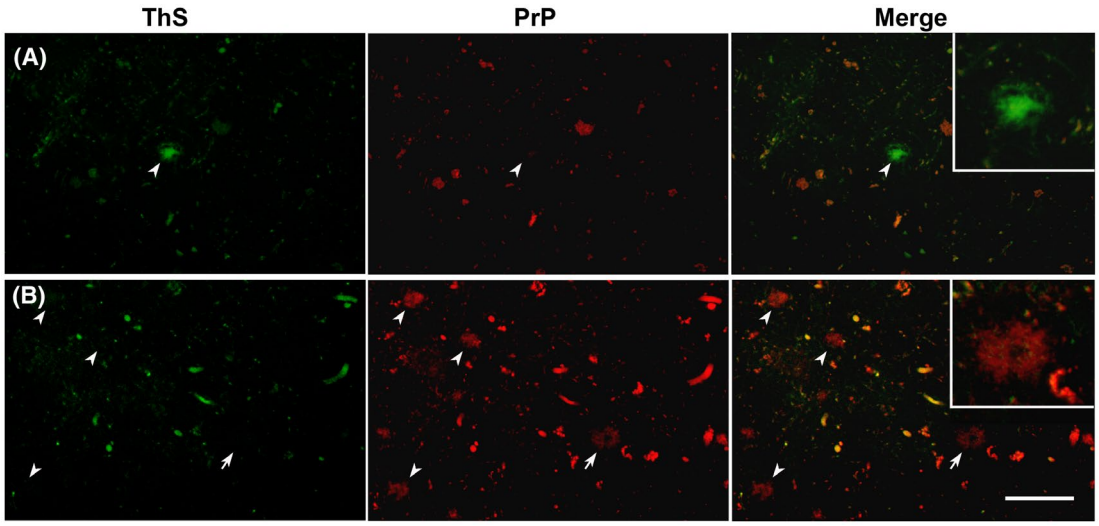

FIGURE 4 Lack of thioflavin S labeling of PrP-positive plaques in non-AD brains. (A) Thioflavin S (ThS) staining (green) for β-pleated Aβ fibrils revealed co-localization with neuritic plaques in a brain tissue obtained by biopsy from an 83-year-old female case clinically diagnosed with dementia (arrowheads). PrP-immunoreactivity (red) was absent in this neuritic plaque. A higher magnification view of the neuritic plaque is shown in the inset. (B) In contrast to the neuritic plaque, ThS staining was not evident in PrP^C-positive plaques (arrowheads). In a higher magnification view of one PrP-positive plaque (arrow), there was no overlap with ThS (inset). Bar: 50 μm

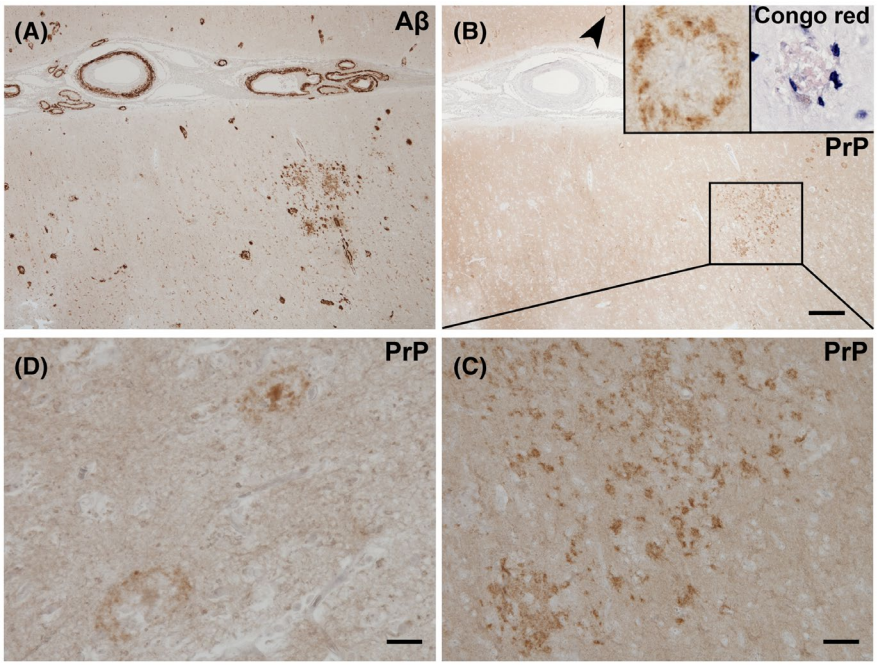

FIGURE 5 PrP^C is present in both diffuse and neuritic plaques of biopsy AD brain tissues. PrP immunoreactivity in plaques reminiscent of both diffuse-type and neuritic-type is evident in aged brain tissue from a representative patient clinically diagnosed with AD. (A) Numerous amyloid deposits are seen in diffuse and neuritic plaques, and in blood vessels, by the Aβ antibody 6E10 in brain tissue from an 84-year-old patient. (B) Only parts of the plaques were detected by PrP antibody 3F4 staining. PrP immunoreactivity is not detectable in blood vessels. Coarse and dense PrP accumulation, similar to dystrophic neurites of neuritic plaques, is evident (arrowhead); a higher magnification is also shown (inset, left). The amyloid core was stained by Congo red in the same plaque (inset, right). (C) In a higher magnification view of the square area of (B) the PrP-positive plaques were mostly diffuse-type plaques, while no remarkable PrP-immunoreactivity was detected in the amyloid cores of this brain section. (D) In another brain section from a biopsy specimen of an 83-year-old patient clinically diagnosed with dementia, dense core PrP-immunoreactivity was detected by 3F4 staining. Bars: 250 μm (A, B), 50 μm (C), 25 μm (D)

However, fewer and fainter immunolabeling of plaques was observed by PrP antibodies 3H2 and T4 and none by antibodies 6H4 and 12F10 (Figure S6). We hypothesize that different labelings with PrP antibodies relates either to epitope blocking, antibody affinity, conformational changes, and/or cleavage of PrP.

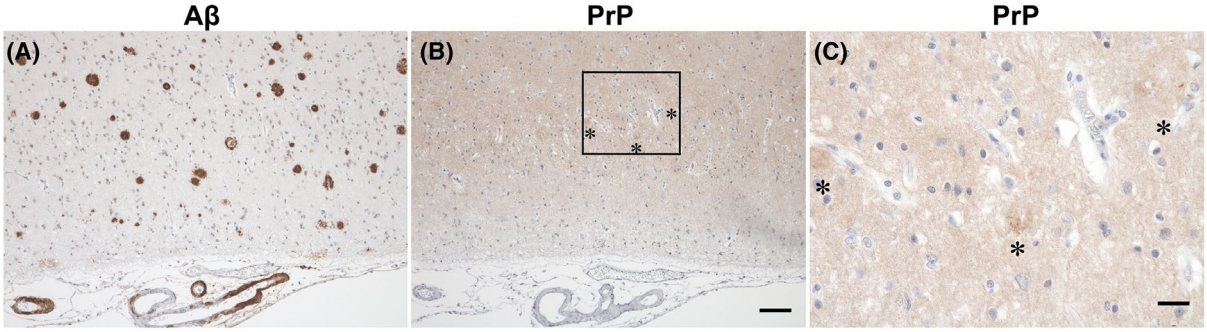

FIGURE 6 PrP antibody 3F4 immunoreactivity was much lower in amyloid plaques of advanced AD brain. (A) Strong immunoreactivity of the Aβ antibody 6E10 was observed in amyloid plaques from brain tissue of a 75-year-old patient clinically and pathologically diagnosed with advanced AD. (B) In contrast, much less 3F4-immunoreactivity was observed in an adjacent section. (C) The squared area in (B) was magnified and the same plaques are visible, respectively (asterisks). Bars: 100 μm (A, B), 25 μm (C)

3.5 | No PrP immunoreactivity as plaques in brain tissue from young and aged patients without amyloid plaques

While intraneuronal Aβ and/or Aβ domain-containing APP were immunohistochemically stained as granular dots by the 6E10 antibody in brain tissues from eight young patients (five biopsies, Patient nos. 15–19; three autopsies, Patient nos. 28–30; Table 1), no obvious plaques were detected in these young cases by either PrP or Aβ antibodies (Figure S7A,B). The intraneuronal accumulation of Aβ/APP was also revealed as granular dots by 6E10 staining in brain tissue specimens from 10 aged patients without dementia (eight biopsies, Patient nos. 7–14; two autopsies, Patient nos. 26, 27; Table 1) (Figure S7C and inset), where amyloid plaques were not seen in five cases (4 biopsies, Patient nos. 11–14; one autopsy, Patient nos. 27; Table 1) (Figure S7C). In these aged brain tissue specimens without amyloid plaques from patients without dementia, no plaque was detected by the 3F4 antibody either (Figure S7D). In contrast, endogenous PrP^C was observed in neurites and neuronal cell bodies by 3F4 antibody at higher magnification (Figure S8). In one case involving an autopsied patient without dementia who had an old cerebral infarction (Patient no. 26; Table 1), numerous amyloid plaques were detected by 6E10 staining (Figure S7E and inset); however, none of these plaques were detected by 3F4 staining (Figure S7F).

4 | DISCUSSION

The finding that PrP^C binds to Aβ oligomers with high affinity has attracted considerable interest (21). Several lines of evidence have demonstrated that the binding of Aβ oligomers to PrP^C is relevant to the activation of downstream proteins, leading to neurotoxicity (22,27,39). Notably, before PrP^C was identified as a receptor for Aβ oligomers by genome-wide unbiased expression cloning, the PrP^C accumulation in neuritic amyloid plaques in AD brain tissue was demonstrated by immunohistochemical staining (33,40). We previously reported that the accumulation of PrP^C was mainly detected within dystrophic neurites of AD brain tissue by immunohistochemical methods and that at times it was also detected in the amyloid cores of some neuritic plaques (35). Since PrP^C is transported to the distal regions of neurites in an anterograde manner (41), we suggested that in AD brain where Aβ and other abnormal proteins aggregate in dystrophic neurites, PrP^C seems to redistribute more in the proximal part of such dystrophic neurites (35).

In this study, our immunohistochemical analyses now demonstrated specific co-localization of Aβ and PrP^C within diffuse-type amyloid plaques in human brain tissue from aged patients. In addition to previous studies that reported the accumulation of PrP^C within amyloid plaques in the brain tissue of patients with AD, we have now detected the accumulation of PrP^C mainly within diffuse-type amyloid plaques in human brain tissue from patients who were not diagnosed with AD. While the accumulation of PrP^C in diffuse plaques was observed in specimens from several patients without dementia, it was more evident in patients with dementia.

In a few brain tissues from patients only clinically, not pathologically, diagnosed with AD we also demonstrated marked immunoreactivity of PrP antibodies in dystrophic neurites and some amyloid cores. Interestingly, in contrast to the brain tissue specimens from the patients who were only clinically diagnosed with AD, in the autopsy brain tissue clinically and pathologically diagnosed with advanced AD, far fewer PrP-positive plaques were detected and the plaques were more faintly stained with PrP antibody in comparison to 6E10-positive amyloid plaques. Our results suggest that PrP^C might preferentially accumulate in plaques of aged brains and in brain tissue of patients with early-stage dementia compared to advanced AD. Alternatively, it is possible that the PrP epitope is more blocked or that PrP^C is cleaved with

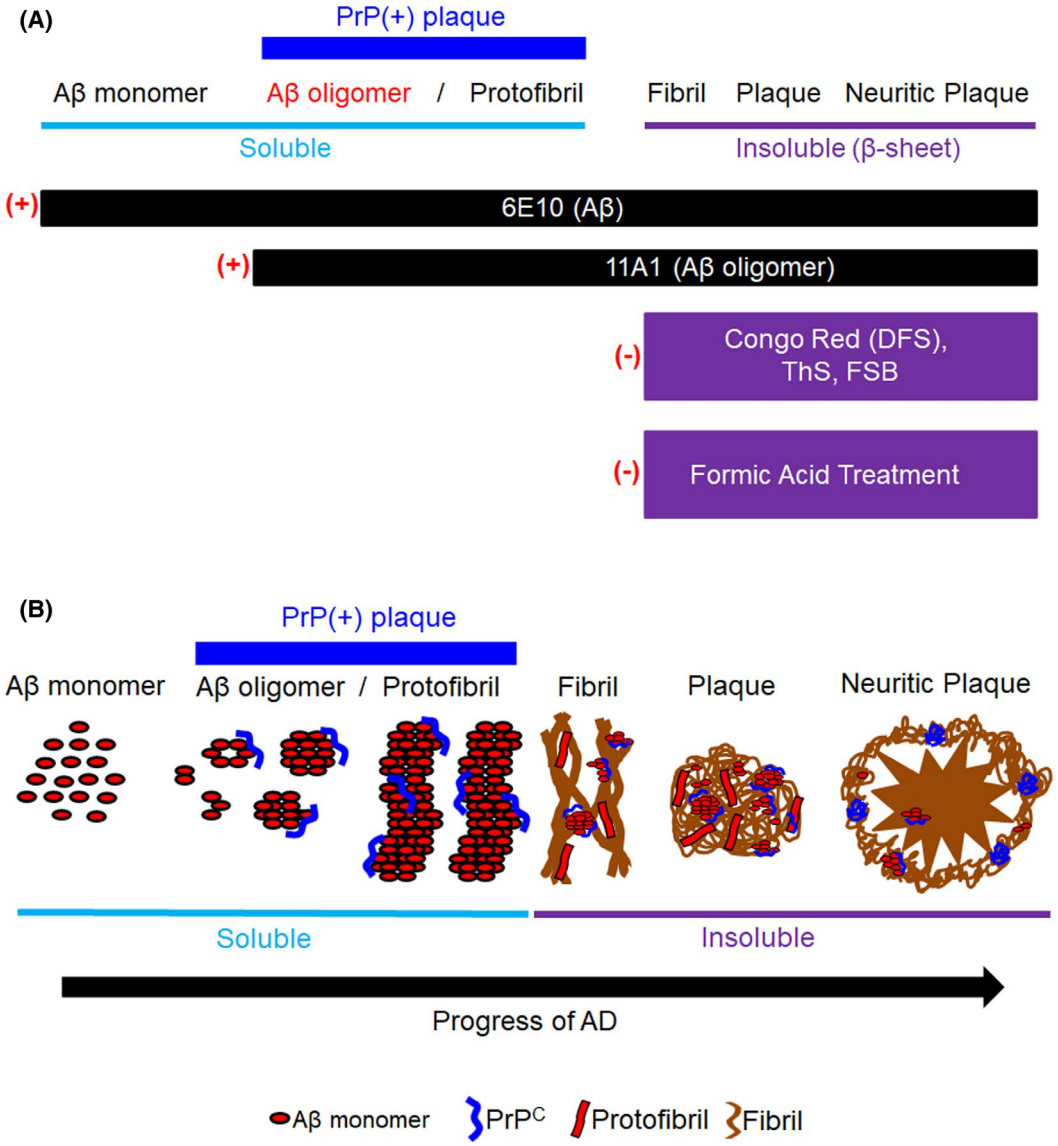

FIGURE 7 Schema for PrP^C labeling with amyloid plaques. (A) Aβ characteristics of PrP (+) plaques (PrP-positive plaques). The PrP (+) plaques were abolished by pretreatment with formic acid and were not stained by ThS, FSB, or Congo Red dyes. In addition, the plaques were immunolabeled by the 11A1 antibody, which detects Aβ oligomers. The PrP (+) plaque thus should be composed of more soluble Aβ oligomers and protofibrils. (B) Relationship between Aβ and PrP^C. The binding affinity of PrP^C might be much higher to Aβ oligomers and protofibrils, and lower to fibrils and more insoluble-types of Aβ. PrP showed markedly lower immunoreactivity in neuritic plaques in brain tissue with advanced AD

increasing Aβ aggregation. We also noted that despite low PrP labeling in advanced AD brain sections, more plaque labeling was detected with PrP antibodies 3H2 and T4, which detect N-terminal and C-terminal region of PrP^C, compared to little to no labeling with antibodies 3F4, 6H4, and 12F10, which detect relatively central regions of PrP^C. In contrast, in non-AD aged tissues, all of the PrP antibodies labeled the diffuse plaques (Figure S2). It is possible that the Aβ oligomer binding site in PrP^C is blocked, PrP^C is cleaved or the PrP conformation is altered within amyloid plaques in advanced AD brain. Our observations are in line with the findings of others that PrP^C plaque load and the amount of PrP^C accumulation is lower in AD than non-AD cases (30,42–44).

We histochemically characterized the PrP^C accumulating plaques. The immunoreactivity of PrP^C in plaques was abolished by pretreatment with formic acid, while that of 6E10 was also significantly decreased. Furthermore, PrP-positive plaques were immunolabeled by an antibody that detects Aβ42 oligomers but was not labeled by ThS, which detects stacked β-sheet fibril structures. Our results support the conclusion that initial PrP^C accumulating diffuse plaques, PrP (+) plaques, are exclusively composed of more soluble Aβ oligomers and/or protofibrils rather than stacked β-pleated fibrils (see schema in Figure 7A). This conclusion is supported by PrP (+) plaques being abolished by pretreatment with formic acid and that they are not stained by amyloid dyes.

In the present pathological study, we did not provide data demonstrating the direct binding of Aβ oligomers and PrP^C. However, since a number of laboratories have reported evidence that the neurotoxicity of Aβ oligomers emerges after binding with PrP^C leading to AD pathology (17,21,30,45,46), this supports that PrP^C and soluble Aβ oligomers bind together in PrP (+) plaques. We propose a model of amyloid plaque formation and the involvement of PrP^C (see schema in Figure 7B). The binding of PrP^C to Aβ may occur at an early phase in the progression of AD or with aging in certain individuals, while PrP^C is not bound to or sequestered by amyloid plaques with the β-sheet structure that is typically observed in the advanced phase of AD. Alternatively, conformational alterations that block the PrP epitope as Aβ aggregates further might block PrP antibody labeling.

Whereas many previous studies have focused on downstream signaling pathways that affect the function and activity of neurons, subsequent to the binding of Aβ oligomers to PrP^C at the neuronal surface (21,27,39,47,48), only a few groups have explored how Aβ oligomers bound to PrP^C at the neuronal surface might act. It was suggested that the internalization of PrP^C may also allow Aβ oligomers to accumulate intracellularly in the cytoplasm of neurons, where they might affect cellular functions, such as protein degradation by the proteasome complex (49). Apolipoprotein E (APOE) has been thought to be involved in the uptake of Aβ by neurons, and the blocking of the interaction between APOE and Aβ by a non-toxic synthetic peptide reduced intracellular Aβ accumulation and Aβ oligomer levels and protected against synaptic protein loss (50,51). The blocking of Aβ internalization through PrP^C might be a therapeutic target for AD therapy.

Recent studies have supported that exosomes play important roles in the pathogenesis of neurodegenerative diseases, especially in AD and prion disease (52–55). Exosomes are small membrane vesicles (30–150 nm in diameter) that are released into the extracellular space and body fluids (20,56). Membranes of multivesicular bodies (MVBs) are invaginated to form intraluminal vesicles (ILVs). Following the fusion of MVBs with the plasma membrane ILVs released into the extracellular space are then called exosomes (55). It was reported that neuronal exosomes are highly enriched in PrP^C, which binds to Aβ oligomers with high affinity (15), and also that prefibrillar Aβ aggregates favorably bind to exosomes (57). Exosomes have been suggested to bind toxic oligomers and convert them to non-toxic fibril types in amyloid plaques to protect from toxicity (58). PrP (+) plaques might be transiently and reversibly formed prior to such non-toxic amyloid plaques. Alternatively, since exosomal proteins are also enriched in plaques in AD brains (55), PrP^C could be transferred to amyloid plaques composed of soluble Aβ oligomers via exosomes or ILVs (6,59). Recently, it was reported that the levels of circulating exosome-bound Aβ in blood samples from AD patients are correlated with amyloid plaque load on positron emission tomography (PET) (57). Investigation of the relationship between PrP (+) plaques and exosomes by exosomal markers might serve to further characterize plaques.

Different types of amyloid plaques have been reported as neuritic plaques, primitive plaques, burnt-out plaques, cotton-wool plaques, and diffuse plaques. And recently coarse-grained plaques in early onset AD were reported (60). We demonstrated the specific co-localization of Aβ and PrP^C within amyloid plaques in human brain tissue from aged patients who were not diagnosed with AD and demonstrated that such PrP^C accumulating plaques are made up of more soluble Aβ oligomers. Not all the diffuse-type plaques, but rather a subset or even just focal parts of the plaques, may be composed of Aβ oligomers with PrP (see Figure 1). We suggest that the PrP (+) plaque is related to aging and/or dementia and that it is another category of amyloid plaque. We, therefore, propose the PrP (+) plaque as a new subtype of amyloid plaque, and more specifically a subset of diffuse plaques. While some neuritic plaques were immunolabeled by anti-PrP antibodies, the mechanisms of Aβ and PrP^C accumulation within diffuse plaques might be different from their mechanisms of accumulation in neuritic plaques. However, clear involvement of PrP (+) plaques in the pathophysiology of AD remains to be elucidated. A better understanding of the role of PrP^C in AD could provide new insights to establish new therapies for AD.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest in association with the present study.

AUTHOR CONTRIBUTIONS

Reisuke H. Takahashi made substantial contributions to the conceptual idea of this project, collection, analysis, and interpretation of data. Reisuke H. Takahashi also designed the experiments and drafted the manuscript. Mayumi Yokotsuka and Yuko Sato contributed to experimental works. Minoru Tobiume, Hideki Hasegawa, and Toshitaka Nagao helped in funding this study. Minoru Tobiume also provided technical advice for experiments. Gunnar K. Gouras edited the manuscript.

DATA AVAILABILITY STATEMENT

All data provided in this study are available from the corresponding author upon reasonable requirement.

ORCID

Reisuke H. Takahashi https://orcid.org/0000-0001-5379-7760

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

FIGURE S1 A schematic diagram of PrP and epitopes detected by respective antibodies used in this study. Antibody 3H2 recognizes the N-terminal and T4 recognizes the C-terminal region of PrP more than other PrP antibodies. One of the binding sites of Aβ oligomers overlaps with the epitope on PrP recognized by antibody 3F4 (21). GPI: glycosylphosphatidylinositol anchor. *The binding sites plotted in the diagram were proposed by Chen et al (23) and Lauren et al (21), residues 23–27 and 95–110, respectively

FIGURE S2 PrP^C accumulation in amyloid plaques is seen with a panel of PrP antibodies. PrP^C accumulation consistent with diffuse-type amyloid plaques with anti-PrP antibodies in brain tissue from an aged patient without dementia. PrP^C immunoreactivity was also detected in sections of aged brain tissue with PrP antibodies 3H2 (A), 6H4 (B), 12F10 (C), and T4 (D). Bar: 250 μm

FIGURE S3 PrP^C accumulating plaques labeled by PrP antibodies in Figure 2 are also stained by Aβ antibody 6E10. PrP^C accumulations are observed in the same area of a serial brain section where amyloid plaques stained by Aβ antibody 6E10 are seen

FIGURE S4 Lack of FSB labeling of PrP-positive plaques in non-AD brains. No co-localization of FSB with PrP-positive plaques was observed, similarly to the ThS staining pattern. (A) FSB staining (blue) was co-localized with neuritic plaques (arrowheads). (B) Conversely, FSB staining revealed no PrP-positive plaques (arrowheads). Bar: 50 μm

FIGURE S5 Negative ThS labeling of PrP accumulating diffuse plaque while positive ThS of neuritic plaque with a very small amyloid core. A diffuse plaque was not labeled by ThS and labeled by 3F4 antibody (arrows), while a neuritic plaque with a very small amyloid core was labeled by both ThS and 3F4 antibodies (arrowheads) from a representative 74-year-old patient without dementia. Bar: 100μm

FIGURE S6 . Low immunoreactivity of PrP antibodies in amyloid plaques of advanced AD brain tissue. (A, D) Compared to the immunoreactivity of Aβ antibody 6E10 in Figure 6A, fainter labeling of PrP antibodies 3H2 (A) and T4 (D) are evident in serial sections of advanced AD brain tissue (arrows). (B, C) Almost no immunoreactivity is evident in plaques in advanced AD with PrP antibodies, 6H4 (B) and 12F10 (C). Bar: 250 μm

FIGURE S7 No PrP-plaque was detected from young and aged brain tissues without dementia. (A, B) The deposition of amyloid plaques was not detected by Aβ antibody 6E10 in these cases (A), and (B) no PrP accumulating plaques were observed in the brain tissue of a 49-year-old patient. (C, D) Even in the brain tissue from an 85-year-old patient without dementia or amyloid plaque deposition (C), no PrP-positive plaque was detected (D). Intraneuronal Aβ/APP immunoreactivity was observed as granular dots by the 6E10 antibody in (A, C) and at higher magnification (C, inset). (E, F) In spite of the remarkable amyloid deposition and large numbers of amyloid deposits (E, inset) in an 85-year-old patient with an old infarction but without dementia (E), no PrP-immunoreactivity was detected. Bars: 250 μm, (inset, E 25 μm)

FIGURE S8 Localization of PrP in neurites and cell bodies of neurons. With higher magnification of Figure S7D, the ubiquitous localization of PrP immunoreactivity by antibody 3F4 is evident along neurites, which appear like lines in parallel (arrows). Additionally, PrP^{C} is seen in cell bodies of neurons evident by stronger brownish labeling (asterisks). Bar: 200 μm

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