Table 2: Most relevant clinical characteristics of the asymptomatic patients.

Asymptomatic (n = 17)	No	Yes
Diabetes	13	4
Hypertension	5	12
Heart disease	6	11
Smoking	9	current 6 and ex 2
Obesity	13	4
Family history of cardiovascular disease	17	0
Peripheral arterial disease	10	7
Statin	13	4
Anti-hypertensives	6	11 (5 CCB)

CCB, calcium channel blockers

with symptoms had 70% lower amounts of calcium (hydroxyapatite) [19]. This is in accordance with other studies suggesting that calcium could make plaques more stable [22], limiting the spread of inflammation [14]. A calcified nodule within or close to the plaque cap can protrude and lead to rupture [23]. However, if the calcified areas coalesce, the interfaces between rigid and distensible areas as well as the mechanical stress decrease [14]. Therefore, depending on their topography in the lesion, calcified areas can function as a protective "shell".

The presence of bone proteins as well as bone and cartilage formation in calcified vascular lesions has suggested that osteogenic mechanisms may play a role in vascular calcification [24]. Interestingly, calpain-2 has been shown to regulate matrix mineralization in a rat growth plate chondrocyte culture model [25], suggesting that calpains could be involved in vascular calcification. Furthermore, it has been suggested that apoptotic bodies derived from vascular smooth muscle cells may act as nucleating structures for calcium crystal formation and thus initiate vascular calcification [26]. A recent paper showed that vascular smooth muscle cell apoptosis in transgenic mice induced features of plaque vulnerability in atherosclerosis [27]. The fact that calpain regulates oxLDL-induced apoptosis [12,28], and possibly other types of vascular cell death, combined with the above-mentioned findings, suggests that this enzyme may be a central regulator of vascular calcification, and play an important role in the development of vulnerable plaques.

Conclusion

Our results suggest that calpain-1 is commonly active in carotid artery atherosclerotic plaques, and that calpain activity is colocalized with cell death and inversely associated with symptoms.

Abbreviations

OxLDL: oxidized low-density lipoprotein.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IG, TCS, ND, LMP, JFF, MPSA, and IPA contributed to the design of the study, LMP and JFF recruited the study participants and collected the samples. IG, MN, TCS, ND, MPSA, and IPA contributed to the collection of data, IG, MN, ND, MPSA, and IPA analysed the data. IG and IPA wrote the draft manuscript, ND and MPSA critically reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This research was supported by funding from foundations at Malmö University Hospital, the Alfred Österlund's Foundation, the Zoèga's Foundation, the Royal Physiographic Society in Lund, and the Swedish Heart and Lung Foundation.

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Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2261/9/26/prepub

BRAIN RESEARCH 1219 (2008) 169-179

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

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Research Report

Plasma antibodies to Aβ40 and Aβ42 in patients with Alzheimer's disease and normal controls

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ARTICLE INFO

Article history:
Accepted 15 February 2008
Available online 4 March 2008

Keywords:
Amyloid
Aβ
Alzheimer's disease
Antibodies
Plasma

ABSTRACT

Antibodies to amyloid β protein (Aβ) are present naturally or after Aβ vaccine therapy in human plasma. To clarify their clinical role, we examined plasma samples from 113 patients with Alzheimer's disease (AD) and 205 normal controls using the tissue amyloid plaque immunoreactivity (TAPIR) assay. A high positive rate of TAPIR was revealed in AD (45.1%) and age-matched controls (41.2%), however, no significance was observed. No significant difference was observed in the MMS score or disease duration between TAPIR-positive and negative samples. TAPIR-positive plasma reacted with the Aβ40 monomer and dimer, and the Aβ42 monomer weakly, but not with the Aβ42 dimer. TAPIR was even detected in samples from young normal subjects and young Tg2576 transgenic mice. Although the Aβ40 level and Aβ40/42 ratio increased, and Aβ42 was significantly decreased in plasma from AD groups when compared to controls, no significant correlations were revealed between plasma Aβ levels and TAPIR grading. Thus an immune response to Aβ40 and immune tolerance to Aβ42 occurred naturally in humans without a close relationship to the Aβ burden in the brain. Clarification of the mechanism of the immune response to Aβ42 is necessary for realization of an immunotherapy for AD.

1. Introduction

AD brains are invariably characterized by two pathological features: initial Aβ amyloidosis characterized by extracellular deposition of Aβ42 (43) and Aβ40, and subsequent tauopathy characterized by intracellular accumulation of neurofibrillary tangles consisting of abnormally phosphorylated tau. Since familial AD-linked mutations of amyloid β protein precursor

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doi:10.1016/j.brainres.2008.02.060

(βAPP), presenilin-1 (PS-1), and presenilin-2 (PS-2) increase the extracellular concentration of Aβ42 (43) and protofibril Aβ, these peptides are likely to be initiating factors in the pathogenesis of all types of AD (Hardy and Selkoe, 2002, Selkoe, 2002). Transgenic mouse models reproducing substantial brain Aβ amyloid support these hypotheses, and the appearance of neurofibrillary tangles (NFT) enhanced by Aβ amyloid in Tg2576 x tau P301L double transgenic mice further indicates that Aβ amyloidosis is the most important target for curing AD (Lewis et al., 2001).

Recent studies suggested that Aβ immunotherapy is the most promising among the many candidate therapies for AD. Schenk and others showed that an Aβ42 peptide vaccine clearly reduced the Aβ amyloid burden in transgenic model mice (Schenk et al., 1999; Weiner et al., 2000; Janus et al., 2000; Morgan et al., 2000). Passive immunization using anti-Aβ antibodies was also shown to be effective for reduction of the Aβ amyloid burden (Bard et al., 2000). These findings suggest peripheral antibodies to Aβ may serve a protective role against AD. A detectable increase in antibodies to Aβ42 was observed in about 25% of patients who received AN1792 in a Phase I study (Orgogozo et al., 2003; Nicoll et al., 2003). Analysis of serum samples by ELISA indicated that 15 of 18 patients experiencing meningoencephalitis in a Phase II study had antibodies against Aβ42. CSF antibodies to Aβ42 were present in 6 of 8 patients tested after the onset of encephalitis. However, titers of antibodies to Aβ42 were not correlated with the occurrence or severity of symptoms or relapses (Orgogozo et al., 2003). An autoantibody to Aβ40 was first detected in human B cell lines from AD patients (Gaskin et al., 1993). Naturally occurring antibodies to synthetic Aβ40 were confirmed by ELISA in the CSF and plasma of non-immunized humans and titers were significantly higher in healthy controls than in patients with AD (Du et al., 2001). Titers of anti-Aβ42 peptide antibodies were lower in AD patients compared with healthy individuals (Weksler et al., 2002), or elevated in AD patients and elder transgenic mice (Nath et al., 2003). Naturally occurring anti-Aβ42 antibodies were detected at very low levels by ELISA in over 50% of elderly individuals and at modest levels in 5% of them. Neither the presence nor the amount of naturally occurring anti-Aβ42 antibodies correlated with the presence, or age of AD onset, or the plasma levels of Aβ40 and Aβ42 (Hyman et al., 2001). Normal levels of antibodies to Aβ42 and Aβ40 were present in both AD and control groups, even in a young population (Baril et al., 2004). Thus, the previous reports suggested complex relationships for naturally occurring antibodies to Aβ.

In the Zurich cohort of a Phase II study, patients who generated antibodies to β-amyloid plaques in the plasma as determined by tissue amyloid plaque immunoreactivity (TAPIR) assay showed significantly slower rates of decline for cognitive functions and daily living activities suggesting that antibodies against β-amyloid plaques may be protective against AD (Hock et al., 2002, 2003; Gilman et al., 2005; Bombois

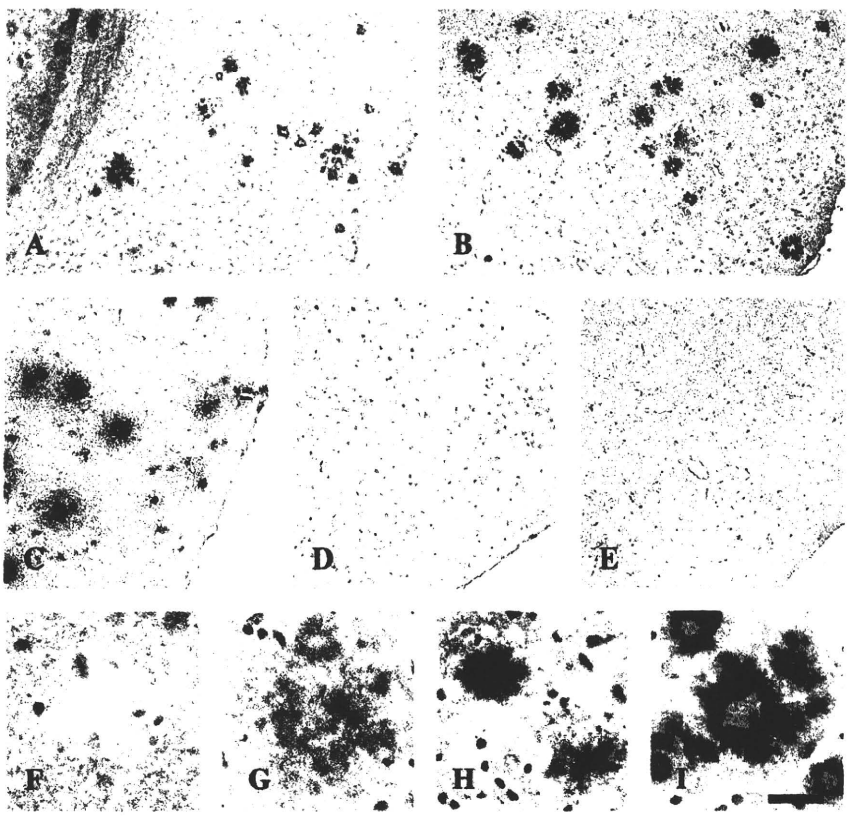

Fig. 1 – TAPIR using plasma and Tg2576 mouse brains. Many senile plaques in Tg2576 brains were labeled prominently in A (AD group, TAPIR grading ++) and B (aNC group, TAPIR grading ++). C: control Aβ immunostaining with Ab9204; no senile plaques were labeled in D (AD group, TAPIR grading –) and E (aNC group TAPIR grading –). F to I are examples of TAPIR grade. F: TAPIR –; G:±; H: +; I: ++. Scale bar=6.25 μm in A–E and 25 μm in F–I.

et al., 2007). Here, we examined 113 AD cases and 155 age-matched normal controls by TAPIR assay in order to clarify the positive rates, antibody characters, correlations with clinical symptoms, and clinical roles of naturally occurring antibodies against β-amyloid plaques. Modification of plasma Aβ40 and Aβ42 concentrations by antibodies to Aβ was also studied based on age- or AD-dependent alterations of plasma Aβ levels.

2. Results

2.1. *High positive TAPIR rate but no difference between AD and aNC groups*

Some plasma samples from AD and aNC groups strongly labeled nearly all amyloid plaque cores (Fig. 1A, B and I; grading ++) as did Ab9201 (Fig. 1C). Other plasma samples from both groups showed negative (Fig. 1D, E, F, grading –), weak (Fig. 1G, grading ±), or positive (Fig. 1H, grading +) labeling. The TAPIR staining was detected by anti-IgG second antibody, but not with anti-IgM or IgA antibodies (not shown), thus TAPIR-positive antibody was shown to be IgG antibody. The specificity of TAPIR-positive antibody was examined by immunoprecipitation of Aβ as described in 2.3. In the AD group, 42 cases (37.2%) were TAPIR –, 20 (17.8%) were ±, 44 (38.9%) were grading +, and 7 (6.2%) were ++. Fifty one of 113 AD patients were ++ and +, suggesting frequent appearance (45.1%) of naturally occurring antibodies to amyloid plaque cores. In the aNC group, 54 cases (34.8%) were TAPIR –, 37 (23.9%) were ±, 44 (28.4%) were +, and 20 (12.9%) were ++. Sixty-four cases of 155 aNC group (41.3%) were TAPIR ++ or +. No significant differences were detected by Mann–Whitney's *U* tests in the positive rates of naturally occurring antibodies to amyloid plaque cores among groups ($p=0.77$), or comparisons between the positive AD group (++ and +), negative AD group (± and –), positive aNC group (++ and +) and negative aNC (± and –) group ($p=0.54$) (Table 1).

2.2. *TAPIR was not correlated with clinical symptoms*

There were no significant differences in gender or mean age in both AD and aNC groups (Table 1). No significant differences were observed in MMS scores and disease duration among the TAPIR –, ±, +, ++ subgroups of AD samples (Table 1 and Fig. 2A, B). There were also no significant differences in the progressive decline of MMS scores among these AD subgroups (Fig. 2C). The presence of naturally occurring antibodies to Aβ as detected by TAPIR may therefore not improve prognosis of AD.

2.3. *TAPIR-positive plasma recognized Aβ40 and FAβ, but Aβ42 very weakly*

As indicated in Fig. 3, freshly prepared Aβ40 and Aβ42 were composed of monomers and dimers. However, formic acid extractable Aβ (FAβ) exhibited polymerization as shown by the higher molecular mass of its oligomers (Fig. 3, left panel). Immunoprecipitation with TAPIR ++/+ plasma obtained from the AD and aNC groups retrieved Aβ40 monomers and dimers as well as higher molecular mass polymers. Immunodetection of monomeric Aβ42 using 6E10 was very weak, whereas no dimeric form of Aβ42 was detected (Fig. 3 right panels). These findings suggest that TAPIR-positive plasma reacts with Aβ, but its reactivity to Aβ42 is very weak.

2.4. *Antibodies to Aβ appeared before Aβ amyloid deposits in the brain*

In order to clarify when these antibodies against Aβ appear, we additionally examined the remaining 50 plasma samples from subjects younger than 43 yeas old in the tNC group. Surprisingly, TAPIR revealed that antibodies to Aβ appeared in a 2 year-old child and also in some young subjects (TAPIR +; Fig. 4A, B and C). TAPIR-positive rates were 57% by 10 years old ($n=7$; 4 TAPIR +), 64% by 20 years old ($n=11$; 6 TAPIR +), 20% by 30 years old ($n=10$; 2 TAPIR +) and 10% by 40 years old ($n=10$; 1 TAPIR +). To confirm further this early appearance of antibodies to Aβ, immunoprecipitation was performed. Essentially identical finding to those seen in the AD and aNC groups were revealed (Fig. 4 D–F). Aβ40 and FAβ monomers and dimers were strongly immunoprecipitated (arrows). However, immunoprecipitation of the Aβ42 monomer was also weak and the Aβ42 dimer was absent in TAPIR-positive plasma from younger controls.

This was also the case in plasma obtained from Tg2576 model mice. Plasma from younger and older Tg2576 mice labeled

Table 1 – Summary of tissue amyloid plaque immunoreactivity (TAPIR) in AD and control groups

	Grade	Cases	rate (%)	Gender (M/F)	Mean age (SD), yr	Mean MMSE (SD)	Mean duration (SD), mo
AD ($n=113$)	–	42	37.2	11/31	75.4 (7.2)	14.7 (7.2)	50.9 (34.4)
	±	20	17.8	5/15	75.0 (8.0)	15.4 (7.3)	39.5 (27.4)
	+	44	38.9	14/30	74.5 (8.2)	14.9 (6.3)	37.5 (24.7)
	++	7	6.2	3/4	77.3 (5.3)	14.7 (6.2)	47.7 (19.7)
aNC ($n=155$)	–	54	34.8	21/33	74.7 (9.5)	29.9 (0.3)	–
	±	37	23.9	16/21	76.0 (8.7)	29.6 (0.5)	–
	+	44	28.4	19/25	77.9 (8.0)	29.7 (0.4)	–
	++	20	12.9	3/17	74.2 (11.8)	29.9 (0.3)	–

In the AD group, 42 cases (37.2%) were TAPIR –, 20 (17.8%) were ±, 44 (38.9%) were +, and 7 (6.2%) were ++; 51 of 113 AD patients were ++ and +, suggesting high rate of TAPIR (45.1%). In the aNC group, 54 cases (34.8%) were TAPIR –, 37 (23.9%) were ±, 44 (28.4%) were +, and 20 (12.9%) were ++; 64 of 155 aNC controls (41.3%) were TAPIR-positive. No significant differences were found in the positive TAPIR rate within each group ($p=0.77$), or in comparisons between the positive AD group (++ and +), negative AD group (± and –), positive aNC group (++ and +), and negative aNC (± and –) group ($p=0.54$). There were no significant differences in gender, mean age, mean MMS score or mean disease duration according to TAPIR grade in both AD and aNC groups. yr: years old; mo: months.

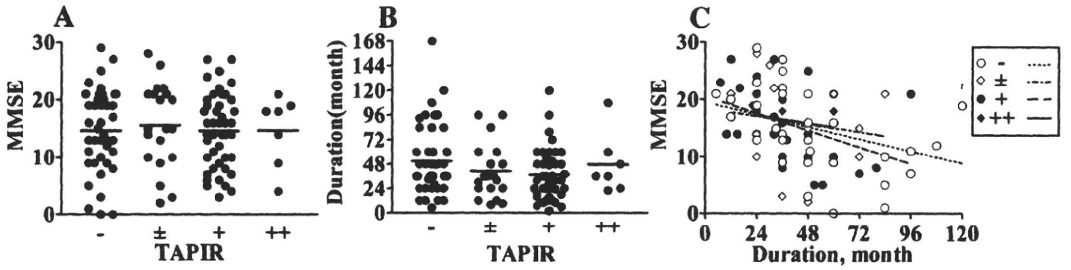

Fig. 2 – TAPIR was not correlated with clinical symptoms. There were no significant differences in MMS scores (A), disease duration (B) or decline of the clinical course of AD according to TAPIR grade. No significant difference in the decline of MMS scores according to duration was shown among AD subgroups (C). $Y=-0.09X+19.54$, $r^2=0.19$, $p=0.01$ in TAPIR – (○); $Y=-0.06X+18.50$, $r^2=18.50$, $p=0.52$ in TAPIR ± (◊); $Y=0.12X+20.59$, $r^2=0.17$, $p=0.02$ in TAPIR + (•); $Y=-0.06X+18.63$, $r^2=0.04$, $p=0.72$ in TAPIR ++ (♦).

amyloid cores in AD brains (Fig. 4G–I). The appearance rate was 1/3 at 4 months old (1 TAPIR +), 3/3 at 8 months old (1 TAPIR ++ and 2 TAPIR +), 1/1 at 16 months old (1 TAPIR ++) and 1/1 at 23 months old mice (1 TAPIR +).

Finally, we summarized age-dependent TAPIR-positive rates (TAPIR grading + and ++) in 10 year increments in both AD and tNC groups (Fig. 4J). TAPIR-positive rates were high in young subjects (1–20 years old), low during adulthood (21–50 years old) and then increased again after 50. No differences were observed between AD and tNC samples from 50 to 91 years old. Thus, the appearance of antibodies to Aβ preceded Aβ amyloid deposition in human and model mouse brains.

2.5. *Levels of plasma Aβ40 and Aβ42 were age-dependently regulated in the tNC group*

To examine the effect of antibodies to Aβ on plasma Aβ concentrations, we measured levels of Aβ40 and Aβ42 in 318 plasma samples by specific ELISA. In the tNC group, plasma Aβ40 levels increased after 40 years of age (Fig. 5A; $p<0.0001$). On the contrary, plasma Aβ42 levels increased between the teens and twenties, then gradually declined with age (Fig. 5B; $p=0.0158$). The Aβ ratio (Aβ40/Aβ42) was stable until ~30 years old and then gradually increased (Fig. 5C; $p<0.0001$).

2.6. *Plasma Aβ ratio is increased in AD*

Significantly increased levels of plasma Aβ40 were observed in the AD group (112±39.51 pmol/L) compared to the aNC group (95.38± 32.30; $p<0.0002$; Fig. 5D). Aβ42 levels were significantly decreased in the AD group (10.29±13.80 pmol/L) compared to the aNC group (12.13±12.29; $p<0.0001$; Fig. 5E). Based on these changes, the Aβ ratio (Aβ40/Aβ42) was more strongly increased in the AD group (14.42±10.00) than in the aNC group (8.34±3.83; $p<0.0001$; Fig. 5F). ROC analysis of the Aβ ratio indicated that the significant cut off value was 9.0, which provided high sensitivity (78.8%) and low specificity (30.3%) for clinical diagnosis of AD. When the mean+2 SD (15.9) of the aNC group was used as a cut off value, the sensitivity was 24% and the specificity was 96%. When AD was divided into 3 subgroups according to clinical stage, increasing Aβ40 levels and Aβ ratio, as well as decreasing Aβ42 levels progressed from the early stage to the advanced stage (Fig. 5G–I). Thus, the plasma Aβ ratio can be used as a specific biomarker for AD although the sensitivity and specificity are lower than those of CSF samples (Kanai et al., 1998; Shoji et al., 2001; Shoji, 2002).

2.7. *TAPIR did not modify Aβ concentration*

Finally, we examined whether antibodies to Aβ could affect levels of plasma Aβ40 and Aβ42. There were no significant differences in the concentrations of plasma Aβ40 or Aβ42, or in the Aβ ratio among AD and aNC classified by TAPIR score (Fig. 6A–C).

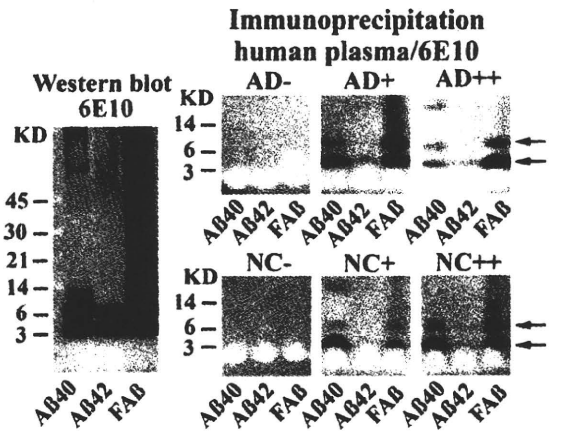

Fig. 3 – TAPIR-positive plasma immunoprecipitated Aβ40 and amyloid Aβ, but Aβ42 very weakly. On direct western blotting of synthetic Aβ40, Aβ42, and FAβ from the AD brain, antibody 6E10 detected monomers and dimers of Aβ40, Aβ42 and brain amyloid Aβ with smear aggregates (left panel). Immunoprecipitations of Aβ40, Aβ42, and FAβ using TAPIR –, +, and ++ plasma from the AD group (right upper panel, AD) or the aNC group (right lower panel, NC) were labeled by antibody 6E10, showing that monomers (arrow) and dimers (arrow) of Aβ40 were recognized by TAPIR-positive plasma (grading + and ++) in addition to Aβ42 monomers, and brain Aβ amyloid monomers and dimers with smear aggregates, which showed weak signals.

3. Discussion

In our study, a high positive rate of TAPIR was found in both AD (45.1%) and aNC (41.2%) groups, but no significant difference was found between these groups. Essentially the same findings were observed even in strongly positive (++) subgroups of AD (6.2%) and aNC (12.9%). Non-parametric analysis revealed that neither MMSE score nor disease duration correlated with TAPIR grade, indicating that the physiological impact of naturally occurring anti-Aβ antibodies is below clinical significance. This is consistent with previous reports describing frequent presence of low levels of antibodies to Aβ40 or Aβ42 peptides as detected by ELISA in plasma and CSF. A large scale study by Hyman et al. showed by ELISA that there were low and modest levels of anti-Aβ42 peptide antibodies in 52.3% and 4.7% of 365 plasma samples from AD and age-matched controls, respectively (Hyman et al., 2001). Neither the presence nor the amounts of anti-Aβ antibodies correlated with the likelihood of developing dementia or plasma levels of Aβ40 and Aβ42 (Hyman et al., 2001; Orgogozo et al., 2003; Moir et al., 2005; Li et al., 2007). Our study indicated that TAPIR-

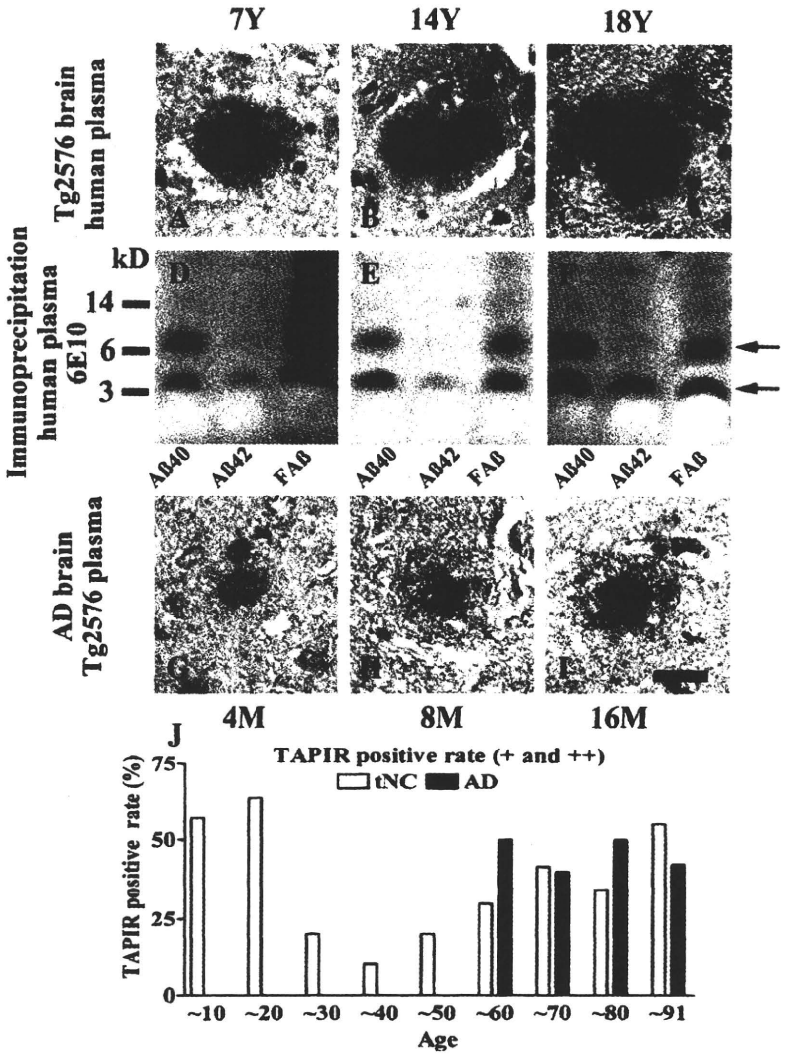

Fig. 4 – Antibodies to Aβ appeared before Aβ amyloid deposits in the brain. TAPIR was positive in 7 years old (TAPIR +; A, 7Y), 14 years old (TAPIR +; B, 14Y), and 18 years old young persons (TAPIR +, C, 18Y). TAPIR-positive plasma strongly immunoprecipitated monomers and dimers (arrow) of Aβ40 and FAβ, and weakly immunoprecipitated monomers of Aβ42 and Aβ amyloid (D, E and F; corresponding plasma of upper panels; D and A 7Y, E and B 14Y and F and C 18Y). Plasma from younger and older Tg2576 mice also labeled amyloid cores in AD brains (G: 4 months old TG; H: 8 months old Tg and I: 16 months old Tg). Bar scale = 15 μm. J: TAPIR-positive rates in the tNC group according to age. Columns show the TAPIR-positive rate (TAPIR grading + and ++) for 10 year increases in the AD (black columns) and tNC (white columns) groups. TAPIR-positive rates were high in young subjects (1–20 years old), low during adulthood (21–50 years old) and then increased again after age 50. No differences were observed between AD and tNC groups in samples from subjects 50 to 91 years old.

positive antibodies to Aβ amyloid plaques also occur naturally and frequently in human plasma and that their titers are not sufficient to prevent development of dementia. High titer of antibodies are necessary to improve the Aβ burden as shown in AD patients treated with an Aβ vaccine (Hock et al., 2002) or an anti-Aβ antibody infusion therapy (Dodel et al., 2002).

TAPIR is a new method to detect anti-Aβ antibodies (Hock et al., 2002, 2003). The fact that cognitive impairment was improved in patients who generated anti-Aβ antibodies after Aβ vaccination leads us to hypothesize that TAPIR-positive anti-Aβ antibodies are substantially different from naturally occurring anti-Aβ peptides antibodies in their specificity for Aβ40 and Aβ42 species or conformational epitopes of Aβ oligomers (Mirra et al., 1991; Kayed et al., 2003). Antibodies labeling Aβ amyloid plaques were more effective for the clearance of the Aβ burden of transgenic mice in passive immunization experiments (Bard et al., 2000). Direct action of the anti-Aβ antibody through the blood–brain barrier without T-cell proliferation as well as microglial clearance via the Fc or non-Fc portion of the antibodies mediated disruption of the plaque structure (Bard et al., 2000; Bacskai et al., 2002; Lombardo et al., 2003). Binding of an IgG2a antibody to the special N-terminal region of Aβ correlated with a clearance response (Bard et al., 2003). Injected antibodies may bind and sequestrate blood Aβ completely and disturb the balance between CSF Aβ and blood Aβ, leading to increased clearance from the brain into the blood (DeMattos et al., 2001; DeMattos et al., 2002). Clearance of diffusible Aβ oligomers that impair cognitive function was considered to be another target for passive immunization (Kayed et al., 2003). Recently a 56-kDa soluble amyloid-β assembly termed Aβ*56 has been shown to disrupt memory (Lesné et al., 2006), and Aβ oligomers have been shown to be increased in CSF from AD patients (Georganopoulou et al., 2005).

These reports all support the hypothesis that naturally occurring TAPIR-positive antibodies to Aβ recognize special Aβ species. Our immunoprecipitation study suggested that

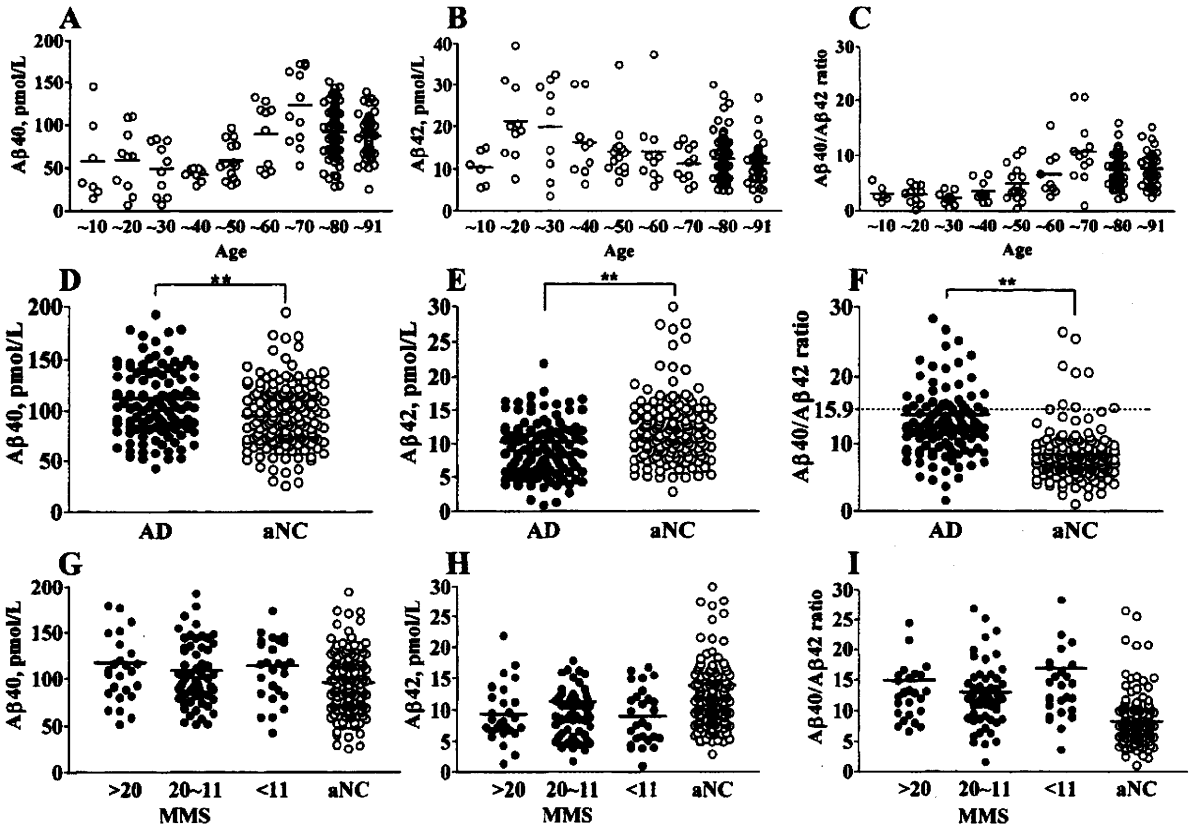

Fig. 5 – Age-dependent regulation of plasma Aβ levels in controls, and their alteration in AD. Plasma Aβ40 and Aβ42 levels showed different age-dependent alterations in the tNC group. Aβ40 levels increased from age 50 and decreased from age 70 (A). Aβ42 levels were high in the teens and twenties, then gradually decreased with age (B). Based on these different changes, the Aβ ratio (Aβ40/Aβ42) progressively increased from age 40 (C). Significantly increased levels of Aβ40 (D: $p=0.0002$) and increased Aβ ratio (F: $p<0.0001$) as well as decreased levels of Aβ42 (E: $p<0.0001$) were shown between the AD and aNC groups. When the mean +2SD of the Aβ ratio in the aNC group was used as a diagnostic marker for AD, the cut off value 15.9 (dot line) provided 24% sensitivity and 96% specificity (F). Constant alterations of plasma Aβ levels in AD were recognized at the early (MMS score >20), moderate (MMS score 20–11), and advanced stages (MMS score <11) (G–I). A, D, G: Aβ40; B, E, H: Aβ42; C, F, I: Aβ ratio. Bars show mean levels.

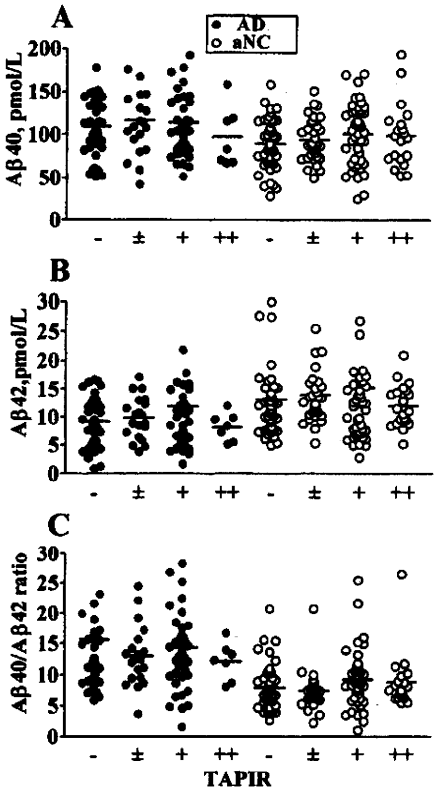

Fig. 6 – TAPIR did not modify Aβ concentration. No significant differences were found in Aβ40 and Aβ42 concentrations as well as Aβ ratios among all TAPIR grades (–, ±, + and ++) in AD (•) and aNC (○) group (A, B and C).

TAPIR ++/+ plasma obtained from AD and aNC subjects retrieved Aβ40 monomers and dimers as well as higher molecular mass polymers. Immunodetection of monomeric Aβ42 using 6E10 was very weak, whereas no dimeric form of Aβ42 was detected under our testing conditions. The absence of anti-Aβ42 dimer antibodies and the relatively low levels of anti-Aβ42 monomers were characteristic of naturally occurring antibodies to Aβ. These findings are considered to be another reason why naturally occurring antibodies to Aβ are not sufficient for prevention of development of dementia.

Our TAPIR assay also showed that anti-Aβ antibodies were naturally present throughout the entire human life span. It is relevant to note that naturally occurring anti-Aβ antibodies were unequivocally detected in young human subjects as well as young Tg2576 mice. In relative terms, the positive rates of anti-Aβ antibodies were highest in young individuals, lowest in those middle-aged and higher in the elderly. The presence of anti-Aβ antibodies in young human subjects was characterized by the subsequent immunoprecipitation study. Anti-Aβ antibodies retrieved Aβ40 monomers and dimers as well as high molecular mass oligomers in FAβ fractions, but they retrieved fewer Aβ42 dimers. To our knowledge, this is the first report showing the relatively selective presence of anti-Aβ40 antibodies, and reduced amounts of anti-Aβ42 antibodies in young individuals. We also found that this was the case in normal elderly as well as AD patients, suggesting that the immune response to Aβ was unchanged in the two groups tested. Impaired spontaneous production of anti-Aβ42 antibodies also took place in elderly human subjects as well as AD patients. It is unknown why these antibodies to Aβ appeared more frequently in the young and the elderly populations and how specific immune tolerance for Aβ42 monomers and oligomers could be present. However, it should be noted that naturally occurring antibodies to Aβ appear in young human subjects and young Tg2576 mice, which do not develop an Aβ burden in their brain. The appearance of naturally occurring antibodies to Aβ is not correlated with the Aβ burden in the brain.

The exact mechanism underlying spontaneous anti-Aβ antibody production remains unknown. Although increased Aβ42 levels have been detected in transgenic animal models (Kawarabayashi et al., 2001), immune hyporesponsiveness to Aβ42 was also shown (Monsonego et al., 2001). Increased T-cell reactivity to Aβ42 was shown to increase in elderly individuals and patients with AD (Monsonego et al., 2003). However, the previous findings and our results could not show increased titers of anti-Aβ42 antibodies in these groups. Thus, hyopoimmunue responses to Aβ42, especially to the Aβ42 oligomer, actually occurred in AD and healthy populations. Since Aβ42 is highly pathogenic and neurotoxic, Aβ42 may be sequestered and spontaneous immune responses to Aβ may be suppressed in human populations. For effective immunotherapy as shown in transgenic mice studies and Aβ vaccine trials (Orgogozo et al., 2003; Hock et al., 2003), it is necessary to further generate antibodies to Aβ42 oligomers as well as monomers and monitor their titers. Furthermore, in order to prevent unexpected adverse reaction as seen in the Phase II trials of AN1792, detection of these spontaneous antibodies to Aβ will be necessary before treatment.

Recent studies have shown that plasma concentrations of Aβ40 and Aβ42 are possible biomarkers (Ertekin-Taner et al., 2000; Fukumoto et al., 2003; Mayeux et al., 1999, 2003; van Oijen et al., 2006; Graff-Radford et al., 2007) and can be used to monitor the effects of special treatments for AD (Dodel et al., 2002; DeMattos et al., 2001, 2002). After administration of an antibody to Aβ, the rapid increase in plasma Aβ was highly correlated with the amyloid burden in the brain (DeMattos et al., 2002), suggesting the possibility that naturally occurring anti-Aβ antibodies may cause increases the plasma Aβ concentration. In order to clarify this effect, we first analyzed age-dependent levels of plasma Aβ40 and Aβ42, and then examined alterations of Aβ40 and Aβ42 levels according to the presence or absence of AD and antibodies to Aβ. In the tNC group, plasma Aβ40 levels increased from age 40. Plasma Aβ42 levels increased between age 10 and 20, then gradually declined with age. The Aβ ratio (Aβ40/Aβ42) was stable until about 30 years and then gradually increased. These natural time courses were identical to those of CSF Aβ40 levels, but completely different from those of CSF Aβ42 levels. CSF levels of Aβ40 and Aβ42 showed U-shaped age-dependent curves, suggesting their correlation with brain development and decline (Kanai et al., 1998; Shoji et al., 2001; Shoji, 2002). The correlation was prominent between the appearance of naturally occurring anti-Aβ antibodies and increased Aβ40 levels in

the CSF and plasma. Increased opportunities for immunological exposure to Aβ40 monomers and oligomers in immature or declining brains in young and elderly indivisuals may be sources for the naturally occurring immune response to Aβ40.

Based on these natural time courses of plasma Aβ concentrations, a comparison between AD and aNC groups was performed that provided intriguing findings. Significantly increased levels of plasma Aβ40, increased Aβ ratio and decreased levels of Aβ42 were revealed in the AD group when compared to the aNC group. Since a clear separation was obtained in the Aβ ratio between the AD and aNC groups, we evaluated the value of the Aβ ratio as a diagnostic or monitor maker of AD. ROC analysis indicated high sensitivity (78.8%) and low specificity (30.3%) for diagnosis of AD. When the mean+2 SD (15.9) of the aNC group was used as a cut off value, the sensitivity was 24% and specificity was 96%. When AD was divided into 3 groups according to clinical stage, the Aβ ratio increased progressively from the early stage to the advanced stages of AD. These findings show that plasma Aβ ratio can be used as an easy, non-invasive, and useful biomarker for diagnosis and monitoring of clinical symptoms of AD, although the sensitivity and specificity are lower than those in CSF samples (Kanai et al., 1998; Shoji et al., 2001; Shoji, 2002). However, naturally occurring antibodies to Aβ did not affect plasma Aβ40 or Aβ42 levels, or the Aβ ratio. There was a possibility that our ELISA system could not detect increased levels of Aβ40 and Aβ42 oligomers. However, all results taken together, suggest that the titer and specificity of naturally occurring anti-Aβ antibodies were not sufficient to elevate plasma Aβ concentrations and increase Aβ clearance from the brain to the peripheral blood with subsequent improvement of clinical symptoms. Higher titers of antibodies to Aβ42 oligomers will likely be necessary to facilitate Aβ clearance from brain amyloid to peripheral blood for AD treatment.

4. Experimental procedures

4.1. *Patients and normal controls*

After informed consent was given, blood samples were collected into 0.1% EDTA from a total of 318 subjects including 113 patients with AD (AD group) and 205 normal controls (total normal control group: tNC group). As age-matched controls (aNC group), 115 samples from subjects over 43 years old were selected from the tNC group. The basic findings for the respective groups are summarized in Table 2. The clinical diagnosis of AD was based on NINCDS–ADRDA criteria (McKhann et al., 1984). Appropriate diagnostic studies including magnetic resonance imaging and single photon emission computed tomography were used to exclude other disorders of dementia. The clinical severity of AD was evaluated using the Mini-Mental State Examination (MMS) (Folstein et al., 1975). AD patients were divided into 3 subgroups according to clinical stages: early stage MMS score >20, moderate stage MMS score 10–20, advanced stage MMS score <10. Controls were judged to be normal based on their MMS score (>28 points) and follow-up with neurological evaluation. After separation of plasma from blood cells, plasma was stored frozen at −80 °C until use.

Table 2 – Summary of the study subjects

	No. of subjects	Gender (M/F)	Mean age (range), yr	Mean MMS Score (SD)	Mean duration (SD), mo
AD	113	32/81	75 (55–89)	14.9 (6.7)	44 (28)
tNC	205	84/121	64 (1–91)	29.8 (0.3)	–
aNC	155	59/96	76 (43–91)	29.7 (0.4)	–
Total	318	116/202	68 (1–91)		

AD: Alzheimer's disease patients; tNC: total normal controls; aNC: age-matched controls over 43 years old selected from the tNC group; M/F: male and female; yr: years old; MMS: Mini-Mental State Examination; SD: standard deviation; Duration: duration from onset, mo months.

4.2. *Tissue amyloid plaque immunoreactivity (TAPIR)*

Five micrometers serial paraffin sections of brains from Tg2576 mice (16–18 months old) or Alzheimer's patients were used. Sections were immersed in 0.5% periodic acid for blocking intrinsic peroxidase and treated with 99% formic acid for 3 min to increase Aβ staining. Sections were then immersed with blocking solution with 5% normal serum in 50 mM phosphate-buffered saline (PBS) containing 0.05% Tween20 and 4% Block Ace (Snow Brand Milk Products, Saporo, Japan) for 1 h; goat serum was used to stain human plasma, and horse serum was used to stain mouse plasma. Sections were incubated at 4 °C overnight with human or mouse plasma diluted with blocking solution (1:100). Sections were then incubated with biotinyzed second antibody (anti-human goat antibody or anti-mouse horse antibody), and horseradish peroxidase-conjugated avidin–biotin complex of Vectastain Elite ABC kit (Vector, Burlingame, CA). Immunoreactivity was visualized by incubation with 0.03% 3, 3′-diaminobenzidine, and 0.02% H_2O_2. Tissue sections were counterstained with hematoxylin. Immunostaining with Ab9204 (Saido et al., 1995) (1:1000, antibody to a synthetic Aβ peptide starting from the amino-terminus of normal L-aspartate) or without the primary antibody were used as positive and negative controls, respectively.

4.3. *Grading of TAPIR*

TAPIR findings were classified into 4 levels: negative –, no senile plaque core (Fig. 1F); weakly positive ±, senile plaque cores were stained weakly and less than 5 cores were stained in each brain section on a slide (Fig. 1G); positive +, ≥5 senile plaque cores were stained clearly in at least one brain section per slide (Fig. 1H); strongly positive ++, most senile plaque cores were strongly labeled when compared to Ab9204 immunostaining (Fig. 1I). Immunostaining findings of diffuse plaques, amyloid angiopathy, positive neurons, degenerative neurites and glial cells were excluded from this grading.

4.4. *Purification of amyloid Aβ (FAβ)*

An autopsy brain fulfilling the CERAD criteria for definite AD (Mirra et al., 1991) was selected. About 2 g of gray matter of the AD brain was homogenized with 4 volumes of TBS (10 mM Tris, 150 mM NaCl, pH 8) with protease inhibitors (1 μg/ml

Leupeptin, 1 μg/ml TLCK, 0.1 μg/ml Pepstain A, 1 mM phenylmethysulfonyl fluoride and 1 mM EDTA), and centrifuged at 100,000 ×g for 1 h. The resulting pellet was extracted with 10 ml of 10% sodium dodecyl sulfate (SDS) in TBS and then with 1 ml of 99% formic acid (FA). The final supernatant was lyophilized, dissolved with 20 μl of 99% dimethylsulfoxide (DMSO), and stored at −80 °C until use (formic acid soluble amyloid Aβ fraction: FAβ) (Harigaya et al., 1995; Matsubara et al., 1999).

4.5. *Immunoprecipation*

20 μl of protein G agarose (Roche diagnostic GmbH, Germany) was washed 3 times with 1 ml RIPA buffer (50 mM Tris, 1% Triton X-100, 0.1% SDS, 0.5% cholic acid and 150 mM NaCl, pH 8.0). Prewashed protein G agarose was mixed with 600 ng synthetic Aβ40, 600 ng synthetic Aβ42 (Sigma, Mo) or 300 ng FAβ in 1 ml of RIPA buffer and incubated at room temperature for 30 min. After centrifugation, the resulting supernatant was mixed again with 20 μl of prewashed protein G agarose and 10 μl of plasma, incubated at room temperature for 3 h, and then centrifuged. The pellet was boiled with 1× NuPage LDS sample buffer containing 0.1 M dithiothreitol for 10 min at 70 °C and separated on a 4 to 12% NuPage Bis-Tris gel (Invitrogen, CA). After electro-transfer, the blot membrane was blocked with 10% skim milk (Snow Brand Milk Products, Saporo, Japan) in TBS with 0.05% Tween 20 (TBST), and incubated with monoclonal 6E10 (specific to Aβ1-16, 1:1000, Signet Lab. Inc. MA) at 4 °C overnight. After washing and incubation with horseradish-peroxidase-conjugated goat anti-mouse IgG (1:2000, Amersham Biosci, Buckinghamshire, UK) at RT for 2 h, the signal was developed by SuperSignal west Dura extended duration substrate (Pierce Biotechology, CA), and quantified by a luminoimage analyzer (LAS 1000-mini, Fuji film, Japan).

4.6. *Quantification of plasma Aβ40 and Aβ42 concentrations by ELISA*

Sandwich ELISA was used to specifically quantify whole plasma Aβ, as previously described (Matsubara et al., 1999). Microplates were pre-coated with monoclonal BNT77 (IgA, anti-Aβ11-28, specific Aβ11-16) and sequentially incubated with 100 μl of samples followed by horseradish-peroxidase-conjugated BA27 (anti-Aβ1-40, specific Aβ40) or BC05 (anti-Aβ35-43, specific Aβ42 and Aβ43) (Kawarabayashi et al., 2001). Synthetic Aβ40 (peptide content: 79.95%, Sigma, MO) and Aβ42 (peptide content:76.58%, Sigma, MO) were used for standard Aβ. The sensitivity was 40 fmol/ml in the Aβ40 assay and 10 fmol/ml in the Aβ42 assay. Both intra-assay coefficients of variation were less than 10% (Matsubara et al., 1999).

4.7. *Statistical analysis*

Comparisons among the groups using Student's t-test, one-way analysis of variance or a non-parametric test with post hoc tests, a receiver-operating characteristic (ROC) curve analysis to determine the cut off value, Mann–Whitney *U* test for appearance rates, and 1st order regression analysis of the relationship between MMS score and AD duration were all performed using SPSS 11.0 (SSPS Inc., IL) and GraphPad Prism, Version 4 (GraphPad Software, San Diego, CA).

Acknowledgments

We thank Yasuko Nogami and Hisae Narihiro for technical assistance, and Dr. TC Saido for kind gift of Ab9204. All animal experiments were performed according to guidelines established in the "Guide for the Care and Use of Laboratory Animals". This study was performed with permission of the ethical committee of Okayama University. Supported by a Grant-in-Aid from the Grants-in-Aid for Primary Amyloidosis Research Committee (M.S.), of the Ministry of Health, Labor and Welfare of Japan and by Grants-in-Aid for Scientific Research (B) (M.S.: 19390233, K.A.: 18390257), (C) (M.I.: 19590980, T.K.: 19590976, A.S.: 18500276, E.M.: 18590968), from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Brain Pathology ISSN 1015-6305

RESEARCH ARTICLE

Transthyretin Accelerates Vascular Aβ Deposition in a Mouse Model of Alzheimer's Disease

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Keywords
Alzheimer's disease, amyloid-β, apoptosis, tau phosphorylation, Tg2576 mouse, Transthyretin.

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Received 12 October 2007; revised: 10 February 2008; accepted 12 February 2008.

doi:10.1111/j.1750-3639.2008.00166.x

Abstract

Transthyretin (TTR) binds amyloid-β (Aβ) and prevents Aβ fibril formation *in vitro*. It was reported that the lack of neurodegeneration in a transgenic mouse model of Alzheimer's disease (AD) (Tg2576 mouse) was associated with increased TTR level in the hippocampus, and that chronic infusion of anti-TTR antibody into the hippocampus of Tg2576 mice led to increased local Aβ deposits, tau hyperphosphorylation and apoptosis. TTR is, therefore, speculated to prevent Aβ pathology in AD. However, a role for TTR in Aβ deposition is not yet known. To investigate the relationship between TTR and Aβ deposition, we generated a mouse line carrying a null mutation at the endogenous *TTR* locus and the human mutant amyloid precursor protein cDNA responsible for familial AD (Tg2576/$TTR^{-/-}$ mouse) by crossing Tg2576 mice with TTR-deficient mice. We asked whether Aβ deposition was accelerated in Tg2576/$TTR^{-/-}$ mice relative to the heterozygous mutant Tg2576 (Tg2576/$TTR^{+/-}$) mice. Contrary to our expectations, the degree of total and vascular Aβ burdens in the aged Tg2576/$TTR^{-/-}$ mice was significantly reduced relative to the age-matched Tg2576/$TTR^{+/-}$ mice. Our experiments present, for the first time, compelling evidence that TTR does not suppress but rather accelerates vascular Aβ deposition in the mouse model of AD.

INTRODUCTION

Insoluble amyloid-β (Aβ) peptides, the main components of brain amyloid plaques, are thought to be the causative agent of Alzheimer's disease (AD) (11). However, Aβ is normally present in a soluble form in plasma and in the cerebrospinal fluid (CSF) (39, 40), suggesting that some other factors may modulate the aggregation of Aβ fibrils. The hypothesis that transthyretin (TTR) might play some role in the pathogenesis of AD originated from the observation that TTR in the CSF binds Aβ, and prevents Aβ fibril formation *in vitro* (36, 37). It was further observed that the levels of both TTR and its oxidized forms in the CSF were lower in patients with AD compared with the age-matched controls (2, 38). The importance of TTR in inhibition of Aβ fibril formation and toxicity *in vivo* was also suggested in two model systems: transgenic *Caenorhabditis elegans* and a transgenic mouse model of AD, Tg2576. Link reported that co-expression of Aβ peptide and TTR in transgenic *C. elegans* led to a reduction in Aβ deposits (22). Tg2576 line has high level of plasma Aβ peptides (14, 18), and develops brain Aβ deposits similar to that seen in patients with AD (15, 35) and behavioral deficits (13, 53). However, it lacks neurofibrillary tangles (NFT) (27, 48, 49) and neuronal loss (15), which are unique characteristics of patients with AD (5). Stein and Johnson reported that the lack of neurodegeneration was associated with increased level of TTR in the hippocampus of Tg2576 (43). They also reported that chronic infusion of an antibody against TTR into the hippocampus of Tg2576 mice led to increased Aβ deposits, tau hyperphosphorylation, neuronal loss and apoptosis in the CA1 neuronal field (42). Carro *et al* reported that reduced Aβ burden after insulin-like growth factor I-treatment of Tg2576 was paralleled by increased brain levels of TTR (6). Giunta *et al* reported the inhibition of Aβ aggregation and toxicity and Aβ-induced apoptotic changes by TTR in cultured cells (10).

All these findings support for the importance of TTR in prevention of Aβ aggregation and toxicity. However, a role for TTR in Aβ deposition is not yet known. To investigate the relationship between TTR and Aβ deposition, we generated a mouse line carrying a null mutation at the endogenous *TTR* locus and the human mutant amyloid precursor protein (APP) cDNA responsible for familial AD (Tg2576/$TTR^{-/-}$ mouse), by crossing Tg2576 mice with TTR-deficient mice generated through gene targeting (9). We asked whether Aβ deposition was accelerated in Tg2576/$TTR^{-/-}$ mice relative to the heterozygous mutant Tg2576 (Tg2576/$TTR^{+/-}$) mice.

METHODS

Animals

Transgenic mice producing human variant APP and lacking endogenous mouse TTR were generated as follows. A male Tg2576 mouse (13) carrying the human mutant APP cDNA with the double mutation K670N and M671L responsible for Swedish familial AD backcrossed to C57BL/6 for 2 generations was mated with $TTR^{-/-}$ female mice backcrossed to C57BL/6 for eight generations (9). The $TTR^{+/-}$ F1 male mice carrying the mutant APP cDNA were mated with $TTR^{-/-}$ female mice. Heterozygous ($TTR^{+/-}$) F2 male mice carrying the mutant APP cDNA (Tg2576/$TTR^{+/-}$) were mated with $TTR^{-/-}$ F2 female mice. The $TTR^{+/-}$ and $TTR^{-/-}$ F3 progenies carrying the mutant APP cDNA (Tg2576/$TTR^{+/-}$ and Tg2576/$TTR^{-/-}$) were used in the present study. The F3 transgenic mice were maintained in cages housing three to six mice each, on separate racks in the same room, kept under a 12-h light cycle. Regular rodent's chow (Oriental Yeast, Tokyo, Japan) and tap water were freely available.

Transgenic mice were killed by cervical dislocation after anesthesia with diethyl ether. The brains were dissected; the right hemi-brains were immediately frozen in liquid nitrogen and stored at −80°C while the left hemi-brains were fixed in 4% buffered paraformaldehyde, and embedded in paraffin. Genotype analysis for each animal was carried out by polymerase chain reaction on DNA, purified from tails, as described (9, 14). The presence and absence of TTR in the serum of Tg2576/$TTR^{+/-}$, and Tg2576/$TTR^{-/-}$ mice, respectively, were confirmed by western blotting analysis as described (51).

All animal experiments were approved by University of Yamanashi Animal Care and Use Committee.

Immunohistochemistry

For brain Aβ detection, the paraffin-embedded left hemi-brain sections (5 μm) were pretreated with 99% formic acid for 3 minutes and immersed in 5% periodic acid for 10 minutes to block endogenous peroxidase. They were then incubated with blocking buffer [5% normal goat serum (Gibco, Carlsbad, CA, USA) in 10-mM phosphate buffer pH 7.4 and 100-mM NaCl with 0.05% Tween-20 (Bio-Rad, Richmond, CA, USA) containing Block Ace (Dainipponseiyaku, Suita, Japan)] for 1 h, with primary antibody [Ab9204 recognizing normal L-aspartate at position 1 (34), 0.1 μg/ml] overnight, and with biotinylated anti-rabbit immunoglobulin G (IgG) antibody (1:200) (Vector Laboratories, Burlingame, CA, USA) for 1 h. Immunoreactivity was visualized with the use of Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA), and 3,3′-diaminobenzidine, tetrahydrocloride (DAB). Tissue sections were counterstained with hematoxylin.

For phosphorylated tau detection, the paraffin sections were pretreated with periodic acid, as described above and then irradiated in 10-mM citric acid buffer pH 6.0 for 15 minutes with microwave oven. After blocking, as described above, the sections were stained with the use of primary antibody AT8, recognizing phosphorylated tau at Ser202/Thr205 (1:500) (Innogenetics, Gent, Belgium) or anti-phosphorylated tau, recognizing phosphorylated tau at Thr231 (Thr231; 1:1000) (Calbiochem, Darmstadt, Germany), and Vectastain ABC Elite kit and counterstained by hematoxylin.

Fragmented DNA of apoptotic cells in the brain was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method with the use of DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA) and DAB according to the manufacturer's instructions.

Quantification of Aβ burden by image analysis

For quantification of Aβ burden, immuno-labeling was examined in the entire cerebral cortex and hippocampal areas of Tg2576/$TTR^{+/-}$ and Tg2576/$TTR^{-/-}$ mice. The amyloid burden was calculated by dividing total area of Aβ deposits by total area of region analyzed (in pixels). Images were captured and analyzed with the use of ImagePro®ver6 software (Media Cybernetics, Silver Spring, MD, USA). Four coronal sections from each of the mice were examined. The burden was expressed as mean ± standard error of the mean.

Protein extraction

Frozen right hemi-brains were sequentially extracted using two-step extraction method, as described previously (18). Initially, the frozen brain samples were homogenized in 2% sodium dodecylsulfate (SDS) (150 mg/ml wet weight) with protease inhibitors (complete protease inhibitor cocktail, one tablet in 50-ml solution; Boehringer Mannheim, Mannheim, Germany) followed by centrifugation at 100,000 *g* for 1 h at 4°C. The supernatant was then removed (termed SDS fraction), and the resultant pellet was sonicated [(35 s at level 10; XL-2000 Microson Ultrasonic Cell Disruptor (Misonix Inc., Farmingdale, NY, USA)] in 70% formic acid in water. After sonication, the samples were centrifuged, as described above, and the supernatant was removed (termed FA fraction). Total protein concentration measurement for SDS fraction was carried out with the use of BCA Kit (Pierce, Rockford, IL, USA).

Western blotting analysis

The SDS fractions of brain extracts (30 μg of protein) were electrophoresed on 4–12% gradient Bis-Tris gels (NuPage, Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride membranes (Tefco, Tokyo, Japan). Membranes were labeled with the use of primary antibody, Saeko (1:1000), recognizing C terminal 30 amino acids of both human and mouse APP (18) overnight at 4°C, incubated with horseradish peroxidase-linked anti-rabbit IgG antibody (Amersham Biosciences, Buckingham, UK) (1:2000) for 1 h, and the immunoreactivity was visualized with the use of Supersignal (Pierce, Rockford, IL, USA). Images were captured by Fuji Bas-1000 imaging analyzer (Fujifilm, Tokyo, Japan), and the

intensity of the bands was quantified with the use of Scion Image (Scion Corp., Frederick, MD, USA).

Sandwich enzyme-linked immunosorbent assay

Amyloid-β 40 and Aβ42 in the brain extracts (SDS and FA fractions) were measured by sandwich enzyme-linked immunosorbent assay (ELISA), as described previously (18, 24, 25). Microplates (Immunoplate I, Nunc, Rockilde, Denmark) were pre-coated with anti-Aβ monoclonal antibody BNT77 (IgA isotype specific for Aβ11-16) that recognizes both Aβ40 and Aβ42, then incubated for 24 h at 4°C with 100 μl/well of samples. The microplates were further incubated for 24 h at 4°C with either horseradish-peroxidase-conjugated BA27 (anti-Aβ1-40, specific for Aβ40) or BC-05 (anti-Aβ35-43, specific for Aβ42 and Aβ43). Color was developed with 3,3′,5,5′-tetramethylbenzidine and evaluated at 450 nm on a microplate Reader (Molecular Devices, Menlo Park, CA, USA). The SDS fractions were diluted 400 times in EC buffer [20-mM phosphate buffer, pH 7.0, 400-mM NaCl, 2-mM EDTA, 0.4% Block Ace (Dainipponseiyaku, Suita, Japan), 0.2% bovine serum albumin, 0.05% CHAPS and 0.05% sodium azide] containing 0.005% SDS. The FA fraction was neutralized by a 1:50 dilution into 1-M Tris-HCl, pH 8.0 and then further diluted 20 times in EC buffer. The program Softmax (Molecular Devices, Menlo Park, CA, USA) was used to calculate Aβ concentration (in picomolar) by comparing the sample absorbance with the absorbance of known concentrations of synthetic Aβ42 or Aβ40 standards (Sigma, St Louis, MO, USA) assayed identically on the same plate. Using the wet weight of brain in the original homogenate, the final values of Aβ in brain were expressed as picomoles per gram wet weight.

Statistical analysis

The difference in the Aβ burden between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice was examined with ANOVA followed by the Student's unpaired *t*-test with GraphPad Prism, Version 4.0 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

RESULTS

There is no significant difference in the brain levels of full-length APP between Tg2576/TTR$^{+/-}$ and Tg2576/TTR$^{-/-}$ mice

Amyloid-β peptides are derived from APP. To determine whether or not TTR affected the level of full-length APP, the groups of two Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ littermates were killed at 16, 18 and 20 months of age, and relative levels of full-length APP in the SDS fractions prepared from the brain were determined by western blotting with the use of Saeko, as described under *Methods*. Significant differences were never detected in the levels of full-length APP among any of the Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice examined (Figure 1). Thus, TTR does not affect the level of full-length APP in the brain of Tg2576 mice.

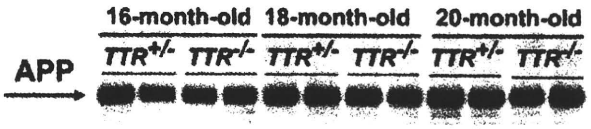

Figure 1. *Western blotting analysis of full-length amyloid precursor protein (APP).* The arrow on the left indicates the location of full-length APP.

Transthyretin deficiency does not increase but decreases the degree of total and vascular Aβ burdens in the brain of Tg2576 mice

Total Aβ burden

To evaluate whether or not TTR affected Aβ deposition, we compared the onset, progression and distribution of amyloid deposition between the brain of Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice, measuring the area occupied by Aβ deposits around the vascular wall of the meninx and cerebral parenchyma (termed cerebral amyloid angiopathy; CAA) and inside the brain parenchyma (termed Aβ plaque), as described under *Methods*. A time-course analysis of the total Aβ deposition in the brain was performed by assessing mice of ages 7–20 months. The number and age of mice examined were shown in Table 1. Aβ deposits were not detected in any of the six 7–11-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice examined. A small amount of Aβ deposits was first observed at 12 months of age in both the mice (data not shown). With advancing age, total Aβ burden increased (Figure 2A), and Aβ deposits were observed in the cerebral cortex, neocortex and hippocampus (Figure 3A), but not in the cerebellum (data not shown) in both the mice. Although there was a trend to reduction of total Aβ burden in 12–17-month-old Tg2576/*TTR*$^{-/-}$ mice relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice, there was no statistically significant difference in the onset, progression and distribution of total Aβ deposition in the entire cerebral cortex between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice (Figure 2A). The size of Aβ deposits in Tg2576/*TTR*$^{-/-}$ mice was also much the same as that in the age-matched Tg2576/*TTR*$^{+/-}$ mice. In 18–20-month-old Tg2576/*TTR*$^{-/-}$

Table 1. The number and age of mice examined by immunohistochemistry. Abbreviation: n = number of mice.

Age (months)	Tg2576/*TTR*$^{+/-}$ (n)	Tg2576/*TTR*$^{-/-}$ (n)
7	2	2
8	2	2
11	2	2
12	2	2
13	5	5
14	6	6
15	6	6
16	6	6
17	3	3
18	6	6
20	2	2
Total	42	42

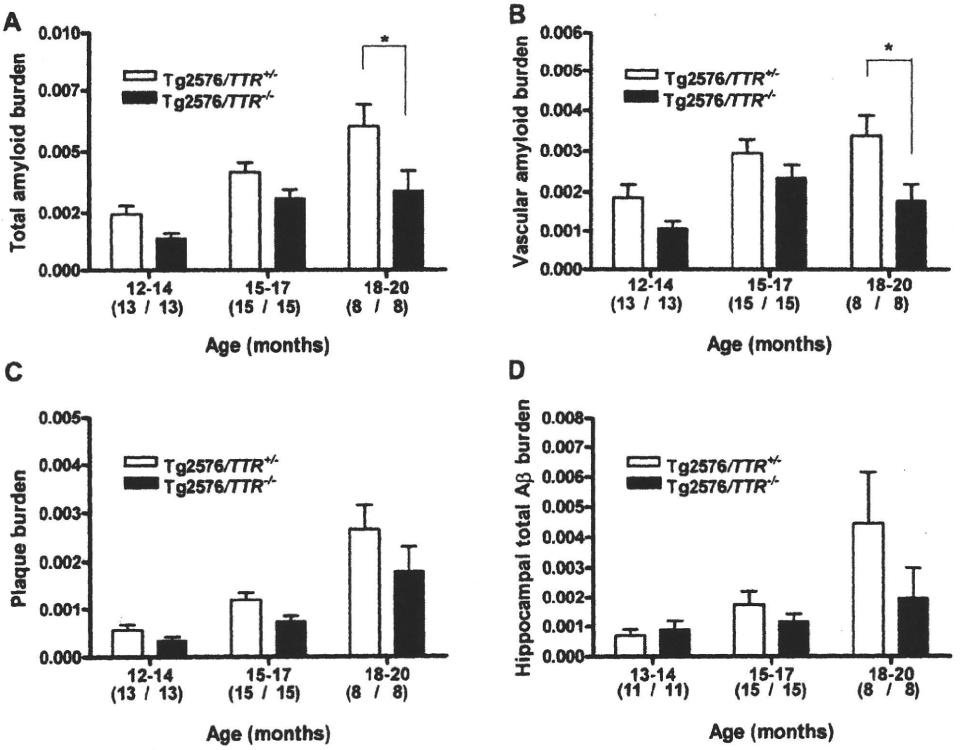

Figure 2. *The Aβ burden in the brain of Tg2576/TTR$^{+/-}$ and Tg2576/TTR$^{-/-}$ mice.* The total Aβ burden (vascular amyloid and plaques) (**A**) vascular Aβ burden (**B**) and Aβ plaque burden (**C**) in the entire cerebral cortex were calculated by dividing total area of Aβ deposits by total area of analyzed cortex. The hippocampal total Aβ burden (**D**) was calculated by dividing area of total Aβ deposits (vascular amyloid and plaques) by area of analyzed hippocampus. All data are expressed as mean ± standard error of the mean. Numbers in parentheses denote numbers of mice examined. *$P < 0.05$. TTR = transthyretin.

mice, however, total Aβ burden was significantly reduced relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.05$) (Figure 2A). Thus, contrary to our expectations, total Aβ burden is not increased, but rather decreased by eliminating TTR in Tg2576 mice.

Vascular Aβ burden

It had been reported that Tg2576 mice developed abundant vascular amyloid while aging, especially in leptomeningeal vessels (31). In order to determine whether the onset and degree of particular form of Aβ deposition were affected by TTR, we separately assessed vascular amyloid and plaque burdens in the brain of Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice, as described under *Methods*.

A time-course analysis of vascular Aβ burden was performed by assessing the mice of ages 7–20 months. A few vascular Aβ deposits were first observed at 12 months of age in both Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice. With advancing age, total vascular Aβ burden increased in both the mice (Figure 2B). Vascular Aβ deposits were detected only in the wall of leptomeningeal vessels of 12–16-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice, while in the 17–20-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice, the deposits were detected in the vascular wall of cerebral parenchyma as well as the wall of leptomeningeal vessels (data not shown). There was no significant difference in the onset, progression and distribution of vascular Aβ deposition in the entire cerebral cortex between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice up to 17 months of age. However, a significant reduction in vascular Aβ burden by 47.1% was found in 18–20-month-old Tg2576/*TTR*$^{-/-}$ mice relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.05$) (Figure 2B). These findings suggested that TTR does not decrease but rather increases the degree of vascular Aβ burden in Tg2576 mice.

Amyloid-β plaque burden

Aβ plaques were first detected in both Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice at 12 months of age, and both the size and number of the plaques increased with advancing age (Figure 2C). Although there was a trend to reduction of total Aβ plaque burden in 12–20-month-old Tg2576/*TTR*$^{-/-}$ mice relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice, there was no statistically significant difference in the onset, degree and distribution of Aβ plaque deposition between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice (Figure 2C). These findings suggested that TTR does not decrease Aβ plaque burden in the brain of Tg2576 mice.

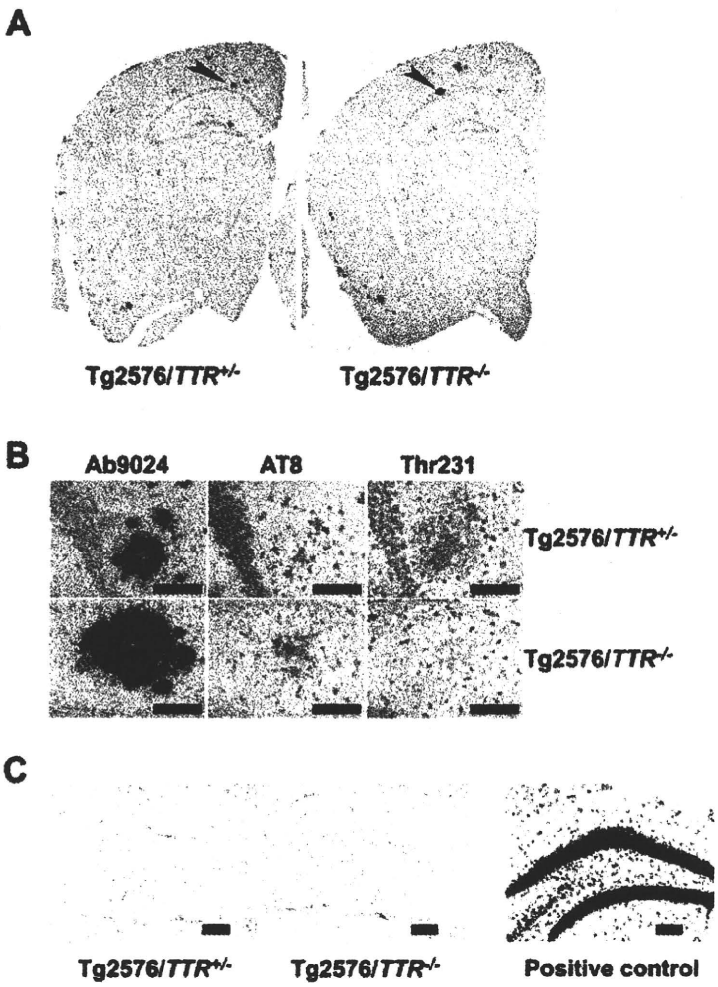

Figure 3. *Immunohistochemistry of Tg2576/TTR$^{+/-}$ and Tg2576/TTR$^{-/-}$ brains.* Immuno-labeling of left hemi-brain sections of 18-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice with Ab9204. **A**. The higher magnification of the hippocampal Aβ plaque with giant cores indicated by an arrowhead in **A** (**B**, *left panels*). Serial sections (5 μm) were labeled with AT8, and anti-phosphorylated tau (Thr231). AT8 and Thr-231 labeled punctate dystrophic neurites in and around Aβ plaques (**B**, *middle and right panels, respectively*). Scale bar; 50 μm. The hippocampal dentate gyrus areas of 18-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice stained with transferase-mediated dUTP nick end labeling. **C**. No apoptotic cells were found in the hippocampus. A DNaseI-treated sample was stained in parallel with the samples as a positive control. Scale bar; 100 μm. TTR = transthyretin.

Transthyretin deficiency does not affect Aβ deposition in the hippocampus of Tg2576 mice

The hippocampus is highly susceptible area to Aβ deposition in both humans (5) and Tg2576 mice (15). To investigate the effect of TTR deficiency on Aβ deposition in the hippocampus, we measured the total Aβ burden in the hippocampus of Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice. The Aβ deposits were first detected in the hippocampus of both the mice at 13 months of age, and showed an age-related increase (Figure 2D). Although the total Aβ burden in Tg2576/*TTR*$^{+/-}$ mice was consistently greater than that in Tg2576/*TTR*$^{-/-}$ mice, the difference was not statistically significant. Thus, the TTR deficiency does not affect Aβ deposition in the hippocampus of Tg2576 mice.

Transthyretin deficiency does not increase but decreases the level of Aβ40 in the brain of Tg2576 mice

Different forms of Aβ, biochemically distinguishable by their solubility properties, are present in varying amounts during the lifetime of Tg2576 mice. Detergent-soluble Aβ (SDS fraction) is present throughout life; however, detergent-insoluble Aβ (FA fraction) is absent up to age 6 months (18). It had been reported in AD that the predominant Aβ peptide present in CAA is Aβ40; however, in brain parenchymal plaques, it is Aβ42 (1, 7, 17, 29, 44). To evaluate whether or not TTR affects the level of different forms of Aβ, we quantified the Aβ40 and Aβ42 in SDS and FA fractions of brain homogenates from Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice by sandwich ELISA, as described under *Methods*. The number and age of 13–20-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice examined were shown in Table 2. Aβ40 and Aβ42 levels in SDS and FA fractions increased with age in both the mice. There was no significant difference in the levels of Aβ40 and Aβ42 in both the fractions between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice up to 17 months of age. In 18–20-month-old Tg2576/*TTR*$^{-/-}$ mice, however, the levels of Aβ40 in both SDS and FA fractions were significantly reduced by 35.2% and by 41.6%, respectively, relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.05$) (Figure 4A,B). The level of Aβ42 in SDS fraction was also significantly reduced by 57.8% in 18–20-month-old

Table 2. The number and age of mice examined by sandwich enzyme-linked immunosorbent assay. Abbreviation: n = number of mice.

Age (months)	Tg2576/*TTR*$^{+/-}$ (n)	Tg2576/*TTR*$^{-/-}$ (n)
13	2	2
14	3	3
15	2	2
16	3	3
17	2	2
18	5	5
20	2	2
Total	19	19

Tg2576/*TTR*$^{-/-}$ mice relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.01$) (Figure 4C). On the other hand, there was no significant difference in the levels of Aβ42 in FA fraction between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice (Figure 4D). The mean level of Aβ42 in FA fraction is much higher than that in SDS fraction. Thus, there was no significant difference in the sum of Aβ42 levels in both the fractions between aged Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice. Thus, TTR deficiency does not increase but rather decreases the level of Aβ40 in the brain of aged Tg2576 mice, a result, which is in good agreement with the immunohistochemistry data, suggesting that TTR increases the vascular Aβ burdens in the brain of aged mice (Figure 2).

Transthyretin deficiency does not affect the distribution and degree of tau phosphorylation in the brain of Tg2576 mice

In contrast to human AD, Tg2576 mice lack NFT, and develop the phosphorylated tau-immunoreactive aberrant structures that are exclusively associated with congophilic Aβ plaques (27, 48, 49). Stein *et al* reported that chronic infusion of an antibody against TTR into the hippocampus of Tg2576 led to an increase of tau phosphorylation within the CA1 neuronal field (42). To investigate whether or not TTR deficiency affected the distribution and degree of tau phosphorylation, the brain slices of 16–20-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice were stained with either

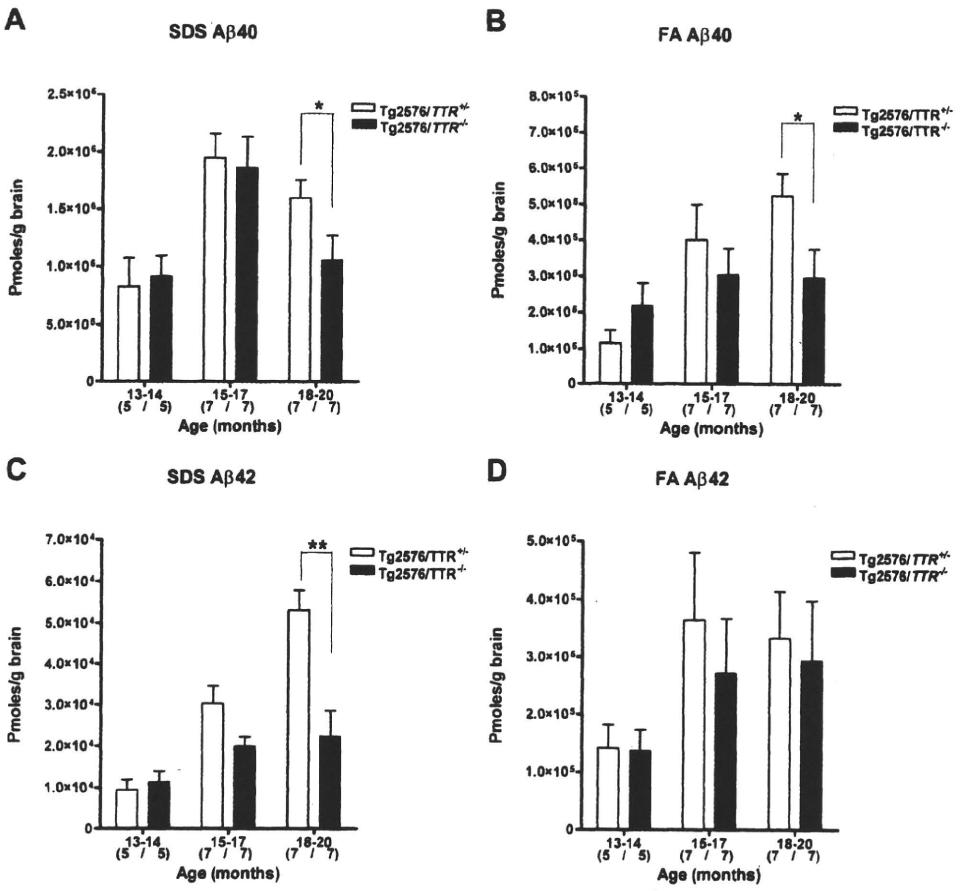

Figure 4. *The Aβ level in the brain of Tg2576/TTR*$^{+/-}$ *and Tg2576/TTR*$^{-/-}$ *mice.* The Aβ40 (**A,B**) and Aβ42 (**C,D**) in Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ brains were quantified by sandwich enzyme-linked immunosorbent assay. The samples were sequentially extracted in 2% sodium dodecylsulfate (SDS) (**A,C**) and 70% FA (**B,D**). All data are expressed as mean ± standard error of the mean. Numbers in parentheses denote numbers of mice examined. $*P < 0.05$, $**P < 0.01$. TTR = transthyretin.

AT8 or Thr231 antibody, as described under *Methods*. Both the antibodies reacted only with the punctate dystrophic neurites (DNs) within the Aβ plaques in hippocampus and cerebral cortex in both the mice (Figure 3B). The abundance of the DNs immunopositive with the antibodies in Tg2576/*TTR*$^{-/-}$ mice was much the same as that in Tg2576/*TTR*$^{+/-}$ mice (Figure 3B). No NFT was detected in any of the mice examined. Thus, TTR deficiency does not affect tau phosphorylation in the brain of Tg2576 mice.

No apoptotic cells are detected in the hippocampus of Tg2576/TTR$^{+/-}$ and Tg2576/TTR$^{-/-}$ mice

Tg2576 mice do not develop severe neuronal loss observed in AD (15). Stein and Johnson suggested that high level of TTR in the hippocampus of Tg2576 mice might protect the mice from severe neuronal loss (43). Furthermore, the same group reported that chronic infusion of an antibody against TTR into the hippocampus of Tg2576 mice led to an increase of neuronal loss and apoptosis within the CA1 neuronal field (42). To determine whether or not TTR deficiency induces apoptosis in the hippocampus of Tg2576 mice, the brain sections from 18–20-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice were subjected to TUNEL immunohistochemistry, as described under *Methods*. Apoptotic cells were never detected in the hippocampus or other parts of brain of any of the mice examined (Figure 3C). These results indicate that TTR deficiency does not induce apoptosis in the brain of Tg2576 mice.

DISCUSSION

To investigate the role of TTR in the Aβ deposition *in vivo*, we generated a mouse line carrying a null mutation at the endogenous *TTR* locus and the human mutant APP cDNA with the Swedish mutation (Tg2576/*TTR*$^{-/-}$ mouse) by crossing Tg2576 mice with TTR-deficient mice generated through gene targeting. We then asked whether Aβ deposition was accelerated in Tg2576/*TTR*$^{-/-}$ mice relative to the heterozygous mutant Tg2576 (Tg2576/*TTR*$^{+/-}$) mice. Contrary to our expectations, the degree of total Aβ deposition, tau phosphorylation and apoptosis in the brain was not increased by eliminating TTR in Tg2576 mice. Moreover, the degree of vascular Aβ burden in the aged Tg2576/*TTR*$^{-/-}$ mice was significantly reduced relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice. Our experiments present, for the first time, compelling evidence that TTR does not suppress but rather accelerates vascular Aβ deposition in the mouse model of AD.

We confirmed that there was no significant difference in the onset, progression and distribution of total Aβ deposition between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice up to age 17 months by immunohistochemistry (Figure 2A). However, total Aβ burden in 18–20-month-old Tg2576/*TTR*$^{-/-}$ mice was significantly reduced relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.05$) (Figure 2A). The result suggested that TTR does not suppress but rather accelerates Aβ deposition in the brain of Tg2576 mice. Although both Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice are smaller than non-transgenic littermates, both of them display no obvious phenotypic abnormalities, and their fertility is normal up to age 10 months. This observation is consistent with the immunohistochemistry data.

We then separately assessed vascular amyloid and plaque burdens in the brain of Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice. Although Aβ plaque burden was much the same between 7–20-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice (Figure 2C), vascular amyloid burden in the aged (18–20-month-old) Tg2576/*TTR*$^{-/-}$ mice was significantly reduced relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.05$) (Figure 2B). The quantification of Aβ40 and Aβ42 in the brain homogenates from Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice by sandwich ELISA demonstrated that TTR deficiency does not increase, but rather decreases the level of Aβ40 in the aged Tg2576 mice (Figure 4). Because the predominant Aβ peptide present in vascular amyloid deposits is reportedly Aβ40 (1, 7, 44), the result is also in good agreement with our immunohistochemistry data (Figure 2), suggesting that TTR increases the vascular Aβ burden in the brain of aged Tg2576 mice.

The reason why vascular amyloid burden is increased by TTR is not clear. Amyloid deposits of all types, including Aβ deposits, contain glycosaminoglycans (GAGs) and serum amyloid P component (SAP). A role for GAGs in amyloidosis is inferred from the observation that small molecules that interfere with GAG/amyloid interactions reduce murine experimental amyloid A (AA) amyloid progression (19). An amyloid-binding protein SAP protects amyloid fibrils from proteolysis *in vitro* (46), and the induction of AA amyloidosis is significantly retarded in the SAP-deficient mice relative to wild-type mice (4, 47). On the other hand, recent evidence indicates that Aβ is mainly cleared out of the brain to blood via transport through the blood-brain barrier, and via the interstitial fluid (ISF) bulk flow along periarterial drainage pathways into the CSF, and from there into the blood (26, 33, 52, 56). It is the CSF and perhaps the ISF and not the brain parenchyma (41) that is enriched in TTR. Thus we think it likely that when Aβ drains from the brain parenchyma along periarterial drainage pathways, it may come into contact with TTR which may protect Aβ deposits from proteolysis like GAG and SAP, thereby, slightly increases vascular amyloid burden over the ages.

Schwarzman *et al* reported that TTR in the CSF binds Aβ, and prevents Aβ fibril formation *in vitro*. They, however, also reported that apoE prevents Aβ fibril formation too (36, 37). It has been well established that apoE promotes assembly of Aβ fibril (23, 32). Thus, TTR may promote the fibrillization of Aβ too. Moreover, Holtzman *et al* found that a transgenic mouse model of AD on an apoE$^{-/-}$ background had significantly reduced Aβ deposition relative to the same mouse model expressing wild-type murine apoE (apoE$^{+/+}$), human apoE3 (apoE3$^{+/-}$) or human apoE4 (apoE4$^{+/-}$) (12). Therefore, TTR null Tg2576 (Tg2576/*TTR*$^{-/-}$) mice may represent mice that are unable to form Aβ fibrils, and the Aβ detected in the brain of the mice could be due in part to apoE.

Stein *et al* reported that chronic infusion of anti-TTR antibody into the hippocampus of Tg2576 mice increased Aβ burden, and led to tau hyperphosphorylation, neuronal loss and apoptosis in the CA1 neuronal field (42). These observations suggest the importance of TTR in inhibition of Aβ fibril formation and toxicity. However, contrary to these reports, our experiments suggested that TTR does not suppress but rather enhances Aβ deposition in Tg2576 mice. The reason for the discrepancy between data of other authors and our data is not clear. TTR is complexed with retinol-binding protein (RBP) and thyroid hormone *in vivo*. In the *in vitro* Aβ aggregation assay, however, recombinant TTR alone, not complexed with RBP or thyroid hormone, was used to examine its ability to inhibit Aβ fibril formation (36, 37). Association of TTR with RBP and thyroid hormone may affect its binding capacity