

Age-Dependent Changes in Brain, CSF, and Plasma Amyloid β Protein in the Tg2576 Transgenic Mouse Model of Alzheimer's Disease

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The accumulation of amyloid β protein ($A\beta$) in the Tg2576 mouse model of Alzheimer's disease (AD) was evaluated by ELISA, immunoblotting, and immunocytochemistry. Changes in $A\beta$ begin at 6–7 months as SDS-insoluble forms of $A\beta_{42}$ and $A\beta_{40}$ that require formic acid for solubilization appear. From 6 to 10 months, these insoluble forms increase exponentially. As insoluble $A\beta$ appears, SDS-soluble $A\beta$ decreases slightly, suggesting that it may be converting to an insoluble form. Our data indicate that it is full-length unmodified $A\beta$ that accumulates initially in Tg2576 brain. SDS-resistant $A\beta$ oligomers and most $A\beta$ species that are N-terminally truncated or modified develop only in older Tg2576 mice, in which they are present at levels far lower than in human AD brain. Between 6 and 10 months, when SDS-insoluble $A\beta_{42}$ and $A\beta_{40}$ are easily detected in every

animal, histopathology is minimal because only isolated $A\beta$ cores can be identified. By 12 months, diffuse plaques are evident. From 12 to 23 months, diffuse plaques, neuritic plaques with amyloid cores, and biochemically extracted $A\beta_{42}$ and $A\beta_{40}$ increase to levels like those observed in AD brains. Coincident with the marked deposition of $A\beta$ in brain, there is a decrease in CSF $A\beta$ and a substantial, highly significant decrease in plasma $A\beta$. If a similar decline occurs in human plasma, it is possible that measurement of plasma $A\beta$ may be useful as a premorbid biomarker for AD.

Key words: Alzheimer's disease; neurodegeneration; Tg2576 transgenic animal model; amyloid β protein; cerebrospinal fluid; plasma

Amyloid β protein ($A\beta$), the principal protein in the senile plaques of Alzheimer's disease (AD), is an ~4 kDa secreted polypeptide that is derived from several isoforms of a large protein referred to as the amyloid β protein precursor (β APP) (Glenner and Wong, 1984; Masters et al., 1985; Kang et al., 1987). Secreted $A\beta$ is readily detected in CSF, plasma, and in medium conditioned by a wide variety of cultured cells (Cai et al., 1993; Citron et al., 1994, 1997; Suzuki et al., 1994; Scheuner et al., 1996; Younkin et al., 1998). In each situation, most secreted $A\beta$ is $A\beta_{1-40}$, but a small percentage (5–15%) is $A\beta_{1-42}$. $A\beta_{1-42}$ is especially important in AD. Synthetic $A\beta_{1-42}$ forms amyloid fibrils *in vitro* much more readily than $A\beta_{1-40}$ (Jarrett et al., 1993), and there is good evidence that $A\beta_{1-42}$ is deposited early and selectively in senile plaques (Iwatsubo et al., 1994).

Early onset Alzheimer's disease can be caused by mutations in the *APP* (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991; Mullan et al., 1992), presenilin 1 (*PS1*) (Sherrington et al., 1995), and presenilin 2 (*PS2*) (Levy-Lahad et al., 1995) genes. Studies of human plasma, human fibroblasts, transfected

cells, and transgenic mice have shown that each of these genetic forms of AD either selectively increases the extracellular concentration of $A\beta_{42}$ (Suzuki et al., 1994; Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996; Citron et al., 1997) or increases both $A\beta_{42}$ and $A\beta_{40}$ (Cai et al., 1993; Citron et al., 1994; Scheuner et al., 1996). Thus, in all of these genetic forms of AD, $A\beta$ metabolism is altered in a way that fosters $A\beta$ aggregation and deposition.

The Tg2576 mouse model of Alzheimer's disease (Hsiao et al., 1996) expresses the Swedish mutation of APP ($APP_{K670N,M671L}$) at high level under control of the hamster prion protein (PrP) promoter. It is well established that this mutation causes concomitant increases in secreted $A\beta_{42}$ and $A\beta_{40}$ (Cai et al., 1993; Citron et al., 1994; Scheuner et al., 1996). As Tg2576 mice age, classic neuritic plaques with Congo red-positive amyloid cores appear that are similar to those seen in Alzheimer's disease (Irizarry et al., 1997). In addition, Tg2576 mice develop age-dependent behavioral deficits as assessed by Y maze, T maze, and Morris water maze testing (Hsiao et al., 1996; Chapman et al., 1999; Westerman et al., 2000).

To exploit the Tg2576 model of AD, it is essential to obtain baseline information on the amount and rate at which various forms of $A\beta$ are deposited in the Tg2576 model compared with human AD. In this study, we obtain this information using an analytic paradigm that combines sandwich ELISAs, immunoblots, and immunocytochemistry based on antibodies to specific domains in the various forms of $A\beta$.

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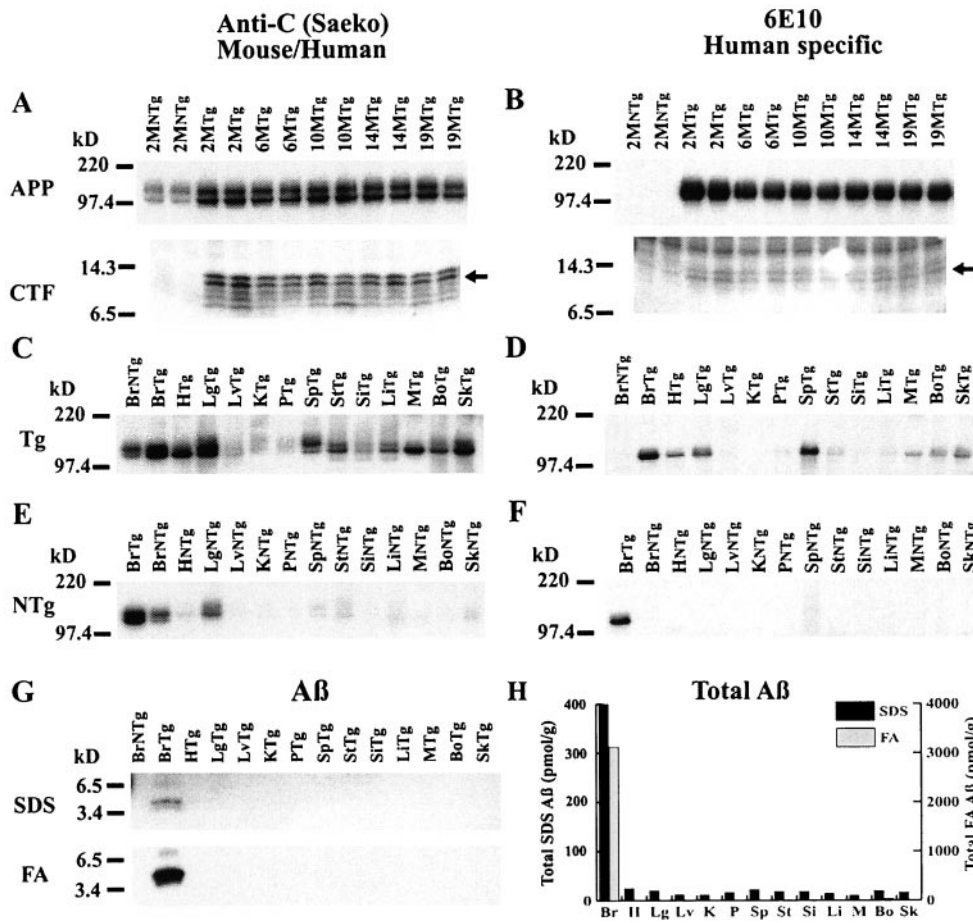


Figure 1. Expression of β APP, CTF, and A β in brain and systemic organs of Tg2576 and nontransgenic mice. Immunoblots in *A–F* were labeled with anti-C (Saeko) (*A, C, E*), which detects both human and mouse β APP, or with 6E10 (*B, D, F*), which specifically detects human β APP. *A, B*, Immunoblots of β APP and CTF in transgenic and nontransgenic mice of various ages (months). Immunoblots were prepared from 16% Tricine gels. *C–F*, Immunoblots of SDS extracts from systemic organs of a 10.9 month Tg2576 mouse (*C, D*) and a 9.3 month nontransgenic mouse (*E, F*). Immunoblots were prepared from 10–20% Tricine gels loaded at 20 mg/lane total protein. *Tg*, Transgenic; *Ntg*, nontransgenic; *Br*, brain; *H*, heart; *Lg*, lung; *Lv*, liver; *K*, kidney; *P*, pancreas; *Sp*, spleen; *St*, stomach; *Si*, small intestine; *Li*, large intestine; *M*, muscle; *Bo*, bone; *Sk*, skin. *G*, Immunoblot of A β in SDS and FA extracts of systemic organs. The A β in SDS extracts (40 μ l) was analyzed by 4G8 immunoprecipitation followed by immunoblotting with 4G8; FA acid extracts (40 μ l), dried and resuspended, were also analyzed by immunoblotting with 4G8. *H*, Total A β (A β 42 plus A β 40) in SDS and FA extracts of systemic organs. A β 42 and A β 40 were analyzed by 3160/BC-05 and 3160/BA-27 ELISAs, respectively.

MATERIALS AND METHODS

Transgenic mice and extraction. Transgenic mice and nontransgenic littermates, bred by mating Tg2576 males with C57B6/SJL F1 females, were killed at 1–25 months (M). Plasma was collected in 0.1% EDTA, and CSF was obtained according to the method of Carp et al. (1971). One hemisphere was frozen in liquid nitrogen, and the other hemisphere was fixed in 4% paraformaldehyde with 0.1 M phosphate buffer, pH 7.6. Plasma, CSF, and frozen brains were stored at -80°C . Frozen hemibrains were sequentially extracted. At each step, sonication (35 sec at level 10; XL-2000 Microson Ultrasonic Cell Disruptor; Misonix Inc., Farmingdale, NY) in an appropriate buffer was followed by centrifugation at $100,000 \times g$ for 1 hr at 4°C . The supernatant was then removed, and the pellet was sonicated in the next solution used in the sequential extraction process. For four-step extraction, sonication of the frozen brain (150 mg/ml wet weight) began in Tris-buffered saline (TBS) (20 mM Tris and 137 mM NaCl, pH 7.6), which contained protease inhibitors (complete protease inhibitor cocktail, 1 tablet in 50 ml solution; Boehringer Mannheim, Mannheim, Germany). The next three sequential extraction steps used 1% Triton X-100 in TBS with protease inhibitors, 2% SDS in water with the same protease inhibitors, and 70% formic acid (FA) in water. For two-step extraction, the initial sonication of brain (150 mg/ml wet weight) took place in 2% SDS with protease inhibitors, and the resultant pellet was then extracted with 70% formic acid in water.

Antibodies. The following antibodies to A β were used: monoclonal, BAN-50 (anti-A β 1–16), BA-27 (anti-A β 1–40), BC-05 (anti-A β 35–43), BNT-77 (anti-A β 11–28), 4G8 (anti-A β 17–24), and 6E10 (anti-A β 1–16); polyclonal, 3160 (anti-A β 1–40), Saeko (anti-C-terminal 30 amino acids of APP) (Kawarabayashi et al., 1996), and five antibodies described by Saido et al. (1995, 1996), which specifically detect N termini of A β , anti-A β N1(D), for the unmodified A β N terminus (N1(D)); anti-L-iso-Asp for isomerized forms of A β N1 (N1(iD)); anti-rectus Asp for stereoisomerized forms of A β N1 (N1(rD)); anti-A β N3-pyroglytamate (N3(pE)); and anti-A β N11-pyroglytamate (N11(pE)).

Sandwich ELISA for A β . Brain extracts were measured by sandwich ELISA as described previously (Suzuki et al., 1994; Gravina et al., 1995).

The following systems were used: (1) BAN-50 capture and BC-05 or BA-27 detection or (2) 3160 capture and BC-05 or BA-27 detection, both of which detect A β 1–42 and A β 1–40, respectively, and (3) BC-05 or BA-27 capture and 4G8 detection, which detect A β x-42 and A β x-40, respectively. Direct comparison of many Tg2576 brains from mice of all ages showed that the amounts of A β 42 and A β 40 detected with 3160 capture ELISAs were essentially the same as when BAN-50 was used for capture. For measurement of plasma and CSF A β , BNT-77 capture and BC-05 detection was used for A β 42, and BAN-50 capture and BA-27 detection was used for A β 40.

The 2% SDS extracts were diluted at least 1:40 so that A β capture took place in EC buffer [0.02 M phosphate buffer, pH 7, 0.4 M NaCl, 2 mM EDTA, 0.4% Block Ace (Dainipponseiyaku, Suita, Osaka, Japan), 0.2% bovine serum albumin, 0.05% CHAPS and 0.05% sodium azide] containing 0.05% SDS. The TBS (at least 1:10) and Triton X-100 (at least 1:20) extracts were also diluted so that A β capture took place in EC buffer containing 0.05% SDS. Formic acid extracts were neutralized initially by 1:20 dilution into 1 M Tris phosphate buffer, pH 11, and then diluted as necessary in EC buffer. The program Softmax (Molecular Devices, Menlo Park, CA) was used to calculate A β concentration (in picomolar) by comparing the sample absorbance with the absorbance of known concentrations of synthetic A β 1–42 or A β 1–40 standards assayed identically on the same plate. Using the wet weight of brain in the original homogenate, the final values of A β in brain were expressed as picomoles per gram wet weight. Nontransgenic tissues were processed identically in parallel with the transgenic tissues.

Immunoblots. To detect SDS-soluble A β around the critical period (4M–10M) when deposition begins, SDS fractions were immunoprecipitated with 3160 by diluting 40 μ l of each extract 40-fold with RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% cholic acid, 0.1% SDS, and 50 mM Tris, pH 8) containing protease inhibitors and immunoprecipitating with protein G-agarose that had been incubated with 1 μ l of 3160. To detect SDS-soluble A β in 21M transgenic and AD brain, SDS fractions were directly applied to the gel. The formic acid fractions were evaporated using a Speed-Vac concentrator (Savant, Holbrook, NY), and dissolved

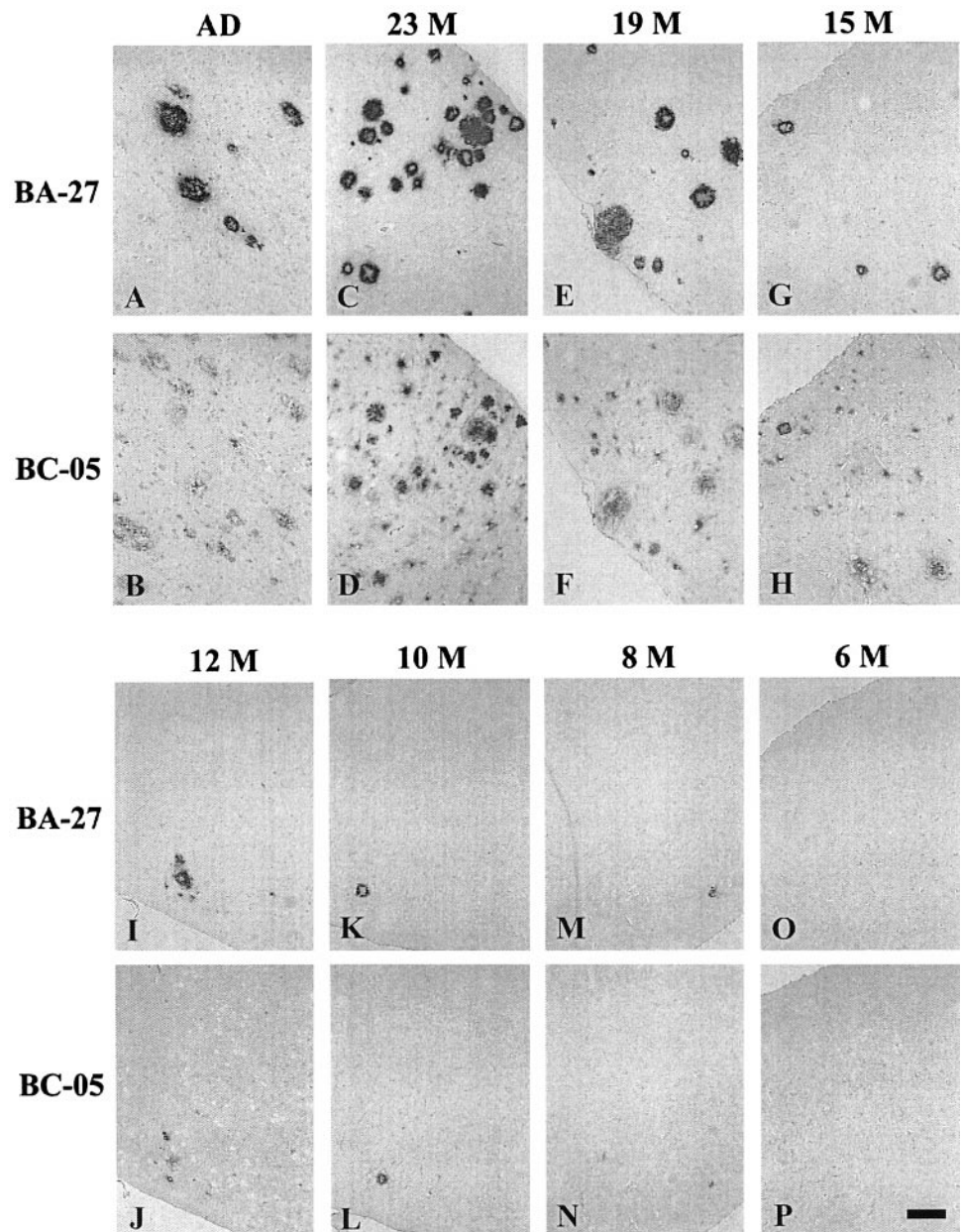


Figure 2. Immunohistochemistry of AD (*A, B*) and aging Tg2576 (*C–P*) brains. Serial sections of temporal cortex from AD brain and Tg2576 brains were labeled with BA-27, which is specific for A β 40, or BC-05, which is specific for A β 42. The age (months) of the Tg2576 brains is shown above the serial sections stained with BA-27 (*top panel*) and BC-05 (*bottom panel*). A β 40 (stained by BA-27) and A β 42 (stained by BC-05) are detected as dense microdeposits from 8 (*M, N*) to 12 (*I, J*) months. From 15 to 23 months (*C–H*), A β deposits increase in number and size and are detected both as cored plaques labeled by both BC-05 and BA-27 and as diffuse plaques, which are selectively labeled by BC-05. Sections are 5- μ m-thick. Scale bar, 15 μ m.

in dimethyl sulfoxide. SDS and FA fractions were separated on 10–20 or 16% Tricine SDS gels (Novex, Wadsworth, OH) and electrotransferred to Immobilon P (Millipore, Bedford, MA) at 100 V for 1.5 hr. Membranes were labeled with primary antibody (BAN50 or 4G8) overnight at 4°C, incubated with horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Arlington Heights, IL) for 1 hr, and detected using Supersignal (Pierce, Rockford, IL). To detect full-length β APP and C-terminal fragments, SDS fractions by two-step extraction were separated on 10% Tricine SDS gels and detected with Saeko anti-C-terminal antibody or 6E10.

Immunocytochemistry. Tissue samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.6, for 8 hr at 4°C. Paraffin sections (5 μ m) were pretreated with 70% formic acid for 5 min and immersed in 0.5% periodic acid for 10 min to block intrinsic peroxidase. They were then incubated with 1.5% blocking serum in PBS for 1 hr, with primary antibodies (BC-05, 0.1 μ g/ml; BA-27, 0.4 μ g/ml; or antibodies to specific N termini of A β , 2.5 μ g/ml) overnight, and with horseradish peroxidase-conjugated secondary antibody (1:100; Dako, High Wycombe, UK) for 1 hr. Immunoreactivity was visualized by incubation with 0.03% 3,3'-diaminobenzidine, 0.065% sodium azide, and 0.02% H₂O₂. To stain anti-A β N1(D), anti-N1(iD), and anti-N1(rD), PBS containing 500 nmol/l NaCl was used to prevent cross-reaction. Methyl green was used for nuclear staining. Sections from seven AD brains were stained in parallel.

RESULTS

Human APP expression in Tg2576 mice is not confined to the brain

SDS extracts from Tg2576 brain were analyzed on immunoblots labeled with anti-C (Saeko), a rabbit polyclonal antibody that recognizes the C terminus of both human and mouse APP (Fig. 1*A*), or with 6E10, a mouse monoclonal antibody specific for human APP (Fig. 1*B*). The APP holoprotein and its 8–14 kDa C-terminal fragments (CTFs) were elevated in the brains of Tg2576 mice as expected and showed no increase in older mice (Fig. 1*A, B*) as reported previously (Hsiao et al., 1996). SDS extracts of other organs from Tg2576 mice (10.9M) and their nontransgenic littermates (9.3M) were analyzed similarly (Fig. 1*C–F*). In nontransgenic mice, the level of endogenous mouse APP holoprotein was highest in brain and lung (Fig. 1*E*), and none of the endogenous proteins in any mouse organ cross-reacted appreciably with the human-specific 6E10 antibody (Fig. 1*F*). In Tg2576 transgenic mice, human APP was present at high

Table 1. Four-step extraction of A β in nontransgenic and Tg2576 transgenic mouse brain

Tg/ NTg	Age (months)	<i>n</i>	A β	TBS	1% Triton-100	2% SDS	70% FA	Total	Total A β (pmol/gm)
NTg	5	3	A β 42	ND	ND	1.3 \pm 0.1 (22)	ND	1.3 (22)	5.9
			A β 40	ND	ND	4.6 \pm 0.2 (78)	ND	4.6 (78)	
Tg	5	5	A β 42	0.4 \pm 0.1 (1)	3.0 \pm 0.5 (7)	6.4 \pm 0.6 (15)	ND	9.8 (23)	42.9
			A β 40	2.1 \pm 0.2 (5)	10.4 \pm 0.9 (24)	20.6 \pm 2.5 (48)	ND	33.1 (77)	
Tg	6	5	A β 42	0.3 \pm 0.1 (0.7)	2.9 \pm 0.3 (7)	8.7 \pm 1.6 (20)	ND	11.9 (27)	43.4
			A β 40	1.7 \pm 0.4 (4)	9.3 \pm 2.0 (21)	20.5 \pm 1.8 (47)	ND	31.5 (73)	
Tg	7	5	A β 42	0.2 \pm 0.0 (0.4)	2.3 \pm 1.4 (4)	11.6 \pm 3.5 (22)	0.9 \pm 0.2 (2)	15.0 (28)	52.9
			A β 40	1.3 \pm 0.1 (2)	8.0 \pm 0.7 (15)	25.5 \pm 1.4 (48)	3.1 \pm 0.8 (6)	37.9 (72)	
Tg	21	3	A β 42	5.4 \pm 1.3 (0.03)	34.8 \pm 6.6 (0.2)	2042.2 \pm 102.1 (10)	482.4 \pm 86.9 (2.4)	2564.8 (13)	19937.1
			A β 40	7.1 \pm 1.4 (0.04)	89.6 \pm 11.8 (0.4)	11,155.6 \pm 624.0 (56)	6120.0 \pm 374.0 (31)	17,372.3 (87)	

Numbers in parentheses show the percent of total A β in the fraction. ND, Not detectable.

level not only in brain but also in spleen and lung (Fig. 1D). Moderate levels of transgenic human APP were present in Tg2576 heart, skin, bone, and muscle, and there was some human APP in pancreas, stomach, and large intestine (Fig. 1D). Thus, transgenic APP expression is not confined to the brain in the Tg2576 mouse model of AD. The A β in various organs of 10.9M Tg2576 mice was analyzed by immunoprecipitation followed by immunoblotting. With this approach, A β was detected only in the brain (Fig. 1G). The more sensitive sandwich ELISA assays (Fig. 1H) were able to detect small amounts of SDS-extractable A β in all systemic organs (10–24 pmol/gm), but the amount of SDS-extractable A β in the brain was much larger (400 pmol/gm). Analysis of SDS-insoluble A β (formic acid extract of the pellet left after SDS extraction) in various organs from 10.9M Tg2576 mice showed that insoluble A β accumulates only in the brain (Fig. 1G,H).

Cored plaques appear early in Tg2576 brain

At 7–8 months, dense cored plaques that contain both A β 40 (BA-27) and A β 42 (BC-05) appeared in the Tg2576 brain (Fig. 2M,N). These early cored plaques were stained by Congo red and showed green birefringence with polarized light (data not shown). They increased between 7 and 10 months but, even at 10 months, only a few cores were present in each section (Fig. 2K,L). At 12–15 months, diffuse plaques appeared that were labeled preferentially by the BC-05 antibody to A β 42 (Fig. 2G–J). Between 15 and 23 months, A β plaques in the Tg2576 brain (Fig. 2C–H) accumulated to levels like those seen in AD brain (Fig. 2A,B) as reported previously (Irizarry et al., 1997). Both meningeal and parenchymal blood vessels in the brain also showed progressive A β accumulation. The A β in blood vessels of the aging Tg2576 brain was preferentially labeled by the BA-27 antibody to A β 40 (data not shown).

Most A β in normal mouse brain requires SDS for extraction

The brains of Tg2576 mice and nontransgenic littermates were extracted sequentially in TBS, 1% Triton X-100, 2% SDS, and 70% FA as described in Materials and Methods. The A β 42 and A β 40 in the supernatants produced by this four-step extraction were analyzed by 3160/BC-05 and 3160/BA-27 ELISAs, respectively (Table 1). In normal nontransgenic and young transgenic mouse brains in which there is no A β deposition, most A β required SDS for solubilization and surprisingly little was ex-

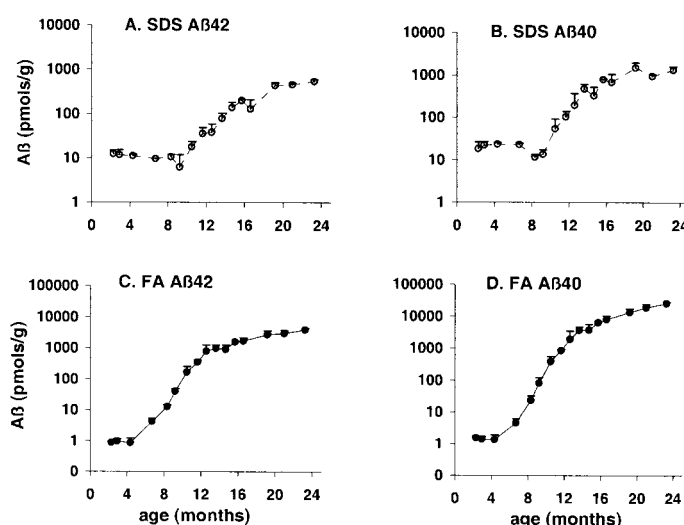


Figure 3. A β in aging Tg2576 brain. A β 42 (A, C) and A β 40 (B, D) were analyzed in Tg2576 brains sequentially extracted in 2% SDS (A, B) and 70% formic acid (C, D). The ELISA assay was 3160/BC05 for A β 42 and 3160/BA27 for A β 40. Note that the y-axes are logarithmic and that there was no detectable A β 40 or A β 42 in the formic acid extract of young (2–5 months) Tg2576 mice.

tracted into TBS or Triton X-100. In 6 month Tg2576 mice, for example, the percentages of total A β extracted into TBS, Triton X-100, and SDS were 5% (0.7% A β 42, 4% A β 40), 28% (7% A β 42, 21% A β 40), and 67% (20% A β 42, 47% A β 40), respectively (Table 1). As expected, there was essentially no insoluble A β in normal mouse brain that required formic acid for solubilization.

A β that requires formic acid for extraction appears at 6–8 months in Tg2576 brain and increases with aging to a level like that seen in AD brain

It is well established that much of the A β deposited as amyloid in AD brain is resistant to SDS extraction and requires formic acid for solubilization (Roher et al., 1993). In the brains of 21 month Tg2576 mice, in which there are numerous amyloid-containing senile plaques (Fig. 2), there also was abundant A β that required formic acid for solubilization (Table 1). To track the time course of formation of this insoluble (formic acid-requiring) A β in

Table 2. Modified and unmodified A β in AD and Tg2576 mouse brain

Brain	<i>n</i>	A β	70% FA (pmol/gm)	Total (pmol/gm)	Modified
Tg2576 21–23 months	5	A β 1-42	3409 \pm 64	26,076	5%
		A β 1-40	22,667 \pm 440		
		A β X-42	1379 \pm 38		
		A β X-40	25,939 \pm 1751		
		A β 1-42	826 \pm 119		
AD Substantial Congo-philic. Angiopathy	6	A β 1-40	5001 \pm 2164	5827	69%
		A β X-42	5089 \pm 409	18,696	
		A β X-40	13,607 \pm 3397		
		A β 1-42	714 \pm 68		
AD Minimal Congo-philic. Angiopathy	9	A β 1-40	32 \pm 7		746
		A β X-42	4663 \pm 421	4879	
		A β X-40	216 \pm 38		

Tg2576 mice, we used a simplified two-step extraction procedure in which brains were first extracted in 2% SDS and then in 70% formic acid. The results of our analysis of the resultant supernatants using 3160/BC-05 (A β 42) and 3160/BA-27 (A β 40) ELISAs are shown in Figure 3. A β 42 and A β 40 first appeared in the FA fraction at \sim 7 months (Fig. 3C,D). By 8–9 months, FA-requiring A β 42 and A β 40 appeared unequivocally in the brain of every Tg2576 mouse examined. Between 6 and 12 months, FA-requiring A β 42 and A β 40 increased exponentially, and both forms continued to increase substantially from 12 to 23 months, reaching levels like those seen in the AD brain (Gravina et al., 1995).

Remarkably, the unequivocal biochemical change that occurred between 6 and 10 months was accompanied by minimal immunocytochemical evidence of A β deposition, although isolated, cored plaques were observed in virtually every section on careful inspection (Fig. 2). Thus, biochemical assessment of brain A β is a sensitive way to quantitate the early AD-like changes that occur in Tg2576 mice.

The A β initially extracted into SDS decreases when FA-requiring A β first appears

The increase in FA-requiring A β that occurred between 6 and 10 months was accompanied by a small decrease in the A β extracted initially into SDS (Fig. 3A,B). Both declines were significant when analyzed by Spearman's rank correlation (A β 42, $p = 0.02$; A β 40, $p = 0.003$). In the four-step extraction (Table 1), in which TBS, Triton X-100, and SDS fractions were obtained before formic acid extraction, it was the TBS and Triton X-100 fractions that significantly decreased as FA-requiring A β appeared (Mann–Whitney comparison of 5M vs 7M; $p = 0.009$ for TBS A β 42, TBS A β 40, and Triton A β 40; $p = 0.05$ for Triton A β 42).

The percentage of A β in SDS and FA extracts of depositing Tg2576 and AD brain is influenced by the extraction procedure

The total amounts of A β 42 and A β 40 extracted by two-step and four-step protocols are virtually identical, although slightly more A β is extracted with the four-step method. In Tg2576 and AD brains, in which there is A β deposition, the relative amounts of A β extracted into SDS and FA with the two methods are considerably different. A much higher percentage of total A β is extracted into SDS in the four-step method. The relevant percent-

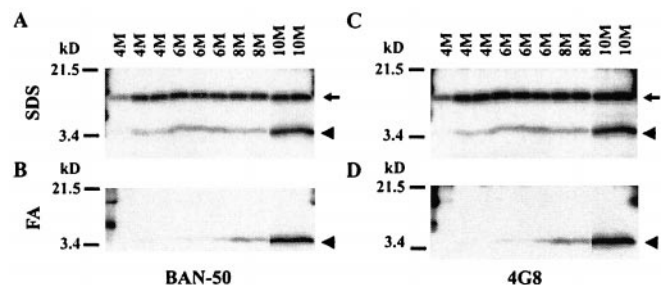


Figure 4. Analysis by immunoblotting of early A β deposition in Tg2576 brain. A β in the SDS (A, C) and formic acid (B, D) extracts from 4–10 month Tg2576 brains was analyzed on immunoblots labeled with BAN-50 (anti-A β 1–16) (A, B) and 4G8 (anti-A β 17–24) (C, D). Proteins were separated on 10–20% Tricine gels, and each lane shows the A β in 40 μ l of the formic acid or SDS extract as described in Materials and Methods. Immunoblotting was first performed with BAN-50. The blots were then stripped and reblotted with 4G8. Note that SDS-soluble A β decreases transiently at 8 months, when SDS-resistant, formic acid-soluble A β appears. The arrows identify CTF β , and the arrowheads identify A β .

ages in 21 month Tg2576 are 66.2% SDS versus 33.1% FA with the four-step extraction and 10.6% SDS versus 89.4% FA with the two-step procedure, and in AD brains, 81.5% SDS versus 18.2% FA with four-step and 13.8% SDS versus 86.2% FA with two-step extraction.

Compared with AD, Tg2576 brain has much less A β that is truncated or modified at its N terminus

In our previous examination of insoluble A β in 27 AD brains (Gravina et al., 1995), A β 42 predominated in 70% and, in 33%, there was essentially no A β 40 deposited. In the 30% in which A β 40 was the predominant species deposited, there was typically prominent congophilic angiopathy. As shown in Table 2, the amounts of total A β 40 and A β 42 deposited in Tg2576 brain most closely resemble the AD brains in which A β 40 deposition predominates. In our previous study (Gravina et al., 1995), ELISAs for A β x-42 (BC-05/4G8) and A β x-40 (BA-27/4G8) showed that most of the A β in AD brain is N-terminally truncated or modified. This is not the case in Tg2576 mice (Table 2). In the brains of the oldest mice examined (21–23 month), the amount of insoluble A β detected by BAN-50 or 3160 capture was 95% of that detected with the BC-05/4G8 and BA-27/4G8 assays that can

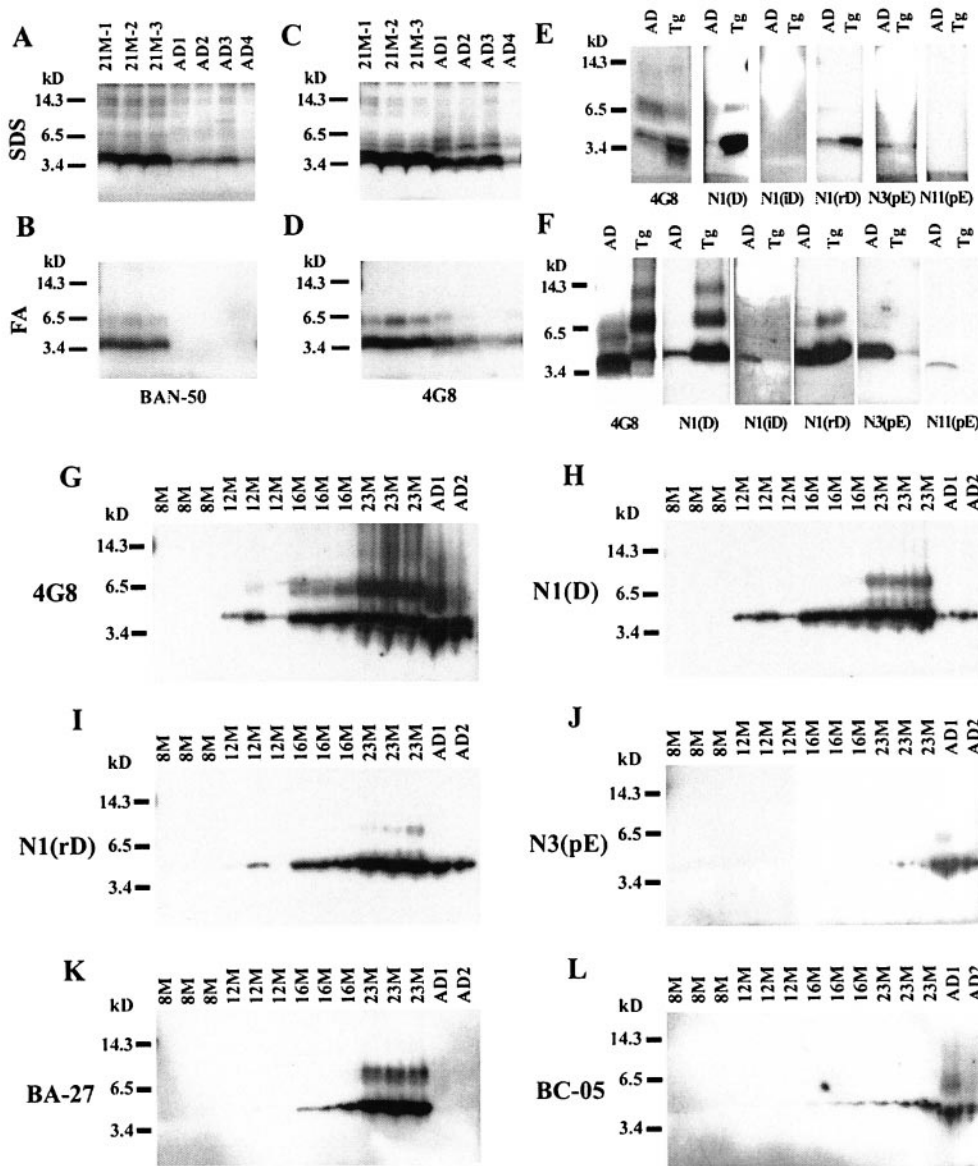


Figure 5. Specific forms of A β in Tg2576 and AD brains. *A–D*, Immunoblot analysis of SDS (*A*, *C*) and formic acid (*B*, *D*) extracts of 21M Tg2576 mouse brains and AD brains labeled with BAN-50 (*A*, *B*) or 4G8 (*C*, *D*). Two microliters of the SDS or FA extract (dried and resuspended) were directly added to each lane; proteins were separated on 10–20% Tricine gels. *E*, *F*, Immunoblot analysis of SDS (*E*) and formic acid (*F*) extracts of AD and 23M Tg2576 brains labeled with the following: 4G8, which detects both N-terminally modified and unmodified A β ; anti-A β N1(D), which detects the unmodified N terminus; anti-N1(iD), which recognizes isomerized forms (L-iso-Asp) of A β N1; anti-N1(rD), which detects stereoisomerized forms (rectus Asp) of A β N1; anti-A β N3(pE), which detects forms beginning with pyroglutamate at position 3; or anti-A β N11(pE), which recognizes forms beginning with pyroglutamate at position 11. The A β in 10 μ l of SDS or FA extracts was examined on each lane. In the SDS extracts, A β was immunoprecipitated with the indicated antibody as described in Materials and Methods before separation and immunoblotting with the same antibody. In the FA extracts, A β was dried and resuspended as described in Materials and Methods before separation and immunoblotting. Proteins were separated on 16% Tricine gels. *G–L*, Time course of accumulation of formic acid-soluble A β in Tg2576 brains (8M–23M) and AD brains labeled with 4G8 (*G*), anti-N1(D) (*H*), anti-N1(rD) (*I*), anti-N3(pE) (*J*), BA-27 (*K*), or BC-05 (*L*). The A β in 10 μ l of formic acid extract was examined on each lane, and proteins were separated on 16% Tricine gels.

detect additional modified–truncated forms of A β . This indicates that, in Tg2576 brain, only 5% of insoluble A β is N-terminally truncated or modified, whereas in AD brain, the corresponding percentage is 69–85% (Table 2). Thus, it appears that N-terminally modified or truncated forms of A β , which predominate in AD brain (Saido et al., 1995, 1996; Hosoda et al., 1998), are minor species in Tg2576 brain that only begin to appear in very old mice.

The results obtained by sandwich ELISAs are confirmed by immunoblotting

Immunoblotting of the SDS and FA fractions obtained by two-step extraction (Fig. 4*A–D*) gave results that were consistent with the results from sandwich ELISAs performed on the same extracts (Fig. 3). SDS-insoluble 4 kDa A β labeled by both BAN-50 (Fig. 4*B*) and 4G8 (Fig. 4*D*) appeared at 6–8 months and increased substantially by 10 months. To analyze the SDS-extractable A β , the SDS extracts were immunoprecipitated with 3160, a rabbit polyclonal antibody to A β 1–40, and the immunoprecipitate was analyzed by immunoblotting with BAN-50 (Fig. 4*A*) or 4G8 (Fig. 4*B*). As expected from ELISA analysis (Fig.

3*A,B*), the total 4 kDa A β extracted into SDS decreased slightly between 6 and 8 months before beginning to increase substantially at 10 months (Fig. 4*A,C*, arrowhead). β APP CTF β , which contains full-length A β , was detected by both BAN-50 (Fig. 4*A*, arrow) and 4G8 (Fig. 4*C*, arrow) in the SDS extracts but not in the formic acid extracts (Fig. 4*B,D*). As expected, CTF β in Tg2576 brain showed no change with aging.

The full-length, unmodified A β in AD compared with 21 month Tg2576 brain was analyzed by immunoblotting two-step SDS and FA extracts (Fig. 5*A–D*). In the FA extracts, BAN-50 (anti-A β 1–16) detected abundant full-length 4 kDa A β in the three Tg2576 brains but almost nothing in any of the four AD brains examined (Fig. 5*B*), a result that is in good agreement with the ELISA data shown in Table 2. In contrast, 4G8 (anti-A β 17–24), which recognizes both full-length A β and A β that is N-terminally truncated or modified, detected substantial amounts of A β in the FA extract of both Tg2576 and AD brains (Fig. 5*D*). Similarly, in SDS extracts, BAN-50 labeled far more 4 kDa A β in Tg2576 than in AD brains (Fig. 5*A*), whereas 4G8 labeled large amounts of A β in both Tg2576 and AD brains (Fig. 5*C*). It is

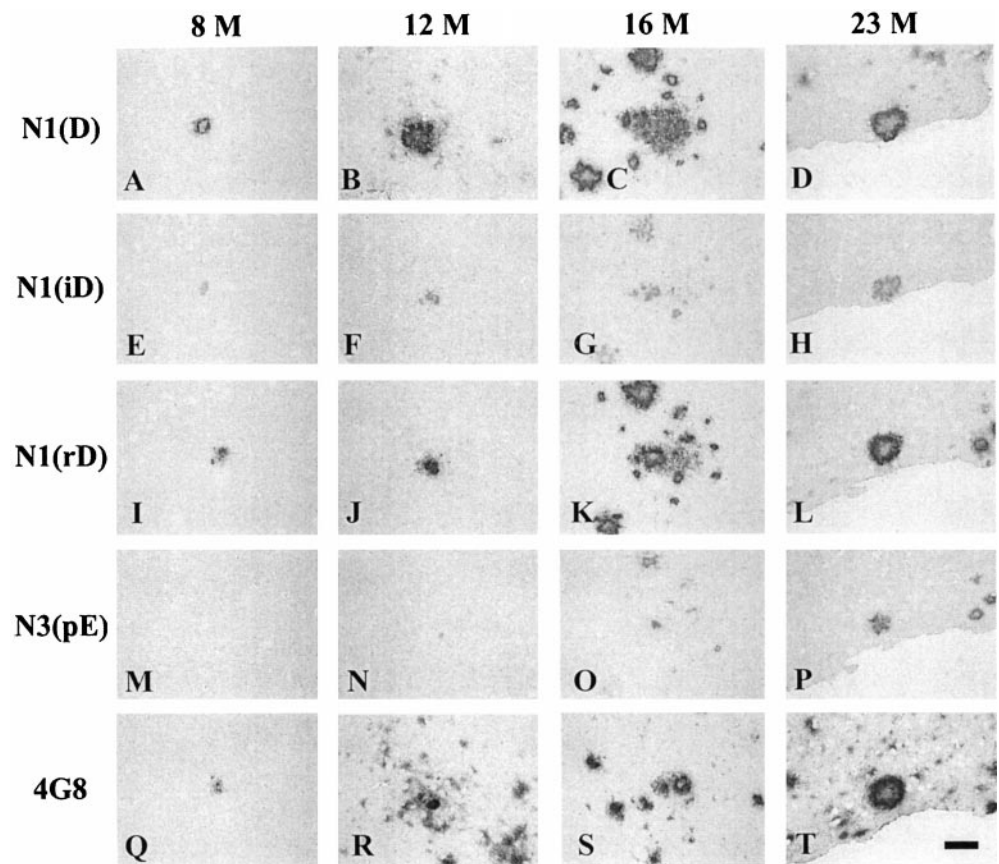


Figure 6. Immunohistochemical analysis of modified forms of A β in aging Tg2576 brain. Serial sections (5 μ m) of Tg2576 cerebral cortex were labeled with anti-N1(D), anti-N1(iD), anti-N1(rD), anti-N3(pE), and 4G8. The age of the mouse brain analyzed is shown at the top of each set of serial sections. Scale bar, 17 μ m.

noteworthy that the A β detected by 4G8 appeared to be slightly smaller in AD than in Tg2576 brain (Fig. 5C, and to a lesser extent D), consistent with the A β in AD brain being truncated at its N terminus.

The immunoblots in Figure 5A–D show that 4G8 and BAN-50 label A β oligomers that are present in both SDS and FA fractions from 21 month Tg2576 brains. Similar oligomers are observed in the SDS and FA extracts of AD brains. As shown in Figures 4A–D and 5G, these oligomers are not detectable at 8 months but are definitely present by 12 months and increase substantially thereafter. Even with prolonged exposure, we have been unable to demonstrate oligomers in either SDS or FA extracts from brains in the critical 6–8 month period when A β aggregation begins.

BA-27, a monoclonal antibody that specifically detects the C terminus of A β 40, and BC-05, which is specific for the C terminus of A β 42, were used to analyze 6–23 month Tg2576 by immunocytochemistry (Fig. 2) and immunoblotting (Fig. 5K,L). This analysis confirmed that A β s terminating at both A β 40 and A β 42 accumulate in aging Tg2576 brain with A β 40 predominating (Figs. 2, 5K,L). In contrast, virtually all A β terminated at A β 42 in the two AD brains examined (Fig. 5K,L), as occurs in ~33% of AD cases (Gravina et al., 1995).

N-terminally modified A β s accumulate in aging Tg2576 brain

Antibodies that detect specific N-terminal modifications of A β (Saido et al., 1995, 1996) were used to analyze 23 month Tg2576 brain by immunoblotting (Fig. 5E–J) and immunocytochemistry (Fig. 6). Comparison of 4G8, which detects virtually all A β regardless of modification, and anti-N1(D), which specifically

detects unmodified A β , confirmed that most A β in AD brains is N-terminally modified or truncated (Fig. 5G,H), whereas most A β in 8–23 month Tg2576 brains is unmodified (Fig. 5G,H and 6, N1(D) versus 4G8). Isomerized A β (N1iD) was labeled well in FA extracts of AD brain (Fig. 5F) and accumulated at a low level in 8–23 month Tg2576 brain (Fig. 6), but the amount of A β in Tg2576 brain was far less than in AD brain (Fig. 5F). Stereoisomerized A β (N1rD) accumulated with aging between 8 and 23 months in Tg2576 brain and, at 23 months, was intensely labeled in Tg2576 brain (Figs. 5I, 6) as it was in AD brain (Fig. 5I). A β N3-pyroglutamate (N3(pE)) is a major form of A β in AD brain but a minor form in aged Tg2576 brain (Fig. 5J). Histochemical analysis (Fig. 6) showed that A β N3-pyroglutamate (N3(pE)) appears late in Tg2576 brain, accumulating between 16 and 23 months. A β N11-pyroglutamate was not detected in Tg2576 brains but was definitely detected in AD brain (Fig. 5F). In both Tg2576 and AD brains, the N-terminally modified A β s were more evident in the SDS-insoluble A β found in FA extracts (Fig. 5F) than in SDS extracts (Fig. 5E). Collectively, these findings indicate that, with the exception of A β N11-pyroglutamate, N-terminal modifications or truncations of A β , which are thought to make A β more insoluble in human brain, also occur in the aging Tg2576 brain. With the exception of stereoisomerized A β , all of these modified forms are, however, far less abundant in Tg2576 than in AD brain.

CSF and plasma A β decline as A β is deposited in the Tg2576 brain

It is well established that A β 42 declines in the CSF of patients with typical late onset AD (Mottter et al., 1995; Nitsch et al., 1995; Kanai et al., 1998; Andreassen et al., 1999), and this decrease

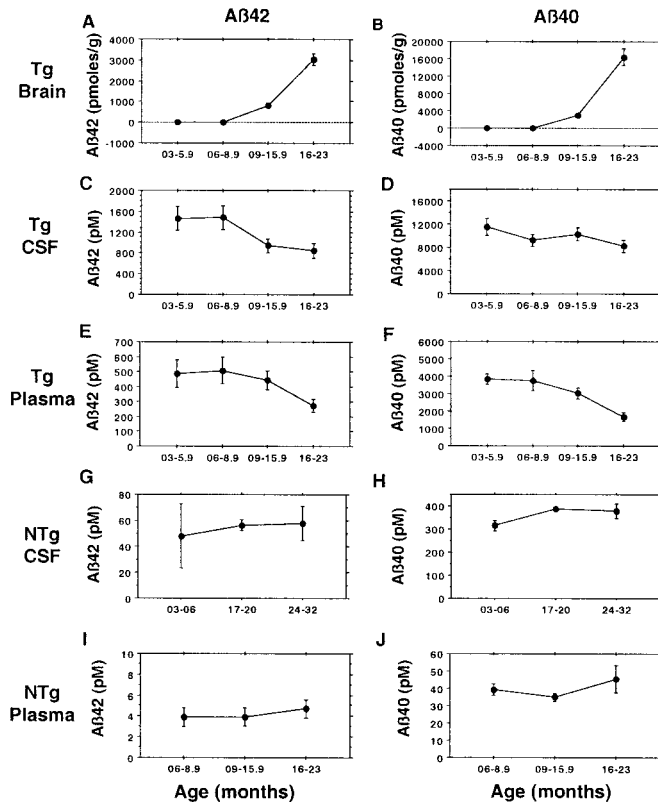


Figure 7. CSF and plasma A β in Tg2576 mice decline as A β is deposited in the brain. Total brain A β 42 (*A*) and A β 40 (*B*) were assayed by 3160/BC05 or 3160/BA27 ELISAs, respectively; see also Figure 2 and Table 2. Tg2576 CSF and plasma A β 42 (*C*, *E*) were assayed by BNT77/BC05 ELISA, and Tg2576 CSF and plasma A β 40 (*D*, *F*) were assayed by BAN50/BA27 ELISA. Both nontransgenic (NTg) CSF and plasma A β 42 (*G*, *I*) and A β 40 (*H*, *J*) were assayed with BNT77 capture. The number of Tg2576 CSF samples assayed for the four time groups are 9, 9, 16, and 11, totaling 45. The number of Tg2576 plasma samples assayed for the four time groups are 30, 18, 64, and 19, totaling 131. The decline for CSF A β 42 is significant ($p = 0.02$), and the declines for plasma A β 40 and A β 42 are highly significant (A β 42, $p = 0.008$; A β 40, $p = 0.006$; Spearman's rank correlation for the 6–23 month age range). The number of nontransgenic CSF samples assayed for the three time groups are 2, 2, and 6, totaling 10. The number of nontransgenic plasma samples assayed for the four time groups are 7, 6, 12, and 6, totaling 31.

could conceivably cause plasma A β 42 to decline because CSF A β , which has a high concentration relative to plasma A β , is normally cleared into blood (Gherzi-Egea et al., 1996). To determine whether CSF and plasma A β decline in Tg2576 mice as A β 42 and A β 40 are deposited in the brain, we analyzed brain (Fig. 7*A,B*), CSF (Fig. 7*C,D*), and plasma (Fig. 7*E,F*) A β 42 and A β 40 in parallel. This analysis showed that, in aged Tg2576 brain, as in human AD brain, there is a decline in CSF A β 42. This decline in CSF A β 42 ($p = 0.03$) occurred in parallel with the marked increases in brain A β 42 and A β 40 that occur between 9 and 23 months, and it was accompanied by a decline in CSF A β 40, although this decrease did not achieve significance. Remarkably, plasma A β 42 ($p = 0.008$) and A β 40 ($p = 0.006$) both showed highly significant decreases (Fig. 7*E,F*) that paralleled the marked accumulation of brain A β and the decline in CSF A β that occurred between 6 and 23 months. To be sure that the declines in CSF and plasma A β that occur in aging Tg2576 mice are linked to A β deposition and not to aging alone, A β 42 and A β 40 were analyzed in CSF (Fig. 7*G,H*) and plasma (Fig. 7*I,J*)

of aging nontransgenic littermates (Fig. 7*G,H*). In these nontransgenic mice, there was no suggestion of a decline in A β because A β 42 and A β 40 both showed a slight upward trend in CSF (Fig. 7*G,H*) and plasma (Fig. 7*I,J*) with aging.

DISCUSSION

As Tg2576 animals age, A β is altered beginning at 6–7 months with the appearance and subsequent increase of A β in the FA-extracted fraction. As insoluble (FA-requiring) A β appears at 6–9 months, the remaining A β in the brain (SDS-extracted in two-step, or TBS- and Triton-extracted in four-step) decreases slightly, suggesting that it is converting to an insoluble form. The definite biochemical change that occurs by 10 months in Tg2576 mice is accompanied by minimal histological evidence of A β deposition, although single plaque cores can be observed in many sections. From studies of trisomy 21 brains (Iwatsubo et al., 1994), it is generally believed that, in human AD, A β deposition begins with the formation of diffuse plaques. In Tg2576 brains, diffuse plaques are not observed in appreciable number until 12 months, 4 months after biochemically detectable alterations in A β have begun in every animal. Thus, there is an early period from 6 to 10 months in Tg2576 mice in which insoluble A β appears accompanied only by rare cored plaques.

Support for the view that there may be a similar early period in human brain comes from a study by Funato et al. (1998), who used the same ELISA system used here. They report that both SDS-dissociable and -insoluble forms of A β accumulate in human cortex, that insoluble A β correlates with amyloid load, and that its biochemical detection precedes plaque formation. They calculate that immunodetection of A β in human cortex requires 400 pmol/gm insoluble A β 42 (or 200 pmol/gm in hippocampus) (Funato et al., 1998). These values are similar to those in 10-month-old Tg2576 mice, the time when plaques become evident.

Our immunohistochemical and biochemical analyses show that, from 10 to 21 months, there is a rapid increase in both diffuse and cored plaques in Tg2576 brain and a coordinate marked increase in SDS-dissociable and FA-requiring A β , with both the biochemical and histological changes rising to levels like those observed in human AD. In most AD patients, very little A β 40 is deposited in the brain, but in ~33%, extraordinary amounts of A β 40 are deposited, and most of these patients show substantial amyloid angiopathy (Gravina et al., 1995). The Tg2576 model is like this latter group of AD patients in that there is marked congophilic angiopathy and the deposition of a large amount of A β 40. It is not clear why large amounts of A β 40 are deposited in the Tg2576 model. It may be that more than one factor contributes and that some combination of species, strain, promoter, expression level, and mutated transgene causes the large amount of A β 40 deposition in this model.

Much of the A β in AD brain is N-terminally truncated or modified (Saido et al., 1995, 1996; Hosoda et al., 1998), and it has been suggested that the formation of SDS stable A β oligomers may be an early event in AD (Enya et al., 1999). Because N-terminally truncated or modified A β [especially forms beginning with pyroglutamate at position 3 (3pE)] and oligomers are resistant to proteolysis (Saido et al., 1996; Kuo et al., 1998), these modifications of A β are thought to be important for amyloid deposition. Our studies indicate that, in Tg2576 brain, it is full-length, unmodified A β that becomes insoluble initially. After this early period, oligomers and most modified forms (e.g., forms beginning at 3pE) appear, but at levels far below those observed in AD brain. It is not clear whether this late development and

relative paucity of modified or oligomeric forms reflects the much shorter time over which A β aggregates in Tg2576 brain or is attributable to fundamental biochemical differences between the human and mouse brain. One intriguing possibility is that the A β modifications observed in the AD brain may play an important pathogenic role and that the relative paucity of these forms in Tg2576 brain may account for the minimal neurofibrillary pathology and neuronal loss observed in Tg2576 brain.

In Tg2576 mice, the APP_{K670N,M671L} transgene is expressed at highest level in the brain, but there is also substantial expression in other organs (Fig. 1), although expression is driven by the hamster PrP promoter. Thus, in Tg2576 mice as in human subjects, plasma A β is likely to be derived from both peripheral organs and brain, where A β may enter the bloodstream either through the normal flow of CSF or by directly crossing CNS endothelium. In a previous study of human plasma (Scheuner et al., 1996), we showed that the Swedish APP_{K670N,M671L} mutation increases both A β 42 and A β 40 and that other FAD-linked APP, PS1, and PS2 mutations selectively increase A β 42. In that report, we suggested that cerebral A β deposition in FAD occurs because of an increase in CNS A β that develops as part of a generalized genetic effect that also increases plasma A β . We emphasized that A β deposition in FAD probably does not occur as a direct effect of increased plasma A β . We have reported previously a transgenic mouse line (NOR β 0304) that expresses an A β containing CTF of APP (Kawarabayashi et al., 1996). Because expression in NOR β 0304 is driven by the β -actin promoter, the transgenic APP CTF is expressed in all organs, and expression is much higher in many peripheral organs than in brain. Significantly, plasma A β is even higher in NOR β 0304 (1400 pM A β 42; 5600 pM A β 40) than in Tg2576, but NOR β 0304 mice do not develop age-dependent A β deposition in brain. Thus, elevated plasma A β can be a good indicator that deposition will occur when it occurs as part of a generalized response in human subjects or in transgenic mice in which expression is under the control of a promoter such as PrP, which causes expression to be highest in brain. Elevated plasma A β does not directly drive deposition, however, because NOR β 0304 mice, which have a β -actin promoter that causes expression to be highest in the periphery, show no deposition, although they have higher plasma A β levels than the Tg2576 line.

We have shown recently that human plasma A β 42 and A β 40 increase with aging over age 65 and that plasma A β 42 and A β 40 are heritable traits that are increased in first degree relatives of patients with typical late onset AD (Younkin et al., 1998). These findings suggest that, in typical late onset AD as in early onset FAD, elevated plasma A β may be associated with the development of AD. If so, one would expect plasma A β to be elevated in typical late onset AD. Our initial analysis of typical AD patients (Scheuner et al., 1996) showed, however, that very few AD patients have high plasma A β 42 when compared with age-matched controls. Other published studies of plasma A β in AD have shown no change or a slight increase in plasma A β 42 (Iwatsubo, 1998; Matsubara et al., 1999). Because it is well established that CSF A β 42 decreases in AD, one way to account for these negative results is to postulate that plasma A β 42 also declines as AD develops. If so, then analysis of symptomatic late onset patients could miss many patients whose disease was initiated by increased A β in the presymptomatic period.

To test the hypothesis that plasma and CSF A β both decline as A β is deposited in the brain, plasma, CSF, and brain A β were analyzed coordinately in aging Tg2576 mice. This analysis

showed that, coincident with the marked deposition of A β 42 and A β 40 in brain, there is not only a decline in CSF A β but also a substantial, highly significant decrease in plasma A β 42 and A β 40. If this also occurs in human subjects, then declining plasma A β could be a useful marker for subjects in whom there is cerebral A β deposition and who are, therefore, at risk for AD. Similarly, elevated plasma A β could be an excellent premorbid biomarker for AD, although it is not useful as a diagnostic marker, if it identifies those who are destined to deposit A β and those who are in the early stages of deposition.

REFERENCES

- Andreasen N, Hesse C, Davidsson P, Minthon L, Wallin A, Winblad B, Vanderstichele H, Vanmechelen E, Blennow K (1999) Cerebrospinal fluid β -amyloid(1–42) in Alzheimer disease: differences between early- and late-onset Alzheimer disease and stability during the course of disease. *Arch Neurol* 56:673–680.
- Borchelt DR, Thinakaran G, Eckman CB, Lee MK, Davenport F, Ratovitsky T, Prada CM, Kim G, Seekins S, Yager D, Slunt HH, Wang R, Seeger M, Levey AI, Gandy SE, Copeland NG, Jenkins NA, Price DL, Younkin SG, Sisodia SS (1996) Familial Alzheimer's disease-linked presenilin 1 variants elevate A β 1–42/1–40 ratio *in vitro* and *in vivo*. *Neuron* 17:1005–1013.
- Cai XD, Golde TE, Younkin SG (1993) Release of excess amyloid β protein from a mutant amyloid β protein precursor. *Science* 259:514–517.
- Carp RI, Davidson AL, Merz PA (1971) A method for obtaining cerebrospinal fluid from mice. *Res Vet Sci* 12:499.
- Chapman PF, White GL, Jones MW, Cooper-Blacketer D, Marshall VJ, Irizarry M, Younkin L, Good MA, Bliss TVP, Hyman BT, Younkin SG, Hsiao KK (1999) Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. *Nat Neurosci* 2:271–276.
- Chartier-Harlin MC, Crawford F, Houlihan H, Warren A, Hughes D, Fidani L, Goate A, Rossor M, Roques P, Hardy J, Mullan M (1991) Early-onset Alzheimer's disease caused by mutations at codon 717 of the β -amyloid precursor protein gene. *Nature* 353:844–846.
- Citron M, Vigo-Pelfrey C, Teplow DB, Miller C, Schenk D, Johnston J, Winblad B, Venizelos N, Lannfelt L, Selkoe DJ (1994) Excessive production of amyloid β -protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. *Proc Natl Acad Sci USA* 91:11993–11997.
- Citron M, Westaway D, Xia W, Carlson G, Diehl T, Levesque G, Johnson-Wood K, Lee M, Seubert P, Davis A, Kholodenko D, Motter R, Sherrington R, Perry B, Yao H, Strome R, Lieberburg I, Rommens J, Kim S, Schenk D, Fraser P, St. George-Hyslop P, Selkoe DJ (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β -protein in both transfected cells and transgenic mice. *Nat Med* 3:67–72.
- Duff K, Eckman C, Zehr C, Yu X, Prada CM, Perez-tur J, Hutton M, Buee L, Harigaya Y, Yager D, Morgan D, Gordon MN, Holcomb L, Refolo L, Zenk B, Hardy J, Younkin S (1996) Increased amyloid- β 42(43) in brains of mice expressing mutant presenilin 1. *Nature* 383:710–713.
- Enya M, Morishima-Kawashima M, Yoshimura M, Shinkai Y, Kusui K, Khan K, Games D, Schenk D, Sugihara S, Yamaguchi H, Ihara Y (1999) Appearance of sodium dodecyl sulfate-stable amyloid β -protein (A β) dimer in the cortex during aging. *Am J Pathol* 154:271–279.
- Funato H, Yoshimura M, Kusui K, Tamaoka A, Ishikawa K, Ohkoshi N, Namekata K, Okeda R, Ihara Y (1998) Quantitation of amyloid β -protein (A β) in the cortex during aging and in Alzheimer's disease. *Am J Pathol* 152:1633–1640.
- Ghersi-Egea JF, Gorevic PD, Ghiso J, Frangione B, Patlak CS, Fenstermacher JD (1996) Fate of cerebrospinal fluid-borne amyloid β -peptide: rapid clearance into blood and appreciable accumulation by cerebral arteries. *J Neurochem* 67:880–883.
- Glenner GG, Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* 120:885–890.
- Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, Mant R, Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M, Hardy J (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349:704–706.
- Gravina SA, Ho L, Eckman CB, Long KE, Otvos Jr L, Younkin LH, Suzuki N, Younkin SG (1995) Amyloid β protein (A β) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at A β 40 or A β 42(43). *J Biol Chem* 270:7013–7016.

- Hosoda R, Saido TC, Otvos Jr L, Arai T, Mann DM, Lee VM, Trojanowski JQ, Iwatsubo T (1998) Quantification of modified amyloid β peptides in Alzheimer disease and Down syndrome brains. *J Neuro-pathol Exp Neurol* 57:1089–1095.
- Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996) Correlative memory deficits, A β elevation, and amyloid plaques in transgenic mice. *Science* 274:99–102.
- Irizarry MC, McNamara M, Fedorchak K, Hsiao K, Hyman BT (1997) APPSw transgenic mice develop age-related A β deposits and neuropil abnormalities, but no neuronal loss in CA1. *J Neuropathol Exp Neurol* 56:965–973.
- Iwatsubo T (1998) Amyloid β protein in plasma as a diagnostic marker for Alzheimer's disease. *Neurobiol Aging* 19:161–163.
- Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y (1994) Visualization of A β 42(43) and A β 40 in senile plaques with end-specific A β monoclonals: evidence that an initially deposited species is A β 42(43). *Neuron* 13:45–53.
- Jarrett JT, Berger EP, Lansbury Jr PT (1993) The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* 32:4693–4697.
- Kanai M, Matsubara E, Ise K, Urakami K, Nakashima K, Arai H, Sasaki H, Abe K, Iwatsubo T, Kosaka T, Watanabe M, Tomidokoro Y, Shizuka M, Mizushima K, Nakamura T, Igeta Y, Ikeda Y, Amari M, Kawarabayashi T, Ishiguro K, Harigaya Y, Wakabayashi K, Okamoto K, Hirai S, Shoji M (1998) Longitudinal study of cerebrospinal fluid levels of tau, A β 1–40, and A β 1–42(43) in Alzheimer's disease: a study in Japan. *Ann Neurol* 44:17–26.
- Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325:733–736.
- Kawarabayashi T, Shoji M, Sato M, Sasaki A, Ho L, Eckman CB, Prada CM, Younkin SG, Kobayashi T, Tada N, Matsubara E, Iizuka T, Harigaya Y, Kasai K, Hirai S (1996) Accumulation of β -amyloid fibrils in pancreas of transgenic mice. *Neurobiol Aging* [Erratum (1996) 17:667] 17:215–222.
- Kuo YM, Webster S, Emmerling MR, De Lima N, Roher AE (1998) Irreversible dimerization/tetramerization and post-translational modifications inhibit proteolytic degradation of A β peptides of Alzheimer's disease. *Biochim Biophys Acta* 1406:291–298.
- Levy-Lahad E, Wijsman EM, Nemens E, Anderson L, Goddard KA, Weber JL, Bird TD, Schellenberg GD (1995) A familial Alzheimer's disease locus on chromosome 1. *Science* 269:970–973.
- Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci USA* 82:4245–4249.
- Matsubara E, Ghiso J, Frangione B, Amari M, Tomidokoro Y, Ikeda Y, Harigaya Y, Okamoto K, Shoji M (1999) Lipoprotein-free amyloidogenic peptides in plasma are elevated in patients with sporadic Alzheimer's disease and Down's syndrome. *Ann Neurol* 45:537–541.
- Motter R, Vigo-Pelfrey C, Kholodenko D, Barbour R, Johnson-Wood K, Galasko D, Chang L, Miller B, Clark C, Green R, Olson D, Southwick P, Wolfert R, Munroe B, Lieberburg I, Seubert P, Schenk D (1995) Reduction of β -amyloid peptide42 in the cerebrospinal fluid of patients with Alzheimer's disease. *Ann Neurol* 38:643–648.
- Mullan M, Crawford F, Axelman K, Houlden H, Lillius L, Winblad B, Lannfelt L (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β -amyloid. *Nat Genet* 1:345–347.
- Murrell J, Farlow M, Ghetti B, Benson MD (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* 254:97–99.
- Nitsch RM, Rebeck GW, Deng M, Richardson UI, Tennis M, Schenk DB, Vigo-Pelfrey C, Lieberburg I, Wurtman RJ, Hyman BT, Growdon JH (1995) Cerebrospinal fluid levels of amyloid β -protein in Alzheimer's disease: inverse correlation with severity of dementia and effect of apolipoprotein E genotype. *Ann Neurol* 37:512–518.
- Roher AE, Lowenson JD, Clarke S, Wolkow C, Wang R, Cotter RJ, Reardon IM, Zurcher-Neely HA, Heinrikson RL, Ball MJ (1993) Structural alterations in the peptide backbone of β -amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J Biol Chem* 268:3072–3083.
- Saido TC, Iwatsubo T, Mann DM, Shimada H, Ihara Y, Kawashima S (1995) Dominant and differential deposition of distinct β -amyloid peptide species, A β N3(pE), in senile plaques. *Neuron* 14:457–466.
- Saido TC, Yamao-Harigaya W, Iwatsubo T, Kawashima S (1996) Amino- and carboxyl-terminal heterogeneity of β -amyloid peptides deposited in human brain. *Neurosci Lett* 215:173–176.
- Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W, Larson E, Levy-Lahad E, Viitanen M, Peskind E, Poorkaj P, Schellenberg G, Tanzi R, Wasco W, Lannfelt L, Selkoe D, Younkin S (1996) Secreted amyloid β -protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat Med* 2:864–870.
- Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Fonci JF, Bruni AC, Montesi MP, Sorbi S, Rainero I, Pinessi L, Nee L, Chumakov I, Pollen D, Brookes A, Sanseau P, Polinsky RJ, Wasco W, Da Silva HAR, Haines JL, Pericak-Vance MA, Tanzi RE, Roses AD, Fraser PE, Rommens JM, St. George-Hyslop PH (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375:754–760.
- Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L Jr, Eckman C, Golde TE, Younkin SG (1994) An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (β APP717) mutants. *Science* 264:1336–1340.
- Westerman MA, Cooper-Blacketer D, Gómez-Isla T, Mariash A, Ashe KH (2000) Age-dependent behavioral deficit in a transgenic mouse model of Alzheimer's disease. *Soc Neurosci Abstr* 26:1318.
- Younkin SG, Eckman CB, Ertekin Taner N, Kawarabayashi T, Yager D, Baker M, Perez-tur J, Houlden H, Hutton M, Younkin LH, Graff-Radford NR (1998) Genetic elevation of plasma amyloid β protein in typical late onset Alzheimer's disease. *Soc Neurosci Abstr* 24:263.