APP Transgenic Mice Tg2576 Accumulate Aβ Peptides That Are Distinct from the Chemically Modified and Insoluble Peptides Deposited in Alzheimer's Disease Senile Plaques[†]

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ABSTRACT: The amyloid (Aβ) peptides generated in Hsiao's APP Tg2576 transgenic (Tg) mice are physically and chemically distinct from those characteristic of Alzheimer's disease (AD). Transgenic mouse Aβ peptides were purified using sequential size-exclusion and reverse-phase chromatographic systems and subjected to amino acid sequencing and mass spectrometry analyses. The mouse Aβ peptides lacked the extensive N-terminal degradations, posttranslational modifications, and cross-linkages abundant in the stable Aβ peptide deposits observed in AD. Truncated Aβ molecules appear to be generated in vivo by hydrolysis at multiple sites rather than by post-mortem C-terminal degradation. In contrast to AD amyloid cores, the Tg mice peptides were soluble in Tris−SDS−EDTA solutions, revealing both monomeric and SDS-stable oligomeric species of Aβ. In contrast to our report on Novartis Pharma APP23 Tg mice [Kuo et al. (2001) *J. Biol. Chem. 276*, 12991], which maintain high levels of soluble Aβ early on with later development of extensive vascular amyloid, Tg2576 mice exhibited an age-related elevation of soluble Aβ with relatively limited vascular amyloid deposition. The transgenic mouse levels of carboxy-terminal (CT) APP fragments were nearly 10-fold greater than those of human brains, and this condition may contribute to the unique pathology observed in these animals. Immunization of transgenic mice may act to prevent the pathological effects of βAPP overproduction by binding CT molecules or halting their processing to toxic forms, in addition to having any effects on Aβ itself. Thus, differences in disease evolution and biochemistry must be considered when using transgenic animals to evaluate drugs or therapeutic interventions intended to reduce the Aβ burden in Alzheimer's disease.

A major hallmark of Alzheimer's disease (AD) pathology is the profuse amyloid-β (Aβ) accumulation in neuritic plaques or as diffuse deposits in the brain parenchyma and the walls of cerebral and leptomeningeal vessels (*1*). The amyloid fibrils are composed mainly of the 40−42 amino acid residue Aβ peptides that are derived from a larger molecule, the β-amyloid precursor protein (βAPP), through the proteolytic action of the β- and γ-secretases (*1*). Aβ peptides are estimated to represent as much as 80% of the total amyloid fibril mass (*2*) in AD with the remaining 20% composed of an assortment of glycoproteins and glycolipids which enhance the amyloid fibril insolubility (*2*, *3*). Amyloid deposition in the AD brain tissue and vascular walls, whether in a soluble oligomeric form or as insoluble fibrillar deposits, provokes glial cell activation and a chronic inflammatory reaction (*4*). As amyloid accumulates in AD, Aβ peptides are degraded, chemically modified, and cross-linked, thereby increasing their relative insolubility, stability, and toxicity (*5*−*8*). Several animal models that partially recreate the AD pathology have recently been developed. Deposits of perivascular Aβ were elicited in the rabbit as the result of the immunotoxic ablation of the basal forebrain cholinergic neurons (*9*). Transgenic mice (Tg) that overexpress mutant βAPP alone or in combination with either presenilin (PS) mutations or transforming growth factor-β1 (TGF-β1) are employed as AD models (*10*−*24*). Most of these models produce substantial Aβ deposition in brain tissue and in the walls of cerebral arteries. Some βAPP Tg mice partially and variably reproduce the neuronal alterations, neuritic plaques, cerebrovascular amyloidosis, astrogliosis, and microgliosis seen in the AD brain, and time-dependent behavioral and

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cognitive changes are evident in these animals. These apparent time-dependent pathological and morphological similarities in the amyloid cascades between Tg mice and humans have encouraged the use of Tg rodents as AD models.

In view of the pathophysiological importance of Aβ deposition in the Tg models and their potential therapeutic relevance to AD, we rigorously characterized and compared the chemical structure of Aβ peptides generated in the Tg2576 mice to that of authentic human AD amyloid. To determine whether the characteristic chemical modifications observed in human AD amyloid occurred after death, we monitored Aβ chemical stability in Tg2576 mice during the post-mortem period. The amyloid peptides generated in Tg2576 mice were chemically distinct from those characteristic of sporadic AD. In addition, we compared the pathophysiological characteristics of the Tg2576 mice with those of the previously studied Novartis Pharma APP23 mice (*27*). The therapeutic implications of these differences as well as the significant differences between rodent and human CNS vascular anatomy and vaccination strategies intended to reverse AD are discussed.

MATERIALS AND METHODS

The βAPP Tg2576 mice examined were developed by Hsiao et al. (*13*). The line which is in the background strain C57B6/SJL contains the human βAPP695 cDNA carrying the double Swedish mutation Lys670Asn, Met671Leu (βAPP770 numbering), which was inserted into the hamster Prion protein (PrP) cosmid vector. The βAPP expression values were almost 10 times higher in the Tg2576 mice (mean relative optical density units = 16.9; range, 14−24) than in the non-Tg mice (mean = 1.7; range, 0.27−4.5) (*25*). Previous quantification of Aβ by europium immunoassay demonstrated that between 8 and 20 months of age the load of amyloid present in the Tg2576 mice brains exponentially increased from less than 1 μg/g of total protein to almost 50 μg/g of total protein (*25*). In addition, the Tg2576 mice exhibit experimentally observable learning and memory deficits (*12*, *26*). All Tg mice brains were removed, flash frozen in a dry ice−ethanol bath, and maintained at −80 °C until the moment of processing.

Preparation of Brain Homogenates for Western Blotting. Four complete brains from 20-month-old Tg2576 mice (two males, two females) and tissues from three temporal cortices from human AD brains (age and gender: 77M, 84F and 91F). The brains from the demented patients fulfilled the diagnostic criteria of AD as dictated by the Consortium to Establish a Registry for Alzheimer Disease (CERAD) (*28*). The tissues were homogenized for 30 s with a polytron tissue homogenizer (Brinkmann) in four volumes of phosphate-buffered saline (PBS), pH 7.4, containing Complete protease inhibitor cocktail from Roche. Samples were diluted directly in NuPage sample buffer (Invitrogen) containing 50 mM dithiothreitol, incubated for 15 min at 37 °C, loaded onto NuPage 12% Bis-Tris gels, and electrophoresed using NuPage MES running buffer. Prestained low molecular weight protein molecular mass markers (Gibco) were used in each gel. Gels were blotted onto nitrocellulose using NuPage transfer buffer with 20% methanol. The blots were boiled 5 min in PBS and then blocked with 5% nonfat dry milk in Tris-buffered saline (TBS). Monomeric and oligomeric Aβ were detected with 6E10 antibody at 10 μg/mL (Seneteck) and anti-mouse HRP secondary antibody at 1:1500 dilution (Amersham). Carboxy-terminal APP fragments were detected (without boiling) using the rabbit polyclonal antibody R57 which recognizes the last 10 amino acids of full-length APP (kindly provided by Dr. P. Mehta) and anti-rabbit horseradish peroxidase (HRP) secondary antibody at 1:1500 dilution (Amersham). All primary and secondary antibodies were diluted in 1% nonfat dry milk in TBS. Blots were developed with ECL reagent (Amersham) and scanned using a Umax Astra 1200S scanner, and band density was determined using Kodak 1D analysis software.

Preparation of Brain Formic Acid Lysates and Size-Exclusion FPLC. The cerebral cortices and hippocampi were dissected from 15 mice (6 male and 9 female). All animals were 20 months old. The total brain tissue, amounting to approximately 2.2 g, was finely minced and homogenized in 30 mL of 90% glass-distilled formic acid (GDFA) at 4 °C using a glass (Dounce) homogenizer. Samples of 10 mL were loaded into 11 mL polyallomer tubes and centrifuged at 288000*g* for 35 min at 4 °C in a Beckman SW41 Ti rotor. The supernatant was collected by aspiration, avoiding the surface lipid layer and the acid-insoluble bottom pellet. Aliquots of 500 μL of the clear formic acid lysate were fractionated by size-exclusion FPLC on a 1 × 30 cm Superose 12 column (Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated, and developed with 80% GDFA. The Superose 12 column output was size-calibrated using a set of proteins of known M_r and using the reverse-sequence Aβ peptide residues 40−1. The proteins were separated at a flow rate of 15 mL/h at room temperature, and the elution flow absorbance was monitored at 280 nm. The fraction corresponding to the retention time interval of 54−64 min (confirmed by calibration to include the 8−3 kDa molecules) was collected in a polypropylene tube containing 5 μL of 2% betaine. Formic acid was removed from the samples by vacuum centrifugation (Savant Instruments, Farmingdale, NY) and stored at −80 °C until use.

For the determination of potential time-dependent post-mortem Aβ degradations, the cerebral cortices and hippocampi of three 20-month-old Tg2576 mice (one male and two female) were finely minced, mixed thoroughly, and divided into three aliquots of equal weight. One sample was lysed immediately with 90% cold GDFA and considered to represent the time zero condition. The second and third samples were allowed to stand at room temperature for 3 and 6 h, respectively, prior to lysis with cold GDFA. Subsequent steps including ultracentrifugation, FPLC, and HPLC separation were as described for all other samples.

Separation of Aβ Peptides by Reverse-Phase HPLC. The Aβ peptides present in the 8−3 kDa size-exclusion fraction were purified by reverse-phase HPLC on a 4.6 × 250 mm Zorbax 300 SB-C8 column (Mac-Mod Analytical, Chadds Ford, PA) using a 500 μL injection loop. Solvent A was 0.1% trifluoroacetic acid (TFA) in water, and solvent B was 0.1% TFA in acetonitrile. The chromatography was developed at 80 °C with a linear gradient from 20% to 40% solvent B in 60 min at a flow rate of 1.0 mL/min with eluate absorbance monitored at 214 nm. The 8−3 kDa fractions obtained from four separate FPLC runs of brain lysate were pooled by dissolution in 350 μL of 80% GDFA. Water was

added to make a final volume of 500 μL, and the sample was HPLC separated. The Aβ 1−40 and Aβ 1−42 chromatographic retention times characteristic for our system were established using synthetic peptides obtained from California Peptide Inc. (Napa Valley, CA) which were further purified in our laboratory by size-exclusion and reverse-phase HPLC prior to their use as molecular markers.

Amyloid Solubility Test. The Tg mice cerebri were coronally sectioned into three approximately equal portions, and each independent cerebrum was gently stirred for 24 h in 50 mL of 50 mM Tris-HCl, pH 7.5, buffer containing 2% SDS and 2 mM EDTA at room temperature, after which all cellular elements were totally lysed. The solutions were then spun at 485000*g* for 3 h. The diminutive pellets were rinsed once in fresh Tris−SDS−EDTA buffer and twice in water, spread on microscopic slides, dried in an oven at 60 °C for 3 h, and stained with thioflavin-S.

Tryptic and CNBr Hydrolysis of Aβ and Separation of Peptides by Reverse-Phase HPLC. The Aβ peptides purified by reverse-phase HPLC were lyophilized, solubilized in 1 mL of 80% GDFA, and dialyzed (SpectraPor no. 6, 1000 MW cutoff) against two changes (2 L each) of distilled water and three changes (2 L each) of 100 mM ammonium bicarbonate. Once equilibrium was reached, the Aβ peptides were digested with 5 μg of TPCK-treated trypsin (Worthington Biochemicals Corp. Freehold, NJ) for 16 h at 37 °C. The insoluble tryptic core was submitted to CNBr cleavage in the presence of 80% GDFA. The resulting tryptic and CNBr peptides were separated by reverse-phase Zorbax SB-C18 column (Mac-Mod Analytical, Chadds Ford, PA) HPLC and characterized by automatic amino acid analysis.

Automatic Peptide Sequencing, Automatic Amino Acid Analyses, and Mass Spectrometry of Aβ Peptides. These procedures were carried out as described in detail by Kuo et al. (*27*).

RESULTS AND DISCUSSION

Expression of the β- and α-secretase-cleaved carboxy-terminal (CT) βAPP fragments and Aβ in 20-month-old Tg2576 mice and in human AD brain samples was quantified and compared by Western blotting (Figure 1). The Tg2576 mice produced about 10-fold more brain βAPP than non-transgenic controls (*25*). The levels of the 12 kDa β-CT and the 10 kDa fragment produced by α-secretase (α-CT) were substantially elevated in Tg2576 mice compared to human tissues (Figure 1A). Our quantification of band intensities revealed almost 10-fold higher levels of CTs in the mouse in comparison to human brains (Figure 1B). The CTs hyperproduction is possibly not the fundamental mechanism provoking AD pathology but could result in the perturbation of normal amyloid processing pathways in Tg mice. However, it is possible that in the brains of patients with the APP Swedish mutations, in contrast to the sporadic type of AD, the amount of the C-terminal fragments may be increased because of the mutations affecting the β-secretase cleavage. Despite the dramatic difference in the content of its direct precursor, β-CT, human and Tg mice had comparable total brain Aβ levels (Figure 1C). The similarity was most striking with respect to the oligomerized Aβ forms revealed by staining with human specific antibody 6E10 (Figure 1D). Both human and Tg mouse tissues possessed bands that

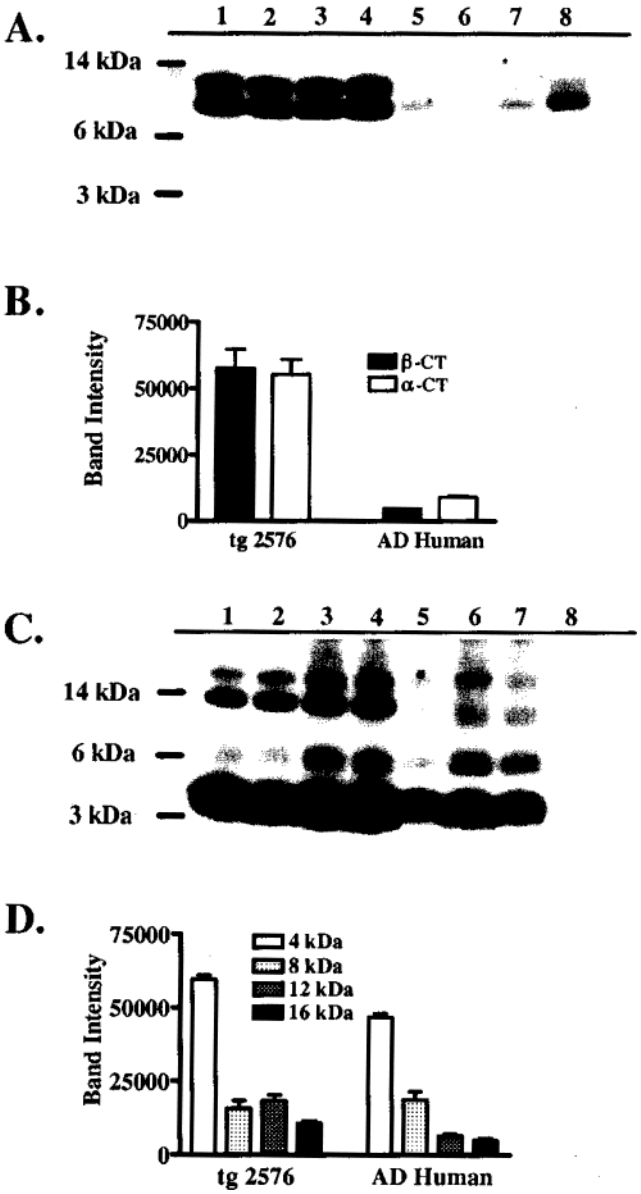

FIGURE 1: Western blots of βAPP carboxy-terminal fragments and Aβ from 20-month-old Tg2576 mice and human AD brains. For a description of specimens and technical details, see Materials and Methods. Equal amounts of proteins were loaded for each tissue, and the staining intensity was compared. (A) R57 staining of carboxy-terminal APP fragments. Lanes: 1−4, Tg2576 mice; 5−7, AD brain; 8, control human brain. (B) Intensity of the β-CT and α-CT bands found in mouse and human brains. Values are the means with standard errors for the mouse and AD tissues. (C) Immunoblots of human Aβ in AD and mouse brains using antibody 6E10. Lanes: 1−4, Tg2576 mice; 5−7, AD brain; 8, control human brain. (D) Intensity of the 4 kDa monomer, 8 kDa dimer, 12 kDa trimer, and 16 kDa tetramer Aβ bands found in mouse and human brain tissues. Values are the means with standard errors for the mouse and AD tissues.

corresponded to low molecular weight Aβ aggregates. The same bands were detected using antibodies to Aβ residues 17−24 as well as to the carboxy terminus of Aβ 1−40 and Aβ 1−42 (data not shown), consistent with the presence of full-length Aβ SDS-stable oligomeric Aβ forms that have been reported previously in the brains of Tg2576 mice (*29*) and in AD brains as well (*30*). However, our experiments revealed that, unlike those in human AD, the oligomeric Aβ species in Tg mice are not formic acid stable.

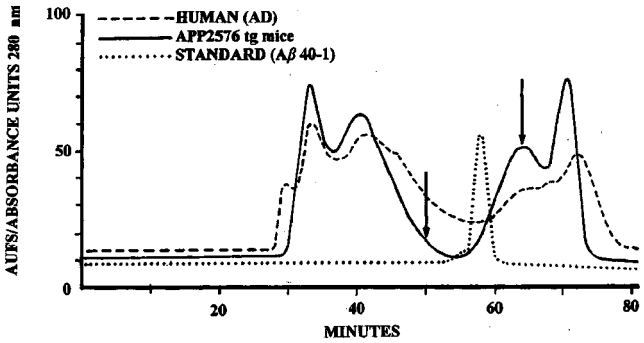

FIGURE 2: Size-exclusion (Superose 12) FPLC chromatographic profiles of the Tg2576 mice and AD brain GDFA lysates developed under denaturing conditions using 80% GDFA. The dotted line depicts the human Aβ 40−1 (M_r 4331 Da) reverse amino acid sequence used as the Aβ molecular mass calibration standard. The vertical arrows indicate the fractions containing peptides ranging from 8 to 3 kDa that were submitted to reverse-phase HPLC.

FPLC size-exclusion separation of the formic acid soluble brain specimens from Tg2576 mice yielded four broad peaks (Figure 2). The 8−3 kDa fraction, which contained the Aβ peptide dimeric and monomeric forms, accounted for about 13.7% of the total amount of 280 nm-detectable brain protein. The 8−3 kDa fractions from four FPLC runs were pooled and submitted to reverse-phase HPLC. The fractions containing the Aβ peptides were eluted between 27.5% and 33% acetonitrile (Figure 3). Of the total amount of protein detected at 214 nm, the UV absorbance for peptide bonds, the Aβ-related peptides represented 7% of the total surface area under the peaks. Aβ peptides detected by 280 and 214 nm UV absorbance in the Tg2576 mice studied represented ~1% of the total brain protein.

The FPLC 8−3 kDa peaks from Tg2576 mice brains were resolved into the Aβ peptides Aβ 1−37, Aβ 1−38, Aβ 1−39, Aβ 1−40, and Aβ 1−42 by reverse-phase HPLC (Figure 3). The formic acid exposure partially modified the Aβ 1−38, Aβ 1−40, and Aβ 1−42 peptides which appeared as doublets, and mass spectrometry confirmed an increase of 28 Da in each of these peptides (Table 1). This suggests the gain of a single formyl group either at the N-terminal residue (Asp) or at one of the two Ser8 or Ser26 OH groups. Reverse-phase chromatography of the purified Aβ 1−40, dissolved in 0.1% TFA solution, revealed that this treatment failed to generate the formylated Aβ peptide. Rechromatography of the separated Aβ 1−40 formylated peptide yielded, on the basis of the retention time, the unmodified Aβ 1−40 peptide, suggesting the loss of the artifactual formyl group. The Aβ 1−40 and Aβ 1−42 were also recovered as oxidized peptides (Figure 3) since they showed an increase of 16 Da (Table 1) per molecule and had a decreased retention time (Figure 3), suggesting the modification of Aβ Met35 to Met sulfoxide. Every C8 reverse-phase chromatographic peak present in the Tg2576 mice samples from an elution time of 37 to 61 min was submitted to mass spectrometry analysis. The only Aβ peptides observed were those listed in Table 1. Special attention was devoted to determine the presence of dimeric Aβ forms which in the AD neuritic plaque amyloid represents ~25% of the total Aβ mass (*5*, *6*, *8*). In contrast, the analysis of the human 8−3 kDa FPLC fraction revealed a complex mixture of Aβ peptides. Examination of these peptides by mass spectrometry demonstrated the presence of intact Aβ 1−40 and 1−42 (Figure 3 and Table 2). In addition, numerous modified and truncated Aβ peptides as well as dimeric Aβ were identified by mass spectrometry (Figure 3 and Table 2). In humans, the irreversible dimeric Aβ probably results from cross-linkage of Tyr10 (*33*; C. Glabe, unpublished observations). Formic acid stable dimeric Aβ forms were not evident in the Tg2576 mice samples. Likewise, a search for the Aβ peptide residues 17−42, recognized as P3 in humans (*34*, *35*), was unsuccessful. A search for these and other forms of modified Aβ, such as pyroglutamyl N-terminal residues or shorter Aβ resulting from N-terminal degradations, abundantly observed in the human AD amyloid (Figure 3 and Table 2), yielded negative results. The Tg2576 mice Aβ 1−40 and Aβ 1−42 peptides

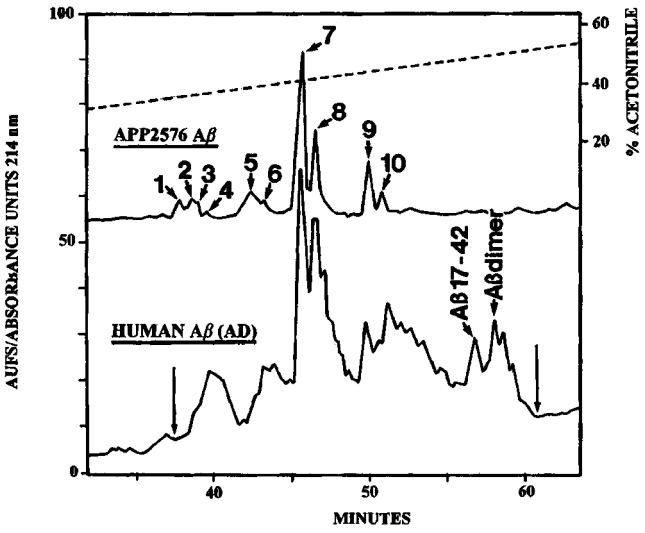

FIGURE 3: Reverse-phase (Zorbax 300 SB-C8) HPLC chromatographic profiles of the Aβ peptides generated in the Tg2576 mice and isolated from AD brains. The chromatographic conditions were exactly the same for the two species. The top trace shows the brain parenchymal and vascular Aβ peptides generated by the Tg mice. The peptides were analyzed by mass spectrometry and automatic amino acid sequence as shown in Table 1. The major fractions, 7 and 8 (containing Aβ 1−40 and Aβ 1−42), were subsequently submitted to tryptic hydrolysis and CNBr cleavage. The resulting peptides were separated by C18 reverse-phase HPLC, and their composition was identified by automatic amino analysis. The bottom trace shows the complex chromatographic profile generated by the degraded and modified brain parenchymal and vascular AD-Aβ peptides. The peptides eluted between 37 and 65 min were identified by mass spectrometry (see Table 2). Most of the AD-Aβ peptides were also present as oxidized forms (M_r + 16). The retention times for the synthetic Aβ 17−42 and human-derived Aβ 1−40 dimeric peptides are indicated. Arrows indicate the interval between 37 and 61 min.

Table 1: Mass Spectrometry and Amino Acid Sequencing of APP2576 Tg Mice Amyloid Peptides[a]

peptide	M_r calcd	M_r obsd	proposed Aβ peptide	N-terminal AA sequence
1	4346.9	4347.8	1−40 ox	ND
2	4075.6	4076.8	1−37	ND
3	4132.6	4133.3	1−38	ND
4	4160.6	4159.0	1−38 f	ND
5	4231.7	4233.2	1−39	ND
6	4531.1	4530.2	1−42 ox	ND
7	4330.9	4331.2	1−40	DAEFRHDSGY
8	4358.9	4358.2	1−40 f	DAEFRHDSGY
9	4515.1	4514.7	1−42	DAEFRHDSGY
10	4553.1	4543.9	1−42 f	DAEFRHDSGY

[a] ND is not determined, ox is oxidized, and f is formulated.

Table 2: Mass Spectrometry of Human Amyloid Peptides

M_r calcd	M_r obsd	proposed Aβ peptide	M_r calcd	M_r obsd	proposed Aβ peptide
4008.5	4008.5	1−36	3315.9	3314.7	10−40[a]
4075.6	4076.7	1−37	3500.0	3501.0	10−42[a]
4132.6	4134.9	1−38[a]	3336.9	3337.7	11−42[a]
4231.7	4230.1	1−39	3318.9	3318.5	11PyE−42[a]
4330.9	4330.0	1−40[a]	2971.5	2972.7	14−42
4515.1	4515.3	1−42[a]	2522.0	2524.2	16−40
3903.4	3904.2	2−36[a]	2762.3	2762.7	16−42[a]
3960.5	3959.0	2−37	2393.8	2393.6	17−40
4045.5	4047.6	2−38	2578.1	2577.1	17−42[a]
4116.6	4118.1	2−39	2280.7	2279.6	18−40[a]
4215.8	4215.6	2−40[a]	2181.6	2179.8	19−42[a]
4400.0	4400.1	2−42[a]	9030.2	9029.4	1−42 dimer
3945.4	3945.4	3−38			
4328.9	4328.7	3−42	636.7	636.9	Tp 1−5
4126.7	4127.8	3PyE−40[c]			(L-isoAsp)[b]
4310.9	4312.0	3PyE−42[a]	636.7	637.4	Tp 1−5
4199.8	4199.4	4−42[a]			(D-isoAsp)[b]
4052.6	4052.1	5−42[a]	636.7	638.1	Tp 1−5
3712.2	3712.7	6−40			(D-Asp)[b]
3896.5	3896.4	6−42[a]	1336.5	1337.4	Tp 1−5
3575.1	3574.7	7−40			(7L-isoAsp)[b]
3759.3	3759.2	7−42[a]	1336.5	1337.9	Tp 6−16
3460.0	3460.7	8−40			(7D-isoAsp)[b]
3644.2	3643.6	8−42[a]	1336.5	1337.5	Tp 6−16
3557.2	355.9	9−42[a]			(7D-Asp)[b]

[a] Also present as 35 Met sulfoxide (M_r + 16) peptides. [b] Recovered from tryptic hydrolysis of Aβ-amyloid cores (*5*). [c] PyE = pyroglutamyl.

were trypsin digested and purified on a reverse-phase column in an attempt to separate peptides containing D-Asp as well as D- and L-iso-Asp at positions 1 and 7. In AD, up to 75% of the core amyloid is modified to iso-Asp 7 (*5*). This structural β-shift in the progression of the α-carbon peptide bonds renders the Aβ more insoluble and resistant to enzymatic degradation (*8*). Neither of these modifications were observed in the Tg2576 mice Aβ since the only recovered Tp peptide peaks corresponded to the nonmodified Aβ sequences of residues 1−5, 6−16, 17−28, 29−40, and 29−42 (data not shown). Cyanogen bromide (CNBr) cleavage at Met35 permitted the recovery of the sequences 29−35 (with residue 35 as homoserine lactone) and 36−42 in the insoluble core left after Tp hydrolysis.

In the Tg2576 mice no significant time-dependent changes in the total amounts of Aβ-40 to Aβ-42 ratios were observed at 0, 3, and 6 h post-mortem, remaining at 87:13, 91:9, and 90:10, respectively. Similar results were obtained with the APP23 Tg mice, where the ratios of Aβ-40 to Aβ-42 at 0, 2, and 4 h post-mortem were 92:8, 92:8, and 91:9, respectively. Not only were the relative ratios maintained, but the areas under their respective chromatographic peaks were also stable between 0, 4, and 6 h of post-mortem delay (data not shown). These observations demonstrated that Aβ 1−40 and Aβ 1−42 species were stable and not subject to significant enzymatic degradation in the immediate postmortem period. In addition, the internal ratios among the yields of Aβ 37/38, Aβ 39, Aβ 40, and Aβ 42 in the Tg2576 mice at 0, 3, and 6 h and APP23 Tg mice at 0, 2, and 4 h demonstrated little variation. In the former, these values were respectively 11:12:67:10, 14:10:69:7, and 13:15:65:7, whereas in the latter, they were 9:4:80:7; 6:4:80:7, and 6:3:83:8. These results suggest that the shorter peptides are probably not post-mortem derivatives of the longer species. The stability and notable absence in both Tg mice strains of the abundant and diverse posttranslational modifications observed in the AD neuritic plaque and cerebrovascular amyloid (*5*) suggest that these modifications are unlikely to represent post-mortem artifacts but are a manifestation of fundamental differences between rodent and human neurochemistry.

One of the most striking differences between the human and Tg mice amyloid was in their relative solubilities. The human fibrillar Aβ in the compact neuritic plaque cores and sheets attached to the walls of the leptomeningeal and intraparenchymal arteries are water insoluble. In addition, these Aβ deposits are stable and do not dissociate in the presence of ionic or nonionic detergents, strong denaturing agents (i.e., urea or guanidine hydrochloride), or chelating molecules, properties which have been exploited to facilitate their chemical isolation (*5*, *36*). In contrast, in the Tg2576 and APP23 Tg mice the amyloid cores were completely soluble in SDS solutions containing EDTA. In the βAPP Tg mice, EDTA increased the solubility of Aβ amyloid, suggesting that, among other factors, heavy metals bound by Coulombic forces are important in maintaining fibrillar stability (*37*, *38*). High-speed centrifugation of the Tg mice brain SDS−EDTA soluble fraction revealed amyloid-laden vascular material but failed to demonstrate the presence of any amyloid cores. The increased solubility of the Tg mice amyloid may also be due to the absence of posttranslational modifications and N-terminal degradations. Furthermore, our chemical analysis revealed no apparent Aβ cross-linked dimers within the Tg mice amyloid. In AD the oligomeric and cross-linked Aβ enhances the stability and insolubility of the amyloid (*5*, *6*, *8*). In a recent publication Kawarayabashi et al. (*31*), using antibodies raised against Aβ 1 iso-Asp, 1D-Asp, and 3-N-terminal pyroglutamyl, reported the presence of small quantities of these modified residues in older Tg2576 mice amyloid. We were unable to confirm these findings. However, trace quantities of these modifications may have escaped detection in our assays or, alternatively, the antibodies recognizing modified residues also cross-reacted with unmodified Aβ molecules. Nevertheless, the amounts and types of chemically modified Aβ detected in the Tg2576 are insubstantial in comparison to that of AD amyloid (Figure 3 and Tables 1 and 2) and unlikely to affect on the overall stability and solubility of the Tg mice amyloid.

Our experiments have revealed that while Tg2576 and APP23 Tg mice share key biochemical similarities, important transgene-specific differences exist in the ultimate pathological manifestations that may offer clues to the initiation of AD amyloidosis. We suggest that intense amyloid deposition in the vasculature and extracellular plaques is initiated when soluble Aβ clearance from the brain is impaired. The APP23 Tg mice have more amyloid deposited in both the parenchyma and the vasculature than do the Tg2576 mice (*17*, *27*, *32*, *39*). In the APP23 Tg mice the amount of vascular amyloid is overwhelming, reminiscent of the heaviest deposits seen in AD individuals having the apoE ϵ4/ϵ4 genotype (*32*). APP23 Tg animals produced high levels of water-soluble Aβ, amounting to over 50 μg/g of brain tissue, that was apparent at the age of 2 months and persisted during the entire life of these Tg mice (*32*). In contrast, the water-soluble Aβ in the Tg2576 mice brains remained at a basal level during the first 8 months of life and increased in an exponential mode thereafter (*25*). This was paralleled by a rapid age-correlated decline in plasma Aβ levels and was

also coincident with an exponential elevation in brain amyloid deposits. Intriguingly, in the APP23 Tg mice, the brain water-soluble Aβ levels were elevated throughout life while the plasma Aβ totals barely departed from those observed in their non-Tg litter mates. It is clear that simple overproduction of Aβ by APP transcription upregulation in neural tissue did not lead to an immediate and profound amyloid deposition initiation in both transgenic animals. We hypothesize that very early in the life of the APP23 Tg mice, the water-soluble Aβ was unable to escape into the circulation. The massive Aβ vascular deposition pattern evident in the APP23 Tg mice suggests that Aβ was entrapped in the periarterial spaces that contribute to the drainage of the brain's interstitial fluid to the cervical lymphatics and the systemic venous circulation (*40*). Aβ in the vascular walls of APP23 Tg mice was histologically evident at 9 months of age. The threshold for amyloid visualization on tissue sections has been estimated to be around 20 ng/ng of protein. Therefore, vascular amyloid deposition probably initiated well prior to this age.

Vaccination of certain Tg mice against Aβ has affected amyloid deposition in these animal AD models (*41*−*45*), leading to efforts to apply similar regimens to humans. The structural differences discovered between APP Tg mice and AD amyloid suggest that a simple and direct extrapolation of these results to Aβ-vaccinated humans may be optimistic. The greatest amyloid load reduction and lack of memory impairment in water maze tests have been obtained by immunizing the Tg mice with the N-terminal Aβ peptide residues 1−14 (44). Stimulating production of analogous human antibodies may be of limited efficacy in AD, because the majority of the Aβ peptides in the human amyloid deposits have a degraded N-terminal region and extensive posttranslational modifications in this domain (Table 2). However, antibodies recognizing the N-terminal region of Aβ could be effective in binding nascent, unmodified potentially cytotoxic peptides as well as acting to prevent amyloid deposition. Our experiments revealed clearly that the amyloid plaque deposits characteristic of Tg mice are "soft" and more readily disaggregated in comparison to those of human AD. The increased solubility of the Tg mouse amyloid is also probably due to the lack of associated molecules such as glycolipids, which are commonly present in the human parenchymal and vascular amyloid (*2*, *3*). However, as is the case with AD, in the Tg mice the vascular wall amyloid remained insoluble in the SDS−EDTA buffer, probably because of its association with the molecules of the extracellular matrix. All of these factors may hinder the antibody-mediated removal of neuritic plaque cores and vascular amyloid in AD patients.

Both Tg2576 and APP23 Tg mice produced substantially larger amounts of CT100 fragments than humans (Figure 1A,B; *46*), and this CT fragment overproduction may result in a unique pathologic condition in the Tg animals. Transgenic mice expressing the APP C-terminal 100 amino acid fragment replicate some AD neuropathology and exhibit cognitive deficiencies (*47*, *48*). Yet to be explored in immunized Tg mice is whether the massively overproduced and potentially neurotoxic human CT molecules are accessible to Aβ antibodies that bind deleterious extracellular epitopes. Antibody neutralization of CTs may explain why in Aβ-vaccinated Tg mice the total Aβ amounts, evaluated by immunoassays or by chromatography, remain nearly equivalent to nonvaccinated Tg mice (*42*), while there is a significant enhancement in memory and cognition as tested by the water maze.

In conclusion, fundamental structural differences exist between the amyloid characteristic of AD and that deposited in the Tg mice. These molecular dissimilarities may be due in part to intrinsic biochemical differences, disparities between species life spans, and variance in the duration of Aβ deposition which in the Tg mice spans approximately 2 years versus a period of possibly decades in humans. More importantly, the induced amyloid cascade in the Tg mice represents a single distressing event in the complex retinue of known and unknown pathological changes that must underlie AD. Despite these limitations, the differences in amyloid deposition exhibited by the APP2576 and the APP23 Tg mice parallel some pathological manifestations observed in AD associated with certain apoE genotypes. Transgenic mice have opened avenues for the testing of AD therapeutic interventions such β- and γ-secretase inhibitors, anti- and depolymerizing Aβ agents, and active and passive anti-Aβ immunizations. Aβ-vaccinated Tg mice show enhanced memory and cognition even though only a miniscule amount of Aβ antibodies reach the brain. While the results obtained through transgenic animal vaccinations against Aβ are significant and encouraging, the efficacy of these protocols in AD patients remains to be established.

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