Comparative Analysis of Aβ Chemical Structure and Amyloid Plaque Morphology of Transgenic Mice and Alzheimer Disease Brains

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ABSTRACT

We have undertaken an integrated chemical and morphological comparison of the amyloid-beta (Aβ) molecules and the amyloid plaques present in the brains of APP23 transgenic (tg) mice and human Alzheimer disease (AD) patients. Despite an apparent overall structural resemblance to AD pathology, our detailed chemical analyses revealed that while the amyloid plaques characteristic of AD contain cores that are highly resistant to chemical and physical disruption, the tg mice produced amyloid cores which were completely soluble in buffers containing sodium dodecyl sulfate. Aβ chemical alterations account for the extreme stability of AD plaque core amyloid. The corresponding lack of post-translational modifications such as N-terminal degradation, isomerization, racemization, pyroglutamyl formation, oxidation and covalently-linked dimers in the tg mouse Aβ provides an explanation for the differences in solubility between human AD and the APP23 tg mouse plaques. We hypothesize that either insufficient time is available for Aβ structural modifications or that the complex species-specific environment of the human disease is not precisely replicated in the tg mice. The appraisal of therapeutic agents or protocols in these animal models must be judged in the context of the lack of complete equivalence between the transgenic mouse plaques and the human AD lesions.

INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized by the presence of extracellular amyloid plaques composed principally of amyloid β (Aβ) surrounded by dystrophic neurites (1). This association and the
realization that the basis of certain early onset familial forms of AD seems to be the enhanced production of one or more Aβ peptides has led to the hypothesis that Aβ is intimately involved in the AD pathogenic process (2). A promising experimental approach to unraveling the role(s) of Aβ in AD pathology has been the construction and characterization of transgenic mice that overexpress the amyloid precursor protein (APP) (3-12). Several transgenic mouse lines have been described that produce Aβ deposits which accumulate in an age-dependent fashion and morphologically resemble the senile plaques characteristic of human AD (3, 6, 8, 12, 37, 38).

The APP23 transgenic (APP23 tg) mice contain an APP751 cDNA with the Swedish familial AD mutation under the control of the neuron-specific Thy-1 promoter and express this human gene at levels 7-fold greater than the endogenous murine APP (12). Longitudinal studies of these mice have revealed that extracellular amyloid deposits become evident as the APP23 tg mice age. These deposits exhibit, at their earliest appearance, the Congo Red birefringence characteristic of the dense core plaques of human AD (12). A gradual progression from a diffuse deposit to a dense plaque is not a feature of the APP23 tg mouse pathology, paralleling our previous finding (13) that the diffuse amyloid deposits of AD do not represent a precursor developmental stage of senile plaques.

A transgenic mouse model system that faithfully mimics every aspect of AD has not been developed. The APP23 tg mouse model reproduces some of the neuropathological changes associated with AD such as amyloid plaques with a core of amyloid, neuritic alterations and neuron loss (15) as well as astrogliosis, microglial activation and deposition of a cerebrovascular amyloid. (14). While an exact analog of
AD is not at hand and may not ultimately be attainable, the understanding, management, and mitigation of AD may be facilitated through the use of the available animal models. Even though the exact role of senile plaques remains undefined, massive amyloid deposition is clearly an AD hallmark and is hypothesized to be seminal in the pathophysiology of this disease. Because there have been no detailed biochemical studies of the amyloid present in the APP23 tg mouse, we have undertaken an integrated chemical and morphological study to examine and compare the biochemical and biophysical properties of the transgenic mouse amyloid plaques to those of AD patients.

MATERIALS AND METHODS

Preparation of brain formic acid lysates.

The generation of APP23 tg mice has been previously described (12). These mice express the human APP751 cDNA with the Swedish double mutation under the control of the neuron-specific mouse Thy-1 promoter fragment. The cerebral cortices, hippocampi and olfactory bulbs which contained the highest concentration of amyloid plaques in the transgenic (APP23) mice (12, 15), representing approximately 165 milligrams of tissue per animal, were carefully dissected from 5 male and 5 female animals at room temperature (RT). The average age of the animals was 22.5 months (range: 22.1 to 22.7 months). The tissue from each brain was finely minced and immediately homogenized in 3.0 ml of 90% (vol/vol) glass distilled formic acid (GDFA) using a glass (Dounce) homogenizer at RT. Samples of 1.5 ml were loaded into 2 ml thick-walled polyallomer centrifuge tubes. The acid-insoluble material was separated by centrifugation in a Sorvall TST 60.4 rotor at 217,000 × g for 1 h at 4° C. The clear supernatant was carefully
collected, avoiding the surface layer of lipid and the small acid-insoluble material deposited at the bottom of the tube.

**Collection of the brain lysate 3-8 kDa mass range molecules.**

Samples (500 µl) of the clear formic acid whole-brain (brain tissue and its vascular network) lysates, were fractionated by size-exclusion FPLC on a 1 cm x 30 cm Superose 12 column (Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated and developed with 80% GDFA. The Superose 12 column fractions were calibrated using a set of proteins of known molecular mass and with the reverse-sequence Aβ peptide residues 40-1. The peptides were loaded into a 500 µl injection loop and separated at a flow rate of 15 ml per h at RT, with the chromatographic eluate absorbance monitored at 280 nm. Fractions of 500 µl were collected into polypropylene tubes every 2 min and those containing the 3-8 kDa molecules (corresponding to a retention time interval of 50-66 min) were pooled into a single tube and 20 µl of 10% (wt/vol) betaine was added. The formic acid was removed by vacuum centrifugation (Savant Instruments, Farmingdale, NY).

**Purification of vascular amyloid.**

To isolate the amyloid deposited in the walls of the cerebral blood vessels, the complete cerebral hemispheres from 2 male and 2 female APP 23 tg mice were each sectioned into 3 coronal portions. The brain tissue was gently stirred for 24 hs in 300 ml of 50 mM Tris-HCl, pH 7.5 containing 2% SDS and 2 mM EDTA at RT. The SDS-insoluble tufts of blood vessels consisting of the extracellular matrix with attached insoluble vascular
amyloid were collected by filtration (20 µm nylon mesh) and washed with Tris–buffer. The insoluble vascular amyloid was solubilized and extracted with 3 ml of 80% GDFA, centrifuged at 217,000 x g for 1 h at 4°C and the supernatant submitted in aliquots of 500 µl to FPLC as described above for the fractionation of brain lysate samples.

**Purification of Aβ peptides from the 3-8 kDa range pool.**

The Aβ peptides present in the 3-8 kDa size-exclusion fractions were separated by reverse-phase HPLC on a 4.6 mmm x 150 mm Source 5RPC column (Amersham Pharmacia Biotech.) using a 500 µl injection loop. Solvent ‘A’ was 0.1% (vol/vol) 30% ammonium hydroxide in water adjusted with GDFA to pH 9.0 and solvent ‘B’ was 60% (vol/vol) acetonitrile and 40% (vol/vol) solvent ‘A’. The chromatography was developed at RT with a linear gradient from 25% to 50% solvent ‘B’ in 100 min at a flow rate of 1.0 ml per min with absorbance monitored at 214 nm. The 3-8 kDa fractions obtained from 4 separate FPLC runs of brain cleared lysate samples were pooled to provide the material for each reverse-phase HPLC separation. To render the peptides soluble, 20 µl of 2 N NaOH was added to each of the polypropylene tubes. After pooling, an additional 20 µl of 6 N NaOH was added, followed by 375 µl of baseline solvent (25% solvent ‘A’ / 75% solvent ‘B’) and a final addition of 25 µl of 6 N NaOH. The Aβ 1-40 and Aβ 1-42 chromatographic retention times were established using the corresponding synthetic peptides obtained from California Peptide Inc. (Napa Valley CA) which had been further purified in our laboratory by HPLC prior to their use as size markers.

**Tryptic hydrolysis and separation of Aβ peptides**
The Aβ peptides separated by reverse-phase HPLC were lyophilized, solubilized in 1 ml of 80% (vol/vol) GDFA and dialyzed (Spectra/Por No. 6, 1000 MW cutoff) against 2 changes of distilled water and 3 changes of 100 mM ammonium bicarbonate. Once equilibrium was attained, the Aβ peptides were digested with TPCK-treated trypsin (10 μg per specimen, Worthington Biochemicals Corp. Freehold, New Jersey) for 16 h at 37°C. The resulting tryptic peptides (Tp) were lyophilized, dissolved in 500 μl of 0.1% (vol/vol) trifluoracetic acid (TFA) in water, thoroughly mixed and centrifuged at 12,000 × g for 10 min to remove any insoluble material. The insoluble tryptic core, mainly consisting of the hydrophobic Aβ C-terminal peptide residues 29-42, was dissolved in 300 μl of 80% GDFA and, after the addition of a small crystal of CNBr, incubated for 16 h at room temperature. The soluble tryptic peptides and the CNBr-cleaved peptides were separated by HPLC at RT on a reverse-phase Spherisorb-ODS2 C18 column (4 mm × 250 mm, 5 μm beads, LKB, Bromma, Sweden). The chromatography was developed with a 90 min linear gradient of 0-20% acetonitrile in 0.1% TFA followed by a second linear gradient 20-60% acetonitrile, 0.1% TFA for 30 min using flow rates of 0.7 ml/ min. Absorbance was monitored at 214 nm. Alternatively, the tryptic peptides were separated on a Source 5RPC column (4.6 mm x 150 mm, 5 μm bead, Amersham Pharmacia Biotech.) using a linear gradient from 0-60% of solvent ‘B’ using the conditions described for the purification of Aβ peptides.

**Automatic amino acid analysis.**

The tryptic and CNBr-derived peptides were submitted to acid hydrolysis on a vapor phase system Work Station (Waters, Milford, MA) in 6 N HCl, 1% (wt/vol) phenol at
150° C for 100 min. After removal of the acid by vacuum centrifugation, the amino acid compositions of the peptide hydrolysates were determined at 570 nm and 440 nm using an automatic injector sampler-HPLC system (Thermo Separation Products, Fremont, CA) and a post-column ninhydrin reaction system (Pickering Laboratories, Inc., Mountain View, CA). The amino acid separations were performed on a sodium cation exchange column (4 mm × 150 mm, 5 µm beads, Pickering Laboratories, Inc.) using the reagents and programs provided by the manufacturer.

**Protein sequencing**

HPLC-purified samples were dissolved in 50 µl 50% (vol/vol) aqueous acetonitrile containing 0.1% TFA. For the major HPLC peaks, approximately 30 µl of the sample was dried on a fiberglass peptide disk (Beckman Coulter, Inc., Fullerton, CA) and used for amino acid sequence determination. Sequencing was performed on a Porton 2090E gas-phase protein sequencer (Beckman Coulter, Inc) equipped with an on-line Hewlett Packard 1090L HPLC. The remainder of the sample was used for mass spectrometry analyses.

**Mass spectrometric analyses of the tryptic digested Aβ peptides**

Mass spectrometry samples were prepared by mixing 5 µl of the dissolved HPLC-separated peptides with a saturated solution of α-cyano-4-hydroxycinnamic acid (also dissolved in 50% aqueous acetonitrile containing 0.1% TFA) and aliquots of approximately 1 ml were dried on the ends of stainless steel sample pins. For precise mass determinations, bovine insulin (MW 5,734.5 for the singly protonated molecular
ion) was added to the mixture of sample and matrix and used as an internal standard. Mass spectra were obtained using a Vestec Lasertec Research mass spectrometer (PE Biosystems, Framingham, MA) operated in the positive ion mode with an accelerating voltage of 23 kV. This instrument incorporates a Laser Science VSL-337ND nitrogen laser that provides 3 ns pulses of 337 nm light of variable intensity. Data were collected using a TDS 520 digital storage oscilloscope (Tektronix, Beaverton, OR) and analyzed on a personal computer using the LabCalc software (Galactic Industries Corp., Salem, NH). Each of the mass spectra reported represents the average of 128 shots.

Morphological characterization of amyloid plaques
Cerebral hemispheres from transgenic mice and control mice as well as cerebral cortex from human AD brains were fixed for 48 h in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, dehydrated by passage through an alcohol and xylene solution series, and embedded in paraffin wax. Sections of 5 μm were taken on a rotary microtome and stained with hematoxylin and eosin for general structure, thioflavine S for amyloid and the Campbell-Switzer silver stain for amyloid diffuse and plaques. Stained sections were viewed with bright-field light microscopy (H & E, Campbell-Switzer-stained sections), fluorescence microscopy and confocal scanning laser microscopy (thioflavine S-stained sections). Human and mouse sections were stained under identical conditions, in the same batch.

RESULTS
**APP23 transgenic mice produced more soluble amyloid plaques.**

One of the most striking characteristics of the amyloid cores present in AD patient brains is their extreme resistance to denaturing agents and detergents such as urea, guanidine hydrochloride and sodium dodecyl sulfate (SDS). During our initial attempt to enrich the amyloid cores from the APP23 tg mouse cerebral cortices using a 2% SDS solution, we noticed that in contrast to the human AD tissue samples, the parenchymal amyloid cores and fibrils completely disappeared. Extended high-speed centrifugation (250,000 × g for 3 h) of the SDS supernatant failed to sediment any dispersed amyloid fibrils in the animal sample. These observations suggested that the plaque cores present in the APP23 transgenic animals were substantially more soluble than those of the AD brain. In contrast to the parenchymal deposits, the amyloid deposited around the blood vessels in the APP23 tg mice after SDS treatment remained intact and as in the case of the AD brain, firmly attached to the vascular walls. We believe that the SDS insolubility characteristic of the vascular amyloid may in part be related to a stronger association between Aβ and the molecules of the extracellular matrix that required formic acid exposure for complete extraction.

Our experiments also revealed that a large proportion of the transgenic mouse parenchymal Aβ was freely soluble in detergent-free aqueous buffers. The APP23 tg mice brains were homogenized at 4° C in an aqueous buffer (20 mM Tris-HCl, pH 7.4) containing a mixture of protease inhibitors (16) followed by centrifugation (100,000 × g, 1 h). The soluble Aβ present in the resulting supernatants was quantified by europium immunoassay (45). At 22 months of age the total water-soluble Aβ and the total water-
insoluble Aβ represented averages of 56.4 µg/g of brain tissue and 750 µg/g of brain tissue, respectively.

Quantification and chemical characterization of the total Aβ present in APP23 transgenic mouse brain tissue. The increased water solubility of the APP23 tg mouse amyloid in comparison to its human AD counterpart suggested that fundamental structural and compositional differences exist between the amyloid plaques formed in transgenic mice and humans. Cortical areas, hippocampi and olfactory bulbs were carefully dissected from the brain and, in order to account for the total amyloid present in the brain parenchyma and vascular walls, the tissue was acid lysed and chromatographed in GDFA. Four discrete peaks were resolved by a size-exclusion column (Fig. 1) of which only the 3-8 kDa range molecules included Aβ. These fractions were pooled, their volumes reduced by vacuum centrifugation, and subjected to chromatography on a Source 5RPC reverse-phase column. A polystyrene/divinyl benzene reverse-phase column was employed in these experiments because, in our experience, Aβ 1-42 exhibits better solubility at alkaline pH (9.0) than at an acid pH (2.0). Of the several chromatographic conditions investigated, a linear gradient from 25-50% acetonitrile gave the best resolution and eliminated most of the contaminating molecules either as unbound material or in the > 50% acetonitrile eluate. A total of ten discrete fractions were recovered (Fig. 2). The two major fractions had retention times identical to the synthetic Aβ markers Aβ 1-40 and Aβ 1-42. All the fractions were submitted to mass spectrometry (Table 1) which revealed the presence of Aβ 1-40 in fraction 7 and Aβ1-42 in fraction 9. Seven steps of automatic amino acid sequencing yielded, in both cases, the authentic Aβ
N-terminal sequence DAEFRHD. Fractions 1 and 3 were identified as Aβ 1-40 with the Met (residue 35), oxidized to Met sulfoxide and Aβ 1-38, respectively. Both peptides also demonstrated the complete Aβ N-terminal amino acid sequence of DAEFRHD. The atomic masses of the molecules in the smaller peaks 2 and 5 corresponded to Aβ 1-37 and Aβ 1-39. We suggest that the Aβ peptides ending in residues 39, 38 and 37 in the tg mice represent soluble products of Aβ degradation. This suggestion is based on our recent analysis of the soluble Aβ peptides from the AD brain which also show a series of shorter C-terminal peptides (A. Roher and Y-M Kuo, unpublished results). Alternatively, the Aβ peptides ending at residues 39, 38 and 37 could represent by-products of γ-secretase-like activity. The loss of C-terminal residues greatly enhances the solubility of the Aβ peptides.

The minor fraction 4 was identified as Aβ 1-42 with an oxidized Met 35. The smaller peaks 6 and 10 were designated as formylated forms of Aβ 1-38 and Aβ 1-42 since their molecular weights were those of the corresponding peptides increased by 28 mass units. This was also the situation with a smaller mass spectrometry signal observed within peak 9 which was identified as formylated Aβ 1-40. The minute amounts of formylated Aβ are probably artifactual modifications resulting from the use of formic acid during the extraction and chromatographic procedures. The minor peak 8 had the same atomic mass as peak 7 (Aβ 1-40). Based on previous experience with chromatographic separations of Aβ we designated this fraction as Aβ with D-Asp either at position 7 or 23 (16, 18) which imparts a slightly delayed retention time on reverse-phase chromatography. Under the same chromatographic conditions the AD Aβ peptide residues 17-42, or P3 fraction, a
component of the diffuse amyloid deposits, has a retention time of approximately 70 minutes. No peaks were evident in this area in the case of the APP23 tg mice amyloid.

Finally, the mass spectra of peak 9 also revealed, in addition to its major component Aβ 1-42, a small signal with an atomic mass of 3,152.8 that may correspond to residues Aβ 11-40 (calculated mass: 3,151.7). This suggestion is supported by the amino acid sequence data that indicated a very small amount of protein, about 3-4% of the main peptide, starting with the sequence EVHHQ (Aβ residues 11-15). The finding of this minor peptide is interesting since in vitro experiments have suggested that the β-secretase, besides generating Aβ peptides starting at residue Asp1, is also capable of cleaving between residues Tyr10 and Glu11 of the Aβ sequence (40, 41). The presence of small quantities of Aβ starting at residue 11 in the brains of AD patients and in the APP23 tg mice also supports this contention (42-44). In addition, mass spectrometry analysis of non-transfected and βAPP-transfected mouse neuroblastoma cells has revealed the presence of Aβ peptides starting at residue 11 in the former but not in the latter (46). Furthermore, about forty Aβ-related peptides were detected in the conditioned media, probably resulting from the action of amino-, carboxy- and endo-peptidases (46).

To detect any post-translational Aβ modifications that were not evident using mass spectrometry, such as isomerization of Asp residues at positions 1 and 7, known to occur in the human Aβ peptides (16), we digested the Source 5RPC chromatography peaks 7 (Aβ 1-40) and 9 (Aβ 1-42) with trypsin. The resulting peptides were separated either by C18 reverse-phase or by 5RPC reverse-phase HPLC (Figs. 3 and 4), acid hydrolyzed, and their amino acid sequences were determined. In contrast to the complex tryptic digest pattern obtained from the human AD Aβ peptide, which revealed up to 14 distinct
peptides due to degradation of the N-terminal domain and to the presence of L-isoAsp, D-isoAsp, L-Asp and D-Asp at positions 1 and 7, and cyclization of Glu at position 3 (16, 19), the tg mouse Aβ tryptic digest map pattern was comparatively simple. The APP23 tg mice (peak 7) yielded only four tryptic digest peptides (Fig. 3), which corresponded to residues 1-5 (DAEFR), 6-16 (HDSGYEVHHQK), 17-28 (LVFFAEDVGSNK) and 29-40 (GAIIGLMVGGVV) of the Aβ peptide. The retention times of these Tp matched those of the Tp derived from synthetic Aβ 1-40 employed as calibration standards (Fig. 3). The C-terminal tryptic peptide of the Aβ 1-42 produced in the tg mice which precipitated as an insoluble core after tryptic digestion was further cleaved by CNBr at Met 35. This procedure generated two heptapeptides corresponding to Aβ residues 29-35 (GAIIGL-homoSer) and residues 36-42 (VGGVVIA). These two peptides were separated by C18 reverse-phase chromatography as previously described (ref. 16: data not shown). Chromatography of Tp at pH 9.0 yielded the expected pattern of four peptides (Fig. 3) and did not reveal the presence of any chemical modifications or N-terminal degradation.

**Characterization of the SDS insoluble vascular amyloid.** Separation of the Aβ peptides present in the cerebro-vascular walls by reverse-phase HPLC on a Source 5RPC column produced only 3 fractions corresponding to Aβ1-40 ox, Aβ 1-40 and Aβ1-42. Mass spectrometry revealed that the relative masses of these peptides were 4345.8, 4329.8 and 4517.0, respectively, which is in agreement with their theoretical expected values.

**Morphological characteristics of APP23 transgenic mice and human amyloid plaques.** As previously reported (12), senile plaques were present in the brains of the
APP23 tg mice and were readily apparent with silver and thioflavine S stains in mature animals (14 and 20 month animals). Most of the plaques were composed of compact amyloid, which fluoresced brightly with the thioflavine S stain (Fig. 5A), indicating the presence of a β–pleated sheet conformation. Old animals also displayed small numbers (<10%) of plaques which in humans have been termed “diffuse” deposits (20-22) (Fig. 5G, arrow). The internal structure of the compact plaques was most apparent after thioflavine S staining, which showed that they were composed of bundles of filaments radiating outward from a central core (Figs. 5A and 5C). These plaques ranged up to 200 µm in diameter, averaging around 80-120 µm. In comparison, human AD compact plaques (Figs. 5B and 5D) are classified as “burned-out” or “naked core” plaques and classical plaques (refs. 20-22). Burned out plaques consist simply of a central amyloid core about 10-20 µm diameter (not shown), while classical plaques have a central core surrounded by an empty region with a halo of fluorescent material with overall diameters averaging around 60-80 µm. Human classical plaques, like the tg mouse plaques, possess wisps of fluorescent material radiating from a central core region (Figs. 5C and 5D). In the tg mice, these wisps were clearly composed of filament bundles, while in the human they had no discernible internal structure.

The Campbell-Switzer stain revealed additional differences between the tg mouse and human AD plaques. Mouse compact plaques took up very little of the stain, appearing light brown in color (Figs. 5E and 5G), unlike compact human plaques, in which the core and halo regions were both stained intensely black (Fig. 5F). Many mouse compact plaques had a delicate halo of black fibrillar material, like that seen in surrounding diffuse plaques (Fig. 5E, arrow) while others, however, did not (Fig. 5G,
asterisk). Classical human AD plaques stained with thioflavine S possessed a relatively empty zone between the intensely stained core and halo (Fig. 5F), while no such empty region was observed in mouse plaques. Diffuse plaques, which were somewhat apparent with the thioflavine S stain, were, as with human diffuse plaques, more readily visualized with the Campbell-Switzer stain (Fig. 5G, arrow). These resembled human diffuse plaques (Fig. 5H, arrow) in their irregular shape and “cotton wool” appearance, although they lacked the fine granular material commonly observed in the diffuse plaques of the AD brain.

**DISCUSSION**

Amyloid plaque cores isolated from AD patients are entirely resistant to proteolytic degradation (23, 24) and only a limited number of powerful chaotrophic agents are capable of completely dissolving the human amyloid fibrils. Chemical characterization of the separated AD amyloid plaque components has revealed the presence of a complex mixture of Aβ and glycoproteins and glycolipids, of which the latter two represent about 20% of the total amyloid core mass (24, 25). The amyloid core insolubility is partially due to the presence of these ancillary molecules (ref. 28 and W. J. Goux and A. E. Roher unpublished results). In addition, the Aβ peptides isolated from the human brain have numerous post-translational modifications, are extensively degraded at the N-terminus and contain a high proportion of cross-linked Aβ molecules resulting from intermolecular linkages between tyrosyl residues (16, 26). Of the total amount of human Aβ solubilized from the amyloid cores by formic acid, monomeric, dimeric and trimeric/tetrameric Aβ molecules represent an average of 55%, 25% and 20%, of the total
amyloid, respectively. All these factors that contribute to the AD amyloid insolubility and resistance to proteolytic degradation are apparently absent in the APP tg mice.

Several transgenic mouse models that reproduce certain aspects of AD have been engineered (3-12, 37, 38). The APP23 transgenic mice develop an intense amyloid deposition in the cerebral vasculature and produce Congo red/thioflavine S birefringent extracellular plaques with associated microglia in the brain parenchyma (14, 27). Our experiments have revealed that the condensed amyloid plaque cores deposited in an age-specific pattern in the transgenic APP23 mice are not precise reproductions of those observed in AD, but differ in fundamental chemical and morphological aspects from the human senile plaques. The majority of the amyloid plaques in the APP23 tg mice consist of compact amyloid cores which morphologically resemble human plaque cores but are much larger and lack the clear zone and halo present in the classical human plaques. Furthermore, in the APP23 mice, there are relatively few diffuse amyloid deposits, which are characteristically abundant in the AD brain.

The increased solubility of the Aβ peptides in the APP23 tg mice relative to that of AD brains is clearly reflected by the amounts of these peptides extracted by detergent-free aqueous buffer per unit of tissue. The averaged yield (n=6, mean age 22 months) of total water-soluble Aβ, representing both 40 and 42 peptides, in the tg mice was 56.4 µg/g of brain. In contrast, the average (n=8, mean age=81 years) water-soluble total Aβ recovered from AD brains, using an identical extraction procedure, amounted to 48 ng/g of cortex (17). This is over a thousand-fold increase in the amount of water-soluble Aβ per unit weight in the APP23 tg mice over that obtained from the AD brain.
Chemical analyses of the Aβ isolated from the APP23 tg mice demonstrated that most of the Aβ molecules initiated at residue Asp1 in both the Aβ 40 and Aβ 42 forms. Only a minute proportion of Aβ 11-40 was detected in the tg mice. In the human, the N-terminal region of brain parenchymal Aβ is extensively degraded either by aminopeptidases, by β-secretase(s), or both. As reported by our laboratory and other investigators, in the human amyloid plaque cores the Aβ molecules initiate at residues 2, 3, 4, 5, 6, 8, 9, 10 and 17 (refs.13, 16, 18, 19, 29-32). In AD only a small proportion of Aβ starts at position 1 L-Asp in the amyloid plaques (~10%) and this fraction is higher in the vascular amyloid (~65%). In human amyloid plaques approximately 50% of the amyloid starts at position 3 in the form of pyro-Glu whereas in the vascular amyloid this form accounts for only 11% (19), while no Aβ molecules with pyro-Glu were detected in the APP23 tg mice. In the human Aβ isolated from the neuritic plaques and vascular walls the N-terminal Iso-Asp accounts for about 20% and 6% of the total Aβ, respectively, while none of this form was detected in the APP23 tg mice. With respect to Iso-Asp at position 7, about 75% of the Aβ in the human amyloid plaques carry this isomerization. This β-shift is present in lesser quantities in the vascular amyloid (~20%). Apparently the APP23 tg mice are free from this β-shift between the peptide bond of residues 7Asp-8Ser that drastically alters the conformation of the α-carbon backbone of Aβ peptide. Finally, only a reduced proportion (~10%) of the APP23 tg mouse Aβ appears to be oxidized at Met 35. An important difference between the tg mice and human experimental subjects is the unavoidable longer post-mortem delay in sample processing for the latter that may account for some of the changes present in the amyloid.
chemistry between the two species. The presence and extent of potential time-dependent post-mortem artifacts affecting amyloid chemistry is currently under investigation.

It is likely that the structural alterations of the Aβ peptides in the human neuritic plaque cores account for their remarkable physical stability as well as for the inability of the phagocytic cells to remove them from the brain during the course of AD progression. We have previously demonstrated that N-terminal truncation of the Aβ molecules results in the net loss of polar residues, thereby increasing the overall hydrophobicity of the peptides and rendering them significantly more water insoluble and resistant to enzymatic degradation (33). This tenet is clearly illustrated by the shorter Aβ 17-42 peptide which, even at low concentrations, avidly precipitates into amorphous aggregates and is abundant in the diffuse amyloid deposits present in the AD brain and Down’s syndrome (13, 36). It is also well established that isomerization of Asp residues results in a greater resistance to and even inhibition of proteolytic degradation in the Aβ peptides as well as in other molecules (33, 34). Oxidation of Met 35 to its sulfoxide form has also been reported to increase the stability of the Aβ peptides (33). The generation of pyroglutamyl at position 3 of Aβ blocks N-terminal degradation of these peptides by aminopeptidases and increases the hydrophobicity of Aβ (32, 33). Lastly, the irreversible oligomerization of Aβ due to stable dimers and trimer/tetramers hinders enzymatic degradation (33).

The average ratio among the four main Aβ fractions found in the APP23 tg mice, Aβ1-40 ox, Aβ1-38, Aβ 1-40, and Aβ1-42, which in this study represent the contribution of both vascular and parenchymal Aβ, was 12: 9: 50: 29. These values contrast with the human neuritict plaque amyloid cores in which the Aβ n-40 and Aβ n-42 have an average ratio of 15:85. The relative ratios of the tg mice vascular Aβ1-40 ox, Aβ 1-40 and Aβ 1-42
peptides were 7: 90: 3, respectively. These data suggest that in the APP23 tg mice the major contributor to the total amount of Aβ 1-40 is the vascular amyloid and that most of the brain’s Aβ 1-42 originates from the cores of parenchymal amyloid.

The values for the human vascular amyloid are more variable since the Aβ n-40 peptides have a range of 40-90%. The larger the amount of vascular amyloid deposited in AD, the greater the amount of Aβ40 peptide in the vessels, reaching a peak in those individuals with ApoE ε4/ε4 genotype (39). Furthermore, our studies have revealed two patterns of vascular amyloid deposition in the ApoE ε4 AD cases. In one of them, there is an overwhelming number of amyloid deposits affecting the form of spherical cores intimately attached to the basal lamina of the capillary network producing an ornate pattern that resembles ‘pussy willows’ (see Figs. 3C and 3D in ref. 29). In the second there is an abundance of arteriolar and small artery (up to 500 µm diameter) amyloid deposits that resemble those observed in the APP23 tg mice. The dominance of Aβ1-40 over Aβ 42 in this strain may reflect the heavy arterial amyloid deposition, an outstanding characteristic of the APP23 tg mice (ref. 14, and Kuo et al. unpublished observations).

One important factor that may explain the almost complete absence of structural changes in the Aβ isolated from the APP23 tg mice is that the alterations commonly observed in the human brain are thought to be time dependent. In the transgenic animals, amyloid is produced at levels that overwhelm the capacity of any endogenous clearance mechanisms and leads to a vastly accelerated depositional process. In the human brain, the process of amyloid accumulation begins several decades prior to the onset of clinical symptoms and continues to occur until the death of the patient. The life span of the
APP23 tg mice is just over two years, which may not be sufficient to manifest the full spectrum of structural Aβ changes observed in terminal AD (15). An additional possibility is that the structural modifications observed in AD do not represent merely a passive and random accumulation of time-dependent changes in Aβ structure, but are actually age- and perhaps species-specific (35), conceivably representing alterations in the expression and activity of key enzymes. It is possible that the processing required to create authentic AD plaques cannot occur in transgenic animals because either the necessary enzyme homologues are not present or the elevated pace of amyloid deposition simply precludes the prerequisite maturational reactions.

Morphological comparison of tg mouse and human senile plaques revealed some similarities and some differences. Both mouse and human possess compact and diffuse plaques (20-22). In AD, the diffuse amyloid exhibits a blend of short fibrils and aggregates of a fine granular material (A. E. Roher, unpublished results). Biochemical analysis of these granular collections revealed the presence of the Aβ sequence of residues 17-42 (13), a peptide apparently absent in the tg mice. The mouse compact plaques displayed a fibrillar internal structure that is not visible in human compact plaques. These plaque structures are significantly larger in the tg mice, being on average about twice the diameter of human classical plaques.

Amyloid plaques may be characterized by either the presence or absence of “dystrophic” neurites. In this respect, it has been reported that compact plaques in these tg mice are associated with dystrophic neurites expressing phosphorylated tau epitopes (12), but without recreation of the paired helical filaments seen in human classical plaques. Another important difference between the plaques observed in the APP23 tg
mice and the AD brain is that in the tg mice the plaques are morphologically homogeneous, whereas in the human AD brains a large variety of subtypes is typically observable.

Other βAPP tg mice strains have been constructed, including some in which there is an accelerated Aβ deposition resulting from the co-expression of mutant presenilin 1 and amyloid precursor proteins (37, 38). The precise degree of similarity of the amyloid deposits in these animals to those of AD awaits experimental investigation. Our studies indicate that the amyloid fibrils deposited in the brain of the APP23 tg mice are chemically and morphologically distinct from the ones accumulated in the AD brain. In conclusion, the appraisal of therapeutic agents or protocols directed toward inhibiting or reversing plaque formation in these animals must be judged in the context of this lack of complete equivalence between the transgenic mouse plaques and the AD lesions.

Acknowledgements: We are in debt to Dr. Dean Luehrs for constructive discussions. This work was partially supported by the state of Arizona Alzheimer’s Disease Research Center.
Table 1

Mass Spectrometry and Amino Acid Sequencing of APP23 tg Mice Amyloid Peptides

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>Mr Calculated</th>
<th>Mr Observed</th>
<th>Proposed Aβ peptide</th>
<th>N-terminal AA sequence</th>
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</thead>
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<tr>
<td>1</td>
<td>4346.9</td>
<td>4345.9</td>
<td>1-40 ox.</td>
<td>DAEFRHD</td>
</tr>
<tr>
<td>2</td>
<td>4075.6</td>
<td>4075.2</td>
<td>1-37</td>
<td>DAE</td>
</tr>
<tr>
<td>3</td>
<td>4132.6</td>
<td>4131.3</td>
<td>1-38</td>
<td>DAEFRHD</td>
</tr>
<tr>
<td>4</td>
<td>4531.1</td>
<td>4533.2</td>
<td>1-42 ox.</td>
<td>N/D</td>
</tr>
<tr>
<td>5</td>
<td>4231.2</td>
<td>4231.7</td>
<td>1-39</td>
<td>N/D</td>
</tr>
<tr>
<td>6</td>
<td>4160.1</td>
<td>4160.6</td>
<td>1-38 f</td>
<td>N/D</td>
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<tr>
<td>7</td>
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<td>DAEFRHD</td>
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<tr>
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<tr>
<td>9</td>
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<td>DAEFRHD</td>
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<tr>
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<td>4358.9</td>
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<td>N/D</td>
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<td>10</td>
<td>4543.1</td>
<td>4543.9</td>
<td>1-42 f</td>
<td>N/D</td>
</tr>
</tbody>
</table>

N/D: Not determined

LEGEND TO FIGURES

**Figure 1.** FPLC-Superose 12 size-exclusion chromatography of GDFA extracted APP23 tg mice brain lysates. The solid line depicts the UV absorbance profile of supernatants derived from high-speed centrifugation of GDFA brain lysates. The hyphenated line represents the peak produced by the Aβ peptide reverse-sequence residues 40-1 (Mr = 4,331 Da) employed as a column calibration standard and had a retention time between 52-60 min. The dotted trace depicts the UV absorbance profile produced by GDFA extracts obtained from human AD amyloid plaque cores. The human-derived amyloid produced 5 fractions (1-5) of which fractions 3, 4 and 5 were composed of Aβ trimers/tetramers, dimers and monomers, respectively. The APP23 tg mice brains yielded
four fractions (A-D). The tubes containing fraction C, (3-8 kDa peptides) were pooled and, after elimination of the acid, submitted to reverse-phase HPLC. The APP23 tg mouse Aβ was contained within the positive slope of fraction C.

**Figure 2.** HPLC-5RPC reverse-phase chromatographic separation of amyloid peptides from APP23 tg mice. Subjecting the Superose 12 size-exclusion column eluate peak C (Fig. 1) to reverse-phase chromatography resulted in ten discrete factions (1-10). These fractions were submitted to mass spectrometry analysis and peptide sequencing. The identification of each fraction is shown in Table 1. The lower chromatography trace is that of the calibration standards, synthetic Aβ 1-40 (A) and Aβ 1-42 (B) carried out under the same experimental conditions which had identical retention times as fractions 7 and 9 from the tg mice.

**Figure 3.** HPLC-C18 reverse-phase chromatographic profiles of tryptic peptides (Tp) derived from HPLC-5RPC reverse-phase chromatography peak 7 as shown in Figure 2. Four Aβ Tp corresponding to amino acid residues 1-5 (peak 1), 6-16 (peak 2), 17-28 (peak 3) and 29-40 (peak 4) were analyzed. The lower chromatogram is that of the four Tp obtained from trypsin digestion of synthetic Aβ 1-40. The retention times of peaks A, B, C and D obtained from synthetic Aβ coincide with those of fractions 1, 2, 3, and 4 obtained from tg mice. All tg mice Tp peaks were acid hydrolyzed and their composition determined by automatic amino acid analysis.
**Figure 4.** HPLC-5RPC reverse-phase chromatographic analysis of tryptic peptides (Tp) derived from HPLC-5RPC reverse-phase chromatography peak 9 shown in Figure 2. The peptides designated as A, B, C and D corresponded to the Aβ peptides residues 1-5, 6-16, 17-28 and 29-42. The low yield of the C-terminal peptide is due to its hydrophobic nature and precipitation after tryptic digestion. Tryptic hydrolysis was also carried out on the Aβ peptide residues 1-38 (peak 3 in Fig. 2). Separation of the resulting peptides using the 5RPC column revealed that the C-terminal Tp (residues 29-38) had a retention time of approximately 50 min which is indicated by a hyphenated trace and designated as peptide E.
Figure 5. Photomicrographs of tg mouse (A, C) and human (E, G) Alzheimer’s disease plaques stained with Thioflavine S and examined with fluorescence microscopy (A-D), or stained using the Campbell-Switzer silver method and examined with brightfield microscopy (E-H). Under conventional fluorescence microscopy (A, B), mouse compact plaques (A) appear as spherical structures composed of filamentous material, while human “classical plaques” (B, arrow) have a more complex structure (see discussion). Calibration bar = 60 µm and serves for both A and B. With confocal scanning laser microscopy (C, D), mouse compact plaques (C) consist of filaments bundles radiating from a central core. Human classical plaques (D) also possess a central core and radiating arms, but filaments are less apparent. Bar in C = 25 µm; bar in D = 15 µm. The cores of mouse compact plaques are relatively unstained by the Campbell-Switzer stain (E, arrow, and G, asterisk), while the cores of human compact plaques are intensely stained (F, arrow points to human classical plaque, observe the target-like appearance in which the “bull’s-eye” is the core). Both mouse (G) and human (H) possess “diffuse” plaques (arrows) which are similar in appearance. Bar in E = 120 µm.
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Figure 1
Comparative Analysis of Aβ Chemical Structure and Amyloid Plaque Morphology of Transgenic Mice and Alzheimer Disease Brains
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