Molecular Pathogenesis of Genetic and Inherited Diseases

Iowa Variant of Familial Alzheimer's Disease

Accumulation of Posttranslationally Modified A β D23N in Parenchymal and Cerebrovascular Amyloid Deposits

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Mutations within the amyloid- β (A β) sequence, especially those clustered at residues 21-23, which are linked to early onset familial Alzheimer's disease (AD), are primarily associated with cerebral amyloid angiopathy (CAA). The basis for this predominant vascular amyloid burden and the differential clinical phenotypes of cerebral hemorrhage/stroke in some patients and dementia in others remain unknown. The A β D23N Iowa mutation is associated with progressive AD-like dementia, often without clinically manifested intracerebral hemorrhage. Neuropathologically, the disease is characterized by predominant preamyloid deposits, severe CAA, and abundant neurofibrillary tangles in the presence of remarkably few mature plaques. Biochemical analyses using a combination of immunoprecipitation, mass spectrometry, amino acid sequence, and Western blot analysis performed after sequential tissue extractions to separately isolate soluble components, preamyloid, and fibrillar amyloid species indicated that the Iowa deposits are complex mixtures of mutated and nonmutated A β molecules. These molecules exhibited various degrees of solubility, were highly heterogeneous at both the N- and C-termini, and showed partial aspartate isomerization at positions 1, 7, and 23. This collection of A β species—the Iowa brain A β peptidome—contained clear imprints of amyloid clearance mechanisms yet highlighted the unique neuropathological features shared by a non-A β cerebral

amyloidosis, familial Danish dementia, in which neurofibrillary tangles coexist with extensive pre-amyloid deposition in the virtual absence of fibrillar lesions. These data therefore challenge the importance of neuritic plaques as the sole contributors for the development of dementia. (Am J Pathol 2010, 176:1841–1854; DOI: 10.2353/ajpath.2010.090636)

Amyloid β (A β) is the major constituent of the fibrils deposited in senile plaques and cerebral blood vessels of patients with Alzheimer's disease (AD) and Down's syndrome. It is an internal processing product of a larger type-I transmembrane precursor molecule APP, which is encoded by a single multiexonic gene located on chromosome 21.¹ Several mutations in the APP gene are associated with early onset familial AD (FAD) [reviewed in Refs. 2 and 3 and AD Mutation Database (www.molgen.ua.ac.be/ADMutations/)]. Many of these mutations, located either 5' or 3' of the nucleotide sequence coding for the A β peptide, result in an overproduction of A β , particularly A β 42, and are clinically associated with AD phenotype. In contrast, mutations located within the A β sequence (Table 1^{4–16}) are typically linked to early onset FAD and primarily associated with cerebral amyloid angiopathy (CAA), although they manifest with either cerebral hemorrhage or dementia. The bulk of the CAA-associated mutations are located in a short stretch—positions

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Table 1. APP Mutations Located within the A β Sequence

Kindred	Nucleotide change	Zygosity	APP codon	A β mutation	Onset (yrs)	Clinical phenotype		Neuropathology			References
						Cognitive impairment	Stroke/hemorrhage	Plaques	NFTs	CAA	
Italian	C > T	Homo	673	A β A2V	36	Yes	No	—	—	—	4
British	A > G	Hetero	677	A β H6R	55	Yes	No	—	—	—	5
Tottori	G > A	Hetero	678	A β D7N	60	Yes	No	—	—	—	6
Flemish	C > G	Hetero	692	A β A21G	35–60	Yes	Yes	Perivascular	Yes	Massive	7
Dutch	G > C	Hetero	693	A β E22Q	30–40	Yes	Yes	Diffuse	Rare	Massive	8
Italian	G > A	Hetero	693	A β E22K	50–60	Mild	Yes	Diffuse	No	Massive	9
Arctic	A > G	Hetero	693	A β E22G	55–60	Yes	No	Compact	Yes	Scarce	10,11
Japanese	delAGA	Hetero	693	A β E22 Δ	40–59	Yes	No	—	—	—	12
Iowa	G > A	Hetero	694	A β D23N	50–60	Yes	Rare	Diffuse	Yes	Massive	13
Piedmont	C > G	Hetero	705	A β L34V	50–70	Yes	Yes	No	No	Massive	14
Italian/Spanish	G > A	Hetero	713	A β A42T	58–68	Yes	Yes	Compact	Yes	Massive	15,16

—, unknown.

21–23—of A β although more recently others have been reported.

The first described A β mutation was found in a Dutch kindred, in which Gln replaced Glu at position 22 (A β E22Q) as a result of a single nucleotide transversion (G for C) at codon 693.⁸ Carriers of the mutation develop recurrent episodes of cerebral hemorrhages that correlated with massive amyloid deposition in the walls of leptomeningeal and cortical arteries and arterioles as well as in vessels in the brainstem and cerebellum.¹⁷ This phenotype was recapitulated, albeit at old age, in transgenic mice carrying the mutation.¹⁸ In addition to the vascular involvement, parenchymal amyloid deposits resembling the diffuse preamyloid lesions seen in AD are also observed in Dutch familial cases, whereas dense-core plaques and neurofibrillary tangles are rare or even completely absent.⁸ Cognitive deterioration generally manifests after the first stroke but in some cases it is the first symptom of the disease and may develop even before the appearance of focal lesions on brain imaging.^{2,19} A similar neuropathological phenotype with comparable clinical presentation to the Dutch form has been more recently reported in a Piedmont kindred in which a G for C transversion at codon 705 results in Leu for Val substitution at position 34 of A β (A β L34V).¹⁴ Neuropathological examination of the few available cases showed severe CAA with compromise of small and medium-size arteries as well as capillaries in all lobes of the brain, particularly the occipital and cerebellar regions. The vascular involvement includes vessel-within-vessel configurations, microhemorrhages, microaneurysms, microthrombi, and lymphocytic infiltration of the vessel walls whereas diffuse and dense-cored plaques as well as neurofibrillary pathology are notably absent. Cognitive impairment is infrequent as a presenting symptom but it is observed after various episodes of intracerebral hemorrhages.²

Two additional genetic variants were described at position 22 of A β in kindreds from Italy and Sweden. The Italian variant presents extensive CAA with hemorrhagic episodes of variable frequency depending on the individual cases. It relates to a G for A point mutation at codon 693 which translates in the replacement of Glu for Lys at position 22 of A β (A β E22K).⁹ This variant is clinically

characterized by a 10–20 year progression of recurrent strokes and mild cognitive decline. The neuropathological findings resemble those in the Dutch kindred with extensive A β deposits in the walls of leptomeningeal and cortical vessels whereas parenchymal compromise is limited to diffuse deposits with absence of mature plaques and neurofibrillary tangles. In contrast, patients from the Swedish kindred develop typical AD pathology without the severe amyloid angiopathy that characterizes other mutations localized within the A β sequence. The disease shows memory impairment at early onset—mean age 57 years—with progressive cognitive decline rather than stroke. The parenchymal compromise is similar (if not identical) to that in AD, including the presence of parenchymal plaque deposits in association with dystrophic neurites and neurofibrillary tangles.^{10,11} In this case the genetic defect, originating in an A to G transition at codon 693, translates into a single amino acid substitution Glu to Gly at position 22 of A β generating the so-called Arctic A β variant (A β E22G).

A familial form of AD present in Flemish patients originates in a different mutation at codon 692, a C to G transversion, resulting in Ala to Gly substitution at position 21 of A β (A β A21G). The patients exhibit cerebral hemorrhages with progressive dementia and AD-like pathology developing in survivors of the multiple hemorrhagic episodes.⁷ Affected brains demonstrate diffuse cortical atrophy, an abundance of vascular and parenchymal A β deposits, and neurofibrillary tangles. Whereas some of the cases present with lobar intracerebral hemorrhage, other members developed presenile dementia. Vascular amyloid is widespread in leptomeningeal and cortical vessel walls, including capillaries. Although diffuse plaques are present, there is a predominance of mature plaques, typically surrounded by τ -reactive dystrophic neurites. Remarkably, these plaques are mostly of perivascular or vasocentric nature appearing to radiate from the affected vessel, a feature that suggests that the AD pathology might be a secondary consequence to CAA. Also presenting with severe vascular compromise in association with compact plaques and neurofibrillary tangles is the G to A mutation occurring at position 42—A β A42T—found in an Italian and a Spanish kindreds.¹⁵ The normal architecture of leptomeningeal arteries and small parenchymal vessels in the cere-

bral hemisphere and cerebellum is severely disrupted by amyloid deposition, presenting thickening and double barreling of the walls, loss of smooth muscle cells, and narrowing of the lumina.

Additional APP mutations have been recently discovered through DNA sequencing and although thus far neuropathological information is not available, their clinical manifestations correlate with early onset familial AD. These include A β H6R present in a family from the United Kingdom⁵ and A β D7N found in a Japanese-Tottori pedigree in patients showing no signs of vascular involvement either clinically or neuroradiologically.⁶ A deletion mutation (E693 Δ), which results in a variant-A β lacking glutamate at position 22, was reported also in Japan in patients showing Alzheimer's-type dementia.¹² The mutation results in a variant-A β with enhanced intracellular accumulation of peptide oligomers in endoplasmic reticulum, Golgi apparatus, early and late endosomes, lysosomes, and autophagosomes.²⁰ Consistent with the non-fibrillogenic property of E22 Δ , a very low amyloid signal was observed in positron emission tomography using Pittsburgh compound-B.^{12,21} The latest intra-A β mutation reported consists of an Ala-to-Val substitution at residue 2 (A β A2V) that leads to AD only in the homozygous state. The genetic defect induces a very aggressive early-onset phenotype with established behavioral changes and cognitive deficits at very early age evolving toward severe dementia with spastic tetraparesis, and complete loss of autonomy in about 8 years. Notably the disease affected two homozygous siblings of the family, whereas six relatives—aged between 21 and 88 years—who carried the mutation in the heterozygous state, were not affected, as deduced by their neuropsychological assessment.⁴

Members of an Iowa pedigree of German descent are carriers of the only APP mutation reported at codon 694, a G to A transition, which predicts a substitution of Asp for Asn at position 23 of A β (A β D23N).¹³ Patients develop early onset, progressive, AD-like dementia with cerebral atrophy, leukoencephalopathy, and occipital lesions constituted by calcified amyloid-laden meningeal vessels. Although small hemorrhages could be identified by magnetic resonance imaging and postmortem examination, no episodes of clinically manifest intracerebral hemorrhage have been reported. In contrast, a second family from Spain carrying the same mutation is associated with symptomatic cerebral hemorrhage in most of the affected members²² suggesting that the presence of the mutation is not in itself sufficient for the induction of a specific clinical phenotype, and that other still undefined factors contribute to the diverse clinical presentation. The neuropathological features of Iowa cases notably resemble our findings—predominant CAA, extensive preamyloid pathology, hippocampal neurofibrillary tangles but very few or no neuritic plaques—in a non-A β cerebral amyloidosis associated with dementia in a Danish kindred,²³ challenging the importance of neuritic plaques as critical components for the development of AD-like pathology and dementia.²⁴

The biochemical composition of the A β lesions in FAD cases associated with A β genetic variants has been assessed in only a few instances, the Dutch deposits being

perhaps the most thoroughly studied.^{25,26} Herein, we present data indicating that A β deposits in carriers of the FAD Iowa variant are complex mixtures of mutated and nonmutated A β species (A β 23N and A β 23D) with very diverse solubility, highly heterogeneous at both N- and C-termini and exhibiting partial isomerization of Asp residues at positions 1, 7 and 23. Complementary *in vitro* studies using multiple synthetic homologues argue that the exacerbated mechanism of fibrillization is primarily driven by the mutation whereas the presence of post-translationally modified isoAsp residues only add a modest contribution to the wild-type A β 40 aggregation proclivity. Overall, the present biochemical data indicates that the A β species composing the lesions certainly contain imprints of amyloid clearance mechanisms and of the putative enzymatic pathways involved.

Materials and Methods

Materials

Monoclonal antibodies 4G8 and 6E10 were purchased from Covance (Princeton, NJ); rabbit polyclonal anti-A β 40 and anti-A β 42 as well as paramagnetic beads pre-coated with anti-rabbit or anti-mouse IgG (Dynabeads M-280) were obtained from Invitrogen (Carlsbad, CA). Sequencing-grade trypsin—pretreated with L-(tosyl-amido-2-phenyl) ethyl chloromethyl ketone to inhibit contaminating chymotryptic activity—as well as Complete Protease Inhibitors mixture were purchased from Roche (Indianapolis, IN). Microreverse-phase ZipTip C4 columns were purchased from Invitrogen, reverse-phase (RP) columns 214TP52 C4 and 218TP52 C18 from Vydac (Hesperia, CA) and Aquasil C18 columns from Thermo Electron (Bellefonte, PA). SDS-PAGE was from Pierce (Rockford, IL), Isoquant Isoaspartate detection kit from Promega (Madison WI), and all chemicals from Sigma-Aldrich (St. Louis, MO).

Wild-type A β 1-40 and A β 1-42, A β 1-40 homologues containing two (positions 7 and 23) or three (positions 1, 7, and 23) isoAsp residues, as well as the D23N variant A β 40 peptides with and without isoAsp 1 and 7 were synthesized using *N*-tert-butylloxycarbonyl chemistry by James I. Elliott at Yale University (New Haven, CT). Peptides were purified by RP-high-performance liquid chromatography (HPLC), their molecular masses were corroborated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and concentrations assessed by amino acid analysis, as described previously.²⁷

Clinical Data

Frozen brain tissue was obtained at autopsy from two cases (IV-1 and IV-2) of the Iowa kindred in which the G to A mutation at codon 694 of APP had been previously identified by DNA sequence analysis,¹³ with pertinent approval from the institutional review board. Case IV-1 was a 68-year-old male with progressive aphasic dementia whose neurological manifestations started at age 48

with brief episodes of confusion that evolved into cognitive decline at age 53. By age 67, the patient demonstrated severe dementia, speech limitations, myoclonus and short-stepped gait, dying a year later from complications of pneumonia.¹³ Case IV-2 was a 66-year-old male that presented with memory impairment and speech changes at age 58 and subsequently developed aphasia, personality changes, myoclonic jerks, memory disturbance, apraxia, and occasional gait disturbance. He died as a consequence of an accident. Postmortem interval until tissue cryopreservation was 7 hours for case IV-1 and 2 hours for case IV-2. Both cases were found to be heterozygous $\epsilon 3/\epsilon 4$ for the ApoE isoforms.²²

Frozen brain tissue from three neuropathologically confirmed AD cases were obtained from the SunHealth Institute Brain Bank (Sun City, AZ). Case 1 was a 84 years old $\epsilon 3/\epsilon 4$ female with 14 years of disease duration and Braak stage VI; case 2 was a 74-year-old $\epsilon 3/\epsilon 4$ male with 12 years of disease duration and Braak stage VI; and case 3 was a 86-year-old $\epsilon 3/\epsilon 4$ female with 9 years disease duration and Braak stage V. Postmortem intervals were 3.5 hours, 2.5 hours and 2.4 hours, respectively.

Immunohistochemical Analysis

Formalin-fixed paraffin-embedded 7- μ m brain tissue sections were evaluated for amyloid load by immunohistochemistry (4G8; 1/200) before biochemical analysis. Antigen retrieval was performed by 10-minute pretreatment with 99% FA before immunostaining. Antibody binding was visualized with ABC complex (DakoCytomation, Carpinteria, CA) and color developed with di-aminobenzidine/ H_2O_2 , followed by hematoxylin counterstaining as described previously.²⁸ Thioflavin-S staining of fibrillar amyloid was performed with a 1% solution (w/v in distilled water) for 30 minutes, followed by differentiation in 80% ethanol to remove fluorochrome excess. Congo red staining was performed using a 1% Congo red alkaline solution in 80% ethanol for 30 minutes, followed by hematoxylin counterstaining, and visualization under polarized light.

Brain Tissue Fractionation

Nonfibrillar and fibrillar A β deposits were sequentially extracted from frozen frontal lobe tissue of both IV-1 and IV-2 cases taking advantage of the differential solubility of preamyloid (usually poorly soluble in PBS solutions but soluble in 2% SDS) in comparison with amyloid materials, typically soluble in 70 to 98% formic acid (FA), as described previously.²⁸ Frontal cortex was divided into gray and white matters, and leptomeningeal vessels carefully removed and analyzed separately. The bulk of microvessels were collected by subsequent filtration through 70- and 30- μ m nylon meshes and combined with the leptomeningeal vessel fraction for analysis. Typically, 1 g of vessel-depleted gray matter and 100 mg of vessels—the common average yield for 1g of gray matter—were separately homogenized in 5 ml of ice-cold PBS containing Complete Protease Inhibitors, as described previously.²⁸

Homogenates were centrifuged (Beckman XL ultracentrifuge, 50.2 Ti rotor; 112,000 $\times g$; 1 hour, 4°C), and the PBS extractions of the remaining pellets repeated until the protein content in the fractions, estimated by absorbance at 280 nm, was <10% of that of the first extraction (typically approximately six times for gray matter and approximately three times for vessels). The remaining pellets were further subsequently extracted with 2% SDS (w/v in 20 mmol/L Tris (pH 7.4)) and 70% (v/v) FA as described previously.²⁸ The protein content in each fraction was quantitated using bicinchoninic acid (Pierce) and bovine serum albumin as standard.

Immunoprecipitation and Biochemical Analysis of Extracted A β Molecules

The PBS- and SDS-extracted fractions used for the immunoprecipitation (IP) studies originated from 100 mg of frontal cortex, whereas the FA fractions, enriched in A β content, corresponded to the material extracted from only 2.5 mg of frontal cortex. A β molecules were immunoprecipitated with paramagnetic beads coated with a combination of 4G8 and 6E10 antibodies, as described previously.^{28,29} Bound materials were eluted with either 5 μ l of acetonitrile-water-trifluoroacetic acid (20:20:1; v/v/v) for MS³⁰ or 10 μ l of Tris-Tricine sample buffer containing dithiothreitol for Western blot (WB).²⁸

Mass Spectrometry Analysis

Extracted peptide-immunoprecipitates were subjected to MALDI-TOF MS at NYU Protein Analysis Facility on a Micromass ToF-Spec-2E MALDI-TOF mass spectrometer in linear mode using a 10 mg/ml α -cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile/0.1% trifluoroacetic acid (TFA) and standard instrument settings.^{28,31} Under these acidic conditions, which render noncovalent binding unobservable,³² the different amyloid molecules were detected as monomeric species, independent of their aggregation propensity. Internal and/or external mass calibration was performed using human adrenocorticotrophic hormone peptide 18–39 (average mass = 2465.68 Da) and insulin (average mass = 5733.49 Da) as standards.

WB Analysis

Samples from each of the extracts, either before (in the case of FA-fractions) or after IP (for SDS- and PBS-solubilized fractions), were separated on 16.5% Tris-Tricine SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). After transference, membranes were boiled for 5 minutes to increase A β immunoreactivity,³³ blocked with 5% nonfat milk in PBS (pH 7.4), containing 0.1% Tween 20, and probed with 4G8, 6E10, anti-A β 40 or anti-A β 42 as primary antibodies^{28,34} followed by incubation with horseradish peroxidase-labeled F(ab')₂ anti-mouse or anti-rabbit IgG (GE Healthcare Life Sciences, Piscataway, NJ).

Fluorograms were developed by enhanced chemiluminescence with ECL Western blotting detection reagent (GE Healthcare Life Sciences) and exposed to Hyperfilm Enhanced Chemiluminescence (GE Healthcare Life Sciences). To verify that the WB experimental conditions were able to detect A β 1-42 oligomeric assemblies, monomeric and oligomerized synthetic A β 1-42 (200 ng) were electrophoresed, transferred to polyvinylidene difluoride membranes, and probed versus C-terminal specific anti-A β 42 antibodies under identical conditions as the tissue extracts.

Amino Acid Sequence Analysis

Automatic Edman degradation was performed on a 494 Procise Protein Sequencer (Applied Biosystems; Foster City, CA) in either: i) the prominent 4 kDa A β monomeric bands from FA extracts transferred to polyvinylidene difluoride membranes after separation on 16.5% Tris-Tricine SDS-PAGE or ii) polybrene-precoated glass filters loaded with HPLC-purified tryptic peptide A β 17-28, as indicated below, for the identification of the amino acid residue at position 23.

Analysis of Amino Acid Heterogeneity at Position 23

FA-fractions containing 10 μ g of protein were lyophilized, redissolved in 100 mmol/L Tris (pH 8), digested with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-trypsin (1:20 w/w) for 2 hours at 30°C, and the resulting peptides separated by RP-HPLC using a Vydac 218TP52 C18 column and a 25 minutes 1 to 40% linear gradient of acetonitrile in 0.1% trifluoroacetic acid (flow rate: 200 μ l/min), monitoring their elution at 214 nm. Tryptic A β fragments in each HPLC fraction were identified by MALDI-TOF MS in linear mode and the fractions containing A β 17-28 were further characterized by a combination of amino acid sequence analysis and MALDI-TOF in reflectron mode to more accurately distinguish the expected one unit mass difference between tryptic peptides containing either Asp or Asn at position 23. For standardization purposes, parallel trypsin digestion experiments were performed *in vitro* using synthetic A β 40 with either Asp or Asn at position 23.

Detection of IsoAsp Residues

The presence of isoAsp residues was tested via Isoquant Isoaspartate Detection Kit in all tryptic fragments generated from FA-fractions and subsequently separated by RP-HPLC. In brief, lyophilized peptides were dissolved in 100 mmol/L phosphate buffer (pH 6.8) containing 1 mmol/L EGTA/0.16% Triton X-100 and allowed to react with protein L-isoaspartyl methyltransferase, which catalyzes the transfer of a methyl group from S-adenosyl-L methionine (SAM) to isoAsp with the concomitant generation of S-adenosyl homocystein (SAH).³⁵ The production of SAH was detected by RP-HPLC using a

150 mm $\times$ 4.6 mm Aquasil C18 column equilibrated in 10 mmol/L potassium phosphate, pH 6.2 and a 20-minute 10 to 40% linear gradient of methanol (flow rate: 400 μ l/min); elution profiles were monitored by absorbance at 260 nm. Synthetic peptide WAGG-isoD-ASGE and SAH (Promega) were used as positive controls whereas A β 29-40, devoid of any Asp residues, served as a negative control.

In Vitro Fibrillization of A β D23N and IsoAsp-Containing Synthetic Homologues

Wild-type A β 1-40 and homologues containing two (positions 7 and 23) or three (positions 1, 7, and 23) isoAsp residues, as well as the D23N variant peptides with and without isoAsp 1 and 7 were dissolved to 1 mmol/L in hexafluoro-isopropanol (Sigma-Aldrich), a pretreatment that breaks down β -sheet structures and disrupts hydrophobic forces leading to monodisperse A β preparations.³⁶ After 2 hours incubation at room temperature, peptides were lyophilized to remove hexafluoro-isopropanol and thoroughly dissolved to 1.5 mmol/L in 01% ammonium hydroxide followed by the addition of deionized water and 2 $\times$ concentrated PBS (pH 7.4) to a final concentration of 1 mg/ml in 1 $\times$ PBS. Reconstituted peptides were incubated at 37°C for up to 3 days and the fibrillization profiles assessed by Thioflavin T binding assay as described previously.³⁷ Briefly, 6- μ l aliquots of each of the peptide aggregation time-point samples were added to 10 μ l of 0.1 mmol/L Thioflavin T (Sigma-Aldrich) and 50 mmol/L Tris-HCl buffer (pH 8.5) to a final volume of 200 μ l. Fluorescence was recorded after 300 seconds in a LS-50B luminescence spectrometer (PerkinElmer, Waltham, MA) with excitation and emission wavelengths of 435 nm (slit width = 10 nm) and 490 nm (slit width = 10 nm), respectively, as described previously.^{38,39} Each sample was analyzed in duplicate.

Results

Main Pathological Features of Iowa Variant Cases

Figure 1 illustrates the extent of A β deposition in patients from the Iowa pedigree, which is characterized by a profuse cerebrovascular compromise (Figure 1A) and abundant presence of preamyloid deposits (Figure 1, B and C, arrows) with limited fibrillar, compact parenchymal plaques, which, in many instances, are vasocentric (double arrows in Figure 1, B and C). The fibrillar nature of the vascular deposits is highlighted by both Congo red (Figure 1, D and E) and Thioflavin-S (Figure 1F) staining, whereas the high anti-A β parenchymal immunoreactivity in combination with the limited fluorescent signal of the Thioflavin-S illustrates the extent of the predominant diffuse parenchymal deposits.

Figure 1. Immunohistochemical analysis of parenchymal and vascular lesions in the Iowa pedigree. Vascular anti- $A\beta$ (4G8) immunoreactivity (**A**); preamyloid lesions (arrows, **B**); vasocentric plaques (double arrow, **C**); Congo red positive, birefringent vascular deposits (**D** and **E**); and thioflavin S fluorescent vascular deposits (**F**). Scale bar represents 200 μ m in **A** and 100 μ m in **B–F**.

Extraction of Soluble and Deposited $A\beta$ Species by Sequential Solubilization

The biochemical characterization of amyloid deposits was performed in frozen brain tissue obtained at autopsy with short postmortem delay. $A\beta$ species were sequentially extracted following protocols previously developed in our laboratory.²⁸ Soluble $A\beta$ peptides are typically recovered in the water-based extracts; oligomeric nonfibrillar $A\beta$ species found concentrated in pre-amyloid lesions

are retrieved by detergent-based solutions, whereas the more insoluble fibrillar $A\beta$ is extracted with FA.

$A\beta$ Species in Water-Soluble Extracts

SDS-PAGE of the immunoprecipitated PBS-soluble $A\beta$ fraction from case IV-2 brain parenchyma showed mainly monomeric with faint dimeric components, which were immunoreactive with 4G8, 6E10, and anti- $A\beta$ 40 but not with anti- $A\beta$ 42 (Figure 2A). Monomers appeared as a doublet of which the lower molecular mass band was the major component. MS identified, in addition to the full-length $A\beta$ 1-40, N- and C-terminally truncated species, particularly at positions 4, 34, and 38 (Figure 2, A and C). Parenchymal extracts from case IV-1 rendered similar results although the $A\beta$ 4-34 derivative was predominant over the other species listed in Figure 2C, perhaps reflecting additional proteolytic degradation resulting from the longer postmortem delay. Vascular PBS-extracts (Figure 2B) presented a similar immunoreactivity with 4G8, 6E10, and anti- $A\beta$ 40 as the parenchymal extracts but were enriched in $A\beta$ species exhibiting a higher degree of polymerization, whereas immunoreactivity with anti- $A\beta$ 42 was also very weak. In addition, most bands in the SDS-PAGE appeared more heterogeneous, suggesting the presence of more than one component. Consistent with these data $A\beta$ 1-40 and C-terminally truncated $A\beta$ 1-38 species coexisted with other N- and C-terminally degraded components, as revealed by MS. $A\beta$ 4-34 was not obvious in the vessel fraction although N-terminally intact $A\beta$ 1-34 was present (Figure 2, B and C). No significant differences between the two cases studied were observed in the vascular $A\beta$ composition either by WB or by MS. Validating the specificity of our detection system and supporting the present finding of a negligible amount of $A\beta$ 1-42 in PBS extracts, synthetic $A\beta$ 1-42 either in monomeric or oligomeric state was highly immunoreactive with the same C-terminal specific anti- $A\beta$ 42 antibody used in Figure 2 (Supplemental Figure 1, see <http://ajp.amjpathol.org>).

$A\beta$ Species in SDS-Extractable Deposits

Overall, immunoprecipitated SDS extracts exhibited a higher degree of $A\beta$ polymerization and less heterogeneity than the water-soluble counterparts, a feature also revealed by MS analysis of extracts from AD cases (see Supplemental Figure 2, see <http://ajp.amjpathol.org>). In parenchyma, monomers, dimers, and higher-order aggregates were labeled by 4G8, 6E10, and anti- $A\beta$ 40. As happened with the PBS-soluble fractions, parenchymal SDS extracts were weakly immunoreactive with anti- $A\beta$ 42. Monomers also appeared as doublets like in the PBS extracts, although in the SDS fractions the upper band seemed to be the main component of the doublet (Figure 3A). Consistent with the presence of more than one band in the monomeric component, MS identified full-length $A\beta$ 1-40 as well as N- and C-terminal truncated species degraded at positions 2, 4, 37, and 38 (Figure 3, A and C).

In the vascular fractions, SDS-soluble $A\beta$ was more abundant than in parenchymal extracts and vastly polymer-

Figure 2. WB and MS analysis of PBS-extracted A β . A β species retrieved from fractions extracted from 100 mg of microvessel-depleted frontal cortex (A); 10 mg of microvessels and leptomeningeal vessels recovered from 100 mg of frontal cortex (B). C: Theoretical and experimental m/z values. WB: * indicates residual Ig light and heavy chains from the IP. MS: ** illustrates nonspecific peaks also present in negative controls.

ized. Antibodies 4G8, 6E10, and anti-A β 40 extensively labeled these oligomeric species whereas anti-A β 42 highlighted only a signal of A β 42 monomers. MS data indicated the presence of A β 1-40 coexisting with A β species truncated at positions 2, 4, and 38 (Figure 3, B and C). Consistent with the faint band in the WB, A β 1-42 was detectable in the IP/MS at lower ratio than in comparable SDS extracts from AD cases analyzed by the same procedure (Supplemental Figure 2, see <http://ajp.amjpathol.org>). Variations between cases IV-1 and IV-2 were not evident.

A β Species in FA-Extractable Deposits

FA-extracts from both parenchyma (Figure 4A) and vessels (Figure 4B) contained highly polymerized A β . Consistent with the pathological features of Iowa cases—extensive CAA in almost complete absence of mature plaques—the amount of A β recovered in the FA vessel fractions was significantly more abundant than in the parenchymal extracts. Although the degree of A β polymerization was similar to that in SDS extracts, the total amount of A β retrieved with FA (>50-fold higher) allowed visualization of A β signals by

WB without prior IP. C-terminal specific labeling by anti-A β 40 or anti-A β 42 indicated that most of the deposited A β consisted of A β _x-40 species with an almost negligible contribution of A β _x-42 components. MS data identified A β 1-40 as well as N- and C-terminal truncated forms, such as A β 2-40, A β 4-40, A β 2-37, and A β 1-38 in both parenchyma and vessels. MS signal of A β 11-42 bearing pyroglutamate at position 11 (A β 11-42pE) was detected in parenchymal deposits, whereas A β 1-42 was also identified in both parenchymal and vascular extracts. Similar A β species were obtained from both cases studied (Figure 4C). Comparison with FA extracts from AD cases further corroborated the minor contribution of A β 42 to the amyloid deposits in the Iowa cases (Supplemental Figure 2, see <http://ajp.amjpathol.org>).

Coexistence of Wild-Type and A β D23N Species in Iowa-Amyloid Deposits

The existence of heterogeneity at position 23 was tested in FA fractions, which contained the bulk of the extracted

Figure 3. WB and MS analysis of SDS-extracted Aβ. Aβ species retrieved from 100 mg of microvessel-depleted frontal cortex (**A**); 10 mg of microvessels and leptomeningeal vessels recovered from 100 mg of frontal cortex (**B**). **C:** Theoretical and experimental *m/z* values. WB: * indicates residual Ig chains. MS: ** indicates nonspecific peaks appearing in the negative controls.

Aβ. MS in linear mode was not useful to distinguish the one mass unit difference between wild-type and D23N; therefore, FA-extracted molecules as well as synthetic homologues of both peptides were subjected to trypsin digestion and the molecular masses of the corresponding RP-HPLC purified peptides was assessed by MS in reflectron mode. As expected by the presence of two Lys and one Arg residues, trypsin cleavage generated four peptides (Figure 5A). Proteolytic fragments Aβ6-16 (T2) and Aβ1-5 (T1) eluted from the RP column at the beginning of the gradient (14 and 15% acetonitrile, respectively), Aβ17-28 (T3) was retrieved at 30% acetonitrile, whereas Aβ29-40 was recovered at the end of the gradient (36% acetonitrile). The main difference in the tryptic fingerprints of extracted and synthetic Aβ digests was observed in Aβ17-28 (T3) (Figure 5B). In the case of the synthetic homologues, T3 showed different retention time depending on whether the peptides contained the mutated (Asn) or the wild-type (Asp) sequence (T3a and T3b, respectively). In the Iowa extracted samples, T3 appeared as a doublet with retention times compatible with the presence of both T3a and T3b at a ~1:1 ratio;

amino acid sequence analysis of both peaks corroborated the coexistence of Asp and Asn at position 23 (Supplemental Table 1, see <http://ajp.amjpathol.org>). MS in reflectron mode confirmed that T3a originated from AβD23N (experimental mass = 1324.60; theoretical mass = 1324.69), whereas T3b resulted from the wild-type-AβD23 peptide (experimental mass = 1325.75; theoretical mass = 1325.67) (Figure 5C). Identical MS results were obtained for the Aβ17-28 peptides derived from the synthetic homologues (data not shown). Similar heterogeneity was present in both parenchyma and vessels in the two cases studied.

Aspartic Acid Isomerization at Positions 1, 7, and 23 of Aβ

Although amino acid sequence analysis of T3a and T3b tryptic peptides established the presence of both Asp and Asn at position 23, recovery calculations (Supplemental Table 1, see <http://ajp.amjpathol.org>) indicated that the yield of Asp23 was lower than that of Asn23

Figure 4. WB and MS analysis of fibrillar Aβ deposits. **A:** Parenchymal FA extracts of frontal cortex obtained from either 0.25 or 2.5 mg of tissue for WB and MS, respectively. **B:** FA extracts from leptomeningeal and microvessel fractions obtained from either 0.025- or 0.25-mg vessels for WB and MS, respectively; **C:** Theoretical and experimental *m/z* values. MS: ** indicates nonspecific peaks appearing in the negative controls.

(Asn23: Asp23 ratio = 2.8: 1 for case IV-1 and 1.3: 1 for IV-2), whereas as judged by the data from RP-HPLC fingerprinting, the ratio appeared to be ~1:1 (Figure 5B). This discrepancy suggested the likely existence of iso-Asp with the concomitant blocking of the Edman degradation chemistry and subsequent decrease in the yield of the amino acid sequence data. On the basis of the sequence recovery, it was estimated that ~65 and ~25% of the Asp23 was blocked in cases IV-1 and IV-2, respectively. Similarly, when directly analyzing the N-terminal sequence recovery of the FA-extracts (Supplemental Table 2, see <http://ajp.amjpathol.org>), the presence of three main sequences was identified corresponding to species starting at position 2, 4 and 1. On the basis of the amino acid recovery for both IV-1 and IV-2 cases, a ratio of ~4:2:1 for species starting at positions 2, 4, and 1, respectively, was estimated. Although Aβ species starting

at residues 2, and 4 were identified in the MS data shown in Figure 4, full-length Aβ40 was overrepresented, suggesting that a proportion of the N-termini were unable to undergo Edman degradation likely due to the presence of isoAsp, as reported in early biochemical studies^{40,41} for position 1 in sporadic AD brains.

To verify the presence of isoAsp at all of the putative Asp sites, tryptic digestion products of FA extracts purified by RP-HPLC were subjected to enzymatic methylation using isoaspartyl-methyl-transferase, which specifically catalyzes the methylation of isoAsp at the α-carboxyl position in the presence of S-adenosyl-L methionine. As a result of the methyl transfer, the latter generates SAH—which can be identified by RP-HPLC— in a 1:1 ratio with respect to the number of isoAsp residues present within the molecule. Figure 5D illustrates the RP-HPLC profiles resulting from the analysis of SAH in the respective tryptic pep-

Figure 5. Biochemical heterogeneity of amyloid lesions. **A:** Location of Iowa mutation on the Aβ1-40 sequence and the T1, T2, T3, and T4 tryptic peptides; **B:** RP-HPLC of tryptic digests of wild-type (D23) and variant (N23) synthetic homologues, as well as of Iowa FA-extracts; **C:** MALDI-TOF MS of T3a and T3b in reflectron mode. Monoisotopic mass of protonated T3 from N23 is 1324.69 Da and from D23 is 1325.67; **D:** Detection of isoAsp residues. **Inset:** Schematic representation of enzymatic reaction for isoAsp identification. RP-HPLC profiles indicate the presence of S-adenosyl-homocysteine in tryptic fragments T1, T2, and T3 but not in T4.

tides. SAH peaks were observed in T1, T2, and T3 fractions, corroborating the presence of isoAsp at positions 1, 7, and 23. In contrast, SAH was not evident in T4 fractions lacking Asp residues.

Effect of Aspartic Acid Isomerization and D23N Mutation on Aβ Fibrillogenesis

Figure 6 illustrates the kinetics of thioflavin T binding of synthetic wild-type Aβ1-40 and homologues containing two (positions 7 and 23) or three (positions 1, 7, and 23) isoAsp residues as well as the D23N variant peptides with and without isoAsp 1 and 7. Wild-type Aβ40, as previously described,²⁷ showed low tendency to form fibrillar/protofibrillar assemblies with thioflavin T fluorescence values that remained low for the 3-day duration of the experiment. The presence of two or three isoAsp residues had an enhancing—albeit modest—effect on fibrillization, without significantly affecting the lag phase and reaching only a slightly higher end-point after 3-days incubation. The combined presence of the D23N mutation together with isoAsp residues at positions 1 and 7, as found in the Iowa brain deposits, strikingly changed the peptide behavior with respect to the wild-type counterpart; the fibrillization kinetics was dramatically accelerated, showing >20-fold higher fluorescence values than the wild-type peptide after only 30 minutes of incubation, and reaching maximum fibrillization at 24 hours. Notably,

Figure 6. Effect of Asp acid isomerization and D23N mutation on Aβ fibrillogenesis assessed by thioflavin-T binding. Fluorescence evaluation (excitation/emission wavelengths 435/490 nm, respectively) of Thioflavin T binding assay of the samples collected at the different time points during the 3-days duration of the experiments was performed as described in *Materials and Methods*. The data are representative of three independent experiments: open circles, AβD23N isoAsp1,7 (solid line); closed circles, AβD23N Asp1,7 (dash line); closed squares, Aβ40 isoAsp7, 23 (solid line); open squares, Aβ40 isoAsp1, 7, 23 (dash line); triangles, wild-type Aβ40 (solid line).

under the conditions tested, the fibrillization kinetics seemed to be primarily driven by the presence of the D23N mutation. Thioflavin-T experiments with the AβD23N homologue bearing Asp residues at positions 1 and 7 rendered overlapping curves with the mutant counterpart containing isoAsp residues.

Discussion

Detailed immunohistochemical analysis of the Aβ deposits in the Iowa variant of AD showed extensive vascular compromise coexisting with pre-amyloid deposits and abundant neurofibrillary tangles in the presence of remarkably few mature plaques with no obvious evidence of intracellular Aβ.² The distribution of Aβ species was notable for the unusual extent of Aβ40 deposition in the parenchymal lesions in clear contrast with the typical Aβ42 predominance in sporadic AD parenchymal plaques, prevalence that was evident in confocal microscopy colocalization studies and further corroborated by quantitative evaluation via ELISA of both components in brain homogenates.^{13,42} The present studies corroborated these findings and biochemically characterized for the first time the Aβ composition of both parenchymal and vascular lesions using a combination of IP, WB, MS, and amino acid sequence analysis following tissue dissection and separation of vessels from parenchyma. Reflecting the complexity of the lesions, fibrillar as well as nonfibrillar components of the Iowa Aβ-peptidome were highly heterogeneous at both N- and C-terminal ends and exhibited a diverse degree of polymerization, going well beyond the classical dichotomy Aβ40-Aβ42. Whereas amyloid deposits were highly aggregated, N- and C-terminal heterogeneity—although present—was not as extensive as that observed in the non-fibrillar Aβ deposits. PBS-extractable materials, in particular, were rich in N- and C-terminal truncated species while showing a less pronounced aggregation. These extracts were enriched in Aβ peptides truncated at Glu3-Phe4 and/or Leu34-Met35 peptide bonds, whereas many of these species

were not even detectable in SDS and FA extracts. Notably, cleavages at these positions are not specific features of the Iowa patients. Both truncations were identified in our studies of brain deposits from sporadic AD cases and have also been reported in sporadic CAA, Lewy Body disease, sporadic and familial AD with presenilin-1 Ile83Met84 deletion mutation, as well as in familial Danish dementia⁴³—a cerebral amyloidosis with deposits of two unrelated subunits, ADan and A β ⁴⁴—suggesting the existence of common proteolytic pathways for A β deposits.

The existence of these N- and C-terminally degraded species in the Iowa deposits correlates not only with findings in AD and other forms of cerebral amyloidosis presenting with A β deposition as reported by our group^{25,28,43} and others,^{40,45,46} but also by proteomic data demonstrating numerous A β fragments in AD cerebrospinal fluid,^{47,48} many of which are common with the species found in the present study. The high solubility exhibited by many of these truncated A β derivatives together with their absence in fibrillar deposits likely reflects the result of clearance mechanisms questioning their importance in the amyloidogenesis process. In this sense, numerous enzymes have been postulated to participate in brain A β catabolic pathways including neprylisin, endothelin-converting enzyme, insulin-degrading enzyme, plasmin, β -Site of APP Cleaving Enzyme (BACE-1), and matrix metalloproteases (MMPs) among others (reviewed in Refs. 49 and 50). Current knowledge attributes to these enzymes the maintenance of the balance between A β production and catabolism, and it has been postulated that defective degradation contributes to the A β accumulation associated with disease. Potential candidates for the major cleavage products present in Iowa cases are insulin-degrading enzyme,⁵¹ neprylisin,⁵² and tripeptidyl peptidase-1⁵³ for peptide bond Glu3-Phe4, while the cleavage at Leu34-Met35 peptide bond likely originates by the action of MMPs⁵⁴ or cathepsin D.⁵⁵ Fragments ending at positions 37 and 38 likely result from the action of γ -secretase, known to exhibit activity at multiple A β C-terminal sites.^{56–58}

Co-deposition of mutated and non-mutated species has been also observed in other cerebral amyloidosis, including the Dutch familial form of AD²⁶ and prion diseases,⁵⁹ as well as in systemic forms of amyloidosis.^{60–64} It is likely that the deposition of the respective mutated species exerts a seeding effect—or a conformational mimicry—enhancing the fibrillization and subsequent co-deposition of the wild-type counterparts, as previously proposed.^{28,65,66} Contributing to the complexity of the lesions in Iowa-variant cases, deposited A β species showed additional heterogeneity with a significant proportion of the peptides containing isoAsp at residues 1, 7, and 23. This posttranslational modification has only been demonstrated at the biochemical level at A β residues 1 and 7 in AD cases.^{40,41} However, immunohistochemical analysis using site-specific antibodies suggested, in conjunction with isoAsp 1 and 7 the additional presence of isoAsp23 in sporadic AD⁶⁷ as well as in the Iowa-FAD⁴², although at a lower ratio than in the other positions. Our studies demonstrate that ~10 to 30% of the total deposited molecules in Iowa brain tissues contain isoAsp23.

The mechanisms leading to the elevated isoAsp23 content in the Iowa variant remain to be determined but it is likely to originate in nonenzymatic Asn deamidation, which typically results in ~30 times faster formation of isoAsp than isomerization at Asp residues.⁶⁸ Thus, the higher content of isoAsp23 in Iowa cases compared with AD may very well result from the presence of the mutation itself, which provides a more efficient starting point, Asn, for isoAsp formation. It is interesting to note that, in general terms, Asn deamidation with the consequent generation of isoAsp—in addition to other post translational modifications—is associated with conformational changes and aging.^{69,70} In this sense, not only do A β molecules bearing isoAsp23 show enhanced *in vitro* fibrillization kinetics compared with wild-type molecules,⁶⁷ but the presence of isoAsp at positions 1 and 7 typically translate into a decreased sensitivity for proteolytic degradation,⁷¹ features that may well contribute to the aggressiveness of the Iowa phenotype. The *in vitro* fibrillization analysis described herein clearly demonstrates that, despite the modest enhancing effect induced by the presence of isoAsp residues, the accelerated kinetics is primarily driven by the presence of the Asn mutation at position 23, which not only exacerbates the fibrillogenic propensity of the peptide but dramatically shortens the lag-phase of the conformational change.

The amino acid change present in Iowa tissues is similar to that of the Dutch mutant; in both cases there is a loss of a negatively charged residue occurring at the immediate location in the molecule (D23N versus E22Q). Perhaps as a result of these similar changes, both D23N and E22Q synthetic homologues display high content in β -sheet secondary structure and rapidly assemble in solution to form typical amyloid fibrillar assemblies.⁷² These conformational properties appear to confer, in turn, to both peptides enhanced toxicity for cerebrovascular smooth muscle cells.^{9,72,73} Notably, peptides carrying both mutations showed enhanced fibrillogenic properties compared with each of the single mutated peptides and exhibited more than double toxicity for cerebrovascular cells.⁷² However, despite the structural similarities between the Iowa- and Dutch-A β peptides, there are clear clinical differences in the *in vivo* phenotypes; whereas the Iowa deposits coexist with neurofibrillary tangles and manifest with memory impairment classic of AD, the Dutch mutant translates in recurrent cerebral hemorrhage episodes in the absence of neurofibrillar pathology. Interestingly, genetically engineered mice carrying both the Dutch and Iowa double mutation show early onset accumulation of fibrillar deposits in cerebral microvessels together with the presence of largely diffuse parenchymal lesions. Thus, the final pathology resulting from the existence of these mutants appears to be a consequence of complex mechanisms that go beyond the mere presence of β -sheet structures and peptide multimerization.

Mounting evidence suggests that plaque burden correlates poorly with the degree of dementia and that soluble intermediate assemblies including protofibrils/oligomers rather than the insoluble amyloid fibrils are more relevant species for neuronal toxicity and dysfunction, in

contrast with earlier studies attributing these effects to the presence of fibrillar components.^{74–79} This concept is strongly supported by the Iowa form of FAD—in which parenchymal neuritic plaques are almost nonexistent—and reinforced by two non- $A\beta$ amyloidoses, familial British and Danish dementias,^{23,80} also presenting with widespread preamyloid lesions and severe CAA. Despite structural differences among ABri, ADan, and $A\beta$, the extensive neurofibrillar degeneration with neurofibrillary tangles identical to those found in AD cases in the virtual absence of compact plaques and the same clinical outcome (AD-like dementia) argue against the sole significance of compact plaques in the mechanism of neurodegeneration.²⁴ These diseases clearly illustrate that the development of dementia is neither exclusive for $A\beta$ nor dependent on the presence of compact plaques. We propose that these disorders constitute excellent models to study early steps of peptide oligomerization/fibrillization as well as the role of preamyloid and vascular involvement in the complex scenario of amyloid-related neurodegeneration

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