Systemic Amyloid Deposits in Familial British Dementia*

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Familial British dementia (FBD) is an early onset inherited disorder that, like familial Alzheimer’s disease (FAD), is characterized by progressive dementia, amyloid deposition in the brain, and neurofibrillary degeneration of limbic neurons. The primary structure of the amyloid subunit (ABri) extracted from FBD brain tissues (Vidal, R., Frangione, B., Rostagno, A., Mead, S., Revesz, T., Plant, G., and Ghiso, J. (1999) Nature 399, 776–781) is entirely different and unrelated to any previously known amyloid protein. Patients with FBD have a single nucleotide substitution at codon 267 in the BRI2 gene, resulting in an arginine replacing the stop codon and a longer open reading frame of 277 amino acids instead of 266. The ABri peptide comprises the 34 C-terminal residues of the mutated precursor ABriPP-277 and is generated via furin-like proteolytic processing. Here we report that carriers of the Stop-to-Arg mutation have a soluble form of the amyloid peptide (sABri) in the circulation with an estimated concentration in the vessels of several peripheral tissues, including pancreas and myocardium. We hypothesize that the high concentration of the soluble de novo created amyloidogenic peptide and/or the insufficient tissue clearance are the main causative factors for the formation of amyloid deposits outside the brain. Thus, FBD constitutes the first documented cerebral amyloidosis associated with neurodegeneration and dementia in which the amyloid deposition is also systemic.

Familial British dementia (FBD)† was originally described in 1933 by Worster-Drought et al. (1) as familial presenile dementia with spastic paralysis. The pedigree has been followed and expanded since then (2), and common ancestors have been identified in case reports by Griffiths et al. (3), and Love and Duchen (4). At present, the Worster-Drought pedigree comprises 343 individuals extending over nine generations dating back to 1780 (5). The disease is clinically characterized by progressive dementia, spastic tetraparesis, and cerebellar ataxia with an age of onset in the fifth decade. Brain MRI scans of FBD patients show the existence of periventricular white matter hyperintensities resemblingBinswanger’s leukoencephalopathy (2, 5). Neuropathological examination of several FBD cases revealed a widespread severe amyloid angiopathy with perivascular deposits, amyloid plaques, and pre-amyloid lesions affecting the hippocampus and occasionally the cerebral cortex, and neurofibrillary tangles (NFTs) in hippocampal neurons (2, 6, 7). Because of its clinical and neuropathological features, FBD has been previously interpreted as an atypical form of familial Alzheimer’s disease (AD) (8), as an example of spongiform encephalopathy (9–11) and also regarded as a specific form of primary congophilic angiopathy (12).

We have recently reported that amyloid ABri, the main component of the parenchymal and vascular lesions in FBD, is a 4-kDa peptide derived from a type II transmembrane precursor molecule coded by a single multixenic gene BRI2 (also known as ITM2B (13)) located on the long arm of chromosome 13. A single substitution in the BRI2 gene (TGA to AGA at codon 267) results in an arginine replacing the normally occurring stop codon in the wild type precursor molecule and a longer open reading frame of 277 amino acids (ABriPP-277) (14). Furin-like proteolytic processing releases the 34-amino acid ABri peptide from the C terminus of the mutated precursor protein (15). Amyloid isolated from leptomeningeal fibrillar deposits shows a high degree of polymerization and a post-translationally modified N terminus (pyroglutamate) (14). Synthetic ABri peptides are able to mimic in vitro the in vivo properties, namely the high tendency to polymerize and to form amyloid-like fibrils in physiologic conditions (16).

The existence of circulating soluble precursors for both systemic and localized amyloid fibrils has been previously proposed (17) as a putative source of most amyloidogenic proteins. In AD, amyloid deposits are composed of Aβ, a 39–43-residue internal proteolytic fragment derived from a larger precursor protein ApPP with a predicted structure of a multidomain, type I transmembrane cell-surface receptor coded by a single gene on chromosome 21 (reviewed in Ref. 18). Aβ species extracted from the tissue deposits are heterogeneous at the N and C terminus and exhibit a high tendency to form aggregates. A soluble form of Aβ (sAβ) has been identified in body fluids, cell conditioned media, and brain supernatants, consisting mainly

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‡ The abbreviations used are: FBD, familial British dementia; NFT, neurofibrillary tangle; AD, Alzheimer’s disease; Ab, antibody; CAPS, 3-(cyclohexylamino)propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CR, Congo red; TS, thioflavin S.
of the first 40 residues of the amyloid peptide. It has been demonstrated in guinea pigs, mice, and rats that the blood-brain barrier has the capability to modulate sAβ transport in and out of the brain (19–21). Although the contribution of circulating sAβ to the deposited Aβ in AD brains is still debatable, the circulating peptide has been postulated to potentially contribute to the amyloid deposition (21). In the present report, we describe that in FBD-affected individuals, the de novo created Aβ fragment is found not only in the circulation but as the main constituent of amyloid deposits in peripheral tissues. The Aβ fragment identified in systemic deposits is indistinguishable from those isolated from the brain. However, the circulating soluble Aβ differs from the deposited peptide in that it lacks the post-translationally modified N-terminal pyroglutamate.

MATERIALS AND METHODS

Immunohistochemistry—Tissue blocks from different organs and skeletal muscle were taken at post mortem, fixed in 10% buffered formalin, and embedded in paraffin. Seven-μm-thick sections pretreated with 99% formic acid were incubated at room temperature with biotinylated anti-ABri Ab 338 (1:200; Dako, Carpinteria, CA) and ABC complex (Dako). Color was developed with diaminobenzinedine/H2O2. Sections were subsequently counterstained in aqueous milk solution, incubated with Ab 338 (1:2000) overnight at 4°C, and used for Western blots and mass spectrometry analysis.

Plasma Samples—Blood samples (7 ml) were collected from 15 normal controls and 20 affected and nonaffected FBD family members. After separation of plasma for immunoprecipitation experiments (see below), peripheral blood leukocytes were used for DNA isolation. After genomic DNA amplification by PCR, carriers of the Stop-to-Arg mutation were confirmed via restriction analysis with XhoI, as described previously (14).

Peptide Preparation—Synthetic Aβ peptide (EASNCFAIRHENKFAVETLICSRTVKKNIIEEN) was synthesized at the W. M. Keck Foundation (Yale University) using solid-phase techniques. The peptide was purified by reverse-phase high performance liquid chromatography (C4; Vydac, Hesperia, CA) and its purity evaluated via amino acid sequence analysis and mass spectrometry. Oxidation of the cysteine residues with the consequent formation of an intrachain disulfide bond was carried out at pH 8.0 in the presence of air. The resulting mixture of oxidized and nonoxidized Aβ peptides was resolved by reverse-phase high performance liquid chromatography using a 40-min linear gradient of 20–80% acetonitrile in 0.05% trifluoroacetic acid and further analyzed by mass spectrometry. Oxidized Aβ exhibited a retention time of 27 min and an average mass of 3953.4 Da (expected 3953.3 Da), while the reduced Aβ peptide displayed a retention time of 30 min and an average mass of 3955.2 Da (expected 3955.3 Da).

Immunoprecipitation—Fifty microliters of paramagnetic beads (Dynabeads M-450; Dynal Biotech, Lake Success, NY) coated with goat anti-rabbit IgG were allowed to interact with polyclonal anti-ABri Ab (1:20; Dako, Carpinteria, CA) and ABC complex (Dako). Color was developed with diaminobenzinedine/H2O2. The resulting pellets were subjected to collagenase (EC 3.4.24.3, Sigma type I) digestion (1% w/w wet pellet) for 16 h at 37°C followed by centrifugation at 100,000 g for 45 min at 4°C, and the remaining insoluble fractions, enriched in amyloid fibrils, were dissolved in 99% formic acid and used for Western blot, N-terminal sequence, and mass spectrometry analysis.

Western Blot Analysis—Aliquots of both the isolated amyloid fractions and the immunoprecipitated components from biological fluids were separated in Tris/Tricine gels under nonreducing and reducing conditions, transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA) using CAPS buffer, pH 11.0, containing 10% (v/v) methanol and immunoreacted with Ab 338 (3 h, room temperature), washed three times with phosphate-buffered saline, and used for Western blots and mass spectrometry analysis.

Western Blot Analysis—Aliquots of both the isolated amyloid fractions and the immunoprecipitated components from biological fluids were separated in Tris/Tricine gels under nonreducing and reducing conditions, transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA) using CAPS buffer, pH 11.0, containing 10% (v/v) methanol and immunoreacted with Ab 338 (3:1:3000) followed by horseradish peroxidase-labeled anti-rabbit IgG (Amersham Pharmacia Biotech). Fluorograms were developed with ECL, exposed to Hyperfilm ECL (Amersham Pharmacia Biotech), and quantified by densitometry using a standard curve (0.5 to 50 ng) of synthetic Aβ.
**Sequence Analysis—**N-terminal sequence analysis of isolated ABri species was carried out by automatic Edman degradation on a 494 Procise Protein Sequencer (Applied Biosystems). Samples were separated on Tris/Tricine SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membranes as described above, stained with Coomassie Blue, and the pertinent bands excised and sequenced.

**Mass Spectrometry—**Molecular masses of both the isolated amyloid fractions and the immunoprecipitated ABri components were determined at the New York University Protein Analysis Facility. Amyloid-containing samples (0.5 µl) were mixed with 0.5 µl of 10 mg/ml o-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in 50% acetonitrile and 0.1% trifluoroacetic acid, air-dried, and analyzed on a Micromass ToFSpec-2E (MALDI-TOF) mass spectrometer in linear mode using standard instrument settings. Internal and/or external calibration was carried out using synthetic Aβ1–42 (average mass = 4513.1 Da), angiotensin I (average mass = 1296.5 Da), and insulin (average mass = 5733.5 Da).

**RESULTS AND DISCUSSION**

A soluble form of ABri (sABri) in serum of patients with FBD was identified using a combination of immunoprecipitation, mass spectrometry, and Western blot analysis. Immunoprecipitation experiments with antibodies specific to the ten C-terminal residues of the ABri peptide (TVKKNIEEN; Ab 338 (14)) followed by Western blot and chemiluminescence identified a 4-kDa soluble component in all tested carriers of the Stop-to-Arg mutation (n = 8), and it was consistently absent in non-carrier family members (n = 12) and normal controls (n = 15) (Fig. 1). In contrast to the brain-deposited ABri (14), sABri was consistently monomeric and devoid of N-terminal pyroglutamate with an observed average mass of 3953.5 ± 0.5 Da (expected: 3953.3 Da for the ABri peptide with oxidized cysteine residues). No obvious N- and/or C-terminal heterogeneity was observed. The experimental average mass was close to the theoretical mass value of the oxidized ABri peptide, suggesting that some of the soluble ABri molecules may contain a single intrachain disulfide bond. Consistent with these data is the mass spectrometry analysis of synthetic full-length ABri peptides either containing or lacking a single disulfide bond between cysteine residues 5 and 22. The observed average mass for the oxidized synthetic ABri was 3953.4 Da versus 3955.2 Da for the reduced peptide (see “Materials and Methods”). A molecular mass of 3955.5 Da was previously reported for a secreted ABri species detected in conditioned medium of N2a cells transfected with full-length ABriPP-277 cDNA containing the Stop-to-Arg mutation, a mass value also consistent with the presence of N-terminal glutamate and suggestive of oxidized cysteines (15).

The origin of the circulating soluble ABri is not clearly established yet; however, based on Northern blot analysis (14) it can be speculated that the brain, kidney, and pancreas might be the most likely sources. The plasma levels in the small number of FBD carriers available (n = 8) was estimated in the range of 20 ng/ml by means of scanning densitometry of the immunoprecipitated bands. The existence of such elevated plasma levels of sABri may imply that, in FBD patients, amyloid deposits could also occur outside the central nervous system. Using conventional immunohistochemical methods, we searched for ABri deposits in systemic organs obtained at autopsy from two FBD cases. As previously demonstrated for cerebral amyloid and pre-amyloid lesions (6, 14), AB 338 labeled Congo red (CR)-positive/thioflavin S (TS)-positive blood vessels in organs such as pancreas (Fig. 2), adrenal gland, lung, myocardium, liver, spleen, and skeletal muscle (not shown). Parenchymal deposits either CR-negative/TS-negative or CR-positive/TS-positive were also seen in organs such as pancreas (Fig. 2), adrenal gland, myocardium, and skeletal muscle (not shown). Western blot analysis and mass spectrometry data of the amyloid material extracted from either vascular or paren-
Chymal lesions of two peripheral organs (pancreas and heart) revealed ABri species identical to those found in the brain lesions. As illustrated in Fig. 3 for the parenchymal deposits in pancreas, the pattern of ABri immunoreactivity (ABri monomers, dimers, trimers, and larger oligomeric species) was comparable with that of the amyloid isolated from the brain. N-terminal sequence analysis of the 4- and 8-kDa components rendered similar results, two minor sequences SNXFAIRHF (corresponding to positions 3–11 of ABri) and EASNXFAIX (corresponding to residues 1–8 of ABri) that represented less than 20% of the protein loaded, suggesting a blocked N terminus for the main deposited component, as previously reported for the leptomeningeal ABri amyloid (14).

Mass spectrometry analysis of the same fractions (depicted in Fig. 4) confirmed that the full-length ABri peptide featuring pyroglutamate at the N terminus with an observed average mass 3935.7 ± 0.3 Da (expected 3935.5 Da) was the main constituent of both brain and peripheral deposits. The experimental mass obtained, 3935.7 Da, was also close to the theoretical mass of the oxidized peptide and differed by 18 units from the ABri species found in circulation (average mass 3953.5 Da), a value accountable for the loss of one molecule of water and the formation of pyroglutamate. Two minor components were also identified: (a) a full-length ABri peptide containing glutamic acid at the N terminus, identical to sABri (observed average mass 3935.3 ± 0.4 Da; expected 3933.3 Da) and (b) an N-terminal truncated ABri fragment starting at serine 3 (observed average mass 3753.4 ± 0.3 Da; expected 3753.3 Da), a product previously identified in cerebrovascular deposits. Collectively, the last two components accounted for less than 20% of the systemic amyloid deposits and less than 10% of the brain lesions, results consistent with the amino acid sequence data. As also indicated in Fig. 4, formylated species of all the above mentioned peptides, probably by-products of the extraction procedure, were present in the samples. Almost identical results were obtained with the microvessels extracts (data not shown). When compared in terms of electrophoretic mobility, pattern of polymerization, immunoreactivity, and molecular mass, the ABri amyloid subunits deposited in the brain were chemically indistinguishable from that in the systemic organs studied, indicating that in both cases, amyloid formation most likely occurs by a similar (if not identical) biochemical mechanism.

The neuropathology of FBD is strikingly similar to that of AD, i.e., both exhibit vascular amyloid and parenchymal amyloid and pre-amyloid deposition and NFTs (22). In AD, parenchymal Aβ deposits are mainly present in the hippocampus and the cerebral cortex, while Aβ vascular lesions are predominantly seen in leptomeningeal and cortical vessels. Degenerating neurons containing NFTs are frequent in limbic areas and in the neocortex. Paired helical filament-containing abnormal neurites, frequently co-localize with amyloid deposits (neuritic plaques). In FBD, however, severe amyloid angiopathy with perivascular plaque formation occurs throughout the central nervous system. Parenchymal amyloid plaques and pre-amyloid deposits together with neurofibrillary pathology predominantly affect limbic structures but rarely the cerebral cortex, clearly indicating that amyloid deposition is of primary importance in the initiation of neurodegeneration. The distribution of NFTs in FBD corresponds to stage IV in the system recommended by Braak and Braak for AD (23). Similar to AD, the NFTs in FBD are ultrastructurally composed of paired helical filaments; moreover, the electrophoretic pattern of abnormal hyperphosphorylated tau isolated from FBD brain tissue is
indistinguishable from that seen in AD (6, 7). The presence of the soluble amyloid peptide sABri in plasma adds another element to the striking pattern of similarities between FBD and AD, although the plasma levels of sABri surpasses by at least 10-fold those of sAβ. Despite this similitude, the ABri amyloid subunit forming the lesions in FBD is completely unrelated in sequence to the Aβ molecule composing the fibrillar deposits in AD.

The N-terminal amino acid of many proteins, hormones, and neurotransmitters is pyroglutamic acid. The pyroglutamyl moeity results from the post-translational modification of either glutamine or glutamic acid. Cyclization of glutamine to pyroglutamic acid involves the nucleophilic attack of the α-amino group on the amidated carboxyl group, and the reaction is catalyzed at neutral pH by an enzyme known as glutamine cyclotransferase (24–26). Less is known about the cyclization of glutamic acid in which the chemical reaction involves the loss of one molecule of water, although an apparently specific enzymatic activity has been recently detected in Aplysia neurons (27). Analysis of the genes codifying the many molecules containing this post-translational modification indicate that glutamine and not glutamate is the common precursor of the N-terminal pyroglutamic acid. However, few exceptions have been reported, among them processing products of pro-opiomelanocortin (28) and fragments of Alzheimer’s Aβ peptide (29–31). In the later case, the use of mass spectrometry and specific antibodies have allowed the identification in the brains of AD patients of deposited modified Aβ species AβpE3 and AβpE11 starting at residues 3 and 11 and containing N-terminal pyroglutamate. In both instances the original amino acid is glutamic acid, as it is the case of the ABri amyloid as well as in the recently described ADan peptide, the main component of cerebral amyloid deposits in familial Danish dementia (32). The mechanism that produce these N-terminal blocked peptides is not entirely clear. In the case of ABri, it is released from its precursor ABriPP-277 by a furin-like proteolytic processing that occurs in the Golgi apparatus. Using N2a cells transfected with ABriPP-277, the release of full-length ABri to the conditioned media was demonstrated (15). The peptide contained glutamate but not pyroglutamate at the N terminus, and it was identical in mass to the circulating sABri described above. Therefore, one likely possibility is that the chemically irreversible conversion from glutamate to pyroglutamate in FBD takes place at the site of deposition; alternatively, the rate of amyloid formation and deposition may preferentially favor the pyroglutamate-containing peptide. The finding that the same ABri species are found in the brain and systemic organ amyloid deposits suggests that the pyroglutamate-forming mechanism is not neuron-specific. It is interesting to note that Aβ and ABri may also share a similar mechanism for the release of the two N-terminal residues that generate Aβ3–40/42 and ABri 3–34. Both molecules contain an acidic N-terminal amino acid followed by alanine (Asp-Ala- ↓ in Aβ and Glu-Ala- ↓ in ABri), a likely substrate for dipetidyl aminopeptidases II and IV (33, 34).

The presence of Congo red/thioflavin S-positive ABri amyloid deposits in systemic organs in individuals with FBD contrasts with the findings of Congo red-negative Aβ immunoreactivity in non-central nervous system tissues deposits in AD patients (35–37). Several elements may converge to produce systemic ABri amyloid deposits in patients with FBD: (i) high levels of the putative circulating precursor, increasing the probability of monomer-monomer interactions resulting in polymerization; (ii) the presence of N-terminal pyroglutamate, which facilitates aggregation and increases the resistance to proteolytic degradation by amino peptidases; (iii) the unlikelihood that specific cellular receptors participate in the physiologic clearance of the deposits, since sABri/ABri are de novo created molecules.

Amyloid deposits simultaneously present in the brain and in systemic organs have been previously found in another autosomal dominant disorder, hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-I). This disease, characterized by massive amyloid deposition of mutant cystatin C Q68 (38) within small arteries and arterioles of leptomeninges, cerebral cortex, basal ganglia, brainstem, and cerebellum (39), also presents with silent amyloid deposits in peripheral tissues such as skin, lymph nodes, spleen, salivary glands, and seminal vesicles (40). Despite these similarities the main clinical hallmark of the disease is cerebral hemorrhage, not dementia, with fatal outcome in the third to fourth decade of life. The results reported here (summarized in Fig. 5) indicate that FBD is the first documented cerebral amyloidosis associated with extensive neurofibrillary degeneration in which significant amyloid deposits are also found in peripheral organs. The extent to

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**Table:**

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**Figure 5:** Schematic summary of the deposited ABri species identified in the brain and pancreas in comparison with soluble ABri identified in plasma. The table at the right-hand side of the figure shows the theoretical and experimental average masses (in daltons) of each of the identified ABri components.
which the systemic amyloid precursor peptide contributes to the mechanism of cerebral amyloid formation remains an open question. The corroboration of an active role of circulating sABri in amyloid deposition in the brain and elsewhere will undoubtedly change the therapeutic approaches proposed for inhibiting amyloidogenesis in the human brain.

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REFERENCES