Hereditary cerebral hemorrhage with amyloidosis in patients of Dutch origin is related to Alzheimer disease

(vascular disease/stroke/β-protein/early senile plaques/Down syndrome)

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ABSTRACT Hereditary cerebral hemorrhage with amyloidosis in Dutch patients is an autosomal dominant form of vascular amyloidosis restricted to the leptomeninges and cerebral cortex. Clinically the disease is characterized by cerebral hemorrhages leading to an early death. Immunohistochemical studies of five patients revealed that the vascular amyloid deposits reacted intensely with an antiseraum raised against a synthetic peptide homologous to the Alzheimer disease-related β-protein. Silver stain-positive, "senile plaque-like" structures were also labeled by the antiseraum, yet these lesions lacked the dense amyloid cores present in typical plaques of Alzheimer disease. No neurofibrillary tangles were present. Amyloid fibrils were purified from the leptomeningeal vessels of one patient who clinically had no signs of dementia. The protein had a molecular weight of ~4000 and its partial amino acid sequence to position 21 showed homology to the β-protein of Alzheimer disease and Down syndrome. These results suggest that hereditary cerebral hemorrhage with amyloidosis of Dutch origin is pathogenetically related to Alzheimer disease and support the concept that the initial amyloid deposition in this disorder occurs in the vessel walls before damaging the brain parenchyma. Thus, deposition of β-protein in brain tissue seems to be related to a spectrum of diseases involving vascular syndromes, progressive dementia, or both.

Amyloid angiopathy of the central nervous system is most commonly associated with normal aging, Alzheimer disease (AD), Down syndrome (DS), and cerebral hemorrhage (1-9). The latter disease can be divided into sporadic and familial cases. The familial form is known as hereditary cerebral hemorrhage with amyloidosis (HCHWA). HCHWA has been described in eight families (12 patients) from an area in western Iceland (Icelandic-type HCHWA, or HCHWA-I) (10, 11) and in four families from two coastal villages in The Netherlands (Dutch-type HCHWA, or HCHWA-D); three families (136 patients) from Katwijk (12) and one family (14 patients) from Scheveningen (13). The clinical picture and pathological findings in the familial cases have been reported to be similar in both countries (11, 12). The apparently healthy and normotensive patients spontaneously develop intracerebral hemorrhages at a younger age and in different localizations than is known for hypertensive strokes. Small arteries and arterioles, most distinctly those in the cortex and leptomeninges, are thickened by the deposition of amyloid fibrils, which in the case of the HCHWA-I patients are composed of a variant of cystatin C (or γ-trace protein), an inhibitor of lysosomal cysteine proteases (14-16).

Since the description of the HCHWA-D patients, some differences have been noted. (i) The HCHWA-I patients suffer the first stroke at the mean age of 27 years, whereas the HCHWA-D patients are approximately 25 years older (11, 12). (ii) The level of cystatin C in the cerebrospinal fluid of HCHWA-I patients is significantly lower than in the HCHWA-D patients and in healthy persons (17, 18). (iii) Immunohistochemically intense staining for cystatin C is found in diseased HCHWA-I brain vessels, whereas the vessels in the HCHWA-D brain only show weak or dubious staining (11, 19). (iv) Pedigree studies have not established a connection between the Dutch and Icelandic families (12). The amyloid angiopathy in AD and in DS is seldom complicated by massive intracerebral hemorrhage. It is caused by the fibrillar deposition of the so-called β-protein, which has a molecular weight of ~4000 and no homology with known protein sequences (20, 21). The amyloid of the characteristic senile plaques and probably of neurofibrillary tangles (NFT), neither of which has been described in HCHWA patients, is composed of the same β-protein (22-24). The amyloid fibrils present in cerebral angiopathy in normal aging and in cases of sporadic cerebral amyloid angiopathy have not been identified.

This paper reports the immunohistochemical studies of five patients with HCHWA-D and the biochemical characterization of the amyloid protein isolated from the leptomeninges of one of these patients.

MATERIALS AND METHODS

Patient. The patient (DW v BO) was a 48-year-old woman from one of the HCHWA families from Katwijk. Her father and two brothers also suffered from intracerebral hemorrhage. She had a stroke approximately 5 months before the hemorrhage that caused her death. Her previous clinical history was only remarkable for emotional lability, which had been present for many years. No signs of dementia were noted.

Histology and Immunohistology. At autopsy, performed within 8 hr after death, half of the brain tissue was fixed in buffered 10% formaldehyde for diagnostic histology and the other half was quick-frozen in liquid nitrogen. Fixed as well as frozen sections were stained with hematoxylin and cosin, periodic acid/Schiff reagent, and 1% Congo red, and by Bielschowsky and methenamine silver staining. Formalin-fixed brain specimens from four patients with HCHWA-D

Abbreviations: AD, Alzheimer disease; DS, Down syndrome; HCHWA, hereditary cerebral hemorrhage with amyloidosis; HCHWA-D, Dutch-type HCHWA; HCHWA-I, Icelandic-type HCHWA; NFT, neurofibrillary tangle(s).

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[two from Katwijk (unrelated to our patient) and two from Scheveningen] were also studied. Sections were incubated with polyclonal antibodies against monomeric amyloid protein of HCHWA-I (15, 16), against a synthetic peptide (SP28) corresponding to the 28 NH2-terminal amino acids of AD-related β-protein (25), against NFT (24), against prealbumin (DAKO-immunoglobulins A/S), against β2-microglobulin (26), against AA protein (27), and against κ and λ immunoglobulin light chains. As second antibody, either peroxidase-conjugated goat anti-rabbit antiserum (E-Y Laboratories, San Mateo, CA), which was visualized with 3,3'-diaminobenzidine and 0.015% H2O2 in 0.05 M Tris/HCl buffer (pH 7.0), or fluorescein-conjugated goat anti-rabbit antiserum (DAKO-immunoglobulins) was used. The sections were extensively rinsed between the different steps with phosphate-buffered saline (0.15 M, pH 7.2). Controls included incubation of the sections with an antiserum against glial fibrillary acidic protein (DAKO-immunoglobulins), use of normal rabbit serum or phosphate-buffered saline as the first-step reagent, and specimens from a patient with AD, from a patient with HCHWA-I, and from a normal age-matched brain.

Isolation of Amyloid Protein. Leptomeninges were dissected from the unfixed brain tissue and amyloid fibrils were extracted according to Pras et al. (28). The tissue was homogenized (Brinkmann homogenizer, type PT 10/35) at speed 4 in 0.15 M NaCl and centrifuged (Beckman Model L) at 100,000 × g for 1 hr at 4°C. This procedure was repeated until the supernatant absorbance at 280 nm was <0.05. The final pellet was then homogenized in distilled water and centrifuged (100,000 × g, 1 hr, 4°C), again until the absorbance remained low. The supernatants with a high absorbance (A280 ≥ 0.60) were pooled and an equal volume of 0.15 M NaCl was added. The resulting precipitate was pelleted (120,000 × g, 1 hr, 4°C). Since the yield of fibrils in this precipitate, as assessed by Congo red staining and electron microscopy, was much lower than that observed in the HCHWA-I preparation, the purification procedure for the less soluble β-protein was applied at this stage (20). The pellet after the distilled-water washes was suspended at 4% (wt/vol) and digested with 1% (wt/wt pellet) collagenase (EC 3.4.24.3, Sigma type I) in 0.05 M Tris/HCl/3 mM NaCl/0.01 mM CaCl2, pH 7.5, for 8 hr at 37°C. After centrifugation (100,000 × g, 1 hr, 4°C) the pellet was suspended at 22% (wt/vol) and homogenized in guanidine-HCl/0.1 M Tris-HCl/0.34 mM EDTA/24 mM dithiothreitol, pH 8.0, stirred for 48 hr at 20°C, and again centrifuged (100,000 × g, 1 hr, 4°C). The layer on top of the supernatant was discarded, and the supernatant, after addition of 25% (vol/vol) 2 M guanidine-HCl/1 M acetic acid, was fractionated on a Sephadex G-100 column equilibrated with 5 M guanidine-HCl/1 M acetic acid, and the fractions containing amyloid protein were pooled, extensively dialyzed against distilled water, and lyophilized.

As a control, leptomeninges from a patient without brain amyloidosis were treated identically. Samples of each step and fraction were checked for the presence of amyloid fibrils by Congo red staining and electron microscopy. The samples for electron microscopy were placed on Formvar-coated nickel grids and negatively stained with 1% uranyl acetate. Furthermore, each sample and fraction was analyzed by NaDodSO4/17% PAGE (29). If the protein bands needed further analysis, they were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) and incubated with one or more of the antibodies that were used for immunohistology. Immunoreactive bands were visualized with 0.05% 3,3'-diaminobenzidine and 0.015% H2O2 in 0.05 M Tris-HCl (pH 7.6). Nonspecific binding was blocked with 0.2% gelatin.

Amino Acid Sequencing. The protein was sequenced in a 470A protein sequencer (Applied Biosystems). The resulting phenylthiohydantoin amino acid derivatives were identified using the on-line 120A PTH analyzer and the standard program (Applied Biosystems).

RESULTS

On pathological examination, the non-atrophic brain showed remnants of an old hemorrhage in the left temporal lobe and a recent hemorrhage in the right parietotemporal lobe. The walls of the small arteries and arterioles in the leptomeninges

Fig. 1. Cerebral cortex of the HCHWA-D patient stained with Bielschowsky’s silver stain. Two arterioles (arrowheads) show a marked thickening of their walls, which stain intensely with silver. A number of silver-positive, senile plaque-like structures can be observed (arrows). Note the lack of the typical amyloid core. (×50.)
and cerebral cortex showed severe congophilic angiopathy (Fig. 1). Bielschowsky's silver stain and staining with methenamine silver demonstrated structures that resembled senile plaques (Fig. 1). They lacked the typical dense central amyloid core, showed a great variety in size, and often shaded off into the surrounding tissue. Preexistent cells could still be recognized amidst the "plaque-like" material and appeared to be vital. These structures were very numerous and were distributed unevenly throughout the cortex. They often were seen in association with capillaries but not with the angiopathic arterioles. NFT were not observed despite silver staining and incubation with the anti-NFT antibody.

Following extraction, the amyloid fibrils were readily denatured and solubilized in 6 M guanidine-HCl. Gel filtration (Fig. 2) yielded two main peaks: the void volume (which was not studied further) and a low molecular weight peak (fraction 4). In NaDodSO4/PAGE, the protein in fraction 4 (Fig. 2 inset, lane 2) had the same apparent size (=4 kDa) as β-protein (lane 3). On immunoblot analysis (data not shown) fraction 4, as well as β-protein, reacted with anti-SP28. The other antibodies did not react with this band, including the one raised against the monomer of the Icelandic cystatin C variant protein.

The amino acid sequence of fraction 4 to position 21 was identical to the NH2-terminal sequence of β-protein of AD and DS (21–23); however, at position 11, glutamic acid was found instead of glutamine as reported for the vascular lesions in AD (20) (Fig. 3). The sequence had NH2-terminal heterogeneity, with approximately 75% of the molecules starting with aspartic acid and 25% starting with alanine (Ala-2). The latter sequence terminated at position 6.

**DISCUSSION**

HCHWA-I and HCHWA-D share many clinical and pathological findings (10–13). Patients of both types, who suffer from recurrent and ultimately fatal intracerebral hemorrhages at a relatively young age, have deposition of large amounts of amyloid fibrils in cerebral arterioles and small arteries. The most significant differences noted in the HCHWA-D patients include the older age of onset, the normal level of cystatin C in cerebrospinal fluid, and the lack of immunohistological crossreactivity with the cystatin C amyloid protein (11, 12, 18, 19). These differences have been interpreted as due to polymorphism of the cystatin C gene (11, 19).

The poor solubility in water of the fibrillar protein of the HCHWA-D patient as compared to the HCHWA-I amyloid protein and the different mobility in NaDodSO4/PAGE analysis suggested more then mere polymorphism. NH2-terminal sequence analysis of the HCHWA-D amyloid protein revealed homology to the β-protein of AD and DS (20–23). However, in contrast to the vascular β-protein found in AD and DS (20, 21), but like the β-protein isolated from senile plaques and NFT (22, 23), this protein had NH2-terminal heterogeneity, indicating that degradation of the protein also occurs in the vessel walls. Further analysis is needed to establish whether or not the AD-related β-protein and the HCHWA-D amyloid protein are identical. Immunoblot analysis and immunohistological studies revealed that the anti-SP28 intensely labeled the blotted amyloid protein and affected vessel walls. This somewhat unexpected result made it imperative to exclude the possibility of AD in this patient. Clinically no signs of dementia were noted; at autopsy no cerebral atrophy was observed, and histopathologically no NFT or typical senile plaques were found. The plaque-like lesions that were visualized by the silver stains and anti-SP28 resembled senile plaques but lacked the central amyloid core. Neumann (30) described a nondenated patient with sporadic cerebral amyloid angiopathy whose brain lacked NFT or typical senile plaques but showed plaque-like lesions similar to those seen in our case. These lesions also can be observed among typical senile plaques in AD and may represent an early form. Tissue sections of brain from four other HCHWA-D patients who had no clinical signs of AD showed the same intense reaction in the vessel walls with anti-SP28. Similar plaque-like structures were found in two of these cases, indicating that these were not isolated findings.

The results of this study indicate that HCHWA-D is not related to HCHWA-I but appears to be related to AD. Moreover, the identical NH2-terminal sequence, similar biochemical properties, and immunological crossreactivity of the amyloid proteins of AD and HCHWA-D suggest that the differences between these proteins, if present, will be minor.

**INDEX TERMS**

CONGENITAL AMYLOIDOSIS; HCHWA-D; CEREBRAL HEMORRHAGE; Histochemistry; Immunohistochemistry; Protein Sequence

![Fig. 2](image-url) Elution profile of HCHWA-D amyloid fibril after Sephadex G-100 column chromatography in 5 M guanidine-HCl/1 M acetic acid. F4, fraction 4. (Inset) NaDodSO4/PAGE of fraction 4 (lane 2), β-protein isolated from vascular amyloid of an AD patient (lane 3), and variant cystatin C protein isolated from a patient with HCHWA-I (lane 4). Markers (lane 1) were bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). Proteins were visualized by staining with Coomassie blue.

![Fig. 3](image-url) NH2-terminal amino acid sequence of HCHWA-D amyloid protein (A, fraction 4, see Fig. 2), AD-related β-protein (B, ref. 20) and DS-related β-protein (C, ref. 21).
In AD, β-protein is not only deposited in the brain vessels (20) but also has been demonstrated in the senile plaques (22), which have been associated with the severity of the clinical symptoms (31), and is probably present in NFT (23, 24). HCHWA-D showed a different pattern: amyloid deposition in the vessels was more prominent, no NFT were seen in this patient, and structures resembling but different from typical senile plaques were observed. These findings suggest that β-protein is first deposited in the vessel walls before entering the brain parenchyma. Structural variants of β-protein and/or different processing of β-protein precursors in particular cell types may be responsible for the distinctive patterns of amyloid deposition in HCHWA-D and AD. Moreover, the finding that amyloid β-protein is present in the walls of small cerebral arteries in cases of sporadic congophilic angiopathy (unpublished observations) suggests that a spectrum of β-protein-deposition diseases exists. The syndromes would be defined by the age of onset, the anatomical distribution, and the quantity and rate of deposition of the amyloid fibrils.

A genetic defect has been proposed as one of the possible causes of AD. Recent studies (32–35) have shown that the gene encoding the AD-related β-protein and the locus for familial AD are located on chromosome 21. Whether the genetic abnormality in HCHWA-D patients is also localized on chromosome 21 remains to be determined.

Our findings imply that HCHWA-D is a distinct type of familial AD with predominant vascular involvement. We propose that this clinicopathological entity be designated familial Alzheimer disease, vascular type.

Note Added in Proof. We have isolated β-protein from the leptomeninges of two AD patients. The amino acid sequence revealed NH2-terminal heterogeneity and glutamic acid at position 11 as in HCHWA-D.

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