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EXPRESSION OF A NORMAL AND VARIANT ALZHEIMER'S β-PROTEIN GENE IN AMYLOID OF HEREDITARY CEREBRAL HEMORRHAGE, DUTCH TYPE: DNA AND PROTEIN DIAGNOSTIC ASSAYS

Frances Prelli, Efrat Levy, Sjoerd G. van Duinen,*
Gerard Th.A.M. Bots,* Willem Luyendijk,** and Blas Frangione[1]***

Department of Pathology and ***Kaplan Cancer Center, New York University Medical Center, New York, NY 10016

*Department of Pathology and **Department of Neurosurgery, University Medical Center Leiden, 2300 R.C. Leiden, The Netherlands

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Amyloid fibrils deposited in cerebral vessel walls in Dutch patients with hereditary cerebral hemorrhage with amyloidosis (HCHWA-D) are formed by polymerization of a 39-residue peptide similar to the β-protein of Alzheimer's disease, Down syndrome, sporadic cerebral amyloid angiopathy and normal aging. Sequence analysis of genomic DNA in HCHWA-D patients demonstrated a point mutation, cytosine for guanine at position 1852 of the precursor β-protein gene, which causes a single amino acid substitution (glutamine for glutamic acid) corresponding to position 22 of the amyloid protein. The normal allele was also present in these patients.

To examine the expression of normal and variant β-protein alleles in HCHWA-D we analyzed all the tryptic peptides obtained from several amyloid fractions from leptomeningeal vascular walls. Amino acid sequence of two peptides (T3a and T3b) with identical amino acid composition revealed that T3a had glutamine and T3b had glutamic acid at position 22. Thus both the normal and variant Alzheimer's β-protein alleles are expressed in vascular amyloid in HCHWA-D and may be detected by tryptic peptide mapping. Moreover, we have developed a diagnostic assay for high risk populations and prenatal evaluation that is based on the existence of the mutation.

Familial cerebral amyloid angiopathy in Dutch patients, also called hereditary cerebral hemorrhage with amyloidosis of Dutch type (HCHWA-D) is an autosomal dominant form of amyloidosis described in four families from two coastal villages in the Netherlands (1,2). The disease is characterized by amyloid deposition in the small leptomeninges, arteries, and cortical arterioles, leading to premature death in the fifth or sixth decade due to recurrent intracerebral hemorrhages. In HCHWA-D, apart from the severe amyloid angiopathy, there is an accumulation of parenchymal plaque-like structure(s) resembling preamyloid lesions found in great abundance in Alzheimer's disease (AD) (3) and Down syndrome (DS) (4). We have proposed that the amyloid deposition in cerebral blood vessels and/or neuropil is an early structural sign of AD and DS. However, very few neuritic plaques and no neurofibrillary tangles have been observed in HCHWA-D patients.

[1]To whom correspondence should be addressed.

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Although there are clinico-pathological distinctions between HCHWA-D and AD, the amyloid fibril extracted from leptomeningeal vessels is related to the β-protein of AD, DS, sporadic cerebral amyloid angiopathy and normal aging (5-13). The vascular amyloid of HCHWA-D and AD is composed of 39 residues, three residues shorter at the carboxyl end than the plaque amyloid protein (A4) of AD (7,10-13). These observations have led to the suggestion that HCHWA-D may be considered a distinct type of familial AD (FAD) with predominantly vascular involvement (10).

Thus far sequence analyses of cDNA clones and genomic DNA encoding the amyloid precursor protein (APP) from AD, DS and FAD patients are identical to data obtained from unaffected individuals (14-17). The mRNAs encoding the 695, 714, 751 and 770 amino acid precursors (14-21) and a shorter form devoid of the transmembrane domain (22) are derived by alternative splicing (20) of a single gene located on chromosome 21 (14-17). The primary structure and properties of the 751 mRNA transcript of APP are analogous to those of a cell-secreted protease inhibitor, nexin II (23,24).

In order to ascertain whether the diverse clinico-pathological manifestations in HCHWA-D and AD may be due to a defect in the APP gene, we recently isolated genomic DNA from the brain tissue of two Dutch patients with HCHWA and sequenced DNA encoding the amyloid β-protein (AP) (25). A point mutation, cytosine for guanine, causing a single amino acid substitution, glutamine for glutamic acid at position 22 of the amyloid protein was found. Moreover, both HCHWA-D patients had the normal allele. To examine the expression of the normal and variant β-protein alleles in the vascular amyloid of HCHWA-D we analyzed not only relevant fractions (11) but also all the tryptic peptides obtained from the amyloid protein extracted from the leptomeninges of two HCHWA-D patients. We developed diagnostic assays to detect the presence of the mutation in both DNA and amyloid fibrils.

MATERIALS AND METHODS

Protein isolation and purification. Leptomeningeal amyloid fibrils were isolated from two HCHWA-D patients (DWvBO, Sh), as previously described (10,11). Briefly, they were solubilized in 6 M guanidine hydrochloride/0.1M Tris/0.17M dithiothreitol, pH 10.2, and stirred for 48 h at room temperature. After addition of 25% (v/v) of 2M guanidine hydrochloride/4M acetic acid, the solution was applied to a calibrated Sephadex G-100 column, 2.5 x 100 cm equilibrated with 5M guanidine hydrochloride/1M acetic acid. The purity and molecular weight of the fractions were determined on 17% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (26). Samples were pretreated with 88% formic acid for 1 h at room temperature, followed by drying under nitrogen. For Western blot analysis (27), electrophoretic transfers of gels were carried out as previously described (28). Polyclonal rabbit antiserum to a synthetic peptide, SP 28 (29), corresponding to the 28 NH_2-terminal residues of AD-vascular β-protein, was used as the first antibody, and peroxidase-conjugated goat anti rabbit antiserum (E-Y Laboratories) was visualized by color development with 3, 3'-diaminobenzidine and hydrogen peroxide.

Enzymatic Digestion. The amyloid protein was pretreated with 88% formic acid (7), dissolved in 0.2M ammonium bicarbonate, pH 8.2, and incubated (enzyme : substrate, 1:100 wt/wt) for 4 h at 37°C with 1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin. Proteolysis was terminated by rapid freezing followed by lyophilization.

High-Performance Liquid Chromatography (HPLC). Tryptic peptides were isolated by reverse-phase chromatography on a μ-Bondapak C_{18} column (0.78 x 30.0 cm, Waters) with a gradient of 0-66% acetonitrile in 0.1% (v/v) trifluoroacetic acid at pH 2.5. The column eluents were monitored at 214 nm.

Amino acid analysis. Intact and tryptic peptides of the amyloid protein were hydrolyzed for 24 h *in vacuo* at 110°C with 0.2 ml 6N HCl containing 0.01% phenol, dried, resuspended in 20 μl of methanol:water:triethylamine solution (2:2:1, v/v) and redried. Precolumn derivatization of the samples was performed by addition of 20 μl of phenylisothiocyanate:water:triethylamine:methanol (1:1:1:7, v/v). After 30 minutes at room temperature they were dried under vacuum and analyzed in a Waters Pico-Tag amino acid analyzer by using the standard Pico-Tag method. The presence of tryptophan was determined by amino acid sequencing.

Sequence Analysis. Sequence analysis was carried out on a 477A microsequencer and the resulting phenylthiohydantoin amino acid derivatives were identified using the on-line 120A PTH analyzer and the standard program (Applied Biosystems).

Isolation and Amplification of DNA Sequences. High molecular weight genomic DNA was isolated from brains of two HCHWA-D patients (DWvBO; CVdV) and from lymphocytes of two unaffected Dutch individuals. Sequences containing exons 14 and 15 (30), which encode the AP, were amplified with Thermus aquaticus (Taq) heat-stable DNA polymerase (31). Amplification reactions in a total volume of 100 μl contained 1 μg of DNA, 0.25 μM of each primer designed on the basis of the intron sequences flanking these two exons (30) and 2.5 units Taq polymerase in reaction buffer (Perkin-Elmer-Cetus) subjected to 25 cycles set at 94°C for 1 min to denature the DNA, at 70°C for 2 min to anneal the primers, and at 72°C for 3 min to extend the annealed primers. Slot blots were performed in duplicate by applying 25 μl of the PCR amplified fragment to nitrocellulose. An oligonucleotide that contains the mutation was synthesized, 5'-labeled with γ-ATP and T4 polynucleotide kinase and hybridized to the blots. Hybridized blots were washed with 2x SSC, 0.1% SDS, (1 x SSC=0.15 M NaCl, 0.015M $Na_3C_4O_7$, pH 7.5) at low stringency (48°C) or high stringency (65°C).

RESULTS AND DISCUSSION

Amyloid fibrils were extracted and purified from the leptomeninges of two HCHWA-D patients as previously described (10,11). HPLC fractionation of the tryptic digest of the amyloid protein yielded four major and three minor peptides (Fig. 1) which were analyzed and sequenced (Fig. 2). Amino acid analysis yielded four peptides T1-T4. Sequence analysis of T1 and T2 corresponded to positions 1-5 and 6-16 of β-protein, respectively. Three peptides, T4a, T4b and T4c, had amino acid compositions identical to positions 29-39, as was confirmed by automated sequence analysis. Since they had varied proportions of methionine sulfoxide, differences in net charge would account for the three HPLC peaks found. Similar results were previously reported for the AD amyloid β-protein (12).

Amino acid compositions of tryptic peptides T3a ($Asp_{1.8}$ $Glu_{0.9}$ $Ser_{1.0}$ $Gly_{1.2}$ $Ala_{1.2}$ $Val_{1.6}$ $Leu_{1.2}$ $Phe_{1.7}$ $Lys_{1.1}$) and T3b ($Asp_{2.1}$ $Glu_{1.0}$ $Ser_{1.1}$ $Gly_{1.2}$ $Ala_{1.3}$ $Val_{1.9}$ $Leu_{1.2}$ $Phe_{1.9}$ $Lys_{1.2}$) were identical to positions 17-28; however, sequence analysis revealed that T3a had glutamine and T3b had glutamic acid at position 22. Although GLN and ASN may undergo deamination during purification and/or sequencing, the recovery of GLN and ASN exclusively at positions 15 and 27, respectively, suggests that the GLU at position 22 expresses the normal β-protein allele, rather than deamination of GLN. HPLC fractionation of the tryptic peptides of vascular AD β-protein yielded only one T3 peptide identical in composition and sequence to the T3b of HCHWA-D. AD β-protein is homogeneous for GLU at position 22 (12). Thus, the tryptic peptide mapping of AP may be utilized for the diagnosis of HCHWA-D.

Recently we cloned and sequenced the two exons encoding the AD amyloid protein from brain tissue of two HCHWA-D patients (DWvBo; CVdV) (25). A point

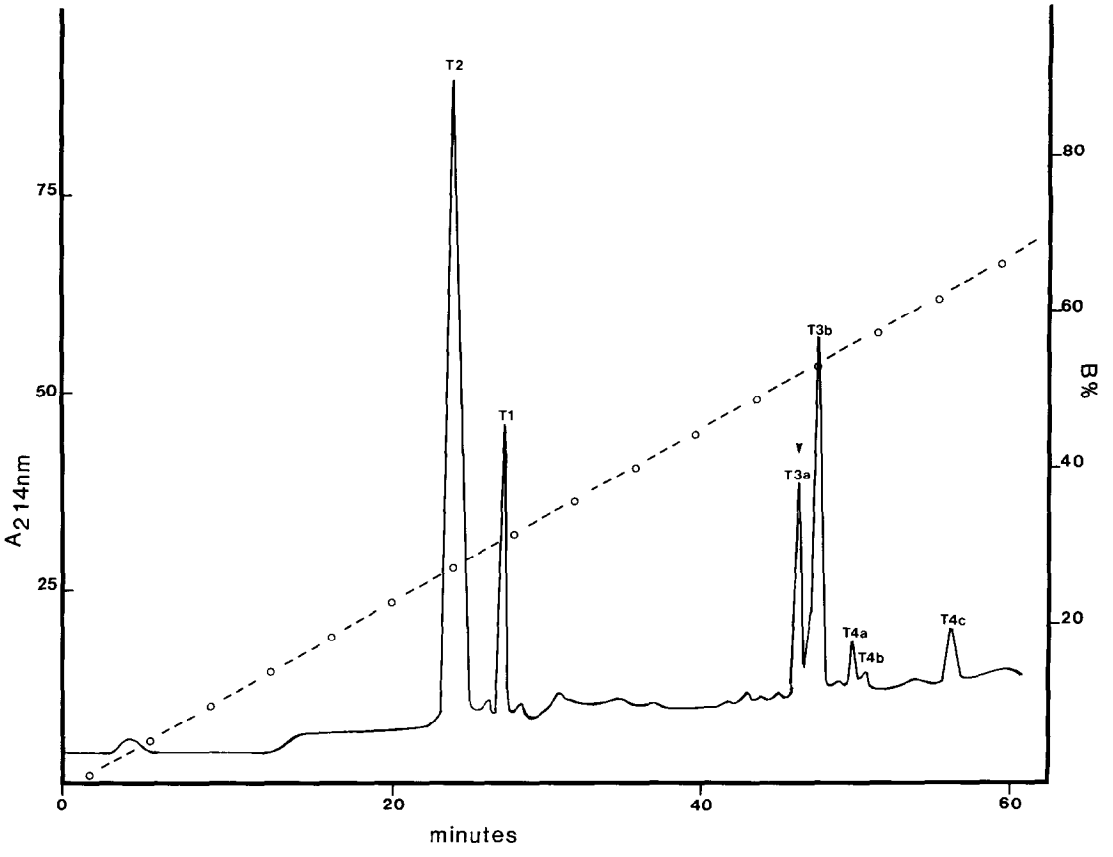
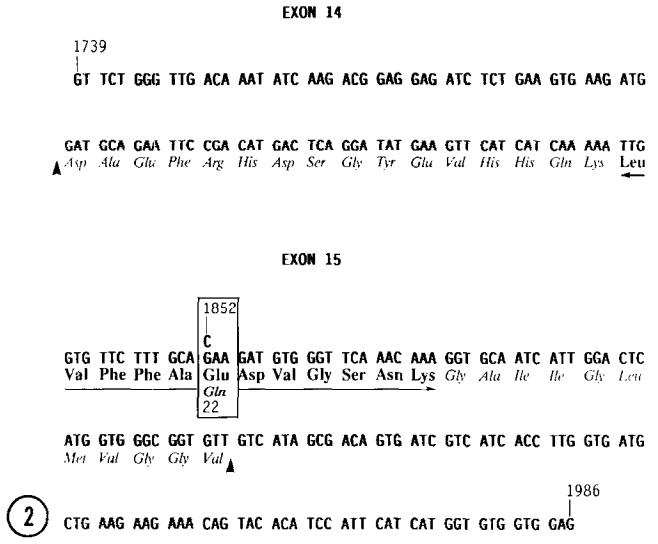


Fig. 1. Reverse phase HPLC tryptic fingerprint analysis of HCHWA-D vascular amyloid on a μBondapak C_{18} column (0.78 x 30 cm) with a 0-66% linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid. T1-T4, tryptic peptides. The arrowhead indicates tryptic peptide 3a of the variant which is absent in AD β-protein (12).

mutation, cytosine for guanine, at position 1852 of the APP gene was found (Fig. 2). The normal allele was also present. We have developed a diagnostic assay for HCHWA-D for high risk populations and prenatal evaluation that is based on the existence of this mutation. This assay involves polymerase chain reaction (31) amplification of genomic DNA sequences encoding AP followed by hybridization analysis using an oligonucleotide that contains the mutated sequence (25). High stringency washes of the blots demonstrated the existence of the mutated allele only in DNA samples isolated from HCHWA-D patients (Fig. 3). While this assay showed polymorphism on the molecular level, tryptic peptide analysis demonstrated that both the normal and variant Alzheimer β-protein alleles are expressed in patients with HCHWA-D, and that both amyloid precursor proteins are processed into amyloidogenic subunits. Moreover, the recent finding that APP and AP coexist in cerebral vessel walls of AD and HCHWA-D patients suggests that the vascular system is a source of APP which is processed *in situ* into aggregating amyloid fragments in both diseases (32).

Within the group of autosomal-dominant forms of amyloidosis, genetic variants of the amyloid protein precursors have been identified, for the different types of familial



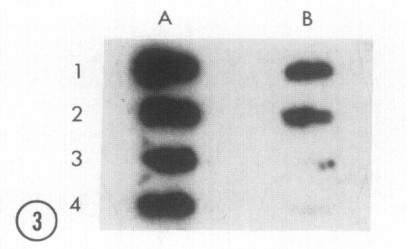


Fig. 2. The amino acid sequence of the cerebrovascular Alzheimer's amyloid β-protein (arrowheads) and the DNA sequence of the two exons (14 and 15) (30) that encode it. The mutation found in HCHWA-D patients (C for G) and the resulting amino acid substitution (Gln for Glu) are boxed. Nucleotides are numbered as for cDNA (17). Tryptic peptides T3a and T3b are underlined. Substituted amino acid is numbered as reference 12.

Fig. 3. High stringency washes of slot blot demonstrating the existence of the mutation in DNA samples. Fragments containing exon 15 (30) were amplified from genomic DNA isolated from tissues of two HCHWA-D patients (1 and 2) and two unaffected Dutch individuals (3 and 4). Duplicate blots were washed in low stringency (lane A) and in high stringency (lane B). The x-ray film was exposed for 1 h.

amyloidotic polyneuropathy (FAP), hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-I) (33), and familial amyloidosis, Finnish type (34). Several mutations have been described for transthyretin (or prealbumin), the serum precursor of the FAP amyloid subunit. In two cases, both the variant and normal prealbumin molecules were present in tissue and/or serum (35,36). Thus far no mutation in the APP gene has been found in DNA isolated from patients with AD, FAD, DS or from unaffected individuals. The mutation found in the AD β-protein gene in HCHWA-D patients may enhance and accelerate amyloid fibril formation and deposition in the cerebral vessel walls resulting in massive cerebral hemorrhage and premature death. Thus, genetic differences at the β-protein locus and/or other related genetic loci may effect the divers clinico-pathologic manifestations of these diseases. Acquired or inherited factors affecting the regulatory mechanisms of expression of the APP mRNAs or the post-translational processing of the precursors may play a key role in the pathogenesis of amyloidosis.

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