Amyloid β Protein Precursor Gene and Hereditary Cerebral Hemorrhage with Amyloidosis (Dutch)

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Human hereditary cerebral hemorrhage with amyloidosis of the Dutch type (HCHWA-D), an autosomal dominant form of cerebral amyloid angiopathy (CAA), is characterized by extensive amyloid deposition in the small leptomeningeal arteries and cortical arterioles, which lead to an early death of those afflicted in their fifth or sixth decade. Immunohistochemical and biochemical studies have indicated that the amyloid subunit in HCHWA-D is antigenically related to and homologous in sequence with the amyloid β protein isolated from brains of patients with Alzheimer's disease and Down syndrome. The amyloid β protein is encoded by the amyloid β protein precursor (APP) gene located on chromosome 21. Restriction fragment length polymorphisms detected by the APP gene were used to examine whether this gene is a candidate for the genetic defect in HCHWA-D. The data indicate that the APP gene is tightly linked to HCHWA-D and therefore, in contrast to familial Alzheimer's disease, cannot be excluded as the site of mutation in HCHWA-D.

DEPOSITION OF AMYLOID β PROTEIN fibrils in the walls of the small vasculature of the leptomeninges and the cerebral cortex is found in a number of brain disorders including Alzheimer's disease, Down syndrome, and CAA (*1*). In contrast to Alzheimer's disease and Down syndrome, extensive vascular amyloid β protein deposition in CAA is responsible for recurrent and often fatal intracerebral hemorrhages (*2*). In most cases, CAA occurs sporadically and accounts for 5 to 10% of the primary nontraumatic brain hemorrhages (*2*). However, a rare autosomal dominant form of CAA has been observed in four families from two coastal villages in the Netherlands and is known as HCHWA-D (*3*). Apart from the severe amyloid angiopathy in HCHWA-D, there is an accumulation of amyloid β protein in the parenchymal senile plaque-like structures (*4*), which resemble the diffuse senile plaques, found in great abundance in Alzheimer's disease (*5*) and Down syndrome (*6*). It has been proposed that extracellular amyloid β protein deposition in the cerebral blood vessels or neuropil or both is an early structural sign of Alzheimer's disease (*5*). However, no compact or neuritic plaques and no neurofibrillary tangles, brain lesions typically found in Alzheimer's disease and Down syndrome, have been observed in HCHWA-D patients (*4*). Nevertheless, the identification of amyloid β protein in the vascular amyloid deposits of HCHWA-D (*4, 7*) and sporadic CAA (*8*) patients suggests that these clinically distinct brain disorders are pathogenically related. These observations have led to the suggestion that HCHWA-D may be considered a distinct type of familial Alzheimer's disease (FAD) with a predominant vascular involvement (*4*).

The amyloid β protein was first isolated from vascular and neuritic plaque amyloid deposits present in the brains of Alzheimer's disease and Down syndrome patients (*9*). The amyloid β protein is derived from a larger amyloid β protein precursor (APP) encoded by a gene located on chromosome 21 (*10*). Genetic linkage analysis has proved the existence of a FAD gene on chromosome 21 in four families with autosomal dominant, early onset FAD (*11*). Localization of the FAD and APP gene approximately equidistant from the D21S1/S11 locus led to the hypothesis that the APP gene was the Alzheimer's disease gene (*12*). However, the finding of several recombinational events excluded the APP gene as the primary site of mutation in FAD (*13, 14*). Furthermore, linkage analyses have indicated that the FAD and APP genes flank the D21S1/S11 locus and are separated by a large genetic distance (*14, 15*).

Using a similar approach, we examined the question of whether the genetic defect in the HCHWA-D families is linked to the APP gene. Samples of DNA were obtained from 20 HCHWA-D patients belonging to two families (AM_1 and AM_2) living in Katwijk (Fig. 1). Despite extensive analysis of the genealogy, we have not proved a direct link between the families, although it is likely that the families may indeed be related. All patients examined showed similar clinical and pathological disease characteristics. The patients sampled for the genetic analysis had suffered from one or more cerebral hemorrhages as confirmed by computed tomography. In seven of the cases the clinical diagnosis was confirmed by pathology evidence obtained at brain biopsy or at autopsy. Of the 20 HCHWA-D patients examined, the mean age of the living pa-

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Table 1. Two-point lod scores (z) for each individual APP RFLP were calculated with the use of the program MLINK (*17*). The frequencies of the APP alleles (1 and 2 in Fig. 1) with Bgl II are 0.36 and 0.64 (*13*), with Nco I are 0.67 and 0.33 (*16*), and with Eco RI are 0.38 and 0.62 (*14*). No significant linkage disequilibrium was observed between all three APP RFLPs in 14 phase-known meioses of unrelated European Caucasians. Hence, lod scores for the combined APP RFLPs were calculated with the use of the program LINKMAP (*17*).

APP RFLP	Pedigree	Lod score (z) at recombination fraction (θ)						
		0.0	0.01	0.05	0.10	0.20	0.30	0.40
Bgl II	AM_1	2.74	2.64	2.24	1.78	0.97	0.42	0.13
	AM_2	0.39	0.36	0.29	0.20	0.09	0.03	0.01
	Total	3.13	3.00	2.53	1.98	1.06	0.45	0.14
Nco I	AM_1	0.59	0.56	0.46	0.34	0.15	0.05	0.02
	AM_2	0.54	0.51	0.42	0.32	0.17	0.08	0.02
	Total	1.13	1.07	0.88	0.66	0.32	0.13	0.04
Eco RI	AM_1	2.49	2.39	2.02	1.58	0.84	0.35	0.11
	AM_2	0.54	0.51	0.38	0.25	0.08	0.02	0.01
	Total	3.03	2.90	2.40	1.83	0.92	0.37	0.12
Combined	AM_1	5.97	5.81	5.18	4.39	2.84	1.45	0.48
	AM_2	1.62	1.56	1.32	1.03	0.53	0.20	0.06
	Total	7.59	7.37	6.50	5.42	3.37	1.65	0.54

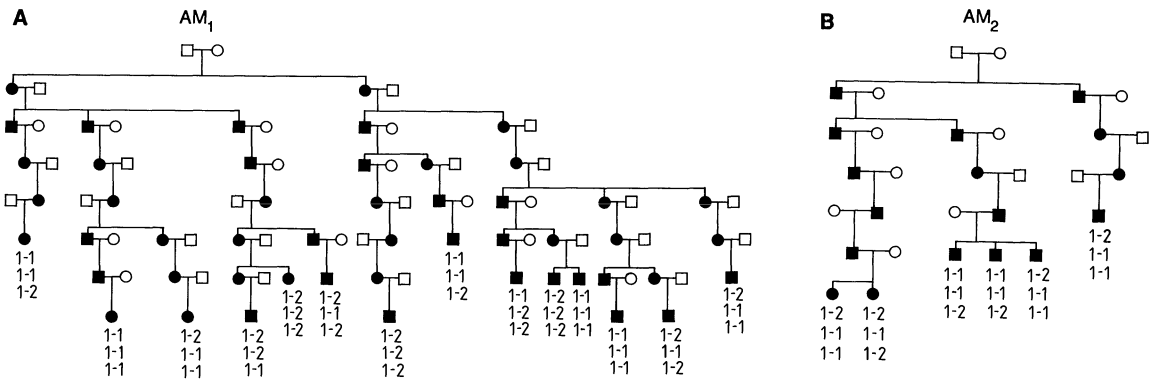

Fig. 1. Details of the APP haplotypes in two pedigrees with HCHWA-D, AM_1 and AM_2. The pedigrees shown are simplified versions of the complete HCHWA-D pedigrees showing solely the number of generations separating the 20 HCHWA-D patients (bottom row) analyzed by DNA, assuming autosomal dominant inheritance and complete penetrance of the disease. Symbols: circle, female; square, male; filled symbol, affected individual. DNA of the HCHWA-D patients was extracted from fresh blood samples or frozen brain material, digested with restriction enzymes, subjected to electrophoresis, blotted on Hybond N (Amersham), and hybridized with the appropriate ^{32}P-labeled fragments of the APP cDNA according to standard DNA analysis techniques. The genotype is indicated below each patient analyzed. Alleles of the APP RFLPs are indicated as 1, polymorphic site absent, and 2, polymorphic site present, for (from top to bottom) the Bgl II, the Nco I, and the Eco RI RFLPs.

tients (n = 15) was 59.2 ± 7.9 years (range 47 to 78 years) and the mean age at death of the deceased patients (n = 5) was 55.2 ± 12.9 years (range 42 to 76 years).

Three informative restriction fragment lengths polymorphisms (RFLPs) were detected by fragments of the APP cDNA, covering nearly the entire coding sequence: (i) a Bgl II RFLP (*13*) recognized by a 150-bp Sau3A1 fragment spanning the amyloid β protein coding sequence, (ii) a Nco I RFLP (*16*) by a 1056-bp Eco RI fragment comprising the amyloid β protein sequence plus part of the 3′ sequence, and (iii) an Eco RI RFLP (*14*) by a 1734-bp Kpn I–Eco RI fragment, located directly 5′ of the amyloid β protein sequence. For each of the three RFLPs examined, there was one identical allele shared by all 20 HCHWA-D patients, regardless of the family to which they belonged. If the three APP RFLPs were combined, there was one identical APP haplotype (111 in Fig. 1). Therefore it is unlikely that crossovers have occurred between HCHWA-D and the APP gene. We also identified the same APP haplotype in one patient belonging to a smaller HCHWA-D family living in Scheveningen, a village close to Katwijk. These results suggest that the mutation responsible for HCHWA-D originated in a common ancestral allele of the APP gene.

The significance of our findings was further evaluated by genetic linkage analysis of the HCHWA-D pedigrees AM_1 and AM_2 with the computerprogram package LINKAGE (*17*). Two-point lod scores for HCHWA-D were calculated for each APP RFLP separately assuming autosomal dominant inheritance of HCHWA-D, with complete penetrance of the disease and a gene frequency of 1 in 1000. Multipoint lod scores were also calculated for HCHWA-D and all three APP RFLPs under the assumption that no crossovers occurred within the APP gene. The linkage data are summarized in Table 1. Conclusive two-point lod scores $z > 3$, were obtained for the Bgl II and Eco RI RFLPs at a recombination fraction $\theta = 0.0$. Less significant two-point lod scores were obtained for the Nco I RFLP since, in contrast with the Bgl II and Eco RI RFLPs, HCHWA-D cosegregates with the common allele of the RFLP (Fig. 1). The multipoint lod score of 7.59 at $\theta = 0.0$ for all three APP RFLPs and the 95% confidence interval $\theta = 0.0$ to 0.045 (peak lod score minus one), confirmed tight linkage of HCHWA-D to APP. The total lod score of 7.59 is likely to be an underestimate because we did not know the exact relationships or the number of generations separating the two HCHWA-D families and treated them independently. In addition, we have used the same Southern blots to test flanking DNA markers of the APP gene. Recombinations were observed between HCHWA-D and the marker loci D21S11 and D21S17, which are located on either side of the APP gene (*18, 19*). These results provided further evidence to our finding that a genetic alteration of the APP gene is likely to be responsible for HCHWA-D.

Although Alzheimer's disease, Down syndrome, and CAA share a common mechanism leading to the accumulation of an apparently identical amyloid β protein in the central nervous system, the exact origin of the deposits is still unknown (*20*). Recent evidence suggests that Alzheimer's disease is not solely restricted to the brain, but is a widespread systemic disorder with accumulation of amyloid β protein in nonneuronal tissues (*21*). The deposited amyloid β proteins may derive either from a common circulating precursor or may be produced locally by cells in the artery walls (*21*). Systemic organs in HCHWA-D patients have not been examined, and no circulating protein carrying the amyloid β protein has yet been defined. However, systemic organs have been shown to be involved in another similar cerebrovascular disease, hereditary cerebral hemorrhage with amyloidosis of Icelandic type (HCHWA-I) (*22*). In HCHWA-I, the subunit of the amyloid fibrils is composed of a variant of the protein Cystatin C, an inhibitor of lysosomal cysteine proteases (*23*). Furthermore, nucleotide sequencing of the Cystatin C cDNA in HCHWA-I patients has revealed a single base substitution responsible for the abnormal proteolytic cleavage and accumulation of altered Cystatin C molecules as amyloid fibrils (*24*). In several other amyloidoses, such as Gerstmann-Sträussler disease (*25*) and familial amyloid polyneuropathy (*26*), point mutations have also been identified as the genetic basis for the amyloid deposition.

In view of our linkage results between the APP gene and HCHWA-D, we postulate that a single base mutation in the coding sequence of APP is responsible for the genetic defect in HCHWA-D, although we cannot exclude the possibility that the HCHWA-D defect is the consequence of an abnormal transcriptional, translational, or posttranslational modification of the APP gene.

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Cleavage of Amyloid β Peptide During Constitutive Processing of Its Precursor

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The amyloid β peptide (AβP) is a small fragment of the much larger, broadly distributed amyloid precursor protein (APP). Abundant AβP deposition in the brains of patients with Alzheimer's disease suggests that altered APP processing may represent a key pathogenic event. Direct protein structural analyses showed that constitutive processing in human embryonic kidney 293 cells cleaves APP in the interior of the AβP, thus preventing AβP deposition. A deficiency of this processing event may ultimately prove to be the etiological event in Alzheimer's disease that gives rise to senile plaque formation.

ALZHEIMER'S DISEASE (AD) IS A neurodegenerative disorder characterized by the presence of extracellular senile plaques and intracellular neurofibriliary tangles in the brains of affected individuals. The central core of senile plaques is comprised of the 4-kD AβP, which is a small fragment of the 90- to 130-kD glycosylated, membrane-bound APP (*1–3*). Three forms of APP that contain AβP are derived from a single gene by alternative mRNA splicing. These putative AβP precursors contain 770 (APP_{770}), 751 (APP_{751}), and 695 (APP_{695}) amino acids and differ from one another primarily by the presence (APP_{770} and APP_{751}) or absence (APP_{695}) of a Kunitz-type protease inhibitor insert (*4–6*).

Proteolytic processing of APP results in the secretion of a large (>100 kD), soluble, NH_2-terminal APP fragment (*3, 7*), identified as protease nexin II (*8*) and the generation of an approximately 11-kD membrane-associated COOH-terminal fragment (*2*). Direct microsequencing has shown that the NH_2-terminus of the secreted APP fragment begins with APP(Leu^{18}) (*9, 10*) and immunochemical studies suggest that the COOH-terminus of the membrane-bound COOH-terminal fragment contains all or nearly all of the COOH-terminal APP sequence (*2, 9*). Immunochemical studies have suggested that some or all of the AβP sequence may be present in the secreted NH_2-terminal APP fragment (*11*), but the exact location of the proteolytic cleavage in APP that gives rise to the NH_2- and COOH-terminal APP fragments has not been identified. To answer this question we have purified these fragments from human embryonic 293 cells stably transfected with cDNA constructs encoding full-length forms of APP_{751} and

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Table 1. Structural characterization of the COOH-terminus of the secreted APP_{695} and APP_{751} forms. Each secreted protein (5 nmol) (*16*) was reduced, treated with pyridylethylene, and cleaved with CNBr. The resulting fragments were purified by RPLC (*13*). The COOH-terminal CNBr fragment was identified in each case by amino acid analysis (**A**) and microsequencing (**B**) with a model 420 amino acid analyzer and a model of 477A gas-liquid phase sequencer (Applied Biosystems), respectively. Fab mass spectrometry of each COOH-terminal CNBr digestion fragment yielded protonated molecular mass ions of 1826.72, which are identical with the calculated mass of the predicted COOH-terminal AβP(1–15) peptide. In (A), 25 and 39 pmol of the COOH-terminal CNBr digestion fragments from the secreted forms of APP_{695} and APP_{751}, respectively, were hydrolyzed and analyzed. In (B), 85 and 128 pmol of the COOH-terminal CNBr digestion fragments from the secreted forms of APP_{695} and APP_{751}, respectively, were subjected to microsequence analyses. PTH-AA, phenylthiohydantoin amino acid.

A Amino acid compositions

Amino acid	Amino acid ratios		
	APP_{695}	APP_{751}	AβP (1–15)
Asp	2.19	1.98	2
Glu	3.42	3.35	3
Ser	0.85	0.91	1
Gly	1.12	1.19	1
His	2.97	2.92	3
Arg	1.12	1.06	1
Thr	0	0	0
Ala	0.98	1.05	1
Pro	0	0	0
Tyr	0.69	0.74	1
Val	0.79	0.89	1
Met	0	0	0
Cys	0	0	0
Ile	0	0	0
Phe	0.88	0.93	1
Lys	0	0	0

B Microsequence analyses

Cycle	PTH-AA	Yields (pmol)	
		APP_{695}	APP_{751}
1	Asp	47.5	73.7
2	Ala	76.5	130.6
3	Glu	55.4	64.7
4	Phe	57.1	87.6
5	Arg	73.2	156.5
6	His	26.1	46.5
7	Asp	37.2	67.3
8	Ser	11.5	18.6
9	Gly	31.1	48.0
10	Tyr	29.7	46.2
11	Glu	22.7	38.3
12	Val	18.3	35.1
13	His	10.3	20.7
14	His	12.3	23.9
15	Gln	5.0	11.0