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ALZHEIMER'S DISEASE: INITIAL REPORT OF THE PURIFICATION AND CHARACTERIZATION OF A NOVEL CEREBROVASCULAR AMYLOID PROTEIN

George G. GLENNER, M.D. and Caine W. WONG

University of California, San Diego (M-012), La Jolla, CA 92093

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SUMMARY: A purified protein derived from the twisted β-pleated sheet fibrils in cerebrovascular amyloidosis associated with Alzheimer's disease has been isolated by Sephadex G-100 column chromatography with 5 M guanidine-HCl in 1 N acetic acid and by high performance liquid chromatography. Amino acid sequence analysis and a computer search reveals this protein to have no homology with any protein sequenced thus far. This protein may be derived from a unique serum precursor which may provide a diagnostic test for Alzheimer's disease and a means to understand its pathogenesis.

Alzheimer's disease is the most frequent cause of dementia in the United States and the fourth most common cause of death, affecting over 2 million individuals. There is no definitive diagnostic test or therapy for this disease, no experimental animal model exists and its pathogenesis is unknown (1). It is characterized pathologically by three intracerebral sites of twisted β-pleated sheet (amyloid) fibrils (2): the intraneuronal neurofibrillary tangles, the extracellar amyloid-containing neuritic plaques and the cerebrovascular amyloid fibril deposits (3). Previous extensive studies have shown that vascular deposits of amyloid fibrils are derived from an abnormal serum protein which is processed by the lysosomal complement of endothelial cells to produce β-pleated sheet (amyloid) fibrils (4). These may differ in specific clinical conditions, e.g. an immunoglobulin light polypeptide chain (5), an acute phase serum protein designated SAA (6), a prealbumin variant (7), a human gamma trace protein (8). The first amino acid sequence analysis of an amyloid fibril protein was reported in this journal in 1970 (9) and since then a variety of others corresponding to different clinical conditions have been defined by sequence analysis (4).

Since cerebrovascular amyloidosis is seen only in Alzheimer's disease, adult Down's syndrome individuals (10) and a familial Icelandic cerebrovascular amy-

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loidosis syndrome (8), it seemed likely that isolation and identification of the cerebrovascular amyloid fibril protein in Alzheimer's disease would make possible the identification of a unique amyloid fibril precursor protein in the serum of these patients. This in turn would lead to a specific diagnostic serum test for Alzheimer's disease.

MATERIALS AND METHODS

Amyloid Fibril Concentration: Human brains obtained at autopsy were frozen at -70°C. Histological sections were taken, stained for amyloid and only those with extensive cerebrovascular amyloidoses were selected for amyloid fibril isolation. Age matched normal brains were used for controls. The meninges were stripped, and gross cortex contaminants removed. The tissue was homogenized in 0.09% sodium chloride-0.1% sodium azide and the homogenate centrifuged in a Sorvall RC-5B (DuPont Instruments) at 12,500 x g for 60 min. at 4°C. The supernatant was discarded. The resultant pellet was made up of two visually distinct layers. The thin brownish top layer was enriched in amyloid protein as monitored by polarization microscopy after Congo red staining. The layers were separated by dissection of the frozen pellet. A second homogenization of the lower layer yielded a significant second crop of amyloid protein. The amyloid enriched top layer was homogenized in 0.05 M TRIS-HCl, 3 mM NaN_3, 0.01 mM $CaCl_2$, pH 7.5 buffer to make an approximate 4% solution (w/v). Solid collagenase (EC 3.4.24.3, Sigma Chemical type I) was added in a 1:100 ratio (weight enzyme: weight pellet) and the resultant mixture was incubated in a Dubnoff shaker at 37°C for 8 hrs. The digestion by collagenase was monitered by Congo red staining with polarization microscopy and by x-ray crystallography (2). After the digestion was completed, the mixture was centrifuged in a Beckman L5-50B ultracentrifuge at 105,000 x g for 60 min. at 4°C. The supernatant was discarded and the pellet frozen at -20°C.

Protein Extraction: The collagenase-treated pellet was solubilized in 6 M guanidine-HCl, 0.1 M TRIS-HCl, 25 mM dithiotheitol, 0.34 mM EDTA, pH 8.0 (22% w/v) and stirred at room temperature for 48 hrs. After 48 hrs. the solution was centrifuged in a Beckman L5-50B ultracentrifuge at 105,000 x g for 60 min. at 4°C. The pellet was separated from the supernatant. The supernatant was placed into 1000 molecular wt. cut off dialysis tubing (Spectra/Por 6, Fisher Scientific) and dialyzed, lyophilized and the resulting powder stored dessicated at -70°C.

SDS-Urea Polyacrylamide Gel Electrophoresis (SDS-urea PAGE): SDS-urea PAGE was done by the Laemmli system (11) modified only by the addition of 8 M urea in the stacking and resolving gel. Slab gels (15%) were made 0.75 mm thick and run at 10 mA constant current. After electrophoresis, gels were stained with Coomassie Brilliant Blue R.

G-100 Sephadex Column Chromatography: The procedure was identical to that employed previously (12) using a 2.5 x 100 cm G-100 calibrated Sephadex column (Pharmacia) equilibrated with 5 M guanidine-HCl, 1.0 N acetic acid with 40 mg of protein applied. The column was calibrated with cytochrome C (horse heart), 12,384 M_r and glucagon, 3,485 M_r. The protein elution profile was monitored at 280 nm with a Beckman 35 spectrophotometer. The protein peak centered at 4,200 M_r was pooled and dialyzed exhaustively against deionized water, lyophilized and stored dessicated at -70°C.

High Performance Liquid Chromatography (HPLC): One hundred µg of the lyophilized protein from peak fractions of the Sephadex column was solubilized into 50 µl of 88% formic acid. This was injected into a Waters HPLC system. The mobile phase was: solvent A: 0.1% trifluoroacetic acid/H_2O, solvent B: 100% acetoni-

trile. The gradient was linear from 10% to 50% solvent B over 60 min. Flow rate was 0.8 ml/min. and the protein peaks were detected at 229 nm with 2.0 AUFS. The stationary phase used was a Vydac 214TP54 C_4 peptide column. Two major protein peaks were found (Fig. 1). One at 35% solvent B and the other at 36% solvent B. These protein peaks were pooled separately, lyophilized and stored at -70°C. For samples that were prepared for amino acid analysis, 10 µl of 20% sodium dodecyl sulfate was added before lyophilization.

Amino Acid Analysis: Samples to be analyzed for amino acid composition were hydrolyzed with 6 M HCl in vacuo at 110°C for 24 hrs. After 24 hrs. the samples were dried in a vacuum chamber with a NaOH trap. The dried samples were solubilized in pH 2.2 citrate buffer and injected into a Beckman 118C Automatic Amino Acid Analyzer for analysis. Norleucine was used for the internal standard.

Amino Acid Sequencing: Samples were dissolved in heptafluorobutyric acid and loaded in a Beckman 890C spinning cup sequencer. The collected anilothiazolone amino acids were converted to phenylthiohydantoin amino acids (PTH-amino acids) with 1 N HCl/MeOH at 50°C for 10 minutes. The PTH-amino acids were dried and redissolved in MeOH. The PTH-amino acids were analyzed on a Beckman 322 HPLC system fitted with a ETH-Permaphase guard column and a IBM 6 µ CN column in line. The eluent was monitored at 254 nm.

RESULTS AND DISCUSSION

By polarization microscopy after staining with Congo red the presence of amyloid fibers can readily be demonstrated by their green polarization color in the isolated meningeal vessels of Alzheimer's disease patients. With this property as our marker we have designed a protocol that affords isolation of a fraction that is highly enriched in amyloid fibers. It appears that collagen is the major contaminant in the crude homogenates. Initial experiments with collagenase showed that the amyloid fibers are resistent to this protease. After collagenase digestion of the crude homogenates with subsequent washes of the insoluble sediments with buffer, our preparations visually appear to be almost homogeneous by polarization microscopy and maintain the β-pleated sheet configuration as determined by x-ray crystallograpy (2). This material is then denatured in 6 M guanidine-HCl, 0.1 M TRIS, 25 mM dithiotheitol, 0.34 mM EDTA pH 8.0. Denaturation of the amyloid fibers is monitored by the loss of the green polarization color. The undissolved materials are removed by centrifugation and the supernatant dialyzed and lyophilized. Six Alzheimer's disease cases and three age matched control cases were examined by SDS-urea PAGE. The lyophilized material revealed a unique band of protein that was not seen in control samples prepared in identical fashion. This protein could be consistently fractionated on a calibrated G-100 Sephadex column with its peak fraction

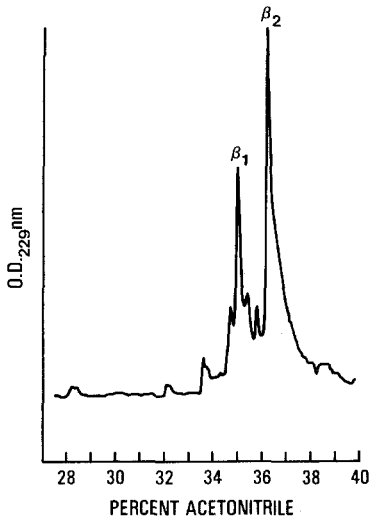

Fig. 1 HPLC of cerebrovascular amyloid fibril protein β from a patient with Alzheimer's disease, previously isolated on Sephadex G-100, reveals two major protein peaks (β_1 and β_2), each of similar amino acid composition and identical amino-terminal amino acid sequence (Table 2).

centered at 4,200 daltons. Because of its uniqueness to amyloid fibril preparations, it was assumed, as shown in numerous, previous studies (4), to be a major protein constituent of the amyloid fibrils. High performance liquid chromatography fractionated two peaks from the G-100 preparation (Fig.1) and these were found to have almost identical amino acid compositions (Table 1). Two other cases of Alzheimer's disease gave identical HPLC profiles.

The vast majority of amyloid fibrils in the systemic diseases are created from a serum protein precursor by proteolysis, probably by the lyosomal complement of phagocytes (4). The finding of proteins fractionated into two peaks by HPLC suggests that one may represent a proteolytic cleavage product of the other. The proteins obtained from these two peak fractions were submitted to automated amino acid sequencing to residue 24 (Table 2). The partial sequences of the two peaks were identical. A computer search of this sequence reveals no homology with any protein sequenced thus far including a human gamma trace serum protein found to constitute the fibrils of a familial Icelandic cerebrovascular amyloidosis (8). This protein, therefore, appears to be a biologic marker for the cerebrovascular amyloid fibril component of Alzheimer's disease.

TABLE 1. Amino Acid Analyses of Chromatographic Fractions

AMINO ACID	Nanomolar Percent			No. of Residues	
	G-100	HPLC			
	β	β_1	β_2	β_1 Peptide	β_2 Peptide
Asp	11.47	11.07	11.11	3	3
Thr	3.15	1.59	1.36	1	1
Ser	5.60	6.35	5.38	2	2
Glu	11.40	12.26	11.16	4	3
Pro	T*	T	T	0	0
Gly	13.65	16.71	15.23	5	5
Ala	8.27	8.08	7.91	2	2
½ Cys	0.94	T	T	1	1
Val	9.47	8.69	11.74	3	4
Met	2.92	2.06	2.48	1	1
Ile	4.29	3.58	3.90	1	1
Leu	7.34	6.68	6.30	2	2
Tyr	2.71	1.89	2.07	1	1
Phe	5.76	6.13	6.95	2	2
His	4.64	5.44	5.94	2	2
Lys	5.01	6.46	5.44	2	2
Arg	3.30	2.97	2.89	1	1
Try	ND**	ND	ND	-	-
Totals	99.92	99.96	99.86	33	33

* Trace; **Trytophan not determined.

The significance of this finding is that when monoclonal antibodies are raised to the amyloid fibril protein reported here, these should detect an antigenically related protein in Alzheimer's disease patients' sera. If the

Table 2. Sequence of Cerebrovascular Amyloid Protein

NH_2-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Gln-Val-
His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val---COOH

serum protein is also unique to this condition, a diagnostic serum (e.g. radioimmunoassay) test for Alzheimer's disease can be devised. This study is in progress. If adult Down's syndrome individuals also are found to have this cerebrovascular amyloid protein (10), they will represent the first predictable model for the study of the pathogenesis of Alzheimer's disease.

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