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Fibril in senile systemic amyloidosis is derived from normal transthyretin

(prealbumin/primary structure)

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ABSTRACT **The amyloid fibril in senile systemic amyloidosis (SSA), like that of familial amyloidotic polyneuropathy, is derived from transthyretin (TTR). SSA, however, is a common disease, affecting to some degree 25% of the population >80 years old. In familial amyloidotic polyneuropathy, the amyloidogenesis has been considered to depend on point mutations leading to TTR variants. We show that the TTR molecule in SSA, on the other hand, has a normal primary structure. Factors other than the primary structure of TTR must therefore be important in the pathogenesis of TTR-derived amyloid.**

The amyloid fibril contains as a major constituent low molecular weight proteins that vary in nature between different types of amyloid (1). Since the original description by Costa *et al.* (2) that transthyretin (TTR) (prealbumin) is a main constituent in the Portuguese type familial amyloid, many other forms of familial amyloidosis have been shown to have TTR as a major subunit protein. Recent studies have revealed that the hereditary factor in the familial amyloidosis is a point mutation in the TTR gene, leading to an amino acid substitution. Several such mutations, each linked to a specific type of amyloid disease, have now been demonstrated. The most widely known amino acid substitution in the TTR molecule is methionine for valine at position 30, found in the Portuguese, Swedish, and Japanese types of familial amyloidosis (3–6).

The most common systemic amyloidosis is senile systemic amyloidosis (SSA), earlier called senile cardiac amyloidosis (7, 8). SSA has been shown to affect ≈25% of persons >80 years old (8), with deposits in many organs. It is usually a benign disease without symptoms. Some individuals, mainly men, are more severely affected with heavy deposits in the myocardium, sometimes giving rise to cardiomegaly and congestive heart failure (7, 9, 10).

Previous studies have shown that a major constituent of the fibrils in SSA is derived from TTR (11, 12). A major question in the understanding of the pathogenesis of the TTR-derived amyloidoses is whether or not an amino acid substitution is a prerequisite for formation of amyloid fibrils. In previous partial characterization of the TTR in three patients with SSA, no deviation from the normal sequence was found (13). However, it has recently been claimed that SSA is a hereditary disease with late onset (14). In the present study, we have characterized the TTR molecule purified from the amyloid fibrils from a patient with typical SSA, and no amino acid substitution was found.

MATERIALS AND METHODS

Heart tissue from one patient (patient 34) with typical SSA was frozen at −20°C. The patient was a male who died when he was 92 years old. Unexpectedly, a large diffusely amyloid-infiltrated heart was found at autopsy. Microscopic analysis revealed small amyloid deposits in many organs.

Purification of Amyloid Fibril Proteins. Amyloid fibrils were extracted (15) from heart ventricles by sequential homogenization 10 times in 0.15 M NaCl, followed by homogenization three times in distilled water. After centrifugation at 20,000 rpm (Beckman JA 20 rotor), most amyloid was found in the pellet material, which was lyophilized and used in the present studies.

Amyloid fibrils were defatted in chloroform/methanol (2:1) and treated with 6 M guanidine hydrochloride in 0.1 M Tris·HCl (pH 8.0) without reduction for 48 hr at room temperature. The material was then centrifuged and the supernatant was applied to a Sepharose CL-6B column, which was eluted with 5 M guanidine in distilled water. This process resulted in a typical pattern with a fairly small V_0 peak, followed by a wide area (designated fraction A) with increased optical density without distinct peaks, and finally an asymmetric late retarded protein peak (designated fraction B) (Fig. 1). The two fractions were dialyzed separately against deionized water and lyophilized. Previous experiments (13–15) have shown that both full-length TTR (called TTR-like protein) and TTR fragments occur in the fibrils in SSA. Under nonreducing conditions, fraction B consists mainly of cysteine-lacking TTR-derived protein fragments, while the TTR-like amyloid fibril protein is eluted in fraction A (13).

For purification of TTR-like protein, material from fraction A was dissolved in 6 M guanidine hydrochloride/0.1 M Tris·HCl buffer, pH 8.0, containing 0.1 M dithiothreitol and was stirred for 2 hr at room temperature. Dithiothreitol was removed by gel filtration through a Sephadex G-25 column (0.9 × 10 cm) and the protein solution was then applied to a Thiopropyl Sepharose CL-6B column (1.6 × 10 cm), equilibrated with 4.5 M guanidine hydrochloride/0.1 M Tris·HCl, pH 8.0 (13). The column was rinsed with the guanidine solution and finally eluted with the equilibration solution, now containing 25 mM L-cysteine. The eluted protein material was dialyzed against deionized water and lyophilized. Further purification was obtained by gel filtration through a Sephacryl S-200 HR column (0.9 × 60 cm) equilibrated and eluted with 5 M guanidine hydrochloride in distilled water.

Fraction B was used for further purification of cysteine-lacking TTR fragments. Lyophilized protein (1 mg/ml) was dissolved in 5 M guanidine hydrochloride in water and injected into an LKB HPLC unit connected to a Vydac 214 TPS column. Proteins were eluted with a 0–70% linear gradient of acetonitrile in 0.1% trifluoroacetic acid and the effluent registered at 224 nm.

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Abbreviations: TTR, transthyretin; SSA, senile systemic amyloidosis; BNPS-skatole, 3′-bromo-3-methyl-2-(2-nitrophenylsulfenyl)indolamine.

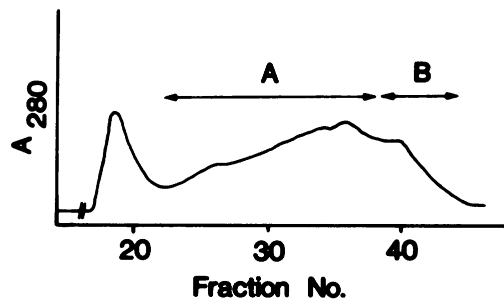

FIG. 1. The gel filtration elution profile of amyloid fibrils dissolved in guanidine hydrochloride and run on a Sepharose CL-6B column. Fractions in A and B were pooled separately for the purification of TTR-like material and TTR-derived fragments, respectively.

Electrophoresis. SDS/PAGE was performed as described (16).

Amino Acid Analysis. TTR-derived polypeptides were hydrolyzed in 6 M HCl and quantitated by amino acid analysis using a Biotronik Wissentschaftlich Gerate, Frankfurt am Main, F.R.G. LC 5000 automatic amino acid analyzer (13).

N-Terminal Analysis. The polypeptides were tested for homogeneity and further analyzed by Edman degradation using a model 477A automatic sequence analyzer from Applied Biosystems, with an on-line 120 A PTH amino acid analyzer (13).

Fragmentation. Polypeptides were cleaved with cyanogen bromide (17) and 3′-bromo-3-methyl-2-(2-nitrophenylsulfenyl)indolamine (BNPS-skatole) (18). Digestion with endoproteinase Asp-N, obtained from Boehringer Mannheim, was performed with SDS-treated polypeptide as described by Drapeau (19).

Purification of Peptides. Peptides were purified on a C_{18} reversed-phase column 218 TP54 (Vydac, CA). A 45-min linear gradient from 0% to 80% of solvent B was used at a flow rate of 1.0 ml/min (20). Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 30% solvent A in acetonitrile.

RESULTS

Purification and Analysis of a TTR-Like Molecule. Previous studies have shown that the TTR-like protein in the amyloid fibrils in SSA and familial amyloidotic polyneuropathy of Swedish type seems to be covalently bound (13, 21). This characteristic made it possible to purify the TTR molecule by initial gel filtration without reduction followed by covalent chromatography and subsequent gel filtration of the cysteine-containing material. In this way, we obtained TTR-like protein that was essentially free from TTR fragments.

N-terminal sequence analysis revealed a ragged N terminus with species starting in positions 1, 2, 3, 4, 5, 6, and 7 of TTR. The contents of these species decreased gradually from 25% (position 1) to 8% (position 7). The initial yield was ≈20%, which is within the expected range when working with lower concentrations of proteins. The TTR sequence to position 14 was confirmed. Cyanogen bromide cleavage of the protein resulted in a free N terminus starting in position 14 of TTR. Thirty-two degradation cycles confirmed positions 14–40 and 43–45. Positions 41 and 42 could not be established. The material left on the glass fiber membrane after 32 degradation cycles was then used for cleavage with BNPS-skatole and taken directly back to the sequencer for analysis. Nineteen degradation cycles confirmed positions 43–51 and 82–98.

TTR-like protein was also digested with endoproteinase Asp-N and the peptides were separated on a C_{18} reversed-phase column. Peptide materials obtained from single peaks were taken for N-terminal analyses. The results confirmed and established the sequences of positions 18–35, 38–41, 42–60, 63–73, 74–88, 89–92, 92–98, and 99–127. The amino acid residues in positions 17, 42, and 62 could not be verified because of background interference. With the partial amino acid sequences of the TTR-derived fragments (see below), the full sequence of the amyloid TTR molecule was obtained (Fig. 2). No amino acid substitution was found.

Amino Acid Sequence Analysis of TTR-Derived Fragments. Reversed-phase HPLC on a C_4 column of the late retarded peak proteins from fraction B, obtained in the first gel filtration, resulted in many closely eluted peaks, which were purified by rerunning in HPLC. Characterization by N-terminal analyses showed that fragments started in positions 46, 49, and 52 of TTR (Fig. 2). Positions 46–78 were established. The amino acid sequence in this region was identical to that reported for TTR (22, 23).

DISCUSSION

All TTR-derived amyloid fibril proteins that have been studied with complete amino acid sequence analysis have shown amino acid substitutions (3–6, 24–29). Such substitutions have therefore been considered to be the primary defect in the amyloidogenesis involving TTR. Regarding the very high frequency of SSA, amino acid substitutions in the TTR in all cases is unlikely, however. The present study shows that TTR with normal primary structure can also make up amyloid

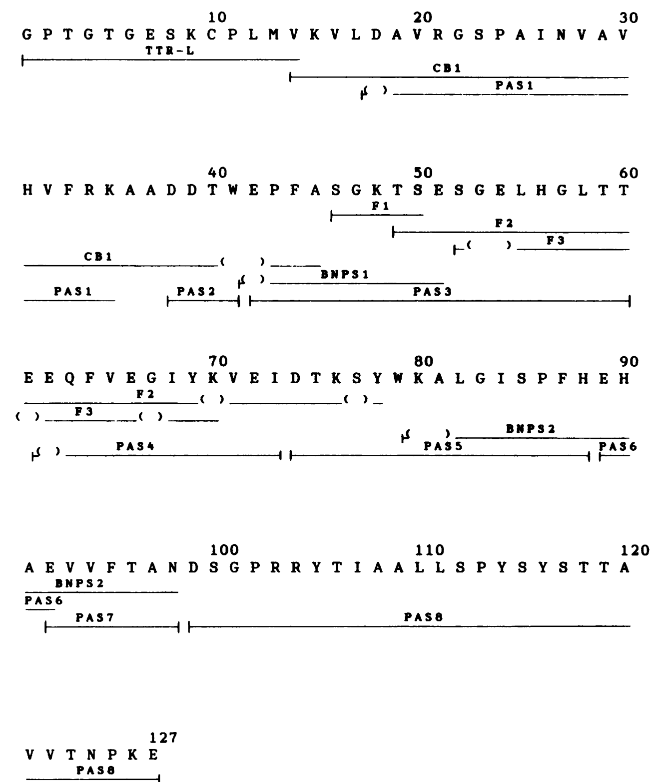

FIG. 2. The amino acid sequence (single-letter code) of an amyloid protein derived from TTR in patient 34. The sequence is identical to that of normal TTR but a large portion of the TTR-like protein started in positions 2–7. TTR fragments started in positions 46, 49, and 52 and constituted a considerable part of the amyloid fibrils. The sequence was obtained by studies of the whole TTR-like protein (TTR-L), TTR fragments occurring in the fibrils (F1, F2, and F3), and peptides resulting from cleavage with cyanogen bromide (CB), endoproteinase Asp-N (PAS), and BNPS-skatole (BNPS). No overlapping between PAS7 and PAS8 was obtained. Parentheses indicate not fully identified amino acid residues.

fibrils. Mechanisms other than amino acid substitutions must therefore be important in fibrillogenesis in TTR amyloidosis.

Cardiomyopathy is the main hallmark of advanced SSA (7, 9, 10). Forms of TTR-derived familial amyloidosis in which cardiomyopathy predominates in the clinical picture also exist. One such form is the Danish amyloidotic cardiomyopathy, where a methionine for valine substitution in position 111 of TTR has been recently shown (28). An isoleucine for valine substitution at position 122 has recently been found in several patients with amyloidotic cardiomyopathy (14, 30–32). It has been claimed that these patients suffer from SSA; however; this conclusion can be questioned since at least one of them was much younger (68 years old; ref. 30) than most patients with SSA. In our material, SSA with advanced cardiomyopathy is almost exclusively observed in individuals >80 years old (ref. 7; B.J. and P.W., unpublished observations). Therefore, amyloidosis with substitution of isoleucine for valine in position 122 should be regarded as a previously unknown form of familial amyloidosis. In a previous study of another patient (HL) with SSA, in which a partial amino acid sequence analysis of the amyloid subunit protein was performed, no substitution at position 122 was seen (11).

An interesting finding in SSA and other forms of TTR-derived amyloid is the occurrence of TTR fragments in the fibrils (13, 21). In many cases of SSA, as well as in some patients with Swedish familial amyloidotic polyneuropathy, such fragments predominate over the full-length TTR molecules (6, 13, 21). This fragmentation is not random, since cleavage has been reported predominantly at positions 46, 49, and 52 (13, 28). In SSA and other TTR-derived amyloid forms, it is possible that cleavage in the TTR molecule precedes the amyloid fibril formation. This process might expose hidden sequences that are prone to aggregate. Another possibility is that cleavage takes place after the fibril has formed. The region with positions 46–52 is in the periphery of the TTR tetramer (33) and is possibly most easily attacked by enzymes. A finding that may indicate that proteolysis is a secondary event is that the proportion of uncleaved TTR in the fibrils varies greatly (unpublished observation).

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