Systemic Senile Amyloidosis

Identification of a New Prealbumin (Transthyretin) Variant in Cardiac Tissue: Immunologic and Biochemical Similarity to One Form of Familial Amyloidotic Polyneuropathy

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Abstract

Isolated amyloid fibrils from threes cases of systemic senile amyloidosis (SSA) contained subunit proteins with molecular masses of 14 (10–20%), 10–12 (60–80%), and 5–6 kD (5–10%) when fractionated under reducing and dissociating conditions. This grouping was identical to that seen in SKO, a case of familial amyloidotic polyneuropathy (FAP) studied earlier. Amino acid sequencing confirmed that SSA subunit proteins were in fact prealbumin (transthyretin). Complete sequence analysis of one SSA preparation revealed the presence of a new variant Pa (TTr) molecule with a single amino acid substitution of isoleucine for valine at position 122. Further studies used an antiserum specific for SKO IV, a subunit protein of SKO previously shown to correspond to carboxy-terminal 78 residues (positions 49–127) of Pa (TTr). Anti-SKO IV reacted with SSA in tissue at equivalent dilutions to anti-Pa (TTr) and with the 10–12-kD fraction of SSA on Western blots; reactivity was blocked by SKO IV, but not by Pa (TTr). SSA is a form of systemic amyloidosis caused by tissue deposition of Pa (TTr) and its fragments, with shared conformational or subunit antigenicity to at least one form of FAP. Identification of a new variant Pa (TTr) molecule in one case suggests further that SSA may be a genetically determined disease expressed late in life.

Introduction

The prealbumin (transthyretin)-related amyloidoses are an expanding spectrum of overlapping clinical syndromes, including several different forms of familial amyloidotic polyneuropathy (FAP)[1] (1–5), at least one type of familial amyloidotic cardiomyopathy (6), sporadic vitreous amyloid (7), and systemic senile amyloidosis (SSA) (8, 9). Among the FAP syndromes, the subunit protein composing amyloid fibrils depositing in different tissues is a variant Pa (TTr) molecule with a single-amino acid substitution at any of six positions in the molecule identified so far (Fig. 1) (1–5, 10–12). Although most studies have reported subunit proteins with the molecular weight of Pa (TTr) monomer (14 kD), smaller fragments have been identified in some instances (6, 7, 14).

Of particular interest has been amyloid isolated from SKO, an Israeli patient affected by FAP. Amyloid fibrils of SKO dissociate to yield three fractions, including Pa (TTr) monomer and fragments corresponding to residues 1–48 and 49–127 of the molecule (1, 10). All fractions have heterogeneous amino termini, suggesting that digestion by endopeptidases, may be important in the processing of Pa (TTr) leading to fibril formation. Both glycine and threonine are found at position 49 in peptides containing this residue (1, 10). Furthermore, an amino acid substitution of isoleucine for phenylalanine at position 33 has been reported to be present in some tissues of SKO but not others (11, 15). In sum, these studies suggest that a structurally abnormal Pa (TTr) molecule may be a prerequisite, but is not sufficient, for amyloidogenesis to occur, and that proteolysis is also important.

We report detailed biochemical studies of amyloid fibril subunit proteins isolated from involved cardiac tissue of individuals affected by immunoglobulin light chain-related (AL), secondary (AA) amyloidosis, and SSA. Amino acid sequence analysis of subunit proteins from one case of SSA has revealed the presence of a new Pa (TTr) variant molecule (Ile^{122}). In addition, a major proteolytic cleavage at position 49 was seen in all SSA preparations, identical to that previously described for the FAP SKO molecules. Furthermore, an antiserum to the carboxy-terminal 49–127 fragment of SKO cross-reacted with several cases of SSA, both by immunoperoxidase staining in tissue sections and on Western blots of solubilized fibril subunit proteins.

Methods

Tissues. Extractions were performed on involved cardiac tissue obtained postmortem from three cases of SSA and from two cases of primary and one of secondary amyloidosis. The presence of extensive cardiac amyloid was confirmed by Congo red staining of fixed tissue and the presence of typical birefringence under polarized light. The diagnosis of secondary amyloidosis was made on the basis of an associated inflammatory disease (tuberculosis), permanganate sensitivity, and reactivity immunohistologically with an antiserum to AA protein. Age-matched autopsy material histologically free of amyloid served as controls. Except for the case of secondary amyloidosis and one case of SSA (obtained in a previous study, reference 26), all material was collected under a protocol approved by our institutional Human Subjects Committee.

Isolation of amyloid fibrils and tissue P component. Fibrils were isolated by a modification of the method of Pras (16), incorporating

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1. *Abbreviations used in this paper:* AL, light chain amyloidosis; CNBr, cyanogen bromide; FAP, familial amyloidotic polyneuropathy; Pa (TTr) prealbumin (transthyretin); SA, secondary amyloidosis; SSA, systemic senile amyloidosis.

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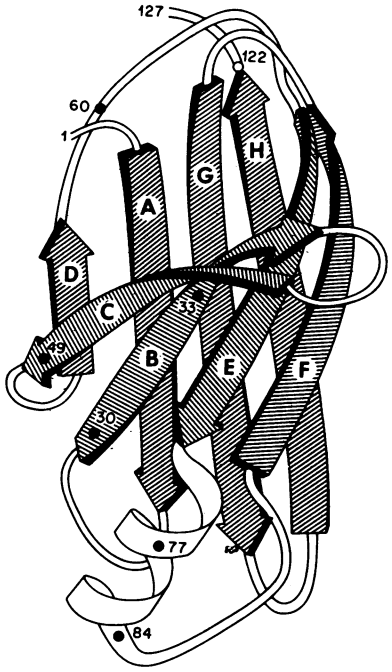

Figure 1. Ribbon representation of Pa (TTr) monomer predicted from x-ray diffraction analysis (13), indicating the location of amino acid substitutions identified to date (1–5, 10–12) for different kindred affected by FAP, and the ILE^{122} substitution found in one case of SSA; A-H ⇦, indicate beta strands in a barrel configuration; ⌓, alpha-helical segments; ⌒, random coils.

0.05 M sodium citrate into the second to sixth saline homogenizations as suggested by Skinner et al. (17) to facilitate isolation of P-component (AP). After six to seven saline-citrate extractions, the remaining residue was homogenized three times in distilled water to yield a cream-colored top-layer preparation after centrifugation at 30,000 rpm for 3 h at 4°C (L5-65; Beckman Instruments, Fullerton, CA). Two to four additional centrifugations at 20,000 rpm for 1 h completed the water extractions, and all top layers were pooled. Top layers and pooled saline supernatants were dialyzed overnight at 4°C against distilled water with 50,000-mol wt cut-off dialysis tubing (Spectrum Medical Industries, Los Angeles, CA). Top layers were diluted in distilled water to give opalescent suspensions, and established to be fibril preparations by the following criteria: (*a*) prompt precipitation on addition of 0.15 M NaCl; (*b*) clarification of the suspension with 0.1 N NaOH; (*c*) metachromatic binding of Congo red when diluted in a 1 mg/ml solution of the dye made up in 0.15 M NaCl; (*d*) birefringence of precipitated material; and (*e*) typical fibrillar ultrastructure by electron microscopy (16). AP was isolated by (*a*) calcium-dependent binding to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) (18); and (*b*) as the first post-void peak of dialyzed and lyophilized saline supernatant subjected to gel filtration on Sephadex G-100 in 5 M guanidine-1 M acetic acid after solubilization in 0.17 M DTT (see below). In later extractions, additional fibril subunit proteins were extracted from the insoluble residue after water homogenization by direct solubilization in guanidine.

Gel filtration. Lyophilized fibrils were dissolved in 6 M guanidine-HCl, 0.1 M Tris, pH 10.2 containing 0.17 M DTT, and then mixed 1:3 (vol/vol) with 2 M guanidine-4 M acetic acid. The solution was centrifuged at 40,000 rpm for 1 h at room temperature to remove insoluble debris and the supernatant gel filtered through a Sephacryl S-300, 50 × 6 cm column equilibrated with 5 M guanidine-1 M acetic acid. Pooled fractions containing subunit proteins were dialyzed against distilled water using low molecular weight (3,500) cut-off dialysis tubing (Spectrum Medical Industries). Subunit proteins were lyophilized and repurified by chromatography on an Ultrogel Ac54, 80 × 2 cm column equilibrated with 5 M guanidine-1 M acetic acid at room temperature.

Gel electrophoresis and Western blots. Purified proteins were characterized on 10–20% gradient 8 M urea slab SDS-PAGE under reducing conditions with 0.1 M DTT (19). Two-dimensional gels were run on a modified Anderson ISODALT II System (20). Individual gels were blotted onto diazotized paper (ABM; Bio-Rad Laboratories, Richmond, CA), as previously described (20).

Isolation of normal prealbumin (transthyretin). Pa (TTr) was purified from 1-liter batches of normal plasma passed through an 18 × 40 cm QAE-A50 Sephadex column equilibrated with 0.001 M NaPO4, pH 8.0 and eluted with a stepwise gradient by a modification of the method of Raz and Goodman (21). Material eluting at 0.4 M NaCl, 0.003 M NaPO4, pH 8.0 was precipitated with 60% ammonium sulfate, 4°C, overnight, and the supernatant dialyzed and lyophilized. This material was found to contain Pa (TTr) tetramer (molecular mass 56 kD), retinol-binding protein, and small amounts of albumin and high molecular weight kininogen. Dissociation to Pa (TTr) monomer (molecular mass 14 kD) required solubilization in 6 M guanidine-HCl, 0.17 M DTT, for 2–3 d, followed by addition of 3 vol 2 M guanidine-4 M acetic acid. The monomer was isolated as a retarded peak when this material was then fractionated on a Sephadex G-100, (83 × 4.5 cm) column. It was homogeneous on SDS-PAGE under reducing conditions, and reacted with identity to normal serum when tested against anti-Pa (TTr) in double diffusion.

Antisera. Rabbit antisera to AA protein, the major subunit protein of human secondary amyloid, P component, FAP SKO IV protein, and Pa (TTr) monomer isolated as outlined above were produced as previously described (1, 10, 20). Anti-kappa and anti-lambda light chain antisera were made to purified Bence Jones proteins. All antisera gave single precipitin lines when tested by immunodiffusion and immunoelectrophoresis against purified proteins and normal serum. Amyloid subunit proteins were tested against monospecific antisera by double diffusion in agar containing 0.1% SDS. Proteins were solubilized with 0.1 N NaOH, and then brought to neutrality with an equivalent volume of 0.1 N HCl before application to the Ouchterlony plate. Before use for immunoperoxidase and Western blot studies, anti-SKO IV was passed three times through a Sepharose 4B absorbant to which Pa (TTr) monomer (prepared as described above) was coupled by activation with cyanogen bromide (22).

Biochemical studies. Amino acid analyses were performed on an automatic analyzer (D-500; Durrum). Samples were hydrolyzed in 0.2 ml 6 N HCl under vacuum for 24 h at 110°C. 40 μl of 1% aqueous phenol solution were added to prevent degradation of tyrosine. The presence of tryptophan was determined by amino acid sequencing. Automated sequence analyses of isolated proteins and of individual peptides were determined by Edman degradation using a liquid phase sequenator (890C; Beckman Instruments) and a 0.1 M Quadrol program. Thiazolinone amino acids were converted to phenylthiohydantoin amino acids (PTH) in a Sequemat P-6 converter at 65°C using methanol/HCl (7:1 vol/vol) and were identified by HPLC performed on a Waters HPLC model ALC/GPC-204 prepacked with an IBM 5 fM octadecyl column eluted with a methanol/water gradient. Low yield peptides were sequenced on a 470A protein sequencer (Applied Biosystems, Inc., Foster City, CA) and the resulting PTH amino acids were identified using the ABS on-line 120A PTH analyzer and standard Applied Biosystems, Inc. program.

Cyanogen bromide (CNBr) cleavage of isolated proteins. Pa (TTr), SSA and SKO amyloid subunit proteins were dissolved in 3 ml 70% formic acid and reacted with 5:1 (wt/wt) CNBr for 24 h, at room temperature with occasional stirring. The sample was then diluted with 30 ml of distilled water and lyophilized. Lyophilized material was washed × 3 with distilled water, centrifuged 30 min at 40,000 rpm in an ultracentrifuge (L5-65; Beckman Instruments, Inc.) the supernatants were discarded, and the final residue was used for amino acid sequence studies.

Isolation of tryptic peptides. Purified Pa (TTr), SSA or SKO proteins pretreated with 70% formic acid, were dissolved (1 mg/ml) in 0.2 M ammonium bicarbonate (pH 8.2) and digested with TPCK-treated trypsin (Worthington Biochemicals, Malvern, PA) at an enzyme/substrate ratio of 1:50 (wt/wt) for 3 h, at 37°C and lyophilized. Tryptic peptides were isolated by reverse-phase chromatography on a μBondapak CF_{18} column (0.78 × 30.0 cm, Waters Associates, Milford, MA)

with a gradient of 0–66% acetonitrile in 0.1% CF_3COOH (Pierce Chemical Co., Rockford, IL), pH 2.5. Some peptides were further purified by chromatography on a second μBondapak C_{18} column (0.38 × 30 cm) with a gradient of 0–66% acetonitrile in 20 mM NH_4OAc, pH 5.6. Column eluents were monitored at 210 nm. Individual peptides were further characterized by amino acid analysis or direct sequencing.

Immunohistology. Immunoperoxidase studies were performed by a modification of the method of Sternberger as described (22). Absorptions to prove specificity were done by incubating antiserum with purified antigen, 1–10 mg/ml, overnight at room temperature. Serial sections from individual blocks were run for comparison.

Results

Isolation of amyloid fibrils and tissue P component. Top-layer material from all cases of cardiac amyloid was found by electron microscopy to contain fibrils. No fibrillar ultrastructure could be detected in equivalent material isolated from age-matched nonamyloidotic controls. The yield of lyophilized top layer was < 1% of the original tissue wet weight. Purified AP had a typical doughnut configuration in cross section by electron microscopy and was found to have subunit molecular mass of 24 kD on SDS-PAGE under nonreducing conditions and to react with identity with anti-AP. No AP could be detected in control nonamyloidotic saline supernatants.

Amyloid fibril subunit proteins. Amyloid fibrils were rigorously solubilized with 0.1 N NaOH, brought to neutrality with 0.1 N HCl and analyzed in double diffusion, with 0.1% SDS incorporated into the agar. Fibrils isolated from cases of SSA did not react in double diffusion with antisera to AA, SAA, AP, kappa, or lambda light chains. However, a faint precipitin line (not shown) was seen with antisera to Pa (TTr). Additional subunit proteins could be obtained by direct extraction in guanidine of the insoluble residue obtained following water homogenization.

Fig. 2 shows a representative profile obtained on Sephacryl S-300 of SSA amyloid fibrils fractionated under reducing and dissociating conditions. A similar profile was obtained with material extracted directly from the insoluble residue in guanidine. By contrast is shown the profile obtained with (*a*) top-layer material from age-matched control cardiac tissue that was devoid of fibrils by electron microscopy; (*b*) amyloid fibrils extracted by an identical protocol from involved cardiac tissue of two cases of primary and one of secondary systemic amyloidosis. Whereas all preparations had a void peak, each of the three types of cardiac amyloid had additional retarded low molecular weight fractions, distinct one from the other as to elution profile and antigenicity. Each SSA amyloid preparation had three peaks, the first of which was reactive with anti-Pa (TTr) in double diffusion. By contrast, both primary cardiac amyloid preparations showed single broad retarded peaks, extending over the molecular mass range of 15–20 kD. One of these reacted weakly with an antiserum specific for lambda light chains of immunoglobulin and had a major band on SDS-PAGE of 18 kD (not shown). The secondary amyloid fibril preparation had a single, well-defined peak of 8.5 kD, homogeneous on SDS-PAGE (Fig. 3, *bottom*) and reactive with antibody to AA protein.

The three SSA Pa (TTr)-positive and -negative peaks were pooled (Fig. 2) and repurified on Ultrogel Ac54 in guanidine to yield three distinct fractions, designated A, B, and C (Fig. 3, *top*). Peak A reacted with anti-Pa (TTr), and had identical (14

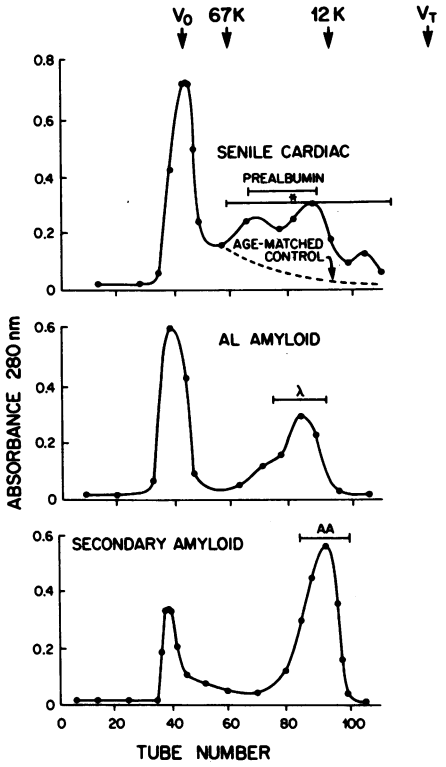

Figure 2. Amyloid fibrils were isolated as described in Methods and fractionated on a Sephacryl S-300 column. (*Top*) Representative elution profile obtained with each of three SSA preparations studied. (*Middle*) One of two cases of primary amyloidosis, this from a patient known antemortem to have monoclonal lambda light chains in urine. (*Bottom*) Fibrils isolated from a patient with secondary amyloidosis (AA) (due to tuberculosis) with cardiac involvement. After dialysis, individual fractions were tested against antisera to Pa (TTr), AA and lambda and kappa immunoglobulin light chain determinants in double diffusion, with 0.1% SDS incorporated into the agar. Specific reactivity is indicated by the horizontal bars (——). Although all three SSA peaks (*) were pooled for repurification on ultrogel AC 54 (Fig. 3), only the first two (prealbumin) reacted with antisera to Pa (TTr).

kD) molecular mass (Fig. 3, *bottom*) as Pa (TTr) monomer. Peak B did not react with anti-Pa (TTr) and was heterogeneous on SDS-PAGE, appearing as a doublet of molecular mass 10–12 kD. It made up 70% of the total subunit protein by weight. Peak C composed 10–20% of the subunit protein, was poorly soluble even in guanidine or 10 M urea, did not react with anti-Pa (TTr), had a molecular mass of 5 kD by gel filtration, and appeared as a diffuse faint band on SDS-PAGE (Fig. 3, *bottom*).

Amino acid sequences of fibril subunit proteins. The identity of each of the three types of cardiac amyloid subunit protein was confirmed by amino acid sequencing of isolated repurified subunit proteins (Fig. 4). The amino-terminal sequence of the cardiac AA protein to residue 11 was consistent with that previously reported for other human AA proteins. The anti-lambda positive, 18-kD cardiac protein was sequenced to residue 27 and placed in the Vλ6 light chain subgroup. This subgroup of immunoglobulin light chain is almost invariably associated with primary amyloidosis (24). Peak A of each of the three SSA preparations had a heterogeneous amino terminus, with major sequences beginning at residues 1, 4, and 6 of Pa (TTr). Partial CNBr cleavage gave an unambiguous

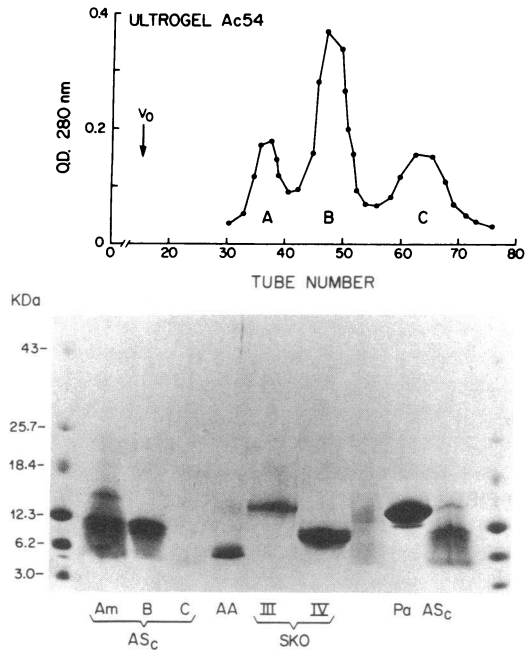

Figure 3. (*Top*) Elution profile of repurified SSA fibril subunit proteins (15 mg), fractionated on an Ultrogel Ac54 column in guanidine-acetic acid. (*Bottom*) 10–20% 15:1 acrylamide/bisacrylamide slab gel polymerized in 8M urea under reducing conditions, showing initial material (Am) pooled from Sephacryl S-300 (Fig. 2), and repurified peaks B and C, compared with (*A*) SKO III and IV proteins isolated from heart, (*B*) AA protein obtained from 2° cardiac amyloid fibrils, and (*C*) Pa (TTr) monomer isolated as described in Methods. Molecular weight markers are insulin (3 kD), bovine trypsin inhibitor (6.2 kD), cytochrome *c* (12.3 kD), β-lactoglobulin (18.4 kD), α-chymotrypsinogen (25.7 kD), and ovalbumin (43 kD).

sequence from residue 14 to 30, identical to that of normal plasma Pa (TTr) reported by Kanda et al. (25). Peak C was not further characterized because of its low yield and poor solubility.

The complete amino acid sequence of peak A from the third SSA preparation is shown in Fig. 5 *A*, and was deduced as follows: automated Edman degradation of peak A gave the sequence of 14 residues from the amino terminus and the CNBr cleavage product (CNBr II) obtained from peak A established an unambiguous sequence to residue 35. Individual tryptic peptides obtained by HPLC (Fig. 5 *B*) were further characterized by amino acid composition and/or Edman degradation (Fig. 5 *A*) and placed by homology to normal Pa (TTr) (25). Incomplete hydrolysis at positions 35–36 (lysyl-alanyl) yielded peptide T5.6 (residues 35–48); and at positions 70–71 (lysyl-valyl) dipeptide T7.8 (residues 49–76). Incomplete cleavage at positions 104–105 (arginyl-tyrosyl), and 126–127 (lysyl-glutamyl) resulted in tripeptide T11.12.13 (residues 104–127) (Fig. 5 *B*). Further purification of peptide T9 revealed a chymotryptic peptide (C1), corresponding to positions 115–127 by direct sequence analysis (Fig. 5 *A*). A single amino acid substitution of isoleucine for valine at position 122 was found present in peptides resulting both from tryptic and chymotrytic splits (Figs. 5, *A* and *B*). Automated Edman degradation of Peak B revealed that it also had a heterogeneous amino terminus beginning at positions 46, 49, and 52 of Pa (TTr). Tryptic peptides isolated by HPLC (Fig. 5 *B*) were analyzed and sequenced (Fig. 5 *A*). Tryptic peptide T7 of peaks A and B had both threonine (80%) and glycine (20%) at the amino terminus by Edman degradation.

Cross-reactivity with SKO FAP proteins. Amino acid sequence studies of amyloid fibril subunit proteins isolated from spleen and thyroid of patient SKO have been previously reported (1, 10). SKO III is the intact Pa (TTr) monomer, SKO IV is the carboxy-terminal 78 residues of the molecule, and SKO V is the first 48 residues.

Two-dimensional gels of dissociated amyloid fibrils isolated from several cases of SSA confirmed the presence of the 14-kD, Pa (TTr) monomer, evident as three closely related spots of decreasing intensity, a heterogeneous, more acidic, fraction corresponding to the B fraction, and low molecular weight peptides (Fig. 6, *top*). The molecular weight and isofocusing coordinates of the SKO IV amyloid fibril subunit protein isolated from heart corresponded to the B fraction seen in the cases of SSA (Fig. 6, *middle*). A rabbit antiserum raised to SKO IV was rigorously absorbed with normal Pa (TTr) monomer. This antiserum still reacted with the SSA fragment corresponding to positions 49–127 on Western blots of two dimensional gels of solubilized tissue proteins (Fig. 6, *bottom*).

Amyloid tissue deposits of formalin-fixed material from each of ten cases of SSA tested reacted with an antiserum prepared to SKO IV, immunoabsorbed with Pa (TTr) (Fig. 7, *left*), thus showing cross-reactivity that did not depend on intact Pa (TTr) monomer. Immunoperoxidase staining was blocked by preabsorption with purified SKO IV (Fig. 7, *middle*) but not by Pa (TTr) (Fig. 7, *right*).

Discussion

Cardiomyopathy due to amyloid infiltration of the heart may be a manifestation of primary or myeloma-associated, secondary, or the neuropathic hereditary forms of systemic amyloidosis (26, 27). The heart may be the predominant or only organ affected in so-called "senile" amyloid cardiomyopathy and rare familial forms of cardiac amyloidosis (28, 29). The former has been estimated to affect ~ 25% individuals over the age of 80 and is an important cause of congestive failure and heart block in this age group (26, 28). The 90% incidence of extracardiac amyloidosis seen in careful retrospective studies underscores the systemic nature of this disorder, which has accordingly been termed SSA. Whereas cardiac involvement in SSA may be massive, amyloid deposits occurring outside the heart are usually small and vascular, the latter providing indirect evidence for an origin from blood (26, 30).

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        1                                   10                                  20
(A)     Asn-Phe-Met-Leu-Thr-Gln-Pro-His-Ser-Val-Ser-Glu-Ser-Pro-Gly-Lys-Thr-Ile-Thr-Ile-Ser-(Cys)-Thr-Arg-Ser-Ser-Gly

        1                                   10
(B)     Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-Glu-Ala-Phe
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Figure 4. Amino-terminal sequences of cardiac amyloid fibril subunit proteins from cases of (*A*) primary and (*B*) secondary amyloidosis.

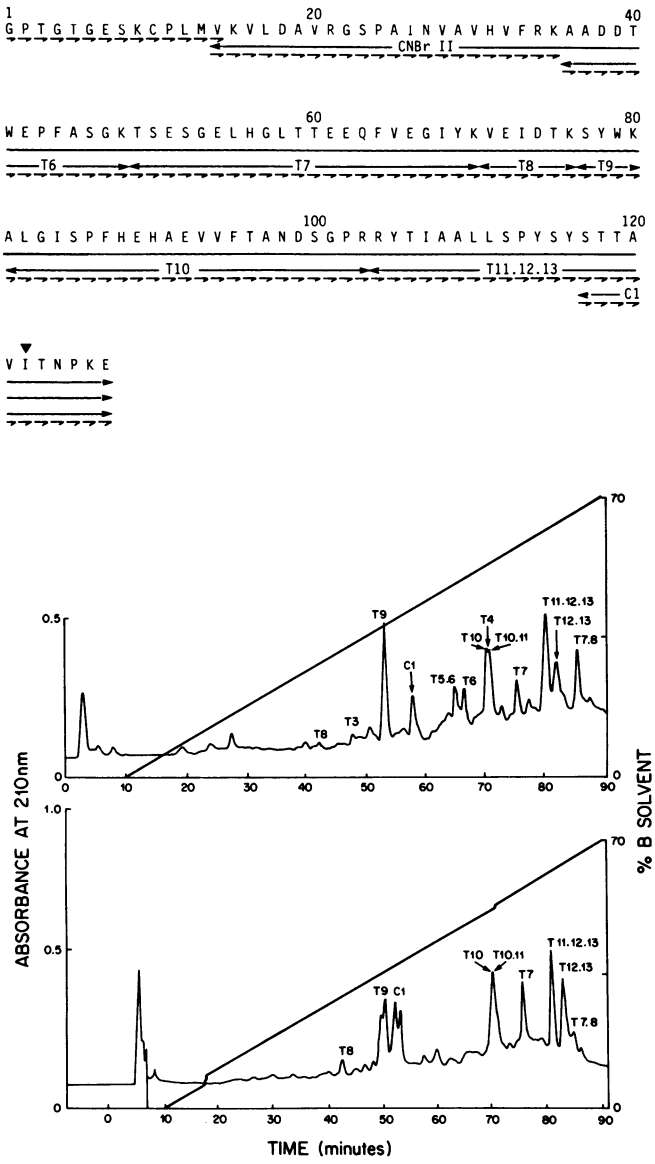

Figure 5. (*Top*) Complete amino acid sequence of third SSA preparation, established by amino acid composition and direct sequencing (→), of subunit proteins and of individual peptides. ▼, Ile[122] substitution found in both A and B fractions. (*Bottom*) HPLC profile of tryptic peptides from SSA amyloid fibrils fractions A and B (Fig. 3).

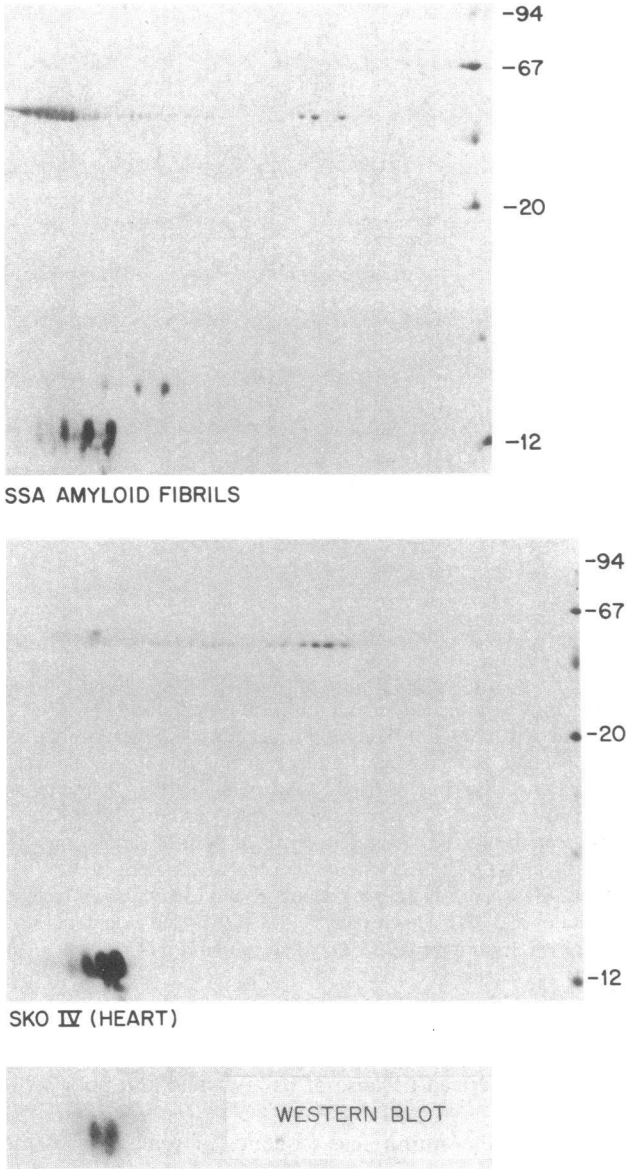

Figure 6. Two-dimensional gels of *top,* amyloid fibril subunit proteins from a case of SSA. (*Middle*) SKO IV isolated from cardiac amyloid (Fig. 3, *bottom*). In both gels, a creatinine phosphokinase carbamylation train is included as an internal charge standard (20). Bottom: Western blot of gel shown at top developed with anti-SKO IV absorbed with Pa (TTr) monomer.

Our studies provide biochemical evidence that SSA is a distinct entity from AA (secondary) and AL (light chain) amyloidosis, using as controls frozen tissue obtained from patients affected by the two other forms of systemic amyloid. The three forms of cardiac amyloid can be distinguished by direct extraction and dissociation of fibrils (Fig. 2), or by the unlabelled peroxidase technique in formalin-fixed tissues using monospecific antisera developed to amyloid-related tissue or serum proteins (Fig. 7).

A series of investigations by Cornwell, Westermark, and associates have shown that amyloid deposits in SSA are Pa (TTr) in nature, both in the heart and other organs (8, 9, 28, 30). Tissue deposits react with anti-Pa (TTr) and anti-FAP antisera, both by immunofluorescence and immunoperoxidase techniques (9, 31). The systemic nature of this disorder is suggested by resemblance to the FAP syndromes in the prevalence of vascular involvement and low levels of Pa (TTr) in blood of affected individuals (32–34). Amyloid subunit proteins of various sizes, including 5-, 9-, and 14-kD (8, 35, 36) and a grouping of 14- and 10–12-kD molecules and low molecular weight peptides (37), have been found in different cases of SSA. Because immunoblot analysis of sera from patients affected by SSA show only intact Pa (TTr) when probed with antisera to Pa (TTr) monomer and to SKO IV[2] proteolytic fragmentation, presumably occurring in tissue, must be a common pathogenic mechanism in this disorder.

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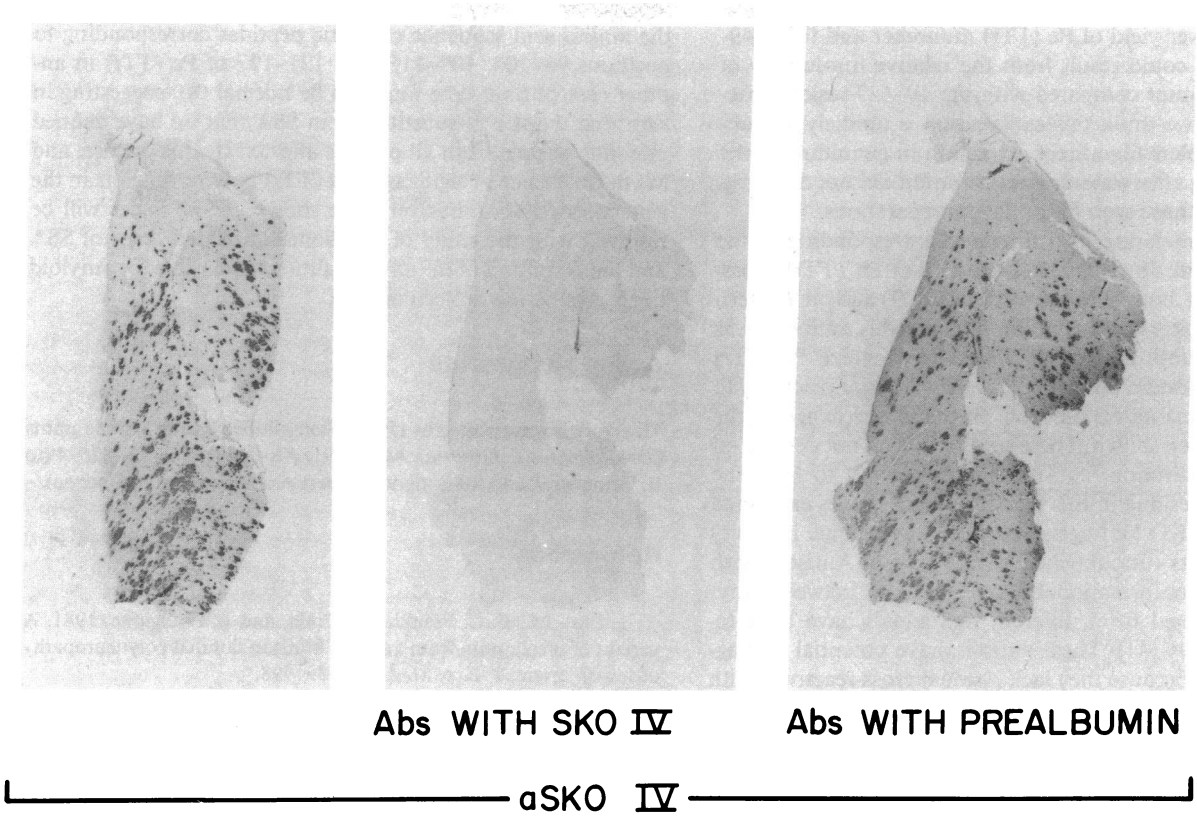

Figure 7. Low power (×4) view of tissue section from a case of SSA, showing immunoperoxidase staining of nodular deposits with an antiserum to SKO IV (1:100 dilution) absorbed with Pa (TTr) monomer (*left*). Peroxidase staining was blocked by absorption with purified SKO IV (*middle*), but not with Pa (TTr) (*right*).

The close relationship between SSA and the FAP syndromes is particularly evident in our fractionation studies of amyloid fibrils isolated from three unrelated individuals affected by SSA. In all instances the predominant component was a fragment of Pa (TTr) corresponding to residues 49–127 (SKO IV or SSA B) (1, 10). Pa (TTr) monomer, molecular mass 14 kD, made up only 10–20% of tissue deposits and differed from the circulating form of the protein (reference 25, and unpublished observation) in exhibiting amino-terminal heterogeneity, with major sequences beginning at positions 1, 4, and 6 of the molecule (Fig. 5 *B*). Similar heterogeneity has been noted by other investigators in different FAP syndromes (2, 5, 14, 38) and may represent tissue digestion by organ-specific endopeptidases (39). A third fraction, corresponding to residues 1–49, is a minor component, variably represented, constituting at best 5–10% of solubilized fibril protein. This fraction, which has only been sequenced from noncardiac amyloidotic tissue of SKO (SKO V), also exhibits considerable amino-terminal heterogeneity and is poorly soluble in most dissociating agents.

In addition to the normal threonine residue at position 49, glycine was also recovered, both by amino terminal sequencing of the whole fraction B, and by Edman degradation of individual tryptic peptides. Similar heterogeneity at this position has been noted in studies of SKO previously reported (1, 10) and by others in another kindred affected by FAP (4). The possibility that the presence of glycine at this position may be due to postranslational modification has been suggested (4); however, the exact basis for this observed heterogeneity remains unclear and will require additional studies. The relationship between a major cleavage at position 49, proteolysis at the amino terminus and polymerization or aggregation of subunit proteins to give fibrils also remains to be defined. An indication that such processing abnormalities may be more common than previously realized are reports of fragments of similar molecular weight in other cases of SSA, in systemic deposits and vitreous opacities of patients with FAP in Northern Sweden, and a case of sporadic vitreous amyloid in the United States (4, 37, 40). In most studies of amyloid fibril subunit proteins from patients with FAP reported to date (2–5, 38), the 14-kD Pa (TTr) monomer has been the only molecular species of the molecule found. Nevertheless, Kametani et al. (14) found an 8-kD protein in addition to a 16-kD protein from a patient from a Japanese kindred with the methionine^{30} variant of Pa (TTr); amino acid sequencing established that the 16-kD molecule corresponded to intact Pa (TTr) and that the 8-kD fraction includes two fragments identical to residues 6–78 of the variant molecule. Further studies will be necessary to establish the prevalence of such fragments in amyloid from specific and different kindred affected by FAP.

It is not clear whether the lower yield of Pa (TTr) monomer and the 1–49 residue fractions seen in our SSA preparations and in SKO result from selective degradation of these fractions or are an artifact of our method for extraction of amyloid fibrils (homogenization in water). We do not know whether each individual amyloid fibril contains all three forms of Pa (TTr) copolymerizing or aggregating in a nonstoichiometric fashion, or whether amyloid fibrils in these patients are in fact heterogeneous and consist of two to three distinct fractions, corresponding to each of the molecular species. If the latter is

the case, the lower yield of Pa (TTr) monomer and the 1–49-residue fraction could result from the relative insolubility of these fibrils in water compared with the 49–127-residue fraction. However, we think this explanation is unlikely, as subunit proteins isolated by direct extraction in guanidine of the pellet remaining after water homogenization did not differ significantly from those seen in top-layer preparations.

An antiserum raised to a fragment corresponding to the carboxy-terminal 78 residues (SKO IV) of Pa (TTr) cross-reacted with SSA both in tissue section (Fig. 7) and on Western blots of solubilized fibrils (Fig. 6). Because this antibody was absorbed with and did not significantly recognize Pa (TTr) monomer on Western blots (Fig. 6, *bottom*) major reactivity to unique determinants exposed on proteolytic cleavage of the molecule has been shown. These specificities do not depend on the ILE^{122} substitution, as SKO IV was found to have the normal valine residue in this position for all tissues examined so far, and all SKO IV fragments reacted with equal intensity on immunoblots (unpublished observations). Antisera with specificity for conformational antigens shared between amyloid fibrils isolated from different individuals have been reported previously (41). These antisera have potential as diagnostic reagents because they lack residual cross-reactivity with normal serum proteins and are thus specific for amyloid deposits in tissue. Our studies in SSA and one form of FAP suggest that some of these reactivities may result from shared subunit antigenicity due to specific proteolysis occurring at the tissue level.

Identification of a new variant Pa (TTr) molecule raises the possibility that SSA may be a genetically determined disorder. Presumably, individuals affected by this disease have an abnormal circulating Pa (TTr) molecule that is present for long periods of time before being expressed clinically as amyloid deposition late in life. Furthermore, although some patients manifest as cardiomyopathy, heart block, or lung disease, many do not develop symptomatology and are only diagnosed postmortem (28, 42). By analogy, individuals affected by FAP types 1 and 2 and related disorders also have low levels of serum Pa and are heterozygote carriers of an abnormal Pa (TTr) molecule with a single amino acid substitution at any of six positions in the molecule identified so far (1–5, 10–12, 32, 33). Different kindreds manifest variable age of onset and progression of disease, which may be late and benign (27, 43, 44). Cardiac involvement is universal in certain forms of FAP and has provided the source of material for extraction and sequencing studies in some instances (4, 5). Recently, one form of familial amyloid cardiomyopathy without associated polyneuropathy has been shown to be due to Pa (TTr) both immunohistologically and by direct amino acid sequence analysis of fibril subunit proteins (6).

The identification of increasing numbers of amino acid substitutions in configurationally disparate parts of the Pa (TTr) molecule (Fig. 1) has made it difficult to define a unified theory for the structural basis of amyloidogenesis in the FAP syndromes and related disorders. Our studies suggest that proteolysis and, in the case of SSA and at least one form of FAP, copolymerization of Pa (TTr) and its 49–127 fragment may be more important pathogenically. Thus (*a*) the ILE^{33} substitution has been found in fibril subunit protein isolated from some tissues of SKO but not others (1, 10, 11, 15); (*b*) SSA is prevalent in the general population and has not yet been shown to be a hereditary disorder by clinical observations; (*c*) the amino acid sequence of peptic peptides corresponding to positions 96–107, 109–115, and 121–127 of Pa (TTr) in another case of SSA were found to be normal (8), suggesting in turn that the ILE^{122} substitution in SSA that we have defined may not be present in all persons affected by this disease; and (*d*) the existence of significant Pa (TTr) polymorphisms in the general population has yet to be shown. These issues will be resolved with the study of additional unrelated cases of SSA and the definition of the abnormalities responsible for amyloid deposition at the molecular level.

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