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Two different types of amyloid deposits—apolipoprotein A-IV and transthyretin—in a patient with systemic amyloidosis

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Certain forms of systemic amyloidosis have been associated with the pathologic deposition as fibrils of three different apolipoprotein-related proteins—apolipoprotein A-I, apolipoprotein A-II, and serum amyloid A. We have previously reported (Bergström *et al*, Biochem Biophys Res Commun 2001;285:903-908) that amyloid fibrils extracted from the heart of an elderly male with senile systemic amyloidosis contained, in addition to wild-type transthyretin-related molecules, an N-terminal fragment of yet a fourth apolipoprotein—apolipoprotein A-IV (apoA-IV). We now provide the results of our studies that have established the complete amino-acid sequence of this ~70-residue component and, additionally, have shown this protein to be the product of an unmutated apoA-IV gene. Notably, the apoA-IV and transthyretin fibrils were not codeposited but, rather, had anatomically distinct patterns of distribution within the heart and other organs, as evidenced immunohistochemically, by variation in the ultra structural morphology and by differences in the intensity of Congo red birefringence. These findings provide the first conclusive evidence that two separate forms of amyloid, each derived from a wild-type amyloidogenic precursor protein, were present in a patient with systemic amyloidosis.
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The systemic amyloidoses comprise a group of often life-threatening protein conformational diseases that are associated with the pathologic deposition as fibrils of at least 12 structurally diverse molecules in vital organs and tissues throughout the body.[1] Most of these proteins are chemically and structurally unrelated,[2] yet the abnormal product, the amyloid fibril, exhibits virtually identical tinctorial and ultra structural features.[3]

The amyloid fibril core has a high degree of β-sheet structure, with the β-strands configured in an orientation perpendicular to the fibril axis and with hydrogen bonds formed between monomeric subunits.[4] Many of the amyloidogenic precursor proteins also display extensive β-structure in their natively folded form (eg, transthyretin, β2-microglobulin and immunoglobulin light chains), but in other cases (eg, apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II)), an α-helix to β-sheet transition must occur.[5] Additionally, some proteins that are natively unfolded also may adopt a β-conformation and form amyloid fibrils.[6]

Transthyretin (TTR) is the protein found in most types of familial amyloidosis. In these diseases, a missense mutation in the TTR gene results in an amino-acid substitution at a particular position in this 127-residue component; notably, more than 85 different alterations have been deemed amyloidogenic.[7] The clinical phenotypes vary, but polyneuropathy and cardiomyopathy predominate. TTR also comprises the pathologic protein found in senile systemic amyloidosis (SSA) where the amyloid fibrils are composed predominately of C-terminal fragments of wild-type (wt) TTR that start at positions 46, 49, and 52.[8] SSA is probably the most

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common of all systemic amyloidoses and in autopsy studies has been seen in 18–25% of individuals over the age of 80 years.[9–11] However, in a Japanese study, only 8% of individuals 80-years old and over had TTR-derived amyloidosis.[12] In most cases, the fibrillar deposits are small, affecting blood vessels in different organs and, seemingly, are not of pathophysiological import. However, in some individuals, mainly elderly males, extensive cardiac deposition of wt TTR can occur that results in cardiomegaly and eventual death due to heart failure.[13,14] Such patients also may have pronounced pulmonary amyloid infiltration, as well as involvement of the renal papillae.[14] Among other proteins associated with the systemic amyloidoses, the high-density apolipoproteins are of special interest, for example, apoA-I and apoA-II that constitute the fibrils found in several familial forms of this disorder.[5,15–20] Another, serum amyloid A, is the precursor protein of fibrils in secondary or reactive (AA) amyloidosis.[21] Why this class of molecules is prone to aggregate and form amyloid fibrils is not known. However, our finding of yet another amyloid-associated apolipoprotein, namely, apolipoprotein A-IV (apoA-IV),[22] suggests that these components share structural features that render them amyloidogenic.

Among individual patients with systemic amyloidosis, the amyloid typically is composed of only one type of protein, although minor populations of other molecules, for example, apolipoprotein E, also may be present in the deposits.[23] However, previously we had identified, through chemical and immunologic analyses of fibrils extracted from the heart of an elderly male who had clinical and pathologic features characteristic of SSA, both apoA-IV and wt TTR.[22] The unexpected detection of apoA-IV provided the first example that this apolipoprotein could be amyloidogenic. We had posited that the two different amyloids were codeposited, based on our demonstration that synthetic apoA-IV polypeptides were inherently fibrillogenic and could serve as seeds to promote *in vitro* TTR fibril formation.[22]

We now report the results of our latest studies where the entire primary structure of the apoA-IV-derived amyloid protein was established. Additionally, it was determined that this component, like the TTR, was a product of an unmutated gene. Most notable was our finding that the apoA-IV and TTR fibrils were not codeposited but, instead, occurred separately within the heart and other organs. Thus, our patient seemingly had two independent systemic amyloid diseases.

Materials and methods

Tissue Specimens

Specimens of heart and other tissues obtained at autopsy from a 92-year-old male with pronounced cardiac amyloidosis (heart weight, 620 g) were frozen and preserved at −25°C and stored for 4 years prior to fibril extraction. Tissue specimens were also fixed in 4% buffered neutral formaldehyde solution and embedded in paraffin.

Immunohistochemistry and Amyloid Identification

Two polyclonal anti-apoA-IV antisera were prepared by immunizing rabbits with a keyhole limpet hemocyanine-linked synthetic peptide corresponding to positions 44–55 of apoA-IV. The antisera were specific for apoA-IV, as evidenced in an enzyme-linked immunosorbent assay (ELISA). Rabbit antiserum to TTR was raised against an *in vitro* expressed protein fragment, corresponding to positions 50–127 of human TTR, coupled to keyhole limpet hemocyanine. This reagent was found to label TTR-amyloid specifically and did not cross-react with apoA-IV or other known amyloidogenic proteins. Deparaffinized 5 μm-thick sections were incubated overnight with antibody diluted 1:3000–1:8000, washed, and the immunoreactivity visualized using the biotin/streptavidin method with 3,3′-diaminobenzidine-tetrahydrochloride as substrate. Immunostained sections also were treated with an alkaline Congo red solution and examined under polarized light for simultaneous demonstration of amyloid birefringence and immunoreactivity.[24,25]

Amyloid Isolation, Purification, and Characterization

Amyloid protein was extracted from fresh-frozen heart and defatted.[8] Fibrils were dissolved in 6 M guanidine HCl/0.1 M Tris-HCl, pH 8.0, containing 0.1 M dithiothreitol. After incubation at room temperature for 48 h, the fibril solution was centrifuged, after which the supernatant was dialyzed against saturated ammonium sulfate followed by deionized water, and then freeze-dried. The lyophilized material was redissolved in 6 M guanidine HCl/0.1 M Tris-HCl, pH 8.0 containing 0.1 M dithiothreitol and applied to a 1.6 × 90 cm Sepharose 6B-CL column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 5 M guanidine HCl (flow rate, 4 ml/h) and the absorbance monitored at 280 nm.

In order to map the heart for areas with apoA-IV amyloid deposits, small pieces of fresh frozen cardiac tissue were taken from six different anatomical sites. This material was squeezed between glass microscopic slides, dried, and the apoA-IV identified immunohistochemically. Based on these results, a 4 g portion was taken from the part of the heart that showed the most pronounced apoA-IV reactivity and the amyloid protein was extracted as described above.

Enzyme-Linked Immunosorbent Assay

The apoA-IV-contained in gel filtration fractions was identified by ELISA. A volume of 50-μl aliquots from each fraction were diluted to 150 μl with 50 mM

sodium carbonate buffer, pH 9.6, containing 0.2% sodium azide and coated onto a 96-well Immulon 2 HB polystyrene microtiter plate (Dynex Laboratories, Alexandria, VA, USA). After washing, the ELISA plate was incubated overnight at room temperature with the anti-apoA-IV antiserum diluted 1:100. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antiserum (Dako, Glostrup, Denmark) was used as the secondary antibody at a dilution of 1:1500. Immunoreactivity was visualized using *p*-nitrophenyl-phosphate (Sigma, St Louis, MO, USA).

Reverse-Phase HPLC

The gel-filtration fractions containing the highest apoA-IV reactivity (as determined by ELISA), were subjected to reverse-phase HPLC (RP-HPLC) on a Brownlee Aquapore BU-300 30×4.6 mm C4 column (Perkin-Elmer, Norwich, CT, USA) using a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.4 ml/min; the absorbance was monitored at 220 nm and the peaks collected manually. Aliquots (25 μl) from each were diluted with 50 mM sodium carbonate buffer, pH 9.6, containing 0.2% sodium azide to a total volume of 100 μl and analyzed for apoA-IV content by ELISA. The apoA-IV-containing fractions were vacuum-dried or concentrated using a Speed Vac (Savant Instruments, Farmingdale, NY, USA).

Amino-Acid Sequencing and Mass Spectrometry

N-terminal amino-acid sequence analysis of RP-HPLC-purified, intact apoA-IV protein by direct Edman degradation was performed as previously described.[26] This material was also digested with trypsin or endoproteinase Asp-N[8,22] (Roche Applied Sciences, Germany) and the resulting peptides subjected to amino-acid sequencing or mass spectrometry[27] using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) (Ultraflex TOF/TOF, Bruekr Daltonics, Bremen, Germany).

Western Blot Analysis

Amyloid fibrils were dissolved in 6 M guanidine/0.1 M. Tris-HCl, pH 8.0, dialyzed, and lyophilized as described above. This material, as well as protein isolated by RP-HPLC, was dissolved in sample buffer containing 3% sodium dodecyl sulfate (SDS) and electrophoresed on SDS-PAGE gels.[28] Proteins were blotted onto a 0.3 μm nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) and exposed first to the anti-apoA-IV antiserum (1:500 dilution) and then to a secondary antibody (horse radish peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) diluted 1:10 000. The reaction was visualized using an enhanced chemiluminescence system (Amersham Biosciences, Uppsala, Sweden).

Electron Microscopy

A small piece of heart from the upper dorsal wall of the left ventricle that had both apoA-IV and TTR immunoreactive deposits (as demonstrated immunohistochemically) was fixed in a solution containing 0.25% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and embedded in Unicryl (British BioCell, Cardiff, UK). Ultra thin sections were placed on formvar-coated nickel grids and immunolabeled with the anti-TTR or anti-apoA-IV antisera diluted 1:200. Immunoreactivity was demonstrated with goat anti-rabbit antibodies labeled with 10-nm gold particles (British Biocell, Cardiff, UK).

Genetic Analysis

DNA was extracted from fresh-frozen heart using the Genomic Prep Cell and Tissue DNA Isolation kit (Amersham Biosciences, Uppsala, Sweden). Exons 1, 2, and 3 of the apoA-IV gene were amplified by PCR with the following primers: 5′-TGTGGCAA GAAACTCCTCCA-3′ and 5′-AGTGCCATCCAAAGA CAGCTT-3′ (exon 1); 5′-CATCATCCAGTCTGCAGC TCA-3′ and 5′-CGTACATTGCATGGCCT TT-3′ (exon 2); 5′-CTTGCCGTGTAAATGCCAAA-3′, 5′-TTCTCC CGCAGCACTCTCT-3′, 5′-TAGCACAGCGCATGGAG AGA-3′, 5′-AGTGACTTCTGCAGCCCT-3′, 5′-TTCCA GAATGAAGAAGAACGCC-3′, 5′-AGGAGTTGACCT TGTCCCTCA-3, 5′-AACAGCTCAAGGCAGAAACTG G-3′ and 5′-AAGGAGGATTCATCCGGCAA-3′ (exon 3). The DNA was dissolved in a $1\times$ PCR buffer (15 mM Tris/HCl, pH 8.0), containing 50 mM KCl, 2.5 μM $MgCl_2$, 200 μM dNTPs, 1.25 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA, USA) and 0.4 μM of each of the sense and antisense primers. The 30-cycle protocol included: 5 min initial heating at 95°C, 30 s denaturation at 95°C, 30 s annealing at 59°C, and an 1 min extension at 72°C. The final extension was performed for 7 min at 72°C. PCR products were separated by electrophoresis on a 1.5% agarose gel and the bands were excised and purified using a Qiaex II gel extraction kit (Qiagen, Valencia, CA, USA) and sequenced (Kiseq, Karolinska Institute, Stockholm, Sweden).

Results

Chemical Nature of the ApoA-IV Fibrils

Amyloid fibrils were extracted from a 4-g portion of fresh frozen heart tissue that contained the most pronounced apoA-IV reactivity, as evidenced immunohistochemically. Western blotting analysis (Figure 1) revealed the presence of an apoA-IV-related component with a molecular mass of ~8 kDa, a value considerably less than that of the

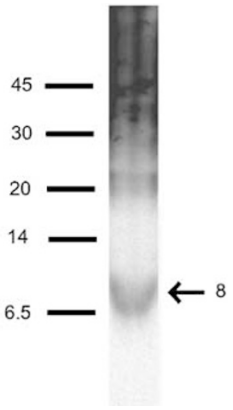

Figure 1 Characterization of apoA-IV amyloid fibrils. The protein extracted from cardiac amyloid deposits was electrophoresed on an SDS-PAGE gel and immunoblotted with a specific anti-apoA-IV antiserum. The molecule weight markers and location of the ~8 kDa component are as indicated.

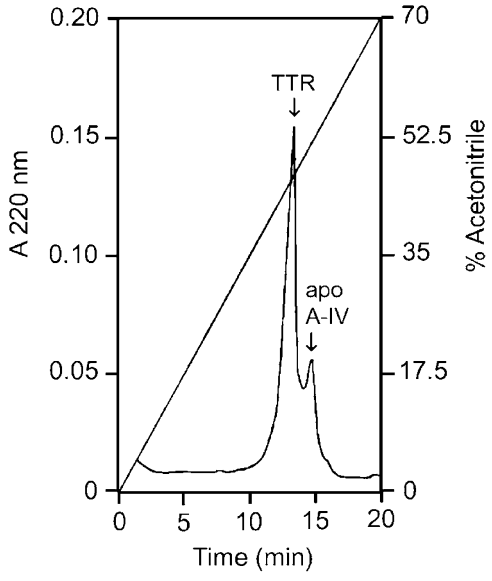

Figure 2 Purification by RP-HPLC of amyloid protein extracted from cardiac tissue. The locations of the TTR- and apoA-IV-related components identified in the column fractions by ELISA are as indicated.

native protein (46 kDa). Further, a faint immunoreactive band (~22 kDa) and a smear with a higher molecular weight were present. The amyloid extract was subjected to gel filtration purification followed by RP-HPLC where two peaks were obtained (Figure 2); the second (and smaller) was found by sequence analysis to contain two N-terminal fragments of apoA-IV, one beginning at position 1 and the other at 11. By direct Edman degradation, the sequence of the first 32 residues of this molecule was established (Figure 3). After digestion with endoproteinase Asp-N, apoA-IV peptides starting at position 1, 5, 13, 44 and 55 were detected (no peptide starting with Asp74 was found). Additionally, the amino-acid sequence from position 32 to 58 was established from analyses of tryptic peptides derived from

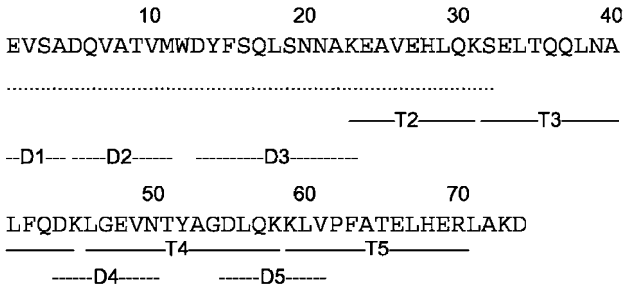

Figure 3 Primary structure of the ~8 kDa apoA-IV amyloid protein. The sequence of this 70-residue component was determined directly from the intact component (···) and from Asp-N (D) and tryptic (T) peptides, as indicated.

apoA-IV containing amyloid extracted from formalin-fixed, paraffin-embedded tissue sections.[22] Through MALDI-TOF spectrometry of trypsin-digested RP-HPLC-purified material, residues located between positions 23 and 70 were identified (Figure 3). Together, these data established that the apoA-IV-related amyloid protein represented an ~70-residue N-terminal fragment of the mature protein and had the amino-acid sequence expected for that portion of the wt molecule.

Characterization of the ApoA-IV Gene

The cDNA encoding the three exons of the apoA-IV gene was cloned from genomic DNA. The predicted protein sequence was identical to that expected for the wt product, that is, it contained no mutations. Although, both Arg and Lys residues have been identified at position 259 in apoA-IV,[29,30] we found only an Arg-specifying triplet (AGG) at this location.

Distribution of ApoA-IV and TTR Amyloid

Examination by polarizing microscopy of Congo red-stained tissue sections revealed that amyloid deposits were present in the heart, lung, kidney, and seminal vesicles, while none were evident in the liver. Notably, two distinct staining patterns were seen within the heart. The first was characterized by bright and glittering birefringence appearing as small patchy deposits that appeared to be located intracellularly within cardiac myocytes as well as both interstitial deposits and larger nodular deposits (Figure 4a–c). This material was immunostained using the specific anti-apoA-IV antiserum (Figure 4b and c). In the second and more predominate pattern, the amyloid deposits were stained less intensely by Congo red and were only weakly birefringent. This material, which was evident in both interstitial deposits and in the form of larger nodules that had a patchy distribution, was immunostained by the specific anti-TTR reagent (Figure 4a and d). Furthermore, TTR immunoreactive deposits of varying size commonly were found in vessel walls. In the cardiac conduction system, apoA-IV-containing amyloid

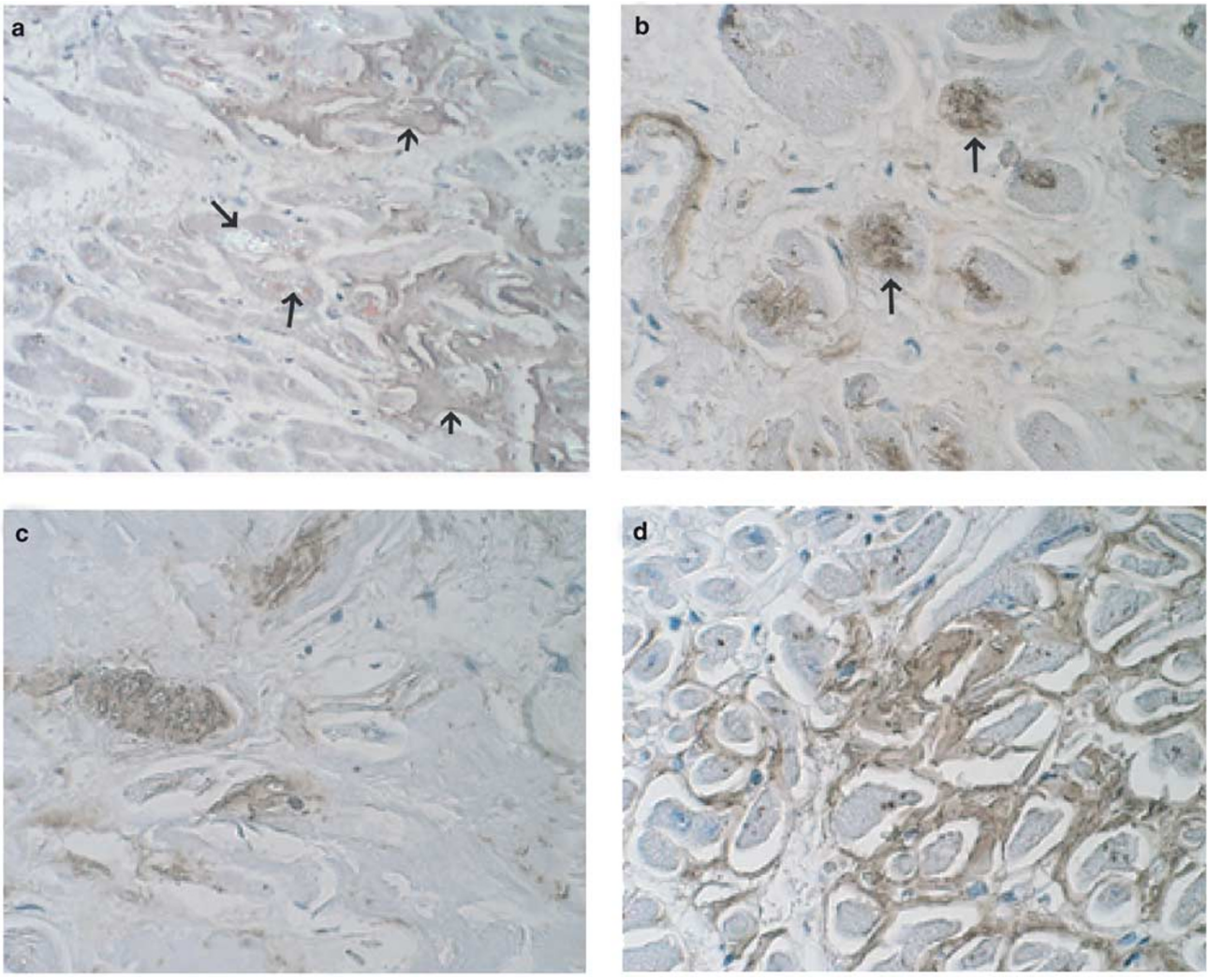

Figure 4 Immunohistochemical demonstration of TTR-and apoA-IV-related cardiac amyloid deposits. Sections in panels (**a**) and (**d**) were immunostained with anti-TTR antibodies and those in panels (**b**) and (**c**), with the anti-apoA-IV reagent. Additionally, the heart tissue in panel (**a**) was counterstained with Congo red and viewed under polarized light. Long arrows indicate apoA-IV amyloid deposits and short arrows ATTR-amyloid (original magnification $\times 280$).

was exclusively located near the atrio-ventricular node, whereas that associated with TTR was deposited adjacent to both the atrio-ventricular and the sinus nodes.

In the lung, apoA-IV amyloid occurred in vessel walls, while that derived from TTR was seen as small deposits in alveolar septa (as characteristically found in SSA). In both the seminal vesicles and kidney, anti-apoA-IV and -TTR antisera labeled anatomically distinct sites in blood vessel walls. Additionally, large amounts of TTR (but not apoA-IV) amyloid were found in the renal papillae. Although both types occasionally were found in the vicinity of each other, only very rarely was apoA-IV and TTR immunoreactivity colocalized.

Electron Microscopy

The two forms of amyloid also could be distinguished ultrastructurally. As evidenced from the immunogold-labeling patterns, the anti-apoA-IV antibody reacted with bundles of parallel, relatively thick fibrils. In contrast, the anti-TTR reagent recognized more slender, nonparallel fibrils running in different directions (Figure 5).

Discussion

In this study, we show for the first time that apoA-IV is a major fibril protein in one form of systemic amyloidosis. Through amino-acid sequencing and mass spectrometric analyses, we now have shown that this amyloid mainly consisted of an $\sim$70-residue N-terminal fragment of apoA-IV, presumably formed through proteolytic cleavage of the native protein. Thus, apoA-IV, like the apolipoproteins serum amyloid A, apoA-I and apoA-II, can be amyloidogenic. Additionally, apolipoprotein E has been found to be a minor component in other

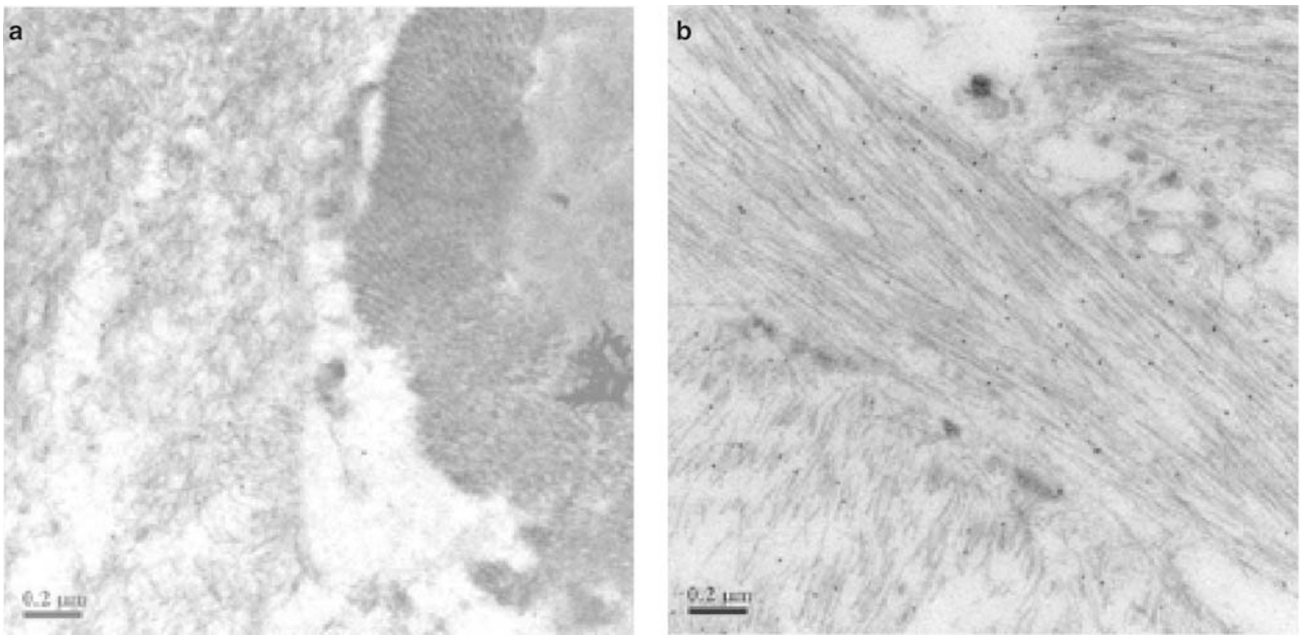

Figure 5 Ultrastructural features of apoA-IV and TTR amyloid fibrils. Electron micrographs of apoA-IV (panel **b**) and TTR (panel **a**) fibrils immunolabeled with 10-nm gold particle-conjugated anti-apoA-IV and anti-TTR antisera, respectively. Bar = 0.2 μm.

chemically diverse forms of amyloid.[23] ApoA-I, apoA-IV and apolipoprotein E have similar molecular structures, including α-helix repeats of 11 or 22 amino acids, often separated by a proline residue.[31,32] These molecules have a critical role in lipid transport and metabolism and their amphipathic helices constitute the lipid binding and lecithin:cholesterol acyltransferase activating regions of the proteins.[33,34] Apolipoproteins may be unusually prone to form amyloid fibrils *in vivo*, possibly as a result of their intrinsic flexibility.[5] They have been shown to adopt a more stable conformation after binding to lipid surfaces; possibly due to a conversion of random coil structure to an α-helical structure.[35]

Studies on apoA-I have indicated that cleavage of the full-length protein and generation of an N-terminal fragment are important steps in amyloidogenesis;[5] most likely, this also occurs in the case of other apolipoprotein-associated amyloidoses. For example, a portion of apoA-I, consisting of the first 93 residues, assumes a random coil configuration in water; however, when it forms fibrils, the protein first has to adopt a helical conformation in order to generate a stable β-sheet structure.[5] A recent study has shown that the binding of apoA-I to lipid is a two-step process that increases the overall α-helicity of the molecule.[32] In this mechanism, which also has been proposed for apoA-IV,[32] the C-terminal amphipathic helices of apoA-I initially bind to the lipid surface, which results in a major conformational change in the N-terminal part of the molecule. This leads to hydrophobic helix–lipid interactions, rather than the intramolecular N-terminal hydrophobic helix–helix interactions that would otherwise occur.[32] The binding of apoA-IV to lipid surfaces also can lead to structural alterations in which hydrophobic buried N-terminal residues are unfolded and reoriented towards the lipid surface.[35] This native plasticity in the N-terminal part of apoA-I and apoA-IV could explain why the N-terminal part of these proteins is prone to start to aggregate. In addition, the N-terminal part of apoA-IV is the most hydrophobic part and contains one of its two native beta sheet structures, ranging from residue 6 to 15.[36,37] In the case of apoA-IV, this apolipoprotein, above all others, exhibits the highest sensitivity to guanidine hydrochloride-induced denaturation, indicating a marginal stability for its native α-helical structure.[38] Therefore, it may be particularly sensitive to environmental perturbations. Furthermore, apoA-IV is the most hydrophilic apolipoprotein and has the lowest affinity for lipid surfaces.[36]

Most forms of apolipoprotein-associated amyloidosis occur systemically, as was found in our patient with the apoA-IV-containing deposits. ApoA-IV is a 46 kDa glycoprotein primarily synthesized by the enterocytes in the small intestine and is important in the absorption, transport, and metabolism of lipids. The plasma concentration varies between 14 and 37 mg/ml, depending on nutritional status.[39,40] A higher than normal concentration of an amyloid fibril protein precursor often is involved in amyloid pathogenesis. No blood specimens were available from our patient for measurement of this component; however, it is important to note that plasma levels of apoA-IV increase with age.[41]

Although other types of apoA molecules, that is, apoA-I and apoA-II, are known to be amyloidogenic,

in all systemic cases these components have been the product of mutated genes.[5,15–20] In contrast, the amino-acid sequence of the apoA-IV fibrils was identical to the corresponding region of the wt protein; furthermore, there were no mutations in the nucleotides of the apoA-IV gene cloned from the patient's genomic DNA.

Characteristically, the systemic amyloidoses are associated with the pathologic deposition of only one biochemical type of amyloidogenic precursor protein, although there has been one report of a case with apoA-I cardiac, laryngeal, and cutaneous amyloidosis in whom TTR also was found in a skin lesion as a minor component.[42] In contrast, our patient was unique in having two distinct kinds of systemic amyloidosis. We originally had postulated that both types of fibrils were colocated, given that a synthetic apoA-IV peptide spontaneously formed fibrils and enhanced *in vitro* TTR fibrillogenicity.[22] Upon further study, we have demonstrated that the apoA-IV and TTR amyloid proteins were deposited independently in anatomically distinct areas within a single organ or blood vessels, as shown immunohistochemically. Further, each could be distinguished from the other on the basis of differences in the extent of Congo red birefringence. Additionally, both types of fibrils had unique ultrastructural morphology.

The finding that TTR and apoA-IV formed discrete deposits and not a diffusely mixed amyloid infiltration is remarkable since both fibril precursors are derived from plasma proteins and not local synthesis. Thus, although amyloid fibrils resemble each other morphologically and in their principle molecular organization, there may be differences that do not allow mixed β-sheet fibrils. This does not exclude the possibility that fibrils of one protein could have served as a seed or nucleus to promote fibrillogenesis of the second. For example, *in vitro* studies have shown that Aβ fibrils can seed IAPP fibril formation.[43] We can only speculate whether the two amyloid forms described in this paper have developed completely separately, or not. If a seeding mechanism was operating here, it is unlikely that ATTR-fibrils seeded wt apoA-IV since we have not found apoA-IV-related molecules in TTR-containing extracts obtained from over 30 patients with SSA.

Thus, we conclude that our patient had two different forms of systemic amyloid—one composed of apoA-IV and the other of TTR.

Acknowledgements

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Duality of Interest

No duality of interest is declared.

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