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Short Communication

Pulmonary Vascular Amyloidosis in Aged Dogs

A New Form of Spontaneously Occurring Amyloidosis Derived From Apolipoprotein AI

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The N-terminus of a mutant form of apolipoprotein AI [apoAI] has previously been shown to be the subunit protein of amyloid fibrils in a human kindred with a form of familial amyloid polyneuropathy (FAP, type III) and in a recently reported kindred with a form of non-neuropathic hereditary amyloidosis. In this study, we demonstrate by amino-acid sequence analysis, that a form of vascular amyloidosis occurring in the lungs of aged dogs is derived from a N-terminal fragment of apoAI and that no amino acid substitution is present in this confirmed sequence. This represents the first documentation of apoAI as a precursor for a form of amyloidosis in animals, and provides the first documentation of apoAI as a precursor for amyloid fibrils in a form of age-associated ("senile") amyloidosis. Secondary structure prediction analysis of the N-terminal regions of normal human and dog apoAI indicated a propensity for β-pleated sheet conformation, and thus amyloidogenesis, in 40 and 45% of the respective sequences. These results suggest that apoAI (like transthyretin) may serve as an amyloid precursor protein for both familial and senile forms of amyloidosis. ApoAI should, therefore, be considered as a potential amyloid precursor when forms of human senile amyloidosis of unknown origin are evaluated. (Am J Pathol 1992, 141:1013–1019)

Amyloid fibrils are derived from any one of at least 15 different proteins (ie, amyloid precursor proteins)[1] present in blood or tissues. The amyloid precursor protein may be either a normal protein or an abnormal variant thereof, and the repetitive monomeric amyloid protein incorporated into the amyloid fibrils may represent the intact precursor or only an incomplete degradation fragment of the precursor. The amyloidogenicity of many of these various precursor proteins is known to be importantly linked to intrinsic molecular regions that have appreciable β-pleated sheet conformation.[2] However, additional factors are involved in the conversion of a precursor protein to amyloid fibrils, and these factors vary with each of the different forms of amyloidosis.

Of the known amyloid precursors, three are apolipoproteins that are associated with high-density lipoproteins (HDL) in plasma. Serum protein AA (ApoSAA)[3,4] is an acute phase reactant[5,6] whose N-terminus constitutes the repetitive subunit protein of the amyloid fibrils in AA amyloidosis (eg, reactive or "secondary" amyloidosis).[7] The N-terminus of a mutant form (glycine → arginine at position 26) of apolipoprotein AI (apoAI) is the subunit protein of the fibrils in a form of familial amyloid polyneuropathy (FAP, type III) in an Iowa kindred of English, Scottish, and Irish descent,[8–10] and also in a more recently reported form of non-neuropathic hereditary amyloidosis in a Massachusetts kindred of Scandinavian descent.[11] The amyloid protein in amyloidosis associated with senescence-accelerated mice (SAM) is a mutant form (proline → glutamine at position 5) of apolipoprotein AII

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(apoAII),[12] which is not enzymatically degraded before its amyloidogenesis.[13]

In this study, we show that the amino acid sequence of the major amyloid protein associated with a relatively frequent form of vascular-associated amyloidosis in lungs of aged dogs corresponds with the N-terminus (positions 1–71) of normal dog apoAI. This represents the first documentation of apoAI as a precursor for amyloid fibrils in animals, and also the first documentation of apoAI as a precursor for amyloid fibrils in a form of age-associated amyloidosis. It is thus evident that apoAI, like transthyretin (prealbumin),[14,15] is unique with respect to its demonstrated involvement as a precursor for both familial and senile forms of amyloidosis.

Materials and Methods

Amyloid Isolation and Purification

Lung from a 14-year-old Springer Spaniel was confirmed to have extensive and generalized vascular amyloid deposits that illicited characteristic green birefringence when viewed with polarized light after staining with Congo red and/or Congo red-Sirius red technique (Figure 1).[16] The deposits were shown not to be consistent with AA amyloid on the basis that they retained Congophilia and birefringence after oxidation with potassium permanganate and dilute sulfuric acid,[17] and also gave no immunoreactivity (PAP: peroxidase antiperoxidase method) with a previously characterized antiserum to purified canine protein AA.[18]

Portions of the lung were frozen and stored at −20° C until they were used for amyloid isolation and purification. Amyloid fibrils were obtained by a modification of the water extraction procedure of Pras et al.[19] Briefly, the thawed tissue was homogenized eight times in normal saline containing 0.05 mol/l sodium citrate, followed by six homogenizations in distilled water. The bottom pellet material obtained from the final homogenization contained the most amyloid and was used for further purification procedures. Lyophilized pellet material was defatted in chloroform and methanol (2:1), and 100 mg of the defatted material was dissolved in 6 ml of 6 mol/l guanidine-HCl in 0.1 mol/l Tris-HCl buffer (pH 8.0) containing 0.1 mol/l dithiothreitol. The dissolved material was gel filtered on a Sepharose 6B-CL column eluted with 5 mol/l guanidine-HCL in distilled water. The elution profile was monitored at 280 nm, and fractions 52–54 were pooled and dialyzed against deionized water and then lyophilized.

Electrophoresis and Isoelectric Focusing

Gradient (10–20%) polyacrylamide slab gel electrophoresis was performed in the presence of sodium dodecyl sulphate (SDS-PAGE).[20] Analytic isoelectric focusing (AIF) was done as previously described.[21]

Protein Cleavage and Amino Acid Sequence Analysis

N-terminal amino acid analysis was completed with an automatic sequence analyzer (Applied Biosystems Model 477A, Foster City, CA), and the amino acid derivatives were determined on line with an Applied Biosystems 120A PTH amino acid analyzer. The amyloid protein was cleaved using BPNS-skatole in excess,[22] with L-tyrosine added in excess to protect tyrosine and histidine residues. The BPNS-skatole cleaved peptides, and also those obtained by cleavage with trypsin, were purified by reversed-phase HPLC for subsequent sequence analysis.

Secondary Structure Prediction

The propensity for β-pleated sheet conformation for local groups of residues in the N-terminal regions of normal human (positions 1–83) and dog (positions 1–71) apoAI, and also the N-terminus of variant human apoAI (positions 1–83) with arginine at position 26, was determined using the Molecular Biology software program (suite 5.4) of IntelliGenetics, Inc, Mountain View, CA. This program utilizes the method of Chou and Fasman,[23,24] which is reported to have up to 70% accuracy in secondary structure prediction.

Immunohistochemistry

The pulmonary amyloid deposits were evaluated (with and without 20-minute pretreatment with concentrated formic acid[25]) (Fisher Scientific, Fair Lawn, NJ) for apoAI immunoreactivity using the PAP method[26] and rabbit antiserum (1:100–1:400) to human apoAI (Calbiochem Corporation, La Jolla, CA). Negative controls included replacement of the primary antiserum with nonimmune serum, and application of the primary antiserum to paraffin-embedded sections of dog systemic amyloid previously confirmed to be of AA type.[18]

Results

Gel filtration of the amyloid fibrils dissolved in 6 mol/l guanidine HCl revealed one major retarded peak (Figure 2) representing approximately 2 mg of protein. SDS-PAGE of this amyloid protein produced several low molecular weight bands (Figure 3A) with M_r varying between approximately 6 and 14 kda. AIF (pH 9.5–3.5) revealed mul-

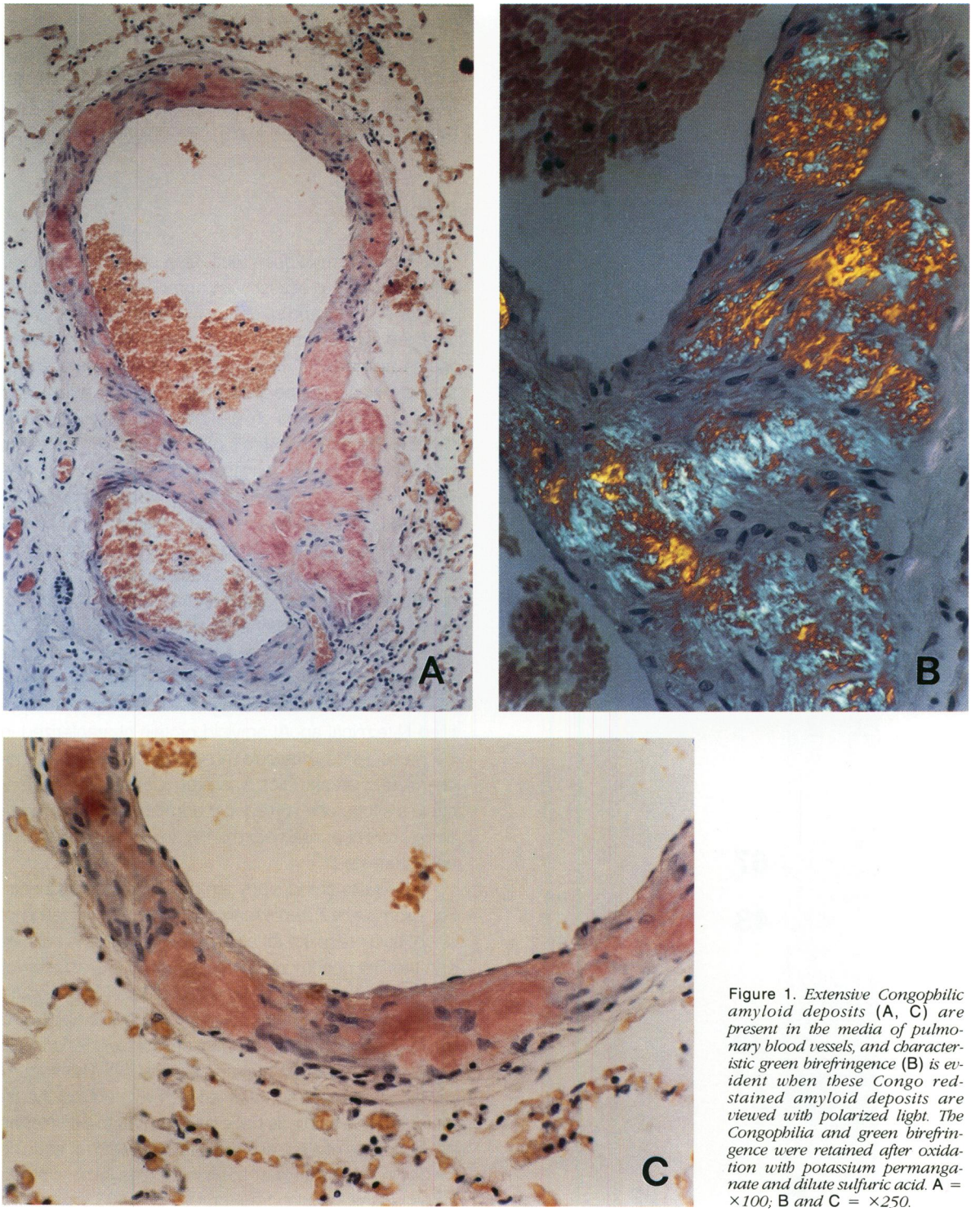

Figure 1. *Extensive Congophilic amyloid deposits* (A, C) *are present in the media of pulmonary blood vessels, and characteristic green birefringence* (B) *is evident when these Congo red-stained amyloid deposits are viewed with polarized light. The Congophilia and green birefringence were retained after oxidation with potassium permanganate and dilute sulfuric acid.* A = ×100; B *and* C = ×250.

tiple protein subspecies with acidic (pH 5.0–3.5) isoelectric points (Figure 3B).

N-terminal sequence analysis of the intact amyloid protein before cleavage elucidated a 17 amino acid sequence that was identical with positions 2–18 of normal canine apoAI. Position 1 was not identified due to background. Sequence analysis of peptide segments obtained by cleavage with BPNS-skatole (identified as BPNS 1, 2, and 3) and trypsin (identified as T 1, 3, 5, 6, 7, 8, and 9) provided additional overlapping sequences conforming to positions 1–71 of canine apoAI (Figure 4). Additionally, a partial sequence, representing positions

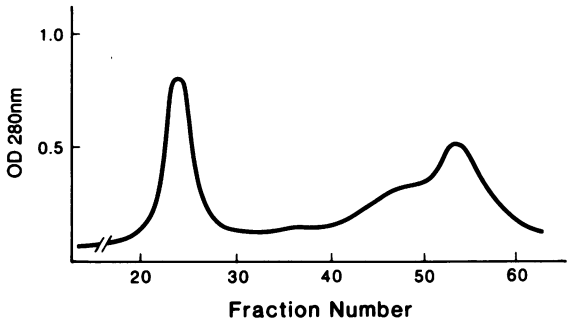

Figure 2. *Elution profile obtained by gel filtration of the dissolved amyloid fibrils on a Sepharose 6B-CL column. Fractions 52–54 (representing the major retarded peak) were pooled, dialyzed, and lyophilized for subsequent analyses.*

77–80 of normal dog apoAI, was obtained from a minor trypsin-cleaved peptide. The entire sequence obtained is identical to that of canine apoAI,[27] with no evidence of amino acid substitutions.

For normal human apoAI, the Chou and Fasman algorithms predicted a propensity for β-pleated sheet conformation at positions 13–22, 29–33, 41–47, 53–57, and 67–72. Identical propensities for β-pleated sheet conformation were obtained for the variant form of human apoAI with substitution of arginine for glycine at position 26. A propensity for β-pleated sheet conformation in dog apoAI

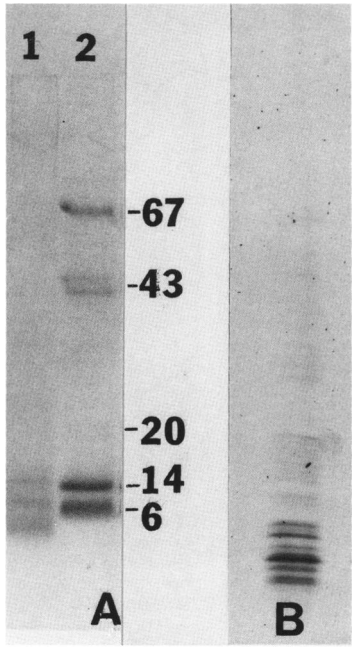

Figure 3. *SDS-PAGE* **(A)** *and AIF (pH 9.5–3.5)* **(B)** *of the amyloid protein (fractions 52–54) isolated from dog lung. SDS-PAGE* **(A)** *reveals amyloid proteins (lane 1) with M_r varying between approximately 6–14 kda. M_r markers (lane 2) are bovine serum albumin (67 kda), ovalbumin (43 kda), soybean trypsin inhibitor (20.5 kda), lysozyme (14.4 kda), and aprotinin (6.5 kda). Heterogeneity is also indicated by multiple protein subspecies with acidic (pH 5.0–3.5) isoelectric points* **(B)** *(anode is down).*

is evident at positions 12–18, 26–32, 40–46, 55–59, and 66–71.

The pulmonary amyloid deposits did not show any immunoreactivity with antiserum to human apoAI, even when the PAP method was performed with tissue sections pretreated for 20 min with concentrated formic acid.

Discussion

Systemic AA amyloidosis, occurring secondary to a variety of disease processes and predominantly affecting the kidneys, liver and spleen, is the most commonly documented form of amyloidosis in dogs and other domesticated animals.[18,28] However, we have repeatedly observed the occurrence of a type of amyloid in aged dogs (usually over 10 years of age) of multiple breeds that is not histochemically or immunohistochemically consistent with AA amyloid (KH Johnson et al, unpublished observations). These amyloid deposits, which predominate within the tunica media of blood vessels in the lung (and sometimes heart), are Congophilic after oxidation with potassium permanganate and dilute sulfuric acid and do not immunoreact with antiserum to canine AA amyloid. However, in subsequent studies of lung tissues from 5 dogs over 10 years of age, we have observed that pulmonary vascular amyloid deposits with these characteristics do immunoreact strongly with antiserum to the purified N-terminal apoAI amyloid protein characterized in the present study. Vascular amyloid with the same histochemical characteristics was previously reported to occur in the lung of a 16-year-old dog[29] but the immunoreactivity and chemical nature of the amyloid deposits were not determined.

The results of this study are new in several respects. For example, this represents the first documentation of apoAI as a precursor for a form of amyloidosis in animals and the first documentation of apoAI as a precursor for amyloid fibrils in a form of age-associated ("senile") amyloidosis. The only other documentation of apoAI-derived amyloidosis are forms of familial amyloidosis reported to occur in two different human kindreds.[9,10] The subunit proteins of amyloid fibrils associated with both of the affected human kindreds have been shown to be N-terminal fragments of a mutant form of apoAI, with an arginine for glycine substitution at position 26. The 9 kda amyloid protein in the Iowa kindred represents an 83 amino acid N-terminal fragment of the mutant apoAI.[9] A slightly larger (11 kda) apoAI N-terminal fragment was identified as the amyloid protein in the Massachusetts kindred, but this amyloid protein was sequenced only through position 33.[10]

The presence of several bands with both SDS-PAGE

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N HUMAN  D E P P Q S P W D R V K D L A T V Y V D V L K D S G R D Y V S Q F E G S A L G K

                         10                  20                  30
N DOG    D E P   Q S P W D R V K D L A T V Y V D A V K D S G R D Y V A Q F E A S A L G K
         [=============================]
           >>>>>>>>>>>>>>>>>>>>>>>>>>>>>

         [----BNPS 1----][-----------------------------------------BNPS 2--------------------------------
           >>>>>>>>       >>>>>>>>>>>>>>>>> >>>>>>>>>>>>>>>>>> >>>>

         [- - - - - - T 1 - - - - - -]   [- - - - - - T 3 - - - - - - ]      [- - - - - - - - - - T 5 - - - - - - -]
         >>>>>>>>>>>>>>>                   >>>>>>>       >>>           >>>>>>>>>>>>>>>>>>>>>>>


N HUMAN  Q L N L K L L D N W D S V T S T F S K L R D Q L G P V T Q E F W D N L E K E T E G L R

         40                  50                  60                  70                  80
N DOG    Q L N L K L L D N W D S L S S T V T K L R E Q I G P V T Q E F W D N L E K E T E V L R

         ----------------------------][---------------------BPNS 3--------------------]
                                        >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>

         [- - T 6 - - ][- - - - - - - - - T 7 - - - - - - - - - - - ][T 8][ - - - - - - - T 9 - - - - - - -]
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Figure 4. *Comparison of N-terminal regions of apoAI from the normal human and normal dog, and the confirmed amino acid sequence of the dog amyloid protein. Human apoAI has an extra amino acid residue (ie, proline at position 4 proline at position 4). The confirmed sequence of the dog amyloid protein (solid line, positions 1–71), which is identical to normal canine apoAI, was obtained by N-terminal sequencing of the intact protein (= = = =) and by sequencing of peptides obtained by cleavage with BPNS-skatole (----) and trypsin (- - - -). Arrowheads (>>>>) indicate confirmed residues within each of the respective peptide segments.*

and AIF suggests the presence of several subspecies of amyloid protein. This may reflect different sites of cleavage of the dog apoAI precursor protein, which is also supported by our demonstration of a minor trypsin-cleaved peptide that had four identified residues conforming to positions 77–80 of normal dog apoAI. The presence of several different subspecies of amyloid protein resulting from variable cleavage sites is also seen in human AA amyloidosis.[21,30,31]

Immunoreactivity with antiserum to human apoAI was not observed even when the vascular amyloid deposits present in sections of lung from this dog were pretreated with concentrated formic acid. This is not surprising in that there is substantial (~15%) amino acid variation evident when either the 71 amino acid N-terminal or complete sequences of human and dog apoAI are compared. Also the apoAI antiserum used in this study was raised to intact human apoAI, and it is not known whether this antiserum contains antibodies corresponding to antigenic epitopes associated with the 71 amino acid N-terminus of dog apoAI.

ApoAI is synthesized in both the liver and small intestine and represents the major protein in HDL.[27] It is the major activator for the enzyme lecithin:cholesterol acyltransferase (LCAT).[32] Human apoAI (243 amino acid residues) is composed of one more amino acid residue than dog apoAI.[27] This difference is reflected by a duplication, in human, of codon 3 which encodes proline. Proline residues are therefore present at both positions 3 and 4 of human apoAI (Figure 4).

It has been suggested that the structure of apoAI does not predict an amyloidogenic protein[10] in that apoAI has been shown to have six 22 amino acid repeats that are able to assume amphiphilic α-helical conformation.[33,34] These repeats are in the C-terminal 75% of the molecule, and the N-terminal 83 amino acid apoAI peptide found in the associated human amyloid fibrils contains one 22 amino acid α-helix at the C-terminus of the peptide.[10,33] The secondary structure for the remainder of the N-terminal peptide was reported to be not well defined. However, we used secondary structure prediction analysis according to Chou and Fasman[23,24] to help further evaluate the potential comparative amyloidogenicity of the N-terminal regions of normal human and dog apoAI. This analysis predicted a propensity for β-pleated sheet conformation in five regions of the N-terminus of both human (positions 1–83) and dog (positions 1–71) apoAI. These regions of predicted β-pleated sheet conformation represent 40% and 44% of the respective human and dog N-terminal apoAI peptide sequences.

The results point to an interesting similarity between apoAI- and transthyretin (TTR)-derived amyloidosis. It is apparent that apoAI, like TTR, may serve as an amyloid precursor in two major categories of amyloidosis. One

category encompasses the familial amyloidoses where mutant precursors lead to amyloid deposition. In the other category, the senile amyloidoses, unknown aging factors are involved in the amyloidogenesis of normal protein precursors.[35] ApoAI should be considered as a potential amyloid precursor when evaluating forms of human senile amyloidosis of unknown origin.

Note Added in Proof

While this article was in press, a form of non-neuropathic systemic amyloidosis in an English family was reported to be derived from N-terminal fragments (88 to 94 residues in length) of a mutant form of apoAI with a substitution of arginine for leukine at position 60 (Proc Natl Acad Sci USA 1992; 88:7389–7393).

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