Identification of Multiple Isoforms Defined by cDNA and Protein Analyses

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Five distinct serum amyloid A (SAA) cDNA clones have been isolated from a library constructed using hepatic mRNA isolated from an individual beagle dog with canine pain syndrome. This implies the existence of at least three SAA genes in the dog genome. One clone predicts a truncated "amyloid A-like" SAA molecule and offers a possible alternative mechanism for the pathogenesis of secondary amyloidosis. Relative to the human and mouse SAA proteins, an additional peptide of eight amino acids is specified by each of the dog cDNA clones. The existence of this peptide in all acute phase dog SAA proteins was confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of acute phase high density lipoprotein and provides support for evidence for gene conversion as a mechanism for maintaining the homogeneity of the SAA gene family within a species. Analysis of hepatic RNA following induction of an acute phase response shows a dramatic increase in SAA mRNA concentration; the SAA transcripts show a transient increase in size early in inflammation due to an increase in polyadenylation.

The acute phase reactants are serum proteins which show a marked increase in circulating levels during the acute phase response to an inflammatory stimulus. SAA,1 a 12-kDa apolipoprotein associated with HDL3, is the major acute phase reactant in many species, including human and mouse, and exhibits a dramatic increase in serum concentration of up to 1000-fold during the acute phase response (1, 2). Chronic or episodic inflammation may result in secondary amyloidosis, characterized by the deposition of amyloid fibrils in a number of organs including the kidney and spleen. Two major constituents of secondary amyloid deposits is amyloid A (AA), a 9-kDa N-terminal product of the limited proteolytic cleavage of SAA by an undefined monocytic membrane-bound protease.

SAA proteins are highly conserved and have been characterized in many species, including human (3-5), mouse (6), dog (7), cat (7), cow (8), and horse (9), although their physiological role(s) remains to be elucidated. Sequence comparisons between species have revealed regions of strict conservation. Residues 33-45 (human numbering) are invariant in SAA from most species so far characterized, and are considered to be important for fibril formation, and therefore of particular relevance to the pathogenesis of secondary amyloidosis. It is notable that the two species in which this region is not strictly conserved exhibit a decreased susceptibility to secondary amyloidosis, i.e. rat hepatic SAA lacks this region (10), and as a possible consequence rats do not develop secondary amyloidosis; the domestic short-haired cat has a single amino acid substitution in this region and is much less susceptible to amyloidosis than the Abyssinian cat which has the conserved sequence (11).

The primary structure of dog AA, isolated from amyloid deposits in the kidney of a 18-year-old mixed breed dog, has been described (7). The sequence reported for dog AA is 93 residues long, 18 residues longer than human AA which has 76, and shows 84% homology with human AA up to residue 69. Dog AA has an eight-amino acid insertion between the residues corresponding to 69-70 of the human sequence, after which the homology is resumed. The presence of an extra peptide in this region is not unique to the dog; although it has never been shown in human or mouse, it has been described in the Abyssinian cat (7), horse (9), and cow (8). The N terminus of dog AA is 1 residue shorter than human AA and does not contain the N-terminal arginine residue which is removed from human SAA/AA by limited proteolysis, yielding the des-Arg form (12).

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Five distinct human SAA cDNA clones have recently been isolated from a library constructed using liver mRNA from a single individual (13) indicating the existence of at least three SAA genes in the human genome. The mouse genome contains three closely linked SAA genes: SAA1, SAA2 (14), and SAA3 (15), plus a pseudogene (14), all of which are located on chromosome 7 (16). All three genes are expressed as mRNA in the liver (17, 18), but to date no translated SAA3 product has been identified. The SAA1 and SAA2 genes show 96% nucleotide sequence homology over their entire length (14), their respective protein products being the major acute phase proteins.
reactants found in mouse serum during inflammation. In contrast, the SAA3 gene shows no homology with SAA1 or SAA2 over the 5'- and 3'-untranslated regions, and intron sequences, and exhibits significant homology only in the third exon and the translated region of the fourth exon (14). Amyloidosis in the mouse is the result of the selective deposition of AA derived predominantly, perhaps exclusively, from the SAA2 isotype (19).

In this paper we report the isolation and characterization of five distinct dog SAA cDNA clones from an individual beagle dog liver library. Analysis of these clones and the expression of dog SAA mRNA and protein during acute inflammation reveal features that have implications for the evolution and biosynthetic control of the SAA gene family.

**MATERIALS AND METHODS**

**Dog cDNA Synthesis and Library Construction**—A cDNA library was constructed from poly(A)⁺ RNA isolated from the liver of a beagle dog with canine pain syndrome, an acute vascular disease which affects young beagle dogs. The library was constructed in the phagemid vector pT7T3 19U (Pharmacia LKB Biotechnology Inc.) using a previously described method (20).

**Isolation of Dog SAA cDNA Clones**—Approximately 10⁵ recombinants were screened on duplicate nitrocellulose filters (Scherlie & Schuell) by a modification of the method of Grunstein and Hogness (21) with the human SAA cDNA clone pAI (22) which had been radiolabeled with [α-³²P]dCTP (Du Pont-New England Nuclear) using an oligolabeling kit (Pharmacia). Unincorporated label was removed by passing over a G-50 "Nick" column (Pharmacia). Filters were prehybridized and hybridized for 1 and 24 h, respectively, in 50% formamide, 6 × SSC, 5 × Denhardt's solution, 0.05% (w/v) sodium pyrophosphate, 0.5% (w/v) SDS, 100 μg/ml sonicated salmon sperm DNA at 42 °C. Following hybridization, the filters were washed subsequently isolated.

Sequence Analysis—The inserts from DSAA plasmid clones were sequenced directly by the dyeode chain termination method (23) from double-stranded template using a Sequenase kit (U.S. Biochemical Corp.) and specific synthetic oligonucleotide primers following alkaline denaturation. Initial sequence data and confirmation of the identity of each clone were obtained using the 30-mer oligonucleotide IPSAA (5'-GATCCCGCATGGGAAGTAG CT-3') (24), designed to a region of mRNA sequence (nucleotides 193-222, SAA2 numbering) shared by the mouse SAA1, SAA2, and SAA3 isotypes (15, 25), as a sequence primer. To complete the sequence of each clone on both strands, five additional 18-mer oligonucleotides designed to anneal to all clones were synthesized.

Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Inc., Foster City, CA), and nucleotide sequence analyses were performed using PGCE software (IntelliGenetics, Mountain View, CA).

**Beanie Liver RNA Extraction**—Liver tissue was obtained from a healthy 6-week-old male beagle-basset cross and from two 1-2-year-old male beagle dogs 6 and 24 h following sterile induction of peritonitis via the intraperitoneal injection of Salmonella typhosa lipopolysaccharide (Sigma) at a dose of 1 mg/kg of body weight. Total RNA was extracted from 0.5-2 g frozen liver samples by the LiCl/urea method (26).

**Northern Blot Analysis**—RNA samples (10 or 20 μg) were size-fractionated by electrophoresis on 1.2% (w/v) agarose, 1.1% (v/v) polyacrylamide gradient gel with a 3% acrylamide stacking gel all containing 0.1% (w/v) SDS. Prior to electrophoresis the samples were treated with 5% (v/v) 2-mercaptoethanol (29).

For electrophoresis, aliquots of 200 μg of dog HDL were lyophilized and delipidated with 0.5 ml chloroform/methanol (21, v/v) (30). The delipidated proteins were resuspended in 1% (w/v) decyl sodium sulfate (Eastman Kodak Co.), 7 M urea, and 5% (v/v) 2-mercaptoethanol. The samples were electrophoresed as described (31) on 0.5-mm polyacrylamide gels containing 7 M urea and 20% (v/v) Ampholines, pH 4-6.5, and 40% (v/v) Ampholines, pH 7-9 (Pharmacia).

**RESULTS AND DISCUSSION**

Canine pain syndrome (CPS) is a naturally occurring, acute necrotizing vasculitis of unknown etiology associated with a range of inflammatory manifestations which affects primarily young dogs and is best described in the beagle (32-34). As part of our aim to understand more fully the molecular mechanisms underlying the inflammatory events that occur during this prevalent veterinary condition, we have initiated a study to examine the role of the major acute phase reactant SAA during the acute phase response in the beagle dog. The ultimate aim of these studies is to better define the pathogenesis of CPS and to improve its diagnosis and treatment.

**Isolation and Sequencing of Dog SAA cDNA Clones**—Preliminary Northern blotting studies had shown the human SAA cDNA probe pAI (22) to cross-hybridize strongly with a single band in beagle liver mRNA identical in size to the human SAA mRNA of approximately 650 bases (data not shown). Screening of 10⁵ recombinants from a beagle liver cDNA plasmid library with the pAI probe yielded approximately 130 positive signals of various intensities. Of these, 30 were selected for colony purification. Candidate dog SAA clones were rescreened with pAI, and plasmid DNA was purified from three clones which showed different hybridization signal intensities: pDSAA15, pDSAA18, and pDSAA32. Sequencing of the clones was completed on both strands via the dyeode chain termination method (23). A further two clones (pDSAA85 and ~86) were selected and fully sequenced following hybridization of 21 of the 30 candidate clones with 18-2 base-long oligonucleotide probes designed to differentiate between the two subclasses of DSAA clones identified.
The SAA protein predicted by clone pDSAA15 corresponds to one of the two previously published AA proteins isolated from the amyloid-laden kidney of a 16-year-old mixed breed dog (7). The reported dog AA protein sequence offers as alternative residues at position 17 leucine and tryptophan in the ratio of 65 to 35%; this may reflect the deposition of AA derived from two similar, possibly allelic, SAA isotypes. Clone pDSAA15 predicts a leucine at this position; however, no SAA cDNA clone corresponding to the Trp-containing AA described by Kluve-Beckerman et al. (7) was identified among the clones isolated from our library. Selective deposition of AA derived from the mouse SAA2 isotype has been described (19). Although it remains unclear which SAA isotype, if any, is selectively deposited in humans, the identification of five variant SAA cDNAs from an individual beagle compared with the presence of only two dog AA proteins, at most, in amyloid-laden kidney suggests the selective deposition in the dog of a particular amyloidogenic SAA gene product.

Of the five cDNA clones reported here, four (pDSAA15, -32, -85, and -86) predict SAA proteins which vary at only four positions: residue 7, Gly/Ser; residue 17, Leu/Trp; residue 70, Leu/Arg; and residue 72, Arg/Lys (Table I; nucleotide changes are also shown). Most SAA species reported to date contain a glycine at residue 8 (human numbering); the serine defined at this position in four of our dog clones has not been observed in any other SAs. The fifth clone, pDSAA18, predicts a truncated SAA protein of 91 residues rather than the full-length 111 residues. Sequence analysis of pDSAA18 revealed a single G to A transition with respect to pDSAA32 at nucleotide 363, resulting in the conversion of a tryptophan codon (TGG) to a termination codon (TGA) at position 92. The proteins predicted by pDSAA16 and pDSAA32 are otherwise identical, as are their respective 3'-untranslated sequences. We believe this to be the first reported instance from any species of a cDNA clone predicting a truncated "AA-like" SAA protein. It is interesting to note that the position of the termination codon corresponds to the tryptophan of the Trp-Gly dipeptide assigned by Kluve-Beckerman et al. (7) to the C terminus of dog AA on the basis of sequence homology with human SAA rather than as part of an overlapping peptide. Given the homology of dog and human SAA/AA it would appear reasonable to have assigned the Trp-Gly dipeptide in this manner. However, the mechanism by which AA is derived

### Table 1

A comparison of the variability found in dog SAA/AA at the protein level is presented (upper case)

<table>
<thead>
<tr>
<th>Residue no.</th>
<th>7</th>
<th>17</th>
<th>70</th>
<th>72</th>
<th>92</th>
</tr>
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<td>G</td>
<td>L/W</td>
<td>L</td>
<td>R</td>
<td>W</td>
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<td>G</td>
<td>L</td>
<td>L</td>
<td>R</td>
<td>W</td>
</tr>
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<td>G</td>
<td>L</td>
<td>L</td>
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DAAKB denotes the AA sequence reported by Kluve-Beckerman et al. (7); DSA denotes the protein sequence specified by cDNA clones isolated from a CPS beagle dog. The single letter amino acid code is used, and the termination codon at position 92 in clone pDSAA18 is denoted by *. The respective codons are given below each residue (lower case), and the nucleotide differences compared with pDSAA15 are underlined.
from SAA during amyloidosis remains a matter of some debate. The current model postulates that AA is the 9-kDa N-terminal product of a limited proteolytic cleavage of SAA by a monocyteic membrane-bound protease. Providing direct evidence to support this theory, Husebekk et al. (37), using polyclonal and monoclonal antibodies, have shown the conversion in vivo of human SAA to AA and its incorporation as human AA into mouse amyloid deposits following the injection of human HDL-SAA into mice with lipopolysaccharide-stimulated amyloidosis. However, Baltz et al. (38), using a similar experimental approach, were unable to demonstrate such a precursor-product relationship between SAA and AA.

If pDSAA18 encodes a translated product which approximates in length to AA, secondary amyloidosis may arise via an alternative mechanism, which does not require proteolytic cleavage of SAA. Therefore, the question of whether a specific “AA gene” exists in the genome of the dog, and possibly other species, is raised.

Although the five dog cDNAs predict SAA proteins with approximately 80% homology to human and mouse SAA, the most apparent difference is the presence in each of the predicted dog sequences of eight amino acids (residues 69-76) which represent an insertion relative to the human sequence between residues 69 and 70, i.e. in the region of variability between the characterized isoforms of human SAA. SDS-PAGE analysis of acute phase dog, human, and mouse HDL (Fig. 2) shows that dog apo-SAA migrates as a single 12.3-kDa species (computer analysis based on amino acid sequence predicts a 12.4-kDa species) of greater molecular mass than either human or mouse, consistent with there being an extra eight-amino acid peptide in all dog SAAs. The insertion of an extra peptide in this position has also been shown at the cDNA level of eight-amino acid peptide in all dog SAAs. The insertion of an extra peptide in this position has also been shown at the cDNA level of all dog SAAs.

Hybridization with IPSAA (Fig. 3a), which detects all dog SAA isotypes, shows that the unstimulated level of SAA mRNA is low, with a signal detected only after overexposure of the blot (track 1). At 6 h (track 2) the SAA mRNA concentration has increased and by 24 h (track 3) has dramatically increased. This is consistent with the well-characterized 1000-fold increase in serum SAA protein concentrations during inflammation (1, 2) and with the kinetics and magnitude of induction of mouse liver SAA mRNA during inflammation (24, 40).

To determine which subclass of SAA gene is being induced during the acute phase response, the DOG15 (Fig. 3b) and DOG32 (Fig. 3c) probes, specific for the 15-like or for the 32-like clones, respectively, were hybridized separately with the three SAA oligonucleotide probes: IPSAA, DOG15, and DOG32 (Fig. 3).

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Multiple Isoforms of Dog SAA

The size of the SAA transcript 6 h after induction of the acute phase response (Fig. 3a, track 2) is 50–100 bases greater than that of the healthy control dog (Fig. 3a, track 1); at 24 h (Fig. 3a, track 3), although the concentration of transcript is markedly greater than at 6 h, the size of the transcript is comparable with that of the control. The increase in transcript size during the acute phase response therefore appears to be transient and to occur relatively early. A similar increase in SAA transcript size has been described during the early stages of inflammation in the mouse (24) and has been shown by RNase H digestion of mRNA annealed to oligo(dT)15 to be due to a transient increase in the length of the poly(A) tail (40). To determine whether the observed alteration in dog SAA transcript size was also the consequence of an increase in polyadenylation an RNase H assay was performed on total liver RNA isolated from the 6- and 24-h inflammation dogs. Following digestion of the poly(A) tail the RNA samples were size-fractionated on agarose/formaldehyde gels and blotted onto nitrocellulose. Blotted RNA was then probed for SAA size-fractionated on agarose/formaldehyde gels and blotted onto nitrocellulose. Blotted RNA was then probed for SAA mRNA subclasses. As is evident from Fig. 3, b and c, the intensity of signal with DOG32 greatly exceeds that generated by DOG15. As both probes were labeled to approximately the same specific activity and showed equivalent background hybridization with 28 and 18 S RNA (data not shown), the difference in signal intensity obtained approximates to differences in the levels of hepatic SAA subclass mRNA. The finding that 75% of 20 randomly picked pDSAA cDNA clones were 22-like had initially suggested that the major acute phase SAA gene(s) is(are) of the 32-like subclass. The Northern blot analysis provides further evidence that the acute phase response involves a marked differential increase in transcription and/or stability of mRNA derived from the 32-like SAA gene(s). It is not possible to distinguish by differential hybridization between the mRNAs corresponding to the two identified members of the 32-like subclass, DSAA18 and DSAA32. However, no truncated AA-like protein product of the gene corresponding to the DSAA18 transcript has been observed in dog sera, we consider it likely that the major transcript during inflammation is that specified by the DSAA32 clone.

In summary, we have identified and characterized five distinct SAA cDNA clones of two subclasses from an individual CPS beagle dog liver library, indicating the presence of at least three SAA genes in the dog genome. The cDNA clones predict SAA proteins of two PI classes, and two PI SAA isoforms are observed experimentally in CPS dog serum. Confirmation of an extra 8-amino acid peptide in all dog

![Fig. 4. RNase H assay of SAA mRNA.](image)
SAAs relative to human or mouse SAAs supports the theory that the maintenance of structural homogeneity of the SAA family within a species occurs via gene conversion. One clone specifies a truncated SAA protein which has identical sequence and which approximates in size to dog AA, thereby offering an alternative mechanism for the pathogenesis of secondary amyloidosis. The dramatic increase in SAA mRNA concentration during the 24 h after sterile induction of inflammation in the beagle dog is accompanied by a transient increase in transcript size due to increased polyadenylation. These studies provide the basis for the analysis of the SAA gene family in the dog and for the investigation of the role(s) of the major acute phase proteins in canine pain syndrome.

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REFERENCES