



Variation of amino acid sequences of serum amyloid a (SAA) and immunohistochemical analysis of amyloid a (AA) in Japanese domestic cats

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ABSTRACT. Amyloid A (AA) amyloidosis, a fatal systemic amyloid disease, occurs secondary to chronic inflammatory conditions in humans. Although persistently elevated serum amyloid A (SAA) levels are required for its pathogenesis, not all individuals with chronic inflammation necessarily develop AA amyloidosis. Furthermore, many diseases in cats are associated with the elevated production of SAA, whereas only a small number actually develop AA amyloidosis. We hypothesized that a genetic mutation in the *SAA* gene may strongly contribute to the pathogenesis of feline AA amyloidosis. In the present study, genomic DNA from four Japanese domestic cats (JDCs) with AA amyloidosis and from five without amyloidosis was analyzed using polymerase chain reaction (PCR) amplification and direct sequencing. We identified the novel variation combination of 45R-51A in the deduced amino acid sequences of four JDCs with amyloidosis and five without. However, there was no relationship between amino acid variations and the distribution of AA amyloid deposits, indicating that differences in SAA sequences do not contribute to the pathogenesis of AA amyloidosis. Immunohistochemical analysis using antisera against the three different parts of the feline SAA protein—i.e., the N-terminal, central, and C-terminal regions—revealed that feline AA contained the C-terminus, unlike human AA. These results indicate that the cleavage and degradation of the C-terminus are not essential for amyloid fibril formation in JDCs.

KEY WORDS: AA amyloidosis, fibril formation, Japanese domestic cat, *SAA* gene

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Amyloid A (AA) amyloidosis is a fatal systemic condition that occurs secondary to chronic inflammatory disorders such as rheumatoid arthritis (RA) [13], tuberculosis, syphilis, chronic pyelonephritis [5], inflammatory bowel disease [24], connective tissue diseases [22], and malignancies [9, 17]. Persistently high concentrations of serum amyloid A (SAA) in the plasma are essential for the pathogenesis of AA amyloidosis [25]. Amyloid fibrils are thought to comprise partially fragmented SAA, which confers on them abnormal molecular conformations [31].

The human *SAA* gene has several polymorphisms. In most human cases of AA amyloidosis, *SAA1*, which has five alleles (SAA1.1 to 1.5), is responsible for the disease. In Caucasian patients with familial Mediterranean fever, the *SAA1.1* allele has been identified as a risk factor for the development of AA amyloidosis [6]. However, Japanese patients with RA who have the *SAA1.3* allele are more susceptible to AA amyloidosis [35]. Two mutation sites (Val52 and Ala57) in the deduced amino acid sequence define amyloidogenicity. The full-length human SAA, which is 104 amino acids long, is never the major species in AA amyloid proteins. The AA protein lacks the C-terminus of the SAA sequence and the main cleavage site is the 76th amino acid residue [34]. However, it has not yet been established whether the cleavage and absence of the C-terminus of SAA are essential for amyloidogenesis.

AA amyloidosis in cats also occurs secondary to chronic inflammatory conditions frequently, especially those resulting from infectious diseases [10]. Hereditary traits of AA amyloidosis have been reported in Abyssinian (Aby) and Siamese (Siam) cats [4, 32]. Three variation sites have been identified between the SAA amino acid sequences of Aby and Siam cats; these variation

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Table 1. JDC cats examined in the present study

Cat No.	Sex	Age	Major pathologic diagnosis at necropsy
1 ^{a)}	SF	13 y	Mandibular abscess and systemic AA amyloidosis
2 ^{a)}	CM	6 y	Bacterial dermatitis and systemic AA amyloidosis
3 ^{a)}	CM	5 y 2 m	B cell lymphoma in cerebellum and systemic AA amyloidosis
4 ^{a)}	SF	12 y 5 m	Chronic renal failure with systemic AA amyloidosis
5	CM	2 y 2 m	Osteochondromatosis and systemic AA amyloidosis.
6	SF	9 y 8 m	Intestinal lymphoma (small cell type) and systemic AA amyloidosis
7	SF	15 y	Pyogenic bronchitis, interstitial pneumonia, acute myocarditis and systemic AA amyloidosis
8 ^{a)}	SF	13 y 3 m	Systemic metastasis of mammary gland carcinoma
9 ^{a)}	SF	16 y	Systemic metastasis of anaplastic plasmacytoma
10 ^{a)}	CM	12 y 2 m	Systemic histiocytosis
11 ^{a)}	F	15 y	Systemic metastasis of mammary gland carcinoma
12 ^{a)}	M	10 y	Systemic metastasis of T cell lymphoma

a) Cats subjected to the DNA sequence analysis. SF, Spayed female; CM, Castrated male; F, Female; M, Male; AA, amyloid A.

sites may be responsible for the differences observed in amyloidogenicity and AA deposition patterns between these breeds. AA amyloidosis also occurs sporadically in Japanese domestic cats (JDCs), and is preceded by high levels of circulating SAA; however, not all cats with the condition develop amyloidosis [28, 29]. Therefore, we hypothesize that, as in humans, genetic mutations in the *SAA* gene may make a large contribution to the pathogenesis of feline AA amyloidosis.

In the present study, we analyzed the genomic DNA sequences of the *SAA* gene in four JDCs with AA amyloidosis and five without. Our objective was to elucidate the amyloidogenic factors responsible for feline AA amyloidosis. In the present paper, we have also included a discussion of the relationship between variations in DNA sequences and the deduced amino acid sequences and patterns of AA deposition. Furthermore, we analyzed AA deposits immunohistochemically in JDCs using antisera against three different parts of the feline SAA protein, namely, the N-terminus, the central region, and the C-terminus.

MATERIALS AND METHODS

Animals and tissue samples

The cats examined in the present study are listed in Table 1. AA-laden and control liver and kidney tissues were fixed in 10% formalin solution, routinely processed, and embedded in paraffin wax. Four-micrometer-thick paraffin sections of every organ of every case (cat Nos. 1–12) were subjected to hematoxylin and eosin (HE) and alkaline Congo red (CR) staining [21], and then subjected to immunohistochemistry and immunofluorescence analysis. The CR-stained sections were examined under a routine or polarized light microscope. The characteristic green birefringent polarization color from CR-stained tissues was regarded as proof of the presence of amyloid.

DNA extraction and polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from fresh liver tissues from four JDCs that had been pathologically diagnosed with AA amyloidosis, and from five JDCs without amyloid deposition (Table 1) using a DNeasy® Blood and Tissue Kit (QIAGEN, Limburg, Netherlands).

Extracted DNA was subjected to PCR amplification for the feline *SAA* gene using PrimeSTAR® Max DNA polymerase (TaKaRa Bio, Otsu, Japan). The specific primers for exons 2, 3, and 4 of the feline *SAA* gene were as follows: exon 2, forward: 5'-CAA CTG CCC TCT TCC TCC AT-3', reverse: 5'-GAC GGA CAC AAA TCC TCT GGT-3'; exon 3, forward: 5'-CTG GAG TCG GTG GCA GAT TT-3', reverse: 5'-CCT TGC TCT GAC CCC ATC AG-3'; and exon 4, forward: 5'-ACC CAG TAA GGG ATC AGC CT-3', reverse: 5'-CAC CCC TGG GAA CGA ACA AA-3'. The PCR was performed in 50 μ l of reaction fluid. The PCR regimen was: 35 cycles of a denaturation step of 98°C for 10 sec; a primer annealing step of 57°C for 5 sec; and an elongation step of 72°C for 3 sec. The PCR products were electrophoresed on an agarose gel and analyzed using a ChemiDoc™ imaging system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The target amplification products were extracted from the gel bands using a QIAquick PCR purification kit (QIAGEN), and sequenced in the forward direction using the BigDye Terminator 3.1 mix (Applied Biosystems, Foster City, CA, U.S.A.) and ABI Prism 3030 Genetic Analyzer (Applied Biosystems).

Antisera preparation

Antigenic peptides of the N-terminal region (MREANYIGAD), the central region (QRGPGGAWAAKV), and the C-terminal region (EWGRSGKDPNHFPR) of feline SAA were synthesized and conjugated with keyhole limpet hemocyanin (KLH). Each synthetic peptide was administered four times to three different conventional rabbits. Forty-nine days after the first administration, whole blood was collected and the antisera were obtained. The antisera were affinity-purified (Eurofins Genomics, Tokyo, Japan) (Fig. 1). The specificity of the antisera against each peptide was assessed by western blot analysis with a recombinant (rb) whole feline SAA protein and each synthetic peptide (Fig. 2). The rb feline SAA protein was produced as previously described [27].

23 AS-No. 1 32 45 AS-No. 2 56
 QWYSFLGEAA QGAWDMWRAY SDMREANYIGADKYFHARGN YDAAQRGPGG AWAAKVSDA
 91 AS-No. 3 104
 RENSQRVTFDF FRHGNSGHGA EDSKADQAAN EWGRSGKDPN HFRPAGLPAK Y

Fig. 1. Amino acid sequence of feline serum amyloid A (SAA). AS-Nos. 1–3 are oligopeptides used as immunogens.

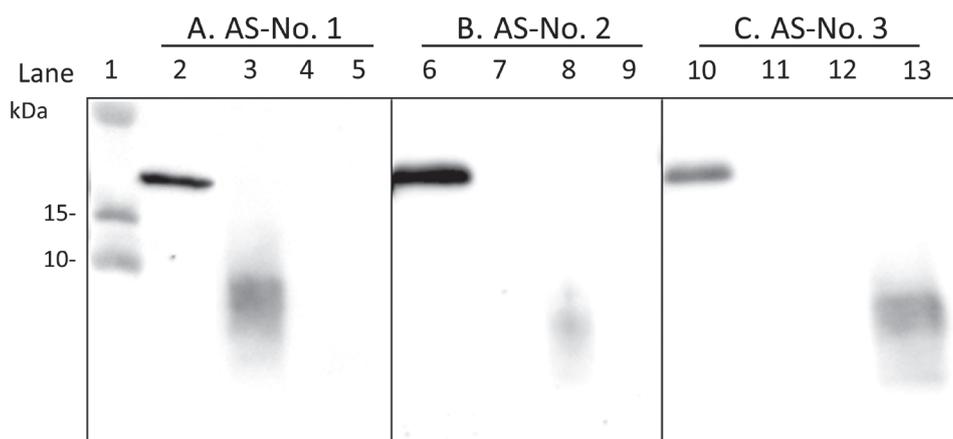


Fig. 2. Western blot analysis. The antisera used were AS-No. 1 (lanes 2–5), AS-No. 2 (lanes 6–9), and AS-No. 3 (lanes 10–13). The electrophoresed antigens were recombinant (rb) feline total serum amyloid A (SAA) (lanes 2, 6 and 10), SAA 23–32 (lanes 3, 7 and 11), SAA 45–56 (lanes 4, 8 and 12) and SAA 91–194 (lanes 5, 9 and 13). Lane 1 represents a size marker.

Table 2. Primary antibodies used for Immunohistochemistry, Immunofluorescence and Western blot analysis

Antibody	Antigen	Species	Dilution	Supplier
AA (clone KM268)	Human SAA 37–47	Mouse	1:50 (IF)	Kyowa Medics
Antiserum				
AS-No.1	Cat SAA 23–32	Rabbit	1:150 (IF) 1:1,000 (WB)	Eurofins Genomics
AS-No.2	Cat SAA 45–56	Rabbit	1:150 (IF) 1:1,000 (WB)	Eurofins Genomics
AS-No.3	Cat SAA 91–104	Rabbit	1:150 (IF) 1:1,000 (WB)	Eurofins Genomics

Immunohistochemistry and immunofluorescence

An immunohistochemical analysis was performed using an Envision polymer system (Dako, Kyoto, Japan). Deparaffinized sections were incubated with 0.1% actinase E at room temperature for 10 min for the reaction with the anti-AA mouse monoclonal antibody. Endogenous peroxidase activity in the sections was blocked with 3% hydrogen peroxide/methanol at room temperature for 5 min. The sections were incubated in 8% skimmed milk/Tris-buffered saline (TBS) at 37°C for 40 min to avoid non-specific reactions. The sections were then incubated at 4°C overnight with the primary antibodies listed in Table 2, respectively. After washing three times in TBS, the sections were incubated with Envision horseradish peroxidase-labeled polymer anti-mouse or anti-rabbit IgG (Dako) at 37°C for 40 min. The sections were washed with TBS and the reaction products were visualized with 0.05% 3,3'-diaminobenzidine and 0.03% hydrogen peroxide in TBS. Counterstaining was performed with Mayer's hematoxylin. Corresponding slides are incubated with TBS in place of the primary antibody, as negative controls.

Four-micrometer-thick paraffin tissue sections were treated with 8% skimmed milk in Tris-buffered saline with 0.1% Tween 20 (TBST) at 37°C for 40 min to avoid non-specific reactions. The sections were then incubated with antisera raised to the synthetic SAA peptides (AS-Nos. 1, 2 and 3) (Table 2) at 4°C overnight. After washing, the sections were incubated with a secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, 1:100 dilution, Life Technologies, Carlsbad, CA, U.S.A.) at 37°C for 60 min. The

Table 3. Tissue distribution and severity of AA deposition in JDCs examined in the present study

Cat No.	Liver	Renal glomerulus	Renal medulla	Thyroid gland	Intestine	Other tissues
1	+++	+++	+	++	ND	Adrenal gland++
2	+++	ND	+	ND	+	Tongue +
3	+++	++	+	++	ND	ND
4	ND	ND	+++	ND	++	Tongue +
5	+++	ND	ND	+	++	Adrenal gland+
6	++	+	+++	+++	++	Adrenal gland+
7	++	ND	ND	ND	+	Adrenal gland+

Amyloid deposition: Renal glomerulus, ND=no amyloid detected, +=mild segmental deposition <25%, ++=moderate segmental to global deposition affecting 25–50% and +++=severe segmental to global deposition affecting >50%; Renal medulla, ND=no amyloid detected, +=minimal to mild deposition affecting <25%, ++=moderate multifocal deposition affecting between 25 and 50% and +++=severe multifocal to diffuse deposition affecting >50%; Liver, Thyroid gland, Intestine and other tissues, ND=no amyloid detected, +=minimal to mild deposition, ++=moderate multifocal deposition and +++=severe multifocal to diffuse deposition.

sections were then incubated in a 1% CR solution for 10 min, and washed in a saturated lithium carbonate solution. To break non-specific bonds, the sections were incubated in 80% ethanol for 5 min. The sections were finally mounted using VECTASHIELD HardSet medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, U.S.A.), and examined using a Carl Zeiss LSM700 confocal laser microscope (Carl Zeiss Micro Imaging, Tokyo, Japan) equipped with a red filter with an excitation peak of 596 nm and an emission maximum of 620 nm [7]. Corresponding slides were incubated with TBS or TBST in place of the primary antibody or antisera, as negative controls.

RESULTS

Histopathology

Table 3 shows the tissue distribution and severity of AA deposition in the cats examined. Amyloid deposits were immunopositive for AA in all cats. Four (Nos. 1–3 and 5) out of seven cats with systemic AA amyloidosis exhibited severe deposition of AA in the liver, whereas two (Nos. 6 and 7) showed moderate deposition. AA was located in the vessel wall of the Glisson's capsule, around the central veins, and in the space of Disse along the sinusoids adjacent to the cords of hepatocytes (Fig. 3a–c). In the areas with severe AA deposition, the remaining hepatocytes showed atrophic changes. Cats with moderate to severe AA deposition in the renal glomeruli also showed minimal to mild deposition in the medullary interstitium (Nos. 1 and 3), whereas the opposite was noted in the others (Nos. 4 and 6). Glomerular amyloid was detected segmentally in the mesangial region (Fig. 4a–c). In cat No. 4, AA deposition was not detected in the liver, but was prominent in the renal medulla (Fig. 5a–c). Furthermore, the thyroid gland, intestines, adrenal gland, and tongue were the favored sites of AA deposition. In the thyroid gland, AA was deposited in the interfollicular interstitium and accompanied by follicular atrophy (Fig. 6a–c). In the intestines, AA was mainly detected in the lamina propria.

SAA protein sequence of JDCs

The nucleotide sequence of the feline *SAA* gene was determined for exons 2–4, with liver samples from nine cats (Nos. 1–4; AA amyloidosis, Nos. 8–12; non-amyloidosis controls) by a direct sequencing method, and the deduced amino acid sequences of the SAA protein were compared with that of Aby cats, which is registered, and Siam cats [16, 20] (Table 4). Three variation sites were found at positions 1, 45 and 51. All control (Nos. 8–12) and two amyloidosis (Nos. 1–4) cats had the same amino acid combination of 45R and 51A, whereas No. 2 had the same amino acids at the same positions as those of the previous Aby case. The kidney was the main target organ in the Aby case of amyloidosis, whereas severe AA deposition was also observed in the liver of No. 2. Although no present JDC cases showed the same sequences at positions 45 and 51 with the previous Siam case, in which the liver was the main target organ, three out of four of the present JDC amyloid cases (Nos. 1–3) exhibited severe AA deposition in the liver. There was an absence of patterns between variations at position 1 and the incidence of AA amyloidosis or deposition patterns.

Immunohistochemistry and immunofluorescence

Antisera of Nos. 1–3 were prepared against residues 23–32, 45–56 and 91–104 of feline SAA, respectively, (Fig. 1). Each antiserum was immunoreacted with rb feline total SAA as well as the corresponding immunogen peptides (Fig. 2). The results of the immunohistochemical analysis demonstrated that CR-positive AA deposits in the liver and kidney were recognized by antisera raised to the peptides within the feline SAA protein (Figs. 7–9). These results indicate that, in contrast to humans, the feline AA protein contains the amino acid residues 23–104 of SAA, suggesting that the cleavage and metabolism of the C-terminal sequence are not essential elements in the formation of amyloid fibrils.

DISCUSSION

We herein identified three amino acid variations at positions 1, 45 and 51 in the feline SAA protein. The common combination

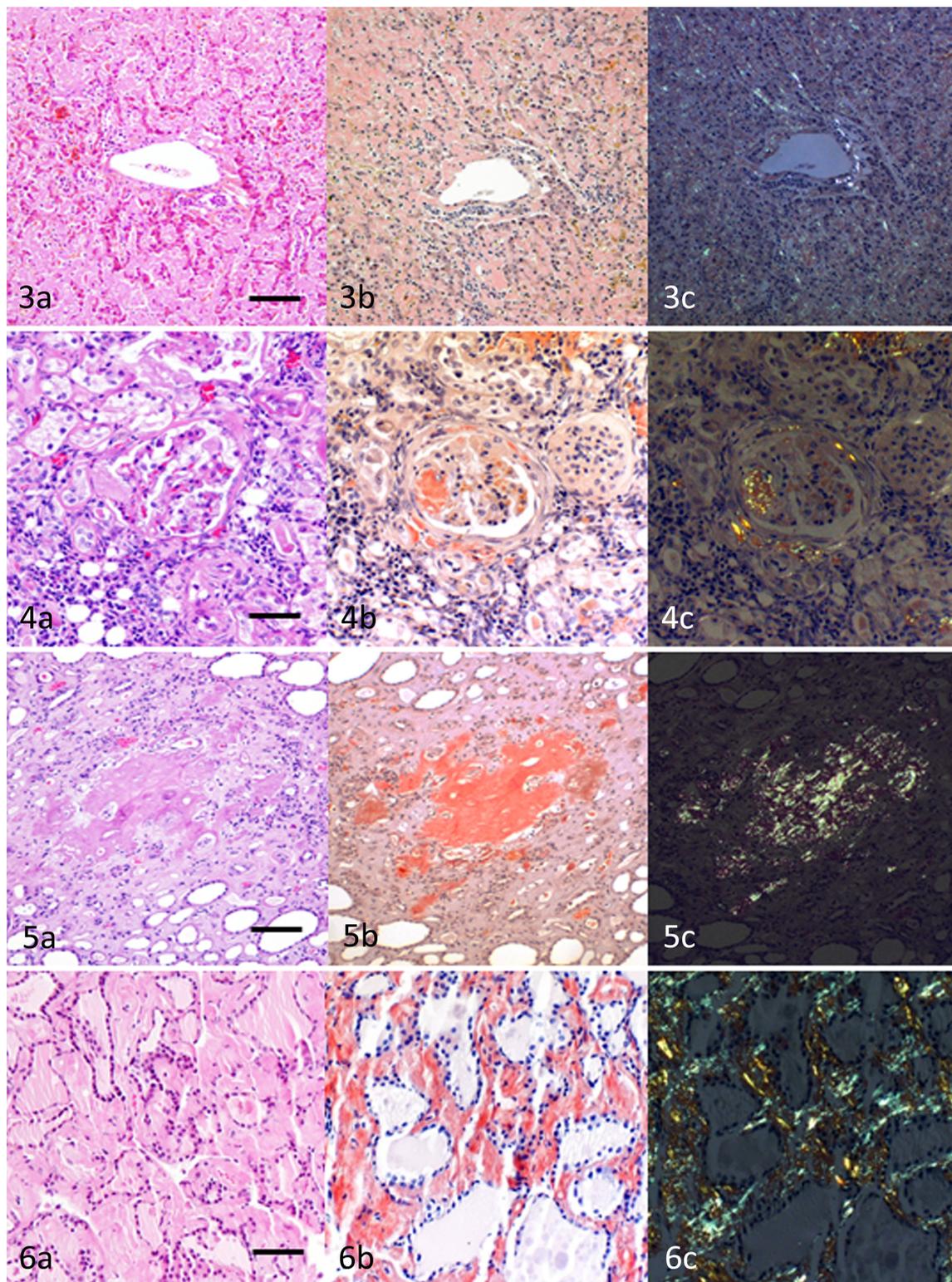


Fig. 3. Amyloid A (AA) amyloidosis, Liver, Cat No. 1, AA deposited around the central vein and in the space of Disse. Hematoxylin and eosin (HE) (a), alkaline Congo red (CR) (b) and CR with a polarized light (c). Bar, 50 μ m.

Fig. 4. Amyloid A (AA) amyloidosis, Kidney, Cat No. 6, AA deposited segmentally in the renal glomerulus. Hematoxylin and eosin (HE) (a), alkaline Congo red (CR) (b) and CR with a polarized light (c). Bar, 25 μ m.

Fig. 5. Amyloid A (AA) amyloidosis, Kidney, Cat No. 4, AA deposited in the interstitium of the medulla. Hematoxylin and eosin (HE) (a), alkaline Congo red (CR) (b) and CR with a polarized light (c). Bar, 50 μ m.

Fig. 6. Amyloid A (AA) amyloidosis, Thyroid gland, Cat, No. 6, AA deposited in the interfollicular interstitium. Hematoxylin and eosin (HE) (a), alkaline Congo red (CR) (b) and CR with a polarized light (c). Bar, 25 μ m.

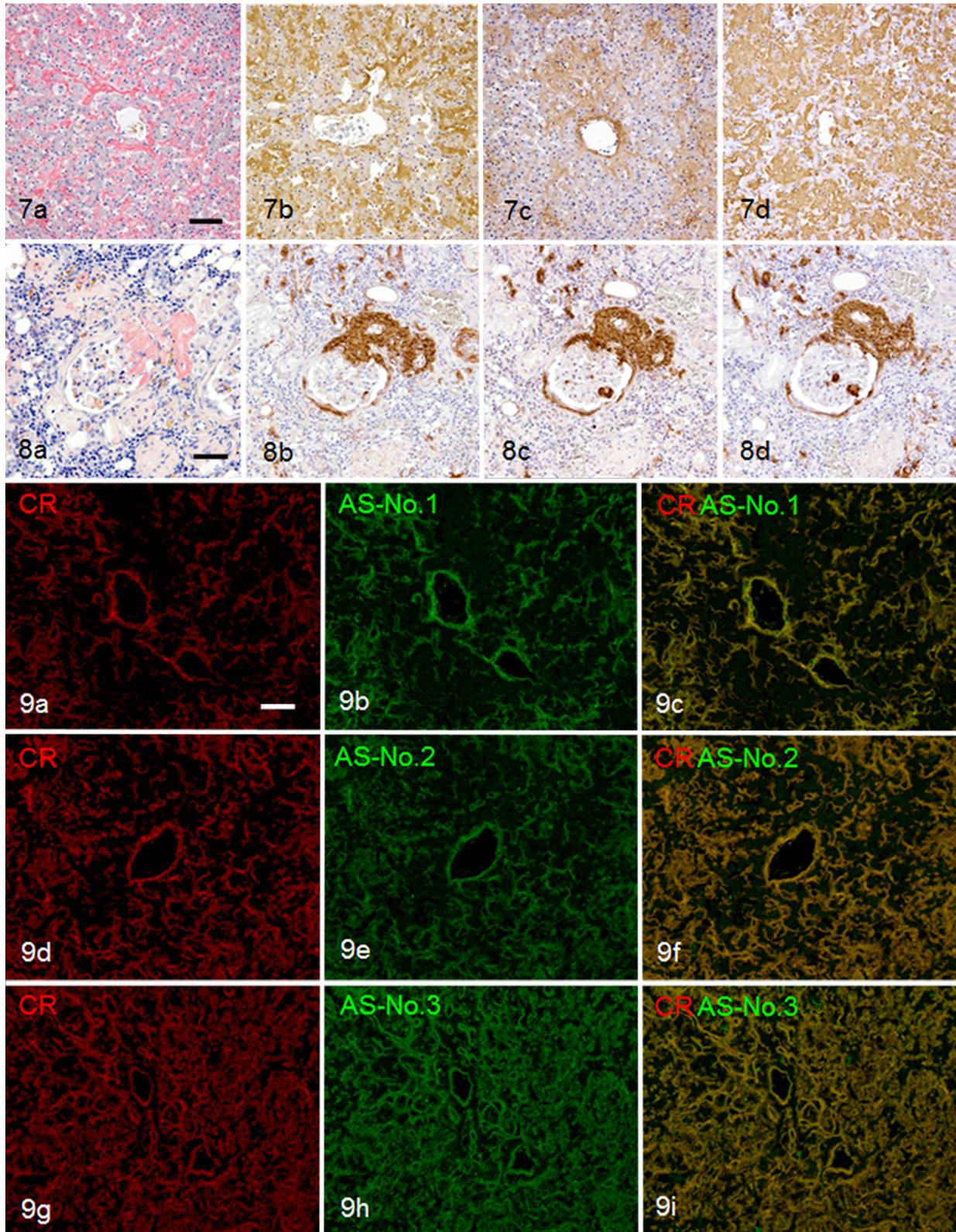


Fig. 7. Amyloid A (AA) amyloidosis, Liver, Cat No. 5, AA deposited mainly in the space of Disse. Alkaline Congo red (CR) (a), and immunostained with AS-No. 1 (b), AS-No. 2 (c), and AS-No. 3 (d). Bar, 50 μ m.

Fig. 8. Amyloid A (AA) amyloidosis, Kidney, Cat No. 6, AA deposited in the glomerulus and in the walls of arterioles. Alkaline Congo red (CR) (a), and immunostained with AS-No. 1 (b), AS-No. 2 (c), and AS-No. 3 (d). Bar, 25 μ m.

Fig. 9. Amyloid A (AA) amyloidosis, Liver, Cat No. 5, Alkaline Congo red (CR)-stained liver sections examined with a confocal laser microscope (a, d and g), and those immunostained with AS-No. 1 (b), No. 2 (e), and No. 3 (h). CR-positive AA deposits also immunoreacted with AS-No. 1 (c), AS-No. 2 (f), and AS-No. 3 (i). Bar, 50 μ m.

Table 5. Amino acid sequence of the SAA protein from various species

	Amino acid sequence																								
	45							51																	
Human	R	G	N	Y	D	A	A	K	R	G	P	G	G	V	W	A	A	E	A	I	S	D	A	R	E
Mouse	R	G	N	Y	D	A	A	Q	R	G	P	G	G	V	W	A	A	E	K	I	S	D	A	R	E
Cat (Aby)	R	G	N	Y	D	A	A	Q	R	G	P	G	G	A	W	A	A	K	V	I	S	D	A	R	E
Cat (Siam)	R	G	N	Y	D	A	A	R	R	G	P	G	G	V	W	A	A	K	V	I	S	D	A	R	E
Cat (JDC)	R	G	N	Y	D	A	A	R	R	G	P	G	G	A	W	A	A	K	V	I	S	D	A	R	E
Chicken	R	G	N	Y	D	A	A	R	R	G	P	G	G	A	W	A	A	K	V	I	S	D	A	R	E
Cow	R	G	N	Y	D	A	A	Q	R	G	P	G	G	A	W	A	A	K	V	I	S	D	A	R	E
Dog	R	G	N	Y	D	A	A	Q	R	G	P	G	G	A	W	A	A	K	V	I	S	D	A	R	E
Mink	R	G	N	Y	D	A	A	Q	R	G	P	G	G	A	W	A	A	K	V	I	S	D	A	R	E
Pig	R	G	N	Y	D	A	A	Q	R	G	P	G	G	A	W	A	A	K	V	I	S	D	A	R	E
Rabbit	R	G	N	Y	D	A	A	Q	R	G	P	G	G	V	W	A	A	K	V	I	S	D	A	R	E

The sequences were aligned based on human SAA on Uniprot.

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