Protein profiling of isolated uterine AA amyloidosis causing fetal death in goats

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ABSTRACT **Pathologic amyloid accumulates in the CNS or in peripheral organs, yet the mechanism underlying the targeting of systemic amyloid deposits is unclear. Serum amyloid A (SAA) 1 and 2 are produced predominantly by the liver and form amyloid most commonly in the spleen, liver, and kidney. In contrast, SAA3 is produced primarily extrahepatically and has no causal link to amyloid formation. Here, we identified 8 amyloidosis cases with amyloid composed of SAA3 expanding the uterine wall of goats with near-term fetuses. Uterine amyloid accumulated in the endometrium, only at the site of placental attachment, compromising maternal-fetal gas and nutrient exchange and leading to fetal ischemia and death. No other organ contained amyloid. *SAA3* mRNA levels in the uterine endometrium were as high as *SAA2* in the liver, yet mass spectrometry of the insoluble uterine peptides identified SAA3 as the predominant protein, and not SAA1 or SAA2. These findings suggest that high local SAA3 production led to deposition at this unusual site. Although amyloid A (AA) amyloid deposits typically consist of an N-terminal fragment of SAA1 or SAA2, here, abundant C-terminal peptides indicated that the uterine amyloid was largely composed of full-length SAA3. The exclusive deposition of SAA3 amyloid in the uterus, together with elevated uterine *SAA3* transcripts, suggests that the uterine amyloid deposits were due to locally produced SAA3. This is the first report of SAA3 as a cause of amyloidosis and of AA amyloid deposited exclusively in the uterus.—Gaffney, P. M., Barr, B., Rowe, J. D., Bett, C., Drygiannakis, I., Giannitti, F., Trejo, M., Ghassemian, M., Martin, P., Masliah, E., Sigurdson, C. J. Protein profiling of isolated uterine AA amyloidosis causing fetal death in goats. *FASEB J.* 29, 911–919 (2015). www.fasebj.org**

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MOST SYSTEMIC AMYLOIDOSES OCCUR when a protein self-assembles into fibrils and accumulates extracellularly, leading to progressive organ dysfunction and failure (1, 2). The aberrant protein may be produced and deposited locally, as seen with pancreatic islet amyloid polypeptide (IAPP) in patients with type II diabetes (3, 4), or may be deposited distantly, such as transthyretin, which is produced by the liver and deposited in the heart in patients with senile systemic amyloidosis (5, 6). Lysozyme, fibrinogen-α (7), and serum amyloid A (SAA) 1 and 2 (8) can also form amyloid deposits at sites distant from production of the parent protein. High protein concentrations promote fibrillization (9) and may explain the localized deposition, yet in many cases, the mechanisms underlying the organ deposition at sites distant to the site of synthesis remain unclear.

Reactive systemic amyloidosis is caused by the overproduction of SAA due to persistent or recurrent inflammation (10, 11). In mammals, there are 3–4 SAA genes that are part of a conserved multigene family (SAA1–SAA4). Recent structural studies indicate that human SAA1.1 is arranged as a 4-α helix bundle that exists as a hexamer (12). SAA1 and SAA2 are acute-phase proteins produced by the liver (13) that circulate as HDL-bound apolipoproteins (14) and are highly up-regulated by proinflammatory cytokines IL-1, IL-6, and TNF-α (15). Elevated serum levels of SAA1 or SAA2 due to chronic or recurring episodes of acute inflammation can provoke SAA polymerization into fibrils (16). Interestingly, transgenic mice expressing high concentrations of mouse SAA1.1 develop extensive amyloid deposits in the absence of inflammation (17). Thus, the overproduction of SAA is necessary for amyloid formation.

SAA3 and SAA4 are not significantly up-regulated in the liver as part of the acute-phase response, except in the pig (18). SAA3 is produced primarily extrahepatically but also

Abbreviations: AA, amyloid A; ACN, acetonitrile; BAC, bacterial artificial chromosome; FA, fluorescent antibody test; HE, hematoxylin and eosin; IAPP, pancreatic islet amyloid polypeptide; IDA, independent data acquisition; IHC, immunohistochemistry; KLH, keyhole limpet hemocyanin; MS/MS, tandem mass spectrometry; SAA, serum amyloid A

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at low levels by the liver (19). SAA3 production has been demonstrated in mouse macrophages (20), bovine endometrial epithelium (21), and in a wide-range of extrahepatic tissues across species (22). However, SAA3 has not been reported as a cause of amyloidosis.

Amyloid A (AA) fibrils in tissue are largely composed of the first 76 N-terminal residues of the precursor SAA1 or SAA2 protein (23, 24), although, shorter and longer fragments as well as full-length SAA have been recovered (25, 26). AA amyloid from SAA1 or SAA2 most often deposits in the kidney, spleen, liver, and vessels, although heart and other organs may also be affected (16). In kidney, AA amyloid in glomeruli is often diagnosed in late stages of disease when glomerular filtration is severely perturbed and renal failure is imminent (27).

Here, we report a highly unusual distribution of AA amyloid from SAA3 in 8 goats representing 2 breeds. Amyloid was restricted to the uterus and associated with late-term fetal death, likely due to the abundant amyloid that diffusely expanded the endometrium at the placental attachment sites. Although in animals there are no reports of isolated uterine amyloidosis, uterine amyloid was described in a cow with systemic amyloidosis (28). In humans, uterine AA amyloid is rare but has also been reported as a component of systemic amyloidosis.

In some mammals, including cattle and horses, SAA3 is secreted by the uterus and other nonhepatic organs (18, 19, 29, 30). To better understand the composition and restricted deposition of uterine amyloid in the goats, we performed HPLC-electrospray ionization-tandem mass spectrometry (MS/MS) analysis on amyloid-positive and control uterine samples to define the sequence of the amyloidogenic protein as well as the most abundant insoluble proteins in the endometrium. We also investigated the levels of *SAA2* and *SAA3* mRNA in the uterus as compared to the liver. This is the first report of AA amyloidosis from SAA3 and of isolated uterine AA amyloid.

MATERIALS AND METHODS

Study population

Here, we studied 8 goats from 2 breeds, Toggenburg ($n = 6$) and Saanen ($n = 2$), ranging from 3 to 7 yr old. Goats were from 3 dairy farms located in adjacent Yolo and Sacramento counties of California, USA. A total of 17 fetuses and 1 live neonate originating from these goats were also examined. There were 4 control goats included that were euthanized for various inflammatory diseases.

Necropsy and diagnostic testing for infectious agents, heavy metals, and selenium

Complete postmortem examinations were performed on all goats. Samples of all major organs were formalin-fixed and paraffin-embedded. Histologic examination was performed on heart, lungs, liver, spleen, esophagus, kidney, ovary, skeletal muscle, mammary gland, rumen, abomasum, pancreas, small and large intestine, brain, lymph nodes, tongue, and adrenal glands. Tissue and/or body fluid samples (serum or fetal fluids) were tested for antibodies against potentially abortigenic infectious pathogens, including bluetongue virus (*Orbivirus* sp.) [capture ELISA (cELISA)], *Brucella* sp. (rose Bengal test), *Coxiella burnetii* (ELISA), 5 serovars of *Leptospira interrogans* (microagglutination test), *Toxoplasma* spp. (latex agglutination), caprine arthritis encephalitis virus (*Retrovirus*) (cELISA), and *Mycobacterium avium* subspecies *paratuberculosis* (Johne's disease) (ELISA). Microbiologic testing was performed for the isolation of aerobic bacteria, *Campylobacter* spp., *Brucella* spp., and the detection of *Salmonella* spp. by PCR, *Chlamydia* spp. by direct fluorescent antibody test (FA), and *Leptospira* by FA. Additionally, the detection and quantification of heavy metals (lead, manganese, iron, mercury, arsenic, molybdenum, zinc, copper, and cadmium) and selenium from liver samples were performed by spectrometry.

In some goats, there were serum antibodies detected against the common organisms bluetongue virus ($n = 2$ goats), *C. burnetii* ($n = 2$ goats), and *Toxoplasma* spp ($n = 1$ goat), although the organisms or associated lesions were not detected within uterine or fetal tissues. *Escherichia coli* was isolated as a rare organism from the uterus of 2 goats, and as part of large numbers of mixed flora in 2 goats, likely due to an open cervix. No bacteria were isolated from fetal tissues or fluids. Assays for *Brucella* spp., *Campylobacter* spp., *Chlamydia* spp., and *Leptospira* spp. were negative, and heavy metals and selenium levels in the liver were normal.

Histology and immunohistochemistry

Five-micrometer-thick formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin (HE), Congo red, or immunolabeled with antibodies against SAA. For SAA immunohistochemistry (IHC), endogenous peroxidase was quenched with 3% hydrogen peroxide, heated in a citrate buffer (pH 6.0) for 20 min in a pressure cooker, blocked, and incubated in rabbit anti-SAA using an affinity-purified antibody against an SAA peptide sequence (DKYFHARGNYDAAQRG) conjugated to keyhole limpet hemocyanin (KLH) (Pierce Custom Antibodies; Thermo Fisher Scientific Incorporated, Waltham, MA, USA). Sections were incubated in biotinylated anti-rabbit IgG antibody, streptavidin-horseradish peroxidase, and 3,3′-diaminobenzidine substrate. Negative controls included preincubating the antibody with the peptide used for immunization, replacing the primary antibody with an IgG rabbit isotype control, and the use of goat uterine samples with no amyloid as well as a cat pancreas with IAPP.

Electron microscopy

A section of uterus at the endometrial caruncle was fixed in Karnofsky's solution. Tissues were postfixed in osmium tetroxide, embedded in Epon Araldite, sectioned at 60 nm on the ultramicrotome, then collected on grids and poststained using saturated uranyl acetate solution and bismuth subnitrate. Grids were analyzed with a Zeiss EM10 electron microscope (Jena, Germany).

Protein isolation and characterization

Uterine caruncles from 4 histologically amyloid-positive and 4 amyloid-negative goats were homogenized in PBS. Proteins were solubilized in sarcosyl (1% final) for 30 min at 37°C and centrifuged for 30 min at 18,000 × *g*. To solubilize proteins in the insoluble fraction, the pellet was resuspended in 10 M urea in 100 mM Tris-HCl (pH 8.0) for 30 min at 37°C and then diluted to 4 M urea (final). Pellet samples were electrophoresed in a 10% Bis-Tris polyacrylamide gel (NuPAGE; Invitrogen, Life Technologies, Grand Island, NY, USA), and the gel was stained with Coomassie Brilliant Blue.

Mass spectrometry sample preparation

Samples from the pellet fraction in 4 M urea were reduced with 100 mM DTT, alkylated with 500 mM iodoacetamide, digested with trypsin for 48 h at 37°C, and centrifuged at 18,000 × *g* for

30 min. The soluble fraction was added to a new tube, and peptides were extracted and desalted using Aspire RP30 desalting columns (Thermo Fisher Scientific Incorporated).

Liquid chromatography-MS/MS

Peptides were analyzed by HPLC coupled with MS/MS using nano-spray ionization. The nano-spray ionization experiments were performed using a TripleTof 5600 hybrid mass spectrometer (AB SCIEX, Framingham, MA, USA) interfaced with nano-scale reversed-phase HPLC (Tempo; Sigma-Aldrich, St. Louis, MO, USA) using a 10 cm–100 μm ID glass capillary packed with 5 μm C18 Zorbax beads (Agilent Technologies, Santa Clara, CA, USA). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–60%) of acetonitrile (ACN) at a flow rate of 250 μl/min for 1 h. The buffers used to create the ACN gradient were Buffer A (98% H_2O, 2% ACN, 0.2% formic acid, and 0.005% TFA) and Buffer B (100% ACN, 0.2% formic acid, and 0.005% TFA). MS/MS data were acquired in a data-dependent manner in which the MS1 data were acquired for 250 ms at m/z of 400–1250 Da, and the MS/MS data were acquired from m/z of 50–2000 Da. For independent data acquisition (IDA) parameters, MS1-time-of-flight was 250 ms, followed by 50 MS2 events of 25 ms each. The IDA criteria were over 200 counts threshold, charge state +2–4 with 4 s exclusion.

The collected data were analyzed using the Paragon Algorithm (31) and Protein Pilot 4.0 (AB SCIEX) for peptide identifications and the proteome of sheep (*Ovis aries*). Protein sequences of SAA and serum amyloid P from the peer-reviewed literature were added from a variety of species with systemic amyloidosis (human, mouse, cat, horse, cow, elk, goat, mink, arctic fox, cheetah, ferret, mallard, flamingo, and Syrian hamster). Predicted SAA protein sequences from SAA gene sequence data from goats were also added. Spectral counts were obtained by normalizing against the whole-protein complex, calculated as previously described (32), and reported as percent total insoluble protein. Only proteins identified with ≥95% confidence were further evaluated. Tryptic peptide alignments were done using Clustal Omega (EMBL-EBI, Cambridgeshire, United Kingdom), and isoforms were identified based on identification of ≥1 different amino acids at a single location in the protein.

Quantitative RT-PCR on liver and uterus for *SAA2* and *SAA3*

Liver and uterus samples were lysed, and total RNA was collected (QIAGEN, Valencia, CA, USA). cDNA was generated from total RNA using a cDNA synthesis kit (Promega, Madison, WI, USA) and used as template for PCR amplification. *SAA2* and *SAA3* transcripts were quantified using real-time PCR with primers for *SAA2* (*SAA2*F, 5′-ACAAATACTTCCATGCCCGC-3′; *SAA2*R, 5′-CTGAATATTTTCTCTGGCATCACTG-3′) and *SAA3* (*SAA3*F, 5′-TGCTGAAGTGATCAGAGAGGC-3′; *SAA3*R, 5′-AAACTGGT CAGCCTTCGTGT-3′) and Power SYBR Green (Applied Biosystems, Grand Island, NY, USA) in a StepOnePlus Real-Time PCR System (Life Technologies), normalized against β-*actin* [primers in (33)] endogenous control. Reactions were run to determine the C_T cycle of each sample as follows: holding conditions at 95°C for 10 min, cycling conditions at 95°C for 15 s, and 60°C for 1 min. The fold change in expression of SAA was determined using the $\Delta\Delta C_T$ method (34). Data were not suitable for parametric tests according to Levene's test, and hence, statistical significance between groups was tested with the Mann–Whitney *U* nonparametric test.

SAA gene sequences

cDNA from liver and uterus samples was amplified using primers designed against sheep and cattle SAA genes as follows: *SAA1* (372 forward, 5′-CTCTCCACAGGCATCATTTTCTGC-3′; 366 reverse, 5′-CTGTGATTCCCTGAAGAGC-3′); *SAA2* (372 forward; 371 reverse, 5′-GTCAGCAGGTCCGAAGTGGTTG-3′; 372 forward and 645 reverse, 5′-GTACTTGTCCAGCAGGTCAGC-3′; 339 forward, 5′-TCACAGGCCTCATTCTCTGC-3′; 341 reverse, 5′-TTGG-GGACTCACTTTGCCAT-3′); SAA3 (372 forward and 367 reverse, 5′-GTACTTGTCAGGCAGGTCAG-3′); and SAA3.2 (368 forward, 5′-CAGCCAGGGATGGGGGACATTC-3′; 367 reverse).

RESULTS

Goats developed extensive endometrial amyloid

Over a 3 yr period, 8 goats (3–7 yr old) showed clinical signs of prolonged gestation, clear vaginal discharge, and agalactia. Goats were diagnosed with fetal death, retention, and mummification ($n = 6$) or had already aborted ($n = 2$). In addition to fetal death, 1 goat also delivered a live kid. There were 4 goats that had experienced at least one prior abortion (**Table 1**). Goats represented 2 distinct breeds, Saanen and Toggenburg, and were closely related within each breed.

An extensive postmortem examination of adult goats and fetuses with histopathologic examination of major organs revealed the uterus as the only amyloid-containing organ. In the normal goat uterus, the endometrium multifocally forms thick protruding discs, or caruncles, which attach to the placenta as sites for fetal nutrient delivery and gas exchange. All 8 goats had pale, chalky-appearing endometrial caruncles with occasional white linear streaks in the stroma. Histologically, the interstitium of the caruncle was expanded by acellular, homogeneous, and eosinophilic deposits that were congophilic and birefringent under polarized light, consistent with amyloid (**Fig. 1**). In some of the caruncles, 80–90% of the stroma consisted of amyloid, whereas in others, amyloid deposits were patchy. Coagulation necrosis and mineralization were present in the caruncles of 2 goats, likely due to a retained placenta. Interestingly, no amyloid was present in the endometrium between caruncles or in the myometrium, endometrial glands, vessel walls, or any subendometrial tissue.

In goats having fetal mummies, there was often a mild-to-moderate endometritis and placentitis with variable superficial neutrophilic infiltrates, surface exudates, and lymphocytes and plasma cells infiltrating the intercaruncular mucosa and the deep submucosa, possibly from secondary ascending bacterial infections from an open cervix. Dead fetuses showed multifocal areas of white matter necrosis and mineralization in the cerebral cortex, consistent with placental ischemia. Serologic and microbiologic tests did not reveal a consistent infectious cause that would explain the fetal loss (please see Materials and Methods for details). There were 6 goats that had mild-to-moderate concurrent inflammatory lesions in other organs, such as mastitis or arthritis.

Amyloid is labeled with anti-SAA antibodies

Because the most common systemic amyloid identified in animals is derived from the SAA protein, we performed SAA immunohistochemical labeling on the uterus of all goats. Many of the antibodies available do not cross-react with goat; thus, we developed an affinity-purified,

TABLE 1. *Goats with uterine amyloidosis*

Animal	Breed	County	Age (yr)	Parity	Prior episodes of abortions	Number of fetuses (condition)[a]
1	Toggenburg	Yolo	5	5	0	2 (aborted)
2	Toggenburg	Sacramento	6	5	1	3 (aborted)
3	Toggenburg	Yolo	4	4	2	3 (2 mummified, 1 live birth)
4	Toggenburg	Sacramento	5	4	0	3 (2 dead, 1 viable)
5	Toggenburg	Yolo	3	3	0	3 (dead)
6	Toggenburg	Sacramento	7	5	2	3 (dead)
7	Saanen	Sacramento	3	2	0	3 (dead)
8	Saanen	Yolo	4	3	1	2 (dead)

[a]The number of aborted fetuses refers to the most recent abortion.

polyclonal antibody in rabbits against a highly conserved SAA peptide conjugated to KLH that would be expected to recognize SAA from many species (epitope DKYFHARGNYDA). IHC revealed multifocal-to-diffuse labeling of the uterine caruncular amyloid, which was strong and granular on the edges of the amyloid deposits. The immunolabeling was abolished by preincubating the target peptide with the antibody, confirming the specificity of the SAA antibody. Isotype control antibodies did not label the amyloid (Fig. 1). Additionally, there was no SAA antibody labeling in the uterus of goats without amyloidosis (Fig. 1*B*) or in a cat pancreas with IAPP (data not shown).

Amyloid ultrastructure

To determine whether the amyloid was composed of fibrils, we performed electron microscopy of thin sections from uterine caruncle. Ultrastructurally, the endometrial amyloid was composed of loosely arranged bundles of unbranching fibrils, 10–15 nm in diameter, which were haphazardly arranged (**Fig. 2**). Extensive sheets of fibrils were observed, consistent with the abundant amyloid seen by light microscopy.

Proteomic analysis of the amyloid by mass spectrometry

To further confirm the identity of the insoluble amyloidogenic protein, we pursued an unbiased approach using mass spectrometry on endometrial samples from 4 goats with uterine amyloid and from 4 goats without uterine amyloid (defined histologically). We centrifuged the endometrial lysates, denatured the insoluble protein fractions in urea, electrophoresed the insoluble fractions by SDS-PAGE, and visualized the proteins with Coomassie Brilliant Blue. A predominant protein band of 12–13 kDa was evident in all 4 amyloid-containing uteri, but not in 4 amyloid-negative samples (**Fig. 3*A***).

The remaining insoluble protein fraction was digested with trypsin and analyzed by HPLC-MS/MS for peptide identification and semiquantification. For 1 sample, proteins

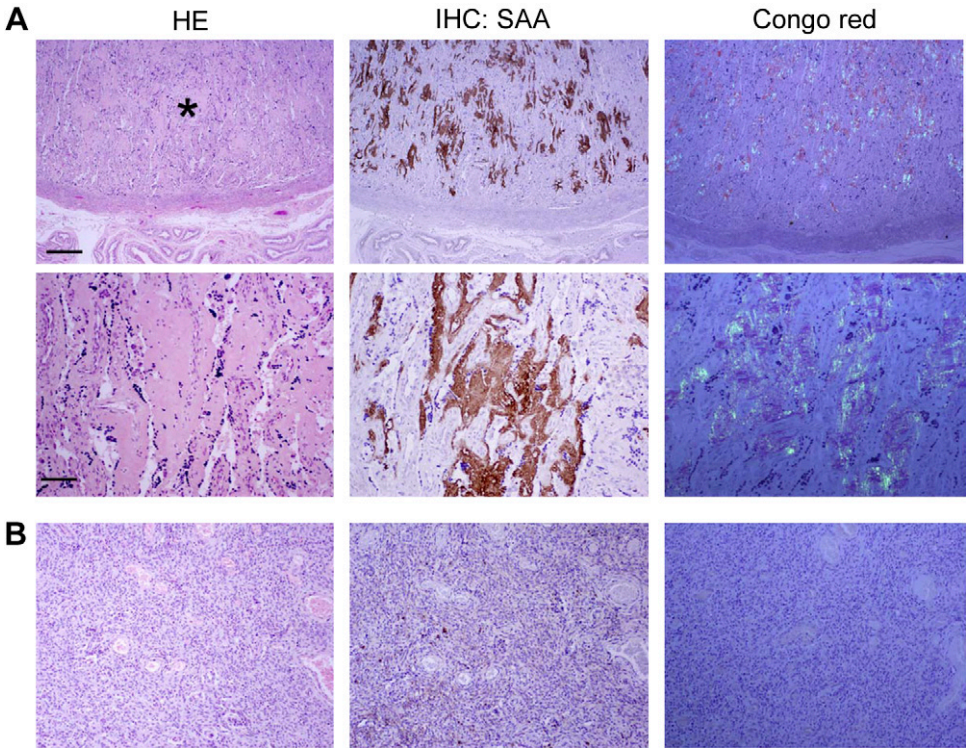

Figure 1. Immunolabeling of endometrial amyloid by an anti-SAA antibody. *A*) High and low magnification of HE-stained goat uterus shows abundant homogeneous, eosinophilic protein throughout the caruncle [denoted by an asterisk (*)]. Amyloid was bound by an anti-SAA antibody and appears green when stained by Congo red and viewed under polarized light. *B*) Goat endometrium with no visible amyloid lacks labeling by the anti-SAA antibody and by Congo red. Scale bars, 1.0 mm (*A*, upper left) and 200 μm (*A*, lower left).

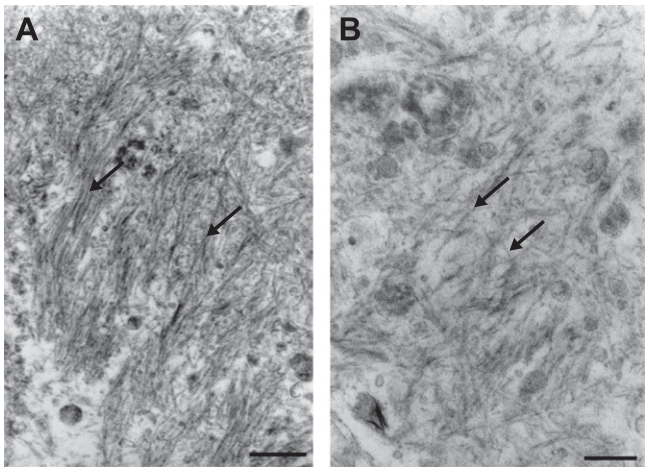

Figure 2. Ultrastructure of a caruncle containing amyloid. Unbranching fibrils, approximately 10–15 nm in diameter (arrows), are arranged in compact bundles of parallel fibrillar structures (*A*) or are haphazardly arranged in loose networks (*B*). Scale bars, 500 nm (*A*) and 250 nm (*B*).

from the single 12–13 kDa gel band were extracted, digested with trypsin, and analyzed by mass spectrometry.

We identified peptides with a query against a sheep database enriched with SAA sequences from other species, including goat SAA, and analyzed peptides identified at ≥95% confidence. For all goats, the most abundant proteins from the insoluble pellet were from SAA3, primarily matching the goat SAA3-predicted gene sequence with less matching elk *SAA* (ACH73015) (Fig. 3*B*). The specific elk SAA gene was not noted but is similar to SAA3.2 of cattle. To compare the abundance of SAA relative to the other proteins identified, we ranked the proteins based on the spectral counts, accounting for differences in protein length. SAA consistently ranked as the most abundant protein in all amyloid-containing samples, ranging from 34 to 45% of the total insoluble proteins. In the gel-extracted sample, SAA was also highly enriched and accounted for 47% of all proteins, whereas the second- and third-most abundant proteins, histone H4-like and hemoglobin subunit β-like, were far less abundant at 17 and 14%, respectively. Except for low levels of fibrinogen, no other known amyloidogenic proteins were identified in any sample. Of 4 goats with no visible uterine amyloid, 3 had no SAA peptides detected, whereas in 1 goat, approximately 10% of peptides identified were SAA3. The goat SAA3 lacked 3 amino acids seen in most other species, which was also evident in the protein sequence deduced from cDNA (**Fig. 4**). The goat SAA3- and alpaca SAA2-predicted sequences lack 3 amino acids at the same positions (caprine SAA3, NP_001272566; alpaca SAA2, XP_006209375).

The amyloid-associated proteins in the insoluble fraction were remarkably consistent, with 8 of the top 20 proteins common to all 4 goats. Of the 8 proteins, 4 (clusterin, cofilin, transgelin, and aldose reductase) were detected exclusively in the amyloid-positive uteri (**Table 2**). The protein chaperone clusterin (apolipoprotein J) accounted for approximately 1–2% of the total protein, after

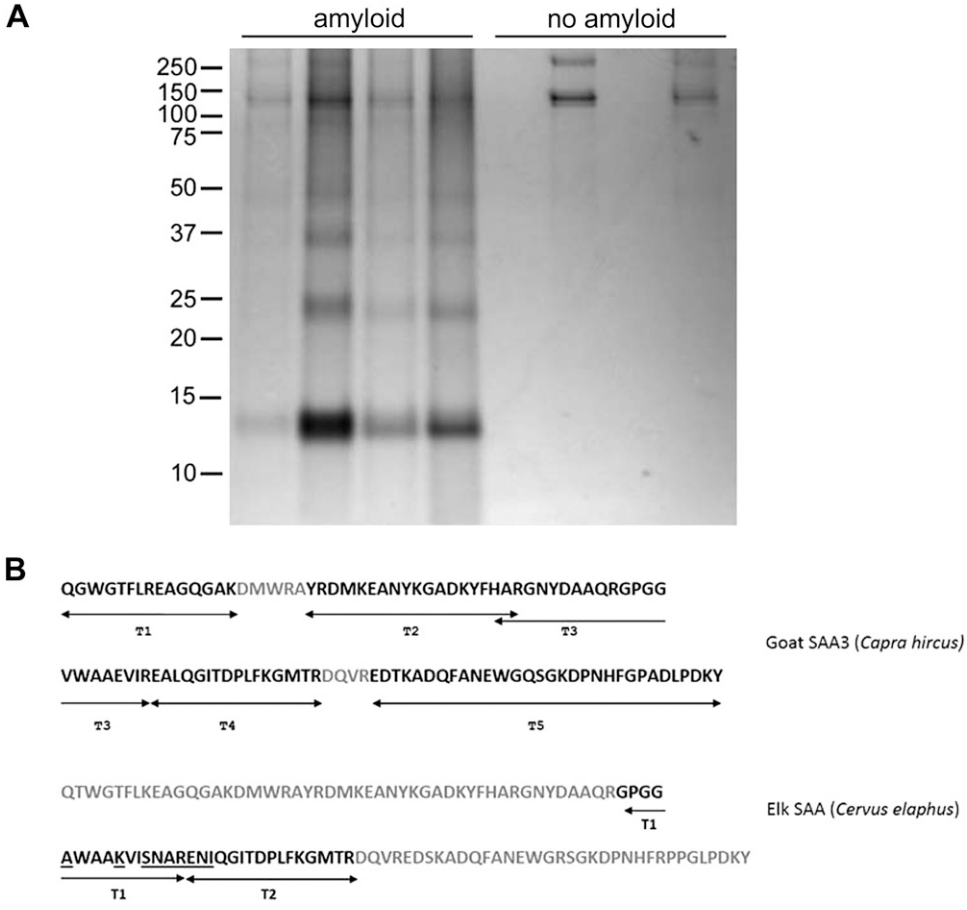

Figure 3. Insoluble proteins from the endometrium of amyloid-containing uteri. *A*) Coomassie Brilliant Blue–stained gel of insoluble proteins shows predominant bands at 12–13 kDa as well as higher molecular weight bands, possibly SAA multimers, in goats with histologically visible amyloid in uteri, but not in unaffected goats. *B*) Alignment of the SAA peptides identified by mass spectrometry analysis in highest abundance (goat SAA3), together with one SAA sequence present in lower abundance (elk SAA; ACH73015). Gray text indicates segments that were not identified in the mass spectrometry analysis from the insoluble protein. Underlined residues signify amino acids that differ from goat SAA3. T1–T5 indicate tryptic peptides identified.

Figure 4. Predicted protein sequences of goat SAA from cDNA that was PCR amplified. *A*) Caprine SAA1 and SAA2 in this study (blue) compared with published ruminant sequences (black). *B*) Caprine SAA3 and SAA3.2. Red text indicates polymorphisms in caprine SAA sequences. The asterisks (*) indicate amino acid positions that contain differences in ovine, caprine, and bovine sequences. Ovine SAA, XP_004019522.1; Bovine SAA, NP_001068728.1; Caprine SAA3.2, ABQ51184.1; Ovine SAA3.2, ABY75296.1; Bovine SAA3, NP_851359.2; Caprine SAA3, ABQ51197.1; var, variant.

normalizing for protein length (Table 2). Clusterin has been found to be elevated in the cerebrospinal fluid in patients with Alzheimer's disease (35) and in brain regions showing Alzheimer's disease pathology (36, 37).

Because serum albumin is abundant in blood (approximately 3 g/dl), we compared the levels of SAA3 with serum albumin in each sample. SAA abundance was consistently higher than albumin in the total insoluble protein preparation and ranged from 15- to 447-fold albumin levels (mean ± SE, 168 ± 99-fold). In the gel-extracted proteins, SAA3 was 472-fold higher than albumin.

AA amyloid deposits often consist of a large N-terminal AA fragment (23, 24). Interestingly, the SAA peptides from the goat uteri extended from the N to the C terminus of the protein in every sample, indicating the presence of full-length SAA in the amyloid. This finding is consistent with the presence of a single band on the Coomassie Brilliant Blue-stained gel in most samples. There were 2 short segments not detected by mass spectrometry, probably due to very short peptide fragments generated during digestion with trypsin (Fig. 3*B*).

SAA gene family of the goat and mRNA levels of *SAA2* and *SAA3* in uterus and liver

The SAA gene family of the goat has not been described. Using primers designed against SAA genes from other ruminants, we amplified the cDNA sequences of goat SAA1, SAA2 (isoforms 1 and 2), SAA3, and SAA3.2 from liver and uterus. The translated gene sequences were identified by comparison with known gene sequences from cattle, goats, and sheep (Fig. 4). The identification of each was also confirmed by one of us (P.M.) that has sequenced goat SAA genes and their orientation by screening a bacterial artificial chromosome (BAC) library and isolating and sequencing 3 overlapping BAC clones (unpublished data).

The distribution of the AA deposits was highly unusual in being restricted to the uterus. We reasoned that SAA3 may be secreted by hepatocytes and assembled into fibrils in the uterus due to a highly favorable environment containing either essential cofactors or an accessible scaffold. Alternatively, SAA may have been secreted in the uterus, leading to high SAA concentrations and local fibril assembly. To help distinguish between these 2 possibilities, we measured mRNA levels of *SAA2* and *SAA3* and housekeeping gene *β-actin* in the uterus and liver of amyloid-affected and -unaffected goats by quantitative RT-PCR. *SAA2* was only expressed in the liver, whereas *SAA3* was expressed at high levels in the uterine caruncle and at low levels in the liver (**Fig. 5**). Together with the lack of SAA2 in uterus by mass spectrometry, these data suggest that the SAA3 was locally produced and deposited in the uterus. Interestingly, the SAA levels in the amyloid-containing

TABLE 2. *The 15 most abundant proteins from the insoluble protein fractions of representative goats with and without amyloidosis*

Rank	Protein[a]	Normalized spectral count (%)[b]
Amyloid positive		
1	SAA3 (*Capra hircus*)[c]	45.5
2	SAA (*Cervus elaphus*)	12.7
3	Actin, cytoplasmic 1	12.5
4	Tubulin α-1B chain, α-1D chain-like, β-5 chain isoform2[c]	7.6
5	Aldose reductase[c]	4.2
6	Cofilin-1	2.7
7	Clusterin isoform 1[c]	2.3
8	Myosin light polypeptide 6 isoform 3, myosin-9-like, myosin-10 isoform 2[c]	1.7
9	Hemoglobin subunit β-like[c]	1.2
10	Transgelin-2 isoform 3[c]	1.1
11	Fibrinogen γ-B chain-like, α chain	1.1
12	Metalloproteinase inhibitor 3 precursor	0.9
13	Heat shock protein β-1[c]	0.9
14	Heterogeneous nuclear ribonucleoprotein A2/B1, k-like isoform 2, F isoform 3	0.7
15	Tropomyosin α-1 chain isoform 2[c]	0.7
Amyloid negative		
1	Tubulin β-5 chain isoform 1, β chain-like, α-1B chain, α-1D chain-like[c]	38.0
2	Collagen α-1(I)[c]	25.1
3	Serum albumin	8.4
4	Actin, cytoplasmic 1	7.2
5	Histone H4-like, H2B type 1-J-like[c]	5.8
6	Hemoglobin fetal subunit β-like, subunit α-1/2-like[c]	16.6
7	Ubiquitin-60S ribosomal protein L40	2.4
8	Dermatopontin[c]	1.6
9	Heat shock protein β-1[c]	1.3
10	Tropomyosin α-1 chain isoform 2[c]	1.1
11	Vimentin[c]	0.8
12	Nucleophosmin[c]	0.7
13	Uncharacterized protein LOC101116248[c]	0.7
14	Elongation factor 1-α 1-like[c]	0.3
15	None	0

[a]All non-SAA proteins are from the *O. aries* database. [b]Spectral counts are normalized to account for differences in protein length. [c]Predicted proteins.

uteri were significantly higher than control uteri with no amyloid ($P < 0.05$, Mann–Whitney *U* test).

DISCUSSION

Here, we present the first report of AA amyloidosis composed of the SAA3 protein. Although AA amyloid composed of SAA1 and SAA2 most commonly accumulates in the spleen, kidney, and liver, other organs can also harbor amyloid, including heart, skeletal muscle, intestine, and mammary gland (38). Nevertheless, reports of AA amyloid in the uterus of humans or animals are exceedingly rare and have not been reported with fetal loss. Among goats, uterine amyloid was composed of SAA3 and lower levels of an AA peptide similar to SAA3.2 of cattle. Extrahepatic SAA3 production has been described in mammary epithelial cells (39), adipocytes (19), and endometrial epithelial cells, the latter of which increase mRNA levels by 3400-fold when stimulated by *E. coli* (21).

SAA1 and SAA2 are up-regulated by IL-1, IL-6, and TNF-α cytokine signaling during chronic inflammatory, neoplastic, or infectious diseases (15). Most goats had focal areas of mild-to-moderate inflammation, including arthritis and mastitis. A central question therefore is, what caused such extensive AA amyloid deposition localized to this aberrant site? Although hepatic-derived SAA1 and SAA2 could form fibrils in the uterine caruncle, this is unlikely because other organs would also be expected to contain amyloid, as seen in patients having systemic AA amyloidosis with uterine involvement. The uterine amyloid contained abundant SAA3, an SAA produced extrahepatically (39), and the *SAA3* mRNA levels were as high in the endometrium as *SAA2* transcript levels in the liver. Collectively, our data showing the highly specific caruncular deposition together with high uterine *SAA3* transcripts suggest that locally produced SAA3 assembled into amyloid.

Most of the goats showed evidence of a mild-to-moderate endometritis; thus, we considered the possibility that

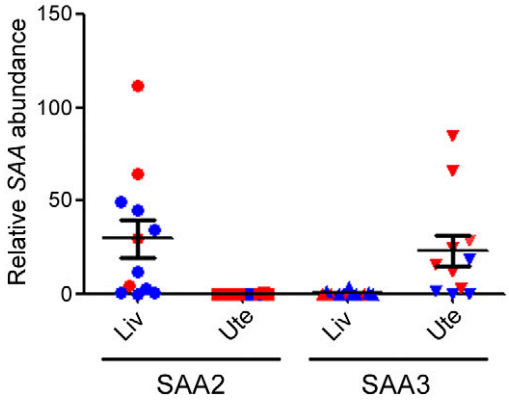

Figure 5. *SAA3* mRNA was more abundant in the uterus (Ute) than in the liver (Liv). *SAA2* and *SAA3* gene expression was measured from uterine and hepatic tissue by reverse-transcription quantitative PCR. *SAA* mRNA abundance was quantified relative to the β-actin mRNA content for each sample using the $\Delta\Delta C_T$ method. Data are presented in a scatterplot with red symbols representing amyloid-positive goats and blue symbols representing amyloid-negative goats.

endometritis could have led to a localized amyloidosis. This scenario seems implausible for several reasons. First, the endometritis appeared to be acute due to the retained placenta, which is inconsistent with amyloid formation presumably weeks earlier. Second, not all goats with uterine amyloidosis had endometritis. Third, goats with endometritis or metritis do not typically develop uterine amyloidosis. A 14 yr retrospective search of goats necropsied within the California Animal Health and Food Safety Laboratory System, a laboratory network for identifying diseases in food animals throughout California, revealed 126 cases of metritis and endometritis, only 1 of which had amyloidosis (F.G., personal communication). In this single case of amyloidosis, the amyloid deposits were systemic and accumulated in the spleen, kidney, and liver, but not in the uterus.

Genetic causes of amyloidosis were considered, although the goats were 2 different breeds. Genetic causes cannot be excluded; however, exogenous or environmental factors should also be considered. Hypoxia and glucocorticoids have been shown to increase SAA3 levels or *SAA3* transcripts in immortalized mouse adipocytes (40) or immortalized macrophages (41), respectively. Prolactin or LPS can induce SAA3 transcription in bovine mammary epithelial cells (42).

Although AA composed of SAA1 or SAA2 often consists of the N-terminal fragment of 76 amino acids (23), here, the AA amyloid presented after electrophoresis as a single band, and C-terminal fragments of SAA3 were identified by mass spectrometry in all goats, suggesting a lack of enzymatic cleavage. Although full-length SAA1 and SAA2 are not commonly reported in AA amyloid, they have been detected as minor components of amyloid fibrils in the spleens of patients with amyloidosis (43). Cleavage of SAA1 and SAA2 is thought to occur prefibril formation (44, 45); however, duck amyloid is composed of full-length SAA (26), suggesting that cleavage may occur postfibril formation. In human Ig light-chain amyloidosis, data indicate that proteolytic cleavage occurs postfibril formation (46). Here, we show that cleavage of SAA3 is not necessary for fibril assembly.

The isolated AA amyloid composed of SAA3 in the gravid uterus is highly unusual and suggests an underlying mechanism for stimulation of SAA3 production within the caruncle or an additional trigger that initiates fibril formation. In human patients, elevated SAA1 occurs frequently but only leads to amyloidosis in a minority of patients; thus, persistently elevated SAA with an additional genetic or exogenous risk factor may be required. In the goats presented here, the underlying mechanism of AA amyloidosis is unclear, and further studies on the cause of this highly localized amyloidosis may reveal insights into hormonal or other noninflammatory inducers of SAA3. FJ

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Protein profiling of isolated uterine AA amyloidosis causing fetal death in goats

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