Cloning and Quantification of Ferret Serum Amyloid A

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ABSTRACT: Serum amyloid A (SAA) is used as a biomarker for infections and inflammation in humans and veterinary medicine. We cloned ferret cDNA encoding SAA from the liver of a ferret via reverse transcription PCR (RT-PCR). The sequence of the cDNA clone revealed that ferret SAA has an open reading frame of 387 bp that encodes 129 amino acids. The deduced amino acid sequence of ferret SAA has 96.1, 89.9, 86.0, 83.8, 83.0, 73.8 and 65.3% similarity to the mink, dog, cat, cattle, horse, human and mouse SAA genes, respectively. Compared to human SAA, the deduced ferret SAA amino acid sequence had an insertion of an 8-amino acid fragment between amino acids 86 and 95. Recombinant ferret SAA (rfrSAA) was expressed using an Escherichia coli strain, BL21 Star. Using Western blot analysis, anti-SAA mAb provided with the multispecies SAA ELISA kit reacted with purified rfrSAA. A significant dose-response relationship was observed between the rfrSAA protein and a commercial multispecies SAA ELISA kit. In contrast, rfrSAA was not recognized with the antibodies included in a commercial human SAA ELISA kit. These results suggest that the structure of ferret SAA is antigentically similar to other domestic animal SAAs, and the multispecies ELISA kit allows for the detection and quantification of ferret SAA in vivo.

KEY WORDS: ELISA, ferret, serum amyloid A (SAA).


Acute phase proteins (APPs), such as serum amyloid A (SAA), C-reactive protein (CRP), and haptoglobin, are circulating proteins that can be used to assess inflammatory responses to infections, traumas, tumors and surgery [2, 4, 8, 11, 15]. By definition, APP serum concentrations change in response to stimulation from proinflammatory cytokines during the disease process. As quantitative biomarkers of disease, APPs can be utilized for diagnosis, prognosis, and monitoring of responses to therapy, as well as for general health screening. While APPs have been widely used as inflammatory biomarkers in both companion and farm animals, there may be differences in the specificities for the APP response among different species. For instance, CRP is a satisfactory inflammatory indicator in dogs, but not cats [3]. Thus, it is important to clarify which APPs are beneficial biomarkers for target animals.

Of the APPs, SAA is clinically or experimentally available in cat, dog, horse, cow, and pig [3]. Therefore, SAA appears to have no great interspecies variability compared to other APPs. Although healthy animals have low serum SAA concentrations (<1 µg/l), its levels rise dramatically by 100–1,000 fold following stimulation, reach a peak at 24–48 hr, and then decline rapidly during the recovery phase. A previous paper reported that white blood cell (WBC) and band neutrophil counts, which are commonly used as indicators of inflammation in veterinary medicine, are insufficient for the detection of inflammation and that another sensitive inflammatory indicator, such as CRP in dogs and SAA in cats, should be utilized for routine measurements [9, 15].

Recently, with an increase in the number of domestic ferrets, reports on tumors, such as adrenal neoplasm and lymphoma, in ferrets have increased [1]. Thus, there is a growing need for diagnosis, prognosis, and monitoring of the response to therapy in ferrets. Furthermore, since ferrets are a superior animal model for acute inflammatory infectious diseases, such as influenza and severe acute respiratory syndrome (SARS), in human medicine [6, 7], a definitive inflammatory indicator is required for sensitive and accurate pathogenetic analyses in ferrets. Although the induction of fibrinogen was found in the lungs of ferrets infected with Pneumonia carinii [14], there are no reports regarding valuable APP markers for evaluating the inflammatory response in ferrets. Therefore, the aims of the present study were to identify the structure of ferret SAA (rfrSAA) and assess the cross-reactivity of commercially available SAA ELISA kits for the measurement of ferret SAA concentrations with recombinant ferret SAA protein (rfrSAA).

Liver tissue was obtained from Japan SLC, Inc. (Hamamatsu, Japan). Total RNA was isolated from the liver samples using ISOGEN (Nippon Gene, Toyama, Japan), according to the manufacturer’s instructions. cDNA was synthesized using murine leukemia virus (MuLV) reverse transcriptase (Applied Biosystems, Branchburg, NJ, U.S.A.). The coding region of the rfrSAA cDNA was amplified via PCR using the synthesized cDNA template. The primer pair was designed based on the previously reported sequence of mink SAA1 mRNA (GenBank accession number: M34953.1). The primer pair was as follows: forward 5'-AGCTCTGCTC-CACTAGCC-3', and reverse 5'-TTAGGTGCCCTC-CACTAGCC-3'. PCR was performed for 35 cycles, with

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each cycle consisting of pre-denaturation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The amplified cDNA was electrophoresed on a 2% agarose gel, visualized with ethidium bromide under UV light, and then purified with a Wizard SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, U.S.A.). Nucleotide sequences of the PCR products were determined via direct sequencing using SMART (http://smart.embl-heidelberg.de/). Multiple alignments of mature frSAA and SAA1 of other animals were generated and analyzed using ClustalW [16].

To express a mature form of frSAA, as a recombinant protein, the sequences encoding the signal peptide were excluded from the target PCR product when the primer pair was designed based on the sequence of frSAA mRNA with a concomitant introduction of CACC at the 5' end of the forward primer (forward 5'-CACCTGGTATTCATTGGT-3', and reverse 5'-TCAGTACTTGTCAGGCAGGC-3'). The PCR product was inserted into the pET100 vector (Invitrogen, Carlsbad, CA, U.S.A.) from which recombinant SAA could be expressed as a His-tagged fusion protein. After this expression plasmid was transformed into the E. coli strain BL21 Star (DE3) (Invitrogen), transformants were isolated and grown overnight in Luria-Bertani (LB) medium with 100 µg/ml of ampicillin. Overnight cultures were diluted 1:20 in LB medium with 100 µg/ml of ampicillin, and grown until an optical density (OD$_{600}$) of 0.7 was reached. Then, 0.5 mM of isopropyl β-D-thiogalactosidase (IPTG) was added into cultures and incubated for 4 hr at 37°C to induce expression of the recombinant fusion protein. To isolate His-tagged SAA, bacterial cultures were centrifuged and lysed, and the protein was purified from the lysate via immobilized metal affinity chromatography on a His-trap HP column (GE Healthcare, Uppsala, Sweden), according to the manufacturer’s instructions. The purified rfrSAA was assessed via SDS-PAGE using 15% polyacrylamide gels (e-PAGE6 ET15L; ATTO Corporation, Tokyo, Japan). The electrophoresed proteins were stained with EzStain AQua (ATTO Corporation) and destained with distilled water.

In the immunoblot analysis, the separated proteins were transferred via electrophoresis onto an Immobilon-P Transfer Membrane (Millipore, Bedford, MA, U.S.A.) after SDS-PAGE. Membrane portions were blocked in Block Ace (Dainihon Pharmaceutical, Osaka, Japan) for 1 hr at room temperature, and the proteins were immuno-detected with horseradish peroxidase (HRP)-labeled anti-SAA mAb provided with the multispecies SAA ELISA kit (Tridelta Development Ltd., Greystones Co., Wicklow, Ireland). Immunostained protein bands were visualized using an Ez-West-Blue solution (ATTO Corporation).

The purified rfrSAA protein concentration was determined using a Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan) and BSA, as a standard. Two-fold serial dilutions (0.03–8 µg/ml) of rfrSAA were used for quantification. ELISAs were performed using purified rfrSAA to investigate the utility of the two commercial ELISA kits, i.e., the multispecies SAA ELISA kit described above and a human SAA ELISA kit (Anogen, Mississauga, Canada). All samples were tested according to the manufacturer’s instructions.

The rfrSAA nucleotide and deduced amino acid sequence were deposited into the GenBank database (Accession No. AB709956). The CDS of ferret SAA was 387 nucleotides, which encoded 129 amino acids (Fig. 1). The rfrSAA had a signal peptide region corresponding to 19 amino acids and a coding sequence for a mature SAA polypeptide containing 110 amino acids. The deduced frSAA amino acid sequence had 96.1, 89.9, 86.0, 83.8, 83.0, 73.8 and 65.3% similarity to the mink, dog, cat, cattle, horse, human and mouse SAA1 genes, respectively. The deduced ferret SAA amino acids are identical to the ferret sequence are indicated by dots. Amino acids that are absent are denoted by a dash. The deduced signal sequence is indicated by a box.

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**Fig. 1.** Alignment of deduced amino acid sequences of Serum amyloid A (SAA) of the ferret and other mammal SAA1 (GenBank accession numbers: human (P02735), mouse (P05366), mink (P18575), cat (NM001040198), dog (P19708), cattle (P35541), and horse (NM001163892)). The amino acid residues that are identical to the ferret sequence are indicated by dots. Amino acids that are absent are denoted by a dash. The deduced signal sequence is indicated by a box.
acid sequence contained an 8-amino acid fragment between amino acids 88 and 95 that is not present in human SAA1.

Using SDS-PAGE, it was found that IPTG induction resulted in the production of a protein of approximately 17 kDa; no such band was observed without IPTG (Fig. 2A). The anti-multispecies SAA mAb provided with the multispecies ELISA kit cross-reacted with the purified rfrSAA on the immunoblot (Fig. 2B).

The multispecies SAA ELISA kit was used for detecting rfrSAA. The absorbance demonstrated a linearly response to rfrSAA within the range of 0.03–1 µg/ml (Fig. 3). In contrast, the anti-human SAA antibodies provided in the human SAA ELISA kit showed no cross-reactivity with rfrSAA (data not shown).

The nucleotide sequences of SAA in many species have already been previously reported. The rfrSAA shares considerably high homology with other mammalian SAA. The rfrSAA shares the highest homology with mink SAA1 (96.1%), which may not be surprising given that ferrets and mink belong to the family Mustelidae. Moreover, the rfrSAA shares the lowest homology with mouse SAA1 (65.3%). Western blot analysis with anti-multispecies SAA mAb revealed a 17-kDa band under reducing conditions, which is the size predicted from the nucleotide sequence of ferret SAA cDNA. Furthermore, when the multispecies SAA ELISA kit detected purified rfrSAA in a dose-dependent manner, rfrSAA was not recognized by the antibodies provided in the human SAA ELISA kit. This may be due to the insertion of 8 amino acid residues between positions 88 and 95 of the amino acid sequence, which may have caused possible changes in the structure of the rfrSAA, as was previously suggested with dog and cat SAAs [10]. This insertion is in accordance with the cat, cattle, dog, horse, and mink SAA amino acid sequences that were previously reported [12, 13]. Although several functions of human SAA were previously characterized [5], there is little information regarding the physiological significance and kinetics of SAA in domestic animals, with the exception of its use as a biomarker of inflammation. Therefore, further investigations that clarify whether the amino acid insertion in SAA of these domestic animals involves a functional change in their SAA are warranted.

In conclusion, we found that a multispecies SAA ELISA kit can specifically quantify rfrSAA, and that the antigenicity and structure of rfrSAA are similar to those of SAAs of other domestic animals. These findings suggest that in vivo concentrations of rfrSAA can be quantified with this ELISA kit, similar to the SAAs of other animals. To determine whether ferret SAA is a significant biomarker in veterinary medicine, future studies that measure SAA concentrations in the circulating blood of healthy and diseased ferrets and monitor SAA kinetics during an inflammatory event are needed.

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REFERENCES


