Verification of Measurement of the Feline Serum Amyloid A (SAA) Concentration by Human SAA Turbidimetric Immunoassay and Its Clinical Application

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ABSTRACT. Serum amyloid A (SAA) is one of the major acute phase proteins in cats that has potential to be used as an inflammatory marker. A previous study showed that the human SAA turbidimetric immunoassay (hSAA-TIA) could be used to measure the SAA concentration in cats. The objectives of the present study were to assess use of hSAA-TIA for determining the feline SAA concentration and to evaluate its clinical application. Recombinant feline SAA protein (rfSAA) was expressed in Escherichia coli and purified for SDS-PAGE and immunoblot analysis with anti-human SAA antibodies. The concentration of rfSAA was determined by ELISA and hSAA-TIA. Plasma SAA concentrations were measured in healthy and diseased cats by hSAA-TIA. The time-courses changes in the SAA and α1-acid glycoprotein (AGP) concentrations in the cats after ovariohysterectomy were investigated. In SDS-PAGE, rfSAA was detected as a clear band that reacted with anti-human SAA antibodies. There was significant correlation between the SAA concentration measured by ELISA and hSAA-TIA. The SAA concentration of the diseased cats (n=263) was significantly increased (P<0.01; 0.0–88.9 mg/l, mean: 7.52 mg/l) compared with that in the healthy cats (n=26; 0.0–0.9 mg/l, mean: 0.14 mg/l). No correlation was observed between SAA and WBC in the diseased cats. The SAA concentration changed more rapidly and remarkably than the AGP concentration after ovariohysterectomy. The present study revealed that hSAA-TIA is useful for determination of the feline SAA concentration. Measurement of the SAA concentration, in addition to the WBC count, would be clinically valuable as a routine test to detect inflammation.

KEY WORDS: acute phase protein, α1-acid glycoprotein, feline, serum amyloid A, turbidimetric immunoassay.

The acute phase response is part of the initial response to inflammatory stimuli such as infection, trauma, tumors and surgery [4]. One of the most characteristic features of this response is increased hepatic synthesis of plasma proteins known as acute phase proteins (APPs), which include serum amyloid A (SAA), C-reactive protein (CRP), α1-acid glycoprotein (AGP) and haptoglobin [5]. In humans, there are dramatic increases in the serum concentrations of CRP and SAA in response to inflammatory stimuli, and these APPs have been used as inflammatory markers [15, 18, 26]. Previous studies have concluded that SAA, AGP and haptoglobin are useful indicators of acute phase status in cats [7, 12, 21].

SAA was first recognized as the precursor of amyloid A (AA) protein, the main fibrillar component in reactive AA-amyloidosis in humans [11]. SAA is an apolipoprotein associated with high density lipoprotein 3 (HDL3), although its physiological role remains to be elucidated. SAA proteins are highly conserved throughout the evolution of mammals, including human [24], mouse [32], dog [23], cat [19, 29], mink [27] and several other mammalian species [9, 20, 25]. Sequence comparisons between species have revealed some regions of strict conservation.

In humans, SAA can increase up to 1,000-fold within 24–48 hr after inflammatory stimuli [10]. Similarly, in cats, SAA can increase markedly in inflammatory states and is recognized as a useful inflammatory marker [21]. High concentrations of SAA have been observed in cats with experimentally-induced inflammation, injury, infectious diseases such as feline infectious peritonitis (FIP) and various other diseases [7, 12, 21].

To implement feline SAA measurement in routine diagnosis and monitoring, reliable, rapid, automated, and readily available assays are required. In 2006, Hansen et al. reported that the SAA concentration in cats could be measured using a commercially available turbidimetric immunoassay (TIA) designed for measuring human SAA [8]. The purposes of this study were to assess the applicability of human SAA-TIA to measurement of the feline SAA concentration by using recombinant feline SAA protein (rfSAA) and to evaluate the clinical usefulness of measuring the SAA concentration routinely in cats with various diseases.

MATERIALS AND METHODS

Expression and purification of recombinant feline SAA protein: Total cellular RNA was isolated from the liver of the cat administered lipopolysaccharide (LPS); these procedures were conducted in accordance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo. Briefly, mRNA was purified using a RNeasy mini spin column kit (Qiagen, Hilden, Germany), and cDNA was synthesized using murine leukemia virus (MuLV) reverse

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transcriptase (Applied Biosystems, Branchburg, NJ) and a GeneAmp RNA PCR core kit (Applied Biosystems). The coding region of the feline SAA cDNA was amplified by polymerase chain reaction (PCR) using the synthesized cDNA template and Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland). The primer pair was designed based on the sequence of a previously reported feline SAA mRNA (GenBank accession number: AB242838) with concomitant introduction of CACC at the 5’ end of the forward primer (5’-CACCTGGTATTCGTTCCCTGGGTGAA-3’, forward; 5’-TCAGTACTTGTCAGCAGGC-3’, reverse). To express the recombinant protein in a mature form, the signal peptide of SAA was excluded when the primer set was designed. The PCR product was inserted into the pET100 vector (Invitrogen, Carlsbad, CA, U.S.A.) in which SAA could be expressed as a His-tag fusion protein. This expression plasmid was transformed into Escherichia coli, BL21 Star (DE3) (Invitrogen), and the transformants were grown overnight in Luria-Bertani (LB) medium containing ampicillin. Overnight cultures were diluted 1/100 in LB medium containing 100 µg/ml ampicillin and grown for 2 hr. Expression of the recombinant fusion protein was induced in culture with 1 mM isopropyl β-D-thiogalactosidase (IPTG) for 5 hr at 37°C. Cultures were centrifuged at 4,000 × g for 20 min at 4°C. Cell pellets were stored at −20°C overnight. The expressed His-tag fusion protein was purified using a QIAexpress Ni-NTA Fast Start kit (Qiagen). Briefly, the cell pellets were resuspended in a native lysis buffer contained in the kit, and the lysates were centrifuged at 14,000 × g for 30 min at 4°C. The supernatant containing the fusion protein was purified under native conditions using the Ni-NTA column in the kit. The supernatant containing the fusion protein was purified using a QIAexpress Ni-NTA Fast Start kit (Qiagen). Briefly, the cell pellets were resuspended in a native lysis buffer contained in the kit, and the lysates were centrifuged at 14,000 × g for 30 min at 4°C. The supernatant containing the fusion protein was purified under native conditions using the Ni-NTA column in the kit.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis:** Samples were analyzed in two 15% polyacrylamide gels (e-PAGEL ET15L; ATTO Corporation, Tokyo, Japan). Prior to electrophoresis, the samples were boiled for 5 min in the presence of 5% (v/v) 2-mercaptoethanol. One of the gels was stained overnight using a BioSource Multispecies SAA ELISA kit (BioSource International, Inc., Camarillo, CA, U.S.A.). Purified rfSAA solution was diluted by 50%, 30%, 10%, 5% and 1%. Samples mixed with biotinylated mouse anti-human SAA monoclonal antibodies were added to the wells of a microtiter plate coated with monoclonal antibodies specific for SAA. After washing to remove all the unbound material, streptavidin-horseradish peroxidase conjugate was added to the wells and then tetramethyl benzidine (TMB) substrate was added. The absorbance of each well at 450 nm was measured using a microtiter plate reader.

**Turbidimetric immunoassay (TIA):** A commercially available TIA for human SAA (LZ-SAA, Eiken Chemical Co., Ltd.) was used for determination of the SAA concentration in the recombinant protein solution and serum/plasma samples. The analysis was performed in duplicate by using an automated chemical analyzer (TBA-80FR NEO2, Toshiba Medical Systems, Tokyo, Japan) according to the manufacturer’s protocol. The calibration curve was obtained using a calibrator for humans supplied with the kit.

**Samples:** Serum or plasma samples were obtained from 263 client-owned cats with various diseases that were referred to and diagnosed at the Veterinary Medical Center of the University of Tokyo (VMC-UT) between April 2006 and July 2007. Cats with multiple diseases or unconfirmed diagnoses and previously treated cats were excluded from the study. Samples from 3 cats who received ovariohysterectomy were obtained before and 8, 24, 48, 72 and 96 hr after surgery. Twenty-six clinically healthy cats were assigned as control cats. All cats were subjected to clinical examination and standard hematological and biochemical tests. Additional diagnostic procedures (e.g., radiography, ultrasonography, endoscopy, exploratory laparotomy, cytology and histopathology) for individual cats were performed depending on the medical condition. All samples were prepared by centrifugation of whole blood and stored at −20°C until analysis.

**AGP analysis:** Measurement of the AGP concentration was performed using a feline-specific single radial immunodiffusion assay (Feline α1AG Plate, The Institute for Metabolic Ecosystem, Miyagi, Japan) according to the manufacturer’s instructions. The reference range of AGP is less than 550 mg/l based on the specification provided by the manufacturer.

**Statistical analysis:** Correlation of the rfSAA concentration measured by ELISA and TIA was analyzed using Pearson’s product-moment correlation coefficient. Correlation between the SAA concentration and other parameters such as WBC count, band neutrophil count and albumin and AGP concentrations was also analyzed using the same method.

**RESULTS**

**Expression and purification of recombinant feline SAA protein:** PCR amplification using feline cDNA as a template yielded a single band at approximately 330 bp on agarose gel electrophoresis (data not shown). The PCR product was inserted into the pET100 vector, and sequence analysis of the vector was performed. The sequence was completely identical to that of previously reported for feline AA protein (GenPept accession number: BAE93428; data not shown).
Under the control of IPTG, the rfSAA protein without the leader peptide was produced as a His-tag fusion protein and purified using the Ni-NTA column.

SDS-PAGE and immunoblot analysis: In SDS-PAGE, a clear band was observed near 16 kDa in elutants (Fig. 1A), which corresponds to the anticipated molecular weight of rfSAA with His-tag. Cross-reactivity was tested between the purified protein and anti-human SAA monoclonal antibodies, which were used in LZ-SAA. As shown in Fig. 1B, anti-human SAA antibodies reacted with purified rfSAA.

ELISA and TIA: The SAA concentrations measured by ELISA and TIA were tested for correlation using rfSAA. Although each SAA concentration value measured by TIA did not correspond to that determined by ELISA, strong correlation was observed ($r=0.99$; Fig. 2).

SAA concentration in cats with various diseases: The SAA concentration increased significantly ($P<0.01$) in the diseased cats ($n=263$; $0.0–88.9$ mg/l, mean: $7.52$ mg/l) compared with that in the healthy cats ($n=26$; $0.0–0.9$ mg/l, mean: $0.14$ mg/l). The reference range for the feline SAA concentration was estimated to be less than $0.82$ mg/l according to the results for clinically healthy cats (mean=$0.14$ mg/l, SD=$0.23$ mg/l).

Table 1 shows the diseases ($n\geq 3$) in which the mean SAA concentration was higher than the reference range. A remarkable increase in SAA concentration was observed in cats with inflammatory and infectious diseases such as acute pancreatitis and FIP. The SAA concentration also increased in cats with neoplastic diseases, such as lymphoma and malignant mesothelioma, or endocrine diseases, such as hyperthyroidism and diabetes mellitus (DM).

Pearson’s product-moment correlation coefficient was used to determine the correlation of the SAA concentration with the WBC count, band neutrophil count and albumin and AGP concentrations in the diseased cats. Strong correlation was observed between the SAA and AGP concentrations ($n=65$, $r=0.71$; Fig. 3). In contrast, the SAA concentration did not correlate with the WBC count ($n=251$, $r=0.25$), band neutrophil count ($n=167$, $r=0.08$) or albumin concentration ($n=137$, $r=-0.27$).

The time-course changes in the SAA and AGP concentrations were examined in the sera of 3 cats before and after ovariohysterectomy (Fig. 4). After surgery, the SAA concentration gradually increased at 8 hr, attained the peak value at 48 hr, and then returned to a normal value within 4...
in contrast, the AGP concentration did not show a gradual increase at 8 hr. It attained its peak value at 48 hr and continued to remain high at 4 days after surgery.

**DISCUSSION**

In the present study, rfSAA with His-tag was successfully expressed and purified using a Ni-NTA column. Anti-human SAA monoclonal antibody, which is the same antibody as that used in the human SAA-TIA kit, could recognize rfSAA. Furthermore, the SAA concentration determined by TIA correlated well with that measured by the multispecies SAA ELISA kit. These results indicate that the human SAA-TIA can detect feline SAA and can be applied to measurement of the feline SAA concentration.

Although strong correlation was observed between the SAA concentrations measured by ELISA and TIA, the SAA concentration determined by ELISA was considerably higher than that determined by TIA. The discrepancy in the SAA concentration between these two assays is probably due to a difference in sensitivity. However, absolute values are considered to be less important than abnormal or change of values in clinical settings. TIA can measure feline SAA easily and rapidly as compared to ELISA, thereby indicating that TIA is suitable for use as a routine laboratory test.

The serum SAA concentration was significantly elevated in the diseased cats compared with that in the healthy cats. Cats with inflammatory and neoplastic diseases such as acute pancreatitis, FIP and lymphoma showed a remarkable increase in SAA concentration. This finding is in accor-
dance with previous reports in which feline SAA increased particularly in cats with inflammatory/infectious diseases [7, 21]. Our results, together with previous findings, indicate that SAA is a clinically useful inflammatory marker in cats [12, 21]. In the present study, however, the SAA concentration also increased in cats with endocrine or non-inflammatory diseases such as DM, chronic renal failure (CRF), hyperthyroidism, and polycystic kidney disease (PKD). A high SAA concentration in cats with DM and CRF has also been reported in a previous study [21]. Increases in the concentrations of APPs such as SAA and CRP have also been reported in human patients with DM [3, 14] and nephrotic syndrome [30]. The increase in the SAA concentration is considered to be caused by an underlying infection and/or vascular endothelial damage. In human patients with hyperthyroidism and PKD, a high concentration of serum interleukin-6 has been reported [2, 16]. Furthermore, SAA is known to be expressed not only in the liver but also in many other tissues, including the kidney and thyroid [28]. These findings may be related to the high SAA concentration in cats with hyperthyroidism and PKD, although the concentration of serum interleukin-6 and expression of SAA protein in the kidney and thyroid were not examined in the present study. In contrast, little or no increase in the SAA concentration was observed in cats with gastroenteritis and bronchopneumonia, in spite of the fact that they are inflammatory diseases. The SAA concentration is thought to be influenced by the severity of the inflammation, such as systemic or focal, or nature of the inflammation, such as acute or chronic. In this regard, the gastroenteritis and bronchopneumonia found in this study may have possessed focal or chronic inflammation.

The WBC and band neutrophil counts are commonly used as indicators of inflammation in veterinary medicine. In the present study, however, a number of cases showed discrepancies between the SAA concentration and WBC/band neutrophil counts. A similar finding has been reported for the CRP and WBC count in dogs [17]. The discrepancy between the SAA concentration and WBC/band neutrophil counts indicates that the WBC/band neutrophil count is insufficient for detection of inflammation and that another indicator of inflammation, such as SAA, should be used for routine measurement. Although albumin is a known negative APP [1], no correlation was observed between the SAA and albumin concentrations in the present study. This is probably due to the influence of many factors such as dehydration and malnutrition.

In the present study, SAA correlated well with AGP, and this finding is in accordance with previous reports [12]. The time-course changes in the SAA concentration in the cats after ovariohysterectomy showed that SAA increased more rapidly and by a higher magnitude and decreased more rapidly than AGP. It has been reported that the half-life of SAA is about 1–2 hr in mice [13] and that the half-life of AGP is about 1–2 days in rats [22]. Although, the half-lives of SAA and AGP have not been reported in cats, a difference of their half-lives might be the cause of the gap between their concentrations in the time-course changes after ovariohysterectomy. The rapid changes in the SAA concentration offer a great advantage for early detection of inflammatory conditions. From this viewpoint, SAA may be a more suitable inflammatory marker in cats. In the chronic phase, however, it is possible that SAA and AGP exhibit completely different behaviors because AGP is secreted not only by the liver but also by leukocytes [6]. Measurement of other APPs, such as AGP, in addition to the SAA concentration would be useful for feline medicine. Further studies evaluating serial SAA and AGP concentrations in cats with various diseases are required to confirm the clinical usefulness of SAA and AGP.

In conclusion, the anti-human SAA antibody used in human SAA-TIA can react with feline SAA, and the human SAA-TIA is useful for measurement of the feline SAA concentration. The SAA concentration increases rapidly when inflammation and or tissue damage occurs, and we observed elevated SAA concentrations in cats with various diseases. It is preferable to measure not only the WBC/band neutrophil count but also the SAA concentration as an inflammatory marker in cats. Measurement of the SAA concentration in cats will be clinically valuable for detection of inflammation as well as determination of disease severity and evaluation of response to treatment.

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