博士論文

論文題目　**Studies on pathophysiological characteristics of feline serum amyloid A**

（猫血清アミロイドＡの病態生理学的特性に関する研究）

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CONTENTS

Chapter 0 (Introductory Chapter)

Serum amyloid A as feline inflammatory and prognostic marker

1. Introduction

Serum amyloid A (SAA) is one of the major acute phase proteins (APPs) in mammals, including humans (Uhlar et al., 1999) and cats (Kajikawa et al., 1999). The acute phase response is a part of initial response to inflammatory stimuli such as infection, trauma, tumors, and surgery (Ebersole et al., 2000). One of the most characteristic features of this response is increased hepatic synthesis of plasma proteins known as APPs that include SAA, C-reactive protein (CRP), α1-acid glycoprotein (α1AG), and haptoglobin (Gabay et al., 1999). SAA gene expression and protein production by hepatocytes are stimulated by inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Weinstein et al., 1987, Jensen et al., 1998), or by bacterial products such as lipopolysaccharide (LPS) (Migita et al., 2004). The produced SAA protein is released into circulation, and the secretion of SAA into blood results in a marked increase in SAA concentration in the plasma. SAA concentration occasionally increases up to 1000-fold above the basal level (Kushner, 1988).

SAA was first recognized as the precursor of amyloid A (AA) protein, the main fibrillar component in reactive AA-amyloidosis in humans (Husby et al., 1988). As AA proteins are proteolytically derived N-terminal fragments of SAA (Rocken et al., 2002), elevated SAA concentration and its metabolism are considered important factors for amyloidogenesis (Lachmann et al., 2007, Magy et al., 2007). Although systemic amyloidosis is relatively uncommon disease in domestic cats, secondary AA amyloidosis has been reported also in cats with chronic inflammatory disease (Beatty et al., 2002). In Abyssinian/Somali and Siamese/Oriental breeds, familial AA amyloidosis has been well

described (Niewold et al., 1999). Abyssinian cats develop the amyloidosis with the kidney as predominat target organ, while in Siamese cats the amyloid depositions are found especially in the liver and gut. In these breeds, genetic factors of SAA were suggested (van Rossum et al., 2004).

In humans, 2 types of SAA proteins, acute phase SAA (A-SAA) and constitutive SAA (C-SAA) were reported (Whitehead et al., 1992). C-SAA was described only in humans and mice (de Beer et al., 1994). A-SAA and C-SAA show several differences in terms of their expression pattern and amino acid sequence. A-SAA levels markedly increase during the acute phase response, whereas C-SAA levels do not show any increase. C-SAA contains an insert of 8 amino acids and shares only 55% identity with A-SAA (Whitehead et al., 1992). Furthermore, only A-SAA is a precursor of secondary amyloidosis (Uhlar et al., 1999). Amino acid sequences of SAA protein were reported also in cats (Ohno et al., 1999, van Rossum et al., 2004), and these sequences are highly conserved compared with human A-SAA. Moreover, the expression pattern and amyloidogenesis of feline SAA is more similar to that of human A-SAA (Ohno et al., 1999). Therefore, it can be considered that SAA reported in cats are comparable to human A-SAA.

Because of the dramatic increase in plasma SAA concentration during inflammation, measurement of plasma SAA concentration is used as an inflammatory marker in humans (Nakayama et al., 1993, Hilliquin, 1995). Measurement of SAA concentration in blood has been used as a marker to evaluate the extent of disorder and the response to treatment in inflammatory diseases such as rheumatoid arthritis (Benson et al., 1979, Scheinberg et al.,

1980). Earlier studies have concluded that SAA, α1AG, and haptoglobin are useful indicators of acute phase status in cats (Giordano et al., 2004, Kajikawa et al., 1999, Sasaki et al., 2003). Moreover, for the measurement of feline SAA, a rapid and automated assay was reported and attracted increased attention (Hansen et al., 2006). However, the clinical usefulness of the measurement of feline SAA remains unclear.

In human medicine, SAA is not only a marker of existing inflammation but also a prognostic marker for several diseases. For example, in patients with arthritis and other inflammatory diseases, median SAA concentration was significantly associated with prognosis and pathogenesis of secondary amyloidosis (Lachmann et al., 2007, Gillmore et al., 2001). The relationships between SAA concentration and prognosis in neoplastic diseases such as gastric cancer (Chan et al., 2007), lung cancer (Liu et al., 2007, Cho et al., 2010), and renal cell carcinoma (Kimura et al., 2001) were also reported. Furthermore, high serum SAA concentration was described as a risk factor in type 2 diabetes (Dalla Vestra et al., 2005). However, there is no report described about the association between SAA concentration and prognosis in cats.

The purposes of this study (chapter 0) were to evaluate the clinical usefulness of measuring SAA concentration and the relationship between serum SAA concentration and prognosis in cats with various diseases.

2. Materials and Methods

2.1. Samples for measurement of SAA concentration

Serum or plasma samples were obtained from 263 client-owned cats that were referred to and diagnosed at the Veterinary Medical Center of the University of Tokyo (VMC-UT), for various diseases from April 2006 to July 2007. Cats with multiple diseases or unconfirmed diagnoses and previously treated cats were excluded from the study. Twenty-six clinically healthy cats were assigned as control cats. All cats were subjected to clinical examination and standard hematological and biochemical profiles. 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.

2.2. Measurement of SAA concentration

A commercially available turbidimetric immunoassay (TIA) for human SAA (LZ-SAA, Eiken Chemical Co., Ltd.) was used for the determination of SAA concentration for serum/plasma samples (Hansen et al., 2006). 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.

2.3. Prognostic analysis

Medical records of cats described above were reviewed. Follow-up data on mortality were collected until March 2012 from medical records, referring veterinarians, and telephone interviews with owners. Cats were excluded from the study if there was only a 1-day follow-up period because of insufficient information for survival analysis. Cats were divided into 2 groups according to serum SAA concentration. The non-elevated SAA group included the cats whose SAA concentration was within the reference range and the elevated SAA group included the cats whose SAA concentration was above the reference range. Furthermore, cats were divided into 3 categories according to diagnosis: neoplastic diseases, inflammatory diseases, and other diseases. The inflammatory diseases category included infectious diseases such as feline infectious peritonitis (FIP). The survival time between both groups was compared. Moreover, the correlation between SAA concentration and survival time was estimated.

2.4. Statistical analysis

Statistical analyses were performed by using a statistical software package (JMP version 10.0.2 Pro; SAS Institute, Cary, NC). The difference between healthy cats and diseased cats in SAA concentration was examined by Mann–Whitney U test. In order to determine differences between normal and elevated SAA groups, the chi-square test was used for categorical data. The Mann–Whitney U test was used to analyze continuous data. Differences in survival between each group were assessed using the Kaplan–Meier product

limit method and the log-rank test. Correlation between SAA concentration and survival time was assessed using the Spearman rank correlation coefficient. Multivariate analyses were performed using the Cox proportional hazards model. Variables with P values < 0.05 were considered statistically significant.

3. Results

3.1. Measurement of SAA concentration

As shown in Fig.1, SAA concentration increased significantly ($P < 0.01$) in cats with various diseases (n = 273, mean = 7.52 μg/ml, median [range]: 0.35 μg/ml [0.0–88.9 μg/ml]) compared to that in healthy cats (n = 26, 0.14 μg/ml, 0.05 μg/ml [0.0–0.9 μg/ml]). The reference range of feline SAA concentration was estimated to be less than 0.82 mg/l according to the result of SAA concentration in clinically healthy cats (mean + 3SD, SD = 0.23 μg/ml).

Table 1 shows the diseases (n ≥ 3) included in this study. A remarkable increase in SAA concentration was observed in cats with inflammatory and infectious diseases such as acute pancreatitis and FIP. SAA concentration also increased in cats with neoplastic diseases, such as lymphoma and malignant mesothelioma, or endocrine diseases including hyperthyroidism and diabetes mellitus (DM).

3.2. Prognostic analysis

For the prognostic analysis, a total of 175 cats with various diseases were included. There were 110 cats in the non-elevated SAA group and 65 cats in the elevated SAA group. Patient characteristics in both groups are summarized in Table 2. Median SAA concentration in each group was 0.15 mg/l (range, 0–0.8 μg/ml) and 18.1 mg/l (range, 0.83–88.3 μg/ml), respectively. Median age at diagnosis was 9 years (range, 0.25–21 years; median age of the non-elevated SAA group, 8 years; median age of the elevated SAA group, 9 years; $P = 0.376$).

Median body weight (BW) was 3.7 kg (range, 0.9–8.15 kg; median BW of the non-elevated SAA group, 3.85 kg; median BW of the elevated SAA group, 3.45 kg; $P = 0.047$). According to diagnostic category, 64, 66, and 45 cats were categorized in neoplastic, inflammatory, and other diseases, respectively. Diagnostic categories did not differ significantly between the non-elevated SAA and elevated SAA groups ($P = 0.202$).

Median follow-up time for all cats was 42 days (range: 1–2,104 days). Seventy-seven cats (70%) in the non-elevated SAA group and 29 cats (44.6%) in the elevated SAA group were alive at the time of last contact. Reasons for premature cessation of hospital visits were not always available from medical records; those cats were censored from the survival analysis at the time of the last arrival. Median survival time for the non-elevated SAA group was 571 days compared with 72 days for the elevated SAA group ($P < 0.01$; Fig. 2A). Median survival time of the elevated SAA group was shorter than that of the non-elevated SAA group also in cats with neoplastic diseases and inflammatory diseases ($P = 0.04$ and $P < 0.01$, Fig.2B and 2C, respectively). No difference in survival time was observed in cats with other diseases ($P = 0.09$, FIg.2D). The correlation between the magnitude of SAA concentration and survival time was examined in cats confirmed dead, and a weak negative correlation was observed ($r_s = -0.37$, $P < 0.01$). With the Cox proportional hazards model, age, BW, diagnostic category, and elevated SAA concentration were included in the multivariate analysis. Elevated SAA concentration was shown to be a significant and independent prognostic factor (hazard ratio: 2.16, 95% confidence interval: 1.32–3.53, $P < 0.01$).

4. Discussion & General introduction for Chapter 1−3

In this study, serum SAA concentration was significantly elevated in cats with various diseases as compared to that in 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 accordance with previous reports in which feline SAA increased particularly in cats with inflammatory/infectious diseases (Giordano et al., 2004, Sasaki et al., 2003). My results, together with previous findings, indicate that SAA is a clinically useful inflammatory marker in cats (Kajikawa et al., 1999, Sasaki et al., 2003). In the present study, however, SAA concentration also increased in cats with endocrine or non-inflammatory diseases such as DM, hyperthyroidism, and renal failure. High SAA concentration in cats with DM and renal failure was also reported in a previous study (Sasaki et al., 2003). Increase in the concentration of APPs such as SAA and CRP was also reported in human patients with DM (Dalla Vestra et al., 2005, Kumon et al., 1994) and nephrotic syndrome (Wasilewska et al., 2007). This increase in SAA concentration was considered to be caused by underlying infection and/or vascular endothelial damage.

In the present study, serum SAA concentration at the time of first examination at the hospital was demonstrated as a significant prognostic factor in cats with various diseases, especially in neoplastic and inflammatory diseases. Although cats with neoplastic diseases were included in this study and neoplastic diseases are generally considered to have poorer prognoses than inflammatory and/or non-inflammatory diseases, SAA concentration was shown as an independent prognostic marker.

There may be two reasons why serum SAA concentration could function as a prognostic marker in patients. First, increased inflammatory markers, such as SAA, suggest that the diseases are poorly controlled, and chronic inflammatory status is considered to affect prognosis directly. The association between SAA concentration and prognosis in human patients with rheumatoid arthritis was reported (Gillmore et al., 2001). Furthermore, elevated CRP concentration was also found to be a prognostic factor in rheumatoid arthritis (Otterness, 1994). Because both SAA and CRP were reported as prognostic markers in patients with inflammatory diseases, the theory that these markers reflect the activity of the diseases is plausible. Second, SAA protein is known as a precursor of AA fibrils and sustained high serum SAA levels may give rise to secondary amyloidosis, so-called reactive AA amyloidosis (Lachmann et al., 2007). The development of AA amyloidosis is considered an important prognostic factor; however, the presence of secondary amyloidosis was not fully examined in the present study because not all cats in this study had postmortem examination.

These theories described above are very simple and well accepted. However, I raised further hypothesis that the pathophysiological characteristics of SAA could influence the SAA concentration and prognosis of diseases.

There are several reports that SAA protein promoted cytokine production (Mullan et al., 2006, Hatanaka et al., 2007, Song et al., 2009) and leukocyte migration (Badolato et al., 1994, Su et al., 1999, Mullan et al., 2006). These functions of SAA protein might affect the prognoses of inflammatory diseases. However, all of these facts were reported in human medicine, and it remains unclear whether it can be applied to cats. Although feline SAA

showed high homology with human A-SAA (81%), feline SAA protein contains an insert of 8 amino acids as dose human C-SAA (Ohno et al., 1999). Therefore, it should be investigated whether feline SAA protein performs similar functions to human A-SAA. In chapter 1, I investigated whether feline SAA protein functions in mediating the immune response as human A-SAA does by using TNF-α production as an indicator.

Tumors are one of the most common problems in feline medicine. In this study, elevated SAA concentrations were observed in cats with neoplastic diseases, and these cats showed shorter survival time than that had non-elevated SAA concentrations. As well as in cats demonstrated in this study, SAA concentration has been reported a useful prognostic marker in neoplastic diseases in humans (Kimura et al., 2001, Chan et al., 2007, Liu et al., 2007). Furthermore, in humans, the relationship between serum SAA concentration and clinical stage of the tumor was elucidated (Ramankulov et al., 2008). Patients with metastatic lesion showed higher serum SAA concentration than patients without metastasis. In general, the clinical stage of tumors is related to the prognosis. Thus, if SAA may affect the tumor metastasis, the effects of SAA could contribute to the shorter prognosis in neoplastic diseases. In chapter 2, direct effects of recombinant feline SAA (rfSAA) protein to invasiveness of feline tumor cells were evaluated. As the indicator of invasiveness, matrix metalloproteinase-9 (MMP-9) expression and production were investigated and effects to the actual invasive capacity of cells were also estimated. As subjects for the study, mammary gland carcinoma cells and lymphoma cells were selected.

SAA is a precursor of AA fibrils, and sustained high SAA levels in the blood may

give rise to secondary amyloidosis. In addition to that, if SAA itself affects to the prognosis of diseases, to control the SAA concentration may become a therapeutic target. Although the biological kinetics, especially degradation of SAA are key factors to control SAA concentration, there are few reports described about the degradation of SAA (Lavie et al., 1978, Kinkley et al., 2006). In some previous studies, the relationship between SAA degradation and amyloidosis was discussed (Migita et al., 2001, Magy et al., 2007); however, the association with SAA concentration was yet described. Furthermore, in cats, there is no report described about degradation of SAA. In chapter 3, I examined about the SAA degradation in cats. Moreover, I also examined what kinds of stimulation or drug affect the phenomena.

Table 1. Diseases (n ≥ 3) included in this study

		SAA concentration (μg/ml)				Number of cats ≥ 0.82 μg/ml	
Diseases	n	Mean	Median	Min.	Max.	n	%
Acute pancreatitis	3	56.87	58.57	23.15	88.90	3	100
Feline infectious peritonitis	14	18.46	8.03	0.05	88.33	7	50
Diabetes melitus	8	14.89	0.44	0.00	80.55	3	38
Lymphoma	31	13.22	1.60	0.00	61.55	17	55
Cholangitis	10	13.08	0.55	0.00	79.28	4	40
Hyperthyroidism	7	12.09	0.58	0.05	64.20	3	43
Hepatitis	3	11.74	0.40	0.30	34.53	1	33
Malignant mesothelioma	6	10.42	1.68	0.50	48.50	5	83
Renal failure	11	9.52	2.15	0.00	40.70	7	64
Squamous cell carcinoma	9	6.62	0.20	0.00	32.65	4	44
Lang carcinoma	5	6.17	0.33	0.00	22.70	2	40
Otitis media	6	5.76	0.15	0.00	33.90	1	17
Polycystic kidney disease	3	5.30	0.50	0.50	14.90	1	33
Rhinitis	7	4.56	0.28	0.08	30.25	1	14
Nasal adenocarcinoma	6	4.10	0.15	0.00	24.00	1	17
Bronchitis	3	2.14	0.25	0.05	6.13	1	33
Gastroenteritis	17	2.00	0.15	0.00	29.00	3	18
Immune-mediated hemolytic anemia	5	1.46	0.20	0.00	4.45	2	40
Esophagostenosis	3	1.21	0.30	0.04	3.30	1	33
Cardiac myopathy	9	0.74	0.43	0.00	2.65	3	33
Stomatitis	3	0.44	0.23	0.10	1.00	1	33
Cystitis	10	0.38	0.23	0.00	1.15	2	20
Hydrocephalus	3	0.37	0.00	0.00	1.10	1	33
Epilepsy	13	0.15	0.05	0.00	0.60	0	0
Osteochondrodysplasia	4	0.15	0.13	0.00	0.35	0	0
Asthma	3	0.13	0.20	0.00	0.20	0	0
Mast cell tumor	8	0.07	0.00	0.00	0.35	0	0

Table 2. Study population and clinical characteristics of the cats included in prognostic analysis

	Non-elevated SAA group	Elevated SAA group	*P*
Cats	110	65	
Median age, years (range)	8 (0.30 - 18)	9 (0.25 - 21)	0.376
Median BW, kg (range)	3.85 (1.2 - 7.8)	3.45 (0.9 - 8.15)	0.047 *
Sex			0.456
Castrated male	49	29	
Intact male	15	4	
Spayed female	36	25	
Intact female	10	7	
Diagnostic category			0.202
Neoplastic disease	35	29	
Inflammatory disease	46	20	
Other disease	29	16	
Median SAA, μg/ml (range)	0.15 (0 - 0.80)	18.1 (0.83 - 88.3)	< 0.01 **

The chi-square test was used for categorical data. The Mann-Whitney U test was used for continuous data. * and ** are $P < 0.05$ and $P < 0.01$, respectively.

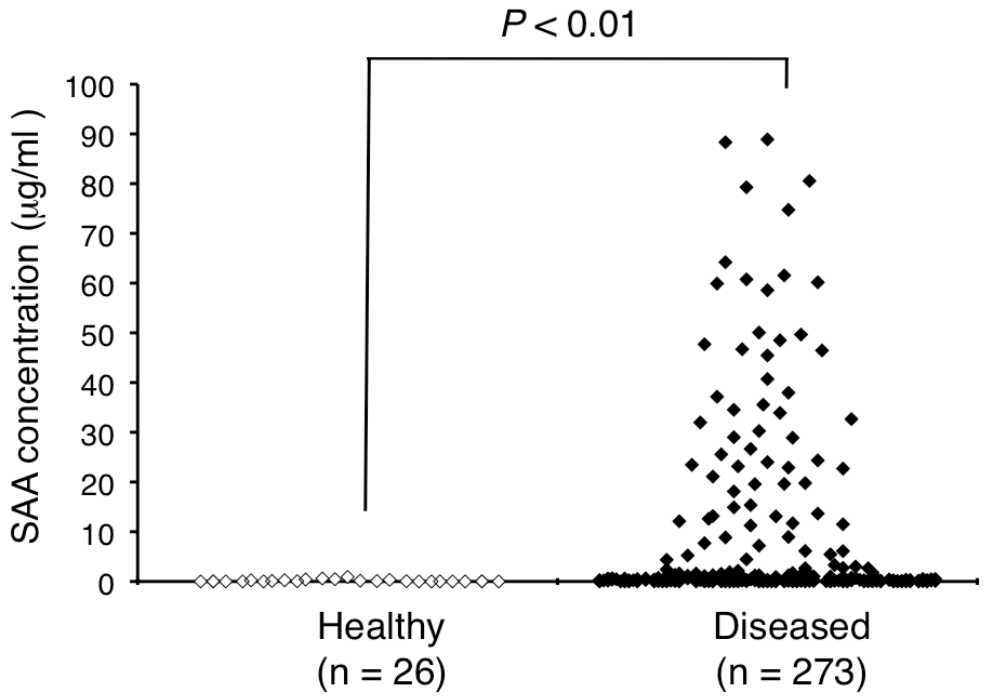

Fig. 1. Serum amyloid A (SAA) concentration in healthy cats (n = 26) and diseased cats (n = 273). SAA concentration was significantly increased in cats with various diseases (mean = 7.52 μg/ml, median [range]: 0.35 μg/ml [0.0–88.9 μg/ml]; $P < 0.01$) compared to that in healthy cats (0.14 μg/ml, 0.05 μg/ml [0.0–0.9 μg/ml]).

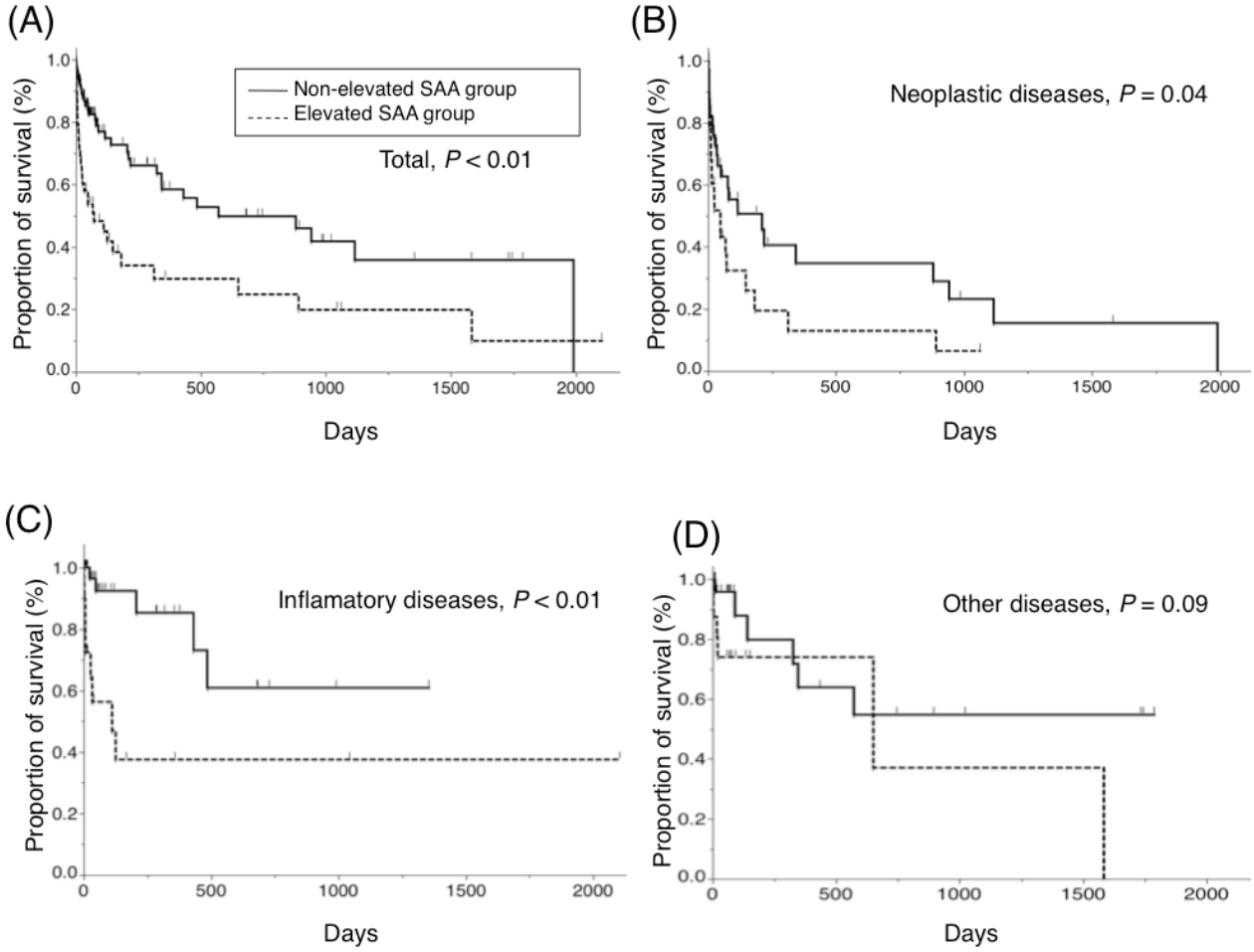

Fig. 2. Kaplan–Meier survival curves of cats categorized according to serum amyloid A (SAA) level. The non-elevated SAA group (< 0.82 μg/ml, solid line) and elevated SAA group (≥ 0.82 μg/ml, broken line) are shown. (**A)** Survival curves of total included cats. The significance of the difference between the curves was calculated by the log-rank test ($P < 0.01$). (**B)** Survival curves of cats in neoplastic diseases category. The significant difference between the curves was observed ($P = 0.04$). (**C)** Survival curves of cats in inflammatory diseases category. The significant difference between the curves was observed ($P < 0.01$). **(D)** Survival curves of cats in other diseases category. No significant difference between the curves was observed ($P = 0.09$).

Chapter 1

Serum amyloid A stimulates the TNF-α production of feline peripheral monocytes

Abstract

Serum amyloid A (SAA) is one of the major acute phase proteins and a biomarker of infection or inflammation in humans and cats. In humans, cytokine-like functions of SAA protein have been determined, and SAA is considered to be an important factor in immune responses. However, there are no reports about the functions of SAA protein in cats. In the present study, the functions of feline SAA protein on peripheral monocytes were investigated by using TNF-α production as an indicator. In feline peripheral blood monocytes, SAA protein stimulated the transcription of TNF-α within 2 h and induced TNF-α secretion in time- and dose-dependent manners. The production of TNF-α by SAA stimulation in feline monocytes was found to be mediated by the activation of nuclear factor-kappa B (NF-κB). Moreover, SAA-stimulated TNF-α production was prevented by a Toll-like receptor 4 (TLR4) antagonist. On the basis of these results, feline SAA was demonstrated to be an endogenous agonist of TLR4 for the stimulation of TNF-α production and secretion by peripheral monocytes. These results suggest that feline SAA can play an important role in the regulation of inflammation and immune responses as it does in humans.

1. Introduction

Serum amyloid A (SAA) is one of the major acute phase proteins (APPs) in mammals, including humans (Uhlar et al., 1999) and cats (Kajikawa et al., 1999). The acute phase response is a systemic reaction to infection and tissue injury as a biological defense, and as part of the acute phase response, APPs including SAA are rapidly synthesized predominantly in the liver (Kisilevsky et al., 1979). SAA gene expression and protein production by hepatocytes are stimulated by inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Weinstein et al., 1987, Jensen et al., 1998), or by bacterial products such as lipopolysaccharide (LPS) (Migita et al., 2004). The produced SAA protein is released into circulation, and the secretion of SAA into blood results in a marked increase in SAA concentration in the plasma. SAA concentration occasionally increases up to 1000-fold above the basal level (Kushner, 1988). SAA is also known as a precursor of amyloid A (AA) fibrils (Husby et al., 1988). Persistently high SAA levels in the blood may give rise to secondary amyloidosis, which is known as reactive AA amyloidosis (Lachmann et al., 2007).

Several studies revealed that SAA has cytokine-like activities in humans. SAA stimulated the production of cytokines such as TNF-α and IL-1β (Patel et al., 1998, Song et al., 2009), chemokines (Furlaneto et al., 2000, Lee et al., 2008), and matrix metalloproteinases by macrophages (Lee et al., 2005). SAA is also a potent chemoattractant for leukocytes such as neutrophils and monocytes (Badolato et al., 1994, Xu et al., 1995). These functions are mediated by some pattern recognition receptors (PRRs). Formyl peptide receptor-like 1 (FPRL1) is most frequently reported as a receptor that mediates SAA functions (Su et al.,

1999) There are many other PRRs identified as SAA receptors, such as CD36 (Baranova et al., 2010), scavenger receptor class B type 1 (SR-B1) (Cai et al., 2005), and Toll-like receptor 2 (TLR2) (Cheng et al., 2008). Recently, Toll-like receptor 4 (TLR4) was reported to be a novel SAA receptor (Sandri et al., 2008). TLR4 is a potential LPS receptor and considered to be an important factor for biological defense (Palsson-McDermott et al., 2004). Moreover, it was reported that SAA protein stimulated the activation of transcription factor nuclear factor-kappa B (NF-κB), which is considered to be one of the important factors for immune responses (Lee et al., 2008, Sandri et al., 2008). From these facts, it is estimated that SAA protein is not just a result of inflammation but also an important factor of the immune response in humans.

Because of the dramatic increase in plasma SAA concentration during inflammation, measurement of plasma SAA concentration is used as an inflammatory marker in humans (Nakayama et al., 1993, Hilliquin, 1995). Measurement of SAA concentration in blood has been used as a marker to evaluate the extent of disorder and the response to treatment in inflammatory diseases such as rheumatoid arthritis (Benson et al., 1979, Scheinberg et al., 1980). Furthermore, in patients with these diseases, median SAA concentration was significantly associated with prognosis (Gillmore et al., 2001). Thus, it seems that cytokine-like functions of SAA are important for the pathophysiologies of these diseases. As shown in chapter 0, SAA concentration is increased in various inflammatory and infectious diseases and measurement of SAA concentration was demonstrated as a prognostic marker also in cats. However, to the best of my knowledge, there is no report that describes the

function of feline SAA.

In humans, 2 types of SAA proteins, acute phase SAA (A-SAA) and constitutive SAA (C-SAA) were reported (Whitehead et al., 1992). C-SAA was described only in humans and mice (Whitehead et al., 1992, de Beer et al., 1994). A-SAA and C-SAA show several differences in terms of their expression pattern, amino acid sequence, and functions. A-SAA levels markedly increase during the acute phase response, whereas C-SAA levels do not show any increase. C-SAA contains an insert of 8 amino acids and shares only 55% identity with A-SAA (Whitehead et al., 1992). Furthermore, only A-SAA is a precursor of secondary amyloidosis (Uhlar et al., 1999) and shows cytokine-like activities (Kumon et al., 2002). Although feline SAA protein contains an insert of 8 amino acids as does human C-SAA, its expression pattern and amyloidogenesis are more similar to that of human A-SAA (Ohno et al., 1999). Moreover, feline SAA showed high homology with human A-SAA (81%), whereas only 59% with human C-SAA. Therefore, it can be considered that feline SAA is comparable to human A-SAA. However, it remains unclear whether feline SAA protein performs similar functions to human A-SAA because of the insert of 8 amino acids.

In this study, we investigated whether feline SAA protein functions in mediating the immune response as human A-SAA does by using TNF-α production as an indicator. In a previous report, it was found that SAA proteins incubated with macrophages were degraded within 6 h (Migita et al., 2001). Thus, it was considered difficult to evaluate the effect of SAA exactly with longer incubation. Because TNF-α increases most rapidly among the inflammatory cytokines (Song et al., 2009), TNF-α production was used as the indicator in

this study. Furthermore, we investigated the involvement of NF-κB and TLR4 in SAA-induced TNF-α production in cats.

2. Materials and Methods

2.1. Recombinant feline SAA (rfSAA)

Total cellular RNA was isolated from the liver of the cat administered lipopolysaccaride (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 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'-CACCTGGTATTCGTTCCTTGGTGAA-3', forward; 5'-TCAGTACTT GTCAGGCAGGC-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, USA) in which SAA could be expressed as a His-tag fusion protein. The plasmid vector was transformed in *Escherichia coli (E. coli)*, OverExpress C43 (DE3) Electrocompetent Cells (Lucigen, Middleton, WI, USA). The transformants were grown in Luria-Bertani medium, and expression of the recombinant protein was induced by culturing with 0.1 mM isopropyl beta-D-thiogalactosidase (IPTG;

Sigma-Aldrich) for 2 h at 37°C. Cultures were centrifuged at 10,000 *g* for 5 min at 4°C, and the cell pellets were stored at -80°C overnight. The expressed protein was purified using TALON Metal Affinity Resin (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The protein concentration of the SAA fraction was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The purification of rfSAA was analyzed by SDS-PAGE (12.5% SDS-polyacrylamide gel) and determined to be greater than 95% by using a software package (Image Lab Software; Bio-Rad Laboratories, Hercules, CA).

2.2. Cell preparation and culture

Feline peripheral blood mononuclear cells (PBMCs) were prepared from fresh, anticoagulated blood of healthy cats by performing Ficoll-Paque Plus (GE Healthcare, Buckinghamshire, UK) density gradient centrifugation. Prepared PBMCs were resuspended in RPMI 1640 (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and antibiotic-antimycotic solution containing penicillin, streptomycin, and amphotericin B (Sigma Aldrich). These cells were plated in a 48-well tissue culture-treated plate at a concentration of 1×10^6 cells/well and cultured at 37°C in 5% CO_2 overnight. The cells were then washed with culture medium to remove the nonadherent cells. Adherent monocytes were maintained in fresh RPMI 1640 containing 10% FBS and antibiotics.

2.3. Stimulation of feline monocytes for TNF-α production

Monocytes were stimulated with rfSAA (0.1–10 μg/mL) or LPS (0.1 μg/mL; Sigma Aldrich) for the indicated time period. For endotoxin control, rfSAA (10 μg/mL) or LPS (0.1 μg/mL) was incubated in fresh RPMI 1640 containing Polymyxin B (PMB, 25 μg/mL; Enzo Life Sciences, Farmingdale, NY) at room temperature for 1 h, after which, the mixtures were added to the monocytes. For pathway analysis of rfSAA, feline monocytes were incubated with NF-κB inhibitor (BAY-11-7082, 10 μM; Cayman Chemical, Ann Arbor, MI) for 30 min or TLR4 antagonist (LPS-RS, 10 μg/mL; InvivoGen, San Diego, CA) for 1 h before the addition of stimulants. To assess the effects of TLR4 antagonist, ultra-pure LPS from *E. coli* (LPS-EK, 0.1 μg/mL; InvivoGen) was used as a control. Cell-free supernatants of culture media were collected by centrifugation and stored at -80°C until analysis. Each experiment was performed at least 5 times by using different cats. The TNF-α concentration was measured by using a commercial ELISA kit for feline TNF-α (R&D Systems, Minneapolis, MN). Each assay was performed in duplicate.

2.4. RNA extraction and quantitative RT-PCR

Monocytes were stimulated with 10 μg/mL rfSAA for the indicated times (0, 2, 4, and 6 h) and then total cellar RNA was extracted by using a commercial kit (Illustra RNAspin Mini RNA Isolation Kit; GE Healthcare). Reverse-transcription was performed using the commercially available reverse transcriptase (ReverTra Ace qPCR RT Master Mix; TOYOBO, Tokyo, Japan), according to the manufacturer's instructions. For quantitative

RT-PCR, the cDNA was amplified with SYBR green (THUNDERBIRD SYBR qPCR Mix; TOYOBO) by using gene-specific primers and a thermal cycler (Thermal Cycler Dice Real Time System; Takara Bio, Shiga, Japan) with the following program: a 10 min preincubation at 95°C, 40 cycles of PCR (5 s at 95°C and 30 sec at 60°C), and dissociation (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). The sequences of the gene-specific primers were as given below: TNF-α sense primer (forward): 5′-GTGGAGCTGACAGACAACCA-3′, TNF-α antisense primer (reverse): 5′-GTGCACATGTGTGGAAGGAC-3′; GAPDH (forward): 5′-GCTGCCCAGAACATCATCC-3′, GAPDH (reverse): 5′-GTCAGATCCACGACGGAC AC-3′. The data were normalized relative to the GAPDH as an endogenous control. These primers were designed based on the previously reported sequences of feline TNF-α (NM_001009835) and feline GAPDH (NM_001009307). Quantification of mRNA transcription was performed using the comparative cycle threshold (Ct) method.

2.5. Immunofluorescence staining for NF-κB and TLR4

Monocytes (1×10^5 cells) were cultured in 4-well chamber slides (Nalge Nunc International, Penfield, NY) overnight. To investigate NF-κB activation, the cells were incubated with rfSAA (10 μg/mL) or LPS (0.1 μg/mL) for 1 h with or without 30 min pretreatment of 10 μM BAY-11-7082. To detect TLR4 cell surface expression, monocytes without any stimulation were prepared. After incubation, the culture medium was discarded, and the cells were washed 3 times with PBS. The cells were fixed in 4% paraformaldehyde (pH 7.4) for 10 min, washed with PBS, and permeabilized in 0.2% Triton X-100 for 10 min.

The cells and the slide surfaces were then blocked with 10% FBS in PBS at room temperature for 30 min. To detect the location of NF-κB, rabbit anti-p65 polyclonal Ab (Santa Cruz Biotechnology, Dallas, TX) was diluted in 10% FBS and added to each well. To detect the TLR4, mouse anti-TLR4 monoclonal Ab (clone HTA125; AbD Serotec, Oxford, UK) was used. After incubation for 1 h, the slides were washed with PBS and diluted Alexa Fluor 488-conjugated anti-rabbit IgG Ab or anti-mouse IgG Ab (Life Technologies, Grand Island, NY) was added to each well. To visualize the nucleus, 4′,6-diamidino-2-phenylindole (DAPI; Roche Applied Science, Indianapolis, IN) was added to the wells at the same time. After 1 h, the slides were washed and covered. The cells were visualized using the 40× objective on a fluorescence microscope.

2.6. Statistical analysis

Statistical analyses were performed using a statistical software package (JMP version 5.0.1J; SAS Institute, Cary, NC). Paired Student's t test was used to compare individual treatment with their respective control values. In all cases, $P < 0.05$ was considered to indicate significance. All data are expressed as mean ± SEM.

3. Results

3.1. TNF-α production in feline monocytes stimulated by rfSAA

To investigate the functions of rfSAA on monocytes, TNF-α production by feline peripheral blood monocytes was measured. The cells were incubated with or without rfSAA (10 μg/mL) for up to 6 h, and samples were obtained at various times (0, 2, 4, and 6 h). As shown in Fig. 1A, the TNF-α concentrations in culture medium without rfSAA remained stable over the entire incubation period. The TNF-α production induced by rfSAA became detectable after 2 h of stimulation and reached a peak at 4 h (Fig. 1A). The rfSAA-induced accumulation of TNF-α transcript was also examined and detected as early as 2 h after stimulation (Fig. 1B). Subsequently, the relationship between TNF-α production and rfSAA concentration was tested. As a result, rfSAA stimulated TNF-α production in a dose-dependent manner (Fig. 1C).

3.2. Effects of LPS on cell activation by rfSAA

Because the rfSAA was produced in *E. coli*, there was a possibility that rfSAA was contaminated by LPS. Therefore, rfSAA and LPS were incubated with PMB before adding each to monocyte culture. PMB is an antibiotic that blocks the biological effects of LPS by binding to lipid A, the toxic component of LPS (Ha et al., 1985), and it is considered a specific inhibitor of LPS (Jacobs et al., 1977). Although PMB significantly inhibited the TNF-α production by LPS, the effect of rfSAA was not prevented by PMB (Fig. 2).

3.3. TNF-α production by rfSAA in feline monocytes via NF-κB activation

To investigate the effect of rfSAA on NF-κB, an immunofluorescence assay of NF-κB p65 was performed. The localization of p65 was detected as a green signal, and the nucleus was detected as a blue signal. In the negative control, the cytoplasm of monocytes were uniformly stained with a green signal (Fig. 3A). With rfSAA stimulation, p65 translocated to the nucleus, and the phenomenon was inhibited by BAY-11-7082 (NF-κB inhibitor) (Fig. 3A). The same effect was observed by LPS stimulation. Subsequently, TNF-α production by stimulated monocytes with or without BAY-11-7082 pretreatment was detected, and the production of cytokines was significantly prevented by BAY-11-7082 pretreatment (Fig. 3B).

3.4. Effect of rfSAA in feline monocytes via TLR4

To determine the involvement of TLR4 in cytokine production by rfSAA, LPS-RS (TLR4 antagonist) was used. Before testing the effect of LPS-RS, the expression of TLR4 on the surface of feline monocytes was examined by immunofluorescence analysis. The results indicated that TLR4 was distributed sparsely on the surface of feline monocytes (Fig. 4A). The cells were preincubated with LPS-RS (10 μg/mL) and then stimulated with rfSAA (10 μg/mL) or ultra-pure LPS (LPS-EK; 0.1 μg/mL) for 4 h. As per LPS-RS alone, there was slight production of TNF-α, but the effect of rfSAA on TNF-α production was completely prevented (Fig. 4B). The effect of LPS-EK on TNF-α production was also completely inhibited by LPS-RS pretreatment (Fig. 4B).

4. Discussion

In the present study, we investigated the effect of feline SAA protein on TNF-α production from feline peripheral monocytes. It was observed that rfSAA stimulated TNF-α production in time- and dose-dependent manners. The production of TNF-α by feline SAA protein was observed to be mediated via TLR4 and NF-κB activation which are downstream components of TLR4 signaling. Thus, to the best of my knowledge, this report provides the first evidence that feline SAA protein is a potent endogenous TLR4 agonist.

The signaling mechanisms responsible for SAA-induced cytokine production in cats were examined in this study. NF-κB is a protein complex that controls the transcription of DNA, such as for genes encoding the inflammatory cytokines and the downstream components of TLR4 signaling. NF-κB family includes several proteins and p65 (RelA) is one of the major components of NF-κB (Kaufman et al., 1992). Without stimulation, p65 is localized at the cytoplasm. When an activation signal such as LPS and cytokines were added, p65 translocated to the nucleus and controlled the transcription of DNA. In my study, NF-κB activation was visualized by immunofluorescence microscopy and rfSAA induced NF-κB activation. Furthermore, a NF-κB inhibitor blocked the SAA-stimulated NF-κB activation and TNF-α production. Thus, the activation of NF-κB by rfSAA was considered critical for cytokine production by feline monocytes.

The present study showed that the effects of feline SAA protein on monocytes were completely inhibited by a TLR4 antagonist, LPS-RS. LPS affects inflammatory cells induced by TLR4 and other adapter proteins (Palsson-McDermott et al., 2004). LPS-RS is LPS from

the photosynthetic bacterium *Rhodobacter sphaeroides* and does not induce TLR4 signaling (Golenbock et al., 1991, Coats et al., 2005). Since LPS-RS is a TLR4 antagonist but a TLR2 agonist, it was considered that slight production of TNF-α with LPS-RS alone was induced by TLR2 signaling. Except for the effect of LPS-RS itself, cytokine production by feline SAA and LPS stimulation were completely inhibited by pretreatment with LPS-RS. Thus, the effects of feline SAA protein on monocytes was mediated by TLR4 signaling.

In a previous report, it was demonstrated that the effects of human SAA protein on human macrophages was inhibited to about 50% by blocking the functions of TLR4 with anti-TLR4 Ab (Niemi et al., 2011). The remaining 50% of SAA functions seemed to be mediated by TLR2 and other receptors (Niemi et al., 2011). In this study, the results suggest the involvement of TLR2 in the functions of feline SAA protein on monocytes. However, the specific effects of TLR2 were not examined in this study. In humans, there are many other receptors described as SAA receptors: FPRL1 (Su et al., 1999, He et al., 2003, Bjorkman et al., 2008), SR-B1 (Cai et al., 2005), and CD36 (Baranova et al., 2010). However, the involvement of these receptors was not examined in this study. Thus, the participation of these receptors on feline SAA-stimulated cytokine production should be examined.

It has been reported that feline SAA protein has relatively high homology with human A-SAA (Ohno et al., 1999, van Rossum et al., 2004). In addition, from other characteristics such as expression in acute phase and amyloidogenesis, it can be considered that feline SAA is compared to human A-SAA. In the present study, it was revealed that the function of feline SAA protein is very similar to that of human A-SAA. The feline SAA

protein contains an insert of 8 amino acids compared with the human A-SAA sequence, as does human C-SAA, and similar findings have been reported for the SAA of cow (Rossevatin et al., 1992), horse (Sletten et al., 1989), and dog (Sellar et al., 1991). Although the 8 amino acid insert has undetermined effects, from the results of the present study, the inserts may have no effect on the functions of SAA protein. However, it is possible that the inserts affect the affinity for receptors or the strength of the functions.

In conclusion, to the best of our knowledge, this is the first report to demonstrate that feline SAA is an endogenous agonist of TLR4 for stimulating TNF-α production and secretion by peripheral monocytes. These results suggest that feline SAA can play an important role in the regulation of inflammation and immune responses, as has been reported for human A-SAA. Further investigation is needed to determine the relationship between further functions of feline SAA and the pathophysiologies of each disease.

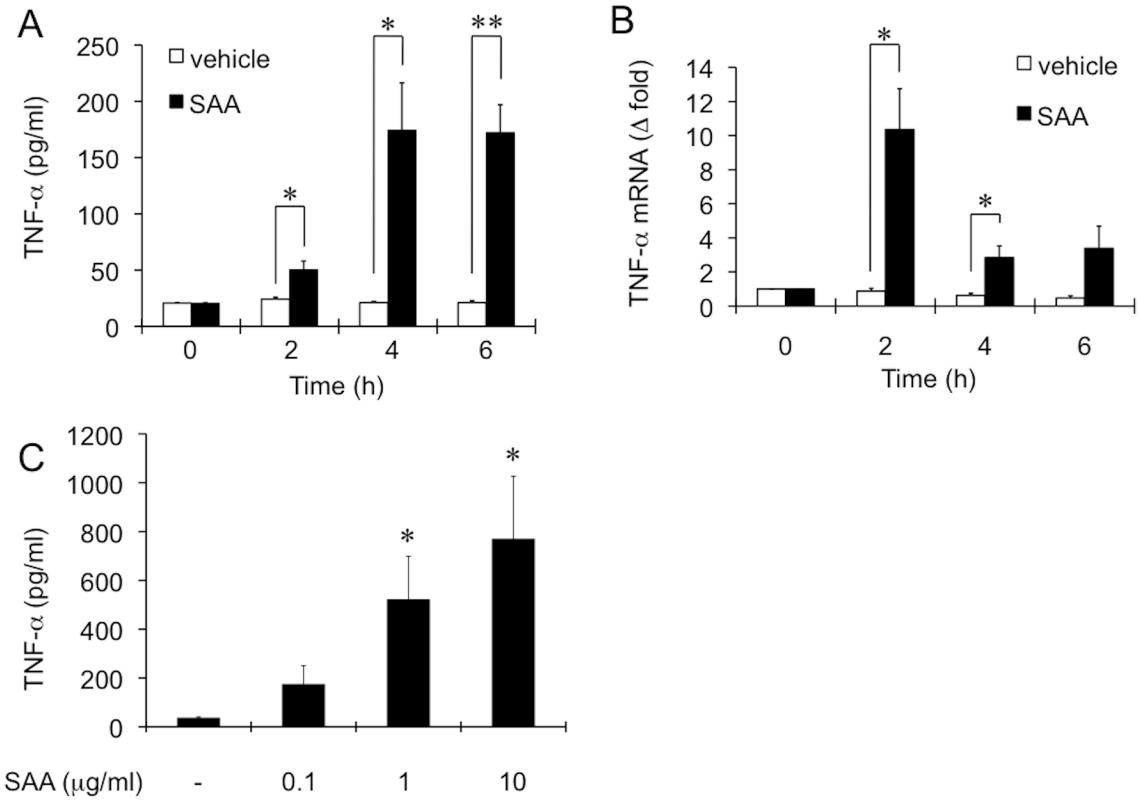

Fig. 1. Recombinant feline SAA stimulates secretion and transcription of TNF-α. **(A)** Induction of TNF-α secretion in rfSAA-stimulated feline peripheral monocytes. The cells were incubated with or without 10 μg/mL rfSAA at 37°C for the indicated time period. The concentration of secreted TNF-α was measured by ELISA. **(B)** Time-dependent changes in TNF-α transcript level in rfSAA-stimulated feline peripheral monocytes. Total RNA was extracted from the cells at the end of each treatment. Real-time RT-PCR was performed, and the relative concentrations of the TNF-α transcript are presented as fold changes. **(C)** Dose-dependent changes in TNF-α secretion in rfSAA-stimulated feline peripheral monocytes. The cells were stimulated by various concentrations of rfSAA at 37°C for 4 h. The concentration of secreted TNF-α was measured by ELISA. The data in this figure represent the mean ± SEM of 5 or 6 independent experiments. *, $P < 0.05$, **, $P < 0.01$, compared with the values obtained from the control (vehicle treated).

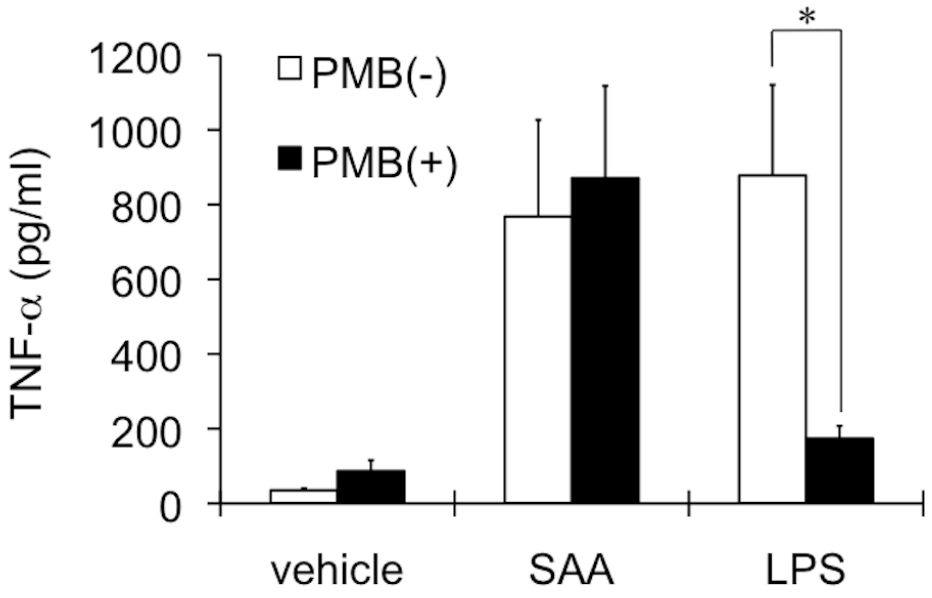

Fig. 2. Effects of LPS in rfSAA-stimulated TNF-α production. LPS from *E. coli* (0.1 μg/mL) or rfSAA produced by *E. coli* were incubated with or without 25 μg/mL polymyxin B (PMB) at room temperature for 1 h before application. Feline peripheral monocytes were stimulated with PMB-incubated rfSAA or LPS at 37°C for 4 h. The concentration of secreted TNF-α was measured by ELISA. The data in this figure represent the mean ± SEM of 6 independent experiments. *, $P < 0.05$, compared with the values obtained from the control (without PMB).

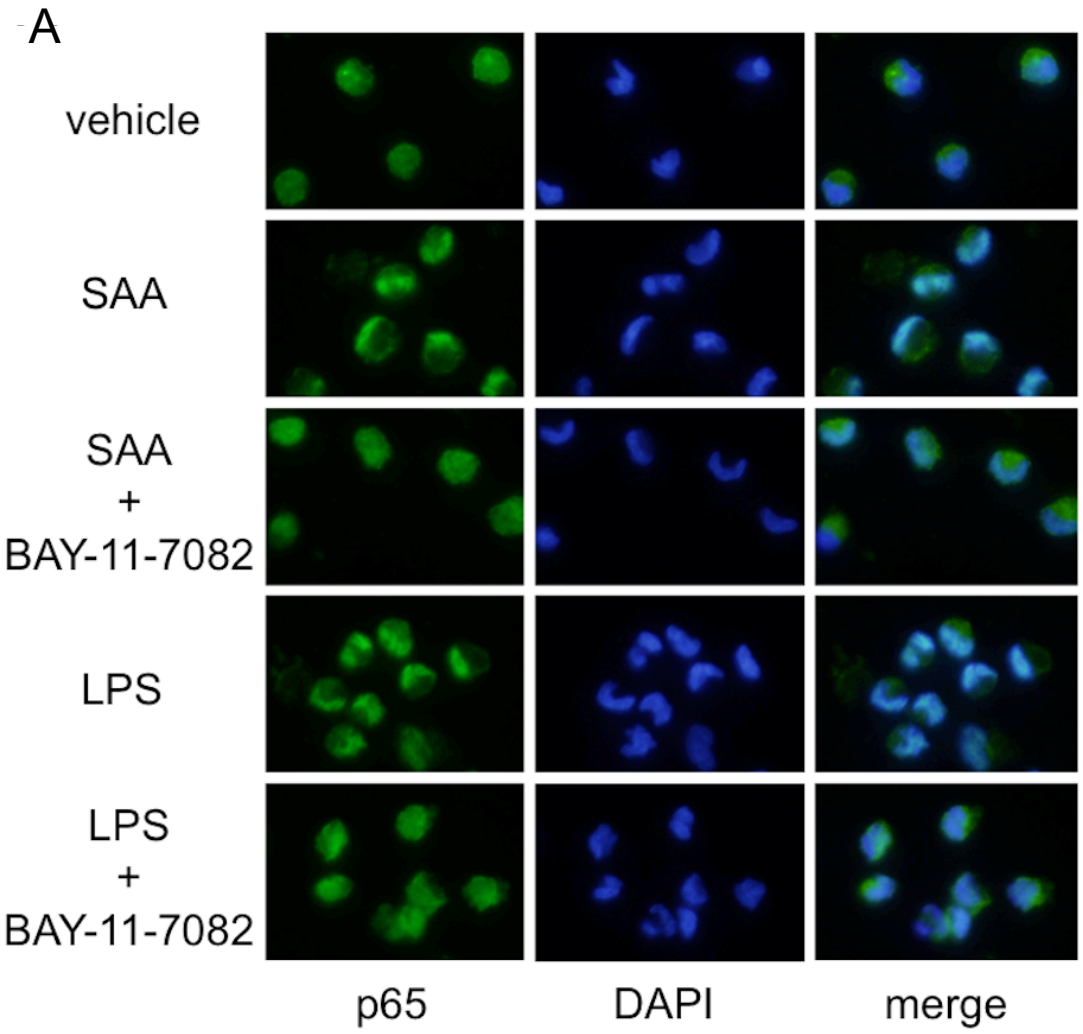

Fig. 3. rfSAA stimulates NF-κB activation, and the activation is required for rfSAA-induced TNF-α production. **(A)** Feline monocytes were stimulated with 10 μg/mL rfSAA or 0.1 μg/mL LPS at 37°C for 1 h with or without pretreatment by NF-κB inhibitor, BAY-11-7082 (10 μM, 30 min, 37°C). The altered localization of NF-κB p65 was detected by immunofluorescence analysis that was considered as an indicator of NF-κB activation. The localization of p65 was detected as a green signal and activated p65 translocated from the cytoplasm to the nucleus. DAPI (blue signal) was used to counterstain the nucleus. Representative photos of each experiment are shown.

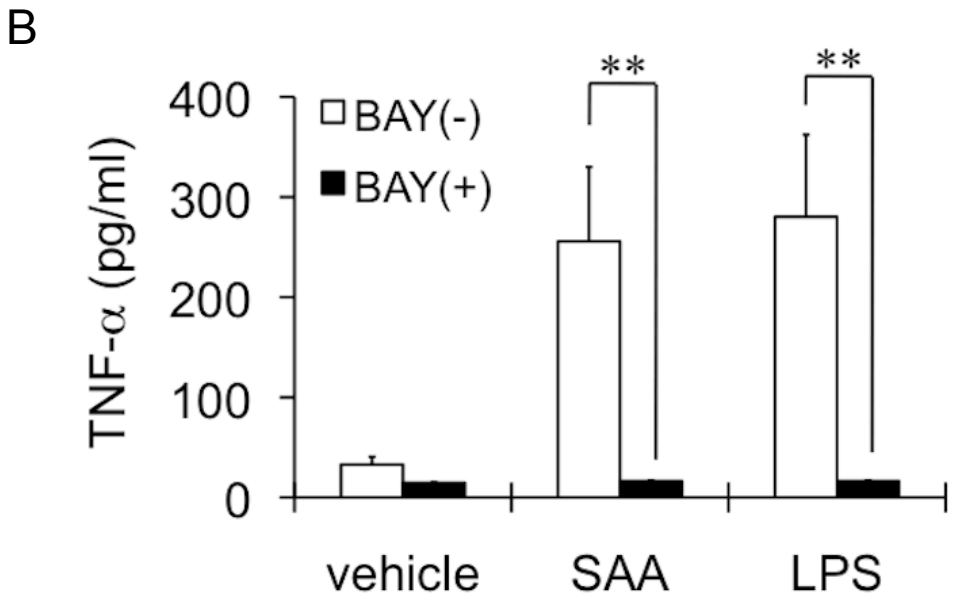

Fig. 3. rfSAA stimulates NF-κB activation, and the activation is required for rfSAA-induced TNF-α production. **(B)** Feline monocytes were incubated with or without 10 μM BAY-11-7082 at 37°C for 30 min before stimulation. The cells were stimulated with 10 μg/mL rfSAA or 0.1 μg/mL LPS at 37°C for 4 h, and the concentration of secreted TNF-α was measured by ELISA. The data represent the mean ± SEM of 6 independent experiments. **, $P < 0.01$, compared with the values obtained from the control (without BAY-11-7082).

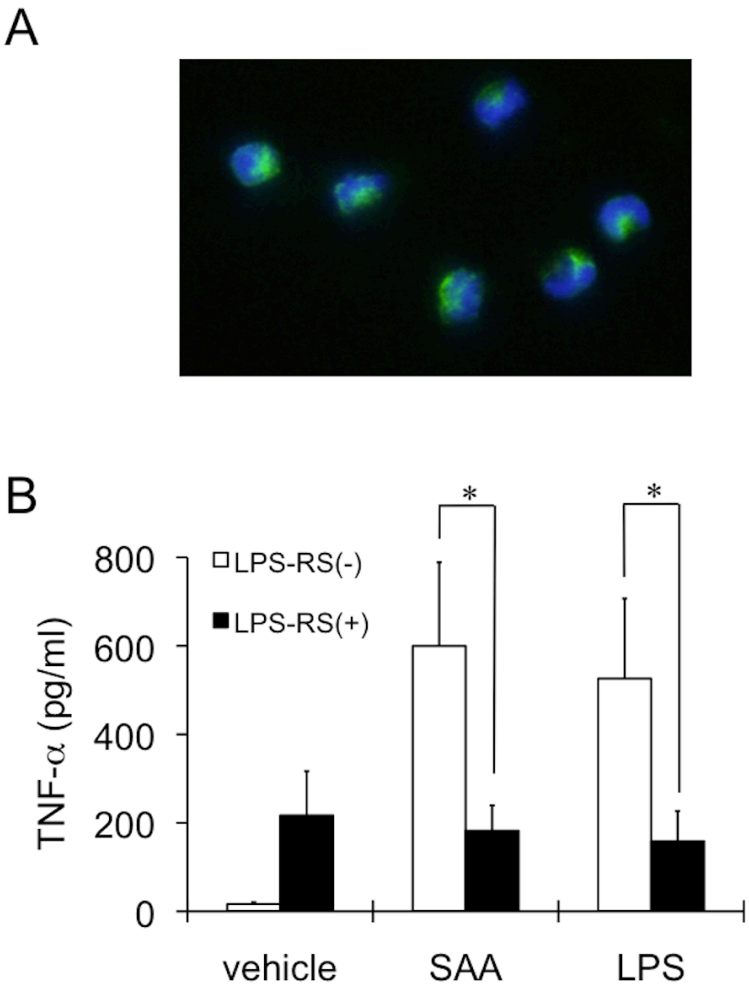

Fig. 4. TLR4 mediated the functions of rfSAA on feline monocytes. **(A)** Cell-surface expression of TLR4 in feline peripheral monocytes was detected by immunofluorescence analysis by using anti-TLR4 Ab. TLR4 was detected as a green signal, and nuclei were detected as a blue signal by DAPI. Representative photo is shown in this figure. **(B)** Feline monocytes were incubated with or without 10 μg/mL TLR4 antagonist, LPS-RS at 37°C for 1 h before stimulation. The cells were stimulated with 10 μg/mL rfSAA or 0.1 μg/mL ultra-pure LPS (LPS-EK) at 37°C for 4 h, and the concentration of secreted TNF-α was measured by ELISA. The data represent the mean ± SEM of 6 independent experiments. *, $P < 0.05$, compared with the values obtained from the control (without LPS-RS).

Chapter 2

Serum amyloid A promotes the invasion of feline tumor cells

Chapter 2-1

Serum amyloid A promotes the invasion of feline mammary carcinoma cells

Abstract

The serum amyloid A (SAA) concentration is higher in mammary tumors with metastases. Thus, the role of SAA in the progression of neoplastic diseases such as mammary tumors is of current interest. In the present study, the direct effects of recombinant feline SAA (rfSAA) protein on invasiveness of feline mammary carcinoma cells were evaluated. As an indicator of invasiveness, matrix metalloproteinase-9 (MMP-9) expression and production were investigated in 4 feline mammary carcinoma cell lines of different origin. In 2 of 4 cell lines, MMP-9 mRNA expression was significantly increased by rfSAA stimulation. Secreted MMP-9 protein in culture media was determined by gelatin zymography, and clear bands of MMP-9 were detected in all 4 cell lines with rfSAA stimulation. A significant increase in semi-quantified MMP-9 levels was observed in 3 of 4 cell lines. The invasive capacities of feline mammary carcinoma cells assessed by matrigel transwell assay showed that rfSAA stimulated cell invasiveness in 2 of 4 cells. Although the extent of responses with rfSAA varied with cell line, the results collectively showed that rfSAA can stimulate invasiveness of feline mammary carcinoma cells. The findings of this study have identified a novel role for SAA in mammary tumorigenesis, and suggest that therapeutic strategies targeting SAA may provide new alternatives in treating tumor invasion and metastasis.

1. Introduction

Serum amyloid A (SAA) is one of the major acute phase proteins (APPs) in mammals, including humans (Uhlar et al., 1999) and cats (Kajikawa et al., 1999). Because of the dramatic increase in serum SAA concentration during inflammation, serum SAA concentration is used as an inflammatory marker in humans and cats (Nakayama et al., 1993). Elevated SAA concentration has been described in various inflammatory and infectious diseases (Hilliquin, 1995). Increased SAA concentration has also been demonstrated in humans with neoplastic diseases (Cho et al., 2010, Wang et al., 2012). Furthermore, the relationship between serum SAA concentration and clinical stage of the tumor has also been shown in earlier studies (Ramankulov et al., 2008). Patients with tumor metastasis have a higher serum SAA concentration than do patients without metastasis. Thus, SAA is also considered as a useful biomarker in monitoring the progression of neoplastic diseases. Besides of the potential of SAA as cancer biomarker, the role of SAA in the progression of neoplastic diseases is of current interest.

An acute immune response may cause an increased risk of peripheral metastases (Hobson et al., 2013). On the other hand, cytokine-like functions of SAA protein were demonstrated in both humans (Song et al., 2009) and cats as shown in chapter 1. SAA protein stimulates the production of various cytokines by macrophages and can play an important role in acute immune response. Therefore, elevated SAA concentration can stimulate tumor metastasis indirectly. The human SAA protein also stimulates matrix metalloproteinase-9 (MMP-9) production by macrophages (Lee et al., 2005). Matrix metalloproteinases (MMPs)

are a family of extracellular matrix degrading proteases that are zinc-dependent and associated with invasion and metastasis during tumor progression because of their ability to degrade extracellular matrix and basement membrane (Stamenkovic, 2000). Type IV collagen is one of the integral components of the basement membrane, and its collagenase, MMP-9, is believed to play a key role in tumor invasion and metastasis (Sehgal et al., 1998). Although these indirect effects of SAA protein in tumor progression have been relatively well-evaluated, there is limited information about the direct activities of SAA protein on the invasiveness of tumor cells (Knebel et al., 2013).

Mammary tumors are common neoplasms in cats, and feline mammary carcinoma shows an age-dependent incidence, histopathology features, and metastasis pattern similar to human breast cancer (MacEwen, 1990). Moreover, human breast cancer and feline mammary carcinoma share the similar biological features at the molecular level (De Maria et al., 2005). Thus, feline mammary carcinoma could be a suitable animal model for human breast cancer. In human patients with breast cancer, elevated serum SAA concentration has been described and the SAA concentration has also been correlated with the stage of the cancer (Zhang et al., 2012). Therefore, it is suggested that SAA affects the progression and metastasis of breast cancer in a direct or an indirect way.

In this chapter, the direct effects of recombinant feline SAA (rfSAA) protein on the invasiveness of feline mammary carcinoma cells were evaluated. As the indicator of invasiveness, MMP-9 mRNA expression and protein synthesis were investigated in 4 feline mammary carcinoma cell lines of different origins. The effect of SAA on the actual invasive

capacity of these cells was also estimated.

2. Materials and Methods

2.1. Cell culture

Feline mammary tumor cell lines (FKN-p, FNN-m, FON-p, and FON-m) were kindly provided by the Laboratory of Veterinary Surgery, the University of Tokyo. Cell lines, FKN-p and FON-p, were established from primary lesions, and FNN-m and FON-m were established from metastatic lesions in feline patients bearing spontaneous mammary tumors. FON-p and FON-m were established from the same patient. Details of each of the cell lines were described previously (Uyama et al., 2005). All cell lines were grown in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Nacalai Tesque, Kyoto, Japan). Cells were maintained at 37°C in 5% CO_2.

2.2. Recombinant feline SAA

The synthesis of rfSAA was carried out as described in chapter 1. The synthesized rfSAA was desalted by using a commercial desalting column (PD-10 Desalting Columns; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The purity of rfSAA was analyzed by SDS-PAGE (12.5% SDS-polyacrylamide gel) and was determined to be greater than 95% by using a software package (Image Lab Software; Bio-Rad Laboratories, Hercules, CA).

2.3. Sequence analysis of feline MMP-9

Feline peripheral monocytes were prepared according to a previously described method in chapter 1. 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. Total cellular RNA was extracted by using a commercial kit (Illustra RNAspin Mini RNA Isolation Kit; GE Healthcare). Reverse transcription was performed using a commercially available reverse transcriptase (ReverTra Ace qPCR RT Master Mix; TOYOBO, Tokyo, Japan), according to the manufacturer's instructions. Template cDNA was amplified by PCR using Taq polymerase (AmpliTaq Gold 360 Master Mix; Applied Biosystems, Foster City, CA) with the following primer pair: 5′-AACCACCACCACACCT GAAT-3′ (forward) and 5′-CAAAGGTCACGTAGCCCACT-3′ (reverse). These primers were designed based on the predicted sequences of feline MMP-9 (GenBank accession number, XM_003983412). The PCR cycles were as follows: pre-denaturing (95°C for 10 min); 30 cycles of denaturation (95°C for 30 sec), annealing (57°C for 30 sec), and extension (72°C for 45 sec); and final extension (72°C for 7 min). The PCR products were electrophoresed through a 2% agarose gel and purified from the gel by using a commercially available kit (Wizard SV Gel and PCR Clean-Up System; Promega Corp., Madison, WI). The purified PCR products were directly sequenced by the dideoxy chain termination method using an ABI prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Gene specific primers described above were used for sequencing. The analysis was performed

in triplicate to avoid errors in sequence analysis. The sequence matched in all 3 analysis were adopted.

2.4. Stimulation of cell lines by rfSAA

Subconfluent (70–80% confluent) cells were detached and resuspended at 1×10^6 cells/ml in serum-free media (RPMI 1640 containing 1% bovine serum albumin; BSA), and added in 100 μl aliquots to a 96-well plate. The cells were incubated for 24 h at 37°C in 5% CO_2. After incubation, culture media were replaced and cells were stimulated with rfSAA (1, 5, and 25 μg/ml) for 24 h. Cells and cell-free supernatants of culture media were collected separately by centrifugation and stored at -80°C until analysis. Each experiment was performed in duplicate and repeated 3 times in separate experiments.

2.5. RNA extraction and quantitative RT-PCR

Total cellular RNA was extracted from the cells and reverse transcription was performed as described above. For quantitative RT-PCR, the cDNA was amplified with SYBR green (THUNDERBIRD SYBR qPCR Mix; TOYOBO) by using gene-specific primers and a thermal cycler (Thermal Cycler Dice Real Time System; Takara Bio, Shiga, Japan) with the following program: a 10 min preincubation at 95°C, 50 cycles of PCR (5 s at 95°C and 30 s at 60°C), and dissociation (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). The reactions were performed with primers specific to feline MMP-9: 5′-GCCCCTACAGTG TCTTTGGA-3′ (forward), 5′-TCCCATCCTTGAAGAAATGC-3′(reverse) or

glyceraldehye-3-phosphate dehydrogenase (GAPDH: NM_001009307): 5′-GCTGCCCAGA ACATCATCC-3′ (forward), 5′-GTCAGATCCACGACGGACAC-3′ (reverse). Data were normalized relative to GAPDH as an endogenous control. Quantification of mRNA transcription was performed using the comparative cycle threshold (Ct) method. Each sample was assessed in duplicate.

2.6. Gelatin zymography

MMP-9 levels in culture media were examined by gelatin zymography. Samples were diluted in sample buffer (125 mM Tris-HCl pH 6.8, 25% glycerol, 5% SDS, 0.2% bromophenol blue) and a dilution containing an equal volume of culture media was subjected to electrophoresis on a 10% SDS-PAGE gel co-polymerized with 0.1% gelatin. Following electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 2 h at room temperature and incubated in enzymatic activation buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.6) for 24 h at 37°C with gentle shaking. The gels were stained with 0.25% Coomassie Brilliant Blue R-250, 50% methanol, and 5% acetic acid for 30 min, and then de-stained in 5% methanol and 7% acetic acid for 1 h. A commercially available zymography marker (Gelatin Zymo MMP Marker; Life Laboratory, Yamagata, Japan) was run on each gel as a positive control. MMP-9 levels were assessed on the basis of gelatinolytic activity, indicated as clear bands against the dark blue background. All gels were analyzed with an imaging analyzer system (Cool Saver version 1.0; ATTO, Tokyo, Japan) and software (CS Analyzer version 2.0; ATTO). To obtain a semi-quantitative value for each sample, the imaging

assessment value of each unknown band was compared with the value of a MMP-9 standard band. The ratio of unknown to standard was calculated, and an arbitrary unit (a.u.) value was assigned to each sample. Each sample was assessed in duplicate.

2.7. Invasion assay

Invasion of cell lines was assessed by the matrigel transwell assay (Knebel et al., 2013). For the analysis, 24-well chambers with 8-μm pore filters (Chemotaxicell; Kurabo, Osaka, Japan) were coated on the upper surface with matrigel (BD Matrigel matrix; Becton–Dickinson, Franklin Lakes, NJ). Matrigel serves as a reconstituted basement membrane *in vitro*. By coating the upper surface of membrane, matrigel occludes the pores of the membrane and blocks non-invasive cells from migrating through the membrane. In contrast, invasive cells secrete proteases that degrade matrigel and enable invasion through the membrane pores. Cells (7×10^4/well) in 200 μl of serum-free medium (RPMI 1640 containing 1% BSA) were added to the upper chamber with rfSAA (1, 5, and 25 μg/ml). The lower wells were filled with 600 μl of media (RPMI 1640 containing 10% FBS). After 24 h, the cells that invaded the matrigel and reached the lower surface of the filter were fixed in methanol, and stained with Wright-Giemsa solution. The upper surface of the filters were scraped twice with cotton swabs to remove non-invading cells. The number of invading cells was counted in 5 high-power (200× magnification) microscope fields per filter, and the ratio of rfSAA stimulated cells to control was calculated. Each experiment was performed in duplicate and repeated 3 times in separate experiments.

2.8. Statistical analysis

Statistical analyses were performed using a statistical software package (JMP version 5.0.1J; SAS Institute, Cary, NC). Student's t test was used to compare individual treatment with their respective control values. In all cases, $P < 0.05$ was considered to indicate significance. All data are expressed as mean ± SEM.

3. Results

3.1. Partial sequence of feline MMP-9

The partial sequence of feline MMP-9 cDNA was determined (GenBank accession number, AB858226). The amino acid sequence of the feline MMP-9 deduced from the partial cDNA sequence determined in this study was shown to have 77%, 66% and 87% identity with those of human, mouse, and dog counterparts, respectively (data not shown).

3.2. Changes in MMP-9 mRNA expression with rfSAA stimulation

The basal level of MMP-9 mRNA expression was significantly higher in FON-p than those in other 3 cell lines (Fig. 1). MMP-9 mRNA expression in FON-p was at least 10 times greater than that of the other 3 cell lines. In FKN-p and FNN-m cell lines, MMP-9 mRNA expression after rfSAA stimulation increased significantly in a dose-dependent manner (Fig. 1). In FON-p, MMP-9 expression was significantly decreased with 1 μg/ml and 5 μg/ml treatment with rfSAA. However, FON-p constantly expressed more MMP-9 mRNA than other 3 cell lines (Fig. 1). Although dose-dependent increase of MMP-9 expression was observed in FON-m cells, statistical significance was not reached (Fig. 1).

3.3. Changes of MMP-9 levels in culture media with rfSAA stimulation

MMP-9 levels in culture media were examined by gelatin zymography and a semi-quantitative value of MMP-9 levels for each sample was calculated. Relatively high level of MMP-9 was observed in the FON-p cell line with no stimulation (Fig. 2A), and there

was significant difference between FON-p and the other 3 cell lines in basal MMP-9 activity (Fig. 2B). In all 4 cell lines, clear bands of MMP-9 were detected by gelatin zymography with 1–25 μg/ml of rfSAA stimulation (Fig. 2A). In FKN-p, FNN-m, and FON-p cell lines, MMP-9 levels increased significantly in a dose-dependent manner (Fig. 2B). Although a dose-dependent increase in MMP-9 level was observed in FON-m cells, statistical significance was not reached (Fig. 2B).

3.4. Tumor cell invasion assay

The invasiveness of tumor cells was assessed by the matrigel transwell assay. FNN-m cells showed relatively higher invasiveness than the other 3 cell lines with no stimulation (Fig. 3A). The number of invading cells increased with rfSAA stimulation in FNN-m, FON-p, and FON-m cell lines (Fig. 3A). In FKN-p cells, however, no difference was observed with or without rfSAA stimulation (Fig. 3A). Moreover, invasion of cells decreased significantly with 5 μg/ml of rfSAA in FKN-p (Fig. 3B). In FNN-m and FON-m cells, cell invasion increased significantly in a dose-dependent manner. Invasions of both cells after 24 h increased more than twice with rfSAA stimulation. Although, a dose-dependent increase in invasion was observed, statistical significance was not reached in FON-p cells (Fig. 3B).

4. Discussion

In the present study, the direct effects of rfSAA on tumor cells were demonstrated. Feline mammary carcinoma cell lines expressed and produced MMP-9 with rfSAA stimulation in a dose-dependent manner. rfSAA also stimulated the actual invasion of the cells through the extracellular matrix component. These findings show that feline SAA may have a role in tumor progression and metastasis. The association between SAA and tumor progression was indicated by several previous clinical studies (Ramankulov et al., 2008, Cho et al., 2010, Wang et al., 2012) and *in vitro* studies (Lee et al., 2005) in humans. SAA also stimulates inflammatory responses (Song et al., 2009) and inflammation is considered as one of the important factors for tumor progression (Hobson et al., 2013). However, these previous studies focused on the indirect effects of SAA on tumor progression while the direct effects remain unexplored.

Although direct effects of SAA such as MMP-9 production and tumor cell invasion were previously reported in 2 human glioma cell lines (Knebel et al., 2013), contradictory effects of SAA were shown in the study. SAA stimulated tumor cell invasion in one cell line; however, tumor cell invasion was significantly suppressed by SAA in the other cell line. In the present study, increased MMP-9 production or increased tumor cell invasion with rfSAA stimulation were observed in 4 feline mammary carcinoma cell lines. The suppressive effect of rfSAA in the cells was almost imperceptible. In humans and animals with mammary tumors, it has been observed that serum SAA concentration is higher in patients with metastasis (Tecles et al., 2009, Zhang et al., 2012). Furthermore, both SAA and MMP-9 were

described as prognostic markers in human patients with breast cancer (Pierce et al., 2009, Sung et al., 2012). Thus, the findings that rfSAA stimulated, not suppressed, MMP-9 production and facilitated invasion of mammary tumor cells are considered as reasonable outcomes.

MMP-9 mRNA expression and protein synthesis were increased in FKN-p cells with rfSAA stimulation. However, rfSAA could not stimulate tumor cell invasion in these cells. Many other proteinases and their inhibitors may be involved in tumor cell invasion, and the expression of the inhibitor, such as tissue inhibitor of metalloproteinases (TIMPs), might suppress cell invasion (Woessner, 1991). Although TIMPs were also described as important factors in the prognosis of breast cancer (Schrohl et al., 2003, Wurtz et al., 2005), the expression of TIMPs was not examined in this study.

FNN-m cells were established from cells in the pleural fluid of a feline patient with thoracic metastasis (Uyama et al., 2005). The cells showed relatively higher invasiveness than the other 3 cell lines with or without rfSAA, and MMP-9 mRNA expression and protein synthesis increased significantly in the presence of rfSAA. Cell lines that had originally high invasive or metastatic capacity might be more susceptible to SAA.

FON-p cells showed unique features compared with the other 3 cells. MMP-9 mRNA expression and protein synthesis in these cells without rfSAA stimulation were significantly higher than in the other cells. Although MMP-9 mRNA expression was suppressed with 1 or 5 mg/ml rfSAA, MMP-9 protein synthesis was stimulated significantly with rfSAA. As MMP-9 mRNA was expressed highly without stimulation, there is a

possibility that MMP-9 mRNA expression was increased more rapidly with rfSAA stimulation and decreased over time.

FON-m cells were established from the same patient as FON-p (Uyama et al., 2005); however, these cells showed different responses to SAA. Although a dose-dependent increase in MMP-9 production was observed in FON-m, the changes were smaller than those observed for FON-p and statistical significance was not reached. The colonized cells after metastasis might be less susceptible to SAA.

SAA protein is mainly synthesized in the liver and released into the blood stream. In humans, it was described that SAA was expressed in various tumor tissues, such as squamous cell carcinoma (Shinriki et al., 2010) and ovarian epithelial tumor (Urieli-Shoval et al., 2010). Moreover, tumor cells themselves can produce SAA protein (Kovacevic et al., 2006). Therefore, in addition to the systemic increase in SAA concentration, the local expression of SAA might affect the tumor cells and their invasiveness through autocrine or paracrine effects. It has also been described that lung cancer cells with forcibly expressed SAA gene showed higher metastatic capacity (Sung et al., 2011). However, SAA expression could not detected in feline mammary carcinoma cell lines used in this study (data not shown). To the best of our knowledge, local expression of SAA has not been examined in feline tumors.

In conclusion, SAA directly stimulates MMP-9 production and tumor cell invasion in feline mammary tumor cells. Thus, a novel role for SAA in mammary tumorigenesis is suggested. The suppression of effects of SAA may provide new therapeutic strategies for

tumor invasion and metastasis. Further investigation is needed to determine the receptors and the signaling pathways of feline SAA in tumor cells.

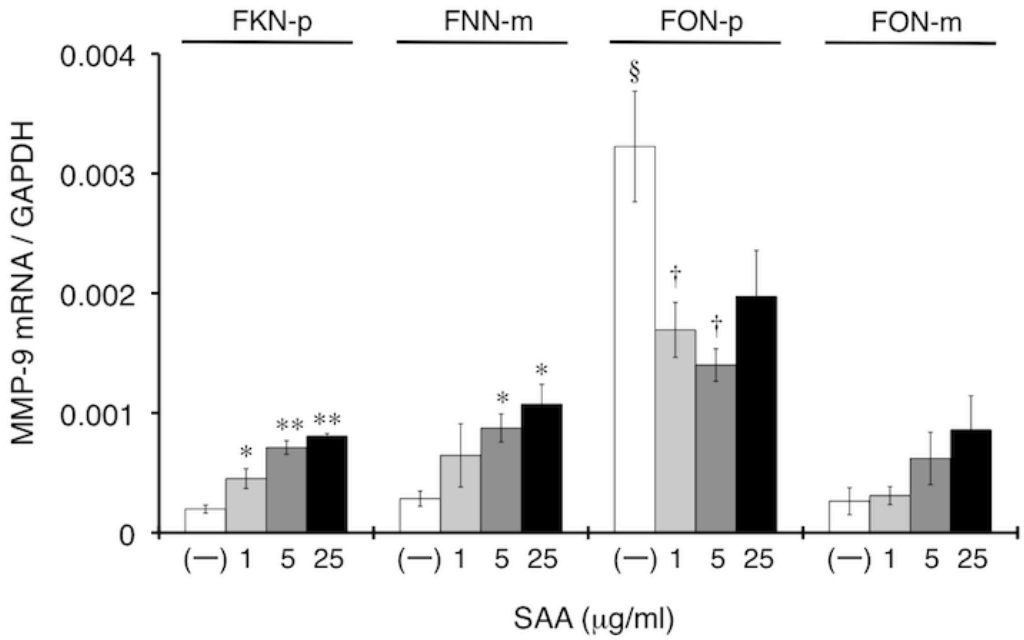

Fig. 1. MMP-9 mRNA expression with rfSAA stimulation. FKN-p, FNN-m, FON-p, and FON-m cells were grown in the absence and presence of 1, 5, or 25 μg/ml rfSAA for 24 h. Total cellular RNA was extracted and the expression of MMP-9 mRNA was examined by quantitative RT-PCR. The expression levels were normalized with that of GAPDH. Each experiment was performed in duplicate and repeated 3 times in separate experiments. Data represent mean ± SEM of 3 experiments. * $P < 0.05$ and ** $P < 0.01$ increased versus control. † $P < 0.05$ decreased versus control. § $P < 0.01$ increased compared with other cells.

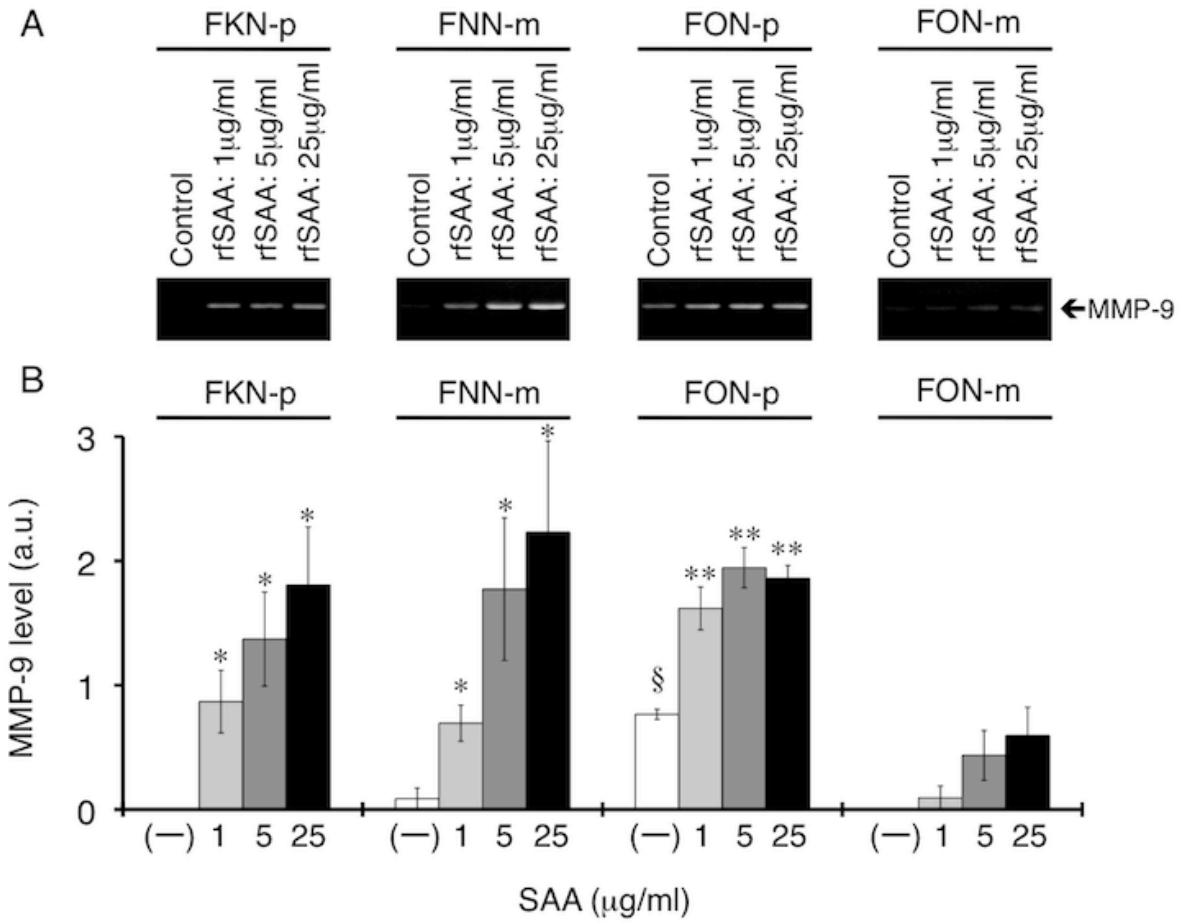

Fig. 2. MMP-9 levels with rfSAA stimulation examined by gelatin zymography. **(A)** FKN-p, FNN-m, FON-p, and FON-m cells were grown in the absence and presence of 1, 5, or 25 μg/ml rfSAA for 24 h. Cell-free culture media were collected and MMP-9 levels were examined by gelatin zymography. MMP-9 levels were assessed on the basis of gelatinolytic activity, indicated as clear bands against the dark blue background. Representative data is shown. **(B)** To obtain a semi-quantitative value for each sample, the expression levels were normalized with that of a commercially available standard. Each experiment was performed in duplicate and repeated 3 times in separate experiments. Data represent mean ± SEM of 3 experiments. * $P < 0.05$ and ** $P < 0.01$ increased versus control. § $P < 0.01$ increased compared with other cells.

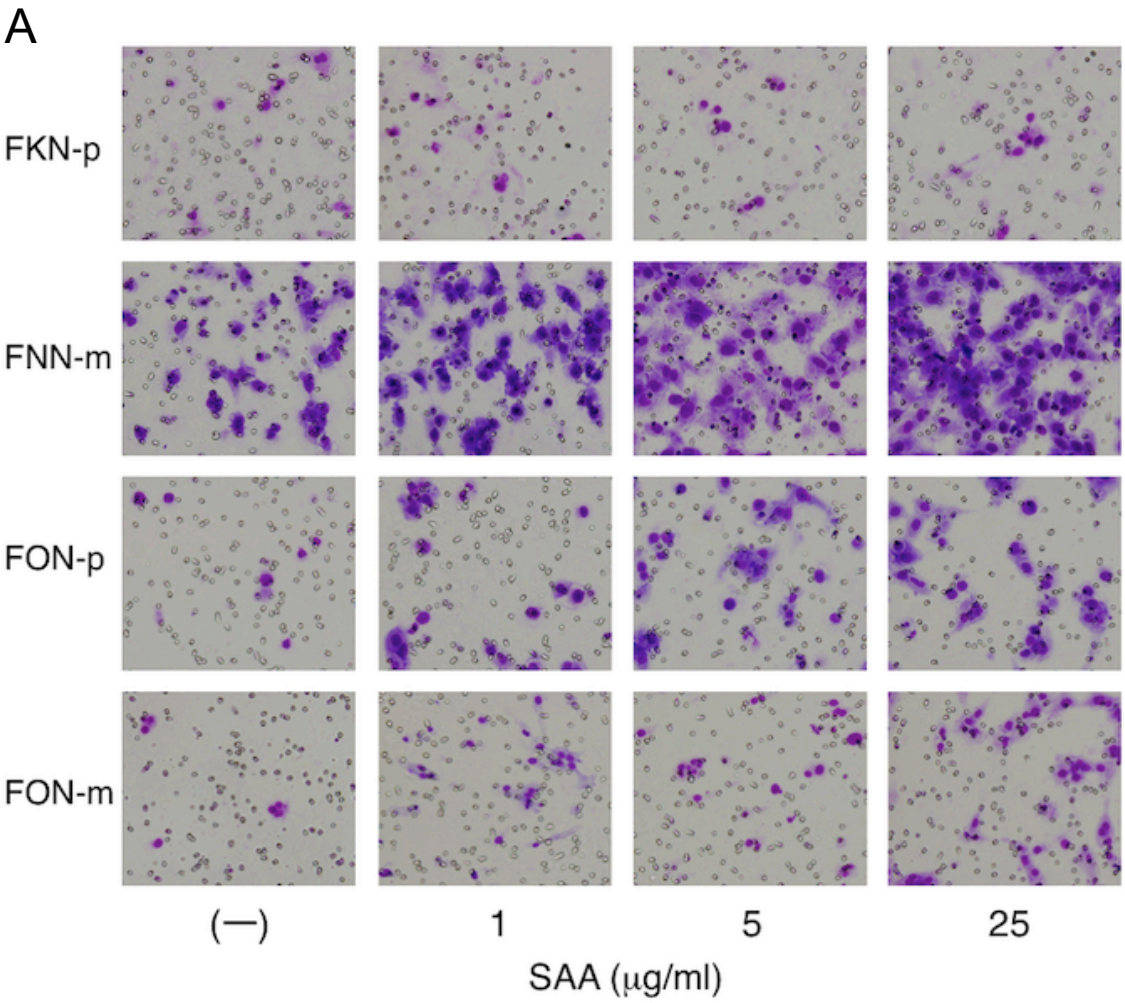

Fig. 3. Tumor cell invasion with rfSAA stimulation. **(A)** Invasiveness of tumor cells was assessed by using the matrigel transwell assay. FKN-p, FNN-m, FON-p, and FON-m cells were grown in the absence and presence of 1, 5, or 25 μg/ml rfSAA on upper surface of the chamber for 24 h. After incubation, cells that reached the lower surface of the filter were fixed and stained with Wright-Giemsa solution. Representative data are shown.

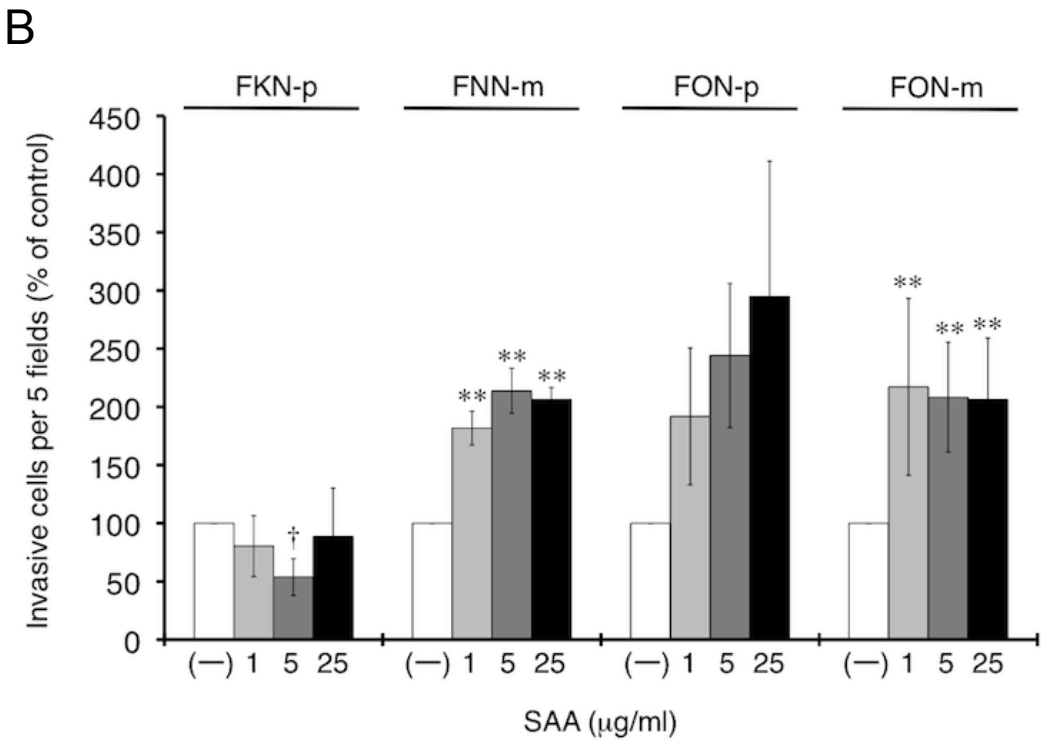

Fig. 3. Tumor cell invasion with rfSAA stimulation. **(B)** The number of invading cells was counted in 5 high-power (200× magnification) microscope fields per filter and the ratio of rfSAA stimulated cells to the control was calculated. Each experiment was performed in duplicate and repeated 3 times in separate experiments. Data represent mean ± SEM of 3 experiments. ** $P < 0.01$ increased versus control. † $P < 0.05$ decreased versus control.

Chapter 2-2

Serum amyloid A promotes the invasion of feline lymphoma cells

Abstract

The serum amyloid A (SAA) concentration was elevated in more than half of cats with lymphoma. Moreover, elevated matrix metalloproteinase-9 (MMP-9) expression was reported in lymphoma cats. In the present study, the association between recombinant feline SAA (rfSAA) and MMP-9 expression were evaluated by using 3 feline lymphoma cells; 3201, MS4, and MCC. The effect of rfSAA on the actual invasive capacity of these cells was also estimated. Only in MCC cells, MMP-9 mRNA expression was significantly increased by rfSAA stimulation. Secreted MMP-9 protein in culture media was determined by gelatin zymography, and clear bands of MMP-9 were detected in the same cell line with rfSAA stimulation. A significant increase in semi-quantified MMP-9 levels was observed with 5 and 25 μg/ml of rfSAA stimulation. The invasive capacities of feline lymphoma cells assessed by matrigel transwell assay showed that rfSAA stimulated cell invasiveness in MCC cells. Although the responses with rfSAA varied with cell line, the results showed that rfSAA can stimulate the MMP-9 expression and invasiveness of feline lymphoma derived cells. The findings of this study have identified a novel role for SAA in progression of lymphoid tumor, and suggest that new therapeutic strategies targeting SAA may be provided.

1. Introduction

As shown in chapter 0, elevated SAA concentration has been described in cats with various inflammatory and infectious diseases. Increased SAA concentration has also been demonstrated in cats with neoplastic diseases. Lymphoma is the common neoplasm in cats and elevated SAA concentration in feline lymphoma patients were observed with a high frequency. Moreover, elevated SAA concentration was demonstrated as a prognostic marker in cats with various diseases including lymphoma.

Matrix metalloproteinases (MMPs) are a family of extracellular matrix degrading proteases that are zinc-dependent and associated with invasion and metastasis during tumor progression because of their ability to degrade extracellular matrix and basement membrane (Stamenkovic, 2000). Type IV collagen is one of the integral components of the basement membrane, and its collagenase, matrix metalloproteinase-9 (MMP-9), is believed to play a key role in tumor invasion and metastasis (Sehgal et al., 1998). Higher expression of MMP-9 and elevated serum MMP-9 concentrations have been described in cats with various tumors including lymphoma (Jankowski et al., 2002). As shown in chapter 2-1, feline SAA may affect to the MMP-9 expression of mammary carcinoma cells. However, there is no information about the relationship between SAA and MMP-9 in cats with lymphoma.

In this chapter, the direct effects of recombinant feline SAA (rfSAA) protein on the MMP-9 mRNA expression and protein synthesis of 3 feline lymphoma derived cells were evaluated. The effect of rfSAA on the actual invasive capacity of these cells was also estimated.

2. Materials and Methods

2.1. Cell culture

Three feline lymphoma derived cell lines were used in this study: 3201 (Snyder et al., 1978), MS4 (Mochizuki et al., 2011), and MCC (Cheney et al., 1990). All cell lines were grown in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Nacalai Tesque, Kyoto, Japan). Cells were maintained at 37°C in 5% CO_2.

2.2. Recombinant feline SAA

The synthesis of rfSAA was carried out as described in chapter 1. The synthesized rfSAA was desalted by using a commercial desalting column (PD-10 Desalting Columns; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The purity of rfSAA was analyzed by SDS-PAGE (12.5% SDS-polyacrylamide gel) and was determined to be greater than 95% by using a software package (Image Lab Software; Bio-Rad Laboratories, Hercules, CA).

2.3. Stimulation of cell lines by rfSAA

Subconfluent (70–80% confluent) cells were detached and resuspended at 1×10^6 cells/ml in serum-free media (RPMI 1640 containing 1% bovine serum albumin; BSA), and added in 100 μl aliquots to a 96-well plate. The cells were stimulated with rfSAA (1, 5, and 25 μg/ml) for 24 h. Cells and cell-free supernatants of culture media were collected separately

by centrifugation and stored at -80°C until analysis. Each experiment was performed in duplicate and repeated 3 times in separate experiments.

2.4. RNA extraction and quantitative RT-PCR

Total cellular RNA was extracted by using a commercial kit (Illustra RNAspin Mini RNA Isolation Kit; GE Healthcare). Reverse transcription was performed using a commercially available reverse transcriptase (ReverTra Ace qPCR RT Master Mix; TOYOBO, Tokyo, Japan), according to the manufacturer's instructions. For quantitative RT-PCR, the cDNA was amplified with SYBR green (THUNDERBIRD SYBR qPCR Mix; TOYOBO) by using gene-specific primers and a thermal cycler (Thermal Cycler Dice Real Time System; Takara Bio, Shiga, Japan) with the following program: a 10 min preincubation at 95°C, 50 cycles of PCR (5 s at 95°C and 30 s at 60°C), and dissociation (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). The reactions were performed with primers specific to feline MMP-9 (GenBank accession number, AB858226): 5′-GCCCCTACAGTGTCTTTG GA-3′ (forward), 5′-TCCCATCCTTGAAGAAATGC-3′(reverse) or glyceraldehye-3-phosphate dehydrogenase (GAPDH: NM_001009307): 5′-GCTGCCCAG AACATCATCC-3′ (forward), 5′-GTCAGATCCACGACGGACAC-3′ (reverse). Data were normalized relative to GAPDH as an endogenous control. Quantification of mRNA transcription was performed using the comparative cycle threshold (Ct) method. Each sample was assessed in duplicate.

2.5. Gelatin zymography

MMP-9 levels in culture media were examined by gelatin zymography. Samples were diluted in sample buffer (125 mM Tris-HCl pH 6.8, 25% glycerol, 5% SDS, 0.2% bromophenol blue) and a dilution containing an equal volume of culture media was subjected to electrophoresis on a 10% SDS-PAGE gel co-polymerized with 0.1% gelatin. Following electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 2 h at room temperature and incubated in enzymatic activation buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.6) for 24 h at 37°C with gentle shaking. The gels were stained with 0.25% Coomassie Brilliant Blue R-250, 50% methanol, and 5% acetic acid for 30 min, and then de-stained in 5% methanol and 7% acetic acid for 1 h. A commercially available zymography marker (Gelatin Zymo MMP Marker; Life Laboratory, Yamagata, Japan) was run on each gel as a positive control. MMP-9 levels were assessed on the basis of gelatinolytic activity, indicated as clear bands against the dark blue background. All gels were analyzed with an imaging analyzer system (Cool Saver version 1.0; ATTO, Tokyo, Japan) and software (CS Analyzer version 2.0; ATTO). To obtain a semi-quantitative value for each sample, the imaging assessment value of each unknown band was compared with the value of a MMP-9 standard band. The ratio of unknown to standard was calculated, and an arbitrary unit (a.u.) value was assigned to each sample. Each sample was assessed in duplicate.

2.6. Invasion assay

Invasion of cell lines was assessed by the matrigel transwell assay (Knebel et al.,

2013). For the analysis, 24-well chambers with 8-μm pore filters (Chemotaxicell; Kurabo, Osaka, Japan) were coated on the upper surface with matrigel (BD Matrigel matrix; Becton–Dickinson, Franklin Lakes, NJ). Matrigel serves as a reconstituted basement membrane *in vitro*. By coating the upper surface of membrane, matrigel occludes the pores of the membrane and blocks non-invasive cells from migrating through the membrane. In contrast, invasive cells secrete proteases that degrade matrigel and enable invasion through the membrane pores. Cells (2×10^5 /well) in 200 μl of serum-free medium (RPMI 1640 containing 1% BSA) were added to the upper chamber with rfSAA (1, 5, and 25 μg/ml). The lower wells were filled with 600 μl of media (RPMI 1640 containing 10% FBS). After 24 h, the cells that invaded the matrigel and reached the lower wells were counted, and the ratio of rfSAA stimulated cells to control was calculated. Each experiment was performed in duplicate and repeated 3 times in separate experiments.

2.7. Statistical analysis

Statistical analyses were performed using a statistical software package (JMP version 5.0.1J; SAS Institute, Cary, NC). Student's t test was used to compare individual treatment with their respective control values. In all cases, $P < 0.05$ was considered to indicate significance. All data are expressed as mean ± SEM.

3. Results

3.1. Changes in MMP-9 mRNA expression with rfSAA stimulation

In MCC cells, MMP-9 mRNA expression after rfSAA stimulation increased significantly in a dose-dependent manner (Fig. 1). In 3201 and MS4 cells, however, MMP-9 expression was not affected by rfSAA stimulation.

3.2. Changes of MMP-9 levels in culture media with rfSAA stimulation

MMP-9 levels in culture media were examined by gelatin zymography and a semi-quantitative value of MMP-9 levels for each sample was calculated. In 3201 and MS4, any bands of MMP-9 were not detected with or without rfSAA stimulation (data not shown). Although the band of MMP-9 was not observed in MCC cell line without stimulation (Fig. 2A), clear bands were detected with 5 and 25 μg/ml of rfSAA stimulation (Fig. 2A). In the cell line, MMP-9 levels increased significantly in a dose-dependent manner (Fig. 2B).

3.3. Tumor cell invasion assay

The invasiveness of tumor cells was assessed by the matrigel transwell assay. In 3201 and MS4 cells, invasion of cells decreased significantly with 1 μg/ml of rfSAA (Fig. 3). In MCC cell, however, cell invasion increased significantly with 25 μg/ml of rfSAA stimulation. Invasions of the cell after 24 h increased more than twice with 25 μg/ml of rfSAA stimulation.

4. Discussion

In the present study, the direct effects of rfSAA on lymphoma cells were demonstrated. In 1 of 3 feline lymphoma cell lines, elevated MMP-9 mRNA expressed and MMP-9 production with rfSAA stimulation were observed in a dose-dependent manner. rfSAA also stimulated the actual invasion of the cell through the extracellular matrix component. These findings show that SAA may have a role in lymphoid tumor progression and metastasis. Although, as shown in chapter 2-1, direct effects of SAA on MMP-9 production and tumor cell invasion were previously reported in feline mammary carcinoma cell lines, there had been no report about hematological tumor.

As shown in chapter 0, elevated SAA concentration was observed in more than half of cats with lymphoma. Moreover, the association between elevated SAA concentration and the prognosis of diseases was reported in cats with various diseases including lymphoma. On the other hand, MMP-9 expression is reported as a prognostic factor of human non-Hodgkin's lymphoma patients (Hazar et al., 2004), and it may apply to feline lymphoma. Thus, from the results of this study, feline SAA may affect to the prognosis of lymphoma through the MMP-9 expression and production.

Three cell lines used in this study are all FeLV negative. 3201 was derived from T-cell lymphoma (Snyder et al., 1978) and MS4 was derived from B-cell lymphoma (Mochizuki et al., 2011). In the previous study with human peripheral blood mononuclear cells, SAA upregulated cytokine mRNA expression on monocytes and did not induce cyokines from lymphocytes (Song et al., 2009). Therefore, 3201 and MS4 could have been

unresponsive to SAA stimulation because lymphocytes might be less susceptible to SAA. MCC was derived from a large granular lymphocyte (LGL) lymphoma patient and shown non-T and non-B immunophenotype (Cheney et al., 1990). LGL lymphoma in cats is considered derived from natural killer (NK) cell or cytotoxic T-cell (Roccabianca et al., 2006). The differences between MCC and other 2 cells on responses to SAA stimulation might be caused by the differences of immunophenotype and surface receptors expression between NK cells and other lymphocytes.

In chapter 2-1 with mammary carcinoma cells, cell lines that had originally high invasive or metastatic capacity showed relatively high responsibility to SAA. 3201 was established from a cat with only thymic lesion (Snyder et al., 1978) and MS4 was established from a cat with two skin lesions (Mochizuki et al., 2011). MCC was established from a cat with two abdominal masses and infiltration of tumor cells to liver, spleen, and mesenteric lymph node (Cheney et al., 1990). Original capacity of tumor cells to invade may affect to the sensitivity to SAA also in lymphoid tumor.

In conclusion, SAA directly stimulates MMP-9 mRNA expression and protein synthesis in feline LGL lymphoma cells. The suppression of effects of SAA may provide new therapeutic strategies for lymphoma progression. Further investigation is needed to determine the receptors and the signaling pathways of feline SAA in tumor cells.

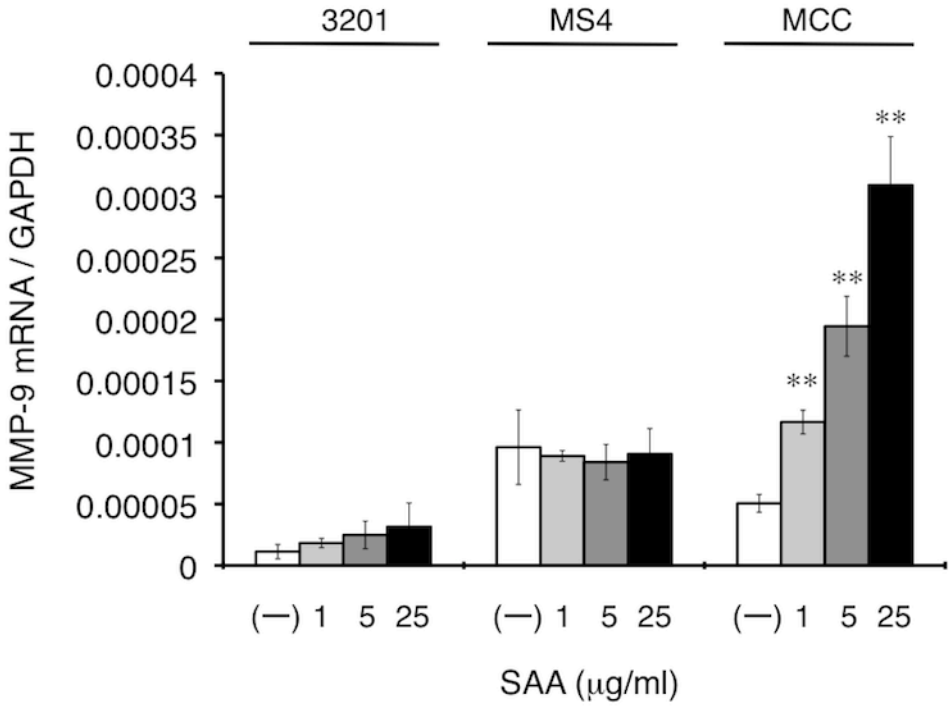

Fig. 1. MMP-9 mRNA expression with rfSAA stimulation. 3201, MS4, and MCC cells were grown in the absence and presence of 1, 5, or 25 μg/ml rfSAA for 24 h. Total cellular RNA was extracted and the expression of MMP-9 mRNA was examined by quantitative RT-PCR. The expression levels were normalized with that of GAPDH. Each experiment was performed in duplicate and repeated 3 times in separate experiments. Data represent mean ± SEM of 3 experiments. ** $P < 0.01$ increased versus control.

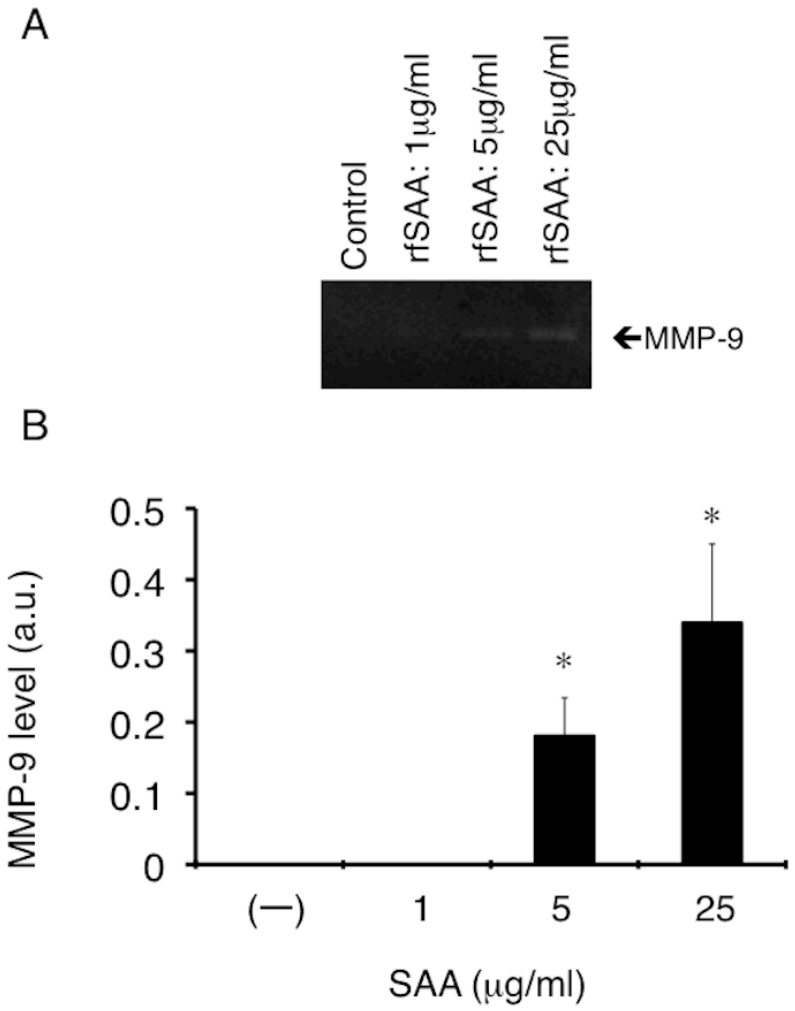

Fig. 2. MMP-9 levels with rfSAA stimulation examined by gelatin zymography. **(A)** MCC cells were grown in the absence and presence of 1, 5, or 25 μg/ml rfSAA for 24 h. Cell-free culture media were collected and MMP-9 levels were examined by gelatin zymography. MMP-9 levels were assessed on the basis of gelatinolytic activity, indicated as clear bands against the dark blue background. Representative data is shown. **(B)** To obtain a semi-quantitative value for each sample, the expression levels were normalized with that of a commercially available standard. Each experiment was performed in duplicate and repeated 3 times in separate experiments. Data represent mean ± SEM of 3 experiments. * $P < 0.05$ increased versus control.

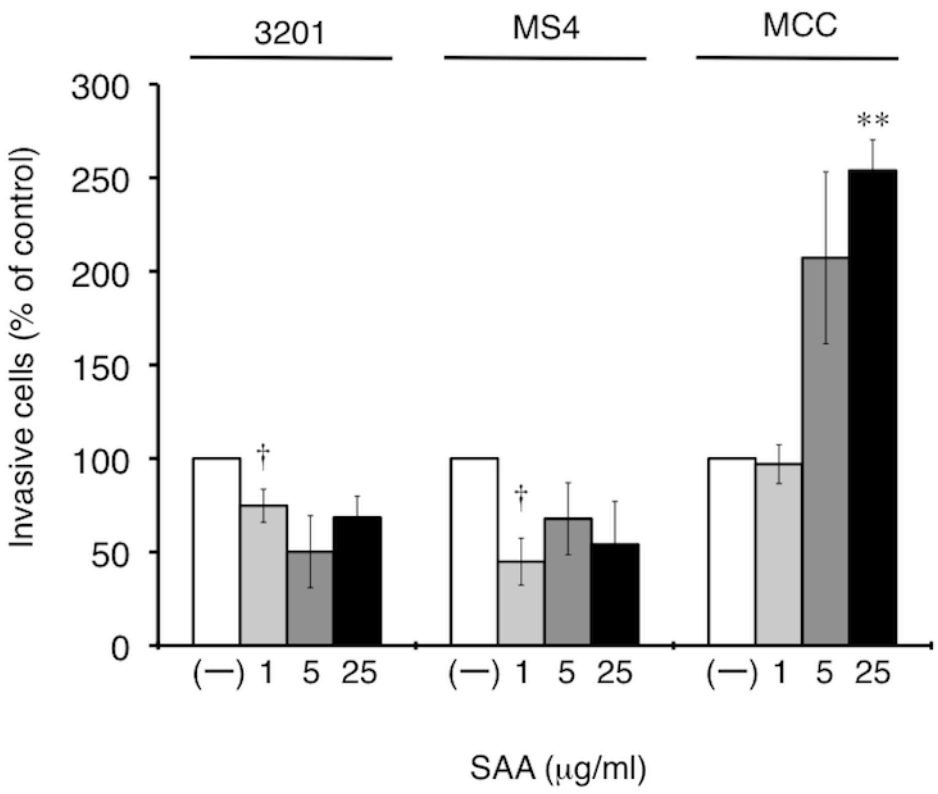

Fig. 3. Tumor cell invasion with rfSAA stimulation. Invasiveness of tumor cells was assessed by using the matrigel transwell assay. 3201, MS4, and MCC cells were grown in the absence and presence of 1, 5, or 25 μg/ml rfSAA on upper wells of the chamber for 24 h. After incubation, cells that reached the lower wells were counted and the ratio of rfSAA stimulated cells to the control was calculated. Each experiment was performed in duplicate and repeated 3 times in separate experiments. Data represent mean ± SEM of 3 experiments. ** $P < 0.01$ increased versus control. † $P < 0.05$ decreased versus control.

Chapter 3

Serum amyloid A uptake by feline peripheral macrophages

Abstract

Serum amyloid A (SAA) is one of the major acute phase proteins in cats and humans. SAA concentrations increase in response to the inflammatory status and secondary amyloid A amyloidosis has been documented in cats. In order to control the SAA concentration, it is important to clarify how the SAA protein is metabolized. Although the details of SAA metabolism in the body remain unknown, human and murine research indicates that macrophages play a key role in SAA uptake. The objectives of this study were to demonstrate SAA uptake by feline macrophages and to evaluate the effects of lipopolysaccharide (LPS) and dexamethasone (Dex) on SAA uptake. The concentration of recombinant feline SAA added to a feline macrophage culture was decreased in a time-dependent manner and was significantly reduced after a 24 h incubation. SAA uptake into feline peripheral macrophages was demonstrated by immunofluorescence microscopy. Pretreatment to macrophages with LPS did not affect this decrease in the SAA concentration, but this was significantly blocked by Dex pretreatment. In conclusion, SAA was incorporated by feline macrophages and pretreatment with Dex inhibited SAA uptake by macrophages in this study. Further investigation is needed to determine the molecules that influence SAA uptake by macrophages and the effect of clinical glucocorticoid usage on the SAA concentration in cats.

1. Introduction

Serum amyloid A (SAA) is one of the major acute phase proteins in many species, including humans (Kushner, 1988, Malle et al., 1996) and cats (Kajikawa et al., 1999,)Sasaki et al., 2003, Giordano et al., 2004). The SAA level is used as a marker of inflammation to assess the existence of inflammatory diseases and the response to therapy (Nakayama et al., 1993). Although physiological roles of the SAA protein in the body are not fully understood, evidence suggests that SAA functions as an exacerbating factor in several diseases. As shown in chapter 1, SAA stimulates the cytokine production by monocytes. Moreover, SAA promotes the tumor cells invasion as shown in chapter 2. Therefore, these things suggest that the SAA protein is produced as a result of inflammation and is an important factor in the progress of inflammatory and neoplastic diseases.

SAA is a precursor of amyloid A (AA) fibrils, and sustained high SAA levels in the blood may give rise to secondary amyloidosis, which is known as reactive AA amyloidosis. The amyloid burden and mortality of human patients with AA amyloidosis correlated significantly with the serum SAA concentration (Lachmann et al., 2007). Furthermore, it was reported that amyloid fibril deposits regressed, and prognosis was improved in patients in whom SAA levels had been lowered to within the reference range by treatment (Gillmore et al., 2001). As shown in chapter 0, the SAA concentration has been shown to increase in many inflammatory diseases and tumors in cats. Although no report has shown a direct association between the SAA concentration and the development of AA amyloidosis in cats, causes of secondary AA amyloidosis have been reported in cats with chronic inflammatory diseases

(van der Linde-Sipman et al., 1997). Therefore, controlling the SAA concentration is believed to be important in managing the primary and intercurrent diseases.

To control the SAA concentration, it is important to clarify how the SAA protein is metabolized. Previous in vitro studies in mice revealed that the SAA protein was incorporated into macrophages immediately after addition to culture medium (Rocken et al., 1998, Kinkley et al., 2006). Another study showed that the SAA protein disappeared in a time-dependent manner when it was added to a monocyte culture (Lavie et al., 1978). This occurs when SAA is cultured with monocytes but not with lymphocytes (Migita et al., 2001). Moreover, this phenomenon was inhibited by inflammatory stimuli, such as IL-1 and IFN-γ. These reports indicate that monocytes/macrophages play a key role in SAA metabolism and that this phenomenon is affected by various factors. However, no report has described SAA protein metabolism in cats.

The aims of this study were to demonstrate the uptake of the SAA protein by feline macrophages and to evaluate the difference in reactivity when the macrophages were pretreated with lipopolysaccharide (LPS) or dexamethasone (Dex).

2. Materials and Methods

2.1. Cell preparation

Feline macrophages were preparedt according to previously described protocols (Mizukoshi et al., 2009, Goto-Koshino et al., 2011). 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, EDTA-treated whole blood from healthy cats was overlaid onto Ficoll-Paque Plus (GE Healthcare, Buckinghamshire, United Kingdom) and centrifuged at 800 *g* for 30 min at room temperature. Peripheral blood mononuclear cells (PBMCs) at the interface were resuspended in Hanks' balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 200 *g* for 10 min to remove contaminating platelets. PBMCs were resuspended in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were plated on a 24-well tissue culture-treated plate (Corning, Lowell, MA, USA) at a concentration of 1×10^6 cells/well, stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), and cultured at 37°C in 5% CO_2. Monocytes were allowed to attach to the plastic bottom of the plate for 24 h, and the plate was subsequently washed with culture medium to remove non-adherent cells. PMA-free culture medium (2 mL) was added to the culture plate, and the cells were cultured for 6 days to induce macrophage differentiation.

2.2. Recombinant feline SAA (rfSAA)

Recombinant feline SAA protein was produced as described in chapter 1. The protein concentration of the SAA fraction was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The purification of rfSAA was analyzed by SDS-PAGE (12.5% SDS-polyacrylamide gel) and determined to be greater than 95% by using a software package (Image Lab Software; Bio-Rad Laboratories, Hercules, CA).

2.3. ELISA for measurement of feline SAA

A rat anti-human SAA monoclonal antibody and a rabbit anti-human SAA polyclonal antibody were provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). Each well of a 96-well plate (Corning) was coated with 2 μg of the anti-SAA polyclonal antibody by incubating overnight at 4°C. After washing the wells with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST), 200 μL of 1% bovine serum albumin in PBST was added to each well and the plate was incubated at room temperature for 2 h to block non-specific binding. After washing the plate with PBST, 100 μL of diluted anti-SAA monoclonal antibody and 50 μL of diluted sample were added to each well. After a 1 h incubation period at 37°C, the wells were washed with PBST and incubated with 100 μL horseradish peroxidase-conjugated anti-rat IgG at room temperature for 1 h. The plate was washed again and incubated with 50 μL of substrate solution (TMB; Nacalai Tesque, Kyoto, Japan) for 15 min. H_2SO_4 (2N, 50 μL) was added to stop color development, and the absorbance was measured at 450 nm and 630 nm, the latter of which was used as the reference. Serially

diluted rfSAA was used as the standard for ELISA.

2.4. Measurement of the SAA concentration in culture medium

Macrophages were cultured in 1 mL culture medium in 24-well culture plates. Each experiment was performed 5 times by using macrophages obtained from 5 healthy cats. On day 7, 10 μg of rfSAA was added to each well for 24 h and the culture media were collected at the following time points: 0 (immediately after the addition), 1, 3, 6, 9, 12, and 24 h after SAA addition. To investigate the effects of pretreatment to macrophages with LPS or Dex, 1 μg/mL LPS (Sigma-Aldrich) or 1 μM Dex (Sigma-Aldrich) was added to the culture on day 6 followed by rfSAA addition on day 7. Culture media were collected before, immediately after, and 24 h after rfSAA addition and were centrifuged. The supernatants were stored at -80°C until analysis. The SAA concentration of the samples was measured by ELISA.

2.5. Immunofluorescence (IF) analysis for SAA localization

Macrophages were cultured in 4-well chamber slides (Nulge Nunc International Co., Naperville, IL, USA) for 6 days. On day 7, 20 μg of rfSAA was added to the culture medium, and the cells were incubated with rfSAA for 1 h. After incubation, the culture medium was discarded, and the cells were washed 3 times with HBSS. Cells were fixed in 4% paraformaldehyde (pH 7.4) for 10 min, washed with PBS, and permeabilized in 0.2% Triton X-100 for 10 min. The cells and the slide surfaces were then blocked with 10% FBS in PBS at room temperature for 1 h. To detect the location of SAA, the rat anti-SAA monoclonal

antibody (Eiken Chemical) was diluted in 10% FBS and added to each well. Concentration-matched rat IgG (R&D Systems, Minneapolis, MN, USA) was used as an isotype control. After incubation for 30 min, the slides were washed with PBS and diluted DyLight 488-conjugated anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added to each well. To visualize the nucleus and cytoplasm, 4′,6-diamidino-2-phenylindole (DAPI; Roche Applied Science, Indianapolis, IN, USA) and propidium iodide (PI; Sigma-Aldrich), respectively, were added to the wells at the same time. After 30 min, the slides were washed and covered. The cells and the location of rfSAA were visualized using 20× objective on a fluorescence microscope.

2.6. Statistical analysis

Statistical analyses were performed using a statistical software package (JMP version 5.0.1J; SAS Institute, Cary, NC). The Dunnett method was used to compare the results among the time points or between pretreatments. The Mann–Whitney *U* test was used to compare the SAA concentrations at 24 h with and without macrophages. Statistical significance was defined as $P < 0.01$ for all analyses.

3. Results

3.1. Establishment of ELISA for feline SAA

A sandwich ELISA for the measurement of feline SAA was established using an anti-SAA monoclonal antibody that showed cross-reactivity with feline SAA as the detection antibody. A typical standard curve using serially diluted rfSAA is shown in Fig. 1. Range of 0.014–0.46 μg/mL was considered as the effective measuring range of this assay with a linear regression line. The intra- and inter-assay coefficients of variation (CV) were calculated by measuring three different serum samples. CVs of < 13% and < 19% were obtained by the intra- and inter-assay, respectively.

3.2. SAA concentration change in culture medium

The SAA concentration was measured at various incubation time points after the rfSAA was added to macrophage cultures. The SAA concentration in culture media decreased gradually over time (Fig. 2). Compared with the concentration at 0 h, significant reductions were observed from 6 h to 24 h ($P < 0.01$). Although the SAA concentration also decreased in culture medium without macrophages (Fig. 2), the concentration with macrophages at 24 h was significantly lower than that without macrophages ($P < 0.01$).

3.3. SAA uptake and localization in cultured macrophages

SAA was detected by an anti-SAA monoclonal antibody and DyLight 488-conjugated secondary antibody (green signal). The cytoplasms of macrophages were

visualized with PI (red signal) and the nucleus was stained with DAPI (blue signal). SAA appeared as bright green spots in macrophages at 1 h (Fig. 3A). These green signals were not detected in the isotype control or in the negative (in which rfSAA not added) control (Fig. 3B, C).

3.4. Effects of pretreatment to macrophages with LPS or Dex on SAA uptake

Macrophages were pretreated with LPS or Dex and incubated with 10 μg/mL rfSAA for 24 h. The changes in SAA concentration with or without pretreatment are shown in Fig. 4. Before rfSAA addition (pre), the SAA concentrations in the culture supernatants did not differ between the pretreatments for 24 h. SAA concentrations increased due to the addition of rfSAA (post) and decreased over a 24-h incubation with macrophages in the presence or absence of pretreatment. LPS pretreatment did not affect the decrease in the SAA concentration. However, Dex pretreatment resulted in a significantly higher SAA concentration at 24 h than that without pretreatment ($P < 0.01$).

4. Discussion

In this study, SAA concentrations in culture medium decreased in a time-dependent manner after rfSAA was added to a feline macrophage culture. Similar results were obtained in a previous study that used human macrophages (Lavie et al., 1978). Although some natural degradation of the rfSAA incubated in culture medium at 37°C without macrophages was also observed, the decreases in the SAA concentration with macrophages were significantly greater than the decreases without macrophages. Thus, the decrease in the SAA concentration was presumably caused by macrophages. Furthermore, the IF assay showed that macrophages isolated from feline peripheral blood immediately incorporated the SAA protein into the cytoplasm. Although SAA degradation products of various molecular weights (4–9 kDa) were detected by immunoblot analysis in previous studies (Lavie et al., 1978, Migita et al., 2001), these were not detected in this study.

A recombinant form of the SAA protein was used in this study. In general, the recombinant form of a protein may not show the same behavior as the endogenous form of the protein. However, the results in this study using recombinant SAA were similar to those of a previous study in which purified SAA was used (Lavie et al., 1978). Combined with the fact that the endogenous SAA protein is not glycosylated (Ehnholm et al., 1985), recombinant SAA appears to reflect the behavior of the endogenous form of SAA.

LPS was used in this study to examine the effect of macrophage pre-stimulation on SAA uptake. LPS is a strong macrophage activator and stimulates the production of cytokines, such as IL-1 and TNF-α (van der Meer et al., 1988). LPS pretreatment did not affect the rate

of SAA loss in culture medium, which is consistent with a previous report (Migita et al., 2001). Although it was reported that SAA degradation was suppressed in a dose-dependent manner in macrophages pretreated with IL-1 or IFN-γ in humans (Migita et al., 2001), the concentrations of these cytokines in the culture media were not determined in the present study.

Dex is a synthetic glucocorticoid that has suppressive effects on macrophages (Joyce et al., 1997, Kiku et al., 2002). Dex was used as a macrophage suppressor in this study. Consequently, the concentration of incubated SAA was significantly higher in culture medium of macrophages pretreated with Dex than that of non-treated macrophages. Although it was previously reported that macrophages produce the SAA protein in the presence of Dex (Urieli-Shoval et al., 1994, Yamada et al., 2000), SAA production by Dex-treated macrophages was not detected in this study. Since Dex itself did not stimulate SAA production from feline macrophages, we conclude that SAA uptake was inhibited by Dex pretreatment. Dex treatment suppresses various macrophage functions and may also suppress the expression of proteins or genes involved in SAA uptake. For example, scavenger receptor class B type 1 (SR-B1) is considered a candidate protein in the process of SAA uptake (Cai et al., 2005), and SR-B1 mRNA levels were suppressed by glucocorticoid administration in corticosterone-insufficient mice (Mavridou et al., 2010). In human and veterinary medicine, glucocorticoids such as Dex are often administered as anti-inflammatory therapy in patients with inflammation frequently associated with high SAA concentrations. Since the concentration of Dex used in this study was near the therapeutic dosage, my results suggest

that glucocorticoid administration can have a harmful effect on control of SAA concentration because it inhibits SAA uptake.

In conclusion, SAA uptake by feline peripheral macrophages was demonstrated in the present study. This uptake was inhibited by pretreating macrophages with Dex, whereas LPS pretreatment had no effect on SAA uptake. Although glucocorticoids are useful and important anti-inflammatory agents, they may be harmful because they reduce control of SAA concentration. Further investigation is needed to determine the molecules that influence SAA uptake by macrophages and the effect of clinical glucocorticoid usage on the SAA concentration in cats.

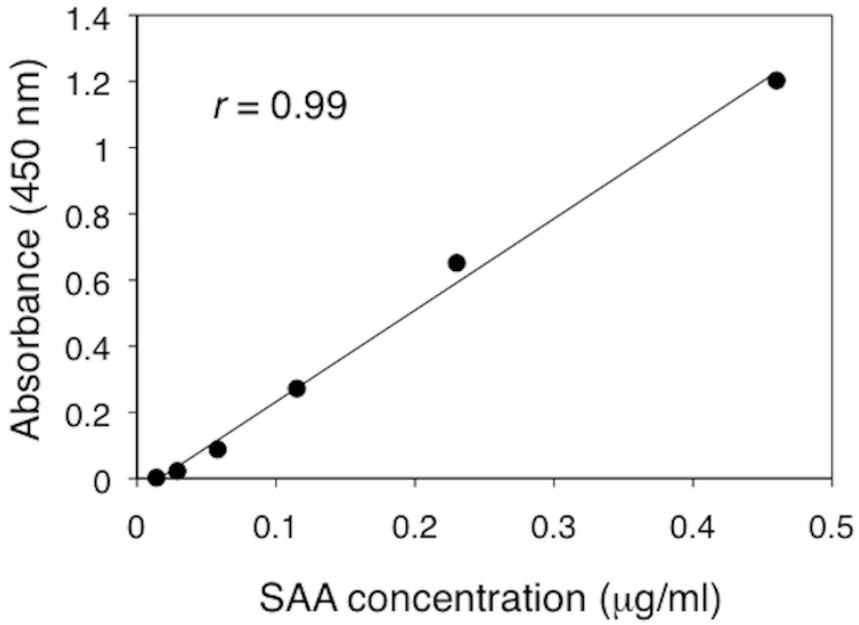

Fig. 1. Measurement of feline SAA by an established sandwich ELISA. Serially diluted rfSAA (0.014–0.46 μg/mL) was used to generate a standard curve.

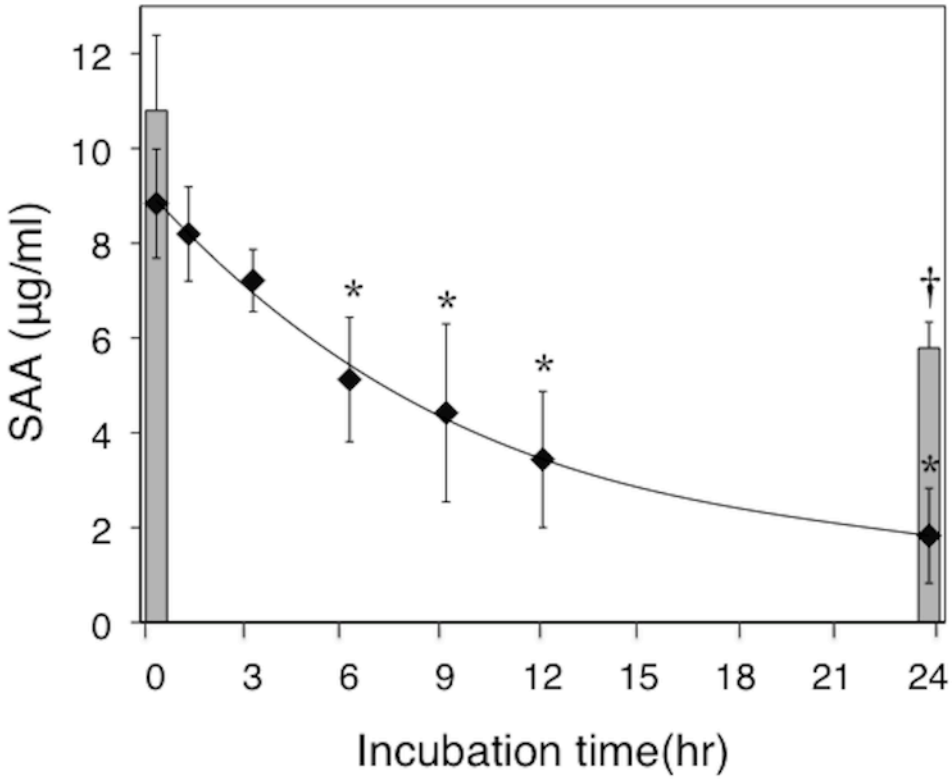

Fig. 2. The concentrations of SAA added to culture medium were measured at various time points. SAA concentrations with macrophages are shown as black diamonds at each time point. The concentrations without cells are shown as gray columns. Each experiment was performed 5 times. Values represent means (SD). The concentration was significantly reduced after 6 h with macrophages compared with that at 0 h (*, Dunnett method, $P < 0.01$). Significant differences were observed between concentrations with and without macrophages at 24 h (†, Mann-Whitney U test, $P < 0.01$).

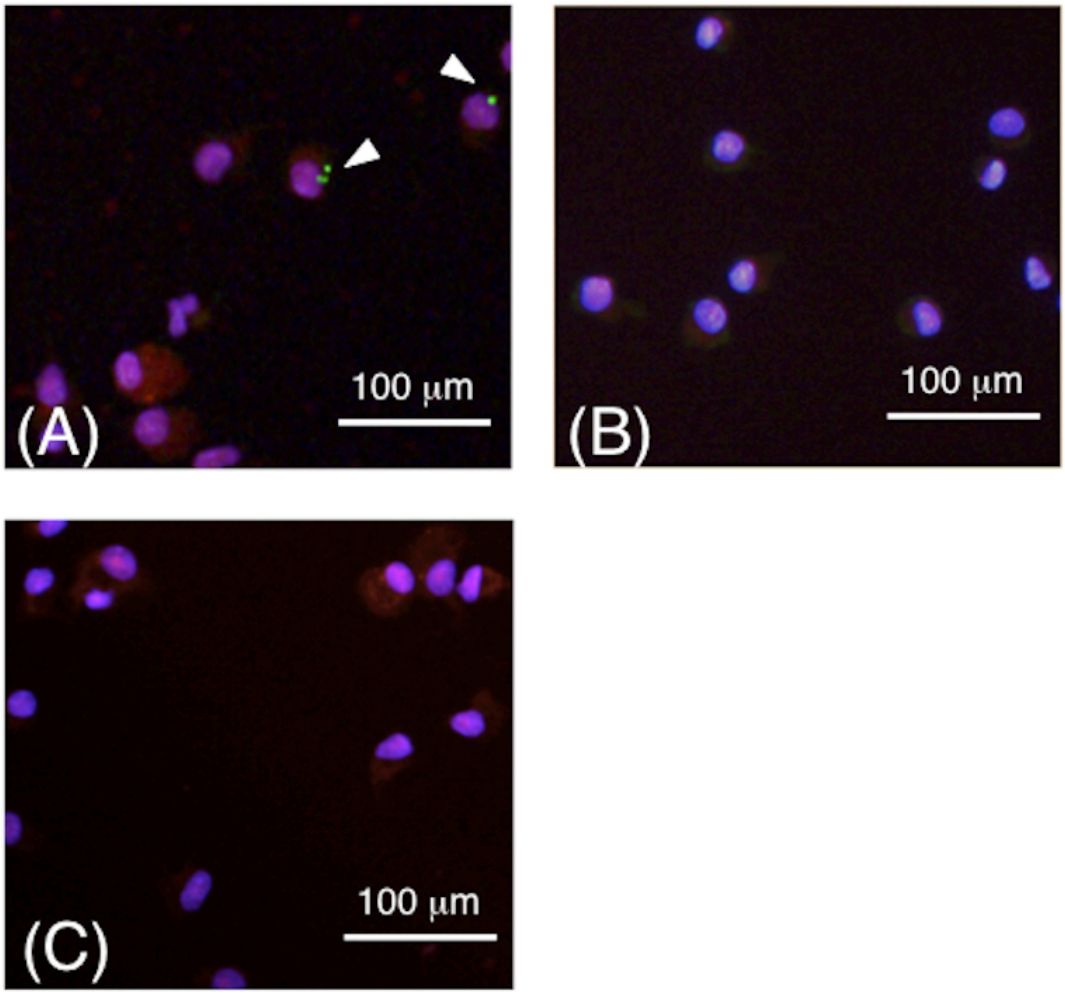

Fig. 3. Immunofluorescence imaging was performed to visualize the uptake and localization of SAA in feline peripheral macrophages after incubation with rfSAA. In each of the panels, PI was used to detect the cytoplasm (red signal) and DAPI was used to identify the nucleus (blue signal). SAA was detected by a rat anti-SAA monoclonal antibody and DyLight 488-conjugated anti-rat IgG (green signal). **(A)** The macrophages were incubated with rfSAA for 1 h, and SAA was detected as bright green spots (white arrowheads). **(B)** Rat IgG was used instead of a primary antibody for the isotype control. **(C)** Macrophages without the addition of rfSAA were used as the negative control. The green signal was not detected in **(B)** and **(C)**.

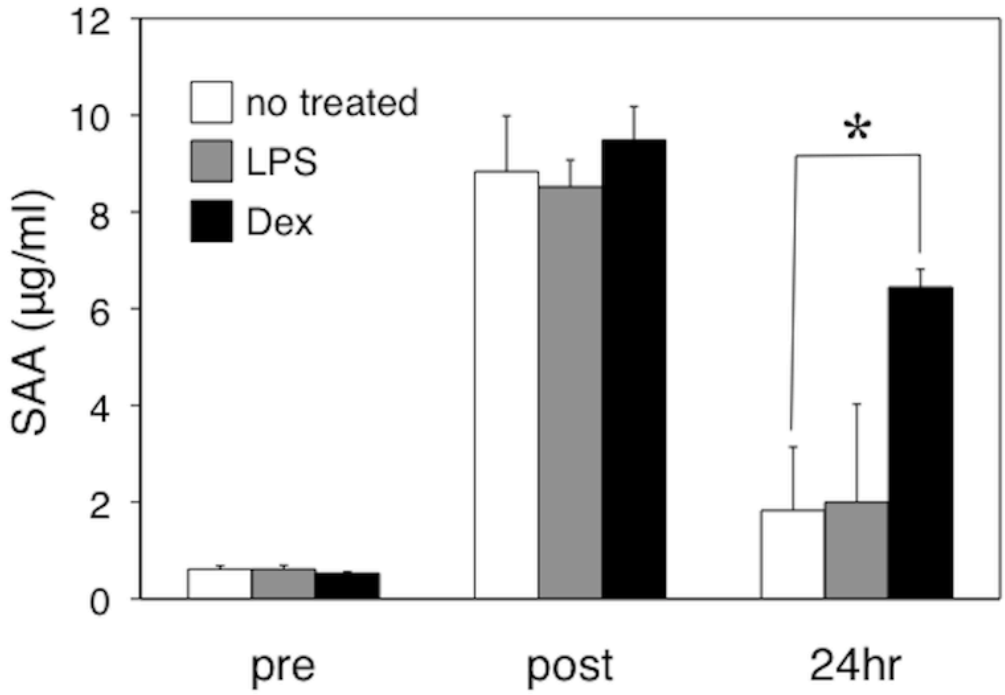

Fig. 4. Concentrations of SAA added to macrophages without pretreatment (white column), with LPS treatment (gray column), and with Dex treatment (black column) are shown. Each experiment was performed 5 times. Values represent means (SD). The SAA concentrations were not different among the groups prior to rfSAA addition. After the addition of rfSAA, the SAA concentration initially increased and then decreased after 24 h in all 3 groups. A significant difference between the non-treated and Dex-treated groups was observed 24 h after incubation (*, Dunnett method, $P < 0.01$).

Conclusion

To obtain the information about existence and magnitude of inflammation is considered clinically useful for both human and veterinary medicine. Inflammatory marker is an examination developed for this purpose and its clinical utility is well investigated. As the inflammatory marker, measurement of the acute phase proteins (APPs) is widely used. Serum amyloid A (SAA) is one of the major acute phase proteins (APPs) in mammals including humans (Uhlar et al., 1999) and cats (Kajikawa et al., 1999). In human medicine, measurement of SAA concentration is used as an inflammatory marker as well as C-reactive protein (CRP) (Gabay et al., 1999). As shown in chapter 0, SAA concentration was increased in cats with various diseases and its clinical utility as an inflammatory marker has been suggested also in cats. Furthermore, SAA is not only an inflammatory marker but also a prognostic marker in both humans and cats. In general, increased inflammatory marker represents that the underlying diseases are more active or severer, and the patient with severer diseases may show shorter survival. Although the theory is simple and may be well accepted, I raised further hypothesis that the pathophysiological characteristics of SAA could influence the clinical settings.

In chapter 1, monocyte activating function of feline SAA was investigated. The receptor and signaling pathway associated with the function were also examined. As the results, feline SAA promoted the TNF-α expression and production by feline peripheral monocytes in a dose-dependent manner. Although the feline SAA protein contains an insert of 8 amino acids compared with the human SAA sequence (Ohno et al., 1999), the inserts may have no effect on the functions of SAA protein. In this study, the receptor and signaling

pathway were also examined and Toll-like receptor 4 (TLR4) and NF-κB were dtermined as important factors related to function of SAA on monocytes. As TLR4 is known as lipopolysaccharide (LPS) receptor (Palsson-McDermott et al., 2004), the activations of monocytes/macrophages evoked by LPS may be caused also by feline SAA. As NF-κB is a protein complex that controls the transcription of genes encoding the inflammatory cytokines, feline SAA may trigger the more cytokines production. Although only TNF-α production by monocytes with feline SAA stimulation could be examined in this study, human SAA stimulate the production of many kinds of cytokines by monocytes, such as IL-1β and IL-6 (Song et al., 2009). SAA is synthesized predominantly in the liver by the stimulation of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and these cytokines are produced mainly by monocytes/macrophages (Jensen et al., 1998). Monocytes stimulated by SAA produce cytokines and the produced cytokines may cause further synthesis of SAA protein. Thus, it is suggested that feline SAA was not only the result of inflammatory reactions but also an important factor for progression of inflammation.

In chapter 2, the direct effects of feline SAA for the invasiveness of tumor cells were examined. From the results in chapter 1, I considered that feline SAA might have more diverse effects on several kinds of diseases. In this study, I used 4 mammary carcinoma cells and 3 lymphoma cells in order to examine the direct effects of feline SAA to neoplastic diseases. Mammary carcinoma is relatively common in cats and considered a serious health problem due to its frequency and highly metastatic properties (MacEwen, 1990). Lymphoma is described as the most common neoplasm in cats (Louwerens et al., 2005) and high

frequency of elevated SAA concentration in lymphoma cats was demonstrated in chapter 0. In all 4 mammary carcinoma cells, elevated matrix metalloproteinase-9 (MMP-9) levels with feline SAA stimulation were observed. In lymphoma cells, the same phenomenon was observed only in one cell line. Higher expression of MMP-9 and elevated serum MMP-9 concentrations have been described in cats with various tumors including mammary carcinoma and lymphoma (Jankowski et al., 2002). MMP-9 was also described as a prognostic marker in human patients with breast cancer (Sung et al., 2012) and non-Hodgkin's lymphoma (Hazar et al., 2004). Taken together with the results of this study, feline SAA may affect to the prognosis of these tumors through the MMP-9 expression and production. Moreover, in 3 of 4 mammary carcinoma cells and 1 of 3 lymphoma cells, feline SAA directly promoted the invasion of cells through the extracellular matrix. Elevated SAA concentration may stimulate the tumor cells invasion and tumor progression, and advanced clinical stage of tumors may reduce the survival time of the patients. Although the invasive responses to feline SAA varied among the cell lines, these responses tended to be higher in the cell lines originally derived from cats with metastasis (Cheney et al., 1990, Uyama et al., 2005). Further investigation is needed to demonstrate what causes the difference in the invasive response among tumor cells.

Through chapter 1 and 2, I pointed out the possibility that feline SAA itself enhances the inflammatory responses and accelerates the progression of tumors. Furthermore, sustained high SAA concentration and its inappropriate metabolism are related to pathogenesis of AA amyloidosis (Lachmann et al., 2007, Magy et al., 2007). Taken together

with these findings, to control the SAA concentration and its metabolism would be a novel therapeutic target. In chapter 3, I examined about the SAA metabolism by feline peripheral macrophages as one of the targets to control the SAA concentration. Macrophages isolated from feline peripheral blood immediately incorporated the SAA protein into the cytoplasm and SAA concentrations in culture medium decreased in a time-dependent manner. Although SAA degradation products of various molecular weights (4–9 kDa) were detected by immunoblot analysis in previous studies (Lavie et al., 1978, Migita et al., 2001), these were not detected in this study. Pretreatment to macrophages with LPS did not affect this decrease in the SAA concentration, but this was significantly blocked by Dexamethazone (Dex) pretreatment. Although macrophage could produce SAA (Urieli-Shoval et al., 1994) and Dex is reported to enhance its production (Yamada et al., 2000), the amount of SAA produced by feline macrophages was negligible in this study. Dex treatment suppresses various macrophage functions and may also suppress the expression of proteins or genes involved in SAA uptake, such as scavenger receptor class B type 1 (SR-B1) (Cai et al., 2005, Mavridou et al., 2010). Glucocorticoids such as Dex are widely known as anti-inflammatory drug in both human and veterinary medicine and used to suppress the inflammatory responses including SAA synthesis. However, from the results of this study, inadequate usage of that drug may have harmful effect on control of SAA concentration because it inhibits SAA uptake.

The effects of SAA on progression of diseases could also be controlled not only by regulating SAA concentration, but by inhibiting SAA functions. Through blocking the receptors for SAA or inhibition of signaling pathways, harmful effects produced by SAA may

be suppressed. However, as shown in chapter 1, SAA affects to cells by the pathways forming the basis of biological defense and immune response, such as TLR4 and NF-κB. Therefore, clinical usage of suppressors for these pathways should be discussed and estimated closely, especially about side effects of those such as immunosuppressive effect.

Through the series of studies, pathophysiological functions and kinetics of feline SAA were evaluated. Originally, feline SAA was focused as an inflammatory marker and elevated SAA concentration was considered to be only results of advanced inflammatory responses. From the results of my studies, the association between feline SAA and pathophysiology of several diseases was suggested. Thus, feline SAA may be a key factor for a tangled vicious cycle of disease progression. For the management of diseases, definitive therapy for the underlying diseases is most important without mentioning. However, radical therapy would not necessarily be accomplished in several diseases, such as feline infectious peritonitis and metastatic tumor. In these cases, serum SAA concentrations are frequently elevated. Thus, if we can control the SAA concentration or function, it may retard the progress of the diseases. It may become a complementary treatment regardless of the type of diseases and achieve the improvement of quality of life. Although there are many problems to solve about the methodology for controlling SAA concentration or function, my studies will serve as a catalyst of further studies. In future, more studies about controlling SAA will be performed and therapeutic strategies against SAA will spread more widely.

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