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Short communication

Effects of serum amyloid A on matrix metalloproteinase-9 production in feline lymphoma-derived cell lines

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ABSTRACT

Serum amyloid A (SAA) concentration and plasma matrix metalloproteinase-9 (MMP-9) levels are increased in cats with lymphoma. In the present study, the association between SAA and MMP-9 production was evaluated using recombinant feline SAA (rfSAA) and three feline lymphoma-derived cell lines: 3201, MS4, and MCC. MMP-9 mRNA expression was significantly increased by rfSAA stimulation only in MCC cells. Secreted MMP-9 protein in culture media was confirmed by gelatin zymography, with clear bands of MMP-9 detected in MCC cells following rfSAA stimulation. A significant increase in semi-quantified MMP-9 levels was observed with 5 and 25 μg/ml of rfSAA stimulation. The infiltrative activities of feline lymphoma cells, assessed by the matrigel transwell assay, showed that rfSAA stimulated cell infiltration in MCC cells, in addition to MMP-9 expression. Although the response to rfSAA stimulation varied between cell lines, the results showed that rfSAA can stimulate MMP-9 production and infiltration of feline lymphoma-derived cells. The findings of this study have identified a novel role for SAA in the progression of some forms of feline lymphoma.

1. Introduction

Serum amyloid A (SAA) is one of the major acute phase proteins (APPs) in mammals, including humans (Uhlar and Whitehead, 1999) and cats (Kajikawa et al., 1999). Acute phase reaction is a systemic response to inflammatory stimulations, such as infection or trauma, and APPs including SAA are synthesized mainly in the liver as part of the reaction (Kisilevsky et al., 1979). SAA gene expression and protein synthesis are stimulated by several inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Jensen and Whitehead, 1998). Synthesized SAA is released into circulation with SAA secretion into blood resulting in a marked increase in the serum concentration of SAA. During inflammation, serum SAA concentration can occasionally increase up to 1000-fold above the basal level (Kushner, 1988).

The dramatic increase in serum SAA concentration during inflammation is used as an inflammatory marker in both humans (Nakayama et al., 1993) and cats (Tamamoto et al., 2009). Increased SAA concentration has also been described in various inflammatory and infectious diseases (Lee et al., 2006; Tamamoto et al., 2008), such as neoplastic diseases in humans and cats (Tamamoto et al., 2008; Cho et al., 2010; Wang et al., 2012). Lymphoma is the most common neoplasm in cats, with increased SAA concentration in feline lymphoma patients observed with high frequency (Tamamoto et al., 2008). Moreover, increased SAA concentration was demonstrated as a prognostic marker in cats with various diseases, including lymphoma (Tamamoto et al., 2013b).

Matrix metalloproteinases (MMPs) are a family of extracellular, zinc-dependent matrix degrading proteases associated with infiltration and metastasis during tumor progression (Stamenkovic, 2000). Type IV collagen is an integral component of the basement membrane and its collagenase, matrix metalloproteinase-9 (MMP-9), is believed to play a key role in tumor infiltration and metastasis (Sehgal et al., 1998). Higher MMP-9 expression and increased serum/plasma MMP-9 concentrations have been described in cats with various tumors, including lymphoma (Jankowski et al., 2002; Tamamoto et al., 2014b). Treatment of cat lymphomas with chemotherapy was however shown to effectively decrease the increased plasma MMP-9 activity (Tamamoto et al., 2014b). Thus,

Abbreviations: SAA, serum amyloid A; MMP-9, matrix metalloproteinase-9; APPs, acute phase proteins; rfSAA, recombinant feline SAA; GAPDH, glyceraldehye-3-phosphate dehydrogenase; TLR4, Toll-like receptor 4.

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increased MMP-9 concentrations and activities are considered as lymphoma progression factors in cats.

We previously reported that feline SAA might affect MMP-9 expression of mammary carcinoma cells (Tamamoto et al., 2014a). However, there is scarce information about the relationship between SAA and MMP-9 in lymphoma. In the present study, the effects of recombinant feline SAA (rfSAA) on MMP-9 mRNA expression and protein synthesis in three feline lymphoma-derived cells were evaluated. The effect of rfSAA on actual infiltration activities of these cells was also estimated.

2. Materials and methods

2.1. Cell culture

Three feline lymphoma-derived cell lines were used: 3201 (Snyder et al., 1978), MS4 (Mochizuki et al., 2011), and MCC (Cheney et al., 1990). All three cell lines are feline leukemia virus (FeLV) negative. Cell line 3201 was derived from T-cell lymphoma and MS4 from B-cell lymphoma. MCC was derived from a large granular lymphocyte (LGL) lymphoma patient and was shown to be a non-T and non-B immunophenotype. 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 using a previously described method (Tamamoto et al., 2008, 2013a). The rfSAA amino acid sequence corresponds to the previously reported sequence of feline SAA and amyloid A (Ohno et al., 1999; Tamamoto et al., 2008). Recombinant protein was produced in *Escherichia coli* and obtained as a hexahistidine-tagged protein. Synthesized rfSAA was desalted 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 12.5% SDS-PAGE and determined to be greater than 95% (Image Lab Software; Bio-Rad Laboratories, Hercules, CA).

2.3. Stimulation of cell lines by rfSAA

Subconfluent (70–80% confluent) cells were 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. 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 in three separate experiments.

2.4. RNA extraction and quantitative RT-PCR

MMP-9 mRNA expression was determined as previously described (Tamamoto et al., 2014a). Total cellular RNA was extracted 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, cDNA was amplified with SYBR green (THUNDERBIRD SYBR qPCR Mix; TOYOBO) using gene-specific primers and a thermal cycler (Thermal Cycler Dice Real Time System; Takara Bio, Shiga, Japan) with the following program: 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). RT-PCR reactions were performed with primers specific to feline MMP-9 (GenBank accession number, AB858226): 5′-GCC CCT ACA GTG TCT TTG GA-3′ (forward), 5′-TCC CAT CCT TGA AGA AAT GC-3′ (reverse) and glyceraldehye-3-phosphate dehydrogenase (GAPDH: GenBank accession number, NM_001009307): 5′-GCT GCC CAG AAC ATC ATC C-3′ (forward), 5′-GTC AGA TCC ACG ACG GAC AC-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, as previously described (Tamamoto et al., 2014a, 2014b). All samples were diluted in sample buffer (125 mM Tris-HCl pH 6.8, 25% glycerol, 5% SDS, 0.2% bromophenol blue) with an equal volume of culture media and then subjected to electrophoresis on a 10% SDS-PAGE gel co-polymerized with 0.1% gelatin. Following electrophoresis, 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. Gels were subsequently 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) including human MMP-9 (MW: 92 or 82 kDa) and MMP-2 (72, 68, or 62 kDa) 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 the MMP-9 standard band. The ratio of unknown to standard protein was calculated, and an arbitrary unit (au) value assigned to each sample. Each sample was assessed in duplicate.

2.6. Infiltration assay

Infiltration of cell lines was assessed by the matrigel transwell assay (Tamamoto et al., 2014a). 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), which serves as a reconstituted basement membrane *in vitro*. By coating the upper surface of the membrane, matrigel occludes the pores of the membrane and blocks non-infiltrative cells from migrating through the membrane. In contrast, infiltrative cells secrete proteases that degrade the matrigel and enable infiltration through the membrane pores. Cells (2×10^5/well) in 200 μl of serum-free media (RPMI 1640 containing 1% BSA) were added to the upper chamber with rfSAA (1, 5, and 25 μg/ml) and the lower wells were filled with 600 μl of media (RPMI 1640 containing 10% FBS). After 24 h, cells that invaded the matrigel and reached the lower wells were counted, and the ratio of rfSAA stimulated to control cells was calculated. Each experiment was performed in duplicate and repeated in three 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 treatments with their respec-