

Elevated Serum Levels of S100 Calcium Binding Protein A8 (S100A8) Reflect Disease Severity in Canine Atopic Dermatitis

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ABSTRACT. A monoclonal antibody to canine S100 calcium binding protein A8 (S100A8) was developed to determine the association between S100A8 and the disease severity of canine atopic dermatitis. Serum S100A8 concentrations were studied in dogs with canine atopic dermatitis (n=213) and healthy dogs (n=213). Statistical correlations between these indices and atopic dermatitis activity were established, and dermatitis severity was assessed according to the CADESI score. Serum S100A8 concentrations were measured with an enzyme-linked immunosorbent assay (ELISA). S100A8 serum levels were significantly higher in canine atopic dermatitis patients than in healthy dogs. A strong positive correlation was identified between S100A8 levels and canine atopic dermatitis patients. Our findings suggested that S100A8 is actively involved in the pathogenesis and clinical picture of canine atopic dermatitis.

KEY WORDS: atopic dermatitis, canine, ELISA, monoclonal antibody, S100A8.

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Canine atopic dermatitis is one of the most common skin diseases affecting dogs; however, the pathogenesis of this disease is not fully understood. Numerous inflammatory cells, including neutrophils, monocytes, and macrophages, may play a role in the pathogenesis of canine atopic dermatitis [7]. The excess migration of recruited phagocytes and enhanced cell surface expression of adhesion molecules result in the development of characteristic inflammatory lesions.

Recent reports have suggested that the S100 protein, S100A8 (MRP-8 in human beings or CP-10 in mice), which is abundant in activated or recruited phagocytes, may have a modulatory role in inflammatory responses [8]. Neutrophils migrate from the blood to inflammatory sites in a multi-step fashion during inflammatory responses and are involved in immune defense [18]. Recent studies have suggested that S100A8 is involved in neutrophil migration [21].

Human S100A8 is a calcium-binding protein member of the S100 protein family [15], is highly expressed in the cytosol of neutrophils and monocytes, and is frequently found at high levels in the extracellular milieu during inflammatory conditions [14]. S100A8 is almost exclusively expressed by cells of myeloid lineage and is constitutively expressed in the cytosol of neutrophils [6]. Monocytes and differentiated macrophages from inflamed tissues also express S100A8 [25]. Many studies have attempted to elucidate S100A8 function in allergic reactions with the majority of published data from human beings [12, 19] or rodent models [4, 12], just a few studies of veterinary field described their involve-

ment in gene (mRNA) levels [10, 24].

In this study, we developed novel monoclonal antibodies recognizing canine S100A8 protein and determined the role of this protein by performing ELISA in the serum of atopic dermatitis patients through the use of novel monoclonal antibodies. We wished to investigate the specificity of increased canine S100A8 serum levels in inflammatory skin disease such as atopic dermatitis and to evaluate S100A8 as a biomarker of disease severity in canine atopic dermatitis patients.

MATERIALS AND METHODS

Production of monoclonal antibodies to canine S100A8

Purification of recombinant canine S100A8: Total RNA was isolated from peripheral blood mononuclear cells (PBMC) via the silica-gel-membrane method, and cDNA samples were synthesized using reverse transcriptase with oligo dT primers. The PCR reaction was performed using a forward primer (5'-GGATCCATGCTGACGGAAGTGGAG-3'), nt 56–73 (the italic sequence is Bam HI adaptor), and a reverse primer (5'-GTCTGACCTACTCCTTGTCGATGTC-3'), nt 308–325 (the italic sequence is Sal I adaptor), in canine S100A8 cDNA (GenBank Accession number FJ752688). The amplified cDNA fragment encoding the mature form of canine S100A8 was cloned into a pDrive vector (PCR cloning kit, QIAGEN, Hilden, Germany) and transformed to *Escherichia coli* (*E. coli*) competent cells (Top10, Invitrogen Corp., Carlsbad, CA, U.S.A.). The plasmids were purified using a commercially available kit (EndoFree Plasmid Maxi Kit, Qiagen, Chatsworth, CA, U.S.A.). The canine S100A8 insert was excised from the pDrive vector using Bam HI and Sal I. The fragment was ligated into

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the Bam HI and Sal I sites of the expression vector, pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ, U.S.A.), to enable the expression of S100A8 as a fusion protein with glutathione S-transferase (GST), using a commercially available kit (DNA ligation kit, Takara, Shiga, Japan). The fusion protein combined with Glutathione Sepharose was separated using a Glutathione Sepharose 4B column (Amersham Biosciences). The fusion protein was incubated with thrombin at room temperature for 16 hr in order to separate the complex between the fusion protein and GST-Glutathione Sepharose from the fusion protein. Recombinant S100A8 protein was then purified again using gel infiltration chromatography (Superose 12HR10/30, Amersham Biosciences). The protein concentration of the purified recombinant S100A8 was determined by BCA protein assay (Pierce Biotechnology, Rockford, IL, U.S.A.) and the purity was examined by SDS-PAGE. After SDS-PAGE, purified enzyme on polyacrylamide gel was transferred to a polyvinylidene difluoride membrane by electroblotting [9] and stained with Ponceau S solution containing 5% acetic acid. The stained portion was excised and used for N-terminal sequencing directly.

Chemotaxis assay: Canine peripheral blood was obtained from healthy beagle dog (Marshall Farms, North Rose, NY, U.S.A.) raised with experimental purpose. Neutrophils were isolated as previously described by Smith [16] and resuspended in HBSS-H [HBSS supplemented with 10 mM HEPES (pH 7.4)] containing 1.3 mM Ca^{2+} and 0.8 mM Mg^{2+} . The purity and cell viability of neutrophil preparations were consistently >98% as assessed by acetic blue staining and trypan blue exclusion, respectively. Neutrophil chemotaxis assays were performed *in vitro* using a modified Boyden chamber technique [17]. Briefly, increasing concentrations of canine recombinant S100A8 (1–1000 ng/ml) or 10^{-6} M fMLP diluted in HBSS-H containing 1.3 mM Ca^{2+} and 0.8 mM Mg^{2+} was added to the lower well of the chambers. Polycarbonate filters (8 μm pore; Neuroprobe, Gaithersburg, MD, U.S.A.) were put in place and the upper chambers were secured. Neutrophils (5×10^4 cells in 200 μl) were added to the upper chambers and allowed to migrate through the membrane for 30 min at 37°C. The upper chambers were washed once with PBS, and the membrane was then fixed in methanol and stained with Wright-Giemsa. Cells that had migrated through the membrane were counted using a microscope in five high-power fields. All responses were assayed in triplicate.

Production of hybridoma: An emulsion of 150 μl complete Freund's adjuvant containing 50 μg of purified canine S100A8 was injected into the peritoneal cavity of six-week-old male BALB/c mice. The injections were repeated after 15, 30, and 45 days using the same amount of antigen with incomplete Freund's adjuvant. Finally, five days prior to fusion, the mice received the same amount of antigen in saline without adjuvant. Among immunized mice, the mouse with the highest blood antibody titer to S100A8 was used in cell fusion. The spleen cells were fused with SP2/0-Ag14 myeloma cells at a ratio of 2:1 in DMEM (Gibco BRL,

Great Island, NY, U.S.A.) with 50% (w/v) polyethylene glycol 1500 (Roche, Molecular Biochemicals, Indianapolis, IN, U.S.A.). Hybridoma cells were selected using hypoxanthine-aminopterin-thymidine (HAT) medium. Positive clones to S100A8 were selected by ELISA, and were subcloned by limiting dilution.

Production and screening of mAbs: Culture supernatants from clones were tested by ELISA as follows. Microtiter plates were coated with 100 μl of 10 $\mu\text{g}/\text{ml}$ recombinant canine S100A8 solution and were kept overnight at 4°C. The plates were blocked with 200 μl of blocking buffer (3% bovine serum albumin in PBS with 0.05% Tween 20) for 2 hr at 37°C and washed three times with PBS-Tween 20. The plates were incubated with 100 μl of culture supernatant fluids for 2 hr at 37°C. After three washes, 100 μl of peroxidase-conjugated goat anti-mouse antibody was added at a dilution of 1:1,500, and the plates were incubated for 1 hr at 37°C. Unbound conjugate was removed through three washes with PBS-Tween 20. Color development was done with 100 μl of substrate solution with *o*-Phenylenediamine (Sigma-Aldrich Co., St. Louis, MO, U.S.A.). The plate was kept for another 20 min at room temperature while being protected from light. The color reaction was stopped with 50 μl of 2.5 M sulphuric acid, and the absorbance of the samples was measured at 450 nm in an ELISA plate reader (Model 550, Bio-Rad, Hercules, CA, U.S.A.). To obtain large amount of mAbs from ascites, we injected 0.3 ml of Freund's incomplete adjuvant into a BALB/c mouse, and in hybridoma cells two weeks later. After two weeks, the ascites fluid was harvested and incubated at 37°C for 1 hr and at 4°C overnight. The supernatants were recovered after centrifugation at 3,000 g for 10 min and then kept at -20°C for storage.

To check the avidity of mAb to canine S100A8, ascites fluids diluted of 1:100 were added into recombinant S100A8 coated plates. After incubation and three times washes, peroxidase-conjugated goat anti-mouse Ab at a dilution of 1:1,500 was added. The mAb with high avidity were selected for this study. The classes and subclasses of selected S100A8 mAb were determined by enzyme immunoassay using an ImmunoPure Monoclonal Antibody Isotyping Kit II (Pierce, Rockford, IL, U.S.A.) containing rabbit antibodies against mouse antibodies for each of the immunoglobulin isotypes. The typing procedure was performed essentially per the instructions of the kit provided by the manufacturer.

In order to examine the possibility of mAb recognizing novel epitopes on canine S100A8 mAb, competitive binding assay was performed. Since there were no commercial products of canine S100A8 antigen or antibodies, we used commercial rat and mouse polyclonal antibody products (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) as a competitor. Each biotinylated mAb (100 ng/ml) and serially diluted competitor mAb (0–5000 ng/ml) were mixed 1:1 by volume and the mixtures (100 $\mu\text{l}/\text{well}$) were added to recombinant canine S100A8-coated plates. Procedures were conducted as above.

Immunoblotting: Ten micrograms of purified recombinant canine S100A8 protein was suspended in 50 μ l of SDS sample buffer and boiled for 10 min prior to electrophoresis. After electrophoretic separation on a 12% acrylamide gel in 120V, protein bands in gel were transferred electrophoretically to a nitrocellulose membrane (HybondECL 0.45 micron, RPN 2020D, Amersham Life Science, Buckinghamshire, England). After transfer, membranes were blocked with a blocking buffer (PBS pH 7.4, 1% BSA, 5% skim milk powder) at 4°C overnight. The membranes were subsequently incubated with mAbs for 1.5 hr at ambient temperature. After three wash steps with wash buffer, the membranes were incubated with sheep anti-mouse peroxidase-conjugate (NA 931, Amersham Life Science) at a dilution of 1:1,500 for 2 hr. After washing three times with PBS, each membrane was incubated for 1 min in DAB solution (50 mM Tris, pH 7.6 containing 0.6 mg/ml diaminobenzidine). For detection, we used the enhanced chemiluminescence reagents, ECL Detection Solutions 1 and 2 (RPN 2209, Amersham Life Science). The exposure time to the x-ray films (Hyperfilm-ECL, Amersham Life Science) varied from 1 min to overnight.

Serum S100A8 analysis

Patients and samples: The study group included 213 dogs with atopic dermatitis (82 females, 39 males, 28 splayed females, and 64 castrated males; mean age was 5 years \pm 2 months). Patients were diagnosed according to the Willemse criteria [23]. Food trial and skin testing were conducted for definitive diagnosis. The patients with positive response to the food trial were excluded from this study. Severity of atopic dermatitis was evaluated with the CADESI-03 (Canine Atopic Dermatitis Extent and Severity Index), in which severity is scored by 4 clinical features (erythema, lichenification, excoriation, and self-induced alopecia) graded at 62 defined body sites on a scale of 0 (none) to 5 (severe) [11]. Dogs suffering from immune-mediated deficiencies, malignancies, infections, and treatment with topical or systemic medications during the 3 months preceding the study were excluded from further analysis. The control group included 213 healthy dogs without a history of allergic or other disease 1 year preceding study analysis (48 females and 165 males; mean age was 4 years \pm 4 months). Blood collection, handling, and storage were performed by the same researcher. Samples were centrifuged at 1,300 g at 4°C for 10 min, and separated serum samples were stored at -70°C until use.

Sandwich ELISA: Before conducting sandwich ELISA, binding inhibition assay for selecting antibody pairs was conducted. Recombinant canine S100A8 was coated onto the wells and then blocked with blocking solution. Then a constant amount of each mAb—inhibitor mAb (1:400) was coincubated with PBST. Assay was conducted as above. Serum S100A8 concentrations were quantified by a sandwich ELISA technique using anti-canine mAbs selected by inhibition assay (3F12 and 5E1). Microtitre plates (96-well; Becton Dickinson, Franklin Lakes, NJ, U.S.A.) were coated with 5E1 anti-canine S100A8 mAbs; the 5E1 mAbs were

applied at concentrations of 3 μ g/ml to plates incubated overnight at 4°C. Plates were washed 3 times with PBS containing 0.05% Tween-20 (PBS-Tween), and were blocked for 1 hr with PBS (200 μ l) containing 1% BSA per well at room temperature. Standard dilutions and samples were subsequently added and incubated for 1 hr at room temperature. Plates were then incubated for 1 hr with the biotinylated 3F12 anti-canine S100A8 mAbs according to standard procedures [20]. After washing, plates were incubated with streptavidin-poly horseradish peroxidase (HRP, Sigma-Aldrich) diluted in PBS containing 2% milk (Sigma-Aldrich) for 40 min at room temperature. After the wells were emptied and washed, tetramethylbenzidine solution (100 μ l) was added to each well, and each plate was incubated for 10 min in darkness. The reaction was stopped by the addition of phosphoric acid (1 mol/l), and optical densities were measured at 450 nm with a Bio-Rad microplate reader (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The ELISA was calibrated with recombinant canine S100A8 in concentrations ranging from 1 to 1,600 ng/ml for detector mAb. The assay was generally performed in triplicate.

Statistical analysis: The Kruskal-Wallis test was used to compare variables between the groups. The atopic dermatitis group was separated into mild, moderate, and severe disease state categories based on the classification methods of reference article (Mild=CADESI<60, Moderate=60<CADESI<120, and Severe=120<CADESI) [11]. Simple regression analysis was used to examine the relationship between the two continuous variables (CADESI scores and serum S100A8 concentrations). Statistic significance was established a *P*-values less than 0.001.

RESULTS

Production of monoclonal antibodies to canine S100A8: Recombinant canine S100A8 was expressed in *Escherichia coli* using pGEX expression vectors for glutathione S-transferase (GST) fusion proteins, purified by affinity chromatography on glutathione-Sepharose. Purity was confirmed by SDS-PAGE (Fig. 1). The N-terminal amino acid sequence of the first 14 residues of canine S100A8 was analyzed by a protein sequencer. The sequence was Met-Leu-Thr-Glu-Leu-Glu-Ser-Ala-Ile-Asn-Ser-Leu-Ile-Glu, which is nearly identical to the predicted sequence of canine S100A8 (GenBank Accession number FJ752688). Chemotactic activity was measured by a bioassay using a modified Boyden chemotaxis chamber. Chemotaxis assay revealed the recombinant canine S100A8 has the potent chemotactic ability to induce migration of canine neutrophils (Fig. 2).

Seventy-two hybridomas were selected on the first fusion, and 11 hybridomas cells were selected on the second fusion. Six hybridoma clones (2F1, 3F12, 5E1, 6A8, 6F7, and 6H6) formed ascites fluid, and identity was confirmed by immunoblotting with recombinant canine S100A8 using developed 6 mAb. We found a band with an approximate molecular weight at 10 kDa (Fig. 3) and concluded all six mAb have a high affinity to recombinant canine S100A8

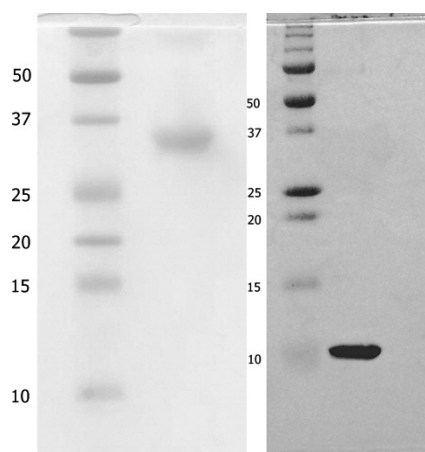


Fig. 1. Purification of recombinant canine S100A8. Recombinant canine S100A8 before and after purification was run on 15 and 12% SDS-PAGE. (Left) 15% SDS-PAGE with colored-marker. Expressed protein with fusion partner located in almost 36 kD (10 kD of target protein + 26 kD of GST). (Right) 12% SDS-PAGE. Purified recombinant canine S100A8 located in almost 10 kD after enzymatic cleavage of the fusion partner.

from the results of avidity assay. To exclude non-specific binding of secondary antibodies in color reaction, we confirmed the specificity of secondary antibodies in our assay. The secondary antibodies to rat IgG1, IgG2a, and IgG2b were used and did not bind to our six mAbs.

The mAb clones showed the distinctive recognition of the recombinant canine S100A8 from the results of competitive binding assay (Fig. 4: other 4 mAb data not shown), respectively. For the serum S100A8 ELISA, two mAb isotypes as

IgG2a/IgG1 kappa were chosen by binding inhibition assay (Fig. 5: other pairs of mAb cross-reaction data not shown), and standard curves for 3F12 and 5E1 were generated with a purified recombinant canine S100A8 (Fig. 6). OD values (450 nm) were increased proportionally with increased concentrations of the recombinant canine S100A8. The relationship was expressed by the equation as follows,

3F12 (detector): $Y = -0.00000161X^2 + 0.00375048X + 0.13990167$, ($R^2 = 0.99705424$, $P < 0.0001$)

5E1 (capture): $Y = -0.00000092X^2 + 0.00326966X + 0.18347719$, ($R^2 = 0.99200031$, $P < 0.0001$)

Serum S100A8 levels are specifically elevated in patients with AD: Serum S100A8 levels were significantly increased in the canine atopic dermatitis patient group (736.71 ± 221.66) compared to healthy control dogs (137.55 ± 18.14), as seen in Fig. 7 ($R^2 = 0.8497$, $P < 0.0001$).

Serum S100A8 levels correlate with disease severity in canine atopic dermatitis: Serum S100A8 levels correlated with CADESI severity scores ($R^2 = 0.8493$, $P < 0.0001$) (Fig. 8). A significant correlation was identified between serum S100A8 levels and CADESI scores in canine atopic dermatitis patients.

DISCUSSION

We prepared recombinant canine S100A8 protein using an *E. coli* expression system, and established six anti-canine S100A8 mAbs in order to examine the association of this calcium binding protein with the pathogenesis of canine atopic dermatitis. The monoclonal antibody 3F12 and 5E1 were chosen for sandwich ELISA as a novel anti-canine S100A8 monoclonal antibody in this study, because it showed the highest reactivity in the preliminary ELISA screening and the best performance in the various kinds of assays including avidity assay, competitive inhibition assay, and binding inhibition assay. The mAbs developed in this

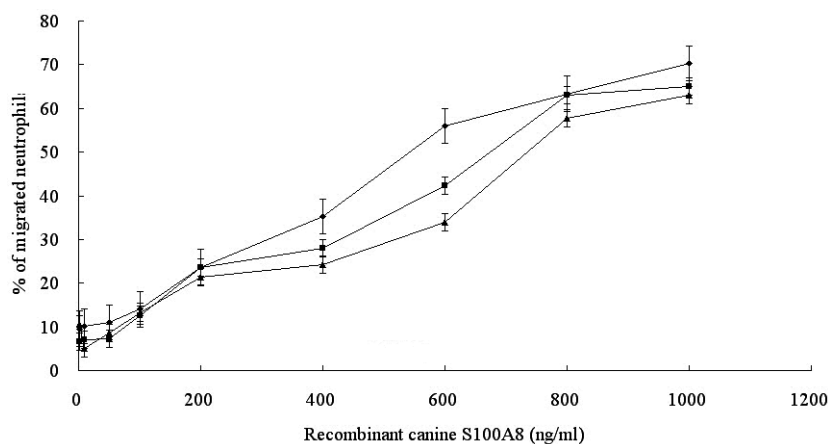


Fig. 2. Migration of canine neutrophil toward recombinant canine S100A8. Concentrations ranging from 1 to 1,000 ng/ml significantly increased migration of all cells tested neutrophils. Results are shown as percentage of migrated neutrophils toward recombinant canine S100A8 and fMLP. Data shown represent mean \pm SEM of three experiments performed on neutrophils from different donors.

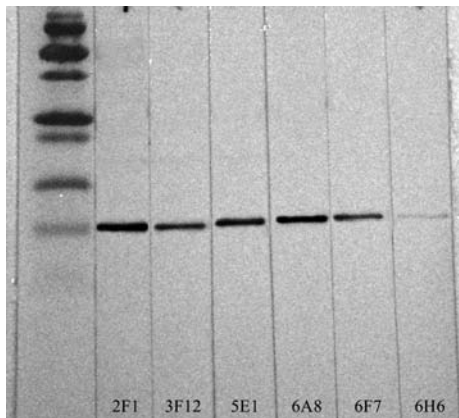


Fig. 3. Six mAbs reacted with purified recombinant canine S100A8 on Western blot assay.

study that recognize discrete epitopes with varying isotypes may provide veterinary researchers with a molecular approach to diverse functional studies and comparative studies of S100A8.

Our data demonstrate that canine atopic dermatitis is

associated with a significant elevation of S100A8 serum levels. A large variety of laboratory and clinical studies in human beings have linked S100A8 measurements to disease severity in atopic dermatitis. This has led to a wide variation in outcome measurements in clinical studies that is hindering evidence-based practice [5]. Atopic dermatitis is characterized by the predominant infiltration of inflammatory cells in lesional skin, and chemotactic factor cells are involved in allergic inflammation by the regulation of cell traffic [1]. Studies conducted using murine myeloid-related proteins (MRPs) demonstrated that murine S100A8 (CP-10) is a potent chemotactic factor (activity at 10^{-13} – 10^{-11} M) for neutrophils [2], and induces sustained *in vivo* leukocyte recruitment [3]. Recent evidence suggests that CP-10 mediate leukocyte retention within microcapillaries and thereby contribute to the initiation of inflammation in vascular beds without activation [2, 14]. Our purified recombinant canine S100A8 showed a clear understanding of activities associated with neutrophil migration to inflammatory sites by the *in vitro* neutrophil chemotaxis assay. Previous veterinary medical studies also indicated the potentiality of canine S100A8 that showed the highest increase of all the genes in microarray analysis [10] and canine S100A8 genes may be

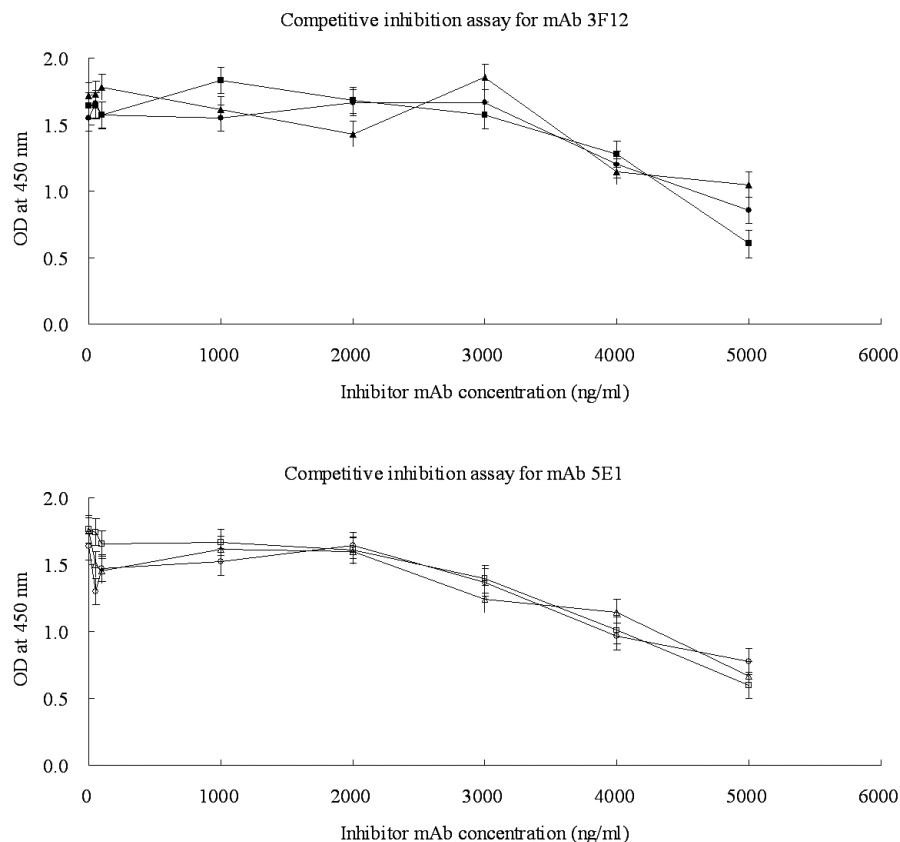


Fig. 4. Competitive inhibition assay results for mAb 3F12 and 5E1 with commercial rat and mouse polyclonal antibody products. Increasing concentrations of rat and mouse polyclonal antibody did not have significant inhibition activity at the concentrations under 3,000 ng/ml.

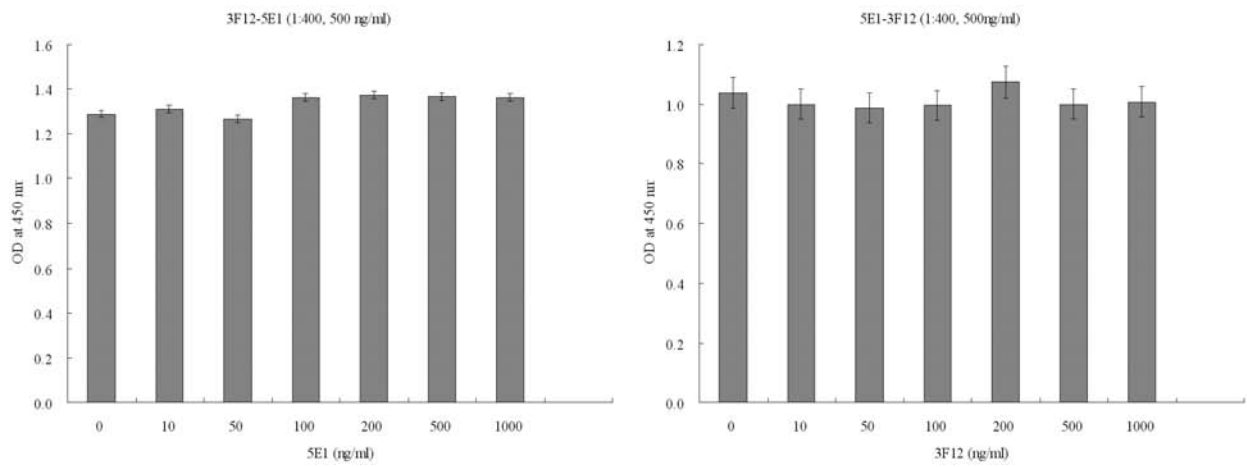


Fig. 5. Binding inhibition assay for selecting antibody pairs in the performance of canine S100A8 sandwich ELISA. No significant inhibition was observed between 3F12 and 5E1. However, 2 pairs of mAb showed inhibitory reactions (6A8/2F1, 6F7/6H6) in the binding inhibition assay (Data not shown).

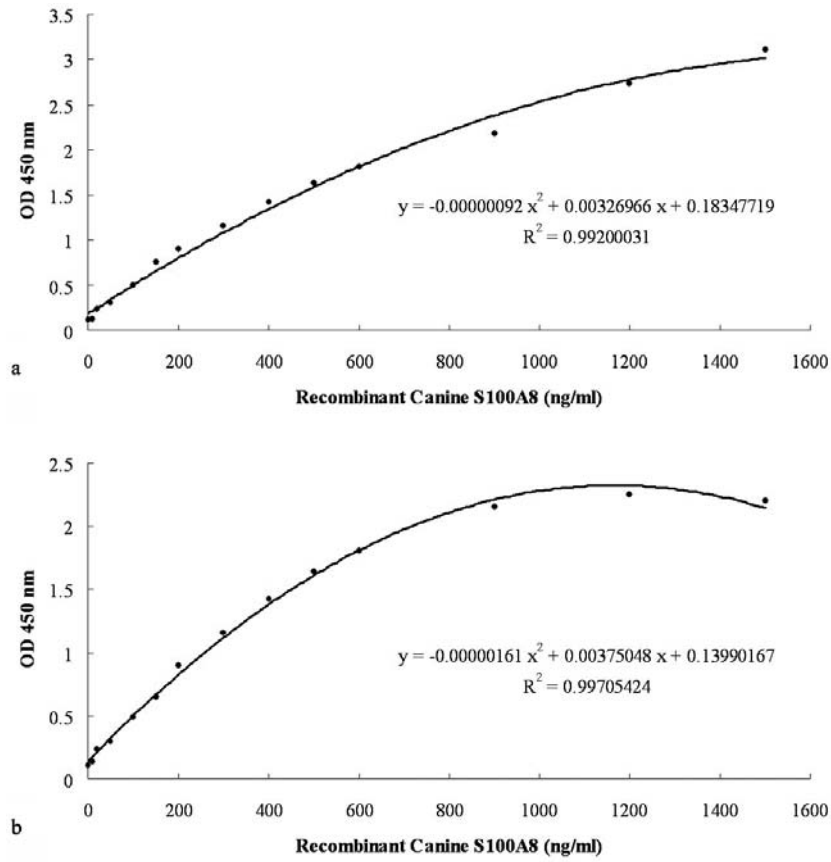


Fig. 6. Calculated schematic-standard curve for 5E1 and 3F12 using a quadratic curve-fitting model. Two standard curves were generated with serial dilutions of purified recombinant canine S100A8, using the 5E1 for capture (a) and the 3F12 (b) for detection. The concentrations were determined by absorbance at 450 nm.

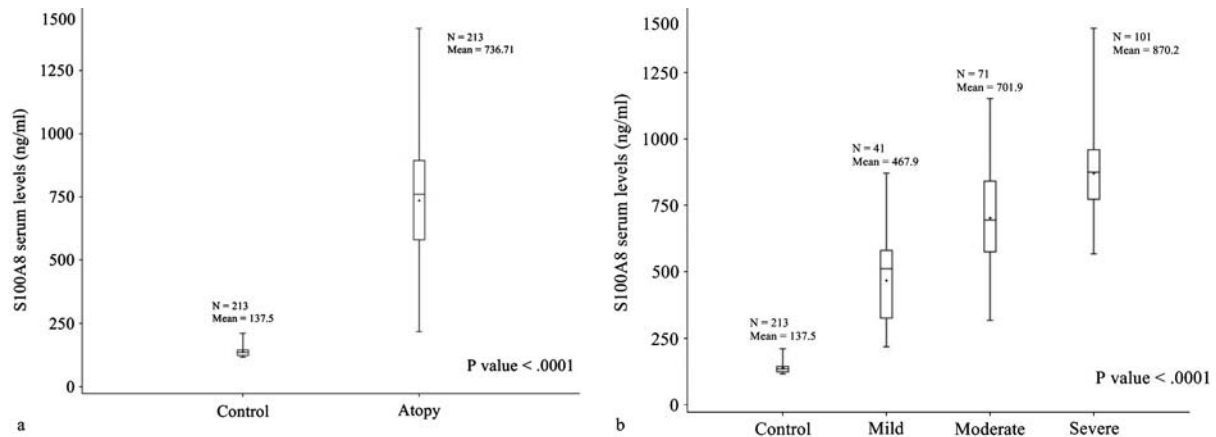


Fig. 7. Canine S100A8 serum levels are elevated in patients with atopic dermatitis including mild, moderate, and severe disease status. Serum S100A8 levels were significantly higher in the atopic dermatitis group than in healthy control ($P < 0.0001$) (a). Second illustration indicates each status of atopic dermatitis group also showing statistically significant differences compared to control group ($P < 0.0001$) (b). However, the serum S100A8 level differences among the mild, moderate, and severe groups were not shown as significant, respectively.

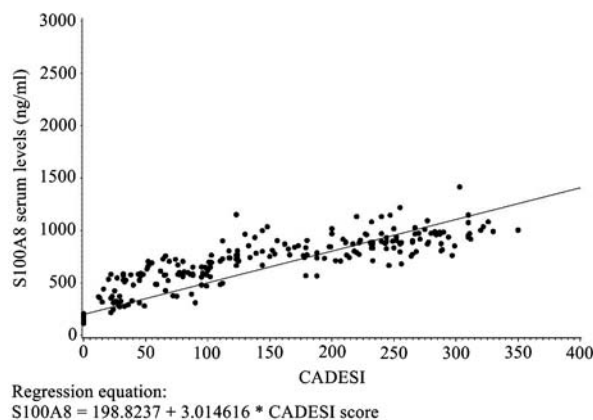


Fig. 8. Serum S100A8 levels in canine atopic dermatitis patients were positively correlated with disease severity. The relationship between serum S100A8 levels ($n=213$) and disease severity (CADESI) is shown.

considered suitable targets for future genetic and protein function studies in human and canine atopic dermatitis [24].

To evaluate a possible role for serum S100A8 levels in canine atopic dermatitis, we determined serum S100A8 levels in phenotypically well-defined groups of canine atopic dermatitis patients. We used nonparametric statistical tests, Kruskal-Wallis nonparametric analysis of variance, was used to assess significant in group comparisons of healthy and atopic dermatitis. We concluded that serum S100A8 levels were elevated significantly in canine atopic dermatitis patients, and linear regression/correlation analysis was used for the significance testing of the correlation between the CADESI score and serum S100A8 concentration. The regression/correlation coefficient was represented by the equation [$S100A8 = a + b \times (\text{CADESI score})$]. Additionally,

the R-value closely approached the value of 1, supporting the correlation between CADESI scores and serum S100A8 concentrations. However, elevated serum levels of canine S100A8 are not disease specific but are sensitive markers suggesting high correlation with S100A8 levels and their function in canine atopic dermatitis. Further comparative studies remain to be investigated with in such as cancer, immune-mediated disease, infectious disease, and other inflammatory disease.

It is likely that canine S100A8 has many pathophysiological roles in the inflammatory processes, such as leukocyte migration. Elevated canine serum S100A8 levels presumably originated from inflammatory cells, including monocytes or neutrophils. Release of S100A8 into the extracellular/extravascular compartment presumably facilitates leukocyte migration and recruitment to sites of skin inflammation in disease states such as atopic dermatitis. In the previous studies about murin CP-10 and human MRP8, release of this protein into the extracellular compartment suggests some kind of specific extracellular function [13]. S100A8 binds to endothelial cell surface heparan sulphate glycosaminoglycans, decrease expression of endothelial cell junction proteins, enhance binding activity of granulocyte CD11b/CD18 integrin receptors to endothelium, and modulate transendothelial migration of leucocytes [13, 22]. Taken altogether, such events presumably deteriorate the clinical condition of canine atopic skin inflammation, suggesting further studies and investigations about pathophysiological process of S100A8 in dogs.

In summary, elevated serum S100A8 concentrations in dogs may serve as a useful biomarker for the monitoring of canine atopic dermatitis clinical activity. S100A8 may be linked to leukocyte recruitment, suggesting that it is one of a key factor in canine atopic dermatitis inflammatory responses. Characterization of canine S100A8 may be useful for the understanding of cellular processes induced in

canine inflammatory diseases.

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