

Evaluation of canine antimicrobial peptides in infected and noninfected chronic atopic skin

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Background – Antimicrobial peptides (AMPs) are small immunomodulatory peptides produced by epithelial and immune cells. β -Defensins (BDs) and cathelicidins (Caths) are the most studied AMPs. Recently, increased cutaneous expression of AMPs was reported in atopic humans and in beagles with experimentally induced atopy.

Hypothesis/Objectives – Our goal was to analyse mRNA expression and protein levels of canine (c)BD1-like, cBD2-like/122, cBD3-like, cBD103 and cCath in healthy and naturally affected atopic dogs, with and without active skin infection, along with their distribution in the epidermis using indirect immunofluorescence.

Animals – Skin biopsies were taken from 14 healthy and 11 atopic privately owned dogs.

Methods – The mRNA levels of *cBD1-like*, *cBD2-like/122*, *cBD3-like*, *cBD103* and *cCath* were quantified using quantitative real-time PCR. The protein levels of cBD3-like and cCath were analysed by relative competitive inhibition enzyme-linked immunosorbent assay, while the distributions of cBD2-like/122, cBD3-like and cCath were detected by indirect immunofluorescence.

Results – Dogs with atopic dermatitis had significantly greater mRNA expression of *cBD103* ($P = 0.04$) than control dogs. Furthermore, atopic skin with active infection had a higher *cBD103* mRNA expression ($P = 0.01$) and a lower *cBD1-like* mRNA expression ($P = 0.04$) than atopic skin without infection. No significant differences in protein levels (cBD3-like and cCath) or epidermal distribution of AMPs (cBD2-like/122, cBD3-like and cCath) were seen between healthy and atopic dogs.

Conclusions and clinical importance – Expression of *cBD103* mRNA was greater, while expression of *cBD1-like* mRNA was lower in dogs with atopic dermatitis that had active infections. Work is needed to clarify the biological mechanisms and possible therapeutic options to maintain a healthy canine skin.

Introduction

Atopic dermatitis (AD) has recently been redefined by the International Task Force on Canine Atopic Dermatitis as 'genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens'.¹ In both humans and dogs, AD is extremely common, affecting up to 30 and 10% of the respective populations.^{2,3} Recently, in both human and veterinary medicine, researchers have shown altered skin barrier integrity to be the main factor involved in the pathogenesis and predisposition to AD.^{4–18} As part of the skin barrier, antimicrobial peptides (AMPs) have been studied in different species, including humans.^{19–22} To date, over 1000 AMPs, subclassified based on their molecular structure, have been identified in diverse

species (e.g. peptides, lipids, histones).^{19,20} Of these peptides, β -defensins (BDs) and cathelicidins (Caths) have received the most research attention. Such peptides have many functions; they have antimicrobial activity against a variety of micro-organisms, are potent angioactive and chemotactic molecules, are involved in wound healing, act as potent 'host defense peptides' able to chemoattract immune cells and respond to danger signals by alerting the adaptive immune system, and they also modulate the innate and adaptive immune responses in higher organisms.^{20,23–25} An increased expression of AMPs (e.g. BDs, LL-37, psoriasin and ribonuclease 7) in some human inflammatory conditions, such as AD and psoriasis, has demonstrated an involvement of such AMPs in inflammatory skin conditions.^{2,26–30}

Few studies have been published on the possible involvement of AMPs in the pathogenesis of AD in dogs. In fact, only two studies have investigated the association of canine AMPs with canine AD. The first used chronically naturally affected atopic dogs and the second used experimentally affected atopic beagles.^{31,32} Results were similar to human studies, showing increased mRNA expression of some canine

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peptides (*cBD1*, *cBD1-like*, *cBD3-like* and *cCath*) in lesional and nonlesional skin of dogs with AD when compared with healthy control dogs;^{31,32} however, decreased mRNA expression of *cBD103* was detected only in naturally affected atopic dogs.³¹

In the above-mentioned study,³² particular care was taken to avoid cutaneous infections in order to reduce confounding factors. Although AMPs are known for their antimicrobial effects, their behaviour in actively infected atopic skin has not been reported. However, the presence of cutaneous infections (bacteria and yeast) is extremely common in AD compared with other skin diseases,^{33–36} and a decreased production of AMPs or production of nonfunctional AMPs has been hypothesized as a possible cause of the higher susceptibility to skin infection in AD.³⁰

To gain more insight into the role of AMPs in canine AD, we analysed the mRNA expression of four cBDs (*cBD1-like*, *cBD2-like/122*, *cBD3-like* and *cBD103*) and *cCath* in healthy and atopic dogs, with and without active skin infection, using a quantitative reverse transcriptase PCR (qRT-PCR). The cBDs and *cCath* used were selected based on previously demonstrated antimicrobial properties and presence in canine skin.^{37–40} In addition, we evaluated their distribution in the epidermis using indirect immunofluorescence (IIF) and their skin protein levels using competitive inhibition enzyme-linked immunosorbent assay (ciELISA).

Material and methods

The study was approved by the Institutional Animal Care and Use Committee. All dogs entered the study with the owners' written informed consent.

Inclusion criteria

Atopic dogs.

The diagnosis of canine AD was based on compatible history, clinical findings and the exclusion of possible differential diagnoses for pruritus (e.g. scabies, demodicosis, food allergy, flea allergy) as previously reported.^{41,42} In particular, for dogs with nonseasonal pruritus a strict food trial with a novel protein source for at least 10 weeks was done. When required, multiple skin scrapings were performed to rule out *Demodex* spp., and a miticidal drug trial was performed to rule out scabies. Dogs included in this group were divided in two subgroups based on the evidence of active skin infection (bacterial, *Malassezia* spp., or both). The diagnosis of infections by bacteria or yeast was based on clinical signs, history and skin cytology with evidence of inflammatory cells and bacteria, yeasts, or both. All the dogs were on commercially available diets at the time of enrolment in this study.

Healthy dogs.

Healthy control dogs were recruited from dogs presented to the Veterinary Teaching Hospital for annual vaccination or were dogs that belonged to the hospital staff. To be included in the study, the dogs had to have no history or presence of any cutaneous or systemic disease.

Exclusion criteria

All dogs.

Dogs were excluded if topical, systemic and depot glucocorticoids had been used for at least 2, 4 and 8 weeks, respectively, or if systemic or topical calcineurin inhibitors had been used for at least

4 weeks. Dogs were also excluded from the study if systemic or topical antibiotic or antifungal medications had been used for at least 2 weeks.

Atopic dogs.

Dogs with AD were excluded from the study if there was a history of administration of allergen-specific immunotherapy, or if there were other allergic conditions (e.g. food and flea allergy) or any other skin (e.g. endocrinopathies or neoplasia) or other systemic disease (e.g. parasitic, metabolic or neoplastic disease).

Healthy dogs.

Control dogs were excluded from the study if there was evidence of superficial or deep bacterial or *Malassezia* spp. infection.

Skin sample collection

Two 8 mm skin biopsy samples were obtained from abdominal skin using local anaesthesia [subcutaneous injection of 1 mL of lidocaine hydrochloride 2% (Hospira Inc., Lake Forest, IL, USA)]. It was not necessary to sedate dogs for this procedure. One skin biopsy sample was immediately divided into quarters and placed in 1.5 mL microfuge tubes, quickly flash frozen in liquid nitrogen, and then stored at -80°C until processed for molecular evaluation (qRT-PCR). The other skin biopsy sample was immediately fixed in 10% neutral buffered formalin for IIF evaluation. The abdominal region was chosen for the skin biopsy site because it is an easily accessible area with low hair density and a common area involved in canine AD.

Quantitative RT-PCR

One quarter of the 8 mm skin biopsy sample was homogenized using a PowerGen 125 (Fisher Scientific, Pittsburgh, PA, USA) using the AllPrep[®] RNA/protein kit (Qiagen, Valencia, CA, USA) reagents and then processed into RNA according to manufacturer's protocol as described.⁴⁰ Total RNA concentrations were determined at 260 nm using UV NanoDrop1000[®] spectrophotometry (Thermo Scientific, Wilmington, DE, USA), and integrity and quality of the RNA was checked using an Agilent 2100 Bioanalyzer (Agilent Biotechnologies Inc., Santa Clara, CA, USA). After DNase treatment using a Turbo DNA-free[™] kit (Invitrogen, Carlsbad, CA, USA), total RNA (0.5 μg) was converted to complementary DNA (cDNA) by reverse transcription of mRNA using SuperScript First-Strand Synthesis System (Invitrogen). Sense and antisense primers for each AMP (Table 1) were generated using Primer Designer software (Scientific and Educational Software Inc., Palo Alto, CA, USA) as reported.⁴⁰ Each primer was designed to cross an exon–exon boundary, maximizing the amplification specificity of the mRNA transcript target and minimizing amplification of any residual contaminating genomic DNA. The primers were generated from previously published GenBank AMP sequences.^{37–39} All primer sequences were subjected to Basic Local Alignment Search Tool (BLAST) comparison to the canine genome build 2.0 for unintended homologies. The relative mRNA expression levels were quantified using SYBR[®] Green (Qiagen) and ABI (Applied Biosystems Inc., Foster City, CA, USA) quantitative RT-PCR methodology. All samples were tested in triplicate 25 μL reactions in an ABI 7500 Real Time PCR System (Applied Biosystems Inc.). The PCR amplifications were carried out as follows: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 15 s and 60°C for 60 s. Amplifications were followed by dissociation (melting) curves to ensure specificity of the primers. The results were analysed using the comparative C_T (cycle threshold) method, and the relative mRNA expression of each AMP was compared using the $\Delta\Delta C_T$ method. This method is effective when the amplification efficiencies of the housekeeping gene and target gene are close to 100%. When this criterion is fulfilled the formula is: $2^{-[\Delta C_T \text{ experimental sample} - \Delta C_T \text{ control sample}]}$, where ΔC_T is the difference between the target gene and the normalizer gene expression.⁴³ All samples were normalized against the gene for canine ribosomal protein L15 (RPLO). This gene was chosen due to the highly stable and consistent expression previously shown for ribosomal genes in canine skin.⁴⁴

Table 1. Primers used

Canine gene	Primer sequences	Amplicon length
<i>cBD1-like</i>	Forward: CGAGTGGAAACTATGCTGT Reverse: GGAATCTGCTGAGATCAGAC	129
<i>cBD2-like/122</i>	Forward: AGTGGGAAACTATGCTGTCT Reverse: GTGCTAAGTGTGAGAATTGC	79
<i>cBD3-like</i>	Forward: CAGACATAAAAACAGACACA Reverse: AGTTGACCATATAGGTGTAG	78
<i>cCath</i>	Forward: CACTGTTGCTACTGCTGCTG Reverse: GTTGAAGCCATTCACAGCAC	98
<i>cBD103</i>	Forward: ACCTTGCCATCCAGTCTCAG Reverse: GGAACAGGCATCAAGAACAG	98
<i>RPLO</i>	Forward: TTGTGGCTGCTGCTCCTGTG Reverse: ATCCTCGTCCGATTCCTCCG	107

Antibody preparation

Polyclonal anti-canine-AMPs were synthesized by the Immunological Resource Center at the authors' Institution.⁴⁰ Briefly, the canine anti-AMP antibodies were generated from previously published GenBank AMP sequences.^{36–38} All amino acid sequences were subjected to BLAST comparison to the canine genome build 2.0 for unintended homologies. Based on the aforementioned genetic and amino acid sequences, the most immunogenic epitopes were chosen for each protein, and synthetic peptides were created. As the amino acid sequence of cBD1-like overlaps with part of both cBD2-like/122 and cBD3-like sequences, it was not possible to generate an appropriate polyclonal antibody against it. Antibody production was carried out using three female New Zealand white rabbits, 3.2–3.6 kg in weight (about 2–3 months old), purchased from Harlan Laboratories, Inc. (Indianapolis, IN, USA). The peptide conjugates were mixed with an adjuvant and subcutaneously injected into the three rabbits. Titermax (Sigma, St Louis, MO, USA) was used for the primary immunization and incomplete Freund's adjuvant for all subsequent immunizations. Rabbits were immunized four times, and their blood was tested for an immune response using an ELISA technique with pre-immune serum as the control. When a satisfactory immune response (positive up to 1:51,200 dilution) had been achieved, animals were exsanguinated and the crude sera were used. The antibodies were tested by Immunodot blotting using the synthetic peptide as antigen, resulting in a positive signal in up to a 1:10,000 dilution. In addition, to verify the specificity of the primary antibodies, the anti-cBD2-like/122, anti-cBD3-like and anti-cCath were tested by immunoabsorption, incubating each antibody with a high concentration (20 µg/µL) of the respective and other synthetic peptides overnight at 4°C before being applied to the tissue sections. An ELISA technique was also used to test possible cross-reactions with each of the peptides synthesized. Multiple attempts to generate anti-canine-cBD103 resulted in unsuitable antibodies for ELISA or IIF technique because only part of the amino acid sequence is predicted.

Indirect immunofluorescence

One half of the 8 mm skin biopsy sample was fixed in 10% neutral buffered formalin solution for no more than 48 h and then placed in phosphate buffer solution (PBS) until processed for IIF.⁴⁰ Briefly, 3 µm sections were processed using the immunohistochemical polymer procedure. The sections were blocked using a casein solution (Power Block®; BioGenex, San Ramon, CA, USA) followed by an extra wash using normal goat serum (BioGenex) as a blocking solution. Epitope retrieval was not necessary for cBD2-like/122 and cBD3-like, whereas it was required for cCath. In the latter case, the method consisted of using boiled sections, performed at 125–130°C under 7.7–10.4 kg pressure for 30 s followed by a 10 s treatment at 90°C. The sections were then stained for 1 h at room temperature using primary polyclonal rabbit antibodies specific for cBD2-like/122, cBD3-like and cCath.³⁹ The primary antibodies were used at 1:200 dilution. Negative controls were established using the pre-immune serum at 1:200 dilution. The sections were washed with a blocking

solution (Power Block®; BioGenex) and then incubated for 30 min at room temperature with a polyclonal goat anti-rabbit antibody bound with a green fluorochrome (Alexa Fluor® 488; Invitrogen) at 1:1000 dilution, according to the manufacturer's recommendations. Finally, DAPI (4',6-diamidino-2-phenylindole; Invitrogen) was used as a counterstain for nuclear detection. Specimens were mounted on glass slides using Vectashield® Mounting Medium (Vector laboratories; Burlingame, CA, USA). The skin sections were examined using an inverted fluorescence microscope (Nikon Eclipse TE 2000-S®; Nikon Inc., Shelton, CT, USA). The images were analysed using MetaMorph® software (version 63r1; Molecular Devices, Sunnyvale, CA, USA). Five representative fields at ×400 magnification were examined for each section, and digital images were recorded.

Relative competitive inhibition enzyme-linked immunosorbent assay

One quarter of the 8 mm skin biopsy specimen was homogenized using a PowerGen 125 (Fisher Scientific) and then the proteins were extracted using the AllPrep® RNA/protein kit (Qiagen) reagents, according to the manufacturer's protocol. The protein concentration was determined at 280 nm using UV NanoDrop1000® spectrophotometry (Thermo Scientific). The ciELISA was then performed as previously described, with some modifications.⁴⁵ The 96-well flat-bottomed microtiter plates (Immunlon II® HB; Fischer, Pittsburgh, PA, USA) were coated with 50 µL per well of 50 ng/mL synthetic cBD3-like or 10 ng/mL synthetic cCath in coating PBS (BioRad, Hercules, CA, USA; pH 7.4) and left overnight at 4°C. After discarding the coating buffer, the plates were blocked with 100 µL per well of 10% fetal bovine serum (Midwest Scientific, St Louis, MO, USA) in PBS for 2 h at room temperature. The plates were then washed three times using a blocking solution containing 10% fetal bovine serum and 0.05% Tween-20 (PBS-T; BioRad). A typical assay consisted of three sets of triplicate wells and three duplicate wells, as follows: (i) triplicate wells (positive controls) receiving 50 µL of specific anti-canine AMP polyclonal serum (1:8000 dilution for anti-cBD3-like and 1:16,000 dilution for anti-cCath) that would give the maximal optical density (OD); (ii) three duplicate wells (negative controls) receiving 50 µL of PBS-T only or 50 µL of secondary antibody only or 50 µL of each specific pre-immune serum that would give the minimal OD; (iii) triplicate wells receiving 50 µL per well of mixtures containing 25 µL (50 µg) of total protein extract (sample) and 25 µL of serum dilution (final protein dilution of 2 µg/µL); and (iv) a series of triplicate wells receiving a mixture of the serum dilution and 10-fold serial dilutions (from 1000 to 0.1 ng/mL) of the appropriate synthetic peptide, used to generate the inhibition standard curves.

The sera dilutions were chosen among those that gave OD values on the linear portion of the curve obtained from a twofold serial titration of each serum on plates coated with the appropriate peptide. The protein extract served as a competitive inhibitor for the binding of the antibodies to the synthetic peptides coating the plates. The plates were incubated for 90 min at room temperature, washed five times using the blocking solution, and 50 µL of a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:4000 dilution; BioRad) was added and incubated in the dark for 1 h at room temperature. The plates were then washed seven times with blocking solution and 100 µL per well of ABTS (Kirkegaard and Perry Laboratories, Fischer, Pittsburgh, PA, USA), a colorimetric substrate, was added. The developed plates were read with an automated MR 500 ELISA reader (Dynatech, Chantilly, VA, USA). The percentage of inhibition obtained from each concentration of peptide was calculated using the average of the absorbance values of each set of triplicate wells and the average of the absorbance values of the positive control wells. To calculate the relative amount of peptide in the 'unknown' wells, the increasing percentage inhibition values were plotted versus the corresponding log concentration of the synthetic peptide used to generate the standard curves. Complete inhibition was obtained using 1000 ng/mL of each synthetic peptide. The cBD2-like/122 and cBD103 protein levels were not quantified owing to a lack of antibodies suitable for ELISA-based quantification. The relative amount of AMPs in the tissue extracts was expressed as the

concentration (in nanograms per millilitre) of its synthetic peptide giving the same percentage of inhibition. This was then transformed into nanograms per square millimetre by dividing the concentration (in nanograms per millilitre) by the surface area of the skin used for the extraction (one quarter of the 8 mm biopsy sample), as follows: $\text{ng/mL}/[3.14(r^2)]$.

Statistical analysis

Mean values and 95% confidence intervals were calculated for all results. The Kolmogorov–Smirnov test of normality was used ($\alpha = 0.05$). Differences between ΔC_T ($C_T\text{AMP} - C_T\text{RPLO}$) of each AMP were compared using Student's unpaired *t*-test. Differences between ΔC_T ($C_T\text{AMP} - C_T\text{RPLO}$) of cCath were compared using the Mann–Whitney *U*-test because they did not follow a normal distribution. Differences between ODs of each AMP were compared using Student's unpaired *t*-test. In the expectation of higher AMP levels for atopic dogs and atopic dogs with active skin infections, we used a one-tailed test, and *P*-values of ≤ 0.05 were considered significant. Statistical analysis was done using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Dogs

A total of 25 dogs were enrolled in this study. Skin biopsy samples were taken from 14 healthy dogs [nine males (three intact) and five spayed females] with a mean age of 4 ± 2.9 years (range, 1–10 years; median, 3 years) and 11 atopic dogs [four males (one intact) and six females (two intact)] with an average age of 5.6 ± 4.1 years (range, 1–15 years; median, 5 years). Of the atopic dogs, four of 11 had active skin infection (three of four bacterial and one of four mixed bacterial and yeast). The majority of dogs were of mixed breed ($n = 9$), five were Labrador retrievers, and one each of the following dog breeds was

included: pug, wirehaired dachshund, Australian shepherd dog, Siberian husky, Samoyed, bull terrier, Jack Russell terrier, miniature pinscher, mastiff, beagle and English setter (Table 2). No significant age or sex differences were seen between groups.

Expression of mRNA

When we compared mRNA expression of each AMP between the dogs with AD and healthy control animals, a statistically significant higher expression (1.9 times) of *cBD103* ($P = 0.04$), but not any other AMP, was shown in atopic dogs (Figure 1a). Likewise, *cBD103* mRNA expression was significantly higher (3.8 times) in dogs with AD that had active skin infection when compared with atopic dogs without skin infection ($P = 0.01$; Figure 1b). This difference was 4.4 times higher when compared with dogs with AD that had active skin infection and healthy control dogs ($P = 0.001$; Figure 1c). In contrast, the *cBD1-like* mRNA expression level was significantly lower (0.48 times) in dogs with AD that had active skin infection compared with dogs with AD without skin infection ($P = 0.04$; Figure 1b).

Expression of protein

Indirect immunofluorescence.

No differences were noted in the distribution of AMPs in the skin of healthy dogs and in dogs with AD demonstrated by IIF. The proteins were homogeneously distributed in skin samples from both groups of dogs. The cBD2-like/122 and cBD3-like were detectable in all layers of the epidermis, whereas cCath was detected predominantly in the stratum granulosum and stratum corneum, as previously reported (Figure 2).^{32,40}

Table 2. Details of the 25 dogs enrolled in the study and the presence of active skin infection

Number	Sex	Age (years)	Breed	Atopic dermatitis	Infection
1	Mc	1	Mixed breed	No	
2	Mc	5	Labrador	Yes	No
3	Mc	10	Mixed breed	No	
4	Mc	6	Mixed breed	Yes	Yes
5	Mc	2	Pug	No	
6	M	1	Labrador	Yes	No
7	M	7	Wirehaired dachshund	No	
8	Fs	4	Mixed breed	Yes	No
9	Fs	4	Mixed breed	No	
10	Fs	1	Labrador	Yes	Yes
11	Fs	2	Australian shepherd	No	
12	F	2	Miniature pinscher	Yes	No
13	F	8	English setter	Yes	Yes
14	Fs	9	Labrador	Yes	Yes
15	M	1	Mixed breed	No	
16	M	8	Mixed breed	No	
17	Mc	4	Mastiff	Yes	No
18	Fs	3	Siberian husky	No	
19	Fs	3	Mixed breed	No	
20	Mc	6	Samoyed	No	
21	Fs	15	Beagle	Yes	No
22	Mc	6	Mixed breed	No	
23	Mc	1	Bull terrier	No	
24	Fs	7	Labrador	Yes	No
25	Fs	3	Jack Russell terrier	No	

Abbreviations: F, female intact; Fs, female spayed; M, male intact; and Mc, male castrated.

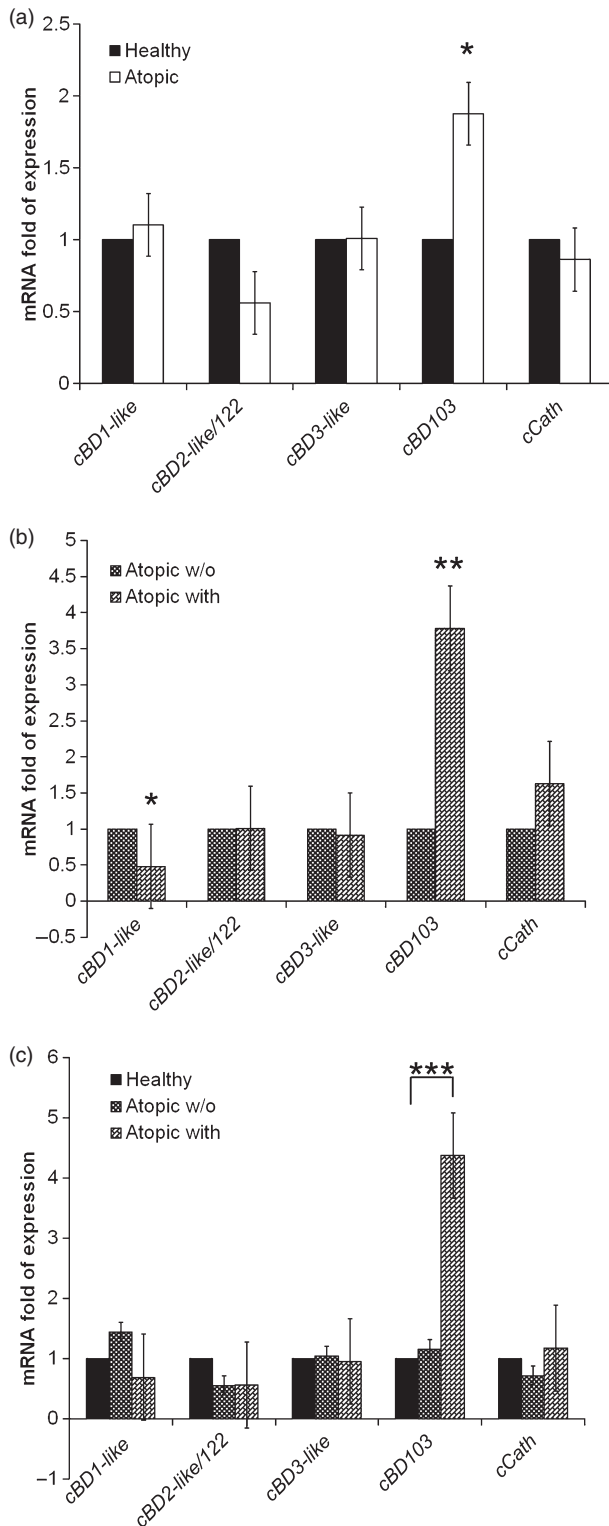


Figure 1. Relative mRNA expression for each antimicrobial peptide [canine β -defensin (cBD) 1-like, cBD2-like/122, cBD3-like, cBD103 and canine cathelicidin (cCath)] in atopic dogs with and without (w/o) skin infection compared with normal control dogs. (a) Comparison between healthy dogs and dogs with atopic dermatitis (AD). (b) Comparison between dogs with AD, with and without active skin infection. (c) Comparison of healthy dogs and dogs with AD, with and without active skin infection. Groups were compared using Tukey's multiple comparison test (* $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$; bars indicate SEM).

Relative competitive inhibition enzyme-linked immunosorbent assay.

Using the ciELISA, we were able to calculate the relative amount of cBD3-like and cCath per square millimetre of skin. The abdominal skin of healthy dogs had an amount of BD3-like corresponding to 1.1 ± 0.95 ng/mm² of synthetic cBD3-like and 1.4 ± 0.99 ng/mm² of synthetic cCath (Figure 3a). Abdominal skin of atopic dogs had an amount corresponding to 1.8 ± 1.8 and 4.7 ± 8.6 ng/mm² of synthetic cBD3-like and synthetic cCath, respectively (Figure 3a). The corresponding amounts of cBD3-like in atopic skin with and without active skin infection were 2.5 ± 1.5 and 1.4 ± 1.9 ng/mm², respectively (Figure 3b). The corresponding amounts of cCath were 5.15 ± 10.54 ng/mm² in dogs without infection and 4 ± 4.9 ng/mm² in dogs with active infection, respectively (Figure 3b). When we compared the amount of cBD3-like and cCath between the AD and healthy control groups, no statistically significant differences were seen (Figure 3a,b). However, a significant increase in cBD3-like and cCath was present when we compared dogs with AD that had active skin infection and healthy control dogs ($P = 0.04$ and $P = 0.05$, respectively; Figure 3c). A trend towards a significant increase of AD without skin infection versus healthy control was also seen ($P = 0.08$; Figure 3c).

Discussion

Our data showed a significant increase of cBD103 mRNA expression levels in skin of dogs naturally affected with AD when compared with healthy control dogs. These results are in accordance with recent studies by Bellardini *et al.*²⁸ and Harder *et al.*,³⁰ in which a clear increase in mRNA expression and protein level of some AMPs, namely LL-37, hBD2 and hBD3/103A (orthologue of cBD103), were demonstrated in the skin of atopic human patients when compared with healthy individuals. The results of the present study are also in accordance with the results of previous study in which we demonstrated increased mRNA expression levels of some AMPs in the skin of beagles with experimentally induced AD.³²

The reason for higher expression and production of AMPs in atopic skin compared with healthy controls is not completely understood. Many hypotheses have been proposed, including the effects of the local immunological milieu, the disruption of the cutaneous barrier, differing rates of protein degradation and the presence of inflammatory cells.^{24–26,46–50} The involvement of cytokines in the regulation of local AMPs is a subject of controversy. Although a strong inverse correlation between human AMPs and the expression of T helper 2 cytokines, such as interleukin-4, interleukin-10 and interleukin-13, has been shown *in vitro*,^{46,48–50} an *in vivo* model has demonstrated higher expression of some AMPs in acute T helper 2-dominant AD lesions.³⁰ In addition, a strong correlation between alterations of the skin barrier, naturally present in AD skin, and production of AMPs has been demonstrated in both mouse and human models.^{50,51} Increased degradation of active AMPs in atopic disease due to hyperactivation of several endogenous as well as exogenous proteases^{52,53} has also been

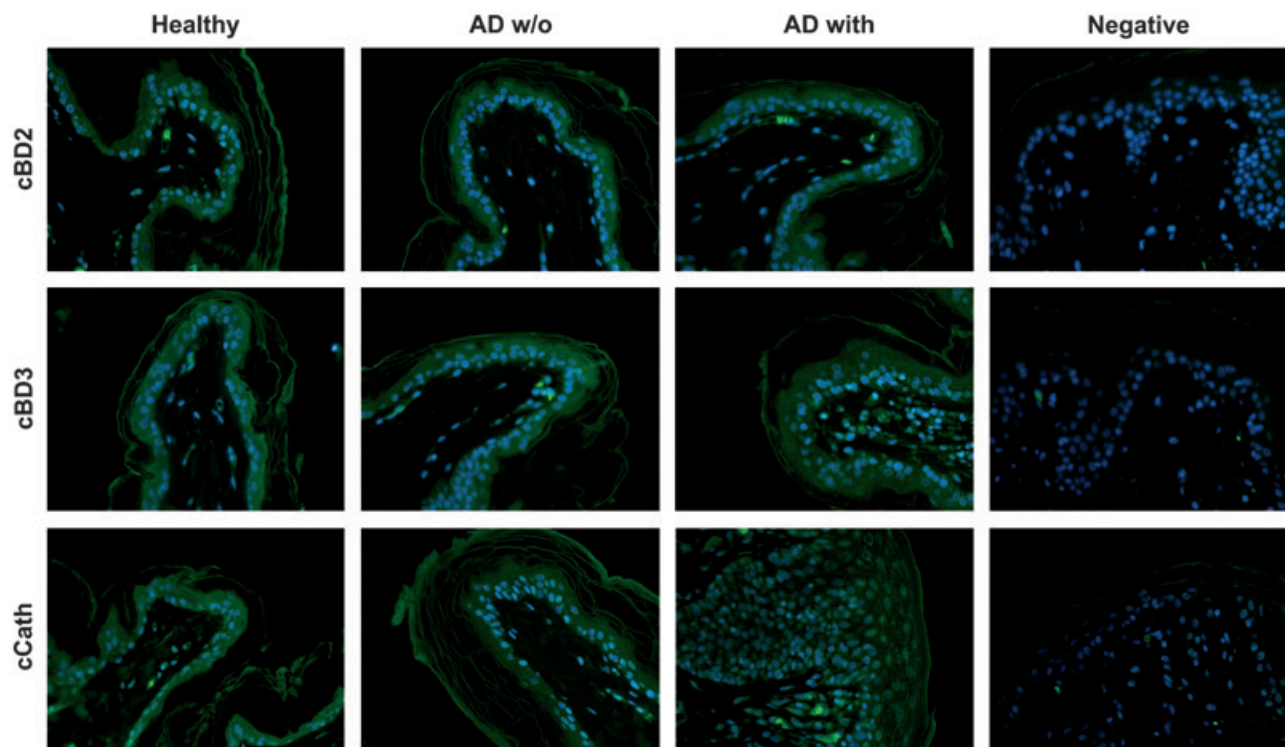


Figure 2. Photomicrographs of canine skin showing indirect immunofluorescence of cBD2-like/122, cBD3-like and cCath. The cBD2-like/122 and cBD3-like fluorescence is diffusely distributed throughout the epidermis, and scattered positive cells are also present in the superficial dermis; cCath is mainly detectable in the stratum granulosum and corneum along with few positive cells in the superficial dermis. The pictures are representative of all samples.

hypothesized in AD skin. In this latter case, the increased mRNA expression could be due to a 'feedback mechanism', in which the keratinocyte increases the genetic signal in an effort to repair the lack of protein expression. The increase in *cBD103* mRNA expression shown in the present study is in disagreement with van Damme *et al.*,³¹ who reported a twofold decrease in mRNA expression of *cBD103* in the skin of dogs naturally affected with AD. Possible explanations for this discrepancy could be differences in skin biopsy location, the different ages of the dogs enrolled in the two studies (older in our study), the treatment with antimicrobials prior to enrolment in the study (avoided in our study), and the potential use of different shampoos and topicals (antimicrobial versus moisturizing products) used before enrolment in the two studies. Different AMP expression has been demonstrated in different body sites in people, with areas more subject to friction and environmental challenge having a higher AMP expression.⁵⁴ Increased AMP expression has also been reported in older versus younger human patients.⁵⁵

We were not able to detect any significant differences in mRNA expression levels of the other AMPs (*cBD1-like*, *cBD2-like/122*, *cBD3-like* and *cCath*) between the two groups. These results are in contrast with a previous study showing an increase of such AMPs in the skin of beagles with experimentally induced AD.³² One explanation for this discord could be the higher variability of mRNA expression found in the present study compared with the previous one. In fact, the use of a canine animal model allowed more efficient control of environmental

and intrinsic variables, such as a shared environment (e.g. indoors versus outdoors), other pets in the household, age and breed.⁵⁶

Atopic dogs, like atopic humans, are more susceptible to bacterial and *Malassezia* skin infection, but only recently has there been an increased interest in the role of AMPs in this predisposition.^{23,31,32,37,38,56} However, no studies have reported expression patterns of AMPs in the skin of atopic dogs with or without active skin infection. The present study is the first to demonstrate higher mRNA expression for *cBD103* and lower expression of *cBD1-like*, but not the other *cBDs* and *cCath*, in the skin of naturally affected atopic dogs with active infections. We showed that the mRNA expression of *cBD103* was 3.8 times higher in infected skin when compared with noninfected skin. This difference increased to 4.4 times when comparing infected AD skin with the skin of healthy control dogs. This increased expression could be the direct effect of the bacterial stimulation, as demonstrated *in vitro*.^{57,58} The significant decrease in *cBD1-like* mRNA expression level in dogs with AD that had active skin infection when compared with dogs with AD without skin infection could be due to less important antimicrobial effects of cBD1-like compared with other defensins; for this reason, cBD1-like might not be actively involved in skin infections.

We designed a semi-quantitative ciELISA for cBD3-like and cCath to detect the relative amounts of AMPs in canine abdominal skin. Although no significant differences were found between AD (total or without skin infection) and healthy skin, or between AD skin with and

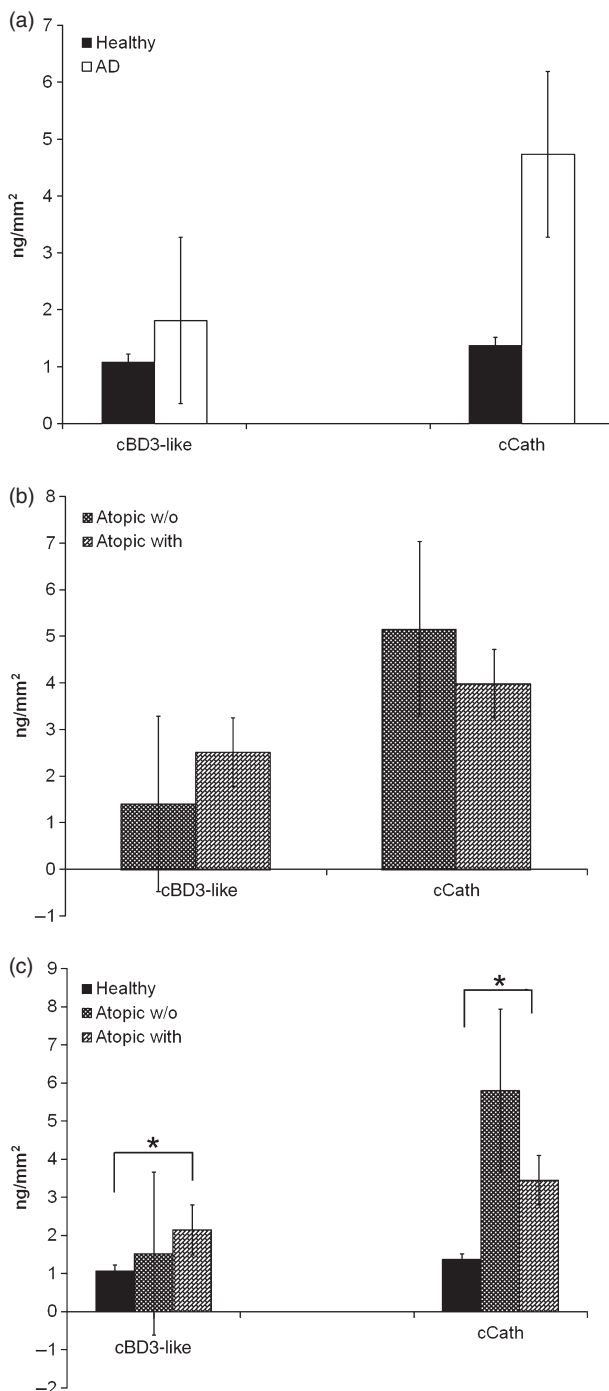


Figure 3. Protein levels for each antimicrobial peptide (cBD3-like and cCath) in atopic dogs with and without (w/o) skin infection compared with normal control dogs. (a) Comparison between healthy dogs and dogs with AD. (b) Comparison between dogs with AD, with and without active skin infection. (c) Comparison of healthy dogs and dogs with AD, with and without active skin infection. Groups were compared using Tukey's multiple comparison test (* $P \leq 0.05$; bars indicate SEM).

without skin infection, a significantly increased amount of cBD3-like and cCath was seen when infected AD skin was compared with healthy control skin. These results may suggest an active stimulation of some AMPs by bacteria and/or yeasts in AD skin, as previously demonstrated.^{27,30,57,58} Unfortunately, at this point in time antibodies to detect cBD103 protein expression are not

available. It would have been interesting to have a comparison between mRNA expression and protein production to confirm the possible major involvement of this AMP in the skin of dogs with AD. Finally, our study showed that there was no difference in cutaneous distribution of the tested AMPs between AD skin, with or without active infection, and healthy skin.

In conclusion, we have demonstrated that, as in people, a higher mRNA expression of *cBD103* (orthologue of *hBD3/103A*) is present in atopic dogs than in healthy control dogs. This difference was more striking when comparing the skin of normal dogs with skin of dogs with AD that had active infection, showing a potentially active role of *cBD103* in cutaneous infection in atopic dogs. In contrast, we showed a lower mRNA expression of *cBD1-like* in AD skin affected by active skin infection when compared with nonactively infected AD skin. These data may indicate that many AMPs may be involved in the cutaneous innate immunity as defense against external micro-organisms. In addition, it is possible that an alteration of the ratio between AMPs is the cause of increased infections in atopic patients. The present study and previous studies have yielded mixed results, raising questions concerning the regulation and the production of AMPs in cutaneous infections, and which AMP ratio is more important in controlling bacterial and yeast infections. Which AMPs are sufficient to control skin infection in healthy and atopic dogs, at what concentrations and what is the action of topicals (e.g. shampoos, foams and sprays) on cutaneous AMP production? More work is needed to clarify the biological mechanisms and the possible therapeutic treatments to maintain a healthy canine skin.

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Résumé

Contexte – Les peptides antimicrobiens (AMPs) sont de petites molécules immunomodulatrices produites par les cellules épithéliales et immunitaires. Les β -défensines (BDs) et les cathélicidines (Caths) sont les AMPs les plus étudiés. Récemment, une expression accrue d'AMPs a été rapportée chez les humains atopiques et chez les beagles atteints d'atopie induite expérimentalement.

Hypothèses/Objectifs – Notre objectif était d'analyser l'expression d'ARNm et le niveau de protéines de c(canine)BD1-like, cBD2-like/122, cBD3-like, cBD103 et cCath chez les chiens sains et naturellement atopiques, avec et sans infection cutanée active, ainsi que leur distribution dans l'épiderme par immunofluorescence indirecte.

Sujets – Les biopsies cutanées ont été réalisées sur 14 chiens sains et 11 chiens atopiques de propriétaires.

Méthodes – Les niveaux d'ARNm de *cBD1-like*, *cBD2-like/122*, *cBD3-like*, *cBD103* et *cCath* ont été quantifiés par PCR quantitative en temps réel. Les niveaux de protéines de cBD3-like et cCath ont été analysés par ELISA inhibitrice compétitive relative alors que les distributions de cBD2-like/122, cBD3-like et cCath ont été détectées par immunofluorescence indirecte.

Résultats – Les chiens atteints de dermatite atopique présentaient une expression de *cBD103* significativement plus élevée ($P = 0.04$) que les chiens contrôles. En outre, la peau atopique avec une infection active avait une expression de *cBD103* mRNA plus élevée ($P = 0.01$) et une expression de *cBD1-like* mRNA plus faible ($P = 0.04$) que les peaux atopiques sans infection. Aucune différence significative des niveaux de protéine (cBD3-like et cCath) ou de distribution épidermique des AMPs (cBD2-like/122, cBD3-like et cCath) n'a été relevée entre les chiens sains et atopiques.

Conclusion et importance clinique – Chez les chiens atteints de dermatite atopique avec une infection active, l'expression de *cBD103* mRNA était plus élevée alors que l'expression de *cBD1-like* mRNA était plus faible. D'autres études sont nécessaires afin de clarifier les mécanismes biologiques et les options thérapeutiques possibles pour maintenir une peau canine saine.

Resumen

Introducción – los péptidos antimicrobianos (AMPs) son péptidos inmunomoduladores pequeños producidos por las células epiteliales y del sistema inmune. Las β -defensinas (BDs) y las catelicidinas (Caths) son los AMPs mas estudiados. Recientemente se ha descrito una expresión cutánea incrementada de AMPs en seres humanos atópicos y en perros de raza Beagle con atopía experimentalmente inducida.

Hipótesis/objetivos – nuestro objetivo fue analizar la expresión de mRNA y los niveles de proteína de los péptidos caninos (c) equivalente de cBD1, equivalente de cBD2/122, equivalente de cBD3, de cBD103 y cCath en perros sanos y en perros con atopía producida de forma natural, con o sin infección activa de la piel, junto con su distribución en la epidermis usando inmunofluorescencia indirecta.

Animales – las biopsias de piel fueron tomadas a partir de 14 perros sanos y 11 atópicos de propietarios particulares.

Métodos – los niveles de mRNA del equivalente de *cBD1*, del equivalente de *cBD2/122*, del equivalente de *cBD3*, de *cBD103* y de *cCath* fueron cuantificados usando PCR cuantitativa en tiempo real. Los niveles de proteína del equivalente de cBD3 y de cCath fueron analizados mediante ensayo de inhibición competitiva de inmunoabsorción ligada a enzimas, mientras que la distribución del equivalente de cBD2/122, del equivalente de cBD3 y de cCath fue detectada por inmunofluorescencia indirecta.

Resultados – los perros con dermatitis atópica tenían expresión significativamente mayor de mRNA de *cBD103* ($P = 0.04$) que perros control. Además, la piel atópica con infección activa tenía una expresión aun más alta de mRNA de *cBD103* ($P = 0.01$) y una expresión más baja de mRNA del equivalente de *cBD1* ($P = 0.04$) que la piel atópica sin infección. No se observó ninguna diferencia significativa en los niveles de la proteína (equivalente de cBD3 y cCath) o en la distribución epidérmica de los AMPs (equivalente de cBD2, equivalente de cBD3 y cCath) entre perros sanos y atópicos.

Conclusiones e importancia clínica – la expresión de mRNA de *cBD103* fue mayor, mientras que la expresión de mRNA del equivalente de cBD1 fue menor en perros con dermatitis atópica que tenían infecciones activas. Se necesitan mas investigaciones para clarificar los mecanismos biológicos y las opciones terapéuticas posibles para mantener una piel sana en perros.

Zusammenfassung

Hintergrund – Antimikrobielle Peptide (AMPs) sind kleine immunmodulatorische Peptide, die von Epithel- und Immunzellen produziert werden. β -Defensine (BDs) und Cathelidins (Caths) sind die am besten untersuchten AMPs. Unlängst wurde eine erhöhte kutane Exprimierung von AMPs bei Menschen mit Atopie und bei Beagles mit experimentell induzierter Atopie beschrieben.

Hypothese/Ziele – Unser Ziel war eine Analyse der Exprimierung von mRNA von caninem *cBD1-like*, *cBD2-like/122*, *cBD3-like*, *cBD103* und *cCath* sowie ihrer Exprimierung auf dem Proteinlevel bei gesunden und natürlich betroffenen Hunden mit Atopie, mit und ohne aktiver Hautinfektion, sowie die Bestimmung ihrer Verteilung in der Epidermis mittels Immunfluoreszenz.

Tiere – Es wurden Hautbiopsien von 14 gesunden und 11 atopischen Privathunden entnommen.

Methoden – Die mRNA Levels von *cBD1-like*, *cBD2-like/122*, *cBD3-like*, *cBD103* und *cCath* wurden mittels quantitativer Real-Time PCR analysiert. Die Proteinlevel von *cBD3-like* und *cCath* wurden mittels relativem kompetitivem Enzymgekoppelten Immunabsorptionsassay analysiert, während die Verteilungen von *cBD2-like/122*, *cBD3-like* und *cCath* mittels indirekter Immunfluoreszenz bestimmt wurden.

Ergebnisse – Hunde mit atopischer Dermatitis zeigten eine signifikant höhere mRNA Exprimierung von *cBD103* ($P = 0.04$) als die Kontrollhunde. Weiters war die Exprimierung von *cBD103* mRNA in atopischer Haut mit aktiver Infektion höher ($P = 0.01$) und *cBD1-like* mRNA Exprimierung in atopischer Haut ohne Infektionen niedriger ($P = 0.04$). Es bestanden keine signifikanten Unterschiede bei den Proteinlevels (*cBD3-like* und *cCath*) oder bei der epidermalen Verteilung der AMPs (*cBD2-like/122*, *cBD3-like* und *cCath*) zwischen gesunden und atopischen Hunden.

Schlussfolgerungen und klinische Bedeutung – Die Exprimierung von *cBD103* mRNA war stärker, während die Exprimierung von *cBD1-like* mRNA bei Hunden mit atopischer Dermatitis, die eine aktive Infektion hatten, niedriger war. Es sind weitere Studien nötig, um die biologischen Mechanismen und die möglichen therapeutischen Optionen zur Erhaltung der Gesundheit der Hundehaut zu erforschen.

要約

背景 – 抗菌ペプチド (AMPs) は表皮と免疫細胞で産生される小型の免疫調節性タンパクである。βディフェンシン (BDs) とカセリシジン (Caths) は最もよく調べられたAMPである。最近、アトピーのヒトと実験的にアトピーを誘発させたビーグルにおいて、AMPの発現が皮膚で報告された。

仮説/目的 – 筆者らのゴールはイヌ (c) BD1 様, cBD2 様/122, cBD3 様, cBD103 と cCath の mRNA 発現とタンパクレベルを健康犬と活発な皮膚感染を示す, または示さない自然発症したアトピー犬の表皮での分布を間接免疫蛍光法で解析することである。

供与動物 – 14頭の健康と11頭のアトピーの飼い犬から採取した皮膚生検材料

方法 – cBD1 様, cBD2 様/122, cBD3 様, cBD103 と cCath の mRNA レベルを定量リアルタイム PCR を用いて定量した。cBD3 様と cCath の蛋白量は相対的競合阻止酵素結合免疫吸着法で解析し, cBD2 様/122, cBD3 様, cCath の表皮の分布は間接免疫蛍光法で検出した。

結果 – アトピー性皮膚炎の犬では cBD103 の mRNA の発現量が対照群の犬と比較し優位に高かった ($P = 0.04$)。さらに, 活発な感染を示すアトピー性皮膚炎の皮膚では, 感染のないアトピー性皮膚炎の皮膚と比較して高い cBD103 の mRNA の発現 ($P = 0.01$)と低い cBD1 様 mRNA 発現 ($P = 0.04$)を示していた。蛋白量 (cBD103 と cCath) あるいは AMP の表皮の分布 (cBD2 様/122, cBD3 様, cCath) は健康犬とアトピー犬で有意差はみられなかった。

結論と臨床的重要性 – 活発な感染を示すアトピー性皮膚炎の犬では cBD1 様 mRNA 発現がより低く, cBD103 の mRNA の発現はより高かった。生物学的なメカニズムの解明と健康な犬の皮膚の維持のための治療オプションを可能にするにはさらなる研究が必要である。

摘要

背景 – 抗菌肽 (AMPs) 是由上皮和免疫细胞产生的小免疫活性肽。β-防御素 (BDs) 和卡特兰杀菌素 (Caths) 是研究最多的AMPs。近期, 有报道发现异位性患者和试验诱发的特异性米格鲁猎犬的皮肤 AMPs 表达增加。

假设/目的 – 我们的目的是分析健康犬和天然异位性患犬, 伴有或没有皮肤感染, 其类 (c) BD1, 类 cBD2/122, 类 cBD3, cBD103 和 cCath 的mRNA表达和蛋白水平, 沿着它们在表皮上的分布使用间接免疫荧光。

动物 – 从14只健康犬和11只异位性私人养犬上进行皮肤活检。

方法 – 用定量实时PCR测量类cBD1, 类cBD2/122, 类 cBD3, cBD103 和 cCath 的 mRNA 水平。相对竞争抑制酶联免疫吸附试验分析类 cBD3 和 cCath 蛋白质水平, 通过间接免疫荧光检测类 cBD2/122, 类 cBD3 和 cCath 的分布。

结果 – 异位性皮肤犬的 *cBD103* mRNA 表达显著高于对照犬 ($P = 0.04$)。此外, 异位性皮肤伴随感染与无感染的相比 *cBD103* mRNA 表达 ($P = 0.01$) 较高, 类 *cBD1* mRNA 表达 ($P = 0.04$) 较低。健康犬和异位性犬相比, 蛋白质水平 (类 cBD3 和 cCath), 或 AMPs (类 cBD2/122, 类 cBD3 和 cCath) 表皮分布没有显著差异。

结论和临床价值 – 在有皮肤感染的异位性皮肤犬上, *cBD103* mRNA 表达较高, 类 *cBD1* mRNA 表达较低。需要更多工作来明确生物学机制和维持犬健康皮肤的治疗方案。