A defective release of host defense peptides is present in canine atopic skin

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\textbf{A R T I C L E   I N F O}

\textbf{Keywords:}
Atopic dermatitis
Keratinocyte
Host defense (antimicrobial) peptides
Canine

\textbf{A B S T R A C T}

The use of dogs as animal model for human atopic dermatitis (AD) is well known. Striking similarities in the pathogenesis of AD have been demonstrated. Similar alteration of host defense peptides (HDP) have been identified in both species. However, the ultrastructural/molecular alterations associated with HDPs secretion in AD have not been elucidated. We were able to use a multidisciplinary approach to investigate the secretion of HDP in canine skin. The contemporary use of indirect immunofluorescence, ELISA and scanning immunoelectron microscopy gave fundamental insights in the pathomechanism of HDP alteration in AD. An increased intracellular expression and a reduced secretion of HDPs is present in atopic skin. An increased presence of HDPs was seen on the surface of atopic skin. These results suggested a defective secretion and an increased adhesion of HDPs to atopic corneocytes might be the reason of the reduced killing activity of HDPs in AD.

\textbf{1. Introduction}

Atopic dermatitis (AD) is a common chronic, inflammatory skin disease affecting up to 20% of children and 10% of adults \cite{1}. Similarly, 10% of the canine population \cite{2} is naturally affected by AD. In both species, AD has a significant impact on quality of life of patients and their caregivers. In addition, recurrent skin infections are common in AD, requiring multiple courses of antimicrobials significantly increasing the risk of bacterial resistance worldwide \cite{3}. An increased colonization/adhesion of \textit{Staphylococci} spp. (i.e. \textit{S. aureus} and \textit{S. pseudintermedius}) is present in both humans and dogs with AD \cite{1,4,5}. For this reason, patients may serve as vectors for resistant bacteria in the community. Alterations of the natural cutaneous immune defenses (e.g. host defense peptides [HDPs]) have been associated with an increase incidence of Staphylococcal infection/colonization in AD \cite{1}. Recently, a decreased secretion of β-defensin (BD)-3 in atopic human skin was associated with a T-helper type 2 cytokines \cite{6}. However, the underlying mechanism of such altered secretion is unknown.

Because of the difficulties to unveil pathological mechanisms in humans, the use of animal models has been increased in the past decades. In this scenario, the use of dogs, as natural animal model for human AD, has been largely accepted \cite{7}. Dogs naturally develop AD and they share the same (micro)environment of their owners \cite{7}. In addition, immunological and structural alterations have been duplicated in both species. Because of the similarity between human and canine staphylococcal infection and structural/immunological abnormalities, dogs are the natural model to investigate alterations present in canine and human atopic skin. Similarly to people, the most studied canine HDPs include cBDs, cathelicidin (cCath), and S100A proteins \cite{8}.

Recently, it has been demonstrated that canine atopic keratinocytes secrete more HDPs than healthy keratinocytes at baseline \cite{9}. However, contrary to healthy keratinocytes, a lack of increased secretion of HDPs is present in atopic keratinocytes after stimulation with immunostimulants. These results suggest intrinsic alterations in atopic keratinocytes \cite{9}. Notably, it has also been demonstrated that although similar HDPs amounts are secreted in skin wash harvested from healthy and atopic dogs, in the latter, a reduced in antibacterial effect was present \cite{10}.

Thus, to further evaluate the mechanisms of HDPs secretion in healthy and atopic dogs, we have collected non-lesional skin explants, to mimic a more realistic scenario, from privately owned, naturally affected atopic dogs and healthy control dogs. The aim of this study is to evaluate potential alterations present in canine AD as translational step to investigate the presence of similar alterations in human AD.

\textbf{2. Materials and methods}

\textbf{2.1. Ethics}

Written informed consent was obtained from each client before enrollment in the study and all procedures were approved by the

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https://doi.org/10.1016/j.cimid.2019.04.008

Received 12 February 2019; Received in revised form 15 April 2019; Accepted 17 April 2019

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Institutional Animal Care and Use Committee at the University of Florida (IACUC protocol # 201509195).

2.2. Dogs

Eleven healthy (2 beagles, 3 mixed breed, 4 German shepherd, 1 Labrador retriever, and 1 Greyhound) and nine atopic (2 German shepherd, 2 pit-bull, 2 mixed breed, 1 Labrador retriever, 1 English setter, and 1 French bulldog) adult, privately-owned dogs were recruited form the Dermatology Service, College of Veterinary Medicine, University of Florida. The healthy dogs had no history or clinical signs of allergies or any other cutaneous disease. Atopic dermatitis was diagnosed based on Favrot’s criteria [11] and following the recently published guidelines [12] for the diagnosis of canine AD. To be enrolled in the study, dogs had not been received topical and/or systemic antimicrobials for at least 2 weeks prior to enrollment, glucocorticoids for at least 4–8 weeks prior to (oral or injectable deposit) enrolment, cyclosporine for at least 2 weeks, and oclacitinib for at least 1 week prior to enrollment.

2.3. Tissue collection

Three 8 mm skin biopsy samples were sterilely obtained from abdominal skin (non-lesional skin in atopic dogs) using local anesthesia [subcutaneous injection of 1 mL of lidocaine hydrochloride 2% (Hospira Inc., Lake Forest, IL, USA)]. Sedation was not necessary for this procedure. Once obtained, the skin samples were put in a sterile tube containing ice-cold sterile phosphate buffer solution (PBS). Once in the laboratory, skin biopsy samples were immediately divided into halves and placed in a 48-well plate with 1.0 mL of keratinocyte growth medium (CnT-09®, CellnTec, Bern, Switzerland). The next day, the medium was changed with fresh medium. After another 24 h, the medium was changed again and five of the skin explants were exposed to bacteria.

2.4. Bacteria preparation

Bacteria were purchased from the ATCC grown overnight on Blood agar plates and subcultured the next day. Then a bacterial suspension at 0.5 McFarland concentration (1 × 10⁸ CFU/mL) was added to the keratinocyte cultures for 1 h, after which the unbounded bacteria were removed by vigorous rinsing and the cultures fixed with 4% paraformaldehyde at room temperature (25 °C) as previously described [6,13,14]. The following bacteria were purchased from the ATCC: Staphylococcus pseudintermedius (ATCC:49444), Staphylococcus aureus (ATCC-29213), Pseudomonas aeruginosa (ATCC-27853), and Escherichia coli (ATCC-25922).

2.5. Enzyme-linked immunosorbent assay (ELISA) (S100A8)

After exposure to bacteria, unexposed and exposed skin explants’ supernatant was collected and immediately frozen in −80 °C until assayed for S100A8. Sandwich ELISAs were performed according to manufacturer’s protocol using NeoScientific canine S100A8 ELISA kits (NeoScientific, Cummings Park Woburn, MA). Skin explant supernatant was tested undiluted. The assays were performed in duplicates. The amount of S100A8 in the supernatant was expressed as ng/mL.

2.6. Relative competitive inhibition ELISA (ciELISA)

After exposure to bacteria, unexposed and exposed skin explants’ supernatant was collected and immediately frozen in −80 °C until assayed for canine β-defensin (cBD)3-like and canine cathelicidin (cCath). The ciELISA was then performed as previously described using customized, canine-specific validated ELISAs [9]. Skin explant supernatant was tested undiluted. The assays were performed in duplicates. The relative amount of cBD3-like and cCath in the supernatant was expressed as the ng/mL of its synthetic peptide giving the same percentage of inhibition [9].

2.7. Indirect immunofluorescence

Once fixed, the skin explants (11 healthy and nine atopic) were processed for indirect immunofluorescence (IF) as previously described [15]. Briefly, 3 µm sections were processed using the immunohistochemical polymer procedure. Epitope retrieval was performed with pH 6 citrate buffer solution for 10 min in boiling water (95–100 °C) followed by 10 min at room temperature. The sections were then blocked using a casein solution (Power Block®; BioGenex, San Ramon, CA, USA) and 10% normal donkey serum. The sections were stained for 1.5 h at room temperature with polyclonal rabbit antibodies specific for cBD3-like, cBD103, and cCath used at 1:200 dilution. Negative controls were established using the pre-immune serum at 1:200 dilution. All the antibodies tested were synthesized by the Immunological Resource Center, University of Illinois at Urbana-Champaign, as previously described [15]. The sections were then washed and incubated for 1 h at room temperature with a polyclonal donkey anti-rabbit antibody bound with a green fluorochrome (Alexa Fluor® 488; Invitrogen) at 1:1000 dilution, according to the manufacturer’s recommendations. Finally, specimens were mounted on glass slides using Vectashield® Mounting Medium with DAPI (4’,6-diamidino-2-phenylindole; Invitrogen) (Vector laboratories; Burlingame, CA, USA). The skin sections were examined and pictures captured using an inverted fluorescent microscope (EVOS; Invitrogen, Carlsbad, CA). Five representative fields at ×400 magnification were examined for each section and digital images recorded. The intensity of the immunofluorescence signal was graded in a semiquantitative manner using an computer imaging software (Image J 1.41; http://rsb.info.nih.gov/ij/). The epidermis was traced and a modified corrected total cell fluorescence (CTCF) index was determined as previously described [16]. Corrected total cell fluorescence was determined as follows: CTCF = integrated density – (area of selected cells × mean fluorescence of background readings). The relative fluorescence per pixel unit was achieved dividing the CTCF by the area analysed and expressed in arbitrary units (a.u.).

2.8. Immuno-scanning electron microscopy (iSEM)

After exposure to bacteria, unexposed and exposed skin explants (three healthy and three atopic) were cut into 2 mm³ pieces and replaced the primary fixative with fresh 4% paraformaldehyde, 1% glutaraldehyde in PBS buffer, pH 7.24. After fixations, explants were PBS washed, incubated with blocking solution (1.5% BSA, 0.5% cold water fish skin gelatin, 0.01% Tween-20 in PBS [pH 7.2] for 1 h. Explants were then incubated for 1 h with polyclonal rabbit antibodies specific for cBD103 and cCath used at 1:500 dilution. Negative controls were established using the pre-immune serum at 1:500 dilution. The explants were washed three times with PBS followed by 1 h incubation with secondary antibody: 18 nm goat-anti rabbit IgG colloidal gold conjugate (Jackson Immuno Research Laboratories, West Grove PA, USA). Specimens were immunolabeled at room temperature. After immuno-gold label, the explants were processed for SEM with the aid of a Pelco BioWave laboratory microwave (Ted Pella, Redding, CA, USA). Tissues were washed in PBS, post fixed with 1% buffered osmium tetroxide, water washed and dehydrated in a graded ethanol series 25%, 50%, 75%, 95%, 100% and critical point dried (Bal-Tec CPD030, Leica Microsystems, Bannockburn, IL, USA). Dried explants were mounted on carbon adhesive tabs on aluminum specimen mounts, and carbon coated (328UHR Cressington, Ted Pella, Redding, CA, USA). Specimens examined with secondary electrons (SE) and backscatter electrons (BSE), digital micrographs acquired with a field-emission SEM (SU-5000, Hitachi High Technologies America, Schaumburg, IL, USA)
2.9. Statistical analysis

Once collected, the data were first tested for normal distribution using the Kolmogorov-Smirnov test (alpha = 0.05). Differences in canine host defense peptides (HDPs) secretion and staining, before and after bacterial exposure, were compared using the Paired Student’s $t$-test (or Wilcoxon Matched single rank when not normally distributed). Student’s unpaired $t$-test (or Mann-Whitney when not normally distributed) were used to evaluate the differences in canine HDPs secretion and staining, between the two groups (healthy vs. atopic) before and after bacterial exposure. $P$ values of $\leq 0.05$ was considered significant. All statistical comparisons were performed using the GraphPad Prism6 Software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. ELISA

First, we evaluated the amount of secreted canine HDPs (cBD3-like, cCath, and S100A8). The results of this experiment show a significantly increased secretion of all the selected HDPs in healthy compared to non-lesional atopic skin at baseline (Fig. 1b, d, f). A significantly higher expression in healthy compared to non-lesional atopic skin was seen after exposure to $P.\ aeruginosa$ (cBD3-like and S100A8) and $E.\ coli$ (S100A8) (Fig. 1b, d). In addition, compared to baseline, a significant decrease in HDPs secretion was seen in healthy skin after exposure to both $S.\ aureus$ (cBD3-like and cCath), $S.\ pseudintermedius$ (cBD3-like, cCath, and S100A8), $E.\ coli$ (S100A8), and $P.\ aeruginosa$ (cCath and S100A8) (Fig. 1). However, only S100A8 was significantly decreased in non-lesional atopic skin after exposure to $E.\ coli$ and $P.\ aeruginosa$ compared to baseline (Fig. 1d).

3.2. Indirect immunofluorescence

We then analyzed the relative fluorescence signal present in the epidermis of healthy and non-lesional atopic dogs before and after exposure to bacteria. The results were diametrical the opposite of the ELISA results (Fig. 1a, c, e). An increased persistence of HDPs (cBD3-like, cBD103, and cCath) was seen in non-lesional atopic when compared to healthy epidermis. These results were seen before and after

Fig. 1. Protein levels for host defense peptides in atopic and healthy canine skin explants before and after exposure to bacteria. Indirect immunofluorescence – cBD3-like, cBD103, and cCath (a, c, e). ELISA – cBD3-like, S100A8, and cCath (b, d, f). cBD: Canine β-defensin; cCath: Canine cathelicidin; SA: Staphylococcus aureus; SP: Staphylococcus pseudintermedius; EC: Escherichia coli; PA: Pseudomonas aeruginosa; a.u.: arbitrary unit. #: comparison with baseline for control group ($# \leq 0.05$; $$\leq 0.01$; $$$\leq 0.001$); ^: comparison with baseline for atopic group ($^\leq 0.05$; ^^$\leq 0.01$; $$$P \leq 0.001$); *: comparison between groups ($^*P \leq 0.05$; **$P \leq 0.01$; $$$P \leq 0.001$); #: comparison with baseline for control group ($#P \leq 0.05$; **$P \leq 0.01$; $$$P \leq 0.001$); : comparison with baseline for atopic group ($P \leq 0.05$; $$P \leq 0.01$). Bars: standard errors.
exposure to *S. aureus* (cBD103), *S. pseudintermedius* (cBD-3like), *E. coli* (cBD103), and *P. aeruginosa* (cBD3-like and cBD103) (Fig. 1a, c). Furthermore, the intensity of the fluorescence signal was similar between before and after bacterial exposure in both groups, except for *P. aeruginosa* and cBD3-like (increased) in healthy skin (Fig. 1a). These results suggest that, similar to people, a defect in HDPs secretion is present in non-lesional atopic canine skin.

### 3.3. Immuno-scanning electron microscopy (iSEM)

Thus to better evaluate the interaction between HDPs and microorganisms, we exposed three healthy and three atopic skin explants to *S. pseudintermedius* and processed them for SEM targeting cBD103 and cCath. Interestingly, cCath tended to form aggregates while cBD103 and *S. pseudintermedius* organisms, we exposed three healthy and three atopic skin explants to 18 nm colloidal-gold-cath. Interestingly, cCath tended to form aggregates while cBD103 and cBD3-like (increased) in healthy skin (Fig. 1a). These results suggest that, similar to people, a defect in HDPs secretion is present in non-lesional atopic canine skin.

In conclusion, the findings of this and previous studies in atopic dogs [9,10] show that similarly to humans [6], canine atopic skin has a defective mechanism of secretion and activation of HDPs. In addition, the results of this study suggest that an increased adhesion of HDPs to corneocytes is present in non-lesional atopic skin compared to healthy skin. These abnormalities could be the reasons why atopic skin, in both species, is more susceptible to infections than healthy skin. Furthermore, this study confirms the usefulness of dogs as animal model for human AD. Further studies, to evaluate the biochemical and structural HDPs alterations occurring in atopic keratinocytes, are needed. These studies could lead to the identification of new therapeutics able to modulate and eventually correct the biochemical/structural alterations of HDPs reducing the need of antimicrobials to fight common skin infections in both humans and animals.

### Conflict of interest

The authors declare that they have no conflict of interests.

### Authors’ contribution

Domenico Santoro: Designed the study, performed the clinical evaluation and the skin biopsy. He also analyzed the data and performed the statistical analysis and wrote the manuscript.

Linda Archer: Performed the skin explant culture and the indirect immunofluorescence.

Karen Kelley: Performed the immune-electron microscopy and gold particle count.
Acknowledgments

The work was supported by AKC Canine Health Foundation Grant Number02182-A, and by University of Florida Foundation Research Grant 2015. We wish to thank Dr Anna de Benedetto for reviewing this manuscript, Mrs. Mary Bohannon to help with recruitment of patients, and the clinical microbiology laboratory at the University of Florida – College of Veterinary Medicine to help with the bacterial cultures.

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