The Study on the Involvement of Periostin in the Pathophysiology of Atopic Canine Skin

イヌのアトピーの皮膚におけるペリオスチンの病態への関与に関する研究

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麻布大学大学院　獣医学研究科

獣医学専攻　博士課程

DV1206　峰重　隆幸

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Abstract

Introduction

  Canine atopic dermatitis (cAD) is a common, pruritic and allergic skin disease of dogs that

shares several clinicopathological features with human atopic dermatitis (hAD). In hAD,

periostin (PO) is assumed to play a critical role in the enhancement and chronicity of allergic

skin inflammation; however, the pathogenesis of cAD involved of PO has been unknown. Also,

the inducer of PO in the canine skin has not been clarified. Therefore in Chapter 1, the author

investigated the expression of the PO protein and mRNA in healthy, atopic, and nonatopic

inflamed skin in dogs. In Chapter 2, the author focused on IL-13 and TGF-β1 which were

assumed to be inducing factor of PO, using skin tissues of cAD as well as canine cultured

keratinocytes and dermal fibroblasts. Next, the function of PO was examined using culture

canine keratinocytes by proliferation assay, microarray and real time quantitative RT-PCR.

Chapter 1: Expression of Periostin in Normal, Atopic, and Nonatopic Chronically Inflamed

Canine Skin

  The aim of this chapter was to examine the expression patterns of PO in healthy, atopic, and

nonatopic chronically inflamed canine skin. Biopsy specimens from 47 dogs with skin disease,

and normal skin tissue from 5 adult beagles were examined by light microscopy,

immunohistochemistry (IHC), and *in situ* hybridization (ISH). In normal skin, PO was localized

just beneath the epidermis and around the hair follicles. In chronically inflamed skin, PO

expression was most intense in the dermis with inflammatory cell infiltrates. In contrast, low

levels of PO were detected in acutely inflamed and noninflamed skin. Conversely, all cAD

tissues characteristically showed the most intense expression of PO in the superficial dermis.

ISH showed that PO mRNA was broadly expressed in the basal epidermal keratinocytes, outer

root sheath cells, and dermal fibroblasts in normal dog skin. High expression of PO mRNA was

observed in fibroblasts in dog skin with chronically inflamed dermatitis. Moreover, in some

chronically inflamed skin specimens, PO mRNA expression was increased in basal

keratinocytes. The severity score of chronic pathological changes and CD3+ cell number in the

dermis were correlated with distribution pattern of PO in the atopic skin. These data suggest that

PO is able to play a role in the pathophysiology of chronic dermatitis, including cAD.

Chapter 2: Involvement of IL-13 in the Induction of Periostin, and Effect of Periostin on

Keratinocytes

From the results of Chapter 1, it was suggested that PO might be involved in the pathogenesis

of chronicity in cAD. Therefore, the author tried to clarify the mechanism of the amplification

and chronicity of dermal inflammation by PO in canine atopic skin in this chapter. By

double-labeled ISH, IL-13 mRNA positive cells were detected near the keratinocytes and

fibroblasts expressing PO mRNA in the dermis of atopic skin. TGF-β1 mRNA positive cell was

not seen in all atopic skins. By *in vitro* assay, IL-13 induced the gene expression of PO in both

canine dermal fibroblasts and keratinocytes. PO enhanced *in vitro* growth of canine

keratinocytes. Moreover, among the PO-induced genes in cultured canine keratinocytes detected

by microarray, the author identified IL-25 and KDAP as possible mediators in canine atopic skin.

Also, real time PCR analysis revealed up-regulation of both IL-25 and KDAP gene expression

in PO-stimulated keratinocytes. These data suggest that IL-13 possibly derived from Th2 cells

stimulates PO production in both keratinocytes and fibroblasts, and then PO may play a critical

role in the pathophysiology of cAD, especially in the enhancement and chronicity of skin lesion

via IL-25 and KDAP.

Conclusion

  The author proposed a novel hypothesis that PO induced by IL-13 contributes to the

enhancement and chronicity of allergic skin inflammation in canine atopic skin. The findings of

the present study provide a molecular basis for considering PO and IL-13 as a potential

therapeutic targets for cAD.

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Contents

General Introduction

Atopic dermatitis (AD) is a chronic, pruritic and allergic skin disease in humans as well as animals, especially dogs. Classically, AD has been believed to associate with high specific IgE against environmental allergens, such as house dust mite (HDM). Recently, AD has been recognized as a multifaceted disease resulting from a complex interaction between environmental and genetic factors.

Canine atopic dermatitis (cAD) has been focused as a spontaneous atopic animal model, because dogs live in human lifestyle environment, and cAD shares several clinicopathological features with human atopic dermatitis (hAD), including age of onset, skin areas affected, severe pruritus, and the immunopathological mechanism.[13, 18, 23] Histologically, the skin lesion in hAD and cAD is characterized by spongiotic dermatitis with mononuclear infiltrate composed of T lymphocytes. [19, 23] In addition, both hAD and cAD are characterized by allergic inflammation due to the release of various cytokines, mainly T helper 2 (Th2) cytokines such as interleukin (IL)-4 and IL-13.[18, 21, 23]

In AD, once allergic inflammation is triggered by exposure to allergens, the skin lesion chronically persists without continuous allergen stimulation. Therefore, chronic inflammation irritation and epidermal hyperplasia are major pathological problems of hAD and cAD. However, the mechanisms underlying chronicity in allergic inflammation and associated skin

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reaction remain unknown.

Periostin (PO) is an extracellular matrix (ECM) protein that belongs to the fasciclin family.[15, 20, 31] PO was first described as osteoblast-specific factor 2 (OSF-2) in 1993 as a homophilic adhesion molecule in bone formation.[25] PO modulates cell function by binding to integrin molecules on the cell surface; thus, providing signals for tissue development and remodeling.[6, 20, 31, 32] The production of PO is induced by transforming growth factor (TGF)-β as well as interleukin-4 and IL-13, which are signature cytokines of the Th2-type immune response in bronchial asthma, suggesting the involvement of PO in allergic inflammation.[7, 14, 31] Recently, Masuoka *et al.*[14] reported that deficiency of PO suppressed allergic inflammation induced by treatment with HDM extract in atopic model mice. They further indicated that PO bound to α_v integrin on mouse keratinocytes *in vitro*, which resulted in the induction of proinflammatory cytokines, including thymic stromal lymphopoietin (TSLP). Thus, PO may play a critical role in the enhancement and chronicity of allergic skin inflammation in hAD and experimental atopic models. However, only one report described a significant alteration of the gene expression of PO in atopic skin compared with healthy skin in dogs by real time reverse transcription polymerase chain reaction (RT-PCR).[29]

In this study, the author tried to clarify the involvement of PO in canine atopic skin using skin tissues of cAD as well as canine cultured keratinocytes and dermal fibroblasts. The findings of

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this study may provide a molecular basis for considering PO as potential therapeutic targets for

cAD.

Chapter 1: Expression of Periostin in Normal, Atopic, and Nonatopic Chronically Inflamed Canine Skin

Introduction

   PO is highly expressed in the skin of hAD patients and is correlated with disease severity on histological examination. Recently, using PO-deficient mice, Masuoka *et al.*[14] reported that a lack of PO results in failed development of allergic skin inflammation caused by sensitization to inhaled allergens. However no reports described the location of PO in canine health and atopic skin.

   Previous *in vitro* analyses demonstrated that dermal fibroblasts produced and secreted PO, which is depending on cytokine stimulation via integrin signaling. In AD lesions, it is suspected that fibroblasts are the main source of PO, although it has not been confirmed by *in vivo* analysis.[14, 22, 26]

   It is important to reveal the localization of PO and PO producing cells in normal and affected skin, because we have to discuss the involvement of PO in canine atopic skin. First, the author examined the expression of the PO protein in healthy, atopic, and nonatopic inflamed skin in dogs. Then, the author examined the localization of PO mRNA by *in situ* hybridization (ISH) in these skin tissues. Finally, the author discussed a part of role of PO in the pathophysiology in canine atopic skin.

Materials and Methods

RNA extraction from normal skin and other tissues, RT-PCR, TA cloning, Sequence analysis

  The normal canine tissues including heart, lung, liver, kidney, spleen, small intestine, large

intestine, ovary, testis, cerebrum, lymphatic node and skin from two adult beagles (one male and

one female, aged 1–7 years) were used as qualitative RT-PCR samples. Total RNA was

extracted from normal tissues using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad,

CA). Extracted total RNA was treated with RNase-free DNase (Promega, Madison, WI) and

used in RT-PCR employing the SuperScript III First-Strand Synthesis System (Invitrogen Life

Technologies). Primer sequences were obtained by determining the predicted mRNA sequences

of Canis lupus familiaris PO (NCBI database accession number: XM_003433308). The primer

sequences were as follows: canine PO forward,

5'CAGCTCAGAGTCTTTGTCTATCGTACAG3'; and reverse,

5'CTCCTGGTGTTAGGTGATAAAGGATG3'. PCR reaction with KOD FX Neo (Toyobo,

Osaka, Japan) was performed as follows: denaturation at 94 °C for 5 min; 30 cycles of

denaturation at 98 °C for 10 s, annealing at 66 °C for 30 s, and extension at 68 °C for 30 s; and a

final extension step at 68 °C for 30 s.

  The gene fragment of skin was cloned into the pTA2 vector (Toyobo) using a TArget

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clone-Plus-TA cloning kit (Toyobo). The partial sequences of PO were cloned into One Shot®

TOP10 Chemically Competent Escherichia coli (Invitrogen Life Technologies). The precipitated

DNA was sequenced and analyzed by TaKaRa Bio (Shiga, Japan) using a 3730xl DNA Analyzer

(Applied Biosystems, Foster city, CA).

Skin Biopsies

  The skin tissues obtained from the chest region of normal 5 dogs (two males and three females,

aged 1–7 years) were used as controls (Azabu university experimental animal committee,

No.100408-3).

  The clinical data and pathological diagnoses of 47 dogs of various breeds included in the

present study are listed in Table 1. Their ages ranged from 4 months to 17 years, and the cohort

included 27 males, 19 females, and one dog for which the sex was not documented. A total of 62

biopsies were taken from the 47 dogs with skin diseases. Skin punch biopsies were fixed in 10%

neutral-buffered formalin, embedded in paraffin, cut, and stained with hematoxylin and eosin

(HE).

  The skin diseases included in the present study were divided into three groups: chronic

dermatitis (inflammatory skin disease with acanthosis and dermal fibrosis) (case Nos. 1–41),

acute dermatitis (case Nos. 42 and 43), and noninflamed skin disease (case Nos. 44–47).

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Chronic skin diseases included cAD (n = 9), sebaceous adenitis (n = 4), superficial pyoderma (n

= 2), pemphigus foliaceus (n = 2), deep pyoderma (n = 2), and sterile pyogranuloma (n = 2).

The pathological diagnosis was based on clinical and light microscopic features. The diagnosis

of cAD was based on the clinical criteria proposed by Terada.[27]

Reverse transcription (RT) for real time PCR was performed on samples from 5 lesion sites of

4 atopic dogs (site Nos. 3, 4, 7, 18, and 19). One half of each skin sample was fixed in 10%

neutral-buffered formalin, embedded in paraffin, cut, stained with HE, and subjected to IHC and

ISH. The second half of each specimen was embedded in optimal cutting temperature (OCT)

compound (Sakura Finetek, Tokyo, Japan), and the block embedded in OCT compound was

snap-frozen and maintained at −80 °C. Ten sections of each specimen, 10 μm in thickness, were

cut on a cryostat at −20 °C and immersed into RNAse-free 1.5-ml microcentrifuge tubes

containing Buffer RLT Plus (Qiagen, Hilden, Germany). Total RNA was extracted from each

specimen using the RNeasy Plus Micro Kit (Qiagen). Extracted total RNA was used in

quantitative RT-PCR employing the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen

Life Technologies).

Immunohistochemistry

Immunohistochemistry (IHC) was performed using the immunoenzyme polymer method and

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the primary polyclonal or monoclonal antibodies shown in Table 2. After incubation with 4%

BlockAceTM (Yukijirushi, Sapporo, Japan) for 10 min at room temperature, dewaxed sections

were incubated overnight with the antibodies against PO at 4 °C. Peroxidase-conjugated

anti-mouse (Histofine Simple Stain MAX-PO (M), Nichirei, Tokyo, Japan) or anti-rabbit

(EnVision+[TM], Dako Corp., Glostrup, Denmark) immunoglobulin (Ig)G were used as secondary

antibodies. After the immunoreaction, the sections were colorized with diaminobenzidine

(DAB) and counterstained with Mayer's hematoxylin. Non-immune mouse or rabbit IgG was

used in place of the primary antibody as a negative control.

Immunofluorescence assay was performed using anti-type IV collagen, anti-laminin, and

anti-PO antibodies. FITC-conjugated goat anti-rabbit IgG (EY Laboratories, San Maeto, CA)

was used this assay. Immunofluorescence was analyzed using an FSX100 fluorescence

microscope (Olympus, Tokyo, Japan).

For quantitative morphometric analysis, the localization and distribution of PO were assessed

within four areas of the skin: the epidermal–dermal junction (EDJ), which constitutes an

anatomic functional unit containing the basement membrane; the perifollicular area (PF),

including the follicular basement membrane and the connective tissue follicle; the superficial

dermis (SD), the top layer of the dermis normally composed of thin collagen fibers; and the

deep dermis (DD), the thin bottom layer of the dermis normally composed of thick collagen

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fibers. Within each area, the expression of PO was assessed semiquantitatively and scored as

follows: 0 = no expression, 1+ = weak expression, 2+ = moderate expression, 3+ = high

expression. In cAD cases, the three distribution patterns of PO in the skin were defined, as

follows (compared with the normal control, which was assigned a normal pattern): basement

membrane (BM) pattern (weak-to-moderate expression in EDJ and PF); superficial pattern (high

expression in EDJ or weak expression in SD); diffuse pattern (moderate expression in SD or

expression in DD); as shown in Table 3. Epidermal thickness was measured; the number of

CD3-positive cells (T cells), CD20-positive cells (B cells), and mast cells in the SD were

counted in 10 randomly selected high-power fields for each sample and their average values

were calculated. Dermal fibrosis was semiquantitatively assessed to evaluate the severity of

chronic pathological changes as follows: no (0), mild (1+), moderate (2+), and severe (3+).

In situ hybridization

  Gene-specific probe sets for canine mRNA consisting of an average of 20 different probe pairs

were custom designed and synthesized by Affymetrix (Santa Clara, CA). PO probes were used

in type 1/Fast Red.

  Single ISH for PO was performed on samples from 21 dogs (case Nos. 1–7, 10, 11, 15, 17, 18,

21, 22, 24, 31, 32, 36, 37, 42 and 43) using the QuantiGene® ViewRNA ISH Tissue Assay

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(Affymetrix) according to the manufacturer's protocol. The signal for PO mRNA was visualized

with fast red and counterstained with Gill's hematoxylin. A canine PO mRNA sense probe or an

ISH reaction solution without the probes was used as a negative control, according to

Affymetrix's recommendations.

Real time quantitative RT-PCR

Quantification of PO and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA

expression was performed using StepOne™ Real-Time PCR Systems (Applied Biosystems) and

TaqMan Universal PCR Master Mix (Applied Biosystems), with sample cDNA in a final

volume of 20 μl per reaction. Canine PO mRNA primers and probes were designed by Applied

Biosystems and supplied as TaqMan Gene Expression Assays Mix containing a 20× mix of

unlabeled PCR forward and reverse primers, as well as a TaqMan MGB probe (Assay ID:

Cf02680558_m1). The PCR reaction of PO mRNA was performed in duplicate for each sample

and mean values of the gene expression were calculated as a ratio to those of GAPDH according

to the ΔΔCT method.

Statistical analyses

Statistical analyses of distribution patterns of PO and histological scores were performed using

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GraphPad Prism (ver. 5.0, GraphPad Software, La Jolla, CA). One-way ANOVA was performed

to analyze the difference between distribution patterns of PO and histological scores, and

significant differences were identified using the Tukey–Kramer test. Comparisons of mRNA

extracted from between atopic and normal dog groups in analysis of PO mRNA expression were

performed by the Mann–Whitney U-test. P values of <0.05 were considered statistically

significant.

Results

Expression of the PO gene in normal skin tissues

  RT-PCR was performed to assess the expression level of the PO mRNA (346 bp) in various

canine tissues, including heart, lung, liver, kidney, spleen, pancreas, small intestine, colon, ovary,

testis, cerebrum, mesenteric lymph node and skin. The PO mRNA was expressed in a wide

range of normal canine tissue (Figure 1a). Canine GAPDH (219 bp) cDNA was amplified as an

internal control (Figure 1b).

  The sequence of PO mRNA extracted from canine skin was registered in the DNA Data Bank

of Japan (DDBJ) (Accession number LC008358). Sequence analysis of the amplification

product showed homology to the partial sequences of predicted canine PO mRNA (100%) and

human PO mRNA (92%).

  ISH revealed that PO mRNA-positive cells were located in the outer root sheath (ORS),

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particularly in the outer cells along the basement membrane, whereas there was a small number

of signals in the ORS inner cells in normal dog skin (Figure 2a). The PO protein was located at

the basement membrane of hair follicles in normal dog skin (score 1+) (Figure 2b). In the

epidermis, PO mRNA was expressed only in the basal epidermal keratinocytes (Figure 2c). The

PO protein was detected at low level in the EDJ (score 1+) (Figure 2d) but not in DD (score 0).

The signals were not detected in tissues that underwent the procedure using the sense probe or

the ISH solution without the probe (Figure 2e). PO protein was not detected in the cells. No

specific staining was observed with non-immune rabbit IgG (Figure 2f).

PO highly deposited in the atopic dermis.

  The results of the immunohistochemical examination of skin diseases are summarized in Table

1. PO protein expression in chronically inflamed skin was most intense in the dermis with

inflammatory cell infiltrates (scores 1+, 2+ or 3+) (Figures 3a, 3b, 4a, 4b, 5a, and 5b), whereas

low levels of PO were detected in acutely inflamed and noninflamed skin diseases. Sebaceous

adenitis was characterized by the destruction of the sebaceous gland and was associated with an

intense expression of PO in the superficial dermis, particularly at PF. In diffuse dermatitis, PO

protein expression was intense throughout the dermis. In sterile pyogranuloma, both

inflammatory cells and PO protein expression were observed in DD and subcutaneous tissue.

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Among the inflammatory skin diseases, all canine AD tissues characteristically showed the most

intense expression of PO in the superficial dermis, particularly at EDJ (2+ or 3+) (Figure 6a).

Expression of type IV collagen and laminin was detected at the EDJ and vascular basement

membrane (Figure 7a, b). PO was more prominently expressed at the EDJ and detected faintly

at the vascular basement membrane (Figure 7c). No staining was observed with non-immune

rabbit IgG (Figure 6c).

Keratinocytes and dermal fibroblasts are the main source of PO in cAD.

 Positive signals for PO mRNA were detected in spindle-shaped stromal cells in the

inflammatory areas (Figures 3c, 4c, and 5c), together with protein expression. These stromal

cells were positive for vimentin and negative for factor VIII-related antigen and smooth muscle

actin, indicating that most of the stromal cells were fibroblasts. In addition, the PO mRNA was

expressed in some adipocytes. In six (case Nos. 3, 5, 10, 15, 24, and 32) out of 21 dogs,

increased PO mRNA expression was observed more clearly in epidermal keratinocytes (Figure

6b). Signals were not detected in tissues that underwent the procedure using the sense probe or

the ISH solution without the probe (Figure 6d).

Real time RT-PCR using total RNA from frozen sections showed no significant difference in PO

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mRNA expression between atopic and control skin groups.

Real time RT-PCR using total RNA from sections of five lesion sites in 4 atopic dogs showed no significant difference in the ratio of PO/GAPDH mRNA expression between atopic and control skin groups (Figure 8).

PO deposition score was correlated with histopathological severity of cAD.

The distribution patterns of PO were correlated with chronic pathological changes and the number of CD3+ cells in the skin tissues of atopic dogs. Chronic pathological changes (epidermal thickening and fibrosis) and the number of CD3+ cells in the skin were significantly different between some distribution patterns of PO, especially in normal and diffuse patterns. Although there were no significant differences in the number of CD20+ cells and mast cells between the different distribution patterns of PO (Table 3 and Figure 9).

Discussion

To the best of my knowledge, this study was the first report showing the expression patterns of PO mRNA and protein in various spontaneous skin diseases of dogs. The author found that, in normal canine skin, similarly to that in human and murine skin, PO mRNA was expressed only in the basal epidermal keratinocytes, outer root sheath cells, and dermal fibroblasts and that PO was deposited just beneath the epidermis and around the hair follicles. Furthermore, the

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distribution pattern of PO mRNA and protein in normal and inflamed skin appeared to be

significantly different. An increased expression of PO was observed in chronically inflamed

skin compared with normal, noninflamed, and acutely inflamed skin. In the present study, the

author confirmed that cAD is characterized by the expression of PO in the superficial dermis,

particularly at EDJ. PO was partially colocalized with other ECM such as type IV collagen and

laminin components of the basement membrane. Moreover, the severity of chronic pathological

changes and infiltration of CD3+ cells in the dermis were different between distribution patterns

of PO, especially between normal and diffuse pattern. In atopic dogs, T cells are major

components of the inflammatory cell infiltrate in the skin.[8] Th2 cytokines play a critical role

in inducing allergic inflammation in addition to promoting IgE class switching.[18, 21, 23]

 The author has identified PO-producing cells within the canine atopic skin and proposes that

these cells are fibroblasts. Conversely, in some atopic canine skin, PO mRNA was notably

expressed in keratinocytes. Nishiyama *et al.*[16] showed that PO mRNA was temporarily

expressed not only in fibroblasts but also in keratinocytes in hair follicles during wound healing.

To the best of our knowledge, to date, no reports have focused on PO production by epidermal

cells in human and canine atopic skin.[14, 22, 26, 31]

 Real time RT-PCR using total RNA from frozen sections, the ratio of PO/GAPDH mRNA

expression did not differ significantly between atopic and normal dog groups. By real time

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RT-PCR using sections, it was impossible to determine the distribution pattern of PO mRNA. As the number of PO non-producing cells (such as lymphocytes) was increased in atopic skin compared with control skin, it was difficult to compare PO gene expression.

In summary, the author has revealed the high expression of PO in canine atopic skin. PO is produced by keratinocyte and fibroblast and may play an important role in canine atopic skin.

Figures and Tables

Periostin (346bp)
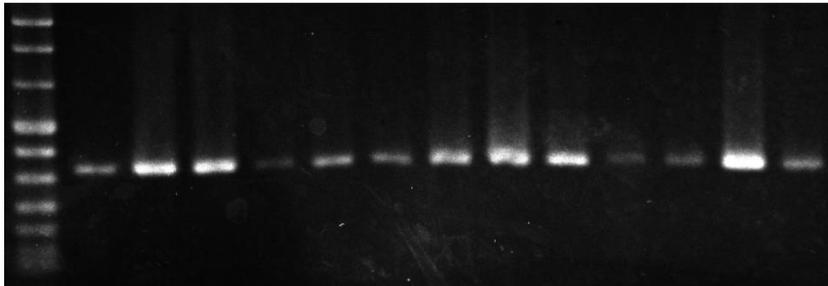
M He Lu Li Ki Sp Pa Si Co Ov Te Ce Ly Sk

1a

GAPDH (219bp)
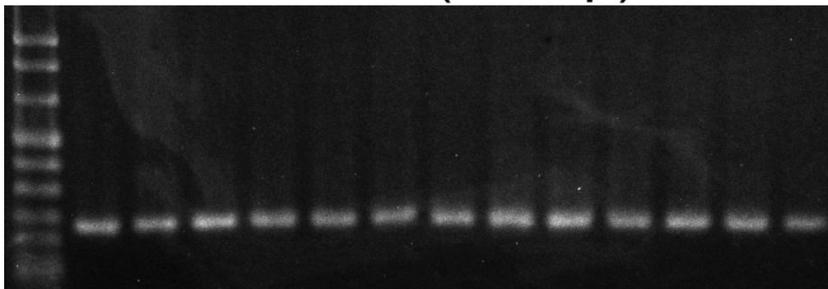
M He Lu Li Ki Sp Pa Si Co Ov Te Ce Ly Sk

1b

Figure.1. Identification of canine periostin (PO) and glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) transcripts in various organs by reverse transcription polymerase chain reaction (RT-PCR).

a. The PO mRNA was expressed in a wide range of normal canine tissue, including heart (He), lung

(Lu), liver (Li), kidney (Ki), spleen (Sp), pancreas (Pa), small intestine (Si), colon (Co), ovary (Ov),

testis (Te), cerebrum (Ce), mesenteric lymph node (Ly) and skin (Sk). **b.** Canine GAPDH cDNA was

amplified as an internal control. M, molecular weight marker.
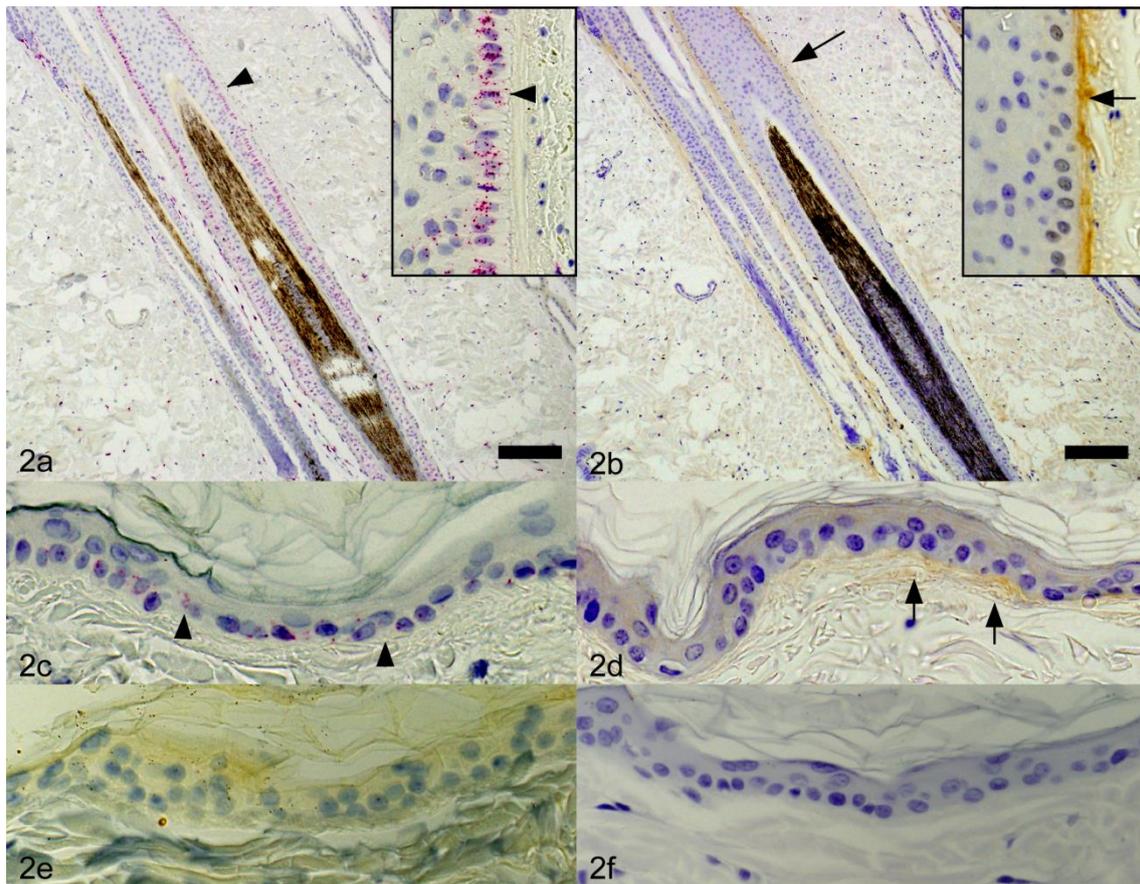
Figure 2. Normal skin, dog. **a.** The red color indicates a positive signal for PO mRNA (fast red) (arrow heads). The PO mRNA was expressed in the outer root sheath cells of the hair follicle, particularly in the outer cells along the basement membrane. *In situ* hybridization (ISH) for PO. *Inset*: high-power magnification view of hair follicles, ISH for PO. Bar = 500 μm. **b.** The brown color indicates positive staining for the PO protein (arrows). The PO protein was localized at the perifollicular area. Immunohistochemical staining (IHC) for PO. *Inset*: high-power magnification view of hair follicles, IHC for PO. Bar = 500 μm. **c.** In the epidermis, PO mRNA was expressed only in the basal epidermal keratinocytes (arrows heads). ISH for PO. **d.** Slight PO protein expression was observed in the dermis, just beneath the basal layer (arrows). IHC for PO. **e.** No signal was observed with the PO mRNA sense probe. ISH for PO (sense probe). **f.** No staining was observed with non-immune rabbit IgG. IHC for non-immune rabbit IgG. 500
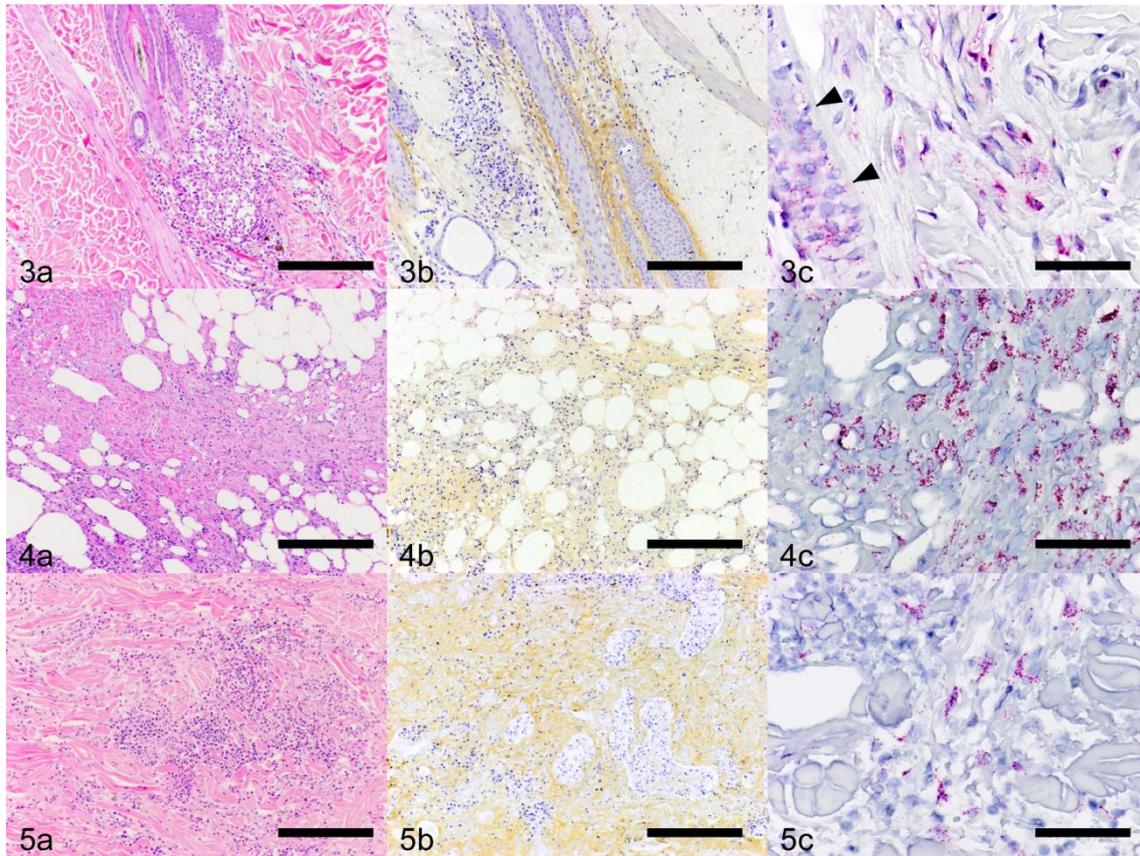
Figure 3. Sebaceous adenitis, skin, dog, case No. 18. **a.** Isthmus perifollicular inflammation was

observed. Hematoxylin and eosin (HE). Bar = 300 μm. **b.** PO protein expression was prominent in

the perifollicular area. IHC for PO. Bar = 300 μm. **c.** The PO mRNA was expressed in outer root

sheath cells (arrows heads) and fibroblasts surrounding hair follicles. ISH for PO. Bar = 50 μm.

Figure 4. Sterile pyogranuloma, skin, dog, case No. 32. **a.** Granulomatous inflammation was

observed in the deep dermis and the subcutis. HE. Bar = 300 μm. **b.** PO was localized in

inflammatory tissue. IHC for PO. Bar = 300 μm. **c.** The PO mRNA was expressed in fibroblasts but

not in inflammatory cells. Positive signals for PO mRNA were also detected in some adipocytes.

ISH for PO. Bar = 50 μm.

Figure 5. Diffuse dermatitis, skin, dog, case No. 22. **a.** Inflammatory cells were detected diffusely in

the dermis. HE. Bar = 300 μm. **b.** PO protein expression was prominent throughout the dermis with

inflammatory cell infiltrates. IHC for PO. Bar = 300 μm. **c.** The PO mRNA was expressed in

fibroblasts but not in inflammatory cells. ISH for PO. Bar = 50 μm.
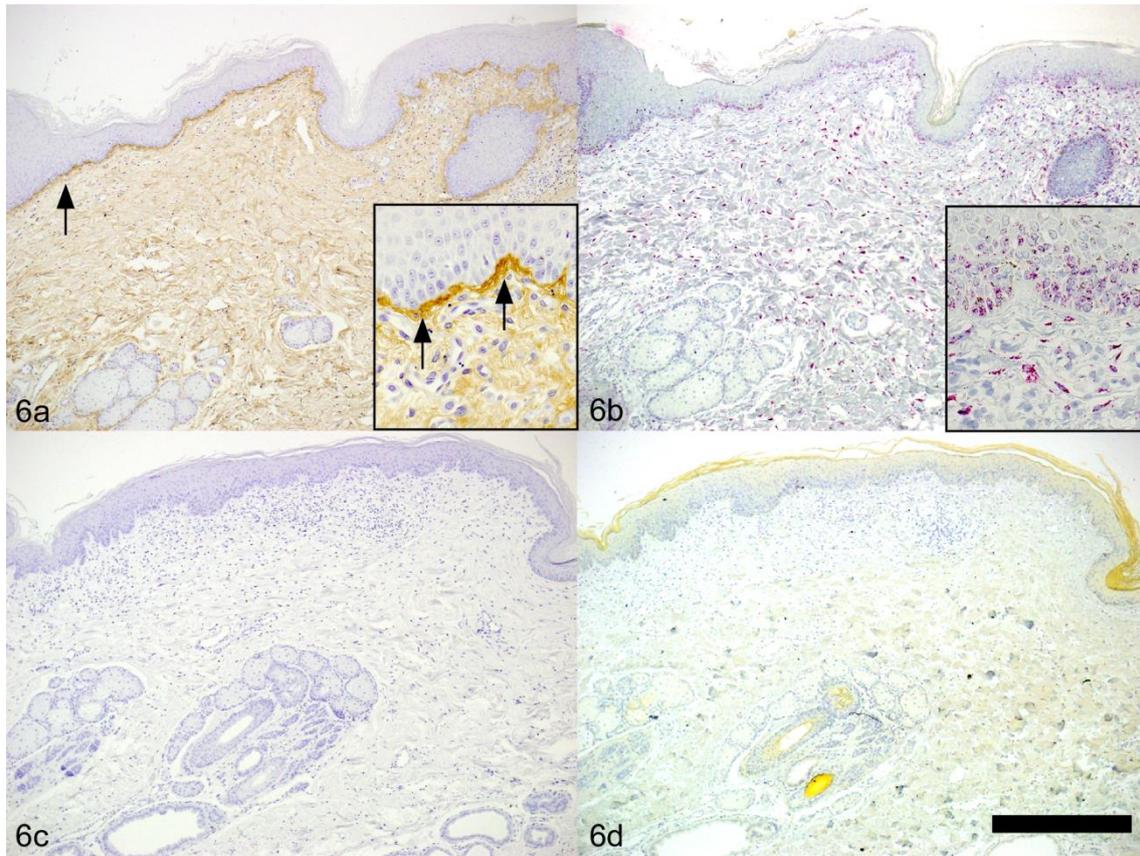
Figure 6. Atopic dermatitis, skin, dog, case No. 5. **a.** The expression of PO was significantly elevated in the superficial dermis, particularly at the epidermal–dermal junction (arrows). IHC for PO. *Inset*: high-power magnification view of the epidermis and superficial dermis, IHC for PO. **b.** The PO mRNA was more prominently expressed in keratinocytes. ISH for PO. *Inset*: high-power magnification view of the epidermis and superficial dermis. **c.** No staining was observed with non-immune rabbit IgG. IHC for non-immune rabbit IgG. **d.** No signal was observed with the PO mRNA sense probe. ISH for PO (sense probe). Bar = 500 μm.
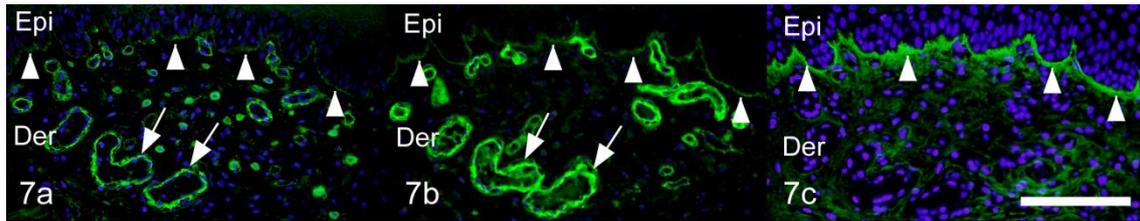
Figure 7. Atopic dermatitis, skin, dog, case No. 5. Epi, epidermis; Der, dermis. **a.** Expression of type IV collagen was detected at the epidermal–dermal junction (EDJ) (arrowheads) and vascular basement membrane (arrows). Immunofluorescence staining (IF) for type IV collagen. **b.** Expression of laminin was detected at the EDJ (arrowheads) and vascular basement membrane (arrows). IF for laminin. **c.** PO was more prominently expressed at the EDJ (arrowheads) and detected faintly at the vascular basement membrane. IF for PO. Bar = 100 μm.
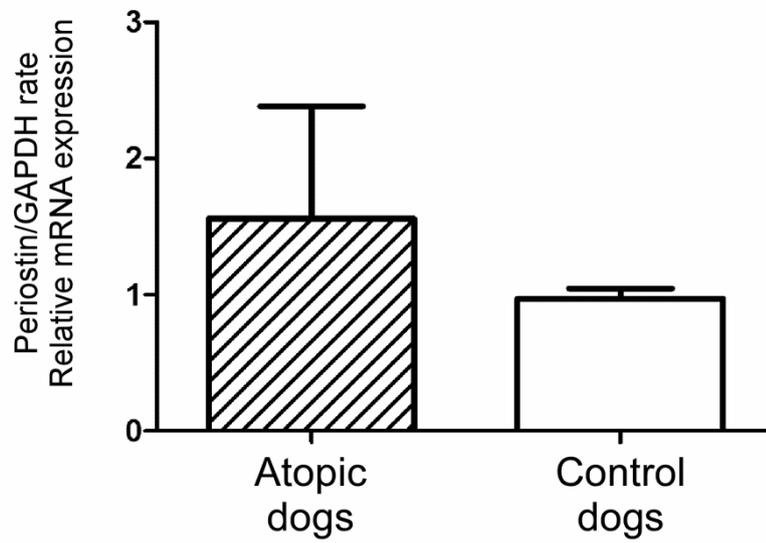
Figure 8. PO/GAPDH mRNA rate in atopic skin (n = 5) and control skin (n = 5). The ratio of

PO/GAPDH mRNA expression did not differ significantly between atopic and normal dog groups.

Real time quantitative RT-PCR.

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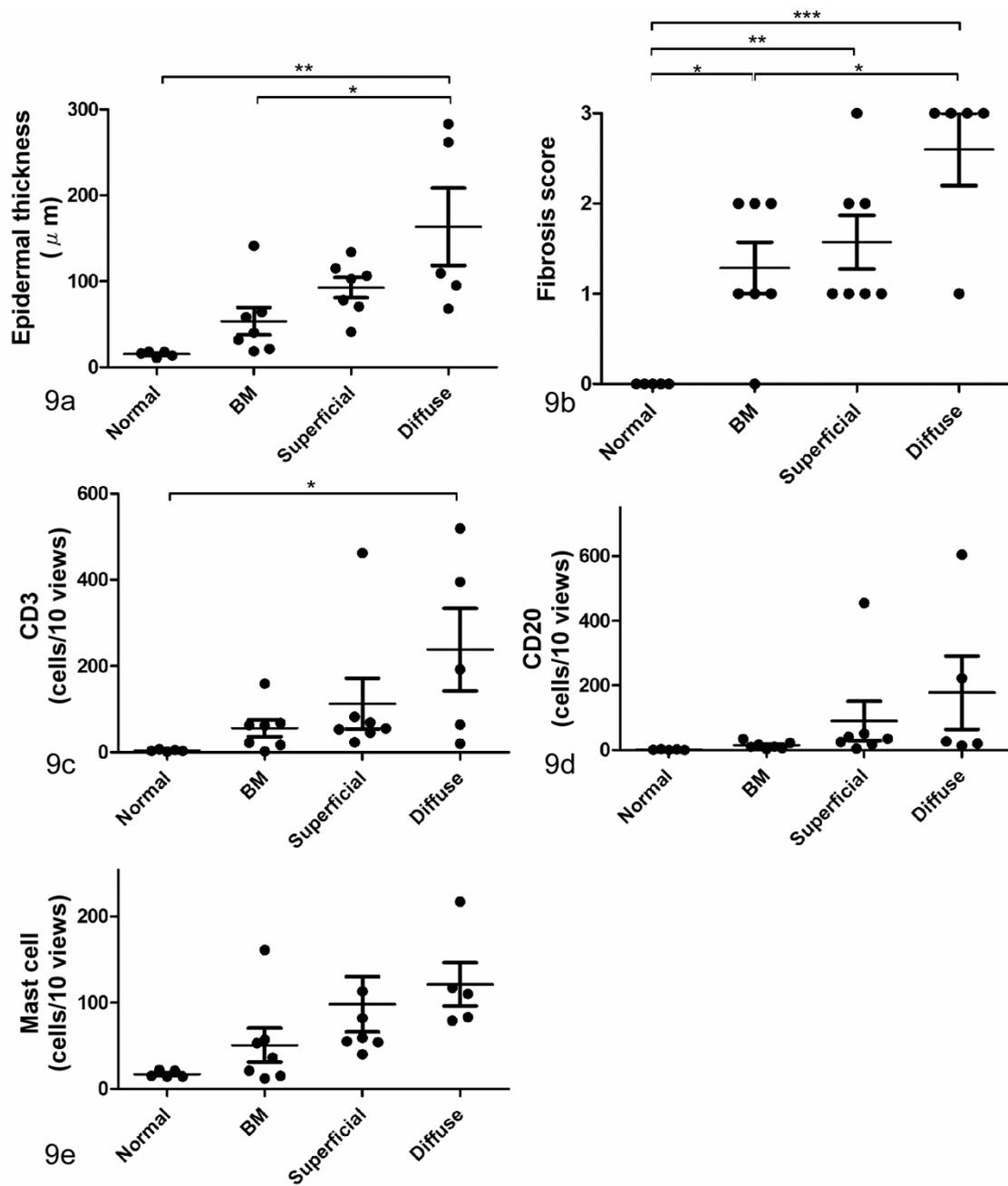
Figure 9. Correlation between distribution patterns of PO and histological severity in the skin tissues

of atopic dogs. Three distribution patterns of PO of atopic dogs were as follows: basement

membrane pattern, superficial pattern and diffuse pattern (compared with the healthy control, which

was assigned a normal pattern). The number of inflammatory cells (CD3+, CD20+, and mast cells)

in the dermis was counted in 10 high-power fields for each sample. Chronic pathological changes

(epidermal thickening and fibrosis) and the number of CD3+ cells in the skin was correlated between some distribution patterns of PO, especially between normal and diffuse pattern. Normal, normal pattern; BM, basement membrane pattern; Superficial, superficial pattern; Diffuse, diffuse pattern. (*p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA). **a.** Epidermal thickness was measured in 10 high-power fields for each sample. A significant positive correlation was observed between epidermal thickness and PO patterns **b.** Correlation between fibrosis scores and PO patterns. **c.** Correlation between the number of CD3-positive cells (T cells) and PO patterns. **d.** Correlation between the number of CD20-positive cells (B cells) and PO patterns. **e.** Correlation between the number of mast cells and PO patterns.

Table1. Characteristics of dogs with skin diseases and the results of immunohistochemical examinations of skin diseases. (continue)

Case No.	Site No.	Site	Breed	Sex/Age[a]	Histological Diagnosis	Comprehensive diagnosis/Remarks[b]	Immunohistochemical score and localization of periostin[c]			
							EDJ[d]	PF[e]	SD[f]	DD[g]
1	1 Face		Welsh Corgi	C/13 y	Superficial dermatitis	AD	3+	2+	2+	0
	2 Back				Superficial dermatitis	AD	3+	1+	2+	0
2	3 Face		Shiba	C/11 y	Superficial dermatitis	AD	3+	2+	1+	0
	4 Armpit				Superficial dermatitis	AD	3+	2+	2+	1+
	5 Hind leg				Superficial dermatitis	AD	3+	2+	1+	0
3	6 Shoulder		Bernese Mountain Dog	M/9 y	Superficial dermatitis	AD/Non-lesion	2+	2+	0	0
	7 Abdomen				Superficial dermatitis	AD/Lesion	2+	2+	1+	0
	8 Abdomen				Superficial dermatitis	AD/Non-lesion	2+	2+	0	0
4	9 Hind leg		Golden Retriever	S/10 y	Superficial dermatitis	AD	2+	2+	1+	1+
	10 Abdomen				Superficial dermatitis	AD	2+	2+	0	0
5	11 Abdomen		Shiba	M/8 y	Superficial dermatitis	AD	3+	2+	2+	0
	12 Abdomen				Superficial dermatitis	AD	3+	2+	0	0
6	13 Shoulder		Chihuahua	M/2 y	Superficial dermatitis	AD	2+	1+	0	0
	14 Back				Superficial dermatitis	AD	3+	1+	0	0
7	15 Neck		Shih Tzu	F/5 y	Superficial dermatitis	AD	3+	2+	1+	0
	16 Armpit				Superficial dermatitis	AD	3+	2+	1+	0
	17 Back				Superficial dermatitis	AD	2+	1+	0	0
8	18 Back		Yorkshire Terrier	S/8 y	Superficial dermatitis	AD	2+	1+	0	0
9	19 Abdomen		Standard Poodle	M/4 y	Superficial dermatitis	AD	2+	2+	0	0
10	20 Abdomen		Papillon	S/10 y	Superficial dermatitis	Superficial pyoderma	2+	2+	0	0
	21 Hind leg				Superficial dermatitis	Superficial pyoderma	2+	1+	0	0
11	22 Hip		Shetland Sheepdog	M/12 y	Superficial dermatitis	Superficial pyoderma/Lesion	3+	2+	1+	0
	23 Hip				Superficial dermatitis	Superficial pyoderma/Non-lesion	2+	1+	0	0

Table 1. Characteristics of dogs with skin diseases and the results of immunohistochemical examinations of skin diseases. (continue)

Case No.	Site No.	Site	Breed	Sex/Age[a]	Histological Diagnosis	Comprehensive diagnosis/Remarks[b]	Immunohistochemical score and localization of periostin[c]			
							EDJ[d]	PF[e]	SD[f]	DD[g]
12	24	Back	Golden Retriever	M/13 y	Demodicosis	Demodicosis	3+	2+	0	0
13	25	Abdomen	Miniature Dachshund	C/10 y	Superficial dermatitis	Pemphigus foliaceus	2+	1+	0	0
14	26	Abdomen	Miniature Dachshund	S/14 y	Superficial dermatitis	Pemphigus foliaceus	2+	1+	0	0
	27	Back			Superficial dermatitis	Pemphigus foliaceus	3+	2+	1+	0
15	28	Hip	Miniature Schnauzer	M/11 y	Superficial dermatitis	Not AD	3+	1+	1+	0
16	29	Neck	Toy Poodle	S/2 y	Superficial dermatitis	Unconfirmed	2+	1+	0	0
	30	Back			Superficial dermatitis	Unconfirmed	2+	1+	0	0
17	31	Face	Maltese	M/8 y	Superficial dermatitis	Not AD	2+	2+	1+	0
18	32	Unknown	Akita	S/5 y	Sebaceous adenitis	Sebaceous adenitis	1+	2+	0	0
19	33	Back	Mixed	M/5 y	Sebaceous adenitis	Sebaceous adenitis	2+	3+	1+	0
20	34	Back	Akita	M/5 y	Sebaceous adenitis	Sebaceous adenitis	1+	1+	0	0
21	35	Back	Akita	M/7 y	Sebaceous adenitis	Sebaceous adenitis	3+	2+	0	0
22	36	Face	Shiba	S/1 y	Diffuse dermatitis	Deep pyoderma	2+	2+	2+	1+
23	37	Nail bed	French Bulldog	S/5 y	Diffuse dermatitis	Deep pyoderma	1+	1+	1+	1+
24	38	Face	Mixed	F/4 y	Diffuse dermatitis	Unconfirmed	3+	2+	1+	2+
25	39	Face	Pomeranian	C/3 y	Diffuse dermatitis	Unconfirmed	1+	2+	1+	1+
26	40	Face	Akita	C/10 y	Diffuse dermatitis	Unconfirmed	2+	2+	2+	2+
27	41	Nail bed	Mixed	M/15 y	Inflammatory granulation tissue	Unconfirmed	1+	2+	1+	1+
28	42	Back	Miniature Dachshund	C/11y	Interface dermatitis	Lesion	2+	2+	0	0
	43	Back			Interface dermatitis	Non-lesion	1+	1+	0	0
29	44	Back	Mixed	F/17 y	Interface dermatitis	Unconfirmed	2+	1+	0	0
30	45	Unknown	Basenji	M/8 y	Interface dermatitis	Unconfirmed	1+	1+	0	0
31	46	Back	Miniature Dachshund	M/15 y	Sterile pyogranuloma	Sterile pyogranuloma	1+	1+	0	1+
32	47	Back	Miniature Dachshund	C/9 y	Sterile pyogranuloma	Sterile pyogranuloma	2+	2+	0	3+

33

Table 1. Characteristics of dogs with skin diseases and the results of immunohistochemical examinations of skin diseases. (continue)

Case No.	Site No.	Site	Breed	Sex/Age[a]	Histological Diagnosis	Comprehensive diagnosis/Remarks[b]	Immunohistochemical score and localization of periostin[c]			
							EDJ[d]	PF[e]	SD[f]	DD[g]
33	48	Back	Miniature Dachshund	M/11 y	Superficial pustular dermatitis	Superficial pustular drug reactions	1+	2+	1+	0
34	49	Unknown	Chihuahua	S/3 y	Superficial pustular dermatitis	Unconfirmed	1+	1+	0	0
35	50	Face	Shetland Sheepdog	M/4 m	Dermatomyositis	Dermatomyositis	1+	1+	0	1+
36	51	Foreleg	Jack Russell Terrier	S/2 y	Folliculitis	Unconfirmed	1+	2+	1+	1+
37	52	Hind leg	French Bulldog	F/5 y	Cutaneous mastocytosis	Cutaneous mastocytosis	1+	1+	1+	0
38	53	Foreleg	Mixed	M/4 y	Vasculitis	Immune-mediated thrombocytopenia with vasculitis	2+	2+	2+	2+
39	54	Hip	French Bulldog	C/10 y	Calcinosis cutis	Calcinosis cutis	2+	2+	2+	2+
40	55	Abdomen	Labrador Retriever	S/3 y	Heat burn	Heat burn	2+	1+	1+	1+
41	56	Abdomen	American Cocker Spaniel	S/7 y	Erythema caloricum	Erythema caloricum	1+	1+	1+	1+
42	57	Abdomen	Maltese	F/9 y	Acute dermatitis	Unconfirmed	1+	1+	0	0
43	58	Abdomen	Maltese	S/12 y	Acute febrile neutrophilic dermatosis	Sweet's syndrome	1+	1+	0	0
44	59	Unknown	Miniature Dachshund	Unknown/7 m	Edema	Unconfirmed	1+	1+	0	0
45	60	Head	Toy Poodle	M/3 y	Follicular dysplasia	Follicular dysplasia	1+	1+	0	0
46	61	Hind leg	West Highland White Terrier	C/7 m	Atrophy of hair follicles	Alopecia	1+	1+	0	0
47	62	Hip	Toy Poodle	F/7 y	Atrophy of hair follicles	Iatrogenic cushing's syndrome	1+	1+	0	0

[a]M, male; F, female; C, castrated male; S, spayed female; y, years old; m, months old [b]AD, atopic dermatitis; Unconfirmed, we could not show clinical diagnosis in some cases, because multiple differential diagnosis could be considered or we couldn't obtain the satisfactory information from veterinary clinicians. [c]0 = no expression, 1+ = mild expression, 2+ = moderate expression, 3+ = high expression in the dermis. [d]EDJ, epidermal–dermal junction [e]PD, perifollicular dermis [f]SD, superficial dermis [g]DD, deep dermis

Table 2. Primary antibodies and immunostaining protocol used in the current study.

Antibody	Clone	Dilution	Source	Antigen retrieval[a]
Periostin	Polyclonal	1:500	Abcam., Cambridge, U.K.	No treatment
Type IV collagen	Polyclonal	1:500	Tokyo University[b]	Pepsin, 37°C, 30 min
Laminin	Polyclonal	1:100	Progen Biotechnik., Heidelberg, Germany	Pepsin, 37°C, 30 min
CD3	Polyclonal	1:50	Dako Denmark A/S., Glostrup, Denmark	Pepsin, 37°C, 30 min
CD20	Polyclonal	1:400	Thermo fisher scientific., California, U.S.A.	No treatment
Factor VIII-related antigen	Polyclonal	Prediluted	Dako Denmark A/S., Glostrup, Denmark	Pepsin, 37°C, 20min
SMA[c]	1A4	1:100	Dako Denmark A/S., Glostrup, Denmark	No treatment

[a]Pepsin = 0.4% pepsin (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) [b]This antibody was kindly supplied by Dr. M. Ito and Dr. K. Arai (Department of Scleoprotein Chemistry and Cell Biology, Tokyo University of Agriculture and Technology). [c]SMA = smooth muscle actin

Table 3. Histological findings and periostin immunolabeling of tissue samples from atopic dogs.

Dog No.[a]	Site No.[a]	Site	Epidermal thickness (μm)[b]	Fibrosis[c]	CD3 (cells/10 views)	CD20 (cells/10views)	Mast cell (cells/10views)	EDJ[d,e]	PF[d,f]	SD[d,g]	DD[d,h]	Periostin patterns[i]
					Histological Findings				Periostin Immunolabeling			
1	1	Face	109.4	3+	395	222	110	3+	2+	2+	0	D
	2	back	68.2	3+	519	604	79	3+	1+	2+	0	D
2	3	Face	78.2	2+	45	35	82	3+	2+	1+	0	S
	4	Armpit	261.9	3+	64	20	117	3+	2+	2+	1+	D
	5	Hind leg	70.5	1+	53	41	55	3+	2+	1+	0	S
3	6	Shoulder	18.7	0	2	6	15	2+	2+	0	0	BM
	7	Abdomen	115.2	1+	462	455	283	2+	2+	1+	0	S
	8	Abdomen	21.3	1+	17	22	12	2+	2+	0	0	BM
4	9	Hind leg	283.2	3+	192	27	217	2+	2+	1+	1+	D
	10	Abdomen	40	2+	63	10	57	2+	2+	0	0	BM
5	11	Abdomen	95.1	1+	20	14	83	3+	2+	2+	0	D
	12	Abdomen	41	1+	23	51	113	3+	2+	0	0	S
6	13	Shoulder	58.4	1+	159	3	161	2+	1+	0	0	BM
	14	Back	106.3	1+	69	25	54	3+	1+	0	0	S
7	15	Neck	134	3+	82	5	40	3+	2+	1+	0	S
	16	Armpit	102.9	2+	55	18	59	3+	2+	1+	0	S
	17	Back	141.4	2+	68	17	53	2+	1+	0	0	BM
8	18	Back	64.1	2+	22	34	21	2+	1+	0	0	BM
9	19	Abdomen	31.9	1+	62	11	36	2+	2+	0	0	BM
Normal dog			15.3 ± 2.7[j]	0	3.8 ± 2.0[j]	1.2 ± 1.2[j]	17.2 ± 3.5[j]	1+	1+	0	0	N

[a]9 Dogs with AD underwent 19 sites all together. [b]Epidermal thickness, measured in 10 randomly selected fields for each sample. [c]Semiquantitative scores for dermal fibrosis. [d]0 = no expression, 1+ = mild expression, 2+ = moderate expression, 3+ = high expression in the dermis. [e]EDJ, epidermal-dermal junction [f]PF, perifollicular area [g]SD, superficial dermis [h]DD, deep dermis [i]Distribution patterns of periostin. N, normal patten; BM, basemant membrane pattern; S, superficial pattern; D, diffuse pattern. [j]mean ± SD

Chapter 2: Involvement of IL-13 in the Induction of Periostin, and Effect of Periostin on

Keratinocytes

Introduction

  From the results of Chapter 1, it was suggested that PO might be involved in the pathogenesis

of chronicity in cAD. Therefore, the author tried to clarify the mechanism of the amplification

and chronicity of dermal inflammation by PO in canine atopic skin in this chapter.

  Firstly, the author focused on IL-13 and TGF-β1 which were assumed to be inducing factor of

PO, using skin tissues of cAD as well as canine cultured keratinocytes and dermal fibroblasts.

It is widely known that TGF-β is a major trigger of PO production of both dermal and

pulmonary fibroblasts.[4, 24, 32] Recently, Masuoka *et al.*[14] reported that IL-13 induced PO

production in the skin of a murine atopic model independently of TGF-β.

  Next, the author examined the function of PO using culture canine keratinocytes by

proliferation assay, microarray and real time quantitative RT-PCR. The keratinocytes acts as

major immune cells as well as a mechanical barrier between the external environment and the

body.[10, 12] Previous studies have reported that cytokines and chemokines such as thymus and

activation-regulated chemokine (TARC/CCL17) are exclusively produced by keratinocytes in

human and canine atopic skin.[10, 12] From the results of immunohistochemistry in Chapter 1,

the author assumed that PO might involve the epidermal thickening and infiltration of

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inflammatory cells which are major pathologic features in canine atopic skin.

Finally, the author discussed the possible involvement of PO in the pathophysiology of cAD.

Materials and Methods

Double-In situ hybridization

Double-ISH for PO and IL-13/ TGF-β1 was performed using the QuantiGene® ViewRNA ISH

Tissue Assay according to the manufacturer's protocol, as described Chapter 1. PO probes were

used in type 1/Fast Red, and IL-13 and TGF-β1 were used in type 6/Fast Blue. Double-ISH

sections were counterstained with Mayer's hematoxylin.

Cell culture of canine keratinocytes and dermal fibroblasts

The author used two types of culture cells, commercially available canine keratinocyte cell line

(CPEK, CELLnTEC Advanced Cell Systems, Bern, Switzerland) and canine dermal fibroblasts.

CPEK was cultured in 25 cm^2 flasks (AGC TECHNO GLASS CO.,LTD., Shizuoka, Japan) in

CnT-09 (CELLnTEC Advanced Cell Systems) with 10% fetal bovine serum (FBS; Hana-nesco

Bio. Co., Tokyo, Japan) and 1% Antibiotic Antimycotic (Thermo Fisher Scientific Inc., Waltham,

MA) until approximately 80% confluence at 37 °C under 5% CO2. The cultured cells were

trypsinized by treatment with 2 ml Trypsin-EDTA and incubated for 7–10 min at 37 °C. Fifth- to

tenth-passage CPEK was used in these experiments.

Primary culture of canine dermal fibroblasts was performed according to the previous report

[1] for the human skin. Briefly, full-depth skin samples obtained from the chest region of

normal dogs were immediately placed in PBS (pH 7.2, 0.01 M) with 1% Antibiotic Antimycotic.

Skin samples were rinsed seven times with PBS. Then skin samples were fragmented into 5

mm^2 pieces. These skin fragments were laid onto the surface of 60 mm x 15mm Petri dishes

with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/ F12, 1:1; Invitrogen

Life Technologies) containing of 10% fetal bovine serum and 1% Antibiotic Antimycotic.

Cultures were incubated at 37°C in a humidified incubator with 5% CO_2. The culture medium

was changed every three days. Satisfactory proliferation of fibroblasts was observed in

approximately twenty days. Fibroblasts of 3rd to 5th passages were used for the experiments.

Real time quantitative RT-PCR

 CPEK and dermal fibroblasts were grown in 24-well plates (AGC TECHNO GLASS

CO.,LTD.); after washing with PBS to remove all sera, then the cells were serum-starved for

24hrs.

 For real time quantitative RT-PCR analysis to estimate PO mRNA expression, CPEK and

fibroblasts were cultured in DMEM/F12 with or without 50ng/μL recombinant canine IL-13

(rcIL-13: R&D Systems, Minneapolis, MN) for 6 or 24 hrs. CPEK were cultured in DMEM/F12

with or without 2µg/mL recombinant human PO (rhPO, R&D Systems) for 2, 6, 12, 24 or 36 hrs.

After stimulation by rcIL-13 or rhPO, total RNA was extracted from cell cultures using the RNeasy Plus Micro Kit. Extracted total RNA was used for RT-PCR employing the SuperScript® VILO™ cDNA Synthesis Kit. Quantification of mRNA expression was performed using StepOne™ Real-Time PCR Systems, TaqMan Universal PCR Master Mix and TaqMan MGB probe for the target genes: PO, GAPDH, IL-25 (assay ID, Cf02643291_m1), keratinocyte differentiation-associated protein (KDAP) (assay ID, Cf02680558_m1), as described in Chapter 1. The PCR reaction was performed in duplicate for each sample and mean values of the target gene expression were calculated as a ratio to those of GAPDH according to the $\Delta\Delta$CT method.

Proliferation Assay

For proliferation assay, CPEK was cultured in with DMEM/F12 supplemented with 0.1% FBS and either rhPO (1 or 4µg/mL) dissolved in PBS or PBS alone for 24 hrs. After stimulation, proliferation assay was performed using a cell-counting kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocol.[11]

Microarray

For DNA microarray analysis, CPEK was cultured with or without 4µg/mL rhPO for 6 or 24

hrs. After stimulation by rhPO, total RNA was extracted from cell cultures using the RNeasy

Plus Micro Kit. The microarray analyses were done by DNA Chip Research Inc. (Yokohama,

Japan) using the Canine (V2) Gene Expression Microarray (4x44k, two-color array) according

to Agilent microarray DNA chip analysis. cRNA was synthesized from the mRNA component

of total cellular RNA. Each cRNA sample was then independently labeled with Cy3 (green) and

Cy5 (red). The fold change was calculated for samples to represent a ratio of expression

between stimulated and unstimulated control samples.

Statistical analyses

Statistical analyses in real time quantitative RT-PCR and proliferation assay were performed

using GraphPad Prism. Comparisons of mRNA between extracted samples from stimulated and

unstimulated cells were performed by the unpaired T test. P values of <0.05 were considered

statistically significant.

Results

IL-13 mRNA positive cells were present near the PO mRNA-positive cells.

By Double-ISH, the IL-13 mRNA-positive small round cells were detected around the PO

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mRNA-positive keratinocytes and fibroblasts in the dermis of atopic skin (Figure 10). TGF-β1

mRNA-positive cell was not seen in all atopic skins.

IL-13 stimulated both keratinocytes and fibroblasts to produce PO.

  By *in vitro* experiments, following 6 and 24hr treatment of rcIL-13, expression of PO mRNA

in keratinocytes was significant increased compared with unstimulated cells (Figure 11a).

Following 24hr treatment of rcIL-13, expression of PO mRNA in dermal fibroblasts was

significant increased compared with unstimulated cells (Figure 11b).

PO enhanced in vitro growth of canine keratinocyte cell.

  The effect of PO on growth of keratinocytes was evaluated using CPEK. *In vitro* growth of

CPEK was enhanced by 4μg/ml of PO treatment (Figure 12).

PO induced gene expression of IL-25 and KDAP of canine keratinocyte cell.

  The microarray data discussed in this article have been deposited in NCBIs Gene Expression

Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series

accession number GSE77041. Among these genes, the author focused on IL-25 and KDAP,

because previous studies reported that these genes were shown to be overexpressed in chronic

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allergic dermatitis.[9, 30]

For validation of microarray results, the author performed real time quantitative RT-PCR for the IL-25 and KDAP. Following 36hr treatment with rhPO, expression of IL-25 mRNA in keratinocytes was significant increased compared with an unstimulated cells (Figures 13). Following 2hr treatment with rhPO, expression of KDAP mRNA in keratinocytes was significant increased compared with an unstimulated cells (Figures 14).

Discussion

Previous studies have shown that cAD is associated with overproduction of Th2 cytokines such as IL-13.[18, 21, 23] By double-labeled ISH, IL-13 mRNA positive cells suspected as lymphocytes were detected near the keratinocytes and dermal fibroblasts expressing PO mRNA in canine atopic skin. This indicates close relationship between these two types of cells in canine atopic skin. These results strongly suggest that IL-13 induce PO production in keratinocytes and dermal fibroblasts in canine atopic skin independently of TGF-β1. To the best of my knowledge, this paper was the first report indicating the relationship between PO and IL-13 in spontaneous atopic skin.

The author revealed a part of functional roles of PO to induce the enhancement and chronicity in canine atopic skin by *in vitro* experiments. Firstly, the author confirmed that rhPO enhanced *in vitro* growth of canine keratinocyte cell. Moreover, among the PO-induced genes detected by

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microarray, the author firstly identified IL-25 and KDAP as possible mediators in spontaneous

atopic skin. Because, qRT-PCR analysis revealed up-regulation of both IL-25 and KDAP gene

expression in PO-stimulated keratinocytes compared to unstimulated control.

IL-25 (also known as IL-17E) is important for Th2-mediated immunity in a murine model of

asthma, and induces the impaired skin barrier observed in AD patients.[5, 9, 28] The level of

IL-25 mRNA has been revealed to be elevated in the lungs of asthmatic patients and in the skins

of hAD patients).[28] Furthermore, compared to PO wild-type mice, PO null mice showed

amelioration of in HDM-induced inflammation and mucous metaplasia, as well as reduced Th2

cytokines including IL-25 mRNA expression.[2] Together with these previous studies, the

author suspects that PO enhances Th2- immunoreaction via IL-25 signaling in canine atopic

skin.

PO has been reported to play a role to enhance proliferation and differentiation of

keratinocytes by *in vitro* and *in vivo* experiments. Deficiency or blockage of PO suppressed

acanthosis by transdermal treatment with HDM extract in atopic model mice. In the present

study, for validation of microarray results, we performed quantitative RT-PCR analysis, and

found temporary up-regulation of keratinocyte differentiation-associated protein (KDAP) gene

expression in rPO-stimulated keratinocytes compared to unstimulated control at 2 hr after

sensitization (Supplemental material 2). KDAP (encoded by *KRTDAP*) is a recently identified

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secretory protein that may play as a soluble regulator for the differentiation of keratinocytes.

Yagihara et al.[30] reported that KDAP was more widely spread in the spinous layers of the

epidermis in chronic allergic dermatitis compared to normal skin in dogs. Acanthosis is a typical

histopathological features of hAD and cAD. We suspected that KDAP induced by PO may play

a role in acanthosis of canine atopic skins.

  Despite treatment with inhaled glucocorticoids or cyclosporin, several dogs of cAD continue to

have uncontrolled skin lesion that requires more intensive therapy.[17] PO may contribute to the

enhancement and chronicity of allergic skin inflammation by activating keratinocytes and

dermal fibroblasts in the absence of environmental allergens as shown in the study. Corren et

al.[3] examined patients with poorly controlled asthma treated with lebrikizumab, which is a

monoclonal antibody to IL-13. Patients with high pretreatment levels of serum PO had greater

clinical improvement than patients with low PO levels. Furthermore, the serum levels of PO

were significantly elevated in hAD patients compared with controls.[14] These facts suggest

close relationship between IL-13 and PO in hAD.

  The author suspects that PO may play an important role in the pathophysiology of cAD. The

data in this study suggest that IL-13 possibly derived from Th2 cells stimulates PO production

in both keratinocytes and fibroblasts, and then PO may contribute to the enhancement and

chronicity of cAD via IL-25 and KDAP (Figure 15). The findings of the present study provide a

molecular basis for considering AD. Based on the results of this study, further *in vivo* studies

using experimental atopic dogs may provide novel therapeutic targets focusing on PO, IL-13

and IL-25 for AD.

Figures and Tables
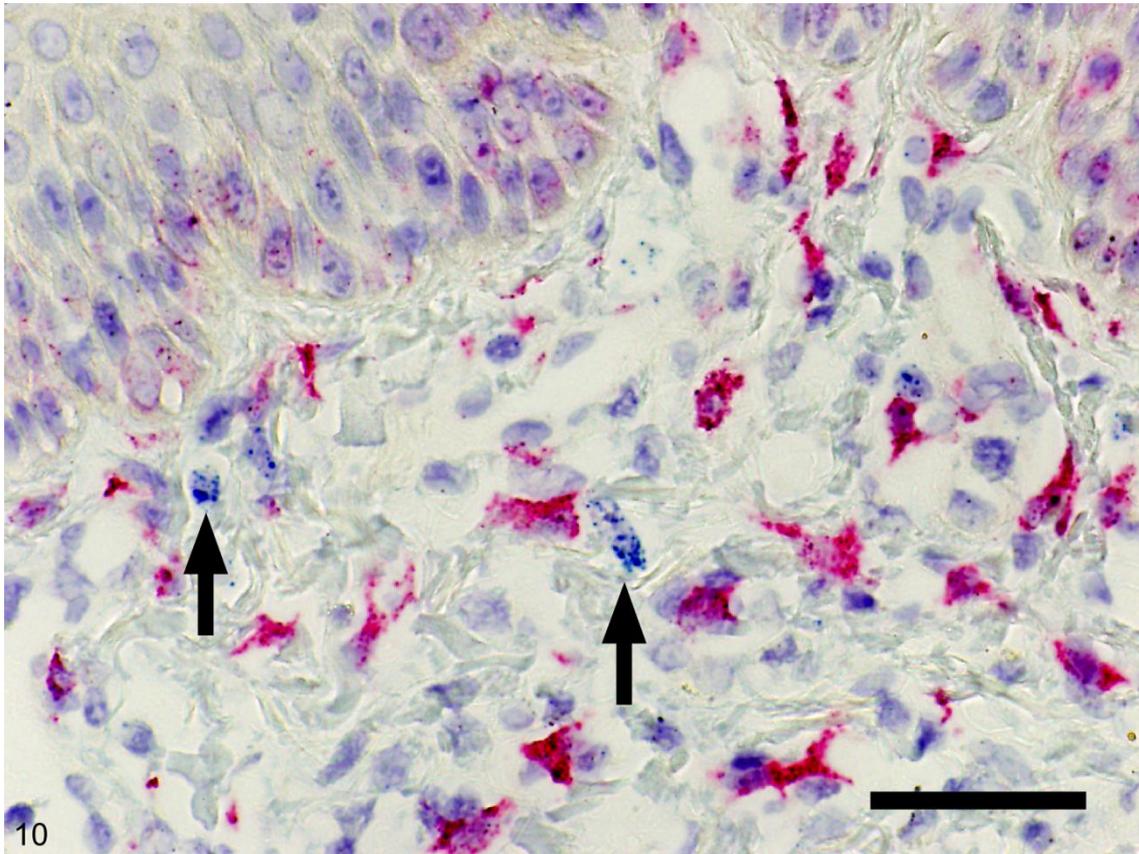
Figure 10. Atopic dermatitis, skin, dog, case No. 5. The interleukin (IL)-13 mRNA-positive cells

(arrows) are detected around the PO mRNA-positive cells in the atopic skin. Double-ISH for PO and

IL-13. Bar = 50 μm.

Keratinocyte

11a

Fibroblast

11b
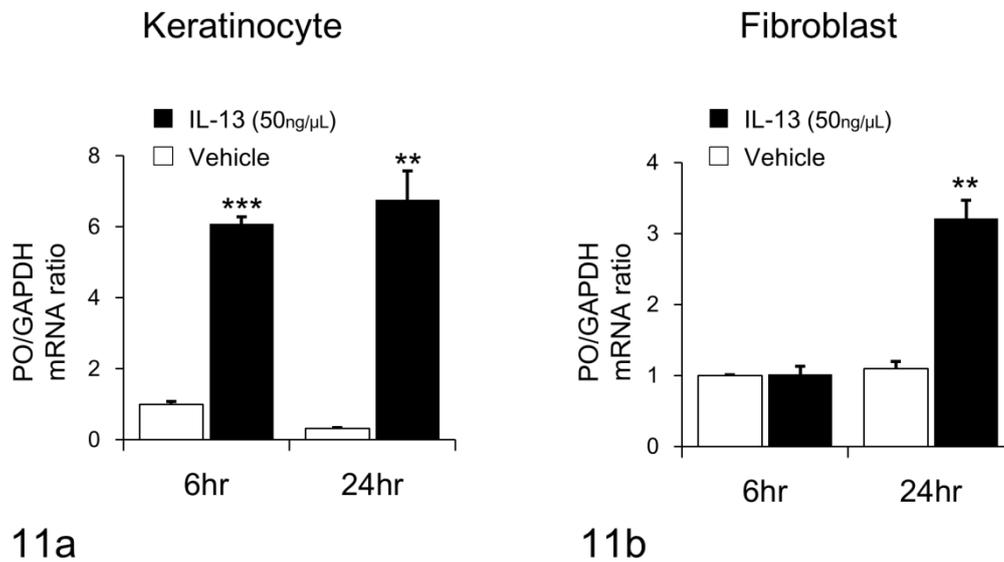
Figure 11. Recombinant IL-13 induced expression of PO mRNA in both canine dermal fibroblasts and keratinocytes. Quantitative analysis of mRNA levels of PO in the cultured keratinocytes and dermal fibroblasts at the indicated time 6 and 24 hrs after sensitization with IL-13. **a.** Following both 6 and 24hrs of IL-13 treatment, PO mRNA expression in keratinocytes was significant increased compared with vehicle control. **b.** Following 24hr of IL-13 treatment, PO mRNA expression in dermal fibroblasts was significant increased compared with vehicle control. Real time quantitative RT-PCR (**P < 0.01, ***P < 0.001; vs vehicle control; unpaired t-test).
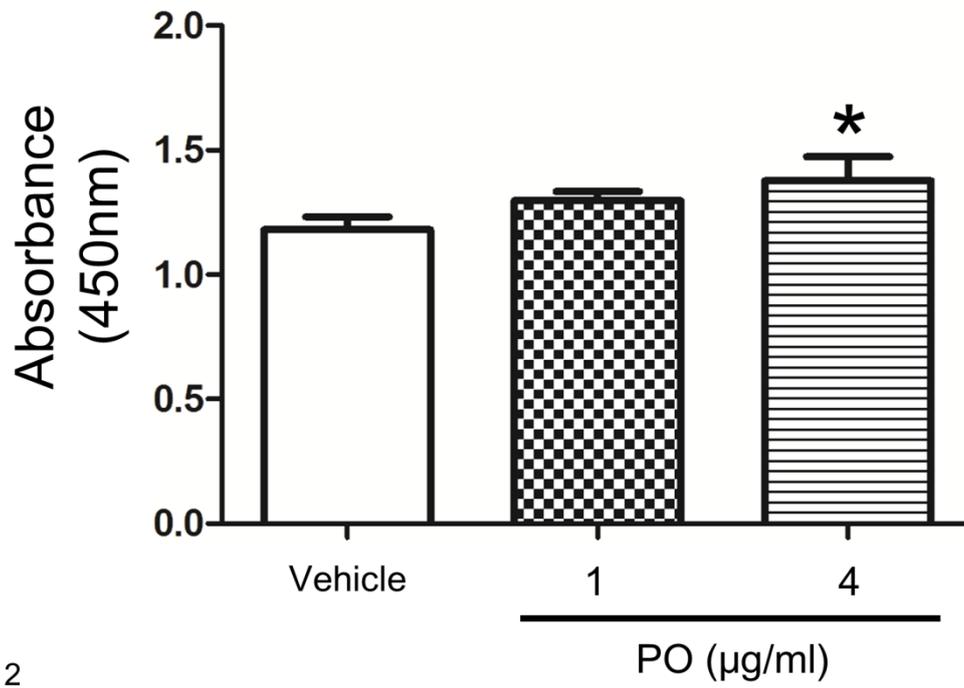
Figure 12. Proliferation assay of canine keratinocyte cell line (CPEK) with or without recombinant PO. *In vitro* growth of CPEK was enhanced by 4μg/ml of PO treatment (*P < 0.05, vs vehicle control; unpaired t-test).
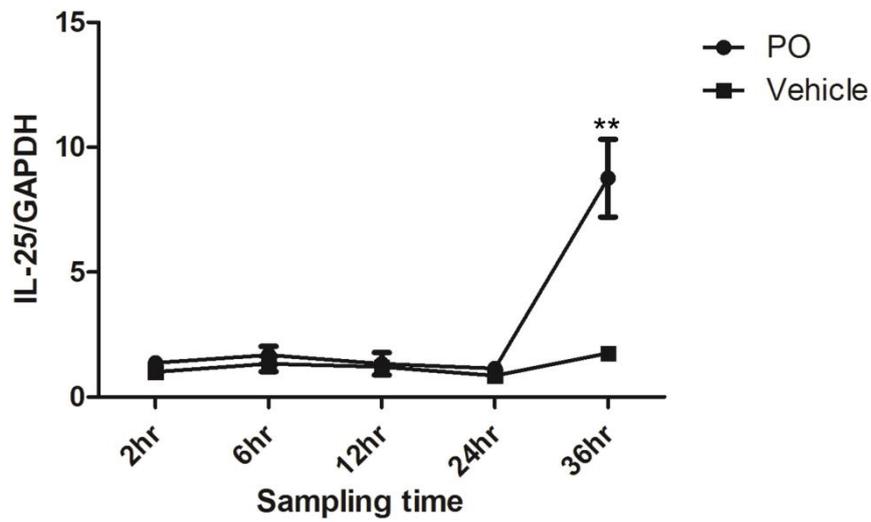
Figure 13. Recombinant PO induced expression of IL-25 mRNA in canine keratinocytes.

Quantitative analysis of mRNA levels of PO in the cultured keratinocytes at the indicated time 2, 6, 12, 24 and 36 hrs after sensitization with PO. Following 36 hrs of PO treatment, IL-25 mRNA expression in keratinocytes was significant increased compared with vehicle control. Real time quantitative RT-PCR (**$P < 0.01$; vs vehicle control; unpaired t-test).

50
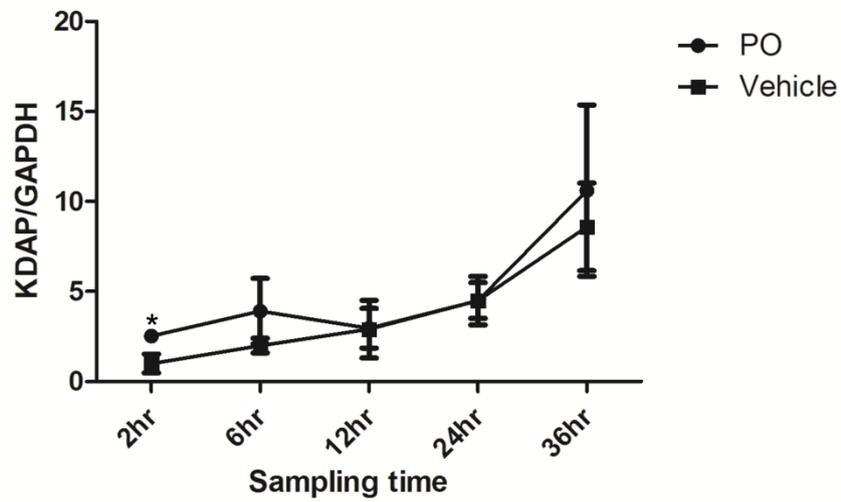
14

Figure 14. Recombinant IL-PO induced expression of KDAP mRNA in canine keratinocytes.

Quantitative analysis of mRNA levels of PO in the cultured keratinocytes and dermal fibroblasts at

the indicated time 2, 6, 12, 24 and 36 hrs after sensitization with PO. Following 2hrs of PO treatment,

KDAP mRNA expression in keratinocytes was significant increased compared with vehicle control.

Real time quantitative RT-PCR (*$P < 0.05$; vs vehicle control; unpaired t-test).
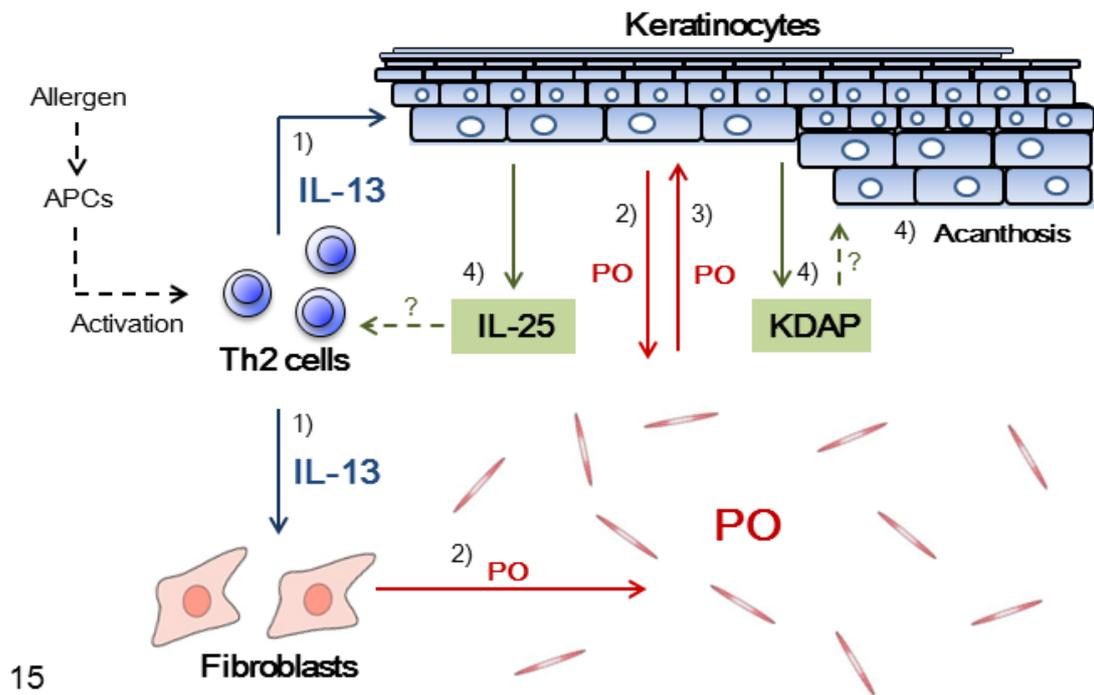
Figure 15. The schema indicates hypothesis on the chronicity of inflammation via PO in atopic skin. The data in this study suggest that IL-13[1] possibly derived from Th2 cells stimulates PO production[2] in both keratinocytes and fibroblasts, and then PO[3] may be involved a critical role in the enhancement and chronicity of cAD via IL-25 and KDAP[4]. APCs, antigen-presenting cells.

Conclusion

  The author proposed a novel hypothesis that PO contributes to enhancement and chronicity of

allergic skin inflammation in canine atopic skin. The new findings on cAD from this study are

summarized as follows.

1. Canine atopic skin characteristically showed intense expression of PO in the superficial

dermis. The severity score of chronic pathological changes and CD3+ cell number in the dermis

were correlated with distribution pattern of PO in the canine atopic skin. These results indicated

that PO could play a role in the pathophysiology of cAD.

2. ISH showed that fibroblasts and keratinocytes were the main source of PO in cAD. To the

best of our knowledge, to date, no reports have focused on PO production by keratinocytes in

both human and canine atopic skin.

3. By double-labeled ISH, IL-13 mRNA positive cells were detected near the keratinocytes and

fibroblasts expressing PO mRNA in the dermis of atopic skin. TGF-β1 mRNA positive cell was

not seen in all atopic skins. By *in vitro* assay, IL-13 induced the gene expression of PO in both

canine dermal fibroblasts and keratinocytes. These results indicated that IL-13 induce PO

production in keratinocytes and dermal fibroblasts in canine atopic skin independently of

TGF-β1.

4. PO enhanced *in vitro* growth of canine keratinocytes, suggesting that PO could be an

important factor of pathogenesis of acanthosis in cAD.

5. PO induced gene expression of IL-25 and KDAP of canine keratinocyte cell. The author

suggested IL-25 and KDAP as possible mediators in canine atopic skin.

  In summary, the author proposes that IL-13 possibly derived from Th2 cells stimulates PO

production in both keratinocytes and fibroblasts, and then PO may play a critical role in the

pathophysiology of cAD, especially in the enhancement and chronicity of skin lesion via IL-25

and KDAP. The findings of the present study provide a molecular basis for considering PO and

IL-13 as a potential therapeutic targets for cAD.

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