A study on periostin involvement in the pathophysiology of canine atopic skin

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ABSTRACT. Atopic dermatitis (AD) is a chronic, pruritic, and allergic skin disease in humans and animals, particularly dogs. Canine AD (cAD) has received attention as a spontaneous animal model of human AD (hAD) because domesticated dogs inhabit a human environment, and cAD shares several clinicopathological features with human AD, including age of onset, skin areas affected, severe pruritus and the immunopathological mechanism [13, 16, 22]. Histologically, the skin lesions in hAD and cAD are characterized by spongiotic and hyperplastic dermatitis with mononuclear infiltrate composed of T lymphocytes [5, 17, 22]. The pathogenesis of both hAD and cAD has been characterized by skin barrier damage and allergic inflammation with T helper 2 (Th2) immune response caused by the release of Th2 cytokines such as interleukin (IL)-4 and IL-13, particularly in the acute lesions [19, 21, 22]. In AD, once allergic inflammation is triggered by exposure to allergens, skin lesions chronically persist without continuous allergen stimulation [14]. However, the mechanisms underlying chronicity in allergic inflammation and the associated skin reaction are unknown.

Periostin (PO) is a matricellular protein that belongs to the fasciclin family [16, 20, 29]. PO was first described as osteoblast-specific factor 2 (OSF-2) in 1993 as a homophilic adhesion molecule in osteogenesis [24]. PO modulates cell function by binding to αvβ3, αvβ5 and αvβ1 integrin molecules on the cell surface; thus, PO provides signals for tissue development, proliferation and remodeling [9, 20, 29, 30]. PO production is induced by transforming growth factor (TGF)-β, IL-4 and IL-13, which are major cytokines of the Th2-type immune response in bronchial asthma, suggesting PO involvement in allergic inflammation [10, 14, 29]. Recently, Masuoka et al. [14] reported that a deficiency of PO suppressed allergic inflammation induced by treatment with house dust mite extract in atopic model mice. They further indicated the binding of PO to α5 integrin on mouse keratinocytes in vitro, which resulted in the induction of proinflammatory cytokines, including thymic stromal lymphopoietin (TSLP) from the keratinocytes [14]. Thus, PO fulfills important roles in the enhancement and chronicity of allergic skin inflammation in hAD and experimental atopic models.

We have previously reported that PO expression was more intense in the canine atopic dermis and correlated with the severity of
chronic histopathological lesions (epidermal thickening and fibrosis) and CD3+ cell number in the dermis, similar to those reported in hAD patients [15]. In the article, in situ hybridization (ISH) showed that fibroblasts and keratinocytes were the main source of PO in cAD [15]. These results suggested that PO may be involved in the pathogenesis of chronicity in cAD.

In the present study, we attempted to clarify PO involvement in canine atopic skin using skin tissues of cAD and cultured canine keratinocytes and dermal fibroblasts. First, we focused on IL-13 and TGF-β1 which were assumed to be inducing factors of PO. Reportedly, TGF-β is a major trigger of PO production from both dermal and pulmonary fibroblasts [23, 30]. However, Masuoka et al. [14] recently reported that IL-13-induced PO production independently of TGF-β in the skin of a murine atopic model.

Further, we examined the effects of PO in cultured canine keratinocytes using a proliferation assay, microarray and real time quantitative reverse transcription polymerase chain reaction (RT-PCR). The keratinocytes act as immune cells and a mechanical barrier between the external environment and body [3, 19]. 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 [12]. From our previous results [15], we assumed that PO may involve in epidermal thickening and the infiltration of inflammatory cells, which are major pathologic features in atopic skin.

Finally, we discussed possible PO involvement in the pathophysiology of cAD. The findings of the present study may provide a molecular basis of PO for further understanding cAD as a model of hAD and for considering PO as a potential therapeutic target for hAD and cAD.

MATERIALS AND METHODS

Skin samples

The diagnosis of cAD was based on the clinical criteria proposed by Terada et al [25]. A total of five biopsies taken from the four atopic dogs were used for immunohistochemistry (IHC) and ISH. Their ages ranged from five to 13 years, and the cohort included three males and one female.

The skin tissues obtained from the chest regions of five normal beagles (two males and three females, aged 1–7 years) were used as control for IHC and ISH, and canine primary dermal fibroblasts were derived from three 12 month-old male beagles. These dogs were purchased from a laboratory animal breeding and supply company (Kitayama Labes Co., Ltd., Ina, Japan) and were confirmed to be healthy by physical examination. They were used for clinical education and euthanized in accordance with the guidelines approved by the Animal Research Committee of Azabu University (No.100408-3). Skin specimens were obtained from the dogs immediately after euthanasia.

Skin specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut, and stained with hematoxylin and eosin (HE). The paraffin sections were provided for IHC and ISH.

Immunohistochemistry

IHC for PO using an immunoenzyme polymer method was performed as previously described [15].

Double-in situ hybridization

Double-ISH for PO and IL-13/ TGF-β1 was performed using the QuantiGene® ViewRNA ISH Tissue Assay (Affymetrix, Santa Clara, CA, U.S.A.) according to the manufacturer’s protocol, as described previously [15]. Type 1/Fast Red was used for PO probes and Type 6/Fast Blue was applied for IL-13 and TGF-β1. Double-ISH sections were counterstained with Mayer’s hematoxylin. To identify the expressing IL-13 mRNA on CD3 positive cells, serial sections were additionally subjected to IHC for CD3 (rabbit anti–human CD3; Dako Denmark A/S., Glostrup, Denmark) as previously described [15]. A ISH reaction solution without the probes was used as a negative control, according to the manufacturer’s recommendations.

Culture of canine keratinocytes and dermal fibroblasts

Two types of culture cells, commercially available canine keratinocyte cell line (CPEK, CELLnTEC Advanced Cell Systems, Bern, Switzerland) and primary canine dermal fibroblasts, were used in the present study.

CPEK was cultured in 25 cm² flasks (AGC Techno Glass Co., Ltd., Haibara, 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, U.S.A.) 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 for the human dermal fibroblasts [1]. Briefly, full-depth skin samples obtained from the chest region of healthy dogs were immediately placed in phosphate buffered saline (PBS; pH 7.2, 0.01 M) with 1% Antibiotic Antimycotic. Skin samples were rinsed seven times with PBS and fragmented into 5 mm² pieces. These skin fragments were placed on a surface of 60 × 15 mm Petri dishes with Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/ F12, 1:1; Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) containing of 10% FBS and 1% Antibiotic Antimycotic. Cultures were incubated at 37°C in a humidified incubator with 5% CO2. The culture medium was changed every three days. Satisfactory proliferation of fibroblasts was observed by approximately 20 days. Fibroblasts of the 3rd–5th passages were used for the experiments.
**Qualitative RT-PCR**

RT-PCR was performed to confirm the expression of the interleukin 13 receptor alpha 1 and αv integrin gene in CPEK and canine dermal fibroblasts. Total RNA was extracted from CPEK and canine dermal fibroblasts using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). Extracted total RNA was used in RT-PCR using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen Life Technologies).

Primer sequences were obtained by determining the predicted mRNA sequences of *Canis lupus familiaris* interleukin 13 receptor, alpha 1 (*IL13RA1*) (NCBI database accession number: XM_538150.5) and *C. lupus familiaris* integrin, alpha V (*ITGAV*) (NCBI database accession number: XM_014110784.1). The primer sequences were as follows: canine IL13RA1 forward, 5′GGAGACTCCTCTTACCATTTG3′; and reverse, 5′CTCATAACCAGTTGTGACTGAAGGG3′, and canine ITGAV forward, 5′GATAGAGCTGTGTTATACAGAGCCAGAC3′; and reverse, 5′CAGATAAGCTACTGTTCCTCACACTGC3′. The PCR reaction with KOD FX Neo (Toyobo, Osaka, Japan) was performed as follows: denaturation at 94°C for 2 min; 35 cycles of denaturation at 98°C for 10 sec, annealing at 65°C for 30 sec, and extension at 68°C for 30 sec; and a final extension step at 68°C for 1 min. The amplification products were separated on 2% agarose gels and stained with Midori Green DNA Stain (NIPPON Genetics Co., Ltd., Tokyo, Japan).

**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 24 hr.

For real time quantitative RT-PCR to evaluate PO mRNA expression, CPEK and fibroblasts were cultured in DMEM/F12 with or without 50 ng/µl recombinant canine IL-13 (rIL-13: R&D Systems, Minneapolis, MN, U.S.A.) for 6 or 24 hr. CPEK were cultured in DMEM/F12 with or without 2 µg/ml recombinant human PO (rPO: R&D Systems) for 2, 6, 12, 24 or 36 hr.

After stimulation by rIL-13 or rPO, 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 (Applied Biosystems, Foster city, CA, U.S.A.), TaqMan Universal PCR Master Mix (Applied Biosystems), and a TaqMan MGB probe for the target genes: PO (Assay ID: Cf02680558_m1; Applied Biosystems), IL-25 (Assay ID, Cf02643291_m1; Applied Biosystems) and keratinocyte differentiation-associated protein (KDAP) (assay ID, Cf02627160_m1; Applied Biosystems) according to the manufacturer’s protocol. 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to the ΔΔCT method.

**Proliferation assay**

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

**Microarray**

For DNA microarray analysis, CPEK was cultured with or without 4 µg/ml rPO for 6 or 24 hr. After stimulation by rPO, total RNA was extracted from cell cultures using the RNeasy Plus Micro Kit. The microarray analyses were performed by DNA Chip Research Inc. (Yokohama, Japan) using the Canine (V2) Gene Expression Microarray (4 × 44k, two-color array; Agilent Technologies, Palo Alto, CA, U.S.A.) 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 PO-stimulated and vehicle control samples.

**Statistical analyses**

Statistical analyses of results of real time quantitative RT-PCR and proliferation assays were performed using GraphPad Prism (ver. 5.0, GraphPad Software, La Jolla, CA, U.S.A.). Comparisons of mRNA between extracted samples from stimulated and unstimulated cells were performed by the unpaired T test. *P*<0.05 was considered to be statistically significant.

**RESULTS**

**PO protein highly deposited in canine atopic dermis**

A small amount of PO protein was localized in the healthy canine dermis at the perifollicular area and exactly beneath the basal layer (Fig. 1a). PO was highly deposited in the canine atopic dermis (Fig. 1b).

**IL-13 mRNA-positive cells were present near PO mRNA-positive cells**

Using Double-ISH, IL-13 mRNA-positive small round cells were detected near PO mRNA-positive keratinocytes and fibroblasts in the dermis of atopic skin (Fig. 2a). Infiltration of IL-13 mRNA-positive cells in the epidermis was also detected in the atopic skin (Fig. 2b), which was consistent with that in CD3-positive cells (data not shown). No TGF-β1 mRNA-positive cells were observed in atopic skins. No signals were detected in the hybridized tissues using the ISH solution without the probe.
IL13RA1 and ITGAV gene expressed in canine keratinocytes and dermal fibroblasts

The mRNAs encoding IL13RA1 and ITGAV expressed in both CPEK and cultured canine dermal fibroblast (Fig. 3a and 3b).

IL-13 stimulated both keratinocytes and fibroblasts to produce PO

Following 6 and 24 hr of treatment with rIL-13, PO mRNA expression in keratinocytes was significantly increased compared with the vehicle control (Fig. 4a). Following-24 hr treatment with rIL-13, PO mRNA expression in dermal fibroblasts was significantly increased compared with the vehicle control (Fig. 4b).

rPO enhanced in vitro growth of canine keratinocytes

The effect of rPO on the growth of keratinocytes was evaluated using CPEK. In vitro growth of CPEK was significantly enhanced by 4 µg/ml of PO (Fig. 5).
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rPO-induced IL-25 and KDAP genes expression of canine keratinocyte cells

The microarray data discussed in the present study have been deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) of the National Center for Biotechnology Information (NCBI) and are accessible through GEO series accession number GSE77041. Among the genes induced by rPO, we focused on IL-25 and KDAP because previous studies reported these genes to be overexpressed in chronic allergic dermatitis [11, 26]. For validation of microarray results, we performed real time quantitative RT-PCR for the IL-25 and KDAP. Following 36 hr-treatment with rPO, IL-25 mRNA expression in CPEK was significantly increased compared with the vehicle control (Fig. 6).
Following 2 hr of rPO treatment, KDAP mRNA expression in keratinocytes was significantly increased as compared with the vehicle control (Fig. 7).

**DISCUSSION**

Here we revealed that IL-13 mRNA-positive cells suspected of being T-lymphocytes located near the keratinocytes and dermal fibroblasts expressing PO mRNA in canine atopic skin. This indicates a close correlation between IL-13 and PO mRNA-positive cells in canine atopic skin. In addition, we confirmed IL13RA1 gene expression in CPEK and cultured canine dermal fibroblasts, and rIL-13-induced PO gene expression in these culture cells. And no TGF-β1 mRNA-positive cells were observed in canine atopic skins. These results strongly suggest that IL-13 induces PO production in keratinocytes and dermal fibroblasts in canine atopic skin independent of TGF-β1. To the best of our knowledge, the present study is the first to indicate the close correlation between PO and IL-13 in spontaneous atopic skin. Corren et al. [4] reported that asthmatic patients with high pretreatment levels of serum.
PO had greater clinical improvement of lung function with administration of lebrikizumab, a monoclonal antibody to IL-13, than patients with low serum PO levels. Furthermore, serum PO levels were significantly elevated in hAD patients compared with healthy humans, suggesting a close correlation between IL-13 and PO in AD [14].

We revealed a subset of the functional roles of PO involved in the enhancement and chronicity of the pathological lesions in canine atopic skin by *in vitro* experiments. In accordance with previous reports [6, 7, 14], we set the concentration of PO at 1–4 µg/ml. First, we confirmed that rPO enhanced *in vitro* growth of canine keratinocytes. In addition, we showed gene expression of integrin αν, which was a receptor of PO, in both cultured canine keratinocytes and dermal fibroblasts. Moreover, among the PO-induced genes detected by microarrays, we first identified IL-25 as a possible mediator in spontaneous atopic skin because quantitative RT-PCR analysis revealed upregulation of IL-25 gene expression in PO-stimulated keratinocytes compared with that in the vehicle control.

IL-25 (also termed IL-17E) is important for Th2-mediated immunity in a murine model of asthma, and impairs the skin barrier in AD patients [8, 11, 27]. The IL-25 mRNA level is elevated in the lungs of asthmatic patients and in the skin of hAD patients [27]. Furthermore, PO null mice showed amelioration of allergic airway inflammation and mucous metaplasia and reduction in Th2 cytokines mRNA expression including IL-25 by a house dust mite (HDM)-challenge compared with PO wild-type mice [2]. Collectively, we suspect that PO enhances Th2-immunoreaction via IL-25 signaling in canine atopic skin.

PO has been reported to play a role in enhancement of the proliferation and differentiation of keratinocytes using *in vitro* and *in vivo* experiments. A deficiency or blockage of PO suppressed acanthosis induced by transdermal treatment with HDM extract in atopic model mice [14]. In the present study, for validation of microarray results, we performed quantitative RT-PCR analysis and found temporal upregulation of KDAP gene expression in rPO-stimulated keratinocytes compared with the vehicle control at 2 hr after sensitization. KDAP (encoded by *KRTDAP*) is a recently identified secretory protein that may act as a soluble regulator for the differentiation of keratinocytes [26, 28]. Yagihara *et al.* [28] reported that KDAP was more widely spread in the spinous layer of the epidermis in chronic allergic dermatitis compared with healthy skin in dogs. Acanthosis is a typical histopathological feature of hAD and cAD [14, 17]. Therefore, KDAP induced by PO may play a role in acanthosis of canine atopic skins.

The data in the present study 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 enhancement and chronicity of cAD via IL-25 and KDAP (Fig. 8). Despite treatment with inhaled glucocorticoids, some atopic patients and dogs develop uncontrolled skin lesions that require more intensive therapy [14, 18]. PO may contribute to the enhancement and chronicity of atopic skin inflammation by activating keratinocytes and dermal fibroblasts in the absence of environmental allergens in cAD and hAD.

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