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Original Article

Modulatory effects of PG102 (*Actinidia arguta*) on inflammatory cytokine mRNA expression in canine atopic dermatitis

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Abstract

Canine atopic dermatitis (AD) is a common, chronic relapsing inflammatory skin disease. An aqueous extract, PG102, derived from *Actinidia arguta* fruit, has been investigated as an anti-inflammatory agent in humans and atopy-like animal models, but its underlying mechanism for canine AD treatment has not been fully understood. This study evaluated the *in vitro* anti-inflammatory effect of PG102 with respect to the representative allergy-related cytokine panel from dust mite allergen extract (DME)-stimulated peripheral blood mononuclear cells (PBMCs) of canine AD patients using quantitative real-time PCR. PBMCs were isolated from eight dogs with definite diagnoses of AD and stimulated with DME for 12 hours in the presence of PG102 (0.1, 1, 10 and 100 μg/ml). The PG102 treatment effectively inhibited the DME-induced TNF-α, IL-4, IL-5, IL-13 and IFN-γ mRNA expression. However, no significant changes in the mRNA expression levels of IL-10 and TGF-β1 were found in the present results. These data suggest that PG102 might be a promising candidate for canine AD therapeutic agent.

Keywords: *Actinidia arguta*, canine atopic dermatitis, cytokine, PBMCs

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Introduction

Canine atopic dermatitis (AD) is a genetically predisposed, chronic relapsing inflammatory skin disease with characteristic clinical features associated with IgE antibodies against environmental allergens (Nambi and Kavitha, 2013). Numerous clinical and immunological similarities exist between canine AD and its human counterpart (Marsella and Girolomoni, 2009). Although the pathogenesis of AD is not fully understood, a complex of immune dysregulation is believed to be associated with increased skin inflammation (Gittler et al., 2013). Recent interest has focused on the T-lymphocyte function abnormalities in AD. Overexpressed Th2 type cytokines such as IL-4 support humoral immunity and IgE production, whereas Th1 cytokines such as IFN-γ promote cell-mediated immunity in canine AD (Nuttall et al., 2002a). TNF-α, a pro-inflammatory cytokine, is also associated in the pathogenesis of canine AD (Maeda et al., 2002). Regulatory cytokines including IL-10 expression by antigen presenting cells (APCs) and T cells have recently been demonstrated to play a key role in Th2 cell development in a murine model of AD (Laouini et al., 2003) as well as that of canine AD (Keppel et al., 2008). These dysregulations of immune complex of AD make it a challenging disease to control.

Due to the chronically relapsing characteristics of canine AD and the unwanted side effect of systemic glucocorticoids, which are mostly used for symptomatic treatment of canine AD, a need for effective steroid sparing agents for controlling canine AD exists. PG102 is a water-soluble extract prepared from the fruit of *Actinidia arguta*, commonly referred to as hardy kiwifruit. Several studies investigated PG102 as an anti-inflammatory agent in human and atopy-like mouse models. Previous studies showed that PG102 improved AD symptoms by decreasing plasma serum IgE level and Th2 cytokine production in murine AD model (Park et al., 2005; Park et al., 2007). Recently, a veterinary clinical trial has shown that PG102 effectively improved clinical symptoms of canine AD and no significant adverse effects were noted (Ho et al., 2009; Marsella et al., 2010). However, the underlying immunological mechanism of PG102 for canine AD treatment has not been fully studied.

The present study evaluated the immune-modulatory effect of PG102 with respect to the representative allergy-related cytokine panels such as proinflammatory cytokines (TNF-α), Th1 cytokines (IFN-γ), Th2 cytokines (IL-4, IL-5 and IL-13), and regulatory cytokines (IL-10 and TGF-β1) from DME-stimulated peripheral blood mononuclear cells (PBMCs) of canine AD patients. The aim of this study was to evaluate the scientific rationale for the anti-allergic medical use of PG102 for the treatment of canine AD.

Materials and Methods

Preparation of PG102: PG102 reagent, extracted from *Actinidia arguta* as described previously (Park et al., 2007), was kindly provided by Viromed Co., Ltd. (Seoul, Korea). The original reagent was composed of 48.4% PG102 and 51.6% water, respectively.

Patient populations: Eight dogs with AD (4 females and 4 males) were used for the study. Mean age was 9.18 ± 2 years. All of the males and two of the females had been neutered. The diagnosis of canine AD included a combination of compatible history and clinical signs of canine AD (Olivry, 2010). The selected patient populations were all sensitive to DME, as determined by a serologic allergen specific IgE test and an intradermal skin test. Glucocorticoids and antihistamines were withdrawn at least 2 weeks prior to the intradermal skin test and blood collection. All dogs had no diseases other than their skin problems.

All experiments were approved by the Institute of Laboratory Animal Resource of Seoul National University (grant number: SNU-100331-1).

PBMC culture: PBMCs were isolated using Ficoll-Hypaque (Sigma, St. Louis, MO) density-gradient centrifugation. PBMCs with final concentration of 5 x 10^6 cell/ml were used. The negative control group (unstimulated condition) contained only PBMCs, whereas 10 μg/ml of DME was added to the stimulated samples. The treatment group contained PG102 at concentration of 0.1-100 μg/ml plus DME in each sample well. Dexamethasone (10^{-8} M) treated group was used as positive control. The plates were incubated for 12 hours at 37°C. In each experiment, triplicate cultures were established for all conditions.

Measurement of cytokine mRNA expression of PBMCs by real-time RT-PCR: Total RNA from cultured PBMCs was extracted by using a commercially available kit (RNeasy Mini kit, Qiagen, Crawley, UK) and cDNA was reverse-transcribed by a Primescript™ 1st strand cDNA Synthesis Kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Transcriptions of cytokine mRNA were quantified by real-time PCR (StepOne, Applied Biosystems, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) for detection according to the manufacturer's instructions. For each of the target genes, canine β-actin, IL-4, IL-5, IL-10, IL-13, TGF-β1, IFN-γ and TNF-α, a pair of oligonucleotide primers was designed based on previously published studies (Fujiwara et al., 2003). Sequences of the primer are shown in Table 1. Amplification conditions were identical for all reactions: denaturation for 10 min at 95°C, 45 cycles of 15 s at 95°C, and 60 s at 60°C. Amplification of a specific product was verified by conducting a melting point analysis after the last cycle by cooling samples to 55°C and then increasing the temperature to 95°C at 0.2°C/sec. Relative expressions of the target cytokine genes were calculated using a comparative CT method previously described (Schmittgen and Livak, 2008). Briefly, the relative transcription of the target cytokines was reported as n-fold difference relative to transcription of the calibrator gene (β-actin) and compared to the unstimulated samples, which were the control group. The amounts of cytokine mRNA were calculated by the 2-ΔΔCT method, (ΔΔCT = (CT Cytokine-CT β-actin) sample - (CT Cytokine-CT β-actin) control), and expressed as n-

fold differences. All samples were examined in triplicate and mean value of ΔΔCT was calculated.

Statistical analysis: Data were expressed as mean ± SEM, and differences between mean values of cytokine mRNA expression levels were analyzed by the unpaired Student *t* test and analysis of variance (ANOVA). *P* values at $P \leq 0.05$ were considered statistically significant

Table 1 Primer sequences and product sizes used for quantitative real-time PCR detection of target cytokines

Target gene	Primer	Sequence (5'-3')	Locus	GenBank accession number	Product size (bp)
β-actin	Forward primer	GACCCTGAAGTACCCCATTGAG	131-152	Z70044	81
	Reverse primer	TTGTAGAAGGTGTGGTGCCAGAT	189-211		
IL-4	Forward primer	CATCCTCACAGCGAGAAACG	117-136	AF054833	83
	Reverse primer	CCTTATCGCTTGTGTTCTTTGGA	177-199		
IL-5	Forward primer	GACTGGTGGCAGAGACCTTGA	110-131	403790	159
	Reverse primer	CGTGGGCAGTTTGGTTCTTC	249-269		
IL-10	Forward primer	CGCTGTCACCGATTTCTTCC	383-402	U33843	78
	Reverse primer	CTGGAGCTTACTAAATGCGCTCT	438-460		
IL-13	Forward primer	GAATCAGGCATCCCTCTGCA	175-195	AF244915	68
	Reverse primer	ATGCCGGCGGTCAGGT	227-243		
TNF-α	Forward primer	GAGCCGACGTGCCAATG	97-113	Z70046	79
	Reverse primer	CAACCCATCTGACGGCACTA	156-175		
TGF-β_1	Forward primer	CAGAATGGCTGTCCTTTGATGTC	635-657	L34956	79
	Reverse primer	AGGCGAAAGCCCTCGACTT	695-713		
IFN-γ	Forward primer	GCGCAAGGCGATAAATGAAC	336-355	AF126247	82
	Reverse primer	CTGACTCCTTTTCCGCTTCCT	397-417		

Results

PG102 suppressed DME-induced pro-inflammatory cytokine: The mRNA expression of TNF-α was observed for changes in pro-inflammatory cytokine. The PG102 treated group showed significant reduction in DME-induced TNF-α production, from 41.0% to 55.8% (Figure 1).

PG102 suppressed DME-induced Th1/Th2 cytokine mRNA expression: IFN-γ mRNA expression was analyzed for Th1 cytokine changes (Figure 2a). In the PG102 treated group, the DME-induced IFN-γ expression was significantly decreased, however, compared to the representative glucocorticoid effect (positive group), PG102 showed less suppressive effect on the amount of Th1 cytokine transcription. The expressions of IL-4, IL-5 and IL-13 were measured to investigate changes in the Th2 cytokine (Figures 2b, 2c and 2d). The DME-induced Th2 cytokine levels (IL-4, IL-5 and IL-13) were significantly decreased by the PG102 treatment.

Effects of PG102 on DME-induced regulatory cytokine mRNA expression: The mRNA expression level of IL-10 (Figure 3a) and TGF-β1 (Figure 3b) were observed for changes in regulatory T cell producing cytokines. In the PG102 treated group, both IL-10 and TGF-β1 showed no statistically significant differences.

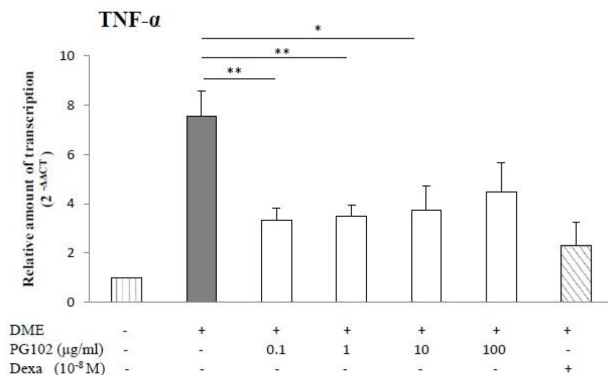

Figure 1 DME-induced TNF-α mRNA expression was significantly reduced by PG102 treatment in PBMCs from canine AD.

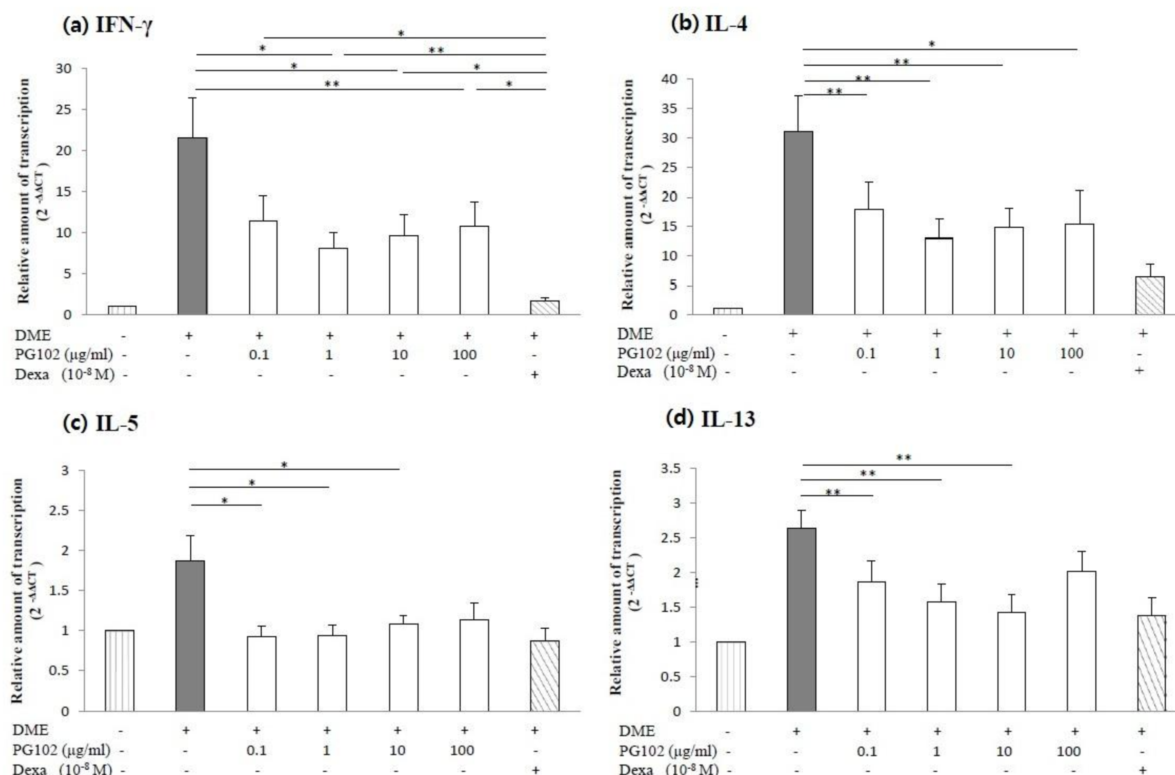

Figure 2a, 2b, 2c and 2d. Effects of PG102 on Th1 cytokine and Th2 cytokine mRNA expression levels in PBMCs from canine AD. DME-induced IFN-γ expression was significantly decreased by PG102 (a). DME-induced Th2 cytokine expressions were inhibited by PG102 (IL-4, b; IL-5, c; and IL-13, d).

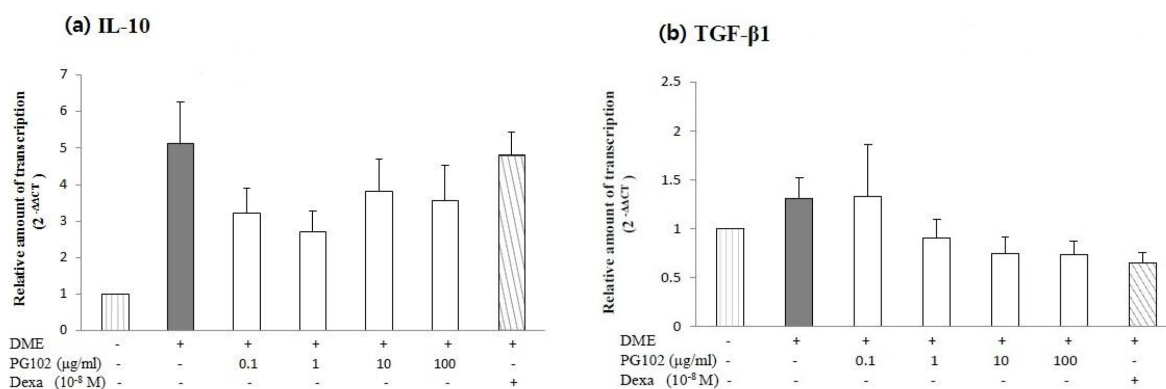

Figure 3a and 3b. Effects of PG102 on regulatory cytokine (IL-10, 3a and TGF-β1, 3b) mRNA expression level in PBMCs from canine AD. IL-10 and TGF-β1 showed no statistically significant differences.

Discussion

The present study assessed the modulatory effect of PG102 on the representative inflammatory cytokines from the PBMC culture of patient subject with definite canine AD diagnoses. It was observed that PG102 effectively inhibited the mRNA expressions of TNF-α, IL-4, IL-5, IL-13 and IFN-γ induced by DME stimulation. The results in this paper support other clinical investigations which suggested that the extracts from *A. argute* would be effective candidates for potent anti-allergic agent (Ho et al., 2009; Marsella et al., 2010). Although several studies of PG102 on its anti-inflammatory effect have been conducted, its immunological mechanisms in canine AD has rarely been investigated. Moreover, there has been no previous study profiling allergy related cytokine panels with PG102 treatment for canine AD.

PBMCs activated by DME used in this study were found to be beneficial to an *in vitro* model system for studying the effect of immune and inflammatory reaction. Previous studies showed that DME stimulation of canine PBMCs induced lymphocyte proliferation and Th1/Th2 cytokine activation (Nuttall et al., 2002b). It was shown that all of the DME-induced Th2 and Th1 cytokines were dramatically reduced by the PG102 treatment in a similar manner to the Dexamethasone group. IL-4 is a key regulator of humoral and adaptive immunity. It is a major cytokine for the production of allergen-specific IgE (Lebman and Coffman, 1988). IL-5 stimulates B cell growth and

is a key mediator in eosinophil activation (Nurse et al., 2000). The increased IL-4 expression in the DME-stimulated PBMCs showed the highest in the present study, demonstrating that IL-4 plays a major role in canine AD. In contrast, the relatively less activation of IL-5 demonstrates that eosinophilia is not a common symptom in canine AD. Th1 cytokine is characterized by the production of IFN-γ, which is involved in cell-mediated immune responses and is necessary for late chronic inflammation in AD (Grewe et al., 1998). A previous study reported that oral administration of PG102 increased Th1 cytokines and suppressed Th2 cytokines in OVA-induced AD-like mouse model (Park et al., 2005). It can be initially assumed that PG102 modulates Th1/Th2 imbalance in canine AD as it was shown in the murine AD-like model. However, in the present study, the DME-induced Th1 and Th2 cytokines were reduced by the PG102 treatment in the PBMCs cultures from canine AD. Both Th1 and Th2 type cytokines contribute to the pathogenesis of AD. House dust mite reactive T cells produce both Th1 and Th2 cytokines, which supports this concept (Wierenga et al., 1991). The development of AD is induced by Th2 type cytokines, while the chronic inflammatory responses are mediated dominantly by Th1 type cytokines (Horikawa et al., 2002). Therefore, for the treatment of AD, both Th1 and Th2 cytokines should be considered as therapeutic targets. The anti-inflammatory effect of PG102 did not appear in a dose-dependent pattern. Nevertheless, the concentration of 0.1-10 μg/ml of PG102 used in this study was effective for suppressing the inflammatory cytokine expression in PBMCs from canine AD. This result may implicate that PG102 causes general immunosuppression, possibly leading to anti-allergic effects in canine AD.

Further studies should be carried out to evaluate the actual molecular mechanism underlying the application of PG102 in canine AD. A previous veterinary study evaluating the therapeutic effect of oral application of PG102 on canine AD showed that clinical improvement was most noticeable after prolonged use of PG102 (Marsella et al., 2010). The reason for the need for longer use may be due to the fact that the mechanism of action requires slow accumulation. Whatever the actual mechanism of PG102 is, the results from this study and other clinical trials suggest that PG102 has great potential as a safe and effective reagent for the treatment of canine AD.

In conclusion, the data in this study indicated that PG102 suppressed allergy-related cytokine from PBMCs. PG102 will be a promising natural immune modulator for the treatment of canine AD.

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บทคัดย่อ

ฤทธิ์แบบต่อเนื่อง (Modulatory Effects) ของ PG102 (Actinidia arguta) ที่มีต่อการแสดง mRNA ของสารสื่อการอักเสบ (Inflammatory Cytokine) ในภาวะผื่นภูมิแพ้ผิวหนังของสุนัข

ยอง อุค ลี[1#] โซล ฮี ปาร์ค[1#] แท โฮ ชุง[2] อิว ฮวา นัม[1] โซล ยอง ฮวาง[1*]

ภาวะผื่นภูมิแพ้ผิวหนังในสุนัข (AD) เป็นโรคผิวหนังอักเสบที่สามารถกลับมาเป็นได้อีกอย่างเรื้อรัง และสามารถพบได้โดยทั่วไป สารสกัดด้วยน้ำ PG102 ซึ่งได้มาจากผล *Actinidia arguta* ได้รับการวิจัยว่าเป็นสารต้านการอักเสบในมนุษย์และสัตว์ทดลองซึ่งมีอาการเหมือนผื่นภูมิแพ้กรรมพันธุ์ อย่างไรก็ตาม กลไกการทำงานของ PG102 ในการรักษาภาวะ AD ของสุนัขยังไม่เป็นที่เข้าใจได้อย่างสมบูรณ์ การศึกษานี้ประเมินฤทธิ์ช่วยต้านการอักเสบของ PG102 ในหลอดทดลอง โดยศึกษาไซโตไคน์ พาเนลของ Peripheral Blood Mononuclear Cells (PBMCs) ที่ถูกกระตุ้นด้วยสารสกัดไรฝุ่นที่ก่อให้เกิดภูมิแพ้ (dust mite allergen extract, DME) ของสุนัขที่เป็นโรค AD โดยการใช้ realtime-PCR เชิงปริมาณ ทำการแยก PBMCs ออกจากสุนัขแปดตัวที่ได้รับการวินิจฉัย อย่างชัดเจนแล้วว่าเป็นโรค AD และกระตุ้นด้วย DME นาน 12 ชั่วโมงในระหว่างที่มี PG102 ปรากฏอยู่ (0.1, 1, 10, 100 μg/ml) ผลการทดลองพบว่า PG102 สามารถยับยั้งการแสดง mRNA ของ TNF-α, IL-4, IL-5, IL-13 และ IFN-γ ที่ถูกกระตุ้นด้วย DME ได้อย่างมีนัยสำคัญ อย่างไรก็ตาม ระดับการแสดง mRNA ของ IL-10 และ TGF-β1 ไม่มีการเปลี่ยนแปลงอย่างมีนัยสำคัญ ข้อมูลเหล่านี้แสดงให้เห็นว่า PG102 อาจจะเป็นสารตัวเลือกที่ใช้ในการรักษาโรค AD ในสุนัขได้

คำสำคัญ: *Actinidia arguta,* ภาวะผื่นภูมิแพ้ผิวหนังในสุนัข, ไซโตไคน์, PBMCs

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