creative commons

COMMONS DEED

저작자표시-동일조건변경허락 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.
- 이차적 저작물을 작성할 수 있습니다.
- 이 저작물을 영리 목적으로 이용할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

동일조건변경허락. 귀하가 이 저작물을 개작, 변형 또는 가공했을 경우에는, 이 저작물과 동일한 이용허락조건하에서만 배포할 수 있습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

수의학석사학위논문

Cytokine responses of PG102, an aqueous extract of *Actinidia arguta* fruit, on PBMCs from dogs with canine atopic dermatitis

아토피 피부염에 이환된 개의 PBMC에서 다래추출물 PG102의 Cytokine 조절 효과

2013 년 2 월

서울대학교 대학원

수의학과 수의내과학(피부과학) 전공

이 용 욱

Cytokine responses of PG102, an aqueous extract of *Actinidia arguta* fruit, on PBMCs from dogs with canine atopic dermatitis

아토피 피부염에 이환된 개의 PBMC에서
다래추출물 PG102의 Cytokine 조절 효과

지도교수 황 철 용

이 논문을 수의학 석사 학위논문으로 제출함
2012 년 10 월

서울대학교 대학원
수의학과 수의내과학(피부과학) 전공
이 용 욱

이용욱의 석사 학위 논문을 인준함
2012년 12월

위 원 장 _____ 윤 화 영 _____ (인)
부위원장 _____ 황 철 용 _____ (인)
위 원 _____ 채 준 석 _____ (인)

Cytokine responses of PG102, an aqueous extract of *Actinidia arguta* fruit, on PBMCs from dogs with canine atopic dermatitis

Yong-Uk Lee

Department of Veterinary Internal Medicine

The Graduate School

Seoul National University

Abstract

An aqueous extract, PG102, derived from *Actinidia arguta* fruit, has been investigated as an anti-inflammatory agent in an animal model, humans, and dog atopy patients. However, its mechanism of action is not well understood. This study evaluated the *in vitro* effects of PG102 on canine peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from eight dogs with atopic dermatitis and stimulated with house dust mite

extract (DME) for 12 hours in the presence of PG102 (0.1, 1, 10, 100 μg/ml). Subsequent quantitative real-time PCR revealed changes in mRNA expressions of representative allergy-related cytokines, such as proinflammatory cytokines (TNF-α), T_{h1} cell cytokines (IFN-γ), T_{h2} cell cytokines (IL-4, IL-5, IL-13), and regulatory cytokines (IL-10 and TGF-β_1) calculated by $2^{-\Delta\Delta C_T}$ and expressed as n-fold differences compared with an unstimulated control. Cytokine suppression by PG102 was compared with that of Dexamethasone, a widely used drug for the control of symptoms of canine atopic dermatitis. PG102 significantly inhibited the expression of TNF-α, IFN-γ and IL-4, IL-5, IL-13, but not the expression of regulatory cytokines (IL-10 and TGF-β_1), when compared to a DME stimulated sample ($P<0.05$). This pattern was similar to that obtained with Dexamethasone except for TGF-β_1. No significant reduction was seen with 100 μg/ml PG102; however, treatment with concentrations of 1 and 10 μg/ml significantly reduced cytokine expression, indicating that 1 and 10 μg/ml of PG102 were effective concentrations for suppressing cytokines in PBMCs. PG102 exerts an anti-inflammatory effect through the inhibition of cytokine production by PBMCs, indicating that it has potential as an alternative therapy for treatment of canine atopic dermatitis.

Key words: PG102, *Actinidia arguta*, canine atopic dermatitis, PBMC, cytokine

Student Number: 2010-23640

CONTENTS

Introduction ····· 1

Materials and Method

1. Reagent preparation ····· 4
2. Test populations ····· 4
3. PBMC preparation ····· 5
4. PBMC culture ····· 6
5. RNA extraction and cDNA synthesis ····· 6
6. Primer preparation and quantitative real-time PCR using SYBR green ····· 7
7. Data analysis of real-time PCR ····· 9
8. Statistical analysis ····· 9

Results

1. Expression of proinflammatory cytokine mRNA ····· 10
2. Expression of T_{h1} cell and T_{h2} cell cytokine mRNA ····· 11
3. Expression of regulatory cytokine mRNA ····· 15

Discussion ····· 16

References ····· 22

국문 초록 ····· 27

Introduction

Canine atopic dermatitis (CAD) is the second most common allergy in dogs, occurring in 10% of the canine population. It is a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features such as pruritus and skin lesions that typically affect the face, feet, and ears.[1-3] CAD shows similar immunological changes and clinical signs to those of human atopic dermatitis and consequently has been proposed as a suitable model of the human condition.[4,5] CAD has been defined as a Type I hypersensitivity associated with IgE and is most commonly directed against environmental allergens. Of the various aeroallergens, the house dust mites (HDMs), *Dermatophagoides farinae* and *D. pteronyssinus,* are the most common allergens and they are known to be associated with the immunopathogenesis of canine atopic dermatitis as well as its human counterpart. [6-9] Recent studies have revealed that both *in vivo* and *in vitro* stimulation by HDMs can induce elevations in HDM-specific IgE and enhanced mRNA transcription of various cytokines and chemokines.[10,11]

Previous studies have demonstrated that the cytokine-mediated response of the immune system to allergens is an important pathogenetic factor. [4,12] Cytokines produced by both T helper 1 (T_{h1}) and T helper 2 (T_{h2}) cells played an important role in the pathogenesis of the CAD. The T_{h2} cytokines mainly promoted isotype switching in the B cells that subsequently produce IgE. Acute skin lesions are characterized by CD4+ T_{h2} lymphocytes and eosinophils and the release of T_{h2} cytokines (IL-4, IL-5 and IL-13).[13,14] However, chronic lesions show a predominance of T_{h1} lymphocytes, macrophages and T_{h1} cytokines (IL-2, IL-12, IFN-γ and IL-18), which correlates with chronicity and severity of

the atopic dermatitis skin lesions. [15,16] Proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), are also increased in patients with atopic dermatitis, although the specificity of their role has not been completely clarified.[17,18] Regulatory cytokines, such as IL-10 and transforming growth factor-beta (TGF-β_1) produced by regulatory T cells, suppress T_{h1} and T_{h2} cytokine responses through mechanisms that depend on cell contact.[19]

Alleviation of CAD symptoms is obtained using anti-inflammatory systemic treatments such as glucocorticoids and cyclosporine, which are effective therapies [20,21]. However, chronic use of glucocorticoid may have the potential for adverse effects such as iatrogenic Cushing syndrome, polyuria/polydipsia, polyphagia, skin thinning, calcinosis cutis, and urinary tract infection.[22] Oral cyclosporine has a high cost, especially in large dogs, and clinical improvement may be relatively late. Antihistamines and essential fatty acids may be unsatisfactory when used in severe cases. [20] Thus, the identification of a safe and effective oral treatment would be valuable as an additional alternative strategy for controlling CAD.

PG102 is a water-soluble extract prepared from the edible fruit of *Actinidia arguta*, commonly referred to as hardy kiwi. PG102, also known as EFF1001 in the USA, has been used for a long time in East Asian traditional medicine.[23] Previous studies on a murine model showed that PG102 improved the symptoms of bronchial asthma, food allergy, and atopic dermatitis by regulating serum IgE revel and the balance of T_{h1}/T_{h2} cytokine production.[24-27] In human patients, serum total IgE levels decreased after 8 weeks of treatment with PG102 when compared with a placebo group and no significant adverse effects were noted in the PG102 experimental group.[28] Recent studies showed that PG102 reduced the CADESI score and pruritus in dogs with atopic dermatitis, but the

mechanism underlying the anti-allergic effect was not determined.[29]

In this study, the anti-inflammatory effect of PG102 was evaluated on representative allergy-related cytokines, such as proinflammatory cytokines (TNF-α), T_{h1} cytokines (IFN-γ), T_{h2} cytokines (IL-4, IL-5, IL-13), and regulatory cytokines (IL-10, TGF-β_1), using HDM-stimulated PBMCs from dogs with CAD. The amount of mRNA was calculated by $2^{-\Delta\Delta C_T}$ and expressed as n-fold differences. The effectiveness of PG102 at concentrations of 0.1, 1, 10, 100 μg/ml was compared with that of Dexamethasone, a steroid that is widely used to control the symptoms of CAD.

Materials and methods

Reagent preparation

PG102 is an extract formulation prepared by boiling *Actinidia arguta* fruit in water. PG102 consists of 48.4% of PG102 and 51.6% of water and this reagent was kindly provided by Viromed Co., Ltd. Before using the reagent, PG102 was diluted to 4 mg/ml with RPMI 1640 medium and filtered through a 0.22μm syringe filter.

Test populations

Eight dogs, diagnosed with atopic dermatitis by Seoul National University Hospital for Animals, were recruited for experimental purposes. Diagnosis was based on compatible history, clinical signs, and exclusion of other causes of pruritus. Their ages ranged from 5 to 12 years (average 9.25 years); four were male and four were female. All of the males and two of the females had been neutered (Table 1). All dogs were sensitive to *Dermatophagoides farinae,* as determined by a serology allergen specific IgE test and intradermal skin test. Glucocorticoids and antihistamines were withdrawn at least 2 weeks prior to the intradermal skin test, serology allergen specific IgE test, and blood collection. All dogs had no diseases other than their skin problem.

Table 1. Characteristics of the dogs with atopic dermatitis

Case No.	Sex	Breeds	Age of onset (years)	Age (years)
1	MC	Cocker Spaniel	2.5	9
2	F	Miniature Schnauzer	2	8.5
3	MC	Shih Tzu	1	11
4	FS	Shih Tzu	2.5	10.5
5	MC	Pekingese	3	7.5
6	MC	Mixed	3	12
7	FS	Shih Tzu	5	10
8	F	Golden Retriever	0.5	5

MC; Castrated Male, F; Female, FS; spayed Female

PBMC preparation

Blood samples (20ml) from the test populations were collected into citrate-phosphate-dextrose-adenosine (CPDA) by jugular venipuncture. Samples were kept cold on ice and processed within 2 hours. The CPDA-preserved blood samples were diluted with an equal volume of PBS and layered on top of 20 ml of Histopaque-1077 (Sigma, St. Louis, MO). This overlaid solution was centrifuged at 400×g for 30 minutes at room temperature. Mononuclear cells at the interface were collected carefully and washed twice with 3 volumes of chilled PBS at 200×g for 10 minutes at 4°C. The isolated PBMCs were suspended in RBC lysis buffer (8.3 g/L ammonium chloride and 0.02M Tris-HCl buffer, pH 7.5) and shaken for 5 minute at 4°C to remove residual red blood cells. Subsequently, PBMCs were washed with PBS and viable cells were counted following trypan blue staining using a modified Neubauer hemocytometer. The PBMC suspension was adjusted to 1×10^7 cells/ml with RPMI1640 medium (Gibco, Rockville, MD) supplemented with 10% FBS (Gibco), 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco) (hereafter

referred to as complete medium). Cell samples showing 90% viability by trypan blue staining were used for the study.

PBMC culture

A 500 µl sample of cell suspension at a concentration of 1×10^7 cell/ml was added to all wells. A 250 µl volume of PG102 stock solution was diluted 10 fold from 400 µg/ml ~ 0.4 µg/ml using complete medium. A 250 µl volume of diluted *D. farinae* crude extract (DME) (Greer Laboratories, Lenoir, NC) solution (40 µg/ml) was added to the appropriate wells; 250 µl of the complete medium was added to control wells resulting in a final cell concentration of 5×10^6 cells. In the control sample (unstimulated condition), only PBMCs were added, whereas 10 µg/ml of DME was added in the stimulated samples. The PG102 stimulated samples contained 100 µg/ml ~ 0.1 µg/ml PG102 solution plus DME in each sample wells. For comparison with PG102, Dexamethasone (10^{-8} M) was used in other wells. The plates were incubated for 12 hours at 37°C in a humidified atmosphere containing 5% CO_2.

RNA extraction and cDNA synthesis

Total RNA was extracted from the PBMCs using a commercially available kit (RNeasy Mini kit, Qiagen, Crawley, UK) according to manufacturer's instructions. The final product was eluted into 30µl of diethyl pyrocarbonate (DEPC)-treated water. The RNA concentration present was determined by measuring the absorbance at 260 nm in a spectrophotometer (IMPLEN Nanophotometer, IMPLEN, Munich, Germany). Each RT reaction used 500 ng ~ 1000 ng of RNA.

Complementary DNA (cDNA) was synthesized using a commercially available kit (Primescript™ 1st strand cDNA Synthesis Kit, TaKaRa, Shiga, Japan). The reaction mixture (20 μl) consisted of 1μl (50μM) oligo dT Primer, 1μl dNTP mix (10 mM of each dNTP), 1μl (200U) Primescript reverse transcriptase and 0.5 μl (20U) RNase inhibitor. The sample was incubated at 42°C for 60 min, and the RT reaction terminated by heating at 70°C for 15 min. All the cDNA samples were subsequently stored at -70°C prior to analyses.

Primer preparation and quantitative real-time PCR using SYBR green

For each of the target genes, canine β-actin, IL-4, IL-5, IL-10, IL-13, TGF-β_1, IFN-γ, and TNF-α, a pair of oligonucleotide primers and oligonucleotide probe were designed based on previously published studies.[30-32] Primers were designed to generate short PCR products (68~159 bp). Sequences of the primer are shown in Table 2.

Quantitative real-time PCR was performed on the Step One Plus™ (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) for detection according to manufacturer's instructions. The PCR was conducted in 96-well optical reactions plates. Each well contained an 11 μl reaction mixture consisting of 5 μl SYBR Green dye, 1 μl of (900nM) of each specific forward and reverse primer, and 4 μl of 10 fold diluted cDNA samples. For every sample, parallel reactions were performed in duplicate in separate wells for detection of cytokine mRNA

Table 2. Sequences of primers used for cytokine quantification by real-time PCR and size of the PCR products

Target gene	Primer	Sequence (5'-3')	Nucleotide position	Gene bank accession number	Length of PCR product (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		

transcription. Amplification conditions were identical for all reactions: denaturation at 10 min at 95°C and 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 /s.

Data analysis of real-time PCR

Relative expressions of the target cytokine genes were calculated using a comparative CT method, as previously described,[33] whereby the relative transcription of the target cytokines is reported as the n-fold difference relative to transcription of the calibrator gene (β-actin) and compared to the unstimulated group as a control sample. The amounts of cytokine and chemokine mRNA were calculated by the $2^{-\Delta\Delta C_T}$ method, ($\Delta\Delta C_T = (C_{T\ Cytokine} - C_{T\ \beta\text{-actin}})_{sample} - (C_{T\ Cytokine} - C_{T\ \beta\text{-actin}})_{control}$). All samples were examined in duplicate and the mean value of $\Delta\Delta C_T$ was calculated.

Statistical analysis

Data were expressed as mean ± standard deviation. The statistical analysis was performed using a t-test and one-way ANOVA for comparing cytokine mRNA expression levels in the PBMCs from different treatments. Differences at $P \leq 0.05$ were considered to be significant.

Results

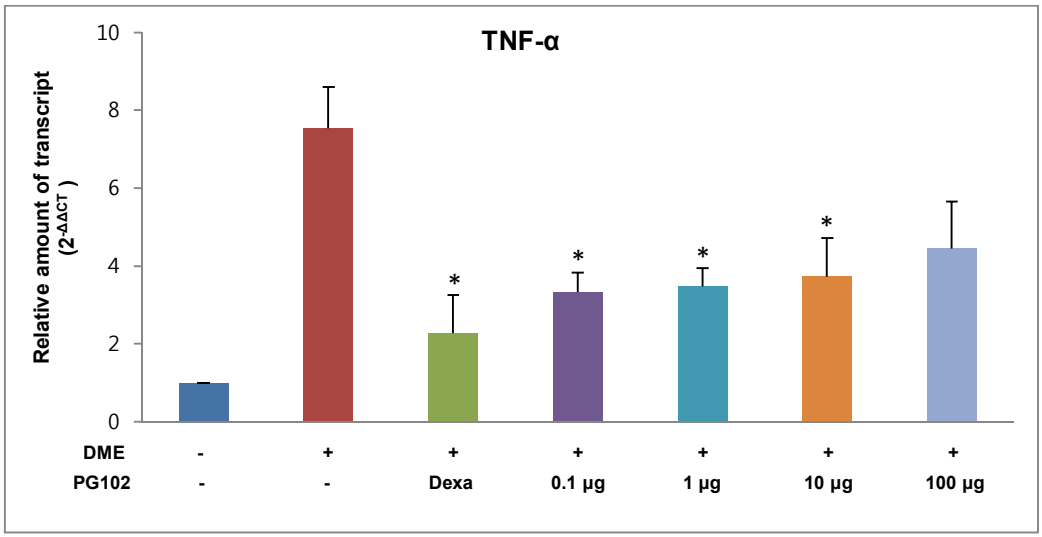


Figure 1. Expression of proinflammatory cytokine (TNF-α)

Expression of TNF-α was observed to investigate quantitative change of proinflammatory cytokines. In the dust mite extract (DME) stimulation group, TNF-α expression was increased 7.55 fold compared with the unstimulated group. In the Dexamethasone induced group, DME induced TNF-α expression was decreased 69.8%. In PG102 groups, DME induced TNF-α expression was decreased by 55.8% with 0.1 μg/ml, 53.9% with 1 μg/ml, 50.5% with 10 μg/ml, and 41.0% with 100 μg/ml PG102. PG102 at 0.1 μg/m was the most effective concentration compared with a Dexamethasone group. Treatments with 0.1, 1, and 10 μg/ml PG102 gave statistically significant (*P<0.05) differences compared with the values for DME alone. Values shown are the means±SE measured in duplicate reactions.

Expression of proinflammatory cytokine mRNA

Expression of TNF-α was selected as a measure of proinflammatory cytokine changes in PG102 stimulated PBMCs. The mRNA of TNF-α in the DME induced group was increased by 7.55 fold. In the Dexamethasone stimulated cells, DME induced TNF-α expression was reduced by 69.8%. In PG102 stimulated groups, a 41.0% ~ 55.8% reduction in DME induced TNF-α expression was observed when compared with the DME induced group. A PG102 concentration of 0.1 μg/ml was the most effective concentration, but was less effective than the Dexamethasone treatment. The values

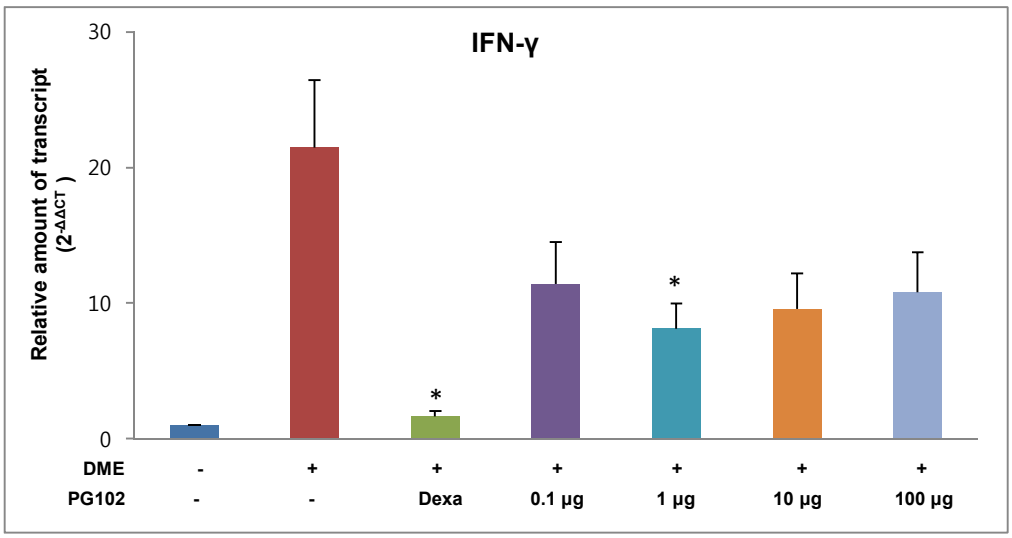


Figure 2. Expression of T_{h1} cell cytokine mRNA

Expression of IFN-γ was observed to investigate quantitative changes in proinflammatory cytokines. In the DME induced cells, IFN-γ expression was increased 21.5 fold compared with the unstimulated group. In the Dexamethasone stimulated cells, DME induced IFN-γ expression was decreased 92.4%. In the PG102 treated cells, DME induced IFN-γ expression was decreased 46.8% with 0.1 μg/ml, 62.3% with 1 μg/ml, 55.4% with 10 μg/ml and 49.7% with 100 μg/ml PG102. A concentration of 1 μg/ml was the most effective concentration when compared with Dexamethasone (*P<0.05). Values shown are the means±SE measured in duplicate reactions.

obtained for 0.1, 1 and 10 μg/ml PG102 treatments were significantly different (p<0.05) when compared with the values for DME stimulated alone (figure 1).

Expression of T_{h1} cell and T_{h2} cell cytokine mRNA

Expression of IFN-γ was observed to investigate T_{h1} cell cytokines and expressions of IL-4, IL-5 and IL-13 were observed to investigate T_{h2} cell cytokines. The mRNA of IFN-γ in the DME induced group was increased by 21.5 fold. The Dexamethasone stimulated cells showed a reduction in DME induced IFN-γ expression of 92.4% when compared with DME stimulated group. In the PG102 stimulated cells, the DME induced IFN-γ expression was decreased by 46.8% ~ 62.3%; 1 μg/ml was the most effective PG102 concentration, but was less effective than Dexamethasone. Differences in cytokine levels

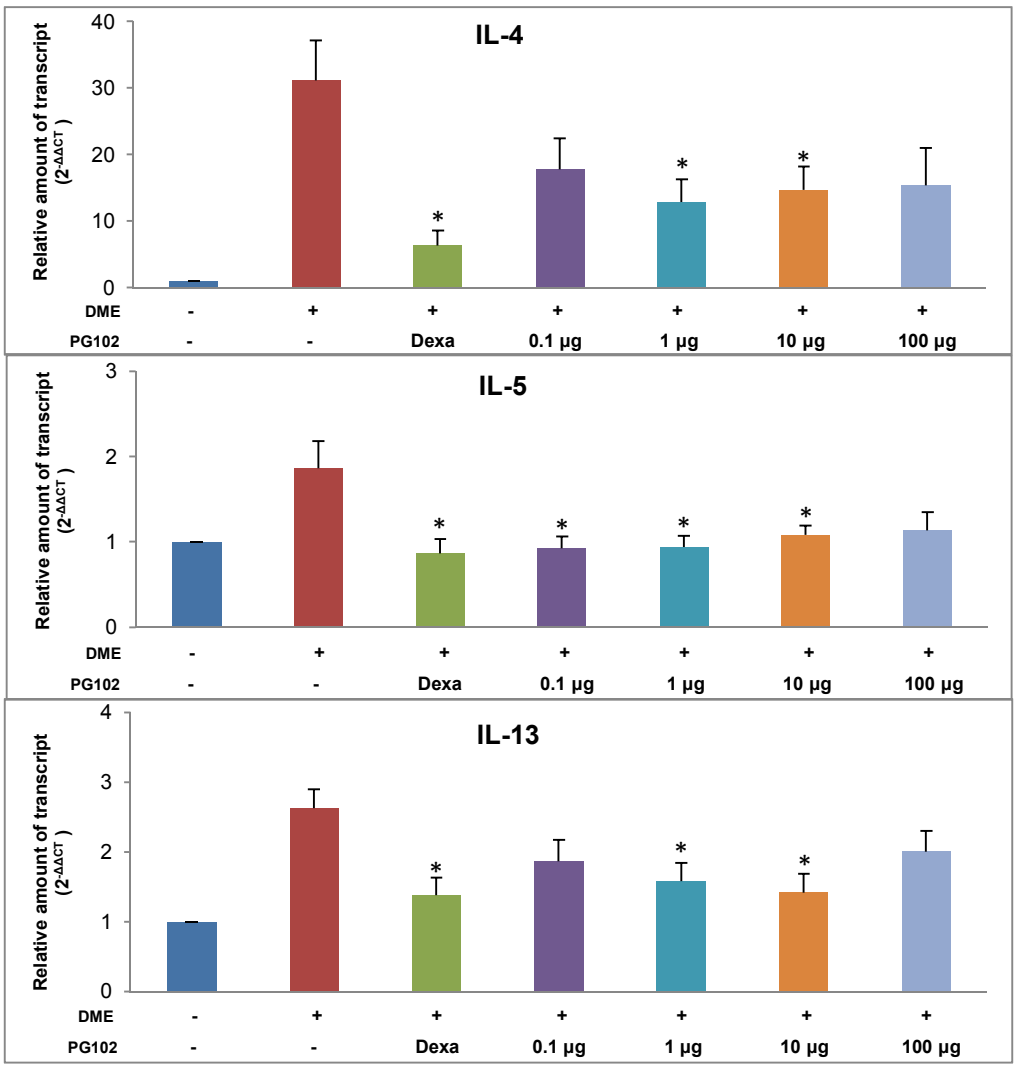
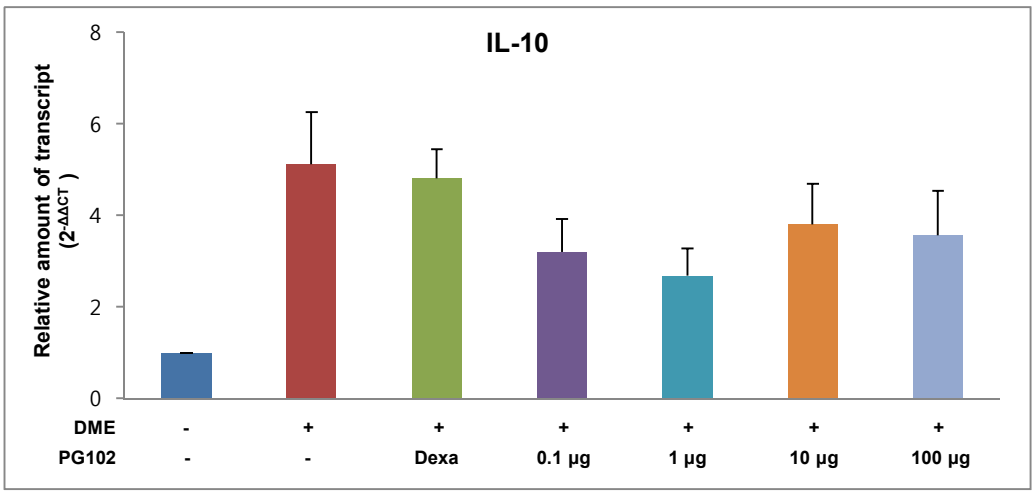


Figure 3. Expression of T_{h2} cell cytokine mRNA

Expressions of IL-4, IL-5 and IL-13 were observed to investigate quantitative change of T_{h2} cell cytokines. In the DME induced cells, T_{h2} cell cytokine expression was increased 31.1 fold for IL-4, 1.87 fold for IL-5 and 2.64 fold for IL-13, when compared with unstimulated group. DME induced IL-4 expression was decreased 79.4% in Dexamethasone stimulation group. In PG102 groups, DME induced IL-4 expression was decreased 42.7% with 0.1 μg/ml, 58.6% with 1 μg/ml, 52.7% with 10 μg/ml and 50.4% with 100 μg/ml of PG102. Differences in cytokine levels in cells treated with Dexamethasone and 1 and 10 μg/ml of PG102 were statistically significant ($*P<0.05$) compared with the values for DME alone. IL-5 expression was decreased 53.4% in Dexamethasone stimulation group. In cells treated with PG102, DME induced IL-5 expression was decreased 50.3% with 0.1 μg/ml, 49.5% with 1 μg/ml, 42.0% with 10 μg/ml and 38.9% with 100 μg/ml of PG102. The differences in cells treated with Dexamethasone, 0.1, 1, and 10 μg/ml of PG102 were statistically significant ($*P<0.05$) compared with the values for DME alone. IL-13 expression was decreased 47.5% in the Dexamethasone stimulated cells. In samples with PG102, DME induced IL-13 expression was decreased 29.0% with 0.1 μg/ml, 39.8% with 1 μg/ml, 46.0% with 10 μg/ml and 23.6% with 100 μg/ml. The differences for Dexamethasone and 1 and 10 μg/ml of PG102 were statistically significant ($*P<0.05$) compared with the values for DME alone. Values shown are the means±SE measured in duplicate reactions.

in cells treated with Dexamethasone and 1 μg/ml PG102 were statistically significant ($p<0.05$) compared with the values for DME alone (figure 2).

A similar pattern of transcription was observed for the T_{h2} cell cytokines IL-4, IL-5, and IL-13. In the DME induced group, the expression was increased by 31.1 fold for IL-4, 1.87 fold for IL-5, and 2.64 fold for IL-13 compared with the unstimulated group. In the Dexamethasone stimulated group, DME induced T_{h2} cell cytokines level was reduced by 79.4% for IL-4, followed by 53.4% for IL-5 and 47.5% for IL-13. For the PG102 stimulated cells, DME induced IL-4 expression was reduced by 42.7%~ 58.6% and 1 μg/ml of PG102 was the most effective concentration. The differences in cells treated with Dexamethasone, 1 and 10 μg/ml of PG102 were statistically significant ($p<0.05$) compared with the values for DME alone. DME induced IL-5 expression was reduced by 38.9% ~ 50.3% and 0.1 μg/ml of PG102 was the most effective concentration. The differences in cells treated with Dexamethasone, 0.1, 1, and 10 μg/ml of PG102 were statistically significant ($p<0.05$) ($p<0.05$) compared with the values for DME alone. DME induced IL-13 expression was reduced by 23.6% ~ 46.0% and 10 μg/ml was the most effective concentration. The differences in cells treated with Dexamethasone, 1 and 10 μg/ml of PG102 were statistically significant ($p<0.05$) compared with the values for DME alone (figure 3).



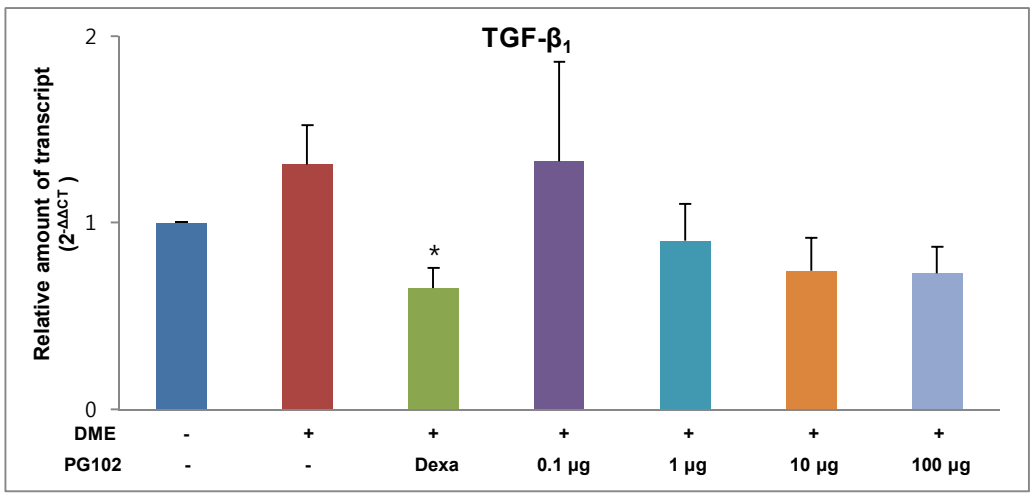


Figure 4. Expression of regulatory cytokine mRNA

Expressions of IL-10 and TGF-β_1 were observed to investigate quantitative change of regulatory cytokine**.** In the DME induced group, IL-10 expression was increased 5.12 folds compared with non-stimulated group. In Dexamethasone stimulation group, DME induced IL-10 expression was decreased 5.8%. In PG102 groups, DME induced IL-10 expression was decreased 37.2% in 0.1 μg/ml, 47.3% in 1 μg/ml, 25.6% in 10 μg/ml and 30.3% in 100 μg/ml of PG102. All groups including Dexamethasone were not statistically significant. TGF-β_1 expression was increased 1.31 folds in DME induced group. In Dexamethasone stimulation group, DME induced TGF-β_1 expression was regulated 50.2%. In PG102 Groups, DME induced TGF-β_1 expression was decreased 31.0% in 1 μg/ml, 43.4% in 10 μg/ml and 44.4% in 100 μg/ml of PG102, but in group of 0.1 μg/ml TGF-β_1 expression was slightly increased 1.3%. Dexamethasone stimulation group was significant ($*P<0.05$) compared with the values for DME alone, but PG102 stimulation group was not significant. Values are shown means±SE measured in duplicate reactions.

Expression of regulatory cytokine mRNA

Expressions of IL-10 and TGF-β_1 were observed to investigate changes in regulatory T cell producing cytokines. Levels of DME induced regulatory cytokine expression was increased by 5.12 fold for IL-10 and 1.31 fold for TGF-β_1. In Dexamethasone stimulated cells, DME induced IL-10 expression was reduced to 5.8% and DME induced TGF-β_1 expression was reduced by 50.2%. In the PG102 stimulated cells, DME induced IL-10 expression was decreased 25.7% ~ 47.4%, but differences for all concentrations were not statistically significant. DME induced TGF-β_1 expression was decreased by 31.0% ~ 44.4% with 1, 10 and 100 μg/ml of PG102, but cells treated with 0.1 μg/ml PG102 showed a slight increase in TGF-β_1 expression of 1.3%. Differences in expression in the Dexamethasone group were statistically significant ($p<0.05$) compared with the values for DME alone, but the differences for the PG102 treated cells were not significant.

.

Discussion

The anti-inflammatory effect of PG102 was evaluated by quantifying the changes in cytokine expression and the results were compared with the effects of 10^{-8}M of Dexamethasone. Based on the IC_{50} value of PG102 components, 0.1, 1, 10 and 100 μg/ml of PG102 were considered as appropriate concentrations. Many reports on CAD have indicated that cytokine-mediated response in the immune system to allergens is an important pathogenic factor. We observed that all concentrations of PG102 inhibited the expressions of TNF-α, IFN-γ, IL-4, IL-5, and IL-13, but expression changes were not significant for IL-10 and TGF-β_1. These results were similar to those seen with Dexamethasone, confirming that PG102 is an effective anti-inflammatory agent.

PG102 is a water soluble extract prepared from *Actinidia arguta*, known as hardy kiwi. This smooth-skinned, grape-sized fruit has been used in Korean traditional medicine for treatment of allergic disorders, diabetes mellitus, osteoporosis, cardiopathy, and gastroenteric troubles. The active component of PG102 has been investigated in several studies. Analysis of the volatile constituents of fresh A. *arguta* fruits revealed more than 240 simple organic compounds, including a variety of monoterpenes.[34] Chromatographic isolation of the fruits yielded several phenolic compounds, including phenolic acids, coumarin, and flavonoids which inhibited several inflammatory pathways.[35] Recent studies reported the isolation of α-linoleic acid, linoleic acid ethyl ester, and stearic acid ethyl ester, and that these compounds reduced IL-4 expression in RBL-2H3 cells.[23] Therefore, specific compounds in *A. arguta* extracts have the potential to act as cytokine production inhibitors in CAD.

The present study investigated the *in vitro* effects of PG102 on PBMCs from atopic dogs, activated by DME, the most common allergen of canine atopic dermatitis.[9] Several human studies have shown that PBMCs from allergic subjects produce strong responses to DME.[36,37] Anti-DME reactive T cell clones provided some evidence that T_{h1} and T_{h2} cytokine polarization had occurred in human,[38] however, increased doses of DME are now known to induce similar quantities of both T_{h1} and T_{h2} cytokine.[39-42] Additionally, allergen-stimulated PBMCs from allergic subjects have been reported to make higher levels of IL-10, a major regulatory cytokine, when compared to PBMCs from non-allergic subjects.[43,44] Several canine studies showed that DME stimulation of canine PBMCs induced lymphocyte proliferation and T_{h1} and T_{h2} cytokine activation.[45,46] In our study, increases in cytokine expression were observed by DME stimulation, confirming the inflammatory effects observed in previous studies.

Glucocorticoids are widely used as immunosuppressive agents to alleviate inflammatory conditions such as atopic dermatitis. One of the major routes by which glucocorticoids influence inflammatory responses is through their effect on cytokine production. In general, glucocorticoids suppress the production of cytokines, most notably those produced by both T_{h1} and T_{h2} cells, such as IL-2, IL-3, IL-4, IL-5, IL-6, and IL-13. Glucocorticoids also suppress the generation of many monocyte-derived cytokines including TNF-α, IL-1, GM-CSF. [47] Similar anti-inflammatory effects were observed in the present study for Dexamethasone, a widely used glucocorticoid therapy in veterinary dermatology practice. The anti-inflammatory effect of PG102 was similar to that of Dexamethasone, confirming the potential use of PG102 as an anti-inflammatory agent.

The pro-inflammatory factor, TNF-α, is an essential cytokine in many pathological conditions including allergic diseases. TNF-α is synthesized by monocytes, lymphocytes,

and other cells in response to inflammatory products and regulates normal T cell expression and secretion. The role of TNF-α has not yet been fully elucidated, but a previous human study found that plasma TNF-α was increased in children with atopic dermatitis, and that the magnitude of the increase was correlated with the severity of the dermatitis.[48] In CAD, TNF-α was increased in lesional skin of atopic dermatitis patients compared with non-lesional patients and normal dogs.[4] In the present study, expression of TNF-α was suppressed by PG102, demonstrating the anti-inflammatory effect PG102; however, differences in response between concentrations was slight, making a dose-dependent pattern difficult to discern.

The expression of cytokine has been reported to shift to a predominantly T_{h2} cell response in allergic diseases, and IgE production from B cells increases under these condition. IL-4 is a key regulator of humoral and adaptive immunity and has many biological roles, including the stimulation of activated B-cell and T-cell proliferation and the differentiation B cells into Plasma Cells. It is a major T_{h2} cytokine for the production of allergen-specific IgE. IL-3 has effects on immune cells that are similar to those of the closely related cytokine IL-4, but it is more importantly implicated as a central mediator of the physiologic changes induced by allergic inflammation in many tissues. The functions of IL-13 considerably overlap those of IL-4, especially with regard to changes induced in hematopoietic cells, but these effects are probably less important than those of IL-4. IL-5 stimulates B cell growth and increases immunoglobulin secretion and is also a key mediator in eosinophil activation. IL-5 has long been associated with the cause of several allergic diseases including allergic rhinitis and asthma. Increases in IL-4 expression in the DME stimulated cells were the highest in the present study, demonstrating that IL-4 plays a major role in CAD, whereas relatively less activation of

IL-5 demonstrates that eosinophilia is not a common symptom in CAD. Some studies have suggested that, following HDM stimulation, IL-13 is produced earlier and for a much longer period than is IL-4. Thus, a relatively lower IL-13 production may have been due to the fact that PBMCs were cultured only for 12 hours. When the sample was stimulated by PG102, all of the T_{h2} cytokines were reduced and a similar effect was observed with Dexamethasone, but the anti-inflammatory effect showed no dose-dependent pattern. Nevertheless, the results suggested that PG102 was effective for treatment of CAD.

T_{h1} cytokines were characterized by the production of IFN-γ, which is involved in cell-mediated immune responses and is necessary for late chronic inflammation in human atopic dermatitis. Several studies reported the detection of IFN-γ in chronic atopic lesions and dominated atopy patch tests, suggesting that these cytokines also participate in the pathogenesis of chronic atopic dermatitis.[15,16] In canine atopy patch tests, IFN-γ expression was significantly induced by HDM antigen and led to the hypothesis that IL-18, a strong IFN-γ inducing factor, promoted the release of IFN-γ.[10] Previous studies have also reported that PG102 up-regulated T_{h1} cytokines to balance T_{h1}/T_{h2} cytokines in serum.[24] However, in the present study, both DME induced T_{h1} cytokines and T_{h2} cytokines were reduced when PBMCs from CAD dogs were treated with PG102. The use of different samples and stimulation methods may have led to these different results. In the present study, expression of IFN-γ was reduced for all concentrations of PG102, demonstrating that PG102 could reduce the levels of cytokines related with the acute phase of CAD as well as cytokines related with the chronic phase of CAD, which is main effect of glucocorticoid therapy.

Regulatory T cells control peripheral immune responses and are likely to play an

important role in determining the incidence and severity of several immune pathologies, including autoimmune, infectious and allergic diseases.[49] Following antigen stimulation, antigen specific regulatory T cells are produced and secrete anti-inflammatory cytokines such as IL-10 and TGF-β_1, which have an immunosuppressive function and reduce the expression of T_{h1} and T_{h2} cytokines. In allergic conditions, a reduction in anti-inflammatory cytokines is reported compared with the normal condition. Allergen immunotherapy for glucocorticoid therapy, which is beneficial for the treatment of allergy and asthma, results in an increased expression of IL-10 in humans and dogs.[50,51] When DME was used in our study, however, the expressions of IL-10 and TGF-β_1 were both slightly increased. These results may represent an attempt of the regulatory T cells to down-regulate an excessive and potentially detrimental inflammatory response. In Dexamethasone stimulated cells, IL-10 production was unchanged; however, TGF-β_1 production was significantly reduced. Previous studies suggested that TGF-β_1 expression in CAD was lower than in normal canine skin samples, which was contrary to the results of our study.[4] Further investigations are needed to elucidate the relationship between the effect of Dexamethasone and TGF-β_1 expression on PBMCs from dogs with CAD. In PG102 stimulated samples, expression changes of IL-10 were not statistically significant and cytokine expression was diverse at each concentration, with no discernible pattern. Differences in TGF-β_1 expression were statistically not significant, although cytokine expression was increased as PG102 was diluted to 0.1 μg/ml. These results demonstrated that the anti-inflammatory effects of PG102 were not related to regulatory cytokine production.

Our study has several shortcomings. There was no placebo or blind control. For accurate results, a normal control consisting of normal dog PBMCs needs to be compared

to PBMCs of dogs with CAD. Cytokine expression was measured only for samples cultured for 12 hours, preventing observation of any time-dependent effects of PG102. A time course study of cytokine expression by canine PBMCs will be useful in further studies. In addition, the $2^{-\Delta\Delta C_T}$ method indicated only the fold changes in gene expression relative to untreated control, not an absolute amount of gene expression.

In conclusion, PG102 inhibited the expression of representative pro-inflammatory cytokines (TNF-α), T_{h1} cell cytokines (IFN-γ), and T_{h2} cell cytokines (IL-4, IL-5, IL-13), but changes in regulatory cytokines (IL-10, TGF-β_1) were not significant. PG102 at concentrations of 1 and 10 μg/ml was effective at reducing the expression of cytokines and was comparable to Dexamethasone. Thus, PG102 shows potential as an alternative therapy for glucocorticoids in the treatment of CAD.

References

1. Hillier A, Griffin CE. The ACVD task force on canine atopic dermatitis (i): Incidence and prevalence. Veterinary Immunology and Immunopathology 2001; 81: 147.
2. Scott DW, Miller WH, Griffin CE. Small Animal Dermatology WB Saunders, 2001. p. 581.
3. Halliwell R. Revised nomenclature for veterinary allergy. Veterinary Immunology and Immunopathology 2006; 114: 207.
4. Nuttall T, Knight P, McAleese S et al. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. Clinical & Experimental Allergy 2002; 32: 789-95.
5. Marsella R, Girolomoni G. Canine models of atopic dermatitis: A useful tool with untapped potential. Journal of Investigative Dermatology 2009; 129: 2351-7.
6. Lian TM, Halliwell REW. Allergen-specific IgE and IgGd antibodies in atopic and normal dogs. Veterinary Immunology and Immunopathology 1998; 66: 203-24.
7. Masuda K, Sakaguchi M, Fujiwara S et al. Positive reactions to common allergens in 42 atopic dogs in Japan. Veterinary Immunology and Immunopathology 2000; 73: 193-204.
8. Yasueda H, Mita H, Yui Y et al. Comparative analysis of physicochemical and immunochemical properties of the two major allergens from Dermatophagoides pteronyssinus and the corresponding allergens from dermatophagoides farinae. International Archives of Allergy and Immunology 1989; 88: 402-7.
9. Hill PB, DeBoer DJ. The acvd task force on canine atopic dermatitis (iv): Environmental allergens. Veterinary Immunology and Immunopathology 2001; 81: 169-86.
10. Marsella R, Olivry T, Maeda S. Cellular and cytokine kinetics after epicutaneous allergen challenge (atopy patch testing) with house dust mites in high-IgE beagles. Veterinary Dermatology 2006; 17: 111-20.

11. Maeda S, Shibata S, Chimura N et al. House dust mite major allergen Der f 1 enhances proinflammatory cytokine and chemokine gene expression in a cell line of canine epidermal keratinocytes. Veterinary Immunology and Immunopathology 2009; 131: 298-302.

12. Sousa CA, Marsella R. The acvd task force on canine atopic dermatitis (ii): Genetic factors. Veterinary Immunology and Immunopathology 2001; 81: 153.

13. Thepen T, Langeveld-wildschut E, Bihari I et al. Biphasic response against aeroallergen in atopic dermatitis showing a switch from an initial Th2 response to a Th1 response in situ: An immunocytochemical study. Journal of Allergy and Clinical Immunology 1996; 97: 828-37.

14. Grewe M, Walther S, Gyufko K et al. Analysis of the cytokine pattern expressed in situ in inhalant allergen patch test reactions of atopic dermatitis patients. Journal of Investigative Dermatology 1995; 105: 407-10.

15. Chen L, Martinez O, Overbergh L et al. Early up-regulation of Th2 cytokines and late surge of Th1 cytokines in an atopic dermatitis model. Clinical & Experimental Immunology 2004; 138: 375-87.

16. Grewe M, Bruijnzeel-Koomen CAFM, Schöpf E et al. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. Immunology Today 1998; 19: 359-61.

17. de Vries IJM, Langeveld-Wildschut EG, van Reijsen FC et al. Adhesion molecule expression on skin endothelia in atopic dermatitis: Effects of TNF-α and IL-4. Journal of Allergy and Clinical Immunology 1998; 102: 461-8.

18. Lee HJ, Lee HP, Ha SJ et al. Spontaneous expression of mRNA for IL-10, GM-CSF, TGF-β, TNF-α, and IL-6 in peripheral blood mononuclear cells from atopic dermatitis. Annals of Allergy, Asthma & Immunology 2000; 84: 553-8.

19. Borish LC, Steinke JW. Cytokines and chemokines. Journal of Allergy and Clinical Immunology 2003; 111: 460-75.

20. Olivry T, DeBoer DJ, Favrot C et al. Treatment of canine atopic dermatitis: 2010 clinical practice guidelines from the international task force on canine atopic dermatitis. Veterinary

Dermatology 2010; 21: 233-48.

21. Olivry T, Rivierre C, Jackson HA et al. Cyclosporine decreases skin lesions and pruritus in dogs with atopic dermatitis: A blinded randomized prednisolone-controlled trial. Veterinary Dermatology 2002; 13: 77-87.

22. Olivry T, Sousa CA. The ACVD task force on canine atopic dermatitis (xx): Glucocorticoid pharmacotherapy. Veterinary Immunology and Immunopathology 2001; 81: 317.

23. Kim DH, Son MW, Kim SH. Fatty acid components of hardy kiwifruit (Actinidia arguta) as IL-4 production inhibitor. Biomolecules & Therapeutics 2011; 19: 126-33.

24. Park EJ, Kim B, Eo H et al. Control of IgE and selective Th1 and Th2 cytokines by PG102 isolated from Actinidia arguta. Journal of Allergy and Clinical Immunology 2005; 116: 1151-7.

25. Park EJ, Park KC, Eo H et al. Suppression of spontaneous dermatitis in NC/Nga murine model by PG102 isolated from actinidia arguta. Journal of Investigative Dermatology 2006; 127: 1154-60.

26. Kim D, Kim S, Park EJ et al. Anti-allergic effects of PG102, a water-soluble extract prepared from Actinidia arguta, in a murine ovalbumin-induced asthma model. Clinical & Experimental Allergy 2009; 39: 280-9.

27. Kim D, Kim SH, Park EJ et al. Suppression of allergic diarrhea in murine ovalbumin-induced allergic diarrhea model by PG102, a water-soluble extract prepared from Actinidia arguta. International Archives of Allergy and Immunology 2009; 150: 164-71.

28. Kim SH, Kim S, Lee SH et al. The effects of PG102, a water-soluble extract from Actinidia arguta, on serum total IgE levels: A double-blind, randomized, placebo-controlled exploratory clinical study. European Journal of Nutrition 2011; 50: 523-9.

29. Marsella R, Messinger L, Zabel S et al. A randomized, double-blind, placebo-controlled study to evaluate the effect of EFF1001, an Actinidia arguta (hardy kiwi) preparation, on CADESI score and pruritus in dogs with mild to moderate atopic dermatitis. Veterinary Dermatology 2010; 21: 50-7.

30. Fujiwara S, Yasunaga S, Iwabuchi S et al. Cytokine profiles of peripheral blood mononuclear cells from dogs experimentally sensitized to Japanese cedar pollen. Veterinary Immunology and Immunopathology 2003; 93: 9-20.

31. Peeters D, Peters IR, Farnir F et al. Real-time RT-PCR quantification of mRNA encoding cytokines and chemokines in histologically normal canine nasal, bronchial and pulmonary tissue. Veterinary Immunology and Immunopathology 2005; 104: 195-204.

32. Wang YS, Chi KH, Chu RM. Cytokine profiles of canine monocyte-derived dendritic cells as a function of lipopolysaccharide- or tumor necrosis factor-alpha-induced maturation. Veterinary Immunology and Immunopathology 2007; 118: 186-98.

33. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(t) method. Nature Protocols 2008; 3: 1101-8.

34. Matich A, Young H, Allen J et al. Actinidia arguta: Volatile compounds in fruit and flowers. Phytochemistry 2003; 63: 285.

35. Lim HW, Park M, Han BH et al. Anti-oxidative and nitric oxide production inhibitory activities of phenolic compounds from the fruits of Actinidia arguta. Natural Product Sciences 2006; 12: 221-5.

36. Chang JH, Chan H, Quirce S et al. In vitro T-lymphocyte response and house dust mite–induced bronchoconstriction. Journal of Allergy and Clinical Immunology 1996; 98: 922-31.

37. Nurse B, Puterman AS, Haus M et al. PBMCs from both atopic asthmatic and nonatopic children show a Th2 cytokine response to house dust mite allergen. Journal of Allergy and Clinical Immunology 2000; 106: 84-91.

38. Parronchi P, Macchia D, Piccinni MP et al. Allergen-and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. Proceedings of the National Academy of Sciences 1991; 88: 4538-42.

39. Hales B, Shen H, Thomas W. Cytokine responses to der p 1 and der p 7: House dust mite allergens with different IgE-binding activities. Clinical and Experimental Allergy 2000; 30: 934-43.

40. Smart J, Kemp A. Increased Th1 and Th2 allergen-induced cytokine responses in children with atopic disease. Clinical & Experimental Allergy 2002; 32: 796-802.

41. Akdis M, Verhagen J, Taylor A et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. The Journal of Experimental Medicine 2004; 199: 1567-75.

42. Heaton T, Rowe J, Turner S et al. An immunoepidemiological approach to asthma: Identification of in-vitro T cell response patterns associated with different wheezing phenotypes in children. The Lancet 2005; 365: 142-9.

43. Matsumoto K, Gauvreau GM, Rerecich T et al. IL-10 production in circulating T cells differs between allergen-induced isolated early and dual asthmatic responders. Journal of Allergy and Clinical Immunology 2002; 109: 281-6.

44. Hales B, Hazell L, Smith W et al. Genetic variation of der p 2 allergens: Effects on T cell responses and immunoglobulin E binding. Clinical & Experimental Allergy 2002; 32: 1461-7.

45. Nuttall T, Pemberton A, Lamb J et al. Peripheral blood mononuclear cell responses to major and minor Dermatophagoides allergens in canine atopic dermatitis. Veterinary Immunology and Immunopathology 2002; 84: 143-50.

46. Neumann C, Gutgesell C, Fliegert F et al. Comparative analysis of the frequency of house dust mite specific and nonspecific Th1 and Th2 cells in skin lesions and peripheral blood of patients with atopic dermatitis. Journal of Molecular Medicine 1996; 74: 401-6.

47. Barnes PJ. Anti-inflammatory actions of glucocorticoids: Molecular mechanisms. Clinical Science 1998; 94: 557-72.

48. Sumimoto S, Kawai M, Kasajima Y et al. Increased plasma tumor necrosis factor-alpha concentration in atopic dermatitis. Archives of Disease in Childhood 1992; 67: 277-9.

49. Hawrylowicz CM. Regulatory T cells and IL-10 in allergic inflammation. The Journal of Experimental Medicine 2005; 202: 1459-63.

50. Keppel K, Campbell K, Zuckermann F et al. Quantitation of canine regulatory T cell

populations, serum interleukin-10 and allergen-specific IgE concentrations in healthy control dogs and canine atopic dermatitis patients receiving allergen-specific immunotherapy. Veterinary Immunology and Immunopathology 2008; 123: 337.

51. Akdis CA, Blesken T, Akdis M et al. Role of interleukin 10 in specific immunotherapy. Journal of Clinical Investigation 1998; 102: 98.

국문 초록

아토피 피부염에 이환된 개의 PBMC에서 다래추출물 PG102의 Cytokine 조절 효과

지도교수: 황 철 용

서울대학교 대학원
수의학과 수의내과학(피부과학) 전공
이 용 욱

개에서의 아토피는 소양감을 주 증상으로 하는 알러지 피부염으로 여러 Cytokine의 변화 (Pro-inflammatory cytokine, T_{h1} cell cytokine, T_{h2} cell cytokine, Regulatory T cell cytokine) 는 개의 아토피에서 증상의 발현과 밀접한 관련이 있는 것으로 알려져 있다. 다래추출물 PG102는 여러 연구를 통해 아토피 실험모델, 사람의 아토피 환자에서 항염증 효과를 가진 것으로 연구되고 있는 물질이며 특히 아토피 개에서의 *in vivo* 실험에서 유의적인 임

상증상 감소를 보인 바 있으나 아직 정확한 항염증 기전이 밝혀져 있지 않다. 본 연구는 아토피 피부염을 가진 개의 Peripheral Blood Mononuclear Cell에서 PG102의 항염증 효과를 평가하고 그 기전을 밝히기 위해 수행되었다. PBMC는 아토피 피부염을 가진 8마리의 개에서 분리되었으며 House Dust Mite Extract(DME)로 자극시킨 아토피 개의 PBMC에 PG102를 농도 별로 첨가한 후 (0.1, 1, 10, 100 μg/ml) 12 시간 배양 한 뒤 각각의 cytokine의 발현을 Real-time PCR을 이용한 $2^{-\Delta\Delta C_T}$ 방법으로 정량화하였다. 실험 결과 PG102는 Pro-inflammatory cytokine(TNF-α), T_{h1} cell cytokine(IFN-γ)과 T_{h2} cell cytokine (IL-4, IL-5, IL-13)의 발현을 적정 농도에서 유의적으로 억제하였지만 Regulatory T cell cytokine(IL-10, TGF-β_1)의 억제는 유의적이지 않았다. 농도 별로 살펴보았을 때 PG102 100 μg/ml에서는 조사한 cytokine의 감소는 유의적이지 않았고 1 μg/ml과 10 μg/ml에서 유의적인 cytokine발현 감소가 확인되었다. 이러한 결과는 PG102의 항 염증 메커니즘이 Cytokine 발현 억제와 관련되어 있는 것을 보여주며 PG102가 개의 아토피 피부염에서 스테로이드의 대체 치료제로서의 가능성이 있다고 생각된다.

주요어: PG102, *Actinidia arguta*, canine atopic dermatitis, PBMC, cytokine

학번: 2010-23640