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OVA로 유도된 천식 모델 생쥐에서 木天蓼子가 조절 T 세포, NK T 세포 및 gammadelta T 세포수 변화에 미치는 영향

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Effects of APF and CsA on the number of regulatory T cells, NK T cells and gammadelta T cells in OVA-induced murine model of asthma.

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ABSTRACT

Objectives : To clarify the effects of Actinidia polygama and CsA on OVA-induced asthma model, we examined the influence of Actinidia polygama fructus extract (APF) and CsA on the number of regulatory T cells, NKT cells and γδ T cells in murine model of asthma.

Methods : All mice were immunized on two different days (21 days and 7 days before inhalational exposure) by i.p. injections of OVA in PBS. Seven days after the second sensitization, mice were exposed to aerosolized ovalbumin for 30 min/day on 3 days/week for 12 weeks and APF (400, 40 mg/kg) were orally administered 3 times a week for 8 weeks.

Results : The suppressive effects of APF on asthma model were demonstrated by the increase the number of regulatory T cells, γδ T cells and by reducing the number of NK T cells.

Conclusion : These results indicate that APF has a deep inhibitory effect on airway inflammation and hyperresponsiveness in murine model of asthma by increase the number of regulatory T cells, and γδ T cells and by reducing the number of NK T cells.

Key words : Actinidiae polygamae fructus (APF), asthma, regulatory T cell, NKT cell, γδ T cell

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Introduction

In recent years, allergic asthma has considerably increased in prevalence worldwide. The disease is characterized by AHR and chronic mucosal inflammation mediated by CD4+ Th2 cells[1,2]. Recent work has shown that following antigen inhalation, CD4+CD25+ T cells play a key immunomodulatory role[3].

It has been reported that Th1 responses are more prone to regulation by CD4+CD25+ T cells than Th2 responses[4]. Nevertheless, CD4+CD25+ T cells can suppress Th2 maturation[5], possibly by inhibiting IL-4 production[6]. CD4+CD25+ T cells play a key role in modulating Th2-mediated pulmonary inflammation by suppressing the development of a Th2 phenotype that is highly effective in vivo at promoting airway eosinophilia. NKT cells were originally identified for their ability to rapidly produce IL-4 and IFN-γ upon stimulation using anti-CD3 antibodies. Allergic airway inflammation is worsened through the activation of NKT cells, and in fact, NKT cells are essential for this pathological response. Mice, which lack NKT cells, show very little airway hyperresponsiveness (AHR) and airway inflammation in response to airway allergen challenge[7]. Peripheral γδ T cells directly involved in IL-4 production will represent an important step for understanding and modulating the development of Th2 responses, particularly in the context of allergic diseases such as bronchial asthma[8]. Whereas some authors favor a key role of γδ T cells for the development of allergic airway inflammation[9], others have demonstrated a down-regulatory effect on AHR and immunoglobulin (Ig) E production[10]. γδ T cells play an important role by inducing Th1 immune responses and thus protect against primary allergic sensitization[11]. McMenamin et al. showed that γδ T cells from ovalbumin-tolerant mice selectively suppress immunoglobulin E antibody production by inducing high levels of IFN-γ[12].

Actinidia polygama (Korean name "gae-darae", Actinidiaceae) is one of the well known herb used in oriental medicine for treatment anti-inflammatory and many allergic diseases[13]. *Actinidia polygama* fructus has long been used as a folk medicine in Korea for treating pain, rheumatic arthritis and inflammation[13]. Kim et al. suggested that AP water-soluble fraction (APW) also showed significant inhibitory activity against the rat paw edema induced by a single treatment of carrageenan and dose-dependently suppressed LPS-induced NO production in RAW 264.7 macrophages without a notable cytotoxic effect and also decreased inducible NO synthase (iNOS) protein expression[14].

However, no study on the anti-asthmatic and anti-inflammatory activity of *Actinidia polygama* has been reported. In order to verify its anti-allergic effects, we have investigated the immunomodulatory and anti-inflammatory activities of the plant. The aim of this study was to evaluate the control activity of *Actinidia polygama* extract on influence the number of regulatory T cells, NKT cells and γδ T cells.

To clarify the effects of *Actinidia polygama* and CsA on OVA-induced asthma model, we examined the influence of *Actinidia polygama* fructus extract (APF) and CsA on the number of regulatory T cells, NKT cells and γδ T cells in murine model of asthma.

Our results have shown that APF and CsA have profound inhibitory effects on asthma model by increase the number of regulatory T cells, γδ T cells and by reducing the number of NK T cells.

Materials and methods

1. Plant material and preparation of extracts

The sample of the fruits of Actinidia polygama were purchased from local market (Busan, Korea) in August, 2004. The plant was identified by Professor Young-Cheol Lee, College of Oriental Medicine, Sangji University in Wonju, Korea, and a voucher specimens (APF) are deposited in our laboratory (Department of Herbology, College of Oriental Medicine, Sangji University Wonju

220-702, Republic of Korea). Plant material (200 g) was extracted three times with H_2O. Then, the extract was filtered and evaporated on a rotatory evaporator (Rotary evaporator, BUCHI B-480, Switzerland) and finally dried by a freeze drier (Freeze dryer, EYELA FDU-540, Japan) to yield the extract APF (20 g). The yield (w/w) of the extract was about 10%.

2. Animals

Seven to eight-week-old male C57BL/6 mice were obtained at Daehan Biolink Co. LTD.(Eumsung, Republic of Korea). All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

3. Digestion of pulmonary tissue, spleen, lymph node and cell preparations

Single cell suspensions from lung tissues, spleen, and lymph node were isolated by mechanical disruption in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 20 mM HEPES, and 2% heat-inactivated fetal bovine serum (FBS, GIBCO, Grand Island, NY). Briefly, lungs were subsequently removed from thoracic cavity. After mincing using sterile scalpels, tissue was incubated in PBS containing 1 mg/ml Collagenase IV and 2 mg/ml DispaseII for 40 min at 37 °C in a sterile polypropylene tube. After incubation, lung tissue was vigorously pipetted up and down to further dissolve remaining tissue clumps and then filtered using 70 ㎛ cell-strainer (Falcon, Le Pont de Claix, France). Total cells were counted manually in a hemocytometer chamber(Fisher). $2\text{-}4\times10^3$ cells were spun onto glass slides (Cytospin centrifuge ,Cellspin, Hanil,, Korea) (400 g for 4 minutes). Differential count was made according to standard morphologic criteria.

4. OVA sensitization and inhalation

By modified protocol as previously described[15], OVA (500 μg/㎖) in PBS was mixed with equal volumes of 10%(w/v) aluminum potassium sulfate(alum; Sigma) in distilled water. Then incubated for 60 min at RT after adjustment to pH 6.5 using 10 N NaOH, and centrifuged at 750×g for 5 minutes. OVA/alum pellet was resuspened to the original volume in distilled water. All mice were immunized on two different days (21 days and 7 days before inhalational exposure) by i.p. injections of 0.2 ㎖ alum-precipitated Ag containing 100 μg of OVA (Sigma-Aldrich Korea, Korea) bound to 4 mg of aluminum hydroxide(Sigma-Aldrich Korea. Korea) in PBS. Seven days after the second sensitization, mice were exposed to aerosolized ovalbumin for 30 min/day on 3 days/week for 12 weeks(at a flow rate of 250 L/min, 2.5% ovalbumin in normal saline) and intratracheallyinjected 250 μg of OVA (on day 8) on the back of the tongue. APF (400, 40 mg/kg) and CsA (10 mg/kg) in solution form were orally administered 3 times a week for last 8 weeks. One day after the last of the OVA exposures, samples (lung cells, spleen cells and lymph node) were collected.

5. Antibodies and flow cytometric analysis.

All antibodies(CD3, CD4, CD25, NK1.1, αβ TCR, and γδ TCR) for flow cytometric analysis were purchased from Becton Dickinson (BD) PharMingen (San Diego, CA). Cells from lung tissues, spleen and lymph node were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS and 0.01% NaN3) for 10 min on ice, and analyzed by two color flow cytometry on a FACScan using Cell Quest software (BD Biosciences, Mountain View, CA).

6. Statistical Analysis.

For statistical analysis of data, P-values were analyzed using a paired Student's t-test software program (Startview 5.1; Abacus Concepts, Berkeley, CA). Results were considered statistically significant when P values were *p<0.05, **p<0.01, ***p<0.001.

Results

1 The effects of APF and CsA on lung, spleen and lymph node cells surface marker

Effects of APF on leukocyte subsets in lungs, spleen and lymph node, there were marked change in numbers of CD4+CD25+ T cells(regulatory Th cells) in lung and lymph node, CD3+NK1.1+(NK T cells) in spleen and lymph node in a murine model of asthma compared to control group. APF and CsA treated group with OVA resulted in further significant reductions in NKT cells(p<0.001), and resulted in significant increase CD3+CD25+, γδ T cells (p<0.001) (Table 1, Fig. 1a, b, c).

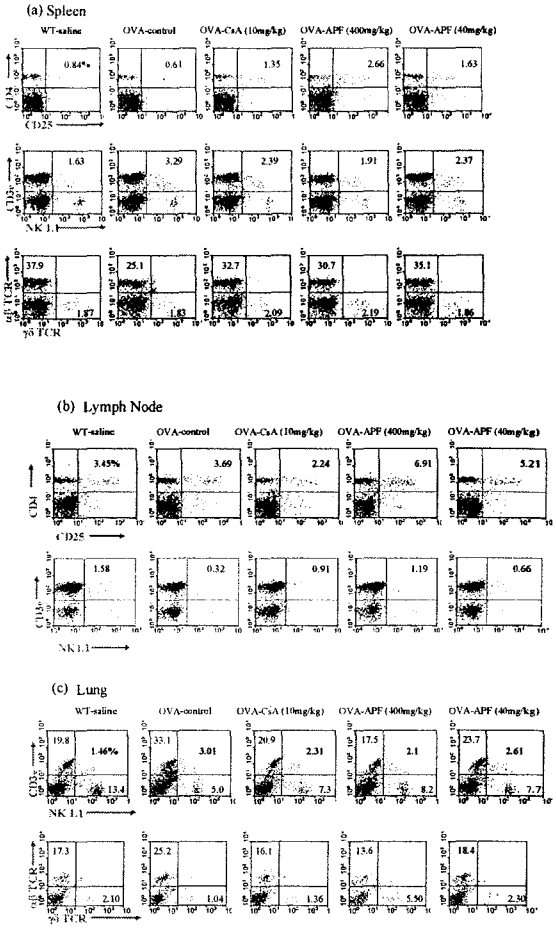
Table 1. Absolute cell numbers in organ of OVA-induced Asthma mice

Cell phenotype	Organ	Normal C57BL/6	OVA-induced asthma mice: Control	CsA	APF (400 mg/kg)	APF (40 mg/kg)
NK1.1+/CD3+ (x 10^5cells)	LN[1]	0.33±0.07	0.23±0.03	0.33±0.05	0.33±0.08	0.55±0.13
	SP[2]	6.6±1.5	34.8±5.8	13.8±4.6	9.0±2.1** *	16.0±5.3** *
	LU[3]	2.7±0.8	27.3±6.3	4.0±1.1	7.6±0.9** *	17.1±5.8** *
CD4+/CD25+ (x 10^4cells)	LN	7.2±1.8	16.9±3.8	8.2±3.3	23.9±4.2*	19.2±4.2
	SP	3.4±0.67	6.4±2.2	7.8±1.5	6.5±2.4	8.9±1.5
	LU	15.6±4.3	19.6±8.2	12.1±2.5	42.6±13.5 ***	18.6±7.8

LN[1]: Lymph node, SP[2]: Spleen, LU[3]: Lung tissue

Each point represents the mean± S.E of 6 mice. Statistically significant value compared with control by T test (*p<0.05 , **p<0.01, ***p<0.001).

Fig.1. Effects of APF and CsA on the number of regulatory T cells(CD4+CD25+), NKT cells(CD3+NK1.1+), alphabeta T cells and gammadelta T cells in OVA-induced murine model of asthma.



Discussion

Actinidia polygama is one of the well known herb used in oriental medicine for treatment anti-inflammatory and many allergic diseases. Anti-inflammatory effects of *Actinidia polygama* in the development of OVA-induced eosinophilia and hyperresponsiveness in murine model of asthma have not been fully investigated in vivo. Cyclosporine A (CsA) has been shown to inhibit single allergen-induced allergic inflammation such as eosinophilic and lymphocytic infiltration and mRNA expression for interleukin (IL)-4 and IL-5. One of the therapeutic objectives in asthma is to

reduce the regional inflammatory response through the reduction of antigen-induced inflammatory cells and inflammatory cytokines production. Kim et al. suggested that AP water-soluble fraction (APW) also showed significant inhibitory activity against the rat paw edema induced by a single treatment of carrageenan[13]. However, no study on the anti-asthmatic and anti-inflammatory activity of *Actinidia polygama* has been reported.

The main question to be addressed is whether APF are involved in the pathology of airway inflammation and asthma. Above of all, regulatory T cells, NKT cells and γδ T cells can regulate airway function. Regulatory T cells can suppress Th2 maturation, and allergic airway inflammation is worsened through the activation of NKT cells, NKT cells are essential for this pathological response. Mice, which lack NKT cells, show very little airway hyperresponsiveness (AHR) and airway inflammation in response to airway allergen challenge. The NKT cell are supposed to be involved in the pathogenesis of allergic inflammation by cytokine regulation, and γδ T cells play a crucial role for the development of allergic airway inflammation and have a down-regulatory effect on AHR and immunoglobulin E production by inducing high levels of IFN-γ.

CD4+CD25+ T cells can suppress the activation and proliferation of other CD4+ and CD8+ T cells in an antigen-nonspecific manner[16,17]. The function of naturally occurring regulatory T cells has been investigated in mouse models of allergic airway disease, and it is noteworthy that these cells do not fully control airway hyperresponsiveness(AHR). Regulatory CD4+CD25+ T cells are important components of the homeostasis of the immune system, as impaired CD4+CD25+ T cell activity can cause autoimmune diseases and allergy[18]. Furthermore, CD4+CD25+ T cells play a key role in regulating airway eosinophilic inflammation. The immunomodulatory properties of CD4+CD25+ T cells do extend to Th2 responses, most notably by limiting the development of a proinflammatory CD4+ Th2 phenotype characterized by reduced cytokine production[18]. Specifically, CD4+CD25+ T cells play a key role in modulating Th2-mediated pulmonary inflammation by suppressing the development of a Th2 phenotype that is highly effective in vivo at promoting airway eosinophilia. While, activation of NKT cells caused further exacerbation of the disease in wild-type mice[7]. γδ T cells play an important role in inflammatory and immune responses in mild asthma.

To clarify the effects of *Actinidia polygama* and CsA on OVA-induced asthma model, we examined the influence of *Actinidia polygama* fructus extract (APF) and CsA on the number of regulatory T cells, NKT cells and γδ T cells in murine model of asthma.

To our results, this is the first study that examines the effects of APF in lung, spleen and lymph node on OVA-induced murine model of asthma after inhaled OVA challenge. We observed that inhalation challenge with APF and CsA administration resulted in a decrease NKT cells, and increase regulatory T cells, γδ T cells when compared with that observed after only OVA challenge.

In summary, APF and CsA have profound effects on airway inflammation and hyperresponsiveness in a murine model of asthma by increase regulatory T cells, γδ T cells and suppression of NK T cells in lung and spleen. The in vivo data, however, seem to be more complex. Future work needs to focus on the regulatory effects of regulatory T cells, NKT cells and γδ T cells in various murine model of asthma. A important area for future studies is the identification of herbal medicines, drugs, cytokines, or costimulatory molecules that induce in vivo growth while preserving the suppressor function of these cells.

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