Anti-inflammatory Activity of Chihyo-san to Protect Respiratory Tissues from Asthmatic Damage

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The present study was carried out to investigate the effect of Chihyo-san (CHS) administration on asthma induced by Alum/OVA treatment in the mice. In CHS-treated animal group, lung weight, which was increased after asthma induction, was significantly decreased, and total number of cells in the lung, peripheral lymph node (PLN) and spleen tissue was significantly decreased in CHS-treated group compared to the asthma control group. The number of immune cells including natural killer (NK) cells in asthmatic animals was largely regulated by CHS treatment, showing a similar pattern as that of CsA-treated positive control group. Levels of mRNAs encoding inflammatory cytokines IL-5, IL-13, TNF-α, and eotaxin were determined by RT-PCR in the lung tissue and showed decreases in CHS-treated group to the similar levels of CsA-treated control group. Histamine level in the serum was significantly lower in CHS-treated group than asthma-induced control group. Both haematoxylin and eosin staining and Masson's trichrome staining results showed decreased number of inflammatory cells, reduced immune cell infiltration, and normalized epithelial cell layering in the bronchial tissue of CHS-treated mouse group. Thus, the present findings suggest that CHS may be useful for protecting bronchial tissues from consistent inflammatory damages that occur in asthma patients.

Key words : Chihyo-san, asthma, Alum/OVA, immune cells, cytokines, bronchial tissues

Introduction

Asthma is defined as a chronic inflammatory disease of airways that is characterized by increased responsiveness of the trachiobronchial tree to a multiplicity of stimuli[33,34]. Asthma shows a pathological manifestation by a narrowing of the air passage and clinically paroxysm of dyspnea, cough, and wheezing. Asthma is very common and estimated that 4% to 5% of the population is affected in most of the countries.

The airway wall in asthmatic patients is infiltrated by different inflammatory cells including T-lymphocytes of the T-helper (Th) type 2 phenotype, eosinophils, macrophages/monocytes and mast cells. This chronic inflammation is associated with airway hyperresponsiveness (AHR) leading to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. Until the late 1980s, oral and inhaled bronchodilators were the mainstay of asthma therapy[11,26]. Their use was aimed at relief of symptoms, and little attention was given to preventive therapy. Inhaled corticosteroids (ICS) were regarded as second medications to be used only when symptoms were intractable and oral corticosteroid use was otherwise inevitable. The evidence that asthma, regardless of its severity, is characterized by extensive inflammation of the airways[8-9,32,41] has warranted the use of ICS in asthma maintenance therapy and systemic corticosteroids in treating severe exacerbations of asthma in the hospital setting. Steroid treatment, especially if high or frequent doses are required, is associated with a range of adverse effects including adrenal suppression and impairment in growth and bone metabolism[44]. These adverse effects and many others, such as skin thinning, easy bruising and cataracts may result from systemic absorption of currently available ICS.

In contrast to steroid medication, use of oriental medicinal drugs has recently drawn attention for the development of therapeutic drugs of asthma. The efficacy of oriental medicinal drugs is a characteristic of a complex mixture of chemical compounds present in the various herbs. The concept of combinatorial medicines has been gaining

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acceptance in the West, as exemplified by the drug cocktail used in the treatment of acquired immunodeficiency syndrome and antibiotics. These therapeutics are mixtures of pure compounds that have been well-characterized. Yet, oriental medicinal drugs represent a much more daunting challenge due to the natural variability of the individual herbs and the chemical complexity of the formulations.

In the present study, possible therapeutic efficacy of Chihyo-san (CHS) as a potential drug of asthma was investigated. CHS is a 15 different mixture of herbal drugs. According to oriental medicinal protocol, most of them are known to be effective for regulating inflammatory, immune, and cardiovascular activities. In clinical oriental medicine, CHS prescription is used for the treatment of respiratory diseases including asthma. However, its effect on asthma treatment has not been demonstrated at the molecular and cellular levels. In the present study, effects of CHS treatment on asthma were investigated using ovalbumin (OVA)-induced asthmatic mouse model. Determination of molecular marker proteins associated with inflammatory activation of immune cells and inflammatory cytokines as well as histological examination of bronchial tissues indicated that CHS treatment could attenuate inflammatory reactions associated with asthma in the mouse.

Materials and Methods

1. Animals and drugs

C57BL/6 mice (18-25 g, obtained from Semtako Co.) were fed food pellets and water. The animals were adjusted for 2 weeks at 22±2℃ in a room of a relative humidity of 50±10%, light intensity of 150~300 Lux and 12 hr of electric light (07:00~19:00) and another 12 hr in the darkness. Only the healthy animals were selected for experiment.

Chihyo-san (CHS) used in this study was obtained from Daejeon University Hospital of Oriental medicine, and was purified before the experimental use. The recipe for CHS per a seal is composed of 6 g of Pinelliae rhizoma (6 g), 4 g of Citri pericarpium, Poria, Perillae fructus, Armeniacae amarum semen, Mori cortex, Platycodi radix, Aurantii fructus each,,3 g of Glycyrrhizae radix and Farfarae flos each, 4 g of Gleditsiae spina, Zingiberis rhizoma recens, Ephedrae herba, Asteris radix, and Friyillariae cirrhosae bulbus each. Two seals of CHS were suspended in 2 liters of distilled water in an autoclave designed for the purpose of herbal drug exaction (S-15000, Saeil Medical) and boiled for 3 hr. CHS filtrate was further concentrated by processing with a rotary vacuum evaporator (Büchi B-480 Co., Switzerland) and freeze-dried for 24 hr using the freeze-drier (Eyela, FDU-540, Japan). The yield was 18.4 g of CHS powder from two seals (120 g) and the product was kept at -84℃ of deep freezer, and resuspended in distilled water immediately before use.

2. Bronchial asthmatic mouse model

Chicken egg ovalbumin (OVA; Grade IV, 500 μg/ml) and 10% (w/v) aluminum potassium sulfate (Alum, Sigma, USA) were dissolved in PBS (pH 6.5). After maintaining for 1 hr at room temperature, the reagent was centrifuged for 5 min at 750x g. OVA/Alum pellets were resuspended in distilled water and 0.2 ml solution containing 100 μg of OVA was intraperitoneally injected into the mouse, and two weeks later, 100 μℓ of OVA (500 μg/ml) were injected into the trachea. To the same animal, 2.5 mg/ml of OVA solution was spray-inhaled into the nasal cavity and respiratory tract for 30 min a day and three times a week for 8 weeks. The control group was treated with either PBS or Alum. Animals were sensitized with OVA/Alum and given oral administration with CHS (400 mg/kg or 200 mg/kg) five times a week. Oral administration of the equivalent volume of distilled water was given to the control group animals.

3. Analysis of fluorescein-labeled cells isolated from the spleen, PLN, and Lung

Spleen, peripheral lymph node (PLN), and lung tissues were dissociated and the cells were isolated after filtration with 100 mesh. After centrifugation for 5 min at 1700 rpm and repeated wash two more times, incompletely digested tissues or debris were removed by passing through the cell strainer. Spleen, peripheral lymph node (PLN), and lung tissues were minced into the small pieces by chopping, treated with collagenase (1 mg/ml in 2% FBS + RPMI 1640) in a 37℃ shaker at 180 rpm for 20 min, and repeated the same procedure three more times to collect the supernatant. Erythrocytes were lysed in ACK solution (8.3 g NH_4Cl, 1 g $KHCO_3$ in 1 liter of demineralized water plus 0.1 mM EDTA) at room temperature for 5 min. The sample was then washed twice with PBS, stained with 0.04% tryphan blue, and used for cell counting. A total of 5 x 10^5 of the spleen cells and lymph node cells were used for immunofluorescence staining. Cells were reacted for 30 min on ice with FITC-anti-CD4 or FITC-anti-CD8, antibodies, washed with PBS three or more times and used for the flow cytometric analysis (Cell Quest program). Positive cell numbers to CD4 and CD8 and the total cell number were determined in the individual tissues.

4. ELISA analysis of serum histamine

The amount of cytokines from mouse serum was measured

by histamine ELISA kit (R&D system). Each antibody was diluted with coating buffer and maintained in microwell at 4℃ overnight. Each well was washed three times and added 100 ㎕ of lung wash solution and serum (diluted 100 times). The samples were reacted for 1 hr at room temperature, washed, and added 100 ㎕ of TMB substrate. After 30 min incubation in a dark place, the samples were treated with 50 ㎕ of stop solution and used for spectrophotometric measurement at 450 nm using ELISA.

5. Real Time Quantitative RT-PCR

1) RNA extraction from lung tissue

Cells were isolated from spleen, PLN and paw joint. The tissues were homogenized in RNAzol^B solution (Tel-Test Inc., Friendswood, USA). Cells were treated with RNAzol^B (500 ㎕) to lyse cells and mixed with 50 ㎕ of chloroform ($CHCl_3$) for 15 min. Cell were placed on ice for 15 min, and centrifuged at 13,000 rpm. Then, the upper later was taken and mixed with 200 ㎕ of 2-propanol gently on ice for 15 min. After phase separation by centrifugation, the pellets were washed with 80% ethanol. After brief spinning and air drying the pellets, RNA pellet was resuspended in 20 ㎕ of DEPC water and heat-treated at 75℃ before the use of the first stranded cDNA synthesis.

2) RT-PCR

Total RNA (3 ㎍) was treated with 3 ㎍ of DNase I (10 U/㎕, 2 U/tube) for 37℃ for 30 min, and denatured for 10 min at 75℃. Denatured RNA was mixed with 2.5 ㎕ of 10 mM dNPTs mix, 1 ㎕ random sequence hexanucleotides (25 pmole/25 ㎕), 1 ㎕ RNasin (20 U/㎕), 1 ㎕ of 100 mM DTT, 4.5 ㎕ 5× RT buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 ㎕ M-MLV RT (200 U/㎕), and H_2O to make the reaction volume to 20 ㎕. The sample was mixed well and incubated for 60 min for the synthesis of first stranded cDNA, and the reaction was stopped by incubating at 95℃ for 5 min. Synthesized cDNA was then used for PCR.

3) Real time quantitative PCR

The PCR was carried out using Applied Biosystems instrument 7500 fast real-time PCR system. The primers used in the present experiment are shown in Table 1. SYBR Green PCR Master mix (ABI) was used for the measurement of inflammatory cytokine expression, human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control, and the primer concentration used was 200 nM. Real-time PCR was performed by following condition: predenaturation for 2 min at 50℃, 10 min at 94℃, 40 cycle progression, then stop reaction for 0.15 min at 95℃ followed by 1 min treatment at 60℃. Quantitative PCR (RQ) data were analyzed by following formula: $y = x(1+e)^n$ (Here, x=starting quantity, y=yield, n=number of cycles, and e=efficiency).

Table 1. Primer sequences for real-time RT-PCR

mouse IL-5	sense	5' agcctaaccctgttggaggt 3'
	antisense	5' gtgatcggcttttcttgagc 3'
mouse IL-13	sense	5' atgcccaacaaagcagagac 3'
	antisense	5' tgagagaaccagggagctgt 3'
mouse TNF-α	sense	5' tgggaggaaaggggtctaag 3'
	antisense	5' acctacgacgtgggctacag 3'
mouse eotaxin 2	sense	5' ctgtgaccatcccctcatct 3'
	antisense	5' cttatggcccttcttggtga 3'
glyceraldehyde-3-phosphate dehydrogenase (G3PDH)	sense	5' tgcgctctagaaaaacctgccaa 3'
	antisense	5' gccccaggctcaaaggtg 3'

6. Pathological tissue examinations

The lung tissue was isolated, fixed with 10% paraformaldehyde solution, and washed with running water for 8 hr. The tissue was then formatted with epoxy, and the tissue sections were prepared by microtome and stained according to the standard procedures of hemotoxylin & eosin (H & E) staining and Masson's trichrome staining.

7. Statistical analysis

The statistical data were represented as mean ± standard error, and comparison amongst data were determined by Student's t-test.

Results

1. Changes in cell numbers and tissue weights

Lung weights were 0.17 ± 0.02 (g) in normal untreated group, 0.40 ± 0.01 (g) in asthma-induced control group, and 0.25 ± 0.01 (g) in cyclosporine (CsA)-treated positive control group. Lung weights in animals treated with 400 mg/kg and 200 mg/kg of CHS were significantly decreased compared with asthma-induced control group, and was similar to those of CsA-treated group(Table 2). The total number of lung cells were 1.43 ± 0.08 ($x10^7$) in normal group, 8.20 ± 0.30 ($x10^7$) in the asthma-induced control group, and 4.73 ± 0.53 ($x10^7$) in CsA-treated positive control group. Measurement of cell numbers in PLN and spleen tissues also showed similar changes after the treatments. CHS treatments at 200 mg/kg and 400 mg/kg decreased cell numbers in lung, PLN, and spleen as summarized in Table 2.

Table 2. Changes in lung weigh and cell numbers of lung, PLN, and spleen tissues

	normal untreated group	asthma-induced	cyclosporine (CsA)-treated	CHS treated	
				200 mg/kg	400 mg/kg
lung weight (g)	0.17±0.02	0.40±0.01	0.25±0.01	***0.26±0.03	***0.29±0.02
lung cell numbers (x 10^7)	1.43±0.08	8.20±0.30	4.73±0.53	5.23±1.03	6.46±1.10
PLN cell number (x10^6)	5.31±0.15	9.82±0.07	8.16±0.48	7.26±0.78	*7.37±0.55
spleen cell number (x10^7)	8.63±0.38	13.10±1.10	9.13±0.13	11.75±1.50	5.25±1.25

*: p<0.05, **: p<0.01, ***: p<0.001 (Student's t-test)

2. Effects of CHS treatment on $CD4^+$ cell numbers

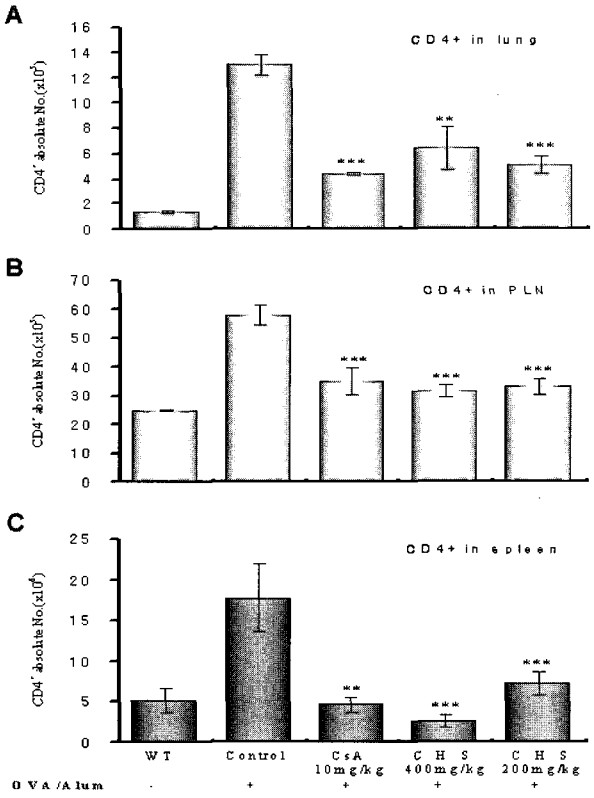

Fig. 1. Effect of CHS extract on $CD4^+$ cell number in the lung, PLN, and spleen of OVA-induced asthmatic mice. C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks for asthma induction. Experimental group animals were treated with 400 mg/kg or 200 mg/kg of CHS, and anti-asthmatic drug cyclosporin A (10 mg/kg) treatment for positive control animals. $CD4^+$ cells in the lung (A), PLN (B) and spleen (C) were analyzed by flow cytometer. The results are expressed as mean ± S.E.M (N=5). **p<0.01, ***p<0.001 (Student's t-test).

In the lung tissue, $CD4^+$ cell numbers were 1.4 ± 0.1 ($x10^5$) in normal group, 1.9 ± 0.4 ($x10^5$) in the negative control group, and 4.4 ± 0.0 ($x10^5$) in CsA-treated positive control group. The cell numbers in the experimental groups were 6.4 ± 1.7 ($x10^5$) and 5.1 ± 0.7 ($x10^5$) at 400 mg/kg and 200 mg/kg of CHS administration respectively, indicating significant decreases at both 400 mg/kg ($p<0.01$) and 200 mg/kg ($p<0.01$) compared with a negative control group(Fig. 1A).

In the PLN, $CD4^+$ cells were 24.8 ± 0.3 ($x10^5$) in normal group, 57.9 ± 3.2 ($x10^5$) in the negative control group, and 35.0 ± 4.5 ($x10^5$) in CsA-treated positive control group. The cell numbers in the experimental groups were 31.6 ± 1.9 ($x10^5$) and 33.1 ± 2.6 ($x10^5$) at 400 mg/kg and 200 mg/kg of CHS administration respectively, indicating significant decreases at both 400 mg/kg ($p<0.001$) and 200 mg/kg ($p<0.001$) compared with a negative control group(Fig. 1B).

$CD4^+$ cell numbers in the spleen were 5.2 ± 1.5 ($x10^6$) in normal group, 17.8 ± 4.2 ($x10^6$) in the negative control group, and 4.6 ± 0.9 ($x10^6$) in CsA-treated positive control group. The cell numbers in the experimental groups were 2.6 ± 0.8 ($x10^6$) and 7.2 ± 1.4 ($x10^6$) at 400 mg/kg and 200 mg/kg of CHS administration respectively, indicating significant decreases at both 400 mg/kg ($p<0.001$) and 200 mg/kg ($p<0.001$) compared with a negative control group(Fig. 1C).

3. Effects of CHS treatment on $CD8^+$ cell numbers

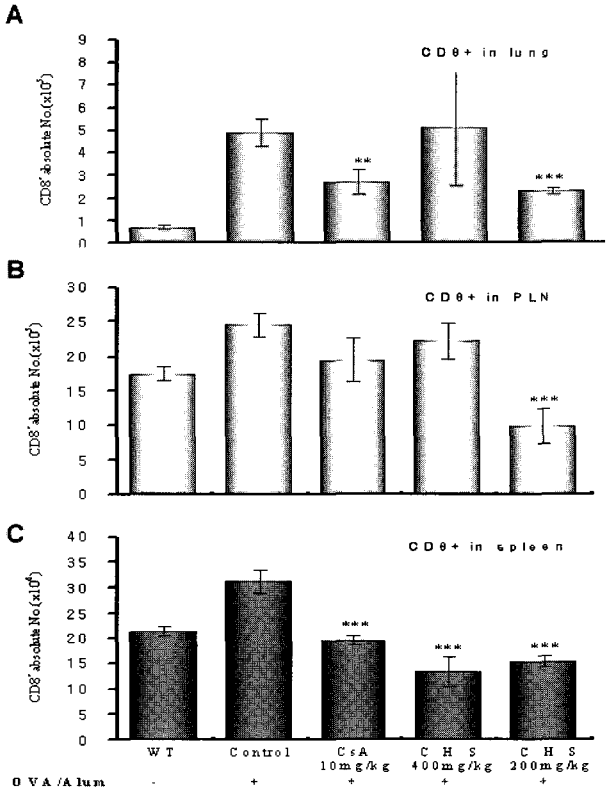

Fig. 2. Effect of CHS extract on $CD8^+$ cell number in the lung, PLN, and spleen of OVA-induced asthmatic mice. C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks for asthma induction. Experimental group animals were treated with 400 mg/kg or 200 mg/kg of CHS, and cyclosporine A (10 mg/kg) treatment for positive control animals. $CD8^+$ cells in the lung (A), PLN (B) and spleen (C) were analyzed by flow cytometer. The results are expressed as mean ± S.E.M (N=5). **p<0.01, ***p<0.001 (Student's t-test).

$CD8^+$ lung cell numbers in the lung were 0.7 ± 0.1 ($x10^5$) in normal group, 4.9 ± 0.6 ($x10^5$) in the negative control group, and 2.7 ± 0.5 ($x10^5$) in CsA-treated positive control group. The cell numbers in the experimental groups were 5.1 ± 2.6 ($x10^5$) and 2.3 ± 0.1 ($x10^5$) at 400 mg/kg and 200 mg/kg of CHS administration respectively, indicating a significant decrease at 200 mg/kg ($p<0.001$) compared with a negative control group (Fig. 2A).

In the PLN, $CD8^+$ cell numbers were 17.5 ± 1.0 ($x10^5$) in normal group, 24.5 ± 1.7 ($x10^5$) in the negative control group, and 19.5 ± 3.1 ($x10^5$) in CsA-treated positive control group. The cell numbers in the experimental groups were 22.2 ± 2.6 ($x10^5$)

and 24.6 ± 3.9 ($x10^5$) at 400 mg/kg and 200 mg/kg of CHS administration respectively, indicating a significant decrease at 200 mg/kg ($p<0.001$) compared with a negative control group (Fig. 2B).

$CD8^+$ spleen cell numbers were 21.3 ± 1.0 ($x10^6$) in normal group, 31.2 ± 2.3 ($x10^6$) in the negative control group, and 19.4 ± 0.9 ($x10^6$) in CsA-treated positive control group. The cell numbers in the experimental groups were 13.4 ± 2.8($x10^6$) and 15.2 ± 1.1 ($x10^6$) at 400 mg/kg and 200 mg/kg of CHS administration respectively, indicating significant decreases at both 400 mg/kg ($p<0.001$) and 200 mg/kg ($p<0.001$) compared with a negative control group(Fig. 2C).

4. Effects of CHS treatment on cytokine production in the lung

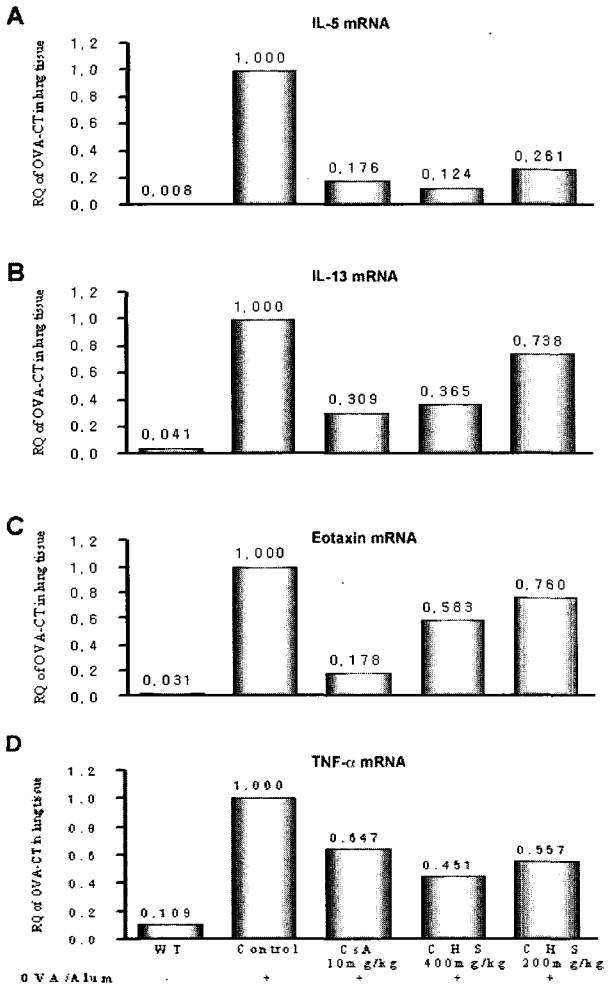

Fig. 3. Effect of CHS extract on mRNA expression in the lung of OVA-induced asthmatic mice. C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks for asthma induction. Experimental group animals were treated with 400 mg/kg or 200 mg/kg of CHS, and cyclosporine A (10 mg/kg) treatment for positive control animals. Mouse lung RNA was isolated and RQ of mRNA expression was analyzed by real-time PCR.

IL-5 mRNA levels in terms of RQ values were 0.008 in normal group, 1.00 in the negative control group, and 0.176 in CsA-treated positive control group. The cell numbers in the experimental groups were 0.124 and 0.261 at 400 mg/kg and 200 mg/kg of CHS administration respectively (Fig. 3A). IL-13 mRNA levels in terms of RQ values were 0.041 in normal group, 1.00 in the negative control group, and 0.309 in CsA-treated positive control group. The cell numbers in the experimental groups were 0.365 and 0.738 at 400 mg/kg and 200 mg/kg of CHS administration respectively (Fig. 3B). Eotaxin 2 mRNA levels in terms of RQ values were 0.031 in normal group, 1.00 in the negative control group, and 0.178 in CsA-treated positive control group. The cell numbers in the experimental groups were 0.451 and 0.557 at 400 mg/kg and 200 mg/kg of CHS administration respectively (Fig. 3C). TNF-α mRNA levels in terms of RQ values were 0.109 in normal group, 1.00 in the negative control group, and 0.647 in CsA-treated positive control group. The cell numbers in the experimental groups were 0.583 and 0.760 at 400 mg/kg and 200 mg/kg of CHS administration respectively (Fig. 3D). These data indicate that CHS inhibited mRNA expression which was induced in the lung of asthmatic animal

5. Effects of CHS treatment on histamine production in the serum

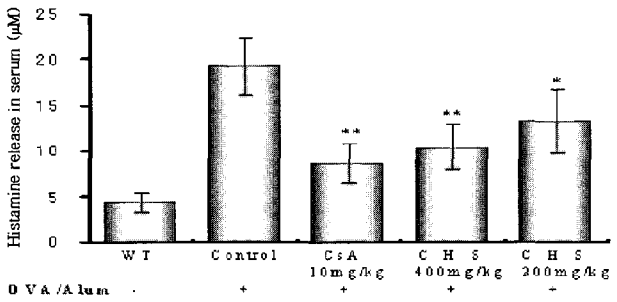

Fig. 4. Effect of CHS extract on histamine release in serum of OVA-induced asthma model in the mouse. C57BL/6 mice were injected, inhaled and sprayed with OVA for 12 weeks for asthma induction. Experimental group animals were treated with 400 mg/kg or 200 mg/kg of CHS, and cyclosporine A (10 mg/kg) treatment for positive control animals. Mouse serum was isolated and histamine release level was analyzed by ELISA. *p<0.05, **p<0.01, (Student's t-test).

Histamine level in the serum was 4.4 ± 101 (μM) in normal group, 19.3 ± 3.1 (μM) in the negative control group, and 8.7 ± 2.2 (μM) in CsA-treated positive control group. The cell numbers in the experimental groups were 10.26 ± 2.6 (μM) and 13.3 ± 3.5 (μM) at 400 mg/kg and 200 mg/kg of CHS administration respectively, indicating a decrease in the experimental groups of both 400 mg/kg and 200 mg/kg ($p<0.01$, $p<0.05$ respectively) compared with a negative control group(Fig. 4).

5. Histological examination of the lung tissues

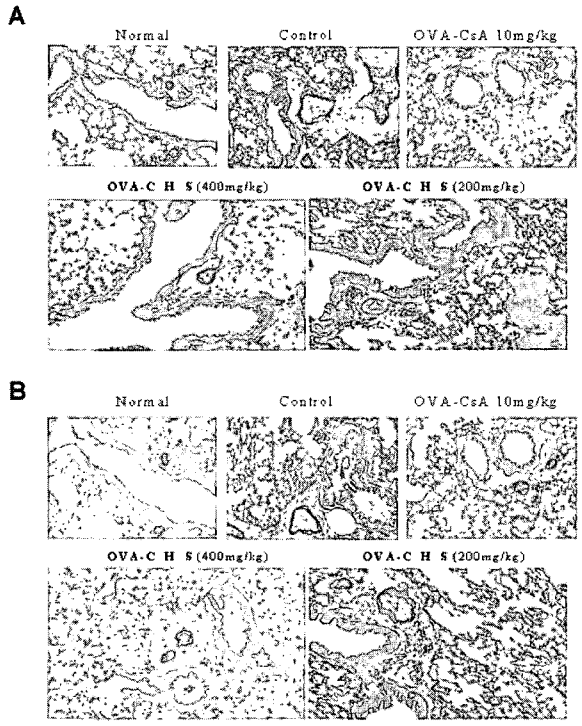

Fig. 5. Histology using H & E staining (A) and Masson's trichromone staining in lung tissue. Normal; C57BL/6 mice. Control; OVA-induced asthmatic mice. OVA-CsA; OVA-induced asthma mice treated with cyclosporine A (10 mg/kg). OVA-CHS; OVA-induced asthma animal treated with CHS (400 mg/kg) or CHS (200 mg/kg).

In normal lung tissue, bronchioles were regularly arranged with columnar cells and single-layered cells were developed along with pulmonary arteries and pulmonary veins. The alveolar lumen showed a structure of interalveolar septum along with capillaries and alveolar epithelial cells. The lung tissues of asthma-induced control group displayed bronchiolar constriction, infiltration of inflammatory cells, multi-layered epithelial cells, and irregular lumen structure. Increased proliferation of smooth muscle cells of the bronchioles and perivascular edema, and irregular sizes of bronchiole caliber were observed. However, proliferation of parenchymal cells and deposition of hyaline materials were not observed. In CsA-treated positive control group, bronchiole constriction and leukocyte infiltration were remarkably decreased and inner lumen of the bronchioles revealed a shape similar to the normal tissue. Also, decreased proliferation of smooth muscle cells and reduced perivascular edema were observed. In the experimental group treated with 200 mg/kg of CHS administration, some bronchiolar constriction and smooth muscle cell proliferation, and moderate levels of leukocyte infiltration were observed. However, perivascular edema was much reduced compared with negative control group. In CHS treated group with 400 mg/kg, low levels of bronchiolar contraction and only a slightly increased smooth muscle cell proliferation were particularly observed. Perivascular edema and leukocyte infiltration were greatly decreased, showing morphologies close to the normal tissue (Fig. 5A, B).

Discussion

The present study was performed to examine the effects of CHS on regulation of cellular and molecular components known to be important for the progression of asthma. For the purpose of this experiment, a mouse model of asthma was developed by treatment of ovalbumin and Alum. The data showed that CHS was effective for regulating parameters related to asthma. Asthma, manifested by airway inflammation, is mediated, in part, by a Th2/Th1 cytokine imbalance that promotes bronchial wall injury[16-18,25]. Asthma, which is characterized by an increase in IL-4, IL-5, IL-13 and IL-9, is considered primarily a Th2-driven process[14,20]. The etiology of airway inflammation, however, remains elusive, encompassing the interplay between genetic predisposition, environmental triggers and dysregulated immune responses[47,48].

The therapeutics in the western medicine include administrations of antiinflammatory drugs (inhaled *corticosteroid;* ICS), *antileukotrienes (cyctyl* leukotriene inhibitor) and several compounds [rapamycin derivative SAR943 (Novartis), Roflumilast (Altana Pharma), a PDE4 inhibitor, PGD2 antagonists (compound 45, S-5751, Shionogi)] have been applied in the clinical fields[3,16]. One of the major problems for using these drugs is the potential side effects and inconsistent effects among patients and according to the severity of disease[5,7].

Alternative approach is to use natural herbal products, which is one of the intense areas of research in the oriental medicine[4,10]. A distinct feature of the oriental medicine from the western medicine is that it emphasizes the balances among numerous factors related to the disease control whereas in the western medicine the relationship between structure and function is primarily focused. Moreover, the oriental medicine considers patients' intrinsic features such as Sasang constitution or predisposition. There have been a few examples demonstrating successful application of oriental medicinal drugs on the treatment of asthma in a randomized trial with patients[15,21,29], and these approaches point to the potential benefits of using oriental medicinal drugs as a complementary therapy reducing the serious side effects of a standard

medication. While these and other studies strongly suggest that oriental medicinal treatment can be efficient for controlling asthma, underlying molecular mechanisms are not generally known.

In the present study, CHS was chosen to examine using the experimental asthma model in the mouse. The animal groups were divided into non-treated normal animal group, OVA-treated negative control group, OVA/CsA-treated positive control group, and CHS-treated experimental group. It was first demonstrated that asthma was properly induced when examined in terms of lung weight and total inflammatory cell numbers in several tissues (; lung, PLN, and spleen) were elevated. CsA, known to attenuate inflammatory responses, effectively decreased inflammatory responses in the respiratory tissues of OVA-induced asthmatic mouse. The first major finding was optimal regulation of immune cell marker proteins in the serum. After inflammation including asthma, immune cell numbers such as $CD4^+$, $CD8^+$ T cells and natural killer cells are increased in the serum[12,13]. In the present study, several criteria were used to determine the responsiveness of the hypersensitized lung cells by Alum/OVA treatment and then changes of the hypersensitivity-related parameters by CHS treatment. In vitro measurement showed significant decreases in granulocyte numbers in CHS-treated asthma-induced mouse group compared to PBS vehicle-treated control animals. Similarly, cell numbers positive to $CD4^+$ and $CD8^+$ were decreased in CHS-treated group compared to negative control group. CD4 and CD8 are surface proteins on helper T cells and are increased as increases in Th2 cells during hypersensivity reaction[2]. Treatment of CHS significantly decreased cells that are positive to these proteins. Determination of total cells in the lung, PLN, and spleen as well as in the granulocytes showed that increased cell numbers by asthma induction were significantly decreased by CHS treatment. These results strongly indicate that CHS may have an alleviating effect on Th2 cell activation induced by allergic asthma, and also decreased levels of other immune cell numbers in the lung, PLN and spleen.

The current study further showed that CHS treatment contributed to regulate levels of inflammation-related cytokine mRNA expression. Real time PCR showed that CHS treatment decreased levels of IL-5, IL-13, TNF-α, and eotaxin mRNA. TNF-α, along with IL-1β is a prototypical inflammatory cytokine responding to and inducing on-going inflammatory reactions including activation of endothelial cells, fibroblast and leukocytes as well as systemic acute-phase reactions[19,22-23,27-28]. During allergic inflammation, Th2 cells are dominant above Th1 cells, and Th2 cells represent the only cell in the immune system that can both directly recognize the allergen peptides via the T cell receptor (TCR) and, at the same time, release interleukins that account for the joint involvement of IgE antibody-producing B cells (IL-4, IL-13), mast cells (IL-4, IL-10), and eosinophil granulocytes (IL-5) in allergic inflammation[35-36,38]. Th2 cytokines IL-4, IL-5, IL-9, and IL-13 can account directly or indirectly for the great majority of pathophysiological manifestations of allergic patients[1,31,39]. IL-4 is able to induce the rolling on, and adhesion to, endothelial cells of circulating eosinophils[6], which can then be attracted into target tissues by both IL-5 and chemokines. IL-13 is responsible for mucus hypersecretion by mucus cells, and induces metaplasia of mucus cells[30]. IL-4 and IL-13 stimulate fibroblast growth and chemotaxis, as well the synthesis of extracellular matrix proteins[40,43]. Eotaxin is eosinophil-specific chemoattractant and generated at a site of allergic inflammation, and has been suggested to contribute to eosinophil accumulation and activation[42]. Eotaxin mRNA, which was induced by asthmatic inflammation in the lung tissue, was greatly decreased by CsA control group. CHS treatment decreased eotaxin levels partly.

Third finding from the present investigation was morphological recovery of bronchial tissues after CHS treatment. In normal lung tissue, bronchioles were regularly arranged with columnar cells and single-layered cells were developed along with pulmonary arteries and pulmonary veins. The alveolar lumen showed a structure of interalveolar septum along with capillaries and alveolar epithelial cells. Major changes after asthma induction were the patterning of bronchiolar constriction, infiltration of inflammatory cells, and epithelial cell laying. Levels of infiltration and bronchial construction and other indicators of inflammatory responses in asthmatic tissues were much improved by CHS treatment.

The present finding is significant because histological changes are one of the most distinct criteria evaluating asthma. Thickening of the lamina reticularis occurs as a prominent histologic finding in the airways of patients with asthma, although it correlates poorly with the duration or severity of asthma. Airway smooth muscle cells secrete increased amounts of fibronectin in response to TGF-β and VEGF[24], whereas fibroblasts from asthmatic subjects secrete four times more proteoglycan than those from normal subjects[46]. Biglycan and decorin also induce morphological and cytoskeletal changes in lung fibroblasts, resulting in cell migration[37,45]. The physiological relevance of alterations in matrix deposition in the bronchial wall of asthmatic patients is unclear, although the deposition of proteoglycans and glycosaminoglycans probably contributes to the altered mechanical properties of the

airway wall and might promote airway obstruction. Together, the present study strongly indicate that CHS may play a role in protecting the lung tissues from asthmatic damage.

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