

## Effects of *Chongyeal-sodok-yeum* on chemokines expression in lung epithelial cells

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### 清熱消毒飲이 사람 폐 상피세포인 A549 세포에서의 chemokine 발현에 미치는 영향

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기관지 천식은 가역적 기도 폐색, 호산구에 의한 만성기관지 염증 및 기관지 수축 물질에 대한 기관지 평활근의 과민반응 등 3가지 주 증상을 갖는 만성 염증성 호흡기 질환의 일종으로서 천식은 항원에 대한 노출이 과민반응으로 이어져 기도 내 염증 유발로 이어지면 조직 내 백혈구 침윤이 일어나게 되어 기도 상피세포의 손상 및 기도 폐색이 일어나게 된다. 이 때 백혈구를 혈액에서 조직으로 끌어오는 것이 chemotactic cytokine, 즉 chemokine이다.

본 실험은 사람의 폐 상피세포를 이용하여 염증유발 매개물질인 TNF- $\alpha$ 와 IL-4를 단독 혹은 병용 투여하여, 폐 상피세포에서 chemokine 중 호중구의 화학주성에 관여하는 TARC, eotaxin, RANTES 의 생성을 유도하였고, 이러한 chemokine의 생성과정에서 清熱消毒飲이 미치는 영향에 대하여 연구하였다.

본 연구를 통하여 清熱消毒飲이 사람의 폐 상피세포에서 TNF- $\alpha$ 와 IL-4로 유발시킨 TARC, eotaxin, RANTES의 생성을 농도 의존적으로 억제하는 효과를 볼 수 있었다.

따라서 清熱消毒飲은 TARC, eotaxin, RANTES와 같은 chemokine 생성을 억제함으로써 천식을 포함한 알레르기 질환 치료 전반에 유의적인 효과를 보일 것으로 사려 된다.

**Key words :** Chongyeal-Sodok-Yeum, Asthma, TARC, Eotaxin, RANTES, IL-4, TNF- $\alpha$

### 1. INTRODUCTION

Asthma is currently considered and defined as a

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chronic inflammatory disorder of the airway mucosa. It was also reported that several chemokines contribute to the allergic inflammatory response associated with this condition<sup>1-4)</sup>.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-4 (IL-4) are known to stimulate

transcription of this gene in lung fibroblasts<sup>5)</sup> and human airway epithelial cells<sup>6)</sup>. It was reported that combination of IL-4 and TNF- $\alpha$  may contribute to allergic disease by recruiting eosinophils, and induce various chemokines in the bronchial epithelial A549 cells<sup>7)</sup>.

Chemokines are small, secreted polypeptides that regulate the tissue-specific recruitment and migration of lymphocytes by signaling through G protein-coupled 7-transmembrane receptors. Some of the most important eosinophils chemoattractant cytokines are IL-5, IL-8, thymus and activation-regulated chemokine (TARC), eotaxin, regulated on activation normal T cells expressed and secreted (RANTES) and TNF- $\alpha$ . Of these, TARC, as a selective chemoattractant of T-helper cells type-2 (Th2) cells, is a reasonable candidate as a key regulator of Th2-mediated inflammation in allergic asthma<sup>8)</sup>. Eotaxin (CCL11) is a CC chemokine that stimulates the migration of eosinophils from the small blood vessels in the lungs by acting on the CC chemokine receptor CCR3<sup>9)</sup>. RANTES is a member of the CC chemokine family and contributes to viral-induced airway inflammation including exacerbations of asthma<sup>10)</sup>.

*Chongyeal-Sodok-Yeum* (CSY), combined preparation of nine herbal medications, has been traditionally used as a therapy for various clinical symptoms associated with inflammation<sup>11)</sup>.

However, the effects of CSY on TNF- $\alpha$ - and IL-4-induced asthmatic allergy were not reported yet. In the present study, inhibitory effects of the CSY on TNF- $\alpha$ - and IL-4-stimulated TARC, eotaxin and RANTES were investigated using human bronchial epithelial A549 cells.

## II. MATERIALS AND METHODS

### 1. Cell culture

A549 cells, a human types II bronchial epithelial cell line<sup>12)</sup> were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO<sub>2</sub> and 95% air in a humidified cell incubator.

### 2. Preparation of Chongyeal-Sodok-Yeum (CSY)

The plant materials were obtained from the Semyung Oriental Medicine Hospital (Jecheon, Chungbuk) and authenticated by Professor Leem, College of Oriental Medicine, Semyung University. The ingredient of CSY include 8 g of *Lonicerae Flos*, 6 g of *Paeoniae Radix Rubra*, 6 g of *Rehmaniae Radix*, 6 g of *Cnidii Rhizoma*, 4 g of *Angelicae Gigantis Radix*, 4 g of *Coptidis Rhizoma*, 4 g of *Gardeniae Fructus*, 4 g of *Forsythiae Fructus* and 4 g of *Glycyrrhizae Radix*.

Table 1. Components of Chongyeal-Sodok-Yeum (CSY)

Drug Name	Herb Name	Dose(g)
Lonicerae Flos	金銀花	8
Paeoniae Radix Rubra	赤芍藥	6
Rehmaniae Radix	生地黃	6
Cnidii Rhizoma	川芎	6
Angelicae Gigantis Radix	當歸	4
Coptidis Rhizoma	黃連	4
Gardeniae Fructus	梔子	4
Forsythiae Fructus	連翹	4
Glycyrrhizae Radix	甘草	4
Total amount		46

An extract of CSY was prepared by decocting the dried prescription of herbs with boiling distilled water (92 g/l). The duration of decoction was about 3 hrs. The decoction was filtered, lyophilized and kept at 4°C. The resulting powder, weighing 14 g (a collection rate of 15.2%), was dissolved in sterile saline.

### 3. MTT cytotoxicity assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) assay kit as per the manufacturer's protocol. In order to determine the cytotoxicity of CSY, cells were treated with CSY at concentrations of 0.5 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml and 50 µg/ml for 24 hrs. Cultures of the control group were left untreated.

MTT labeling reagent (10 µl) was added to each well, and the plates were incubated for 4 hrs. The solubilization solution (100 µl) was then added to each well, and the cells were incubated for another 12 hrs.

The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm and a reference wavelength of 690 nm. Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) × 100.

### 4. Measurement of TARC, eotaxin and RANTES production

TARC, eotaxin and RANTES were measured by ELISA R&D Systems (Minneapolis, MN, USA) as

described<sup>13)</sup>.

Briefly, 96-well plates were coated with 0.1 µg/ml polyclonal mouse anti-human TARC, eotaxin and RANTES, as capturing antibodies, and stored overnight at 4°C. The following day, the plate was washed with 50 mM PBS/Tween 20 and nonspecific binding was blocked by treatment with 1% BSA for 1 hr. After washing, a standard series of diluted human recombinant TARC, eotaxin and RANTES proteins and supernatant samples was added and incubated for 2 hrs. Next, the plates were then incubated for 1 hr with 1 µg/ml goat anti-human secondary antibody. The sections were subsequently incubated for another 1 hr with an avidin-biotin-horseradish peroxidase complex (1:50; Vector Laboratories, Burlingame, CA, USA). Bound horse redox peroxidase (HRP) was visualized with 3,3',5,5'-tetramethylbenzidine (TMB) containing hydrogen peroxide. The plate was incubated at room temperature with shaking, and the reaction was stopped through the addition of 1 M H<sub>2</sub>SO<sub>4</sub>. The absorbance of the content of each well was then measured at 450 nm.

### 5. Statistical analyses

The results are expressed as the mean standard error mean (S.E.M.). The data were analyzed by one-way ANOVA followed by *t*-test using SPSS (Version 11.5). Difference was considered statistically significant at *P* < 0.05.

## III. RESULTS

### 1. Effect of CSY on cell viability

The viabilities of cells treated with CSY at

concentrations of 0.5  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$ , 5  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$  and 50  $\mu\text{g/ml}$  for 24 hrs were  $100.04 \pm 0.66\%$ ,  $99.52 \pm 3.29\%$ ,  $101.01 \pm 2.28\%$ ,  $103.10 \pm 1.44\%$ ,  $105.72 \pm 0.56\%$  and  $104.28 \pm 1.51\%$  of the control value, respectively, indicating that CSY in itself does not possess overtly toxic effects on A549 cells.

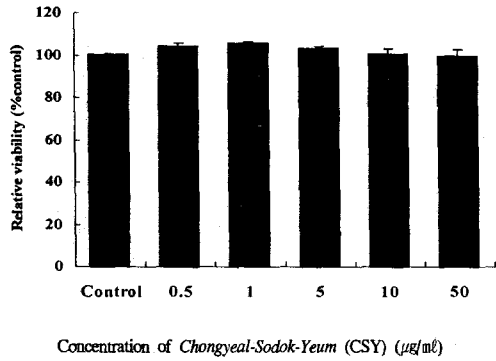


Fig. 1. Effects of Chongyeal-Sodok-Yeum (CSY) on cell viability.

Results are represented as mean standard error mean (S.E.M.).

A549 cells were treated with CSY at concentrations of 0.5  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$ , 5  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$  and 50  $\mu\text{g/ml}$  for 24 hrs.

No change in viability was observed following treatment with CSY.

## 2. Effects of CSY on TARC release

From TARC immunoassay, the amount of TARC concentration was 0 or negligible in the control or 5 ng/ml TNF- $\alpha$  only or 5 ng/ml IL-4 only treatments for 24 hrs. However, this figure increased to  $14.08 \pm 1.23 \text{ pg/ml}$  by treatment with TNF- $\alpha$  and IL-4, while decreased to  $12.62 \pm 1.06 \text{ pg/ml}$ ,  $9.30 \pm 0.58 \text{ pg/ml}$  and  $7.83 \pm 0.39 \text{ pg/ml}$  by the treatment with CSY at 0.5  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$  and 5  $\mu\text{g/ml}$ , respectively.

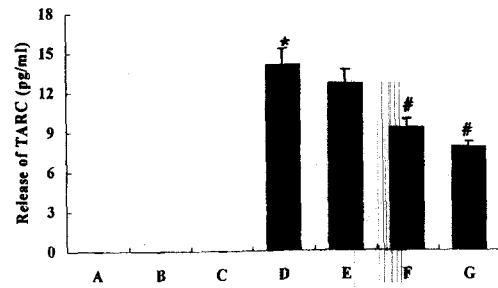


Fig. 2. Measurement of TARC production in bronchial epithelial A549 cells.

- A: Control
  - B: TNF- $\alpha$  treated group
  - C: IL-4 treated group
  - D: TNF- $\alpha$  and IL-4 treated group
  - E: TNF- $\alpha$ , IL-4 and 0.5  $\mu\text{g/ml}$  CSY treated group
  - F: TNF- $\alpha$ , IL-4 and 1  $\mu\text{g/ml}$  CSY treated group
  - G: TNF- $\alpha$ , IL-4 and 5  $\mu\text{g/ml}$  CSY treated group
- \* represents  $P < 0.05$  compared to the control  
# represents  $P < 0.05$  compared to the TNF- $\alpha$  and IL-4 treated group

## 3. Effects of CSY on eotaxin release

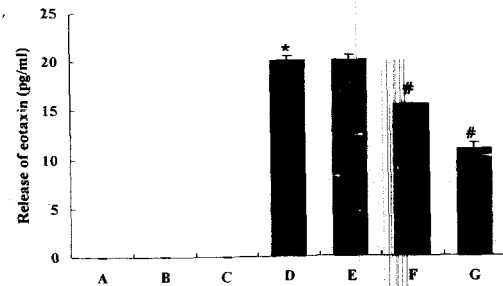


Fig. 3. Measurement of eotaxin production in bronchial epithelial A549 cells.

- A: Control
  - B: TNF- $\alpha$  treated group
  - C: IL-4-treated group
  - D: TNF- $\alpha$  and IL-4 treated group
  - E: TNF- $\alpha$ , IL-4 and 0.5  $\mu\text{g/ml}$  CSY treated group
  - F: TNF- $\alpha$ , IL-4 and 1  $\mu\text{g/ml}$  CSY treated group
  - G: TNF- $\alpha$ , IL-4 and 5  $\mu\text{g/ml}$  CSY treated group
- \* represents  $P < 0.05$  compared to the control  
# represents  $P < 0.05$  compared to the TNF- $\alpha$  and IL-4 treated group

From eotaxin immunoassay, the amount of eotaxin concentration was 0 or negligible in the control or 5 ng/ml TNF- $\alpha$  only or 5 ng/ml IL-4 only treatment for 24 hrs. However, this figure increased to  $20.01 \pm 0.42$  pg/ml by treatment with TNF- $\alpha$  and IL-4, while decreased to  $15.43 \pm 0.05$  pg/ml and  $10.94 \pm 0.64$  pg/ml by the treatment with CSY at 1  $\mu$ g/ml and 5  $\mu$ g/ml, respectively.

#### 4. Effects of CSY on RANTES release

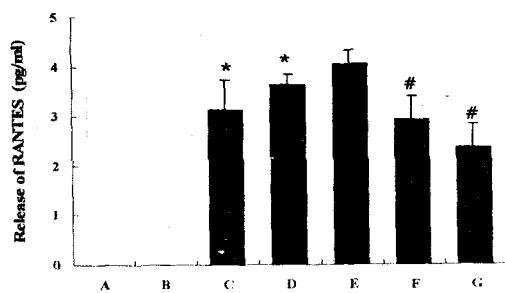


Fig. 4. Measurement of RANTES production in bronchial epithelial A549 cells.

- A: Control
  - B: IL-4 treated group
  - C: TNF- $\alpha$  treated group
  - D: TNF- $\alpha$  and IL-4 treated group
  - E: TNF- $\alpha$ , IL-4 and 0.5  $\mu$ g/ml CSY treated group
  - F: TNF- $\alpha$ , IL-4 and 1  $\mu$ g/ml CSY treated group
  - G: TNF- $\alpha$ , IL-4 and 5  $\mu$ g/ml CSY treated group.
- \* represents  $P < 0.05$  compared to the control  
# represents  $P < 0.05$  compared to the TNF- $\alpha$  and IL-4 treated group

From RANTES immunoassay, the amount of RANTES concentration was 0 or negligible in the control or 5 ng/ml IL-4 only treatment for 24 hrs. The amount of RANTES concentration was  $3.13 \pm 0.60$  pg/ml in the 5 ng/ml TNF- $\alpha$  only treatment for 24 hrs. However, this figure increased to  $3.63 \pm 0.21$  pg/ml by treatment with TNF- $\alpha$  and IL-4,  $4.06$

$\pm 3.44$  pg/ml by the treatment with TNF- $\alpha$ , IL-4 and 0.5  $\mu$ g/ml CSY, while decreased to  $2.94 \pm 0.46$  pg/ml,  $2.37 \pm 0.45$  pg/ml by the treatment with CSY at 1  $\mu$ g/ml and 5  $\mu$ g/ml, respectively.

#### IV. DISCUSSION

Airway inflammation plays a central role in the pathogenesis of asthma. The large and medium airways of patients with asthma show evidence of chronic inflammation, including leukocyte infiltrates in bronchial tissue, excessive mucus production, epithelial damage, basement membrane thickening and smooth muscle hypertrophy<sup>1-4,14</sup>. Asthma is associated with atopy and recruitment of eosinophils to the airways, leading to the hypothesis that its pathogenesis is driven by a Th2 response to inhaled antigens<sup>15</sup>.

Numerous studies have demonstrated that TNF- $\alpha$  and IL-4 attributes contribute to the inflammatory conditions present in airway of asthmatic subjects. TNF- $\alpha$  is expressed in asthmatic airways and may play a key role in amplifying atopic inflammation through the activation of various transcription factors such as nuclear factor- $\kappa$ B (NF- $\kappa$ B). TNF- $\alpha$  is expressed primarily by the alveolar cells and tissue macrophages, mast cells, and bronchial cells, and increases the production of several chemotaxins<sup>16-17</sup>. IL-4 is critical for the synthesis of IgE by B lymphocytes and to the development of Th2 cells. IL-4 receptor blocking antibodies inhibit allergen-induced airway hyperresponsiveness and pulmonary eosinophilia in a murine model<sup>18</sup>.

In addition, co-treatment of TNF- $\alpha$  and IL-4 were reported to synergize in the secretion of various chemokines<sup>4,19-20</sup> reported that several chemokines

and their receptors involve with pathogenesis of allergic asthma. Several members of the C-C branch of chemokines exhibit chemoattractant properties toward eosinophils, and these include TARC, eotaxin and RANTES. Numerous studies suggested that TNF- $\alpha$  and IL-4 treatment stimulates production of TARC<sup>21)</sup>, eotaxin<sup>18)</sup> and RANTES<sup>22)</sup>. Present results also shown that TNF- $\alpha$  and IL-4 treatments enhances TARC, eotaxin and RANTES releases in human bronchial epithelial A549 cells.

CSY or its components possesses anti-inflammatory effects<sup>11)</sup> and is composed of nine herbs. Of the CSY ingredients, *Paeoniae Radix Rubra* is one of the important medicinal herbs that are widely used for the treatment of various allergic diseases by suppressing secretions of monocyte chemoattractant protein (MCP)-1 and -3 in human nasal fibroblasts<sup>23-24)</sup>. However, no study on the effect of CSY on the TNF- $\alpha$ - and IL-4-stimulated TARC, eotaxin and RANTES generation has been made yet.

In the results of present study, the crude extract of CSY suppressed the TNF- $\alpha$ - and IL-4-stimulated TARC, eotaxin and RANTES production in the human bronchial epithelial A549 cells.

Based on the present results, CSY may be useful in the treatment asthmatic allergy by inhibiting TARC, eotaxin and RANTES chemokines.

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