Effects of Hwangryun-Hae-Dok-tang on TNF-α and IL-4 Stimulated TARC, eotaxin, RANTES in the Human Bronchial Epithelial A549 Cells

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Allergic diseases are the result of Th2-dominated responses to single or multiple environmental allergens. Th2 cytokines regulate these mechanisms of allergic disease at many levels, including initiation, progression, and persistence. The effect of Hwangryun-Hae-Dok-Tang (HRHDT) on tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) stimulated inflammation was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide assay, thymus and activation-regulated chemokine (TARC), eotaxin, regulated on activation normal T cells expressed and secreted (RANTES) immunoassay on the human bronchial epithelial microglial cells. From the present study, the crude extract of Hwangryun-Hae-Dok -tang suppressed the TNF-α and IL-4 stimulated TARC, eotaxin, and RANTES production in the human bronchial epithelial A549 cells. Based on the present results, Hwangryun-Hae-Dok-tang may be useful in the treatment asthmatic allergy by inhibiting TARC, eotaxin, and RANTES chemokines.

Key words : Hwangryun-Hae-Dok-tang, Allergy, TARC, Eotaxin, RANTES

Introduction

Allergic diseases are the result of Th2-dominated responses to single or multiple environmental allergens. Th2 cells represent indeed the only cell in the immune system that can both directly recognize the allergen peptides via the T cell receptor and at the same time, release interleukins (ILs) that account for the joint involvement of IgE antibody-producing B cell, mast cells and eosinophil granulate in allergic inflammation[1].

The role of Th2 cells in allergic inflammation is not limited to their capacity to induce the production of allergen-specific IgE antibodies by B cells and to promote the infiltration of target tissues by mast cells and eosinophils. It is of note that the Th2 cytokines IL-4, IL-5, IL-9 and IL-13 can account directly or indirectly for great majority of pathophysiological manifestations of allergic patients[2]. Of these, IL-4 is indeed able induce the rolling on, and adhesion to, endothelial cells of circulating eosinophils[3], which can then be attracted into target tissues by both IL-5 and chemokines. And TNF-α has been shown to activate the inflammatory cells, up-regulate the adhesion molecules on endothelium and circulating leukocytes, increase the production of chemotaxins[4-5].

It was also reported that combination of IL-4 and TNF-α may contribute to allergic disease by recruiting eosinophils, and induce various chemokines in the bronchial epithelial A549 cells[6]. These findings indicate that IL-4 and TNF-α can account for all the hallmarks of allergic inflammation[2].

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-α. Of these, TARC, as a selective chemoattractant of T-helper cells type-2 cells, is a reasonable candidate as a key regulator of Th2-mediated inflammation[7]. Eotaxin is a CC chemokine that stimulates the migration of eosinophils from the small blood vessels by acting on the CC chemokine receptor CCR3[8]. RANTES is a member of the CC chemokine family and contributes to viral-induced inflammation[9].

Hwangryun-Hae-Dok-Tang (HRHDT), combined preparation of four herbal medications, has been traditionally used as a therapy for various clinical symptoms associated with gastrointestinal disorders, inflammation, and cardiovascular diseases[10-12].

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However, the effects of HRHDT on TNF-α and IL-4 induced allergic inflammation were not reported yet. In the present study, inhibitory effects of the HRHDT on TNF-α and IL-4 stimulated TARC, eotaxin, and RANTES were investigated using human bronchial epithelial A549 cells.

Materials and Methods

1. Cell culture

A549 cells, a human types II bronchial epithelial cell line[13] 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℃ in 5% CO_2 and 95% air in a humidified cell incubator.

2. Preparation of Hwangryun-Hae-Dok-Tang(HRHDT)

The plant materials were obtained from the Semyung Oriental Me- dcine Hospital (Jecheon, Chungbuk) and authenticated by Professor Leem, College of Oriental Medicine, Semyung University. The ingre- dient of HRHDT (Table 1) include 20 g of , Coptidis rhizome, Scu- tellariae radix, Phellodendri cortex and Gardeniae fructus. An extract of HRHDT was prepared by decocting the dried presc- ription of herbs with boiling ditilled water (100 g/ ℓ). The duration of decoction was about 3 hrs. The decoction was filtered, lyophilized and kept at 4°C. The resulting powder, weighing 16 g (a collection rate of 16%), was dissolved in sterile saline.

Table 1. Components of Hwangryun-Hae-Dok-Tang(HRHDT)

Herb Medical Name	Herbs	Scientific Name	Dose(g)
Coptidis rhizome	黃連	Coptis japonica	5
Scutellariae radix	黃芩	Scutellaria baicalensis	5
Phellodendri cortex	黃栢	Phellodendronamurense	5
Gardeniae fructus	梔子	Gardenia jasminoides	5
		Total amount	20

3. MTT cytotoxicity assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit as per the manufacturer's protocol. In order to determine the cytotoxicity of HRHDT, cells were treated with HRHDT at concentrations of 0.5 ㎍/㎖, 1 ㎍/㎖, 5 ㎍/㎖, 10 ㎍ /㎖, and 50 ㎍/㎖ for 24 hrs. Cultures of the control group were left untreated. Ten ㎕ of the MTT labeling reagent was added to each well, and the plates were incubated for 4 hrs. One hundred ㎕ of the solubilization solution 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 ㎚ and a reference wavelength of 690 ㎚. 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[14]. Briefly, 96-well plates were coated with 0.1 ㎍/㎖ 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 ㎛ 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 ㎍/㎖ 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 reddish peroxidase (HRP) was visualized with 3,3',5,5'-tetramethyl- benzidine (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_2SO_4. The absorbance of the content of each well was then measured at 450 ㎚.

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 $P < 0.05$.
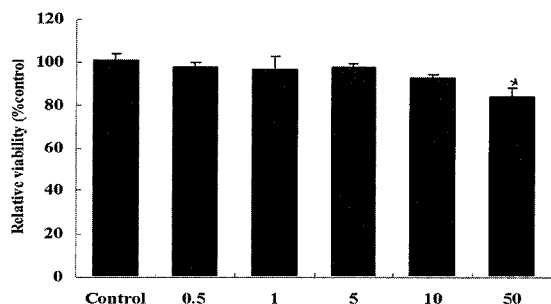
Results

1. Effects of HRHDT on cell viability

The viabilities of cells treated with HRHDT at concentrations of 0.5 ㎍/㎖, 1 ㎍/㎖, 5 ㎍/㎖, 10 ㎍/㎖, and 50 ㎍/㎖ for 24 hrs were 100.72 ± 3.07%, 97.78 ± 2.11%, 97.07 ± 6.11%, 97.66 ± 1.75%, and 83.73 ± 4.17% of the control value, respectively, indicating that HRHDT in itself does not possess overtly toxic effects on A549 cells.

2. Effects of HRHDT on TARC release

From TARC immunoassay, the amount of TARC concentration was 0 or negligible in the control or 5 ng/㎖ TNF-α only or 5 ng/㎖ IL-4 only treatments for 24 hrs.

However, this figure increased to 14.08 ± 1.23 pg/mℓ by treatment with TNF-α and IL-4, while decreased to 12.50 ± 1.11 pg/mℓ, and 10.16 ± 1.00 pg/mℓ by the treatment with HRHDT at 1 μg/mℓ and 5 μg/mℓ, respectively.

Fig. 1. **Effects of Hwangryun-Hae-Dok-Tang(HRHDT) on cell viability.** Results are represented as mean ± standard error mean (S.E.M.). A549 cells were treated with HRHDT at concentrations of 0.5 μg/ml, 1 μg/ml, 5 μg/ml, 10 μg/ml, and 50 μg/ml for 24 hrs. No change in viability was observed following treatment with HRHDT.
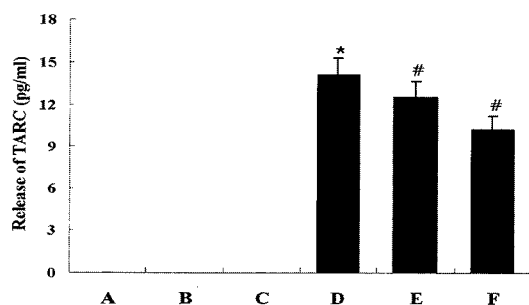

Fig. 2. **Measurement of TARC production in bronchial epithelial A549 cells.** A: Control B: TNF-α treated group C: IL-4 treated group D: TNF-α and IL-4 treated group E: TNF-α, IL-4 and 1 μg/ml HRHDT treated group F: TNF-α, IL-4 and 5 μg/ml HRHDT treated group *represents P < 0.05 compared to the control #represents P < 0.05 compared to the TNF-α and IL-4 treated group

3. Effects of HRHDT on eotaxin release

From eotaxin immunoassay, the amount of eotaxin concentration was 0 or negligible in the control or 5 ng/mℓ TNF-α only or 5 ng/mℓ IL-4 only treatment for 24 hrs. However, this figure increased to 20.01 ± 0.42 pg/mℓ by treatment with TNF-α and IL-4, while decreased to 17.58 ± 0.21 pg/mℓ, and 16.02±0.82 pg/mℓ by the treatment with HRHDT at 1 μg/mℓ, and 5 μg/mℓ, respectively.

4. Effects of HRHDT on RANTES release

From RANTES immunoassay, the amount of RANTES concentration was 0 or negligible in the control or 5 μg/mℓ IL-4 only treatment for 24 hrs. The amount of RANTES concentration was 3.13 ± 0.60 pg/mℓ in the 5 μg/mℓ TNF-α only treatment for 24 hrs. However, this figure increased to 3.63 ±

0.21 pg/mℓ by treatment with TNF-α and IL-4, while decreased to 1.38 ± 0.22 pg/mℓ by the treatment with HRHDT 5 μg/mℓ, respectively.
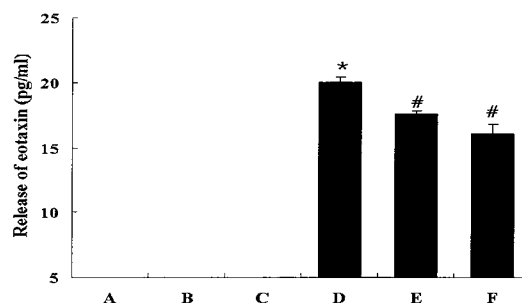

Fig. 3. **Measurement of eotaxin production in bronchial epithelial A549 cells.** A: Control B: TNF-α treated group C: IL-4 treated group D: TNF-α and IL-4 treated group E: TNF-α, IL-4 and 1 μg/ml HRHDT treated group F: TNF-α, IL-4 and 5 μg/ml HRHDT treated group *represents P < 0.05 compared to the control #represents P < 0.05 compared to the TNF-α and IL-4 treated group

Fig. 4. **Measurement of RANTES production in bronchial epithelial A549 cells.** A: Control B: IL-4 treated group C: TNF-α treated group D: TNF-α and IL-4 treated group E: TNF-α, IL-4 and 1 μg/ml HRHDT treated group F: TNF-α, IL-4 and 5 μg/ml HRHDT treated group *represents P < 0.05 compared to the control #represents P < 0.05 compared to the TNF-α and IL-4 treated group
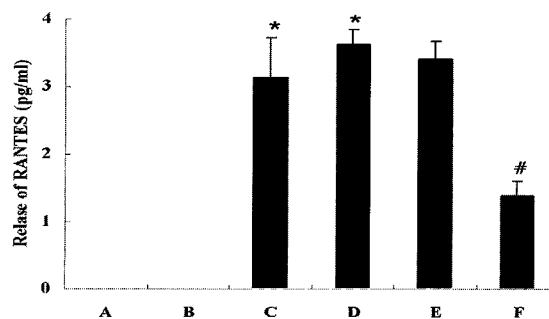
Discussion

A first step in the development of allergic diseases and inflammation is allergen sensitization and the production of IgE. The factors regulating these processes, cytokines and chemokines provide insight into mechanisms of disease at many levels, including initiation, progression and persistence. Therefore these processes become eventual therapeutic targets[15].

Cytokines and chemokines are protein or glycoprotein molecule synthesized and secreted by cells. cytokines as a group possess a broad spectrum of bioactivities and have been found to play a part in cell growth, repair, inflammation and the immune response[16].

Human mast cells express a number of cytokines including TNF-α, IL-4, IL-5, IL-6 and IL-8[17-20]. IL-4 is expressed preferentially by MCTC mast cells[21]. There is also evidence that human mast cells produce chemokines. The human mast cell line

HMC-1 is a source of multiple chemokines including regulated on activation, normal T cell expressed and secreted(RANTES) et al[22].

TNF-α has been shown to activate the inflammatory cells, up-regulate the adhesion molecules on endothelium and circulating leukocytes, increase the production of chemotaxins[4-5].

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 inflammation in a murine model[23].

Numerous studies suggested that TNF-α and IL-4 treatment stimulates production of TARC[24], eotaxin[25] and RANTES[26]. In addition, co-treatment of TNF-α and IL-4 were reported to synergize in the secretion of various chemokines[25,27].

Bisset et al. reported that several chemokines and their receptors involve with pathogenesis of allergic diseases[28]. Several members of the C-C branch of chemokines exhibit chemoattractant properties toward eosinophils, and these include TARC, eotaxin, and RANTES.

Present results also show that TNF-α and IL-4 treatments enhances TARC, eotaxin and RANTES releases in human bronchial epithelial A549 cells.

HRHDT or its components exert anti-inflammatory effects mainly on the early stages of inflammation[12] and is composed of four herbs. Of the HRHDT ingredients, Scutellariae radixis one of the important medicinal herbs that is widely used for the treatment of various inflammatory diseases[29] and allergic disease[30]. Recently, Fukutake et al. reported that berberine in Coptidis rhizome selectively inhibited COX-2 enzyme activity and that Phellodendri cortex also decreased its activity[31].

However, no study on the effect of HRHDT on the TNF-α and IL-4 stimulated TARC, eotaxin and RANTES generation has been made yet. In the results of present study, the crude extract of HRHDT suppressed the TNF-α and IL-4 stimulated TARC, eotaxin, and RANTES production in the human bronchial epithelial A549 cells. Based on the present results, HRHDT may be useful in the treatment asthmatic allergy by inhibiting TARC, eotaxin, and RANTES chemokines.

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