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Original Article / 원저

Inhibitory Effect of *Semen Sinapis Albae* on Immediate Hypersensitivity Reaction

Kyou-Young Lee* · Chul-Hee Hong
Department of Opthalmology and Otolaryngology and Dermatology,
College of Korean Medicine, Sangji University

백개자의 즉시형 과민 반응에 대한 억제 효과

이규영 · 홍철희*
상지대학교 한의과대학 안이비인후피부과학교실

Abstract

목적 : 본 연구에서는 백개자 열수추출물이 활성화된 대식세포 및 사람 비만세포주, HMC-1에서 염증 반응을 효과적으로 억제하는가를 관찰하고자 하였다.

방법 : 대식세포에 여러 농도의 백개자 열수추출물을 가한 뒤 LPS로 염증을 유도하여 NO 생산, iNOS와 COX-2 단백질 발현을 관찰하였으며 HMC-1에도 여러 농도의 백개자 열수추출물을 가한 후 PMACI로 염증을 유도하여 histamine 분비와 NF-κB 활성 및 IκB-α의 인산화, MAPKs pathway에 대한 저해효과를 관찰하였다.

결과 : 백개자 열수추출물은 대식세포에서 LPS로 유도된 NO 생성 및 INOS, COX-2 단백질 발현을 농도 의존적으로 저해하였으며 HMC-1에서 PMACI로 유도된 histamine의 분비와 p38 MAPK, ERK, JNK의 인산화 반응 및 IκB-α의 인산화와 NF-κB의 활성을 저해하였다.

결론 : 백개자 열수추출물은 대식세포 및 비만세포의 활성을 저해함으로써 알레르기 질환의 치료에 사용될 잠재성이 크다고 사료된다.

Key words : *Semen Sinapis Albae*; Human Mast Cell (HMC-1); Macrophage; Immediate Hypersensitivity Reaction; Allergic Disease

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Ⅰ. Introduction

Mast cells are broadly distributed throughout mammalian tissues and play a critical role in a wide variety of biological responses. Typically, mast cells have been considered not only in the association of immediate-type hypersensitivity, but also in late reactions like inflammatory responses, which are mast cell dependent[1,2]. Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants[3]. This is related to cytokines and pro-inflammatory mediators secreted from macrophage. An allergic reaction is the result of an inappropriate immune response triggering inflammation[4]. A common example is hay fever, which is caused by a hypersensitive response by skin mast cells to allergens[5]. In allergic inflammation, humans produce immunoglobulin E (IgE) against allergen infiltration resulting in activation of mast cells which release histamine, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and NF-κB[6]. Histamine is produced by basophils and by mast cells found in nearby connective tissues[7]. Histamine binds to H1 receptors in the target cells to contract gut and bronchus smooth muscle and to increase venule permeability and rheum. Histamine increases the permeability of the capillaries to white blood cells and some proteins to allow them to engage pathogens in the infected tissues[8]. TNF-α is secreted from the allergic mechanism of mast cells, macrophage and T cells, causes the expression of adhesion factor to vascular endothelial cells, and accumulates white blood cells, resulting in inflammation response[9,10]. IL-6 causes chronic inflammatory response, activating T cells and producing IgE[11]. IL-8 acts as a chemotactic factor for neutrophil, eosinophil, and T-lymphocyte, activating inflammatory response[12]. NF-κB functions as a transcription factor binding to the NF-κB response element located at the promoter of target genes regulating TNF-α, IL-6, and IL-8[13,14]. Mast cell-derived pro-inflammatory cytokines play an important role in the development of acute-and late-phase allergic inflammatory reactions. Therefore, inhibition of the production and secretion of these mediators is likely to provide a major mechanism to enhance efficacy in the treatment of inflammatory diseases[15-17].

Mitogen-activated protein kinases (MAPKs) activated by various different stimuli regulate the transcriptional activity of mammalian cellular physiology. Those extra cellular stimuli commence specific biological responses involving differentiation, proliferation and apoptosis through the activation of MAPK signaling cascades, which constitute three major subfamilies, such as extra cellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK). These three types of MAPKs play an significant role in the signaling of apoptosis and cytokine expression[18,19]. NF-κB is a substantial transcription factor required for the expression of many inflammatory genes. NF-κB plays a serious role in the expression of cytokines such as TNF-α, IL-6, which mediate response to

교신저자 : 이규영, 원주시 상지대길 80
상지대학교 부속 한방병원 안이비인후피부과
(Tel : 033-741-9266, E-mail : lky0706@naver.com)
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inflammatory stimuli in the allergic inflammatory responses[20]. For this reasons, NF-κB is an obvious target of anti-inflammatory treatment[16,21,22].

Semen Sinapis Albae (SSA, white mustard seed) is *Cruciferae Sinapis* Alba(L.) or Brassica juncea(L.) Czern. et Coss.'s mature seed and mainly has effects on Lung Meridian[23]. SSA is believed by doctors of traditional oriental medicine to have the action of reliving dyspnea and cough by eliminating cold-phlegm, reducing nodulation and reliving pains by removing the obstruction of collaterals. As the crude drug has a strong stimulation effect on skin and mucous membrane, it is often stir baked and used as the medicine in treating cough, asthma and distending pain of the chest caused by cold-phlegm; arthralgia accompanied with numbness, and deep abscess diseases[24]. According to studies about SSA, SSA seems to have anti-arthritic and immunomodulating effect[25] and combined administration of SSA and *Raphanus sativus* L.(RS) appears to inhibit inflammatory and allergic action of airway, leading to alleviation of asthma[26]. Through this studies we could presume the anti-allergenic inflammatory effect of SSA. However, the anti-allergenic inflammatory activities of the hot water extract from the *Semen Sinapis Albae* (WSSA) and the possible mechanism of action focusing on the expression of proinflammatory cytokines are still unknown. In the present study, to clarify the mechanism of WSSA that accounts for its anti-allergenic inflammatory effect, we examined the potential role of WSSA on gene expression of inflammatory cytokines, and the possible mechanisms of action in PMA plus Calcium Ionophore A23187 (PMACI)-stimulated human mast cells (HMC-1).

II. Materials and Methods

1. Materials for Experiments

(1) Reagents

Phorbol 12-myristate 13-acetate (PMA), Calcium ionophore A23187 (Calcymycin; C29H37N3O6), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT), L-N6-(1-iminoethyl)lysine (L-NIL), LPS (Escherichia coli, serotype 0111:B4), Triton X-100, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), and liquefied in DMSO. Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY). Iscove's modified Dulbecco's medium (IMDM) was purchased from Life Technologies (Grand Island, NY). iNOS, COX-2, p65, IκBα, phospho-ERK, phospho-p38, phospho-JNK, ERK, p38, JNK, Poly ADP Ribose Polymerase (PARP), β-actin monoclonal antibodies, and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

(2) Preparation of WSSA

SSA was purchased from Daehyo Co. (Suwon, Korea), and water extract of SSA was prepared by decocting the dried prescription of herbs with boiling distilled water. The amount of dried prescription of herbs was 500 g and the duration of decoction was about 3 h. The decoction was filtered, lyophilized and kept at 4℃. Dilutions

were made in distilled water then filtered through 0.2 ㎛ syringe filter.

(3) Cell Culture

The RAW 264.7 murine macrophage cell and HMC-1 were grown at 37℃ in DMEM and IMDM supplemented with 10% FBS, penicillin (100 units/㎖) and streptomycin sulfate (100 ㎍/㎖) in a humidified atmosphere of 5% CO_2. RAW 264.7 cells were treated with various concentration of WSSA (0.25, 0.5, and 1 ㎎/㎖) for 1 h, or with positive controls (L-NIL), and then stimulated with LPS (1 ㎍/㎖) for the indicated time. HMC-1 cells were treated with various dose of WSSA (0.25, 0.5, and 1 ㎎/㎖) for 1 h prior to stimulation with (PMACI) for the indicated time.

2. Experiment methods

(1) MTT assay

RAW 264.7 cells and HMC-1 cells were plated at a density of 1×10^5 cells/well in 96 well plates. To determine the appropriate concentration of WSSA, which has no effect on cell viability, cytotoxicity studies were performed at 24 h following treatment of cells with various concentrations of WSSA. Viabilities were determined using colorimetric MTT assays, as described previously[27].

(2) Nitrite Determination

RAW 264.7 cells were plated at 1×10^5 cells/well in 24 well-plates and then pretreated with various concentrations (0.25, 0.5, and 1 ㎎/㎖) of WSSA for 1 h. After 1 h, the cells were stimulated with LPS (1 ㎎/㎖) for 24 h. Nitrite levels in culture media were determined using the Griess reaction assay and presumed to reflect NO levels[24]. Briefly, 100 ㎖ of cell culture medium was mixed with 100 ㎖ of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl], incubated at room temperature for 10 min, and then the absorbance at 540 ㎚ was measured in a microplate reader (Perkin Elmer Cetus, Foster City, CA). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the serial dilution standard curve of sodium nitrite.

(3) Histamine assay

HMC-1 cells were pretreated WSSA for 1 h and then stimulated with PMACI for 6 h. Level of histamine was analyzed by using a commercial enzyme immuno assay kit (Cayman Chemical).

(4) Western blot analysis

Cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride, and 0.5 mM Na orthovanadate) containing 5 ㎎/㎖ each of leupeptin and aprotinin and incubated with 20min at 48℃. Cell debris was removed by micro centrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacture's instruction. Forty

micrograms of cellular protein from treated and untreated cell extracts was electroblotted onto a PVDF membrane following separation on a 10% SDS-polyacrylamidegel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4℃, followed by incubation for 4 h with a primary antibody. Blots were washed four times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Blots were again washed three times with TTBS, and then developed by enhanced chemiluminescence (Amersham Life Science, Buckinghamshire, UK).

(5) Nuclear extraction

HMC-1 cells were plated in 60 ㎜ dishes (5×10^5 cells/㎖), and treated with WSSA (0.25, 0.5, and 1 ㎎/㎖) for 1 h, and then stimulated with PMACI for 1 h, washed once with PBS, harvested into 1 ㎖ of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously[27]. Cell pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 ㎎/㎖ aprotinin) and incubated on ice for 15 min. Cells were then lysed by adding 0.1% Nonidet P-40 and vortexed vigorously for 10 s. Nuclei were pelleted by centrifugation at 12,000× g for 1 min at 4℃ and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate).

(6) Statistical analysis

Datas are presented as mean ± standard deviation (SD). Datas for the treatment groups were compared using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. All statistical analyses were performed using SPSS v.13.0 statistical analysis software (SPSS Inc., USA). The statistical significance of differences was accepted at the level of $p < 0.05$.

Ⅲ. Results

1. Cell viability of WSSA on RAW 264.7 macrophages

Cell viability of WSSA was examined by using MTT assay in RAW 264.7 macrophage cells. We found that WSSA (0 - 1 ㎎/㎖) did not affect cell viability on RAW 264.7 macrophage cells (Fig. 1).

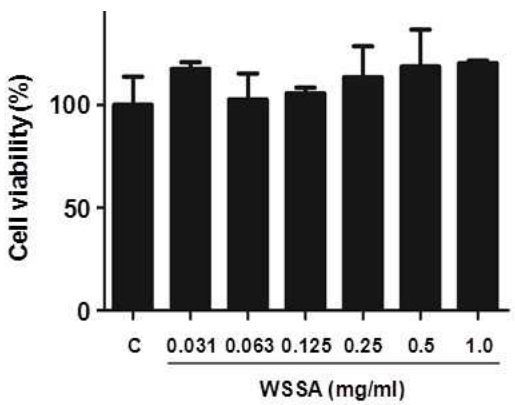

Fig. 1. Effect of WSSA on cell viability in RAW 264.7 macrophages cells

RAW 264.7 macrophages cells were treated with different concentrations of WSSA for 24 h, and their viability were determined using MTT assay. Values represent mean ± S.D. of three independent experiments.

2. Effect of WSSA on NO production in LPS-stimulated RAW 264.7 cells

We observed inhibitory effect of WSSA on NO production induced by LPS in RAW 264.7 cells. WSSA treated for 1 h, cultured with LPS (1 ㎍/㎖) for 24 h and then measured NO production in the cell supernatants. WSSA inhibited NO production in a dose-dependent manner (Fig. 2).

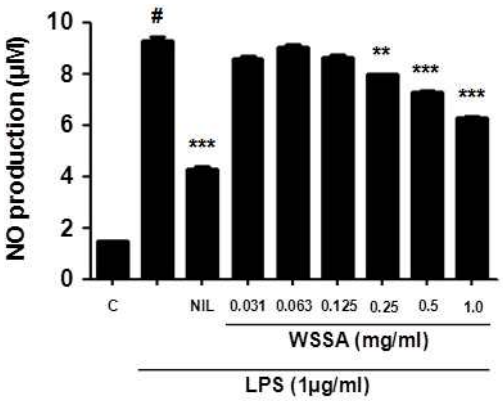

Fig. 2. Inhibitory effect of WSSA on the LPS-induced NO production in Raw 264.7 cells

Raw 264.7 cells were treated with WSSA for 1 h prior to the addition of LPS (1 ㎍/㎖), and the cells were further incubated for 24 h. NIL(20 ㎛M) were used as positive control in these assay. Values represent mean ± S.D. of three independent experiments (significant as compared to LPS alone, ** p 〈 0.01, *** p 〈 0.001, significant as compared to CON, # p 〈 0.05).

3. Inhibitory effects of WSSA on protein expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells

The effects of WSSA on iNOS and COX-2 protein expression were examined by Western blot analysis in RAW 264.7 cells. As shown in Fig. 3, the expression of iNOS and COX-2 proteins were significantly up-regulated in response to LPS (1 ㎍/㎖), and WSSA inhibited the expression of these proteins in a dose-dependent manner.

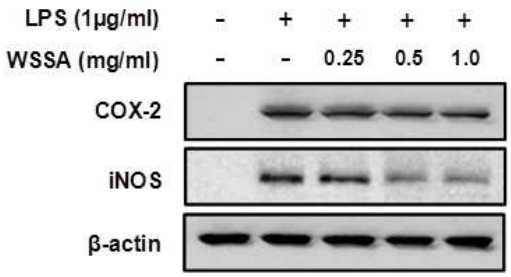

Fig. 3. Inhibitory effect of WSSA on the LPS-induced COX-2 and iNOS expressions in Raw 264.7 cells

Raw 264.7 cells were treated with WSSA for 1 h prior to the addition of LPS (1 ㎍/㎖), and the cells were further incubated for 24 h. The whole cell extracts were prepared for Western blot analysis. Expressions of COX-2 and iNOS proteins were determined by Western blot analysis using specific anti-COX-2 and anti-iNOS antibodies. β-actin was used as a loading control.

4. Cell viability of WSSA on HMC-1 cells

Cell viability of WSSA was examined by using MTT assay in HMC-1 cells. We found that WSSA (0 - 1 ㎎/㎖) did not affect cell viability on HMC-1 cells (Fig. 4).

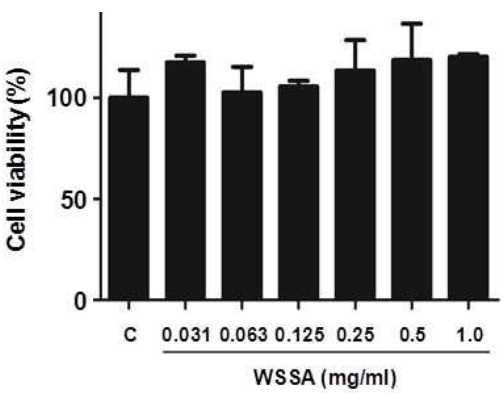

Fig. 4. Effect of WSSA on cell viability in HMC-1 cells

HMC-1 cells were treated with different concentrations of WSSA for 24 h, and their viability were determined using MTT assay. Values represent mean ± S.D. of three independent experiments.

5. Effect of WSSA on histamine release in PMACI-stimulated HMC-1 cells

HMC-1 cells are activated by PMACI and secrete inflammatory mediators such as histamine, serotonin, hydrolase, heparin, and prostaglandin. In this study, the effect of WSSA on histamine release was investigated in PMACI-stimulated HMC-1 cells (Fig. 5). WSSA decreased histamine release in PMACI-stimulated HMC-1 cells.

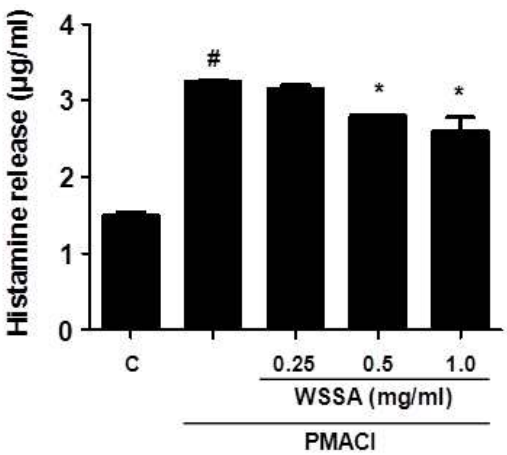

Fig. 5. Effect of WSSA on histamine release in PMACI-stimulated HMC-1 cells

The HMC-1 cells were pretreated with indicated concentrations of WSSA (0-1.0 ㎎/㎖) for 1 h and then incubated with PMACI for 6 h. Histamine release was measured by enzyme immunoassay. Each data point presents the mean ± SD of three independent experiments (significant as compared to PMACI alone, * $p < 0.05$, significant as compared to CON, # $p < 0.05$).

6. Effect of WSSA on NF-κB activation and IκBa degradation in PMACI-stimulated HMC-1 cells

NF-κB is a major transcription factor that regulates the expressions of pro-inflammatory proteins induced by PMACI. To investigate the effect of WSSA on the transcriptional activity of NF-κB, the prevention effect of WSSA on the PMACI-stimulated nuclear translocation of NF-κB was investigated by Western blot analysis in HMC-1 cells. HMC-1 cells were pre-treated with WSSA for 1 h, stimulated with PMACI for 1 h, and nuclear and cytosolic fractions were seperated. WSSA markedly suppressed the PMACI-stimulated nuclear translocations of p65 (Fig. 6). Moreover, WSSA inhibit the PMACI-stimulated degradation of IκBa in HMC-1 cells.

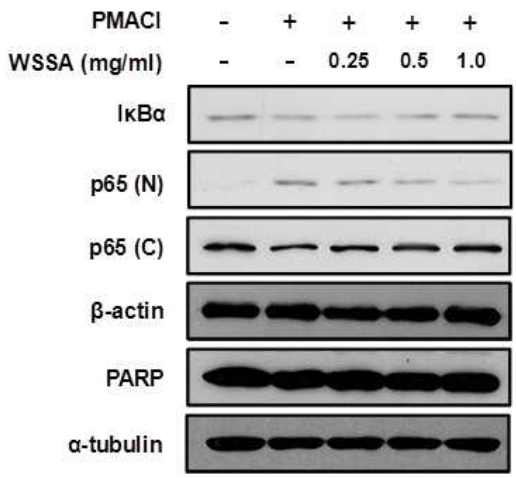

Fig. 6. Inhibitory effect of WSSA on the PMACI-stimulated NF-κB activation in HMC-1 cells

The level of nuclear p65 protein and IκBα protein was monitored 1 h after treatment of cells with PMACI with or without WSSA pretreatment. Expressions of IκBα and p65 proteins were determined by Western blot analysis using specific anti-IκBα and anti-p65 antibodies. The β-actin and PARP were used as a loading control.

7. Inhibitory effects of WSSA on the MAPKs pathway in PMACI-stimulated HMC-1 cells

The signaling pathways of MAPK molecules are important mediators by activated mast cells during allergic inflammation. Therefore, to investigate the effect of WSSA on signaling pathways in PMACI-stimulated HMC-1 cells, the phosphorylations of three MAPK, ERK, JNK, and

p38, were analyzed by Western blot analysis. The phosphorylated ERK, JNK, and p38 MAPK were significantly elevated in HMC-1 cells after treatment with PMACI, whereas the levels of total ERK, JNK, and p38 MAPK did not show significant change (Fig. 7). However, compared to the cells treated with PMACI only, the cells pretreated with WSSA showed significantly lower PMACI-induced phosphorylations of ERK, JNK, and p38 MAPK. This result indicates that phosphorylations of ERK, JNK and p38 MAPK were effectively blocked by WSSA in PMACI-stimulated HMC-1 cells.

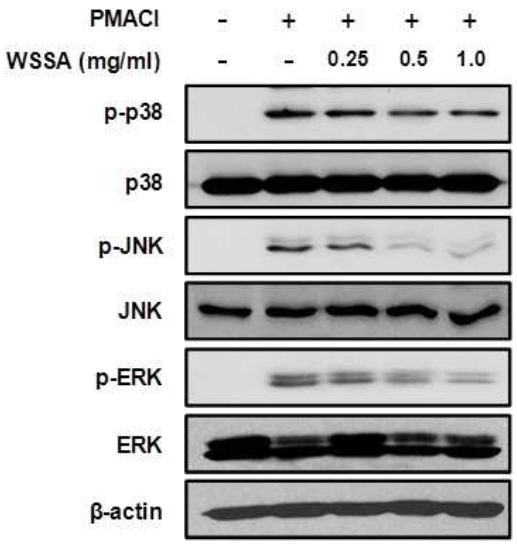

Fig. 7. Inhibitory effect of WSSA on the MAPKs pathway in PMACI-stimulated HMC-1 cells

Cells were treated with WSSA for 1 h prior to the addition of PMACI and the cells were further incubated for 30min. Expressions of phosphorylated-ERK (p-ERK), ERK, p-JNK, JNK, p-p38 and p38 were determined by Western blot analysis using specific antibodies.

Ⅳ. Discussion

Allergic Rhinitis (AR) is characterized by inflammation of the nasal mucosa with hypersensitivity resulting from seasonal or perennial responses to specific environmental allergens and by symptoms like nasal rubbing, sneezing, rhinorrhea, lacrimation, nasal congestion and obstruction, and less frequently cough[28]. AR is frequently involved in acute and chronic airway disease such as bronchial asthma, which is also caused by hypersensitivity to antigens, resulting in greater local inflammation as well as bronchoconstriction, vasomotor change, and mast cells play critical roles in the pathogenesis of allergenic responses in AR. Thus, mast cells express the immunoglobulin Fc epsilon receptor I (Fc ε RI) that triggers specific IgE responses to antigen. After IgE-dependent stimulation, mast cells release allergenic mediators such as histamine, α-hexosaminidase, cytokines, chemokines and arachidonic acid derivatives, mediating acute and chronic inflammation[29]. Therefore, treatment options for AR consist of allergen avoidance, symptomatic treatment and allergenic-specific immunotherapy to further improve the control of allergic responses[30]. Although therapeutic agents are greatly required, the current treatment of AR is limited to anti-histamine, nasal corticosteroid, anti-leukotriene and anti-allergen imunotheraphy, which are still not ideal, and it is important to continue to study the pharmacology of this disease in the search for better drugs[31].

Mast cell activation has been reported for a significant proportion of allergic inflammation.

Because mast cell contains potent mediators, including histamine, heparin, proteinases, leukotrienes, and multifunctional cytokines, its potential contributions to processes of inflammation and matrix degradation have recently become evident[32,33]. In the present study, WSSA attenuated PMACI-induced release of histamine from mast cells by inhibiting their degranulation. These results demonstrated that WSSA induced mast cell stabilization, and had anti-histamine activity against the PMACI-induced allergic response.

WSSA inhibited PMACI-induced activation of MAPKs in HMC-1 cells. The MAPK cascade is one of the important signaling pathways in immune responses[19]. Because of their essential role in intracellular signaling network, MAPK pathways are appropriative targets for pharmacological treatment of inflammatory disorders[34]. According to the current study, WSSA suppressed the activation all three MAPKs and inhibition of MAPKs by WSSA plays a leading part in controlling inflammatory process. Crosslinking of IgE receptors on mast cells initiates a complex series of phosphorylation events via the activation of Src, Syk, and Rho family protein tyrosinekinases[33]. Clarifying the effect of WSSA on the upstream of MAPKs would be important subject in this study.

NF-κB activation in tune leads to the coordinated expression of many genes that encode proteins such as cytokines, chemokines, enzymes, and adhesion molecules involved in mediator synthesis and the further amplification and perpetuation of the inflammatory reaction[22]. Because suppression of NF-κB activation has been linked with anti-inflammation, we postulated that WSSA mediates its effects at least partly through suppression of NF-κB activation. Activation of NF-κB requires phosphorylation and proteolytic degradation of the inhibitory protein IκBα[35]. In PMACI-stimulated mast cells, WSSA decreased the degradation of IκBα and nuclear translocation of p65 NF-κB. These data demonstrate that WSSA might attenuate activation of NF-κB involved inflammatory cytokines.

A closer look at the result of Inhibitory effect of WSSA on the PMACI- stimulated NF-κB activation and MAPKs pathway in HMC-1 cells, we could find out that the inhibitory effect of WSSA is fully expressed especially at concentrations of 1.0 ㎎/㎖. This provide suggestion that in clinical use of WSSA, it is better to use WSSA in high concentration.

In view of the increasing prevalence of allergic diseases such as asthma, allergic rhinitis, and eczema worldwide[15,22,33], there is a need for novel and safe treatments of the underlying inflammation of these mast cell-mediated disease[15,34]. Mast cells play differential roles in the inflammation by initiating and orchestrating immune responses by the release of various chemokines and cytokines via differential intracellular signal transduction pathways[34]. In conjunction with this study regarding the cross-talk between different signaling pathways for the fine control of pro-inflammatory cytokines on HMC-1 cells[33,34], our present results therefore provide further new insight that the activation of HMC-1 cells are under fine, diversified and complicated intracellular regulation. The

consequences obtained in this study manifestly provide new evidence that WSSA contributes importantly to the prevention or treatment of mast cell-mediated inflammatory diseases.

V. Conclusions

We examined the potential role of WSSA on gene expression of inflammatory cytokines, and the possible mechanisms of action in PMACI-stimulated human mast cells (HMC-1). Results obtained are as follows.

1. WSSA (0 - 1 ㎎/㎖) did not affect cell viability on RAW 264.7 macrophage cells.
2. WSSA inhibited NO production in a dose-dependent manner.
3. WSSA inhibited the expression of iNOS and COX-2 proteins in a dose-dependent manner.
4. WSSA (0 - 1 ㎎/㎖) did not affect cell viability on HMC-1 cells.
5. WSSA decreased histamine release in PMACI-stimulated HMC-1 cells.
6. WSSA markedly suppressed the PMACI-stimulated nuclear translocations of p65. Moreover, WSSA inhibited the PMACI-stimulated degradation IκB-α in HMC-1 cells.
7. Compared to the cells treated with PMACI only, the cells pretreated with WSSA showed significantly lower PMACI-induced phosphorylations of ERK, JNK, and p38 MAPK.

Through these results we can conclude that WSSA is a potential medicine for the treatment of allergic diseases through the down-regulation of mast cell activation.

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