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Sargassum hemiphyllum Inhibits Atopic Allergic Reaction via the Regulation of Inflammatory Mediators

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Abstract. *Sargassum hemiphyllum* (SH) has long been used in Korean folk medicine for the therapeutic treatment of various allergic diseases. The effects of SH in previous experimental models, however, have been inconclusive. We studied the effects of methanol extract of SH on mast cells. Our experiments showed that SH significantly inhibited compound 48/80-induced histamine and β-hexosaminidase release from rat peritoneal mast cells. SH inhibited interleukin (IL)-8 and tumor necrosis factor (TNF)-α release induced by phorbol 12-myristate 13-acetate and A23187 from HMC-1, and it also showed an inhibitory effect on the anti-dinitrophenyl IgE antibody-induced passive cutaneous anaphylaxis reaction. In addition, SH inhibited the increase of TNF-α-induced NF-κB protein levels, transcription factor of TNF-α from 293T cells. A period of 48 h exposure to SH had little effect on HMC-1 cell viability. Our results suggest that SH has an inhibitory effect on the atopic allergic reaction and thus this may be useful in the treatment of allergic inflammatory diseases, such as atopic dermatitis.

Keywords: *Sargassum hemiphyllum*, histamine, cytokine, mast cell, atopic dermatitis

Introduction

The brown seaweed, *Sargassum hemiphyllum* (SH), can be found living off the shores of Cheju Island and Busan, East China Sea, Korea, and it is claimed to be a valuable folk medicine for treating allergic disorders. It has been described to possess bioavailability (1, 2), but its action mechanism is poorly understood.

Atopic dermatitis (AD) is a common pruritic inflammatory skin disease that often begins in infancy and frequently affects subjects with personal or family histories of atopic disease (3). The prevalence of AD has steadily increased during the past few decades (4). The mast cell is a tissue-based inflammatory cell of bone marrow origin that responds to the danger signals of innate and acquired immunity with both the immediate and delayed release of inflammatory mediators (5). In the skin, mast cells appear in the greatest number near blood vessels, hair follicles, and sebaceous glands. Human skin contains approximately 10^4 mast cells per cubic millimeter (6). A higher number of mast cell profiles can be found in AD lesional skin, as compared with nonlesional skin (7).

Mast cells release various mediators upon stimulation. Preformed mast cell mediators include histamine, proteases, adenosine, acid hydrolases, and proteoglycans (8). A report that mast cell mediators other than histamine are involved in compound 48/80-induced pruritus in AD patients has been published (9). In pathological skin conditions, histamine is involved in the induction of itching, flaring, and edema (10, 11).

Mast cell activation brings about the process of degranulation, which results in the fusion of the cytoplasmic granule membranes with the plasma membrane.

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Both histamine and β-hexosaminidase stored in mast cells are effectively released by positively charged substances such as compound 48/80 (12). The synthetic compound 48/80 is known to be one of the most potent secretagogues (13). Theoretical studies on compound 48/80-induced mast cell degranulation have been continuously performed by Kim et al. (8). Mast cells are also capable of synthesizing various cytokines. After stimulation, human mast cells have been shown to produce interleukin (IL)-4, IL-5 (14 – 16), and tumor necrosis factor-α (TNF-α) (17). In addition, human derived cell lines with appearances similar to mast cells are known to produce IL-6 and IL-8 (12). The release of these cytokines may be of major importance in the development of many inflammatory skin disorders (18). Inhibition of secretion from these mast cells cytokines can aid in the development of a useful therapeutic strategy for allergic inflammatory diseases such as AD.

In this study, we investigated the effects of SH on histamine and β-hexosaminidase release from the rat peritoneal mast cells (RPMCs), which were activated by compound 48/80. We evaluated the effects of SH on phorbol 12-myristate 13-acetate (PMA) and A23187-induced IL-8 and on TNF-α secretion from human mast cells (HMC-1). We also investigated the effect of SH on the anti-dinitrophenyl (DNP) IgE antibody-induced skin anaphylactic reaction in a murine model by oral administration. In addition, SH inhibited increase of NF-κB protein levels, transcription factor of TNF-α. Finally, we checked the NF-κB protein levels of 293T cells by the luciferase assay, and we checked the cytotoxicity of SH on HMC-1 by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.

Materials and Methods

Cells and reagents

Human leukaemic mast cell line-1 (HMC-1) was maintained in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. Human embryonic kidney-293 cell line and its derivative 293T cells, which express the SV40 large T antigen, were obtained from the American Type Culture Collection. The cells were maintained in RPMI1640 supplemented with 10% FBS. Compound 48/80, metrizamide, PMA, A23187, *p*-nitro-phenyl-*N*-β-D-glucosaminide, and *o*-phthaldialdehyde were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant IL-8 and biotinylated anti-human IL-8 were purchased from Pharmingen (San Diego, CA, USA). Recombinant TNF-α and biotinylated anti-human TNF-α were purchased from R&D systems Inc. (Minneapolis, MN, USA). MTT and cell culture media, IMDM with glutamix-1, were purchased from Gibco BRL (Grand Island, NY, USA).

Animals

The original stock of Wistar rats and ICR mice was purchased from the Damul Experimental Animal Center (Daejeon, Korea), and the animals were maintained in the College of Pharmacy, Wonkwang University. The rats were housed five to ten per cage in a laminar airflow room, which was maintained at a temperature of 22 ± 1°C and a relative humidity of 55 ± 10% throughout the study. The animal experiments were approved by the Animal Research Committee of Wonkwang University.

Plant materials and extraction procedure

The specimens of SH were collected along the shore of Cheju island and Busan, East China Sea, Korea. An alga taxonomist, J.S. Yoo, from the Research Institute of Marine Science and Technology, Korea Maritime University, Korea, confirmed the taxonomic identification of these specimens. The dried samples (30 g) were extracted with 300 ml of methanol for 24 h at room temperature. This step was repeated with sediment (19). The methanol extracts (total of 600 ml) were concentrated in 2 g plant samples using a rotary evaporator. The residues obtained were stored in a freezer at −80°C.

Preparation of RPMCs

RPMCs were separated from the major components of rat peritoneal cells (i.e., macrophages and small lymphocytes) according to the method described by Hong et al. (20) and Yurt et al. (21). In brief, peritoneal cells suspended in 1 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM $NaHCO_3$, 2.7 mM KCl, 0.3 mM NaH_2PO_4) containing 0.1% gelatin (Sigma) were layered onto 2 ml of 0.225 g/ml metrizamide (density, 1.120 g/ml; Sigma) and centrifuged at room temperature for 15 min at 400 × *g*. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% bovine serum albumin) containing calcium. RPMC preparations were about 95% pure, as assessed by means of toluidine blue staining. More than 97% of the cells were viable, as judged by trypan blue uptake.

Histamine assay

Purified RPMCs were resuspended in Tyrode buffer A containing calcium for the treatment with compound 48/80. RPMCs suspensions (2×10^5 cells/ml) were

preincubated for 10 min at 37°C before the addition of compound 48/80 for stabilization. The cells were preincubated with the SH for 30 min and then incubated for 15 min with compound 48/80 (6 μg/ml). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at $400 \times g$ for 5 min at 4°C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at $400 \times g$ for 5 min at 4°C. The histamine content was measured by means of the *o*-phthalaldehyde spectrofluorometric procedure of Shore et al. (22). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) with a spectrofluorometer. The inhibition percentage of histamine release was calculated using the following equation:

$$\% \text{ inhibition} = (A - B) \times 100 / A,$$

where A is the histamine release without SH, and B is the histamine release with SH.

β-Hexosaminidase assay

β-Hexosaminidase in the supernatant was measured using a previously reported method (12). Briefly, 50 μl of each sample was incubated with 50 μl of 1-mM *p*-nitrophenyl-*N*-β-D-glucosaminide dissolved in a 0.1 M citrate buffer, pH 5, in a 96-well microtiter plate at 37°C for 1 h. The reaction was terminated with 200 μl/well of 0.1 M carbonated buffer, pH 10.5. The plate was read at 405 nm in an enzyme-linked immunosorbent assay (ELISA) reader. The inhibition percentage of β-hexosaminidase release was calculated using the following equation:

$$\% \text{ inhibition} = (A - B) \times 100 / A,$$

where A is β-hexosaminidase release without SH and B is β-hexosaminidase release with SH.

IL-8 and TNF-α assay

HMC-1 cells (3×10^5 cells/ml) were incubated with SH (0.01, 0.1, and 1.0 mg/ml) at 37°C for 30 min. Thereafter, the cells were stimulated by PMA (50 nM) and A23187 (1 μM) at 37°C for 8 h. IL-8 and TNF-α concentrations in the culture supernatant were determined by means of a modified ELISA method, as previously reported (23). Sandwich ELISA for IL-8 and TNF-α was carried out, in duplicate, in 96-well ELISA plates (Nunc, Roskilde Denmark) coated with each of 100 μl aliquots of mouse anti-human IL-8 and TNF-α monoclonal antibodies at 1.0 μg/ml in PBS at pH 7.4 and was incubated overnight at room temperature. The plates were washed in PBS containing 0.05% Tween-20 (Sigma) and blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3 for 2 h. After additional washings, sample or recombinant IL-8 and TNF-α standards were added and incubated at 37°C for 2 h. After 2 h of incubation at 37°C, the wells were washed and then biotinylated anti-human IL-8 and TNF-α, 0.2 μg/ml each, was added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and the plates were incubated for 30 min at 37°C. Wells were washed again and ABTS substrate (Sigma) was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant human IL-8 and TNF-α (R&D Systems) in serial dilutions.

Passive cutaneous anaphylaxis (PCA) reaction

ICR mice were passively sensitized according to the previously described method (12). The dorsal skin of each mouse was injected intradermally with 0.5 μg of anti-DNP IgE. This was followed 48 h later by an injection via the tail vein of 1 μg Ag of DNP-HSA in PBS containing 1% Evans Blue. Then, after 40 min, the mice were sacrificed and reaction sites were excised for a determination of extravasated dye. The amount of dye was determined colorimetrically after extraction with 1.0 N KOH and a mixture of acetone and phosphoric acid, based on the method of Katayama et al. (24). SH was suspended in PBS at appropriate concentrations and was administered orally for 1 h, before challenging DNP-HSA.

Transfection and luciferase report assay

For transient transfection, 293T cells were seeded at 5×10^5 in a 12-well plate 1 day prior to transient transfection (90 – 95% cell confluency). Cells were transfected with a serum- and antibiotics-free RPMI 1640 medium containing 4 μl/ml of LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA, USA), and a 1.6 μg/ml NF-κB luciferase reporter construct, which were kindly provided by Professor Jang-Soo Chun, of the Kwangju Institute of Science and Technology, Korea. After 5 h of incubation, the medium was replaced with a RPMI 1640 medium containing 10% FBS and antibiotics. Cells were allowed to recover at 37°C for 20 h and were subsequently stimulated as indicated. Cell lysates were prepared and assayed for luciferase activity using a Luciferase Assay System (Promega, Madison, WI, USA), according to the manufacturer's instructions (25).

MTT assay

HMC-1 cells were cultured at a density of 5×10^4 cells/ml in 4-well plates (Nunc). Then the medium was

removed and fresh medium, along with various concentrations of SH, was added to cultures in parallel. After incubating for 48 h, an MTT assay was performed, following the method of Scudiero et al. (26). To determine the cell viability, 50 μl of MTT (5 mg/ml) was added to each well and cells were cultured for an additional incubation period of 4 h. After washing out the supernatant, the insoluble formazan product was dissolved in DMSO. Then, the optical density of 96-well culture plates was measured, using an ELISA reader at 540 nm. The optical density of formazan that formed in the untreated control cells was taken as 100% of viability.

Statistical analyses

The results were expressed as the mean ± S.E.M. Statistical significance was compared between each treated group and the control using a one-way ANOVA. Results of $P<0.05$ were considered statistically significant.

Results

Effects of SH on histamine and β-hexosaminidase release from RPMCs

The inhibitory effects of SH on compound 48/80-induced histamine and β-hexosaminidase release from RPMCs are shown in Table 1. RPMCs (2×10^5 cells/ml) were preincubated with the SH at 37°C for 15 min prior to the challenge with compound 48/80. SH (100 μg/ml) significantly inhibited compound 48/80-induced histamine release from RPMCs ($P<0.05$). Inhibition rates of histamine release were 45.92% with an SH concentration of 100 μg/ml. The release of β-hexosaminidase decreased significantly with a 100 μg/ml concentration of SH ($P<0.05$). The inhibition rate of β-hexosaminidase release was 43.78% with an at the SH concentration of 100 μg/ml.

Effects of SH on IL-8 and TNF-α secretion from HMC-1 cells

In addition to the release of preformed mediators, such as histamine and β-hexosaminidase, activated mast cells can produce other inflammatory mediators and several proinflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α, as well as the transforming growth factor (TGF)-β (21 – 22, 27). Modulation of these cytokine secretions from mast cells can support an effective therapeutic strategy for allergic inflammatory disease. The HMC-1 cells were stimulated with PMA and calcium ionophore A23187. To assess the effects of SH on PMA and A23187-induced IL-8 and TNF-α secretion, the cells were pretreated with SH (0.01 – 1.0 mg/ml) for 30 min prior to stimulators. Results showed that pretreatment of the cells with SH resulted in the inhibition of both IL-8 and TNF-α secretion. SH significantly ($P<0.05$) inhibited the PMA and A23187-induced IL-8 release by 25.94% with an SH concentration of 1.0 mg/ml. In addition, when TNF-α is secreted by PMA and A23187 stimulation, the inhibitory effects of SH were found to be significant and dose-dependent: 19.13% with a dose of 0.1 mg/ml and 34.68% with a dose of 1.0 mg/ml (Fig. 1).

Effect of SH on PCA reaction

It has been well-documented that the PCA reaction is a typical model for mast cell-dependent immediate hypersensitivity and can be used to assess the anti-allergic activities of potential drugs (12). These previous findings led us to investigate the effect of SH on the PCA reaction. When orally administered for 1 h, SH produced a marked inhibitory effect (49.71% and 51.12% at the doses of 0.1 and 1.0 g/kg, respectively) on the PCA reaction induced by anti-DNP IgE and DNP-HSA (Table 2). The results were statistically significant ($P<0.05$).

Table 1. Effect of SH on compound 48/80-induced histamine and β-hexosaminidase release from RPMCs

SH (μg/ml)	Compound 48/80 (6 μg/ml)	Histamine release intensity	β-Hexosaminidase optical density (O.D)
None (saline)	–	2.06 ± 0.05	0.084 ± 0.001
None (saline)	+	26.75 ± 0.91	0.169 ± 0.004
10	+	24.15 ± 1.169	0.099 ± 0.003
100	+	15.41 ± 0.169*	0.095 ± 0.004*

RPMCs (2×10^5 cells/ml) were preincubated with SH at 37°C for 30 min and then incubated for 15 min with compound 48/80 (6 μg/ml). Each datum represents the mean ± S.E.M. of three experiments. *$P<0.05$, significantly different from the control value.

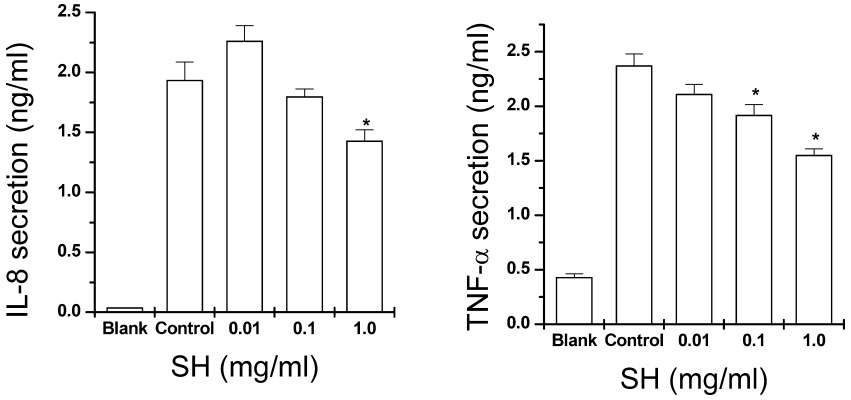

Fig. 1. Effect of SH on PMA- and A23187-induced IL-8 and TNF-α secretion from HMC-1. Each datum represents the mean ± S.E.M. of three experiments. **P*<0.05, significantly different from the control value. Blank, none (saline).

Table 2. Effect of SH on IgE-induced passive cutaneous anaphylaxis in mice

SH (g/kg)[a]	Amount of dye (μg per site)[b]	Inhibition (%)
None (saline)	1.555 ± 0.275	—
0.01	0.802 ± 0.171	48.42
0.1	0.782 ± 0.042	49.71*
1.0	0.760 ± 0.079	51.12*

[a]SH dissolved with distilled water was orally administered to mice 1 h before the challenge. [b]Each value indicates the mean ± S.E.M. (n = 6). **P*<0.05, significantly different from the saline value.

Effects of SH on NF-κB-mediated transcriptional activity in 293T cells

The effects of pretreatment with SH on TNF-α (1 ng/ml)-induced NF-κB activation in 293T cells were investigated. Cells (1×10^5) were transiently transfected with the NF-κB luciferase reporter plasmid. Then the cells were incubated for 24 h with SH (100 μg/ml) or SH (100 μg/ml) plus TNF-α (1 ng/ml). NF-κB activation was determined by means of the luciferase activity assay. TNF-α induced severe NF-κB activation, which was blocked by SH (Fig. 2). These observations indicate that SH suppressed proinflammatory cytokine expression via the suppression of NF-κB activation.

MTT assay to determine cytotoxicity of SH on HMC-1 cells

HMC-1 cells (3×10^5 cells/ml) were incubated with SH (0.001 – 1 mg/ml) at 37°C. After incubation for 48 h, cell viability was measured by means of an MTT assay. SH (0.001 – 1 mg/ml) exerted no cytotoxic effect for 48 h (Fig. 3).

Characterization of the principal components of SH

SH was standardized by means of nuclear magnetic

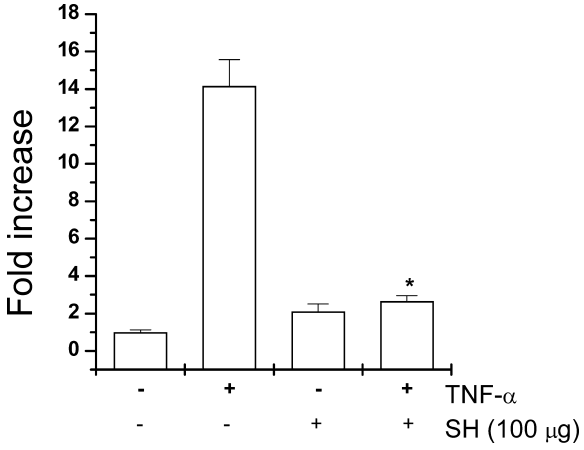

Fig. 2. Effect of SH on the NF-κB reporter gene expression in 293T cells. These data are representative of three independent experiments. Data represent means ± S.E.M. (n = 5). **P*<0.05, significantly different from the TNF-α (+) and SH (–) group.

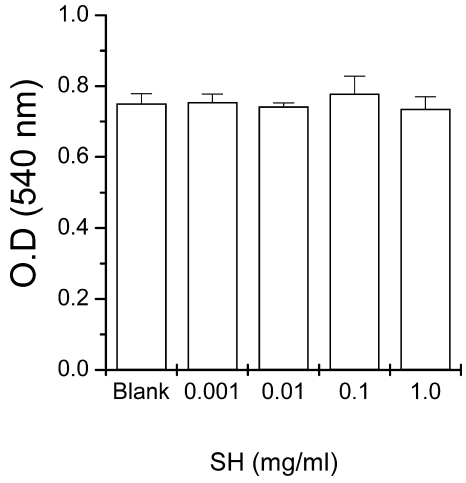

Fig. 3. MTT assay to determine cytotoxicity of SH on HMC-1 cells. Cell viability was evaluated by the MTT colorimetric assay. Each column represents the mean ± S.E.M. (n = 3). Blank, none (saline).

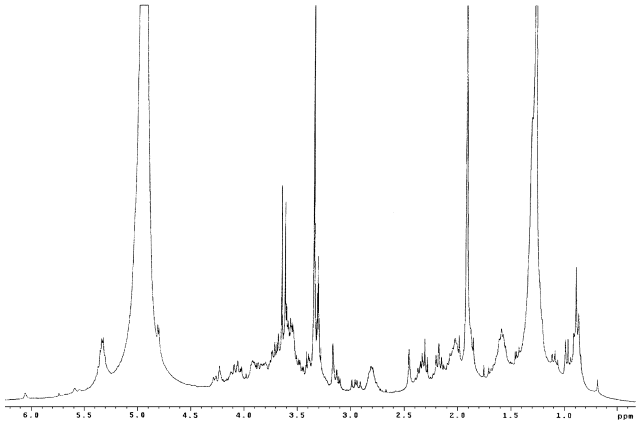

Fig. 4. NMR spectra of SH.

resonance (NMR) spectrometry (Fig. 4). NMR spectra were measured in MeOH-d_4 using a standard pulse sequence on a 300 MHz Varian Unity spectrometer. All chemical shifts were recorded with respect to MeOH-d_4 (δ 3.3) as an internal standard. In the ^{1}H NMR spectrum of the methanol extracts, several signals in the region of δ 4.4 – 3.3 as well as one strong signal of about δ 1.25 indicated the possibility that compounds having sugar moieties and long aliphatic chains exist as major components. The methanol extracts were partitioned between methylene chloride and water. The organic layer was further partitioned between 85% aqueous MeOH and *n*-hexane. The aqueous lower layer was concentrated to a sticky residue. Repeated column chromatography of this residue with silica gel revealed a mixture of compounds that were considered to be SQDG (sulfoquinovosyldiacylglycerides) and SQMG (sulfoquinovosylmonoacylglycerides). SQMS has a sugar moiety and a long aliphatic chain, while SQDG has a sugar moiety and two aliphatic chains. This result agrees well with the interpretation of ^{1}H NMR spectral data for the methanol extracts of SH. Investigations on further separation for more detailed bioactivity test are currently in progress.

Discussion

Atopic dermatitis is a chronic inflammatory skin illness, with remissions and exacerbations, itch, and an association with allergic rhinitis and asthma. There is a complex interrelationship of genetic, environmental, pharmacological, and psychological factors that contribute to the development and severity of the illness. Different manifestations of immunological disorders are an increment in the liberation of proinflammatory mediators by mast cells (28). The important role of mast cells in immediate-type allergic reactions is well recognized. Several papers have described the involvement of mast cells in the skin rash in AD (29, 30).

Stimulation of mast cells with compound 48/80 initiates the activation of a signal transduction pathway that leads to histamine and β-hexosaminidase release. There have been some reports that compound 48/80 is able to activate G proteins (31 – 33). The report that compound 48/80 increased the permeability of the lipid bilayer membrane, by causing a perturbation of the membrane (34), indicates that the membrane permeability increase may be an essential trigger for the release of mediators from mast cells. Inhibition of the release of prestored mediators in mast cells can be achieved by several different mechanisms such as membrane alteration, inhibition of protein kinase C, and dysfunction of cytoskeleton.

The recent discovery that mast cells are active cytokine-producing cells suggests a close link between mast cells and other cells of the immune system (35 – 40). The regulation of these cytokines secretions from mast cells can support an effective therapeutic strategy for allergic inflammatory diseases such as AD. TNF-α is elevated in patients with AD (41). TNF-α influences the development of skin inflammation by induction of adhesion molecules, including endothelial E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 expression (42 – 47). TNF-α can induce production of the chemokine IL-8 by a variety of cell types, such as monocytes and epithelial and endothelial cells (46). IL-8 is a chemoattractant for neutrophils, macrophages, and T lymphocytes (48) and facilitates the migration of these cells into inflamed skin (49, 50). IL-8-producing inflammatory cells are found in the dermis of atopic patients (51). Lin et al. (52) reported that stromal cell-derived factor-1 stimulated production of IL-8. In the present study, SH decreased IL-8 and TNF-α secretion on the PMA- and A23187-stimulated HMC-1 cells. SH inhibited increase of NF-κB protein levels, transcription factor of TNF-α. These results show that SH is not only associated with the production of the chemokine IL-8, but also with the inhibition of TNF-α-mediated allergic inflammatory reactions. In IgE-dependent allergic disorders, including anaphylaxis, allergic asthma, rhinitis, and atopic dermatitis (53, 54), mast cell activation induces many of the acute changes observed. The secretory response of mast cells can be induced by the aggregation of their cell surface-specific receptors (Fcε RI) for IgE by a corresponding antigen (55, 56). The SH-administered mice were protected from the IgE-mediated local allergic reaction. The mechanism of the protection against anti-DNP IgE, while not clear at present, may be suggested only in some particular conditions. It is conceivable that SH inhibits the initial phase of immediate type allergic reactions, probably

through interference with the degranulation system.

In this study, SH also inhibited TNF-α-stimulated NF-κB activity. NF-κB has been previously implicated in allergic inflammation (57). The majority of proteins encoded by NF-κB target genes participate in the host immune responses. These include a large number of cytokines (58). The suggestion that NF-κB dysregulation may be a critical factor in mediating susceptibility to AD is supported by the findings that RelB-deficient mice show a phenotype and histopathological changes resembling AD (59).

In conclusion, the results obtained in the present study provide evidence that SH significantly contributes to the treatment of atopic allergic reactions such as AD. Future studies are necessary to isolate and characterize the active chemical constituents involved.

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