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Regulatory Effect of Th-2 Cytokine Production in Mast Cells by 02PS15

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Abstract – 02PS15 extracts (BuOH, H_2O, and crude extracts) significantly inhibited IL-4 and IL-6 secretion from the phytohemagglutinin (PHA)-plus phorbol 12-myristate 13-acetate (PMA)-induced peripheral blood mononuclear cells ($P<0.05$). 02PS15 extracts (BuOH and crude extracts) also significantly inhibited the histamine release from rat peritoneal mast cells ($P<0.05$). Significant reduced levels ($P<0.05$) of PMA- and A23187-induced IL-8 were observed in the human mast cell line, HMC-1, with 02PS15 extracts (BuOH and crude extracts). 02PS15 extracts (BuOH and crude extract) downregulated the expression of IL-6 and IL-8 in the activated HMC-1. These results suggest that 02PS15 has the inhibitory effect of atopic allergic reaction and this might be useful for clinical application to treat several allergic diseases such as atopic dermatitis.

Keywords □ histamine, cytokines, atopic allergic reaction

INTRODUCTION

02PS15 is the specimens of brown alga *Sargassum thunbergii* that is distributed widely in southern coastal area of Korean Peninsula. Few data on secondary metabolite from this brown alga has been reported though it was chemically investigated. As a part of our search for bioactive compounds from marine organisms, we have collected the brown alga *S. thunbergii* along the shore of Busan.

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 (Hopkin, 1998). The prevalence of AD has steadily increased during the past few decades (Taylor and Metcalfe, 2001). Th-2-dependent cytokines IL-4, IL-5, IL-6, and IL-13 support humoral immunity and IgE production, which is characteristic of AD (Abbas *et al.*, 1996; Romagnani *et al.*, 1997).

Th-2-mediated inflammations like asthma and atopic dermatitis have a strong infiltration of mast cells, and it has previously been suggested that Th-2 cytokines are part of the factors that regulate the recruitment of mast cells in Th-2-mediated inflammations (Olsson *et al.*, 2004).

The mast cell is tissue-based inflammatory cell of bone marrow origin that responds to danger signals of innate and acquired immunity with immediate and delayed release of inflammatory mediators (Kim, 2000). In the skin, mast cells appear in greatest number near blood vessels, hair follicles, and sebaceous glands. Human skin contains approximately 10^4 mast cells per cubic millimeter (Allansmith *et al.*, 1989). In AD lesional skin, an increased number of mast cell profiles found as compared with nonlesional skin (Shin *et al.*, 1997).

Mast cells release various mediators upon stimulation. Preformed mast cell mediators include histamine, proteases, adenosine, acid hydrolases, and proteoglycans (Kim and Cho, 1999). A report that the mast cell mediators other than histamine are involved in compound 48/80-induced pruritus in AD patients has been published (Kim *et al.*, 1999). In pathological skin conditions, histamine is involved in the induction of itching, flaring, and edema (Cooper, 1994).

Mast cell activation brings about the process of degranula-

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tion that results in the fusion of the cytoplasmic granule membranes with the plasma membrane. The synthetic compound 48/80 is known to be one of the most potent secretagogues (Na *et al.*, 2002, Galli, 1993). Mast cells are also capable of synthesizing various cytokines. Human derived cell lines with mast cell like appearance are known to produce IL-6 and IL-8. The release of these cytokines may be of major importance in the development of many inflammatory skin disorders (Trefzer *et al.*, 2003). Inhibition of these cytokines secretion from mast cells can provide us with a useful therapeutic strategy for allergic inflammatory disease such as AD.

In this study, we have investigated whether 02PS15 extracts inhibit Th-2 cytokine in peripheral blood mononuclear cells and mast cell ($P<0.05$).

MATERIALS AND METHODS

Plant materials and extraction procedure

The specimens of brown alga *Sargassum thunbergii* were collected by hand at the coast of Youngdo Island in November 2002, South Sea, Korea. A voucher specimens are deposited at the Herbarium of the Division of Ocean Science, Korea Maritime University, Busan, Korea under curatorship of Jong-Su Yoo. Shade-dried samples (250 g) were chopped into small pieces and repeatedly extracted for 2 days with Acetone-CH_2Cl_2 (1:1) (1.5 L×2) and MeOH (1.5 L×2). The combined crude extracts (12.5 g) were evaporated under vacuum to give a dark brown oil that was partitioned between CH_2Cl_2 and water. The organic layer was re-partitioned with 85% aqueous MeOH and *n*-hexane. The aqueous fraction was also further fractionated with *n*-BuOH and H_2O, successively, to produce the *n*-hexane (3.6 g), 85% aqueous MeOH (8.8 g), *n*-BuOH (1.2 g) and H_2O (8.0 g) fraction.

Peripheral blood mononuclear cells (PBMCs) isolation and culture

PBMCs from heparinized venous blood were isolated by Ficoll-gradient centrifugation, washed three times in phosphate-buffered saline (PBS) solution, and resuspended in RPMI 1640 medium (Gibco BRL) supplemented with 2 mmol/l-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10% FBS inactivated for 30 min at 56°C. PBMCs were adjusted to a concentration of 2×10^6 cells/ml in 30 ml falcon tube, and 100 μl aliquots of cell suspension were placed in a 4-well cell culture plate. PBMCs were cultured for 24 h in 95% humidified air containing 5% CO_2 (37°C), in the presence or the absence of 02PS15 extracts supernatants were collected by centrifugation and stored at -20°C.

Cells and reagents

Human leukaemic mast cell line-1 (HMC-1) was maintained in Iscoves modified Dulbeccos medium (IMDM) with 10% fetal bovine serum (FBS) 37°C in 5% CO_2. Compound 48/80, metrizamide, PMA, A23187, and o-phthaldialdehyde were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant IL-6 and IL-8, biotinylated IL-6 and IL-8, and anti-human IL-6 and IL-8 were purchased from Pharmingen (Torreyana Road, San Diego, CA). Recombinant IL-4, biotinylated IL-4, and anti-human IL-4 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).

Preparation of rat peritoneal mast cells

Rat peritoneal mast cells (RPMCs) were separated from the major components of rat peritoneal cells (i.e., macrophages and small lymphocytes) according to the method described by Yurt *et al.* (Yurt *et al.*, 1977). In brief, peritoneal cells suspended in 1 ml of Tyrode buffer B 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, 0.1% bovine serum albumin) containing calcium. RPMCs preparations were about 95% pure as assessed by 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 02PS15 for 30 min, and then incubated for 15 min with compound 48/80 (6 mg/ml). The reaction stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at 400×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×g for 5 min at 4°C. The histamine con-

tent was measured by the *o*-phthalaldehyde spectrofluorometric procedure of Shore *et al.* (Shore *et al.*, 1959). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer.

The inhibition percentage of histamine release was calculated using the following equation:

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

where *A* is histamine release without 02PS15 and B is histamine release with 02PS15.

IL-8 assay

HMC-1 cells (3×10^5 cells/ml) were stimulated with 02PS15 (100 μg/ml) at 37°C for 30 min prior to addition of PMA (50 nM) and A23187 (1 μM) for 8 h. Culture supernatants were assayed for IL-8 protein levels by ELISA method. The cytokines measured by a modified ELISA. Sandwich ELISA for IL-8 was carried out in duplicate in 96-well ELISA plates (Nunc, Denmark) coated with each of 100 μl aliquots of mouse anti-human IL-8 monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) 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, St. Louis, MO, USA) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for 2 h. After additional washes, sample or recombinant IL-8 standards was added and incubated at 37°C for 2 h. After 2 h of incubation at 37°C, the wells were washed and then each of 0.2 μg/ml of biotinylated anti-human IL-8 was added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37°C. Wells were again washed 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 (R&D Systems) in serial dilutions.

RT-PCR analysis

Total RNA was isolated from HMC-1 cells according to the manufacturers specification using easy-BLUE RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.5 μg) was heated at 65°C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37°C using cDNA synthesis kit (AmershamPharmacia, USA). PCR was performed with the following primers for human (h) IL-6 (5' ATG AAC TCC TTC TCC ACA AGC GC 3'; 5' GAA GAG CCC TCA GGC TGG ACT G 3') and IL-8 (5' CGA TGT CAG TGC ATA AAG ACA 3'; 5' TGA ATT CTC AGC CCT CTT CAA AAA 3'). The GAPDH (5'CAA AAG GGT CAT CAT CTC TG 3'; 5'CCT GCT TCA CCA CCT TCT TG 3') was used to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 56°C for hIL-6 and 60°C for IL-8 and GAPDH, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

MTT assay

Cells were plated out at a density of 5×10^4 cells/ml in 4-well plates (Nunc, Sweden) and allowed an overnight period for attachment. Then the medium was removed and fresh medium, along with various extracts of 02PS15, was added to cultures in parallel. After incubating for 8 h, MTT assay was performed by the method of Scudiero *et al.* (Scudiero *et al.*, 1988). To determine the cell viability, 50 μl of MTT (5 mg/ml) was added to each well and cells were cultured additional incubation for 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, optical density of 96-well culture plates was measured using an ELISA reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

Statistical analysis

The results were expressed as the mean ± standard error of the mean (S.E.M.). Statistical significance was compared between each treated group and control by the Independent *t*-test. Results with $P<0.05$ were considered statistically significant.

RESULTS

Effects of 02PS15 extracts on PHA and PMA-induced IL-4 and IL-6 production from PBMCs

We examined that 02PS15 extracts were able to stimulate potential mediators such as IL-4 and IL-6 in isolated PBMCs. PBMCs were cultured with various extracts of 02PS15 for 30 min and then stimulated with PHA and PMA for 24 h. ELISA measured the amount of IL-4 and IL-6 secreted by the cells. As shown in Fig. 1, PHA and PMA stimulate the production of IL-4 and IL-6 (27 ± 1.29 pg/ml for IL-4 and 23.22 ± 1.74 ng/ml for IL-6) from PBMCs. The amount of IL-4 and IL-6 were significantly inhibited in the BuOH and crude extracts of 02PS15 pretreated cells than PHA and PMA-treated cells (6.5 ± 0.5 pg/

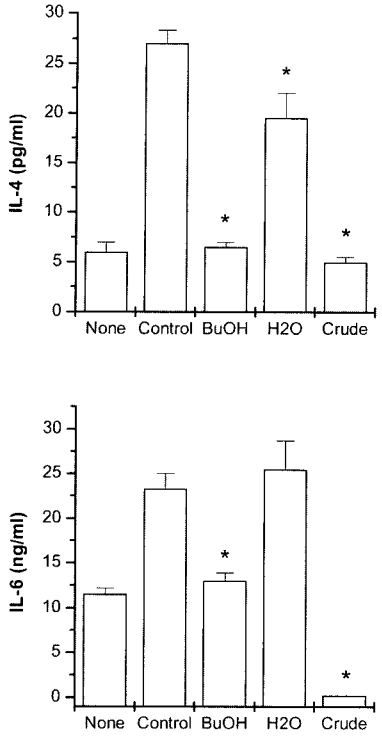

Fig. 1. PBMCs were stimulated with PHA (25 μg/ml) and PMA (50 ng/ml) in the presence of 02PS15 extracts. The supernatants were harvested after 24 h of culture. IL-4 and IL-6 secreted into the medium are presented as the mean ± S.E.M. of six independent experiments. *P<0.05, different from PHA and PMA treated control value.

ml and 5.0 ± 0.5 pg/ml for IL-4, 12.92 ± 0.92 ng/ml and 0.20 ± 0.02 ng/ml for IL-6, P<0.05) (Fig. 1).

Effects of 02PS15 extracts on histamine release from RPMCs

The inhibitory effects of 02PS15 extracts on compound 48/80-induced histamine release from RPMCs are shown in Fig. 2. RPMCs (2×10^5 cells/ml) were preincubated with the 02PS15 extracts at 37°C for 30 min prior to the challenge with compound 48/80. BuOH and Crude extracts of 02PS15 (100 μg/ml) significantly inhibited compound 48/80-induced histamine release from RPMCs (P<0.05, compared with compound 48/80 stimulated value). Inhibition rates of histamine release were 59.62% at the treated BuOH extracts of 02PS15 and 49.85% at the treated Crude extracts of 02PS15.

Effects of 02PS15 extracts on IL-8 secretion from HMC-1 cells

As well as histamine release, activated mast cells can produce proinflammatory cytokine such as IL-8. The HMC-1 cells

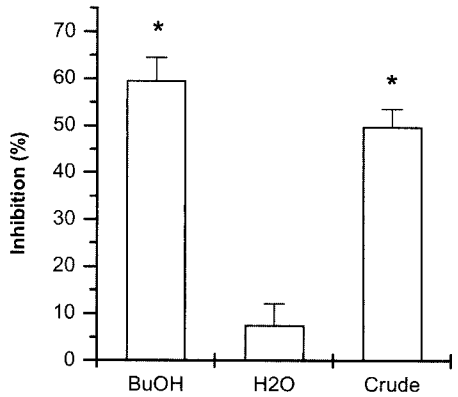

Fig. 2. RPMCs were preincubated with various extracts (BuOH, H_2O, crude extract) of 02PS15 at 37°C for 30 min prior to incubation with compound 48/80 for 15 min. Each datum represents the mean ± S.E.M. of six independents experiments. *P<0.05, different from compound 48/80 stimulated value.

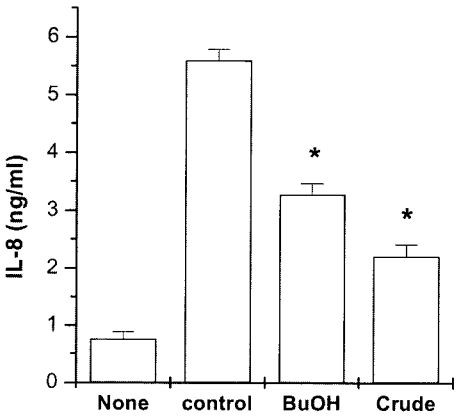

Fig. 3. HMC-1 cells were stimulated with PMA (50 μg/ml) and A23187 (1 ng/ml) in the presence of 02PS15 extracts. The supernatants were harvested after 8 h of culture. IL-8 secreted into the medium are presented as the mean ± S.E.M. of six independent experiments. *P<0.05, different from PMA and A23187 treated control value.

were stimulated with PMA and calcium ionophore A23187. To assess the effects of 02PS15 extracts in PMA and A23187-induced IL-8 secretion, the cells were pretreated with 02PS15 extracts (100 μg/ml) for 30 min prior to stimulators. These results showed that pretreatment of the cells with 02PS15 extracts inhibited IL-8 secretion. BuOH and crude extracts of 02PS15 significantly inhibited secretion of IL-8 by 41.50% and 60.28% respectively (Fig. 3). But the inhibitory effects of 02PS15 H_2O extracts against IL-8 secretion stimulated by PMA and A23187 were not investigated.

Expression of cytokine by 02PS15 extracts in HMC-1 cells

HMC-1 cells were treated with PMA (50 ng/ml) and

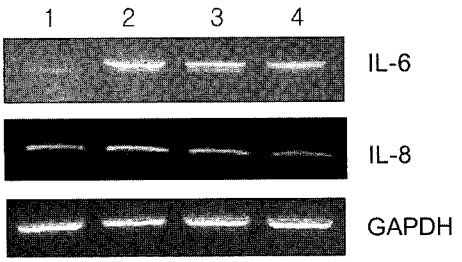

Fig. 4. HMC-1 cells were treated with PMA (50 ng/ml) and A23187 (1 μM) for 2 h. The mRNA levels of IL-6 and IL-8 were measured using RT-PCR. 1, unstimulated cells; 2, PMA + A23187; 3, PMA+A23187+BuOH extract; 4, PMA+A23187+ crude extract.

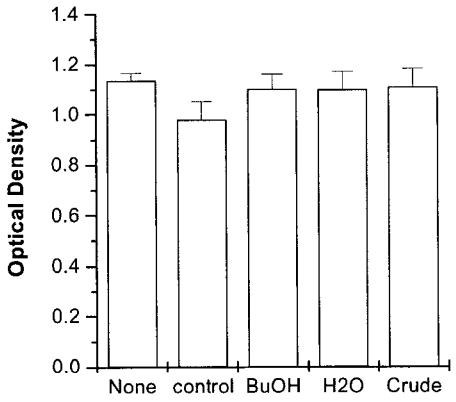

Fig. 5. Cytotoxic effects of 02PS15 on HMC-1 cells in various extracts (BuOH, H_2O, crude extract). The cell viability of HMC-1 cells was measured by MTT assay.

A23187 (1 μM) for 2h. As shown in Fig. 4, constitutive expression in unstimulated cells was found for IL-6 and IL-8. Enhanced levels of IL-6 and IL-8 mRNA were inhibited after treatment with BuOH and crude extract of 02PS15.

Effects of 02PS15 extracts on the cytotoxicity of HMC-1 cells

HMC-1 cells (3×10^5 cells/ml) were stimulated with 02PS15 extracts (BuOH, H_2O, and Crude extracts, 100 mg/ml) at 37°C for 30 min prior to the addition of PMA (50 nM) and A23187 (1 μM) for 8 h. After incubating for 8 h, cell viability was measured by MTT assay. 02PS15 extracts exerted no cytotoxic effect for 8 h (Fig. 5).

DISCUSSION

We found that 02PS15 extracts significantly inhibited IL-4 and IL-6 secretion from PBMCs and inhibits histamine release induced by compound 48/80 from RPMCs. Significantly higher levels of IL-4 are expressed in human atopic dermatitis (Leung, 2000). PBMCs from patients with AD produced significantly higher levels of IL-6 compared to health non-atopic controls (McHugh *et al.*, 1994). McHugh *et al.* showed that early and sustained production of large amounts of IL-6 typifiles the immune response to specific allergens in atopic patients. They assumed that IL-6 from monocytes and other cells contributes directly and indirectly (via induction of IL-4) to a predominantly Th-2 cytokine environment.

Different manifestations of immunological disorders are an increment in the liberation of proinflammatory mediators by mast cells (Sosa Vazquez *et al.*, 2001). The important role of mast cells in immediate-type allergic reactions is well recognized. After activation by immunological or non-immunological stimuli, mast cells release various preformed mediators including histamine, protease, adenosine, acid hydrolases, and proteoglycans. The report that the mast cell mediators other than histamine are involved in compound 48/80-induced pruritus in AD patients has been published (Rukwied *et al.*, 2000).

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 (Gordon *et al.*, 1990; Plaut *et al.*, 1989; Bradding *et al.*, 1992). The regulation of cytokines secretion from mast cells can provide us with a useful therapeutic strategy for allergic inflammatory disease, such as AD. IL-6 is known to potentiate IgE production and it is related with allergic manifestation. IL-8 is a chemoattractant for neutrophils, macrophages, and T lymphocytes (Baggiolini *et al.*, 1994) and facilitates the migration of these cells into inflamed skin (Neuber *et al.*, 1995; Euber *et al.*, 1991). IL-8-producing inflammatory cells are found in the dermis of atopic patients (Van Joost *et al.*, 1992). In addition, synthesis of IL-8 has already been described in the HMC-1, but only after stimulation by chemical compounds such as calcium ionophore and PMA (Moller *et al.*, 1998). 02PS15 extracts showed a significant inhibitory effect on IL-8 secretion, the major factor of mast cell-mediated inflammation, from HMC-1 cells. These results suggest that 02PS15 extracts contributes to the regulation of atopic allergic reaction. In addition, 02PS15 extracts (BuOH extracts and Crude) effectively downregulated the expression of IL-6 and IL-8 in the activated HMC-1 cells by RT-PCR.

In conclusion, the results obtained in the present study provide evidence that 02PS15 extracts significantly decreased IL-4, IL-6, and IL-8 secretion in PBMCs and mast cells. These results are similar with the inhibition of Th-2 cytokine increase that is occurred in the atopic allergic reaction such as AD.

Future studies on the isolation and characterization of the active chemical constituents are needed.

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