A Citrus Flavonoid, 6-Demethoxytangeretin, Suppresses Production and Gene Expression of Interleukin-6 in Human Mast Cell-1 *via* Anaplastic Lymphoma Kinase and Mitogen-Activated Protein Kinase Pathways

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Citrus species has been traditionally used in Korea for the treatment of coughing, sputum and dyspepsia. Of the known citrus flavonoids, 6-demethoxytangeretin was reported to exert anti-inflammatory activity. In order to determine the anti-allergic activity of 6-demethoxytangeretin, we examined whether or not 6-demethoxytangeretin was able to suppress activation of the human mast cell line, HMC-1, induced by phorbol 12-myristate 13-acetate (PMA) plus A23187. Interleukin-6 production and relevant gene expression in activated HMC-1 cells were determined by enzyme-linked immunosorbent assay (ELISA) and quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Also, the involvement of the anaplastic lymphoma kinase (ALK) and mitogen-activated protein kinases (MAPKs) in activated HMC-1 cells were studied. 6-Demethoxytangeretin suppresses interleukin-6 production, tumor necrosis factor- α gene expression, ALK and MAPKs in HMC-1 cells stimulated by PMA plus A23187. Therefore, it was evident that 6-demethoxytangeretin suppressed activation of HMC-1 cells by PMA plus A23187 by inhibiting the activity of ALK and MAPKs and subsequently suppressing gene expression, which suggest that 6-demethoxytangeretin may be involved in the regulation of mast cell-mediated inflammatory responses.

Key words 6-demethoxytangeretin; human mast cell-1; interleukin-6; anaplastic lymphoma kinase; mitogen-activated protein kinase

Mast cells are broadly distributed throughout mammalian tissues and play a critical role in a wide variety of biological responses. In general, immediate-type hypersensitivity reactions that involve allergic rhinitis and asthma are mediated by various chemical mediators released from mast cells.¹⁾ Activated mast cells release pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-4, IL-6, IL-8 and inflammatory mediators including histamine, leukotrienes, serotonin, prostaglandins.^{2,3)} Cytokines, such as TNF- α , IL-4, IL-6 and IL-8 are released in a coordinate network and play an important role in chronic inflammation or allergy. As such, the pattern of cytokine expression largely determines the nature and persistence of the inflammatory response.⁴⁾ Thus, the importance of mast cell in immunomodulation has been discussed in recent reviews and reports.^{5–7)}

Phorbol 12-myristate 13-acetate (PMA) has been used as a biomedical research tool in models of carcinogenesis. PMA, together with calcium ionophore (A23187), is also used to stimulate T-cell activation, proliferation, and cytokine production.⁸⁾ PMA directly affects phosphatidylinositol kinase or indirectly regulates its activity in mast cell.^{9–12)}

6-Demethoxytangeretin (6DMT) is, one of methoxyflavones in *Citrus* species, known to be involved in various pharmacological activities including neuritogenic activities and anti-inflammatory activities. Recent studies have reported that 6DMT regulates cAMP/protein kinase A (PKA)/extracellular

signal-regulated kinase (ERK)/cAMP response element-binding protein (CREB) signaling and expression of matrix metalloproteinase 9.^{13,14)} However, its anti-allergic effect on human mast cell-1 (HMC-1) remains unknown. In the present study, 6DMT was evaluated for its inhibitory effect on the IL-6 production, and activation of anaplastic lymphoma kinase (ALK) and mitogen-activated protein kinase (MAPK) in PMA plus A23187-induced HMC-1 along with mRNA expressions of cytokines.

MATERIALS AND METHODS

Cell Culture A human mast cell line, HMC-1, was obtained from the Korea Research Institute of Bioscience and Biotechnology (South Korea) and grown in IMDM Medium containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin sulfate. Cells were incubated in a humidified 5% CO₂ atmosphere at 37°C.

Drugs and Chemicals IMDM, penicillin, and streptomycin were purchased from Hyclone (Logan, UT, U.S.A.). Bovine serum albumin, phorbol 12-myristate 13-acetate (PMA) and A23187 were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-mouse IL-6 antibody and biotinylated anti-mouse IL-6 antibody were purchased from BD Biosciences (BD Pharmingen, San Diego, CA, U.S.A.). p-Signal transducer and activator of transcription (STAT)3, STAT3, p-Janus family kinase (JAK), JAK, p-PLC γ 1, PLC γ 1, p-AKT, AKT, p-ALK, ALK, β -actin, p-ERK, ERK, p-c-Jun N-terminal kinase (JNK), JNK, p-p38, p38 antibodies were purchased from Cell Signal-

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ing Technology, Inc. (Danvers, MA, U.S.A.). TNF- α , GSF2, IL-6, IL-6R, IL-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide primers were purchased from Bioneer Corp. (Daejeon, Korea). Sinensetin (1), nobiletin (2), tangeretin (3), 3-methoxytangeretin (4), 3-methoxynobiletin (5), and 6-demethoxytangeretin (6, 6DMT) were purified from the peels of *Citrus unshiu* fruits and identified by measuring NMR and MS spectroscopic data.

Determination of Interleukin-6 Levels Cells were seeded at 1×10^6 /mL per well in 24 well tissue culture plates and pretreated with the indicated concentration of compounds for 30 min before stimulation. After 24 h, the supernatant was decanted into a new micro centrifuge tube, and the amount of interleukin-6 (IL-6) was determined using the enzyme-linked immunosorbent assay (ELISA) kit according to the procedure described by the manufacturer (BD Bioscience, U.S.A.). All subsequent steps took place at room temperature, and all standards and samples were assayed in duplicate.

Immunoblot Analysis Protein expression was assessed by western blot analysis according to the standard procedure. Briefly, HMC-1 was cultured in 60 mm culture dishes (2×10^6 /mL) and then pretreated with various concentrations of 6DMT (0.8, 4, 20 μ M). After 30 min of pretreatment, PMA (50 nM) plus A23187 (1 μ M) was added to the culture medium, and the cells were then incubated at 37°C for 30 min. Following incubation, the cells were washed twice in ice cold phosphate buffered saline (PBS) (pH 7.4). The sample treated cell pellets were then resuspended in lysis buffer on ice for 15 min, after which the cell debris was re-moved by centrifugation. Protein concentration was then determined using Bio-Rad protein assay reagent according to the manufacturer's instructions. Protein (20–30 μ g of whole cell) was mixed 1:1 with 2 $\times$ sample buffer (20% glycerol, 4% sodium dodecyl sulfate (SDS), 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris [pH 6.8]), loaded onto a 8 or 15% SDS-polyacrylamide gel electrophoresis (PAGE) gel, and run at 150 V for 90 min. Cell proteins were transferred to an ImmunoBlot polyvinylidene difluoride membrane (Bio-Rad) with a Bio-Rad semidry transfer system according to the manufacturer's instructions. The polyvinylidene difluoride membrane was then incubated with primary antibody (Ab) (dilutions 1:500–1:1000) in 5% milk in Tris-buffered saline with 0.1% Tween 20 overnight. The blots were washed three times with Tris-buffered saline with 0.1% Tween 20 and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary anti-immunoglobulin G (IgG) Ab (dilution 1:2000–1:20000). The blots were washed again three times with Tris-buffered saline with 0.1% Tween 20, and immunoreactive bands were developed using the chemiluminescent substrate ECL Plus (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total cellular RNA was isolated using a Trizol RNA extraction kit according to the manufacturer's instructions. Briefly, total RNA (1 μ g) was converted to cDNA by treatment with 200 units of reverse transcriptase and 500 ng of oligo-dT primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), and 1 mM deoxyribonucleotide triphosphates (dNTPs) at 42°C for 1 h. The reaction was then stopped by incubating the solution at 70°C for 15 min, after which 1 μ L of the cDNA mixture was used for enzymatic amplification. PCR

reactions were performed using 1 μ L cDNA and 9 μ L master mix, containing iQ SYBR Green Supermix (Bio-Rad), 5 pmol of forward primer, and 5 pmol of reverse primer, in a CFX384 Real-Time PCR Detection System (Bio-Rad) as follows: 3 min at 95°C followed by 40 cycles of 10 s at 95°C and 30 s at 55°C and plate read. The fluorescence signal generated with SYBR Green I DNA dye was measured during annealing steps. Specificity of the amplification was confirmed using a melting curve analysis. Data were collected and recorded by CFX Manager Software (Bio-Rad) and expressed as a function of threshold cycle (C_T). The relative quantity of the gene of interest was then normalized to the relative quantity of hypoxanthine phosphoribosyltransferase ($\Delta\Delta C_T$). The sample mRNA abundance was calculated by the equation $2^{-(\Delta\Delta C_T)}$. Specific primer sets used are as follows (5' to 3'): IL-6 CCA CAC AGA CAG CCA CTC AC (forward), TGA TTT TCA CCA GGC AAG TCT (reverse); GAPDH GAAGGTGAAGGTCTGGAGTCA (forward), AATGAAGGGGTCATTGATGG (reverse); IL6R CTCAGTGTCACCTGGCAAGA (forward), AGTGATGCTGGAGGTCTTG (reverse); TNF- α TCA GCC TCT TCT CCT TCCTG (forward), GCCAGAGGGCTGATTAGAGA (reverse); CSF2 TCTCAGAAATGTTTGACCTCCA (forward), AGGGCAGTGCTGCTTGATG (reverse); IL-8 CTGCGC CAACACAGAAATTA (forward), CTCTGCACCCAGTTT TCCTT (reverse). Gene-specific primers were custom-synthesized and purchased from Bioneer, Daejeon, Korea.

RT-PCR Total cellular RNA was isolated using a Trizol RNA extraction kit according to the manufacturer's instructions. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Specific primer sets used are as follows (5' to 3'): IL-6 AAAGAGGCACTGGCA GAAAA (forward), AAAGCTGCGCAGAATGAGAT (reverse); GAPDH GTCAGTGGTGGACCTGACCT (forward), AGGGGAGATTCAAGTGTTG (reverse); TNF- α AGC CCA TGT TGT AGCAAACC (forward), GGAAGACCCCTC CAGATAG (reverse); IL-8 AGGGTTGCCAGATGCAAT AC (forward), GTG GAT CCT GGCTAGCAGAC (reverse). Gene-specific primers were custom-synthesized and purchased from Bioneer, Daejeon, Korea.

Statistical Analysis Data from the experiments are presented as the mean $\pm$ S.E.M. The level of statistical significance was determined by ANOVA followed by Dunnett's *t*-test for multiple comparisons. The *p*-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Six flavonoids were isolated from *Citrus unshiu* and identified as sinensetin (1), nobiletin (2), tangeretin (3), 3-methoxytangeretin (4), 3-methoxynobiletin (5), and 6-demethoxytangeretin (6, 6DMT) by comparing these spectroscopic data with the published data. Culture supernatants were assayed for IL-6 levels using the ELISA method. Of the isolated compounds 1–6, compounds 1, 3 and 6 displayed the inhibitory activity against IL-6 production in stimulated HMC-1 with IC₅₀ values, 8.00, 4.07 and 4.01 μ M, respectively (Fig. 1A). The cell viability test with compounds in the human mast cell (HMC-1) revealed no toxicity up to 20 μ M (data not shown). Effect of compounds on IL-6 mRNA expression induced by PMA (50 nM) plus A23187 (1 μ M) was examined using RT-PCR. As shown in Fig. 1B, treatment of PMA plus A23187

Fig. 1. Effect of Compounds from *Citrus unshiu* on the Release of IL-6 (A), the Expression of TNF- α , IL-6, IL-8 mRNA (B), Densitometry Quantification of TNF- α , IL-6, IL-8 mRNA Relative Fold Changes of TNF- α (C), IL-6 (D) and IL-8 (E) in PMA (50nM) Plus A23187 (1 μ M)-Stimulated HMC-1

Statistical significance: * p <0.05 as compared to the PMA plus A23187 treated group. Values shown are the mean $\pm$ S.E. of duplicate determinations from three separate experiments.

increased the mRNA expression of TNF- α , IL-6 and IL-8 whereas compounds **1**, **3** and **6** suppressed this increase. Of the compounds tested, 6DMT (**6**) exhibited the most potent inhibitory activity against TNF- α , IL-6 and IL-8 production in stimulated HMC-1 (Figs. 1C–E).

Cytokines play several roles in inflammatory responses such as leukocyte proliferation and activation.¹⁵⁾ Colony stimulating factor 2 (CSF2) is a cytokine for the *in vitro* generation of immune cells and is thought to control the development of inflammation.¹⁶⁾ Also, inhibition of interleukin-6 receptor prevents IL-6 signal transduction to inflammatory mediators that summon B and T cells.¹⁷⁾ PMA plus A23187 induce the expression of genes for inflammatory cytokine, such as tumor necrosis factor- α (TNF- α), IL-6 and IL-8 in mast cells.¹⁸⁾ We performed real-time RT-PCR analysis of the expression of mRNA related to inflammation and immunity in

HMC-1 cells that has been treated with PMA plus A23187 for 6h. In the presence of PMA plus A23187, five genes including those for inflammatory cytokine, interleukins, CSF2 and TNF- α , showed significantly increased expression in HMC-1 (Fig. 2). We pre-incubated HMC-1 with 6DMT for 30min, and then incubated the cells for 6h with PMA plus A23187. The increased levels of gene expression observed in the HMC-1 cells in the presence of PMA plus A23187 were substantially reduced by treatment with 4 or 20 μ M of 6DMT (**6**) (Fig. 2).

Mitogen-activated protein kinase (MAPK) signaling cascades have been shown to be important in the activation, proliferation and degranulation of various immune cells.^{19,20)} In addition, MAPKs regulate expression of pro-inflammatory genes.²¹⁾ It has been documented that activation of HMC-1 cells by PMA plus A23187 is associated with phosphorylation of MAPKs.²²⁾ In order to elucidate the underlying mechanisms

Fig. 2. Effect of 6DMT on Expression of TNF- α , CSF2, IL-6, IL-6R and IL-8 in PMA (50 nm) Plus A23187 (1 μ M)-Stimulated HMC-1

Statistical significance: * $p < 0.05$ as compared to the PMA plus A23187 treated group. ** $p < 0.005$ as compared to the PMA plus A23187 treated group. Values shown are the mean $\pm$ S.E. of duplicate determinations from three separate experiments.

Fig. 3. Effects of 6DMT on the Phosphorylation of MAPKs in Stimulated HMC-1

HMC-1 was treated with the indicated concentrations of 6DMT for 30 min prior to being incubated with PMA (50 nm) plus A23187 (1 μ M) for 30 min. Whole cell lysates were then analyzed by Western blot. Equal amounts of protein (20 μ g) were then separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies.

of 6DMT, we examined the effects of 6DMT on activation of MAPKs. The stimulation of HMC-1 cells with PMA plus A23187 resulted in an increased phosphorylation of all three types of MAPKs, p38, JNK, ERK after 30 min post treatment. 6DMT apparently suppressed phosphorylation of ERK1/2, JNK1/2 and p38 in HMC-1 cells (Fig. 3).

Application of the stimulants to release inflammatory mediator from mast cells, such as combination of A23187 and PMA, as an activator of PLC and PKC, released inflammatory mediator in our experimental system.²³⁻²⁵ Activated PLC γ cleaves the membrane-bound lipid phosphatidylinositol-4,5-bisphosphate into diacylglycerol, a stimulator of protein kinase C and inositol 1,4,5-trisphosphate the ligand for the IP $_3$ receptor calcium channel in the ER (endoplasmic reticulum).²⁶ In addition, it is reported that PMA and A23187 are engaged in both the JAK/STAT pathways.^{27,28} Anaplastic lymphoma

Fig. 4. Effect of 6DMT on the PMA (50 nm) Plus A23187 (1 μ M)-Induced Activation of NPM-ALK Pathway in HMC-1

HMC-1 was pretreated with 6DMT for 0.5 h and then stimulated for 0.5 h to detect phosphorylated NPM-ALK, JAK2, STAT3, AKT and PLC γ 1.

kinase (ALK) is a receptor tyrosine kinase with a transmembrane domain and an extracellular domain.²⁹ Growth factor or activators induce phosphorylation of both ALK and the downstream effectors, STAT, JAK, PLC γ , and AKT.³⁰ The effect of 6DMT on ALK, JAK2, STAT3, AKT and PLC γ 1 phosphorylation induced by PMA plus A23187 was examined using immunoblot analysis. As shown in Fig. 4, when treated

with 6DMT, phosphorylation of nucleophosmin (NPM)-ALK, JAK2, STAT3, AKT and PLC γ 1 was suppressed in a dose dependent manner.

Regulators of the ALK pathway, such as suppressors of cytokine and protein inhibitors of activated ALK proteins, function to modulate the inflammation, immune response and maintain homeostasis.²⁹⁾ PLC γ is a downstream target of NPM-ALK that contributes to its mitogenic activity.³¹⁾ In addition, NPM/ALK exerts its activity through activation of multiple signaling cascades prompted us to investigate the role of Jak2/SAT3 pathway.³²⁾ NPM/ALK constitutively activates the PI3K-Akt pathway and that this pathway plays an important role in the NPM/ALK.³³⁾ Thus, JAK/ STAT, AKT and PLC γ contribute NPM/ALK activation that plays a role in allergic inflammation.

From these observations, ALK and MAPKs seemed to be involved in the production of cytokine in PMA plus A23187 stimulated mast cell.³⁴⁾ 6DMT is thought to exert its anti-allergic activity by inhibiting the release or expression of TNF- α , CSF2, IL-6, IL-6R and IL-8 via suppressing ALK and MAPKs activation stimulated by PMA plus A23187 in human mast cells.

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