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6-Methoxyluteolin from *Chrysanthemum zawadskii* var. *latilobum* Suppresses Histamine Release and Calcium Influx *via* Down-Regulation of FcεRI α Chain Expression

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Mast cells and basophils are important effector cells in immunoglobulin-E (IgE)-mediated allergic reactions. Using the human basophilic KU812F cells, we assessed the inhibitory effects of 6-methoxyluteolin, isolated from *Chrysanthemum zawadskii*, in the FcεRI-mediated allergic reaction. We determined that 6-methoxyluteolin inhibited anti-FcεRI α chain antibody (CRA-1)-induced histamine release, as well as elevation of intracellular calcium concentration $[Ca^{2+}]_i$ in a dose-dependent manner. Moreover, the inhibitory effects of 6-methoxyluteolin on the cell surface expression and the mRNA level of the FcεRI α chain were determined by flow cytometric analysis and reverse transcription-polymerase chain reaction (RT-PCR), respectively. Therefore, these results show that 6-methoxyluteolin is a potent inhibitor of histamine release and calcium influx *via* down-regulation of the FcεRI α chain.

Keywords: *Chrysanthemum zawadskii* var. *latilobum*, 6-methoxyluteolin, histamine, calcium influx, FcεRI

The worldwide prevalence and severity of allergic diseases has increased dramatically over the past decade, especially in developed countries; thus, it is essential that we find preventative strategies to suppress individuals' sensitization to environmental allergens and the onset of allergic diseases [1]. Mast cells and basophils play an important role as effector cells in IgE-mediated allergic reactions. The aggregation of FcεRI by multivalent allergen–IgE complexes or by anti-FcεRI antibody to its receptor triggers degranulation of activated mast cells and basophils, resulting in IgE-mediated allergic responses such as asthma, atopic dermatitis, and allergic rhinitis [2–5]. Elevation of the intracellular calcium concentration and the release of inflammatory mediator such as histamine and β-hexosaminidase induced by FcεRI cross-linking and various stimuli are considered an essential mechanism in the process of mast cell and basophil degranulation [6, 7].

FcεRI is a high-affinity tetrameric IgE receptor that is expressed on the surfaces of mast cells and basophils, and is composed of 1 α, 1 β, and 2 γ chains. Among the 3 FcεRI subunits, the α chain mostly extends out to the extracellular region, binds directly to the Fc portion of the IgE antibody with high affinity, and is a specific component of FcεRI [8]. Thus, inhibition of degranulation by the down-regulation of FcεRI expression may lead to attenuation of the IgE-mediated allergic reaction.

Chrysanthemum zawadskii var. *latilobum,* known as "Gu-Jeol-Cho" in Korea, is a perennial herb of the Compositae family, and has been used as a traditional medicine for the treatment of pneumonia, bronchitis, cough, common cold, pharyngitis, bladder-related disorders, gastroenteric disorders, and hypertension. This plant has a variety of pharmacological properties, including cancer protective, oxidative, inflammatory, and liver-protective effects, and it has the ability to differentially regulate Nrf2-mediated genes [9–16]. Moreover, *C. zawadskii* is flavonoid-rich, and linarin and acacetin isolated from this plant have protective effects against cancer and inflammation (linarin, acacetin). However, the properties of 6-methoxyluteolin isolated from *C. zawadskii* has not yet been studied.

Many studies have recently shown the anti-allergic effects of bioactive compounds such as catechin, (-)-epigallocatechin-3-0-gallate, kaempferol, and phlorotannins on FcεRI-mediated allergic reactions [17–21]. We previously reported that the methanol (MeOH) extract of *C. zawadskii* exhibited anti-allergic activity by suppressing FcεRI expression [22]. In the present study, we investigated the

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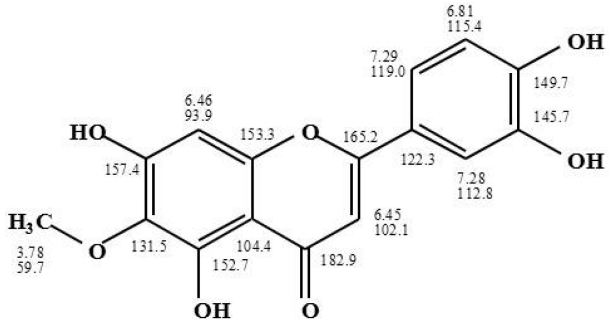

Fig. 1. Chemical structure and NMR data (^{1}H and ^{13}C) of 6-methoxyluteolin isolated from *C. zawadskii*.

inhibitory effects of 6-methoxyluteolin (Fig. 1) isolated from *C. zawadskii* on FcεRI-mediated allergic reactions.

Materials and Methods

Materials and Chemicals

NMR spectra were determined using a JNM-ECA 600 (^{1}H-NMR 600 MHz; ^{13}C-NMR 150 MHz) FT-NMR spectrometer (Jeol, Tokyo, Japan) with tetramethylsilane as an internal standard. The chemical shifts were referenced to the respective residual solvent peaks (δH 3.30 and δC 49.0 for CD_3OD) and values expressed in ppm were recorded. Electrospray ionization-mass spectrometry (ESI-MS) spectra were obtained using an Agilent 1100 liquid chromatography (LC)/MS spectrometer (Agilent, Santa Clara, CA, USA). Column chromatography was performed on a Sephadex LH-20 (GE Healthcare Bioscience, Sweden). Thin-layer chromatography (TLC) was conducted using a Merck Kieselgel 60 F_{254} plate (0.25 mm), and the spots were detected under ultraviolet light using 50% H_2SO_4. All solvents used in column chromatography were of reagent grade from commercial sources.

Extraction and Isolation of Plant Materials

The whole plant of *C. zawadskii* used in this study was purchased from Herbal Medicine Merchandise, Jecheon Hanbang Yakcho (http://www.jchanbang.com), South Korea. A voucher specimen was deposited at the author's laboratory. Lyophilized whole plant powder (1 kg) of *C. zawadskii* was extracted using MeOH (3 × 9 L) at room temperature for 2 days. The extract was suspended in water and partitioned sequentially with *n*-hexane, $CHCl_3$, and EtOAc. 3-Methoxyluteolin was isolated from the EtOAc layer, which inhibited the histamine release. The EtOAc layer was applied to a Sephadex LH-20 column with 70% MeOH, which yielded fractions I and II. The fractions were further fractionated on a high-performance liquid chromatography system (Agilent 1100; Agilent) using an octadecyl silane column (10 × 150 mm, 5 μm; Tosoh, Japan) at a flow rate of 3 ml/min by using a 5~100% aqueous methanol gradient system (0.04% trifluoroacetic acid) as the mobile phase. 6-Methoxyluteolin (8.1 mg) was isolated at a retention time of 20.2 min from fraction II.

Cell Culture, Treatment, and Stimulation

The human basophilic KU812F cells line was obtained from the American Type Culture Collection, maintained in RPMI-1640 (HyClone, Logan, UT, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and 10 mM HEPES (Sigma, St. Louis, MO, USA), at 37°C with a humidified atmosphere with 5% CO_2, and cells were passaged every 3–4 days. Cells were treated with compound under serum-free RPMI-1640 medium, and the cells were stimulated with 10 μg/ml of CRA-1 (Kyokuto, Tokyo, Japan) in Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2$) for 30 min at 37°C.

Cell Viability Assay

Cell viability was assayed using the Celltiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. KU812F cells were seeded in 96-well plates and incubated with serum-free medium in the presence of 6-methoxyluteolin. After treatment for 24 h, reagents were added, and cells were incubated for 4 h. Dehydrogenase enzymes in live cells convert MTS tetrazolium compound into colored formazan products; the absorbance was measured at 490 nm using a microplate reader (Molecular Devices, VersaMax, Sunnyvale, CA, USA). Relative cell viability was calculated compared with the absorbance of the untreated cells.

Measurement of Histamine

Histamine content was measured by a spectrofluorometric assay [23]. The treated and stimulated cells were centrifuged, and the supernatants were treated with 1 N NaOH and 0.2% OPA (Sigma) for 40 min on ice. The reaction was terminated by addition of 3 N HCl. The fluorescence intensity was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm on a microplate fluorescence reader (BioTek, FLx800, Winooskin, VT, USA).

$[Ca^{2+}]_i$ Measurement

$[Ca^{2+}]_i$ was measured using the Fura 2-AM (Sigma) calcium reactive fluorescence probe. KU812F cells were suspended in Tyrode solution, and incubated with 2.0 μM Fura 2-AM at 37°C for 30 min. The cells were then washed 3 times, resuspended in fresh buffer, and stimulated with 10 μg/ml of CRA-1. The Fura 2 fluorescence was monitored at an excitation wavelength of 360 nm and an emission wavelength of 528 nm.

Flow Cytometric Analysis

The expression of cell surface FcεRI was measured using indirect immunofluorescence and flow cytometry. In brief, KU812F cells (1×10^6 cells) were incubated with CRA-1 (10 μg/ml) for 60 min on ice. The cells were then stained with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-mouse IgG (20 μg/ml) (Jackson ImmunoResearch Lab., Baltimore, PO, USA) for 60 min on ice, followed by washing with ice-cold phosphate-buffered saline for flow cytometry. Mouse IgG antibody (10 μg/ml) replaced CRA-1 as a negative control.

RT-PCR

Total cellular RNA was isolated using TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using an $oligo(dT)_{20}$ primer (Gibco BRL) and MMLV reverse transcriptase (Promega). The resultant cDNA samples were subjected to PCR amplification in the presence of specific sense and antisense

primers. Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a control. Primer sequences used in this study were as follows: FcεRI α chain, sense 5'-CTTAGGATGTGGGTTCAGAAGT-3' and antisense 5'-GACAGTGGAGAATACAAATGTCA-3'; and G3PDH, sense 5'-GCTCAGACACCATGGGGAAGGT-3' and antisense 5'-GTGGTGCAGGAGGCATTGCTGA-3'. PCR was performed as follows: denaturation at 94°C for 30 s; annealing at 55°C for 30 s; and extension at 72°C for 1 min, with 18 cycles for FcεRI α chain and G3PDH genes. The amplified PCR products were analyzed using agarose gel electrophoresis.

Statistical Analysis

All measurements were performed independently in at least triplicate. Data were expressed as the mean ± SD. Statistical differences between control and compounds groups were determined by the Student's *t*-test. Differences were considered significant at $p<0.05$ or $p<0.005$.

RESULTS AND DISCUSSION

Structure Identification of 6-Methoxyluteolin

Yellowish powder, positive ESI-MS *m/z* 317 $[M+H]^+$, $C_{16}H_{12}O_7$, ^{1}H-NMR (600 MHz, CD_3OD) δ 7.29 (1H, d, J = 8.2, H-6'), 7.28 (1H, s, H-2'), 6.81 (1H, d, J = 8.2, H-5'), 6.46 (1H, s, H-8), 6.45 (1H, s, H-3), 3.78 (3H, s, OCH_3). ^{13}C-NMR (150 MHz, CD_3OD) δ 182.9 (C-4), 165.2 (C-2), 157.4 (C-7), 153.3 (C-8a), 152.7 (C-5), 149.7 (C-4'), 145.7 (C-3'), 131.5 (C-6), 122.3 (C-1'), 119.0 (C-6'), 115.4 (C-5'), 112.8 (C-2'), 104.4 (C-4a), 102.1 (C-3), 93.9 (C-8), 59.7 (C-OCH_3) (Fig. 1) [24].

Cytotoxic Effects of KU812F Cells

To experimentally assess the nontoxic concentrations of the 6-methoxyluteolin, its effects on cell viability were determined using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium inner salt cell viability assay. We confirmed that at the concentrations (5~100 μM) examined, 6-methoxyluteolin had no significant effect on cell proliferation (Fig. 2). Therefore, this compound (at a concentration of 5~100 μM) was proven to have no cytotoxic effects, and could be used in further studies.

6-Methoxyluteolin Inhibits FcεRI-Mediated Histamine Release

Mast cells, basophils, and dendritic cells play pivotal roles in allergic disorders. The activation of mast cells or basophils initiates a series of biochemical events that result in the release of biologically active mediators that cause allergic reactions. A key mechanism for the stimulation of these cells is the interaction between the antigen and IgE bound to a high-affinity IgE receptor, FcεRI, on the cell surface. A series of biochemical events then results in the release of performed mediators from granules and the generation of newly synthesized mediators. Histamine is a potent inflammatory mediator that is stored in the secretory granules, and is released in immunologically activated mast cells and basophils. Thus, histamine in the medium is utilized as a marker of the degranulation of mast cells and basophils [25–28].

To investigate the inhibitory effects of 6-methoxyluteolin on FcεRI-mediated histamine release, we assessed its effects on the levels of histamine release in KU812F cells stimulated with CRA-1. As shown in Fig. 3, 6-methoxyluteolin reduced FcεRI-mediated histamine release in KU812F cells in a dose-dependent manner.

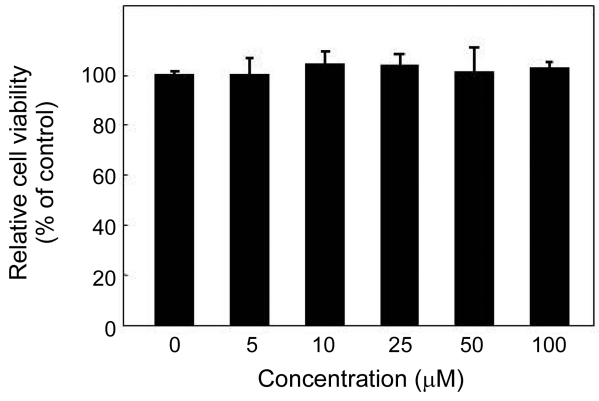

Fig. 2. Viability of KU812F cells in the presence of 6-methoxyluteolin.

KU812F cells were cultured in the presence of 6-methoxyluteolin (0, 5, 10, 25, 50, and 100 μM) for 24 h under serum-free conditions. The cell viabilities were determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium inner salt assay. Each determination was made in triplicate and the data are expressed as mean ± SD.

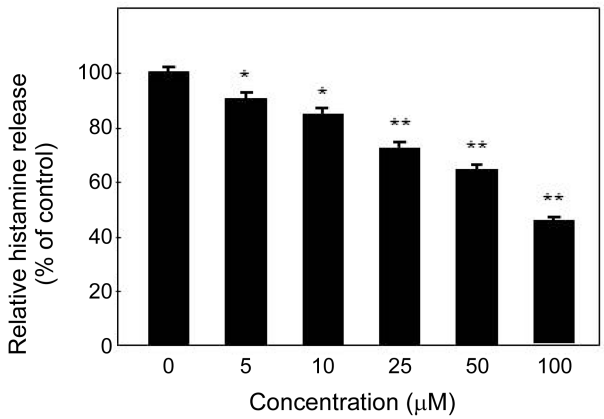

Fig. 3. Effects on FcεRI-mediated histamine release.

KU812F cells were cultured in the presence of 6-methoxyluteolin for 24 h and the cells were stimulated for 30 min with CRA-1 in Tyrode buffer. Histamine content released from stimulated cells was determined using a spectrofluorometric method. Each value represents the mean ± SD of 3 different experiments. *Values are significantly different from those of the control (*$p<0.05$ or (**$p<0.005$).

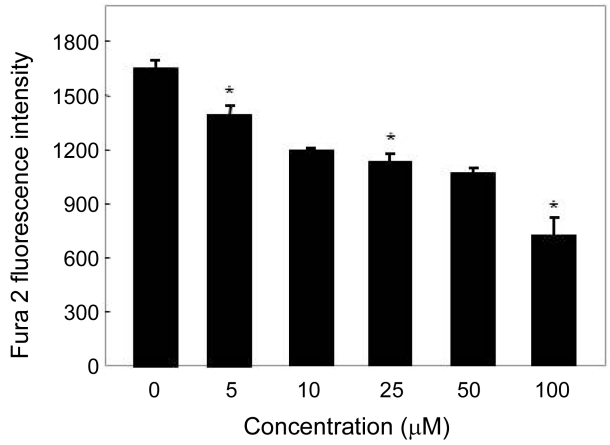

Fig. 4. Effects on FcεRI-mediated $[Ca^{2+}]_i$ elevation.
The pretreated cells with 6-methoxyluteolin were incubated with Fura 2-AM and stimulated for 30 min with CRA-1. $[Ca^{2+}]_i$ was determined spectrofluorometrically. Each value is expressed as the mean ± SD of 3 different experiments. *Values are significantly different from those of the control (*$p<0.05$).

Effects on FcεRI-Mediated $[Ca^{2+}]_i$ Elevation

$[Ca^{2+}]_i$ elevation contributes to degranulation, eicosanoid production, and cytokine production through FcεRI activation [29].

To determine the effects of 6-methoxyluteolin on calcium influx, KU812F cells were labeled with a calcium-specific fluorescence probe, Fura 2-AM, and stimulated with CRA-1. This compound inhibited the elevation of $[Ca^{2+}]_i$ in CRA-1-stimulated cells in a dose-dependent manner (Fig. 4). These results suggest that the inhibitory effects of 6-methoxyluteolin on FcεRI-mediated $[Ca^{2+}]_i$ elevation contribute to the inhibition of degranulation.

FcεRI-induced calcium signaling in mast cell and basophil activation is regulated by various PLCγ, which generates diacylglycerol. Cooperation of diacylglycerol and intracellular calcium signaling is activated by protein kinase C, which then activates other pathways such as the NF-κB pathway, ultimately leading to mast cell degranulation [29]. Therefore, further research on the regulation of transcriptional factors in FcεRI-induced downstream signaling by 6-methoxyluteolin must be performed.

Effects on FcεRI Expression

The FcεRI α chain mostly extends out to the extracellular region, binds directly to the Fc portion of the IgE antibody with high affinity, and is a specific component of FcεRI [25]. The role of FcεRI in an IgE-mediated allergic reaction caused by 6-methoxyluteolin was assessed using flow cytometric analysis and RT-PCR.

To evaluate the suppression of cell surface expression of FcεRI, KU812F cells were treated with different concentrations of 6-methoxyluteolin for 24 h under serum-free conditions, and the cell surface expression of FcεRI was assessed by indirect immunofluorescence and flow cytometry using CRA-1. The FcεRI expression on the cell

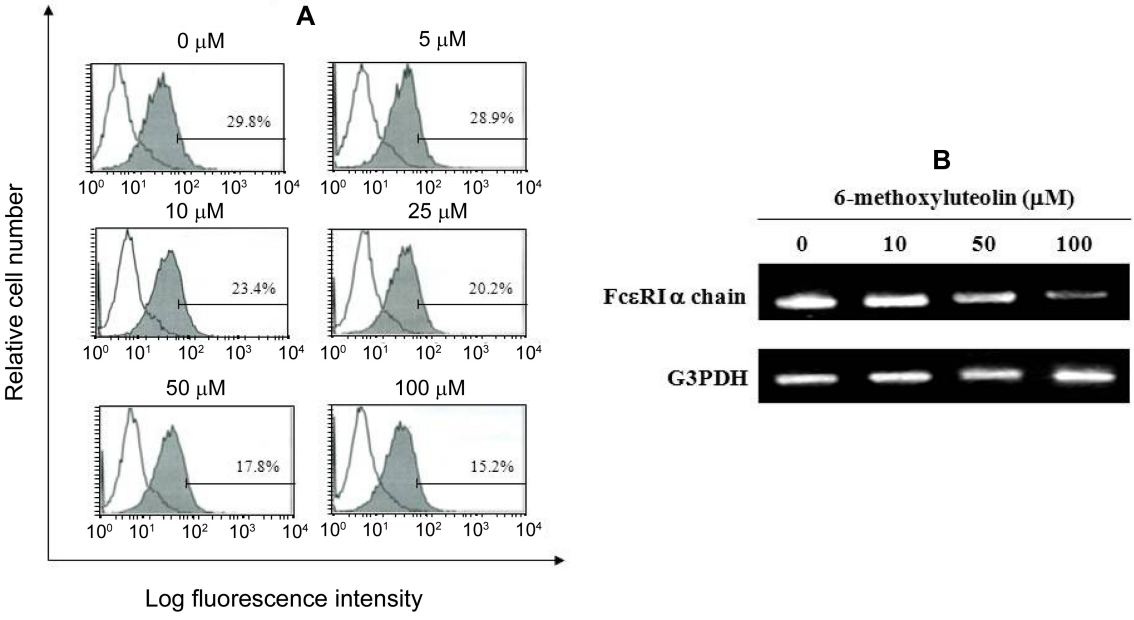

Fig. 5. Effects on FcεRI α-chain expression.
(**A**) Effects on the cell surface FcεRI α-chain expression. Pretreated cells were incubated with CRA-1, followed by staining with the FITC-conjugated $F(ab')_2$ goat anti-mouse immunoglobulins. The stained cells were then analyzed using flow cytometry. The number in the figures indicates the percentage of CRA-1-positive cells. (**B**) Effects on the FcεRI α-chain mRNA level. Total cellular RNA was extracted from the pretreated cells, and the FcεRI α-chain mRNA level was analyzed using RT-PCR.

surface was reduced from 29.8% to 28.9%, 23.4%, 20.2%, 17.8%, and 15.2% by treatment with 6-methoxyluteolin at 0, 5, 10, 25, 50, and 100 μM, respectively (Fig. 5A). Moreover, the mRNA level of the FcεRI α chain of non-treated cells was clearly detected, and the level of the corresponding mRNA in the 6-methoxyluteolin-treated cells was shown to be reduced (Fig. 5B).

FcεRI is a high-affinity IgE receptor that is expressed on the surfaces of mast cells and basophils. It plays a key role in allergies by triggering IgE-mediated allergic reactions [26–29]. KU812F cells are a human basophilic cell line that expresses the cell surface IgE receptor FcεRI. Expression of the cell surface IgE receptor FcεRI was suppressed in KU812F cells by 6-methoxyluteolin.

These results suggest that 6-methoxyluteolin may exert its anti-allergic properties *via* down-regulation of FcεRI expression and a subsequent reduction in $[Ca^{2+}]_i$ elevation and histamine release.

Acknowledgments

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