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ORIGINAL CONTRIBUTION

Dietary flavones suppresses IgE and Th2 cytokines in OVA-immunized BALB/c mice

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Abstract *Background* The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Previously, we have shown that the two flavones, chrysin and apigenin could suppress the expression of the high affinity IgE receptor FcεRI in human basophilic KU812 cells. We also demonstrated that dietary apigenin decreased IgE level in C57BL/6N mice sera. *Aim of the study* To evaluate the anti-allergic effect of the two flavones *in vivo*, we evaluated the effect of the two flavones, chrysin and apigenin, on the immune system in BALB/c mice sensitized with ovalbumin (OVA). *Methods* Mice were fed experimental diets containing either of the flavones for 3 weeks and immunized with OVA. After the experimental feeding period, measurement of Igs concentration in the mice sera was performed using a sandwich ELISA. Cytokines expression in mice sera was assessed using a cytokine array. Furthermore, cytokines mRNA levels in spleen lymphocytes from mice sensitized with OVA were measured by RT-PCR. *Results* The total IgE level in mice fed one of the two flavones were suppressed, whereas levels of IgG, IgM, and IgA were not affected. The production of interleukin (IL)-4, which is known as one of Th2 cytokines and regulates the production of IgE, was down-regulated by the chrysin or the apigenin diet. Moreover, OVA-induced mRNA expression of Th2 cytokines in spleen lymphocytes from mice sensitized with OVA, such as IL-4 and IL-13 were down-regulated by the chrysin or the apigenin diet. *Conclusions* The results suggest that the diet containing one of the two flavones might suppress the up-regulation of serum IgE induced by OVA-immunization through the suppression of Th2-type immune response.

Key words chrysin – apigenin – Th2 cytokines – BALB/c mice

Introduction

The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Flavonoids are plant polyphenolic compounds, which have a diphenylpropane skeleton (C6C3C6) structure, which include monomeric flavanols, flavanones, flavones, and flavonols. Some flavonoids have been found to possess various clinically relevant properties such as anti-tumor, anti-platelet, anti-ischemic, and anti-

EJN 658

inflammatory activities [1, 2]. Our previous report demonstrated that the two flavones, chrysin and apigenin, could suppress the expression of the high affinity IgE receptor FcεRI in human basophilic KU812 cells [3]. Furthermore, we had shown that dietary apigenin reduced serum IgE level in C57BL/6N mice [4]. Recently, it has been reported that some flavonoids including chrysin and apigenin exhibited weak peroxisome proliferator-activated receptor γ (PPARγ) agonist activities in an in vitro competitive-binding assay [5]. On the other hand, we have found that the FcεRI expression is negatively regulated by the PPARγ ligand, prostaglandin D_2 metabolite 15 deoxy-$D^{12,14}$ prostaglandin J_2 (15d-PGJ_2) [6]. We also reported that PPARγ ligands inhibit IL (Interleukin)-4-induced IgE class switching in human B cell line DND39 [7]. These reports suggest that the chrysin and apigenin may potentially have anti-allergic activity *in vivo*.

In this paper, to evaluate the anti-allergic effect of the two flavones *in vivo*, we examined the effects of a diet added to either chrysin or apigenin on Igs and cytokines levels in BALB/c mice immunized with ovalbumin (OVA).

Materials and methods

Chemicals

Chrysin (purity of 97%) and apigenin (purity of 95%) were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chem. Co. (St. Louis, MO), respectively. Safflower oil was purchased from Rinoru Oil Mills (Nagoya, Japan). Ovalbumin (OVA, Grade V) and aluminum hydroxide hydrate (alum) were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental animals and diets

Male 8-week-old BALB/c mice were obtained from CHARLES RIVER LABORATORIES JAPAN, INC. They were kept at the Biotron Institute of Kyushu University in a 12-h light/12-h dark cycle (light on 8 AM–8 PM) in an air-conditioned room (20°C and 60% humidity under specific pathogen-free conditions). This experiment was carried out according to the guidelines for animal experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Japanese government. After preliminary breeding for 1 week, the mice were divided into each group and provided with one of the following diets 5 g/day for 3 weeks. The experimental diets were manufactured according to the AIN-93M standard [8] containing 0.025% chrysin or 0.025% apigenin. For the basic dietary fat source, we used high-oleic acid safflower oil. After maintaining this feeding schedule for 3 weeks, the mice of all groups were provided with a control diet for 2 weeks. Blood samples were collected from the abdominal aorta under light anesthesia with diethylether at the end of the feeding period. Serum was obtained by centrifugation at $1{,}000 \times g$ for 15 min at 4°C and stored at −80°C until use. Body weight was measured before the experimental feed started and after in ended. The heart, kidney, liver, lung, spleen, epididymal adipose tissues were excised and weighed. We performed same experiment twice. Results of Tables 1, 2 and Fig. 1 were derived from the first experiment. Results of Table 3, Figs. 2 and 3 were derived from the second experiment.

Ovalbumin immunization

Mice received the first dose of OVA (100 μg/mouse) with an alum adjuvant by intraperitoneal (i.p.) injection on day 7. After 7 days (day 14), a second dose was injected the same as first injection.

Table 1 Effects of the flavones diet on the growth parameters of BALB/c mice sensitized with OVA

	Control	Chrysin	Apigenin
Body weight (g)			
Initial	25.2 ± 0.5	25.5 ± 0.4	25.5 ± 0.4
Final	30.8 ± 0.9	31.0 ± 0.8	30.0 ± 0.6
Organ weight (g)			
Heart	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
Kidney	0.49 ± 0.02	0.48 ± 0.01	0.48 ± 0.02
Liver	1.56 ± 0.04	1.57 ± 0.06	1.48 ± 0.03
Lung	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00
Spleen	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.00
EWAT	0.66 ± 0.05	0.69 ± 0.05	0.63 ± 0.05

Data are means ± SEM for 5–6 mice in each group. EWAT, epididymal white adipose tissue

Table 2 Total serum immunoglobulins level in BALB/c mice sensitized with OVA

Ig	Control	Chrysin	Apigenin
IgM (mg/ml)	5.2 ± 0.2	5.4 ± 0.2	5.5 ± 0.1
IgG (mg/ml)	27.7 ± 2.4	23.5 ± 3.0	24.9 ± 3.3
IgA (mg/ml)	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
IgE (ng/ml)	87.5 ± 6.9	68.2 ± 4.2*	65.5 ± 9.4*

Serum concentration of Igs from OVA-sensitized mice fed flavones was measured by ELISA. Data are means ± SEM for 5–6 mice in each group. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's test. Mean values are significantly different from those of the control group: $^*P < 0.05$

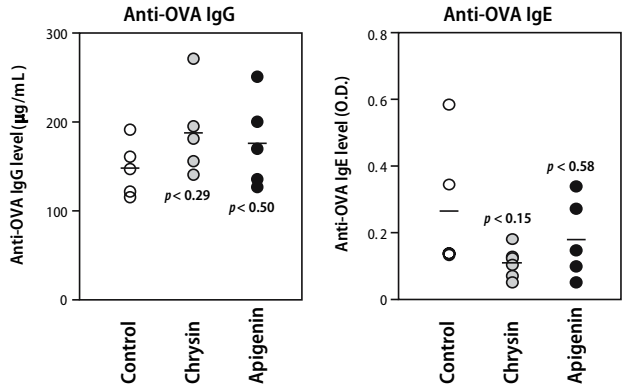

Fig. 1 Serum OVA-reactive IgG and IgE level in OVA-immunized mice. OVA-reactive IgG and IgE level in the serum of BALB/c mice fed chrysin or apigenin diets. OVA-reactive IgE data are expressed as optical density (OD) values. Each data point represents a mean for 5–6 mice

Table 3 Effects of the flavones on cytokines expression in mice sera

Cytokines	Relative intensity compared to the control group	
	Chrysin	Apigenin
CTACK	0.681 ± 0.030**	0.734 ± 0.021**
Eotaxin	0.710 ± 0.042**	0.752 ± 0.046*
G-CSF	0.741 ± 0.071*	0.776 ± 0.065*
IL-4	0.702 ± 0.037**	0.674 ± 0.035**
Leptin	0.761 ± 0.015**	0.804 ± 0.024*
MCP-1	0.714 ± 0.006***	0.695 ± 0.024***
TPO	0.767 ± 0.017***	0.837 ± 0.009**
VEGF	0.700 ± 0.011***	0.719 ± 0.009***

The relative level of cytokines was determined by intensity. The densities of signals were normalized with background and positive control. Data are means ± SD. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's test. Mean values are significantly different from those of the control group: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. CTACK, cutaneous T-cell-attracting chemokine; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; MCP, monocyte chemoattractant protein; VEGF, vascular endothelial growth factor

Measurement of immunoglobulin levels

To analyze immunoglobulin levels, a sandwich enzyme-linked immunosorbent assay (ELISA) was performed as described in our previous paper [4]. Measurement of anti-OVA IgG was also determined by ELISA. A total of 100 μl of OVA (100 μg/ml) in 50 mM sodium carbonate buffer, pH 9.6, was added to each well of a 96-well plate, and incubated for 1 h at 37°C. Then, 300 μl of 1% BSA-PBS was added and kept at 37°C for 2 h; samples (50 μl) were added to each well for 1 h at 37°C. The wells were treated with a solution of POD-conjugated goat anti-mouse IgG (H+L) to detect IgG and incubated for 1 h at 37°C. Plate washing between each step and the coloring reaction was performed. The antibody level for each sample was calculated by comparison with internal standards. Anti-OVA IgG standard was obtained by genetic engineering methods reported previously [9].

Anti-OVA IgE was measured by ELISA. A total of 100 μl of OVA (100 μg/ml) in 50 mM sodium carbonate buffer, pH 9.6, was added to each well of a 96-well plate, and incubated for 1 h at 37°C. Then, 300 μl of 1% BSA-PBS was added and kept at 37°C for 2 h; samples (50 μl) were added to each well for 1 h at 37°C. The wells were treated with a solution of biotin-conjugated rat anti-IgE monoclonal antibody (BD Bioscience, Franklin Lakes, NJ). After that, streptavidin POD-conjugated (Zymed) diluted by 1% BSA-PBS was added to each well. Plate washing between each step and the coloring reaction were performed.

Cytokine array

As described in our previous paper [4], 32 cytokine proteins in mice sera were assessed using a commercially available RayBio Mouse Cytokine Array II (Ray Biotech, Inc., Norcross, GA). The density of each band was quantified using a computer program obtained from the U.S. NIH.

Preparation and culture of spleen lymphocytes

Immediately after the excision, splenocytes were isolated from the spleen. Cells were suspended into RPMI 1640 medium (Nissui, Tokyo, Japan) and washed two times. Then 5 ml of the cell suspension was added to Lympholyte-mouse (Cedarlane, Hornby, Canada) to isolate the lymphocytes. Red cells were lysed with the ammonium-chloride potassium carbonate buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 10 mM EDTA 2Na, pH 7.4), and lymphocytes were washed two times with RPMI 1640 medium.

Reverse transcription-polymerase chain reaction (RT-PCR)

The spleen lymphocytes, 2×10^6 cells/ml, were cultured in RPMI 1640 medium containing 5% fetal calf serum (PAA Laboratories GmbH, Austria) with 100 μg/ml OVA and incubated at 37°C for 24 h. Total RNA was extracted from lymphocytes using TRI_{ZOL} (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. First-strand cDNA was synthesized from total RNA (2 μg) with a $(dT)_{20}$ primer and 20 U of Moloney mouse leukemia virus (MMLV)-reverse transcriptase (Amersham Pharmacia Biotech). Denaturation at 95°C for 1 min was followed by primer annealing at 60°C for 1 min and extension at 72°C for 1 min. A final extension phase of 7 min was added. Specific primer se-

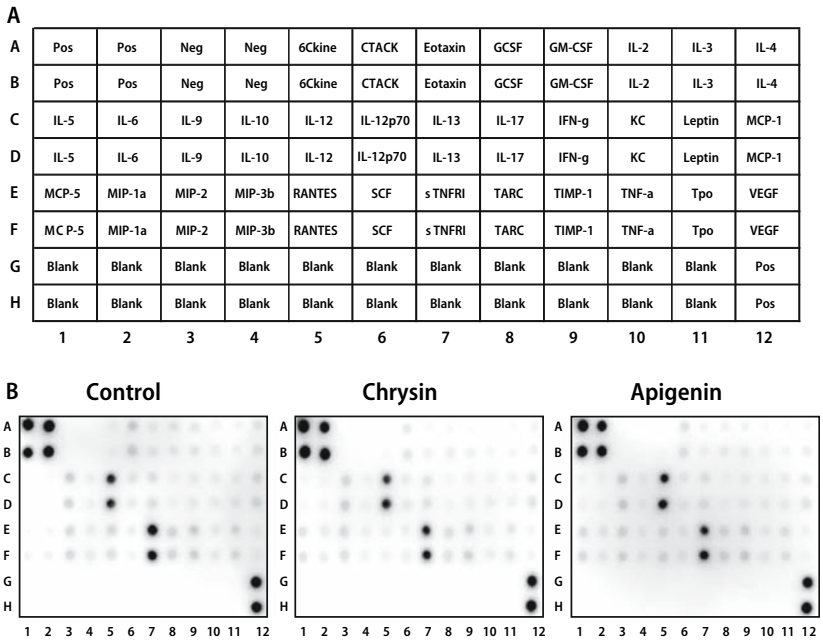

Fig. 2 Images of Mouse Cytokine Array II membranes. A template of the mouse cytokines in the array. Pos, positive control; Neg, negative control; CTACK, cutaneous T-cell-attracting chemokine; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; KC, CXC ligand 1; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T cell expressed and secreted; SCF, stem cell factor; sTNFR, soluble tumor necrosis factor receptor; TARC, thymus and activation-regulated chemokine; TIMP, tissue inhibitor of metalloprotease; TNF, tumor necrosis factor; Tpo, thrombopoietin; VEGF, vascular endothelial growth factor (**A**). Membranes were assayed with mice sera obtained from each dietary group (**B**)

quences for each gene were as follows: mouse IL-2, sense 5′-ATGTACAGCATGCAGCTCGCATC-3′, and antisense 5′-GGCTTGTTGAGATGATGCTTTGACA-3′; mouse IL-4, sense 5′-ATGGGTCTCAACCCCCAGC-TAGT-3′, and antisense 5′-GCTCTTTAGGCTTTCCAG GAAGTC-3′; mouse IL-10, sense 5′-ATGCAGGACTTT AAGGGTTACTTGGGTT-3′, and antisense 5′-ATT-TCGGAGAGAGGTACAAACGAGGTTT-3′; mouse IL-12 p35, sense 5′-GCCAGGTGTCTTAGCCAGTC-3′, and antisense 5′-GCTCCCTCTTGTTGTGGAAG-3′; mouse IL-12 p40, sense 5′-GAGGTGGACTGGACTCCCGA-3′, and antisense 5′-CAAGTTCTTGGGCGGGTCTG-3′; mouse IL-13, sense 5′-AGTTCTACAGCTCCCTGGTT CTC-3′, and antisense 5′-GGATGGTCTCTCCTCAT-TAGAAGG-3′; mouse IFN-γ, sense 5′-AACGCTACACA CTGCATCT-3′, and antisense 5′-AGCTCATTGAATG CTTGG-3′; mouse β-actin, sense 5′-TGGAATCCTGTG GCATCCATGAAAC-3′, and antisense 5′-TAAAACGC AGCTCAGTAACAGTCCG-3′. The PCR cycle numbers used for the amplification of the respective cDNAs are 35 for 7 cytokines and 23 for β-actin. Specific PCR fragments were separated on an agarose gel electrophoresis and visualized by SYBR Green I (Invitrogen, Carlsbad, CA). The density of each band was quantified using a computer program obtained from the U.S. NIH.

Statistical analysis

Data were analyzed using one-way analysis of variance followed by Dunnett's test. Mean values were significantly different from those of the control group at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Results

Body and organ weight

We examined whether the flavone intake affects the weight of the body and organs. As shown in Table 1, there was no significant difference in body and organs weight during the study. These results suggest that the chrysin or the apigenin diet did not affect body and organs weight in OVA-immunized mice.

Immunoglobulin level in mice sera

The mouse experimental allergic model was generated by sensitizing BALB/c mice with OVA. Alum group as a negative control was sensitized with an alum/PBS. It was confirmed that total IgG and IgE level from mice immunized with OVA were significantly higher than that from mice immunized with alum only (data not shown). As shown in Table 2, total IgE level in the chrysin and the apigenin diet group was lower than the control group. After OVA-immunization, we could detect no significant difference in the serum levels of IgG, IgM, and IgA among any of the dietary groups.

Antigen-reactive antibody production in mice sera

The serum antigen-reactive antibody level was elevated by antigen immunization. To investigate whether the flavone diet could affect the development of antigen-reactive antibodies in OVA-immunized mice,

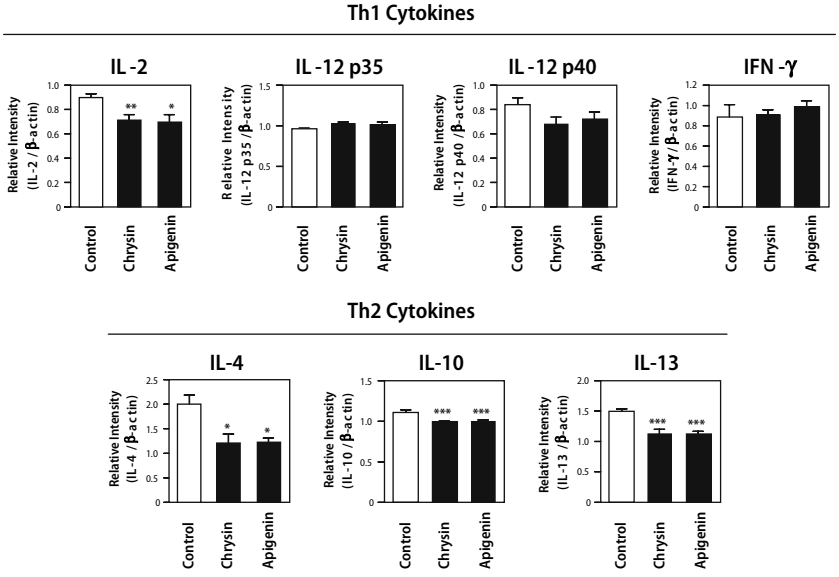

Fig. 3 Effects of the flavones diet on cytokines mRNA expression from spleen lymphocytes in OVA-immunized mice. After the spleen lymphocytes were cultured with 100 μg/ml OVA for 24 h, total mRNA isolation from the spleen lymphocytes in OVA-immunized BALB/c mice fed the chrysin and the apigenin diet was performed, and then IL-2, IL-4, IL-10, IL-12 p35, IL-12 p40, IL-13, IFN-γ and β-actin mRNA were analyzed by RT-PCR. Data are means ± SEM for 5–6 mice in each group. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's test. Mean values are significantly different from those of the control group: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$

serum OVA-reactive IgG and IgE levels in the OVA-immunized mice were determined using indirect ELISA. Antigen-reactive IgG and IgE levels were significantly increased in the control group as compared with the alum group (data not shown). OVA-reactive IgG and IgE levels in the sera of mice immunized with OVA did not significantly change both the chrysin and the apigenin groups.

Cytokines level in mice sera

To clarify the mechanisms involved in the suppression of the IgE production in mice fed the flavone diet, we performed same experiment again and evaluated for cytokines level in Mouse Cytokine Array II, an assay that can detect a panel of cytokines (Fig. 2). Among the 32 cytokines assessed, any cytokines were not up-regulated or down-regulated over 2 times in both chrysin and apigenin groups as compared with the control group. However, CTACK, eotaxin, G-CSF, IL-4, Leptin, MCP-1, TPO, and VEGF levels were down-regulated in the chrysin diet or the apigenin diet groups as shown in Table 3. IL-4 is known as one of Th2 cytokines, which induces the IgE production. Thus, our data suggested that down-regulation of the IgE production induced by OVA-immunization may result from the decrease of Th2 cytokines including IL-4 in mice sera.

Quantification of the cytokines levels from spleen lymphocytes

The IgE production is induced by Th2 cytokines and is negatively regulated by Th1 cytokines. To determine the regulatory effect of the chrysin or the apigenin diet on Th1/Th2 cell responses in OVA-immunized mice, Th1 cytokines such as IL-2, IL-12 p35, IL-12 p40 and IFN-γ, and Th2 cytokines such as IL-4, IL-10, and IL-13 levels in spleen lymphocytes re-stimulated with OVA from the same mice. To begin with, we examined the productions of the four cytokines in the culture supernatants from spleen lymphocytes by ELISA. IL-4, IL-10, and IFN-γ were not detected, and no significant difference was observed in the IL-12 production (data not shown). Next, we measured Th1 and Th2 cytokines mRNA levels in spleen lymphocytes stimulated with OVA by RT-PCR. As shown in Fig. 3, a remarkable down-regulation of IL-2, IL-4, and IL-13 mRNA level was observed in the chrysin diet and the apigenin diet groups. In the case of IL-10, the changes of mRNA level in both chrysin and apigenin groups were very small though there was a significant difference statistically. On the other hand, there was not any significant difference in IL-12 p35, IL-12 p40, and IFN-γ mRNA expression. Although we also examined mRNA expression of two chemokines, eotaxin, and MCP-1 in spleen lymphocytes, eotaxin was not detected, and no significant difference was observed in MCP-1 (data not shown). These results suggest that dietary chrysin or apigenin down-regulate the expression of Th2 cytokines such as IL-4 and IL-13 induced by OVA-stimulation in spleen lymphocytes.

Discussion

We demonstrated that the two flavones diet reduced the total IgE level in the sera of OVA-sensitized mice.

IgE is one of the major mediators of the immediate hypersensitivity reaction that underlie atopic conditions such as seasonal allergy, food allergy, asthma, and anaphylaxis [10]. In many individuals, the level of total serum IgE correlates roughly with the severity of these allergic diseases [11].

$CD4^+$ helper T cells are divided into two subpopulations (Th1 and Th2) based on different patterns of cytokine secretion [12–15]. The balance of these two types of cells is considered to be important in maintaining homeostasis in the host. Once this balance is disturbed, various immunological diseases, such as allergies, can occur due to circumvention of the host defense mechanism. Th2 cells produce IL-4, which is the major inducer for class-switching to IgE biosynthesis in B lymphocytes. Elevated serum IgE level induced by IL-4 is associated with eosinophil infiltration into the airway. A diet containing chrysin or apigenin inclined to reduce the serum level of IL-4 as shown in Table 3. Moreover, an increased IL-4 mRNA expression in spleen lymphocytes re-stimulated with OVA was suppressed remarkably in the chrysin and the apigenin groups as shown in Fig. 3. A diet including flavonoid astragalin has been considered to have an inhibitory effect on IL-4 synthesis from spleen cells and serum IgE elevation in mice [16]. These findings suggest that flavonoid compounds including chrysin and apigenin suppress both IL-4 production and thus IgE production.

In the case of IgE class switching, IL-4 activates the ε germline promoter, which leads to the expression of ε germline transcription (εGT) through the tyrosine phosphorylation pathway to activate the signal transducers and activators of transcription 6 (STAT6) in B cells [17, 18]. Previously, we have shown that strictinin, which is a member of the plant polyphenol family known as ellagitannins, suppressed IL-4-induced εGT expression and STAT6 tyrosine phosphorylation *in vitro* and antigen-specific IgE production *in vivo* [19]. Recently, it has been reported that some flavonoids including chrysin and apigenin exhibit weak PPARγ ligand activities [5]. In addition, we also reported that PPARγ ligand 15d-PGJ_2 inhibits IL-4-induced IgE class switching in human B cell line [7]. Chrysin and apigenin may suppress the IgE production by acting as the PPARγ ligand, although further experiments are necessary.

Dietary chrysin or apigenin suppressed Th2 cytokines such as not only IL-4 mRNA expression but also IL-13 mRNA expression in spleen lymphocytes stimulated with OVA. In addition, some chemokines level in mice fed chrysin or apigenin was decreased as shown in Table 3. Chemokines are relevant in allergy and asthma not only for their role in regulating leukocyte recruitment, but also for other activities, such as cellular activation, inflammatory mediator release, promotion of Th2 inflammatory responses, and regulation of IgE synthesis [20]. Furthermore, IL-2 mRNA expression in spleen lymphocytes stimulated with OVA was decreased by feeding the chrysin or the apigenin diet. IL-2 activates monocytes and macrophages producing chemokines. Thus, the reduction of chemokines level such as CTACK, eotaxin, and MCP-1 in the sera may be caused by the suppression of IL-2 expression.

In this study, after the two flavones diet for 3 weeks, a control diet was provided for 2 weeks. Regardless of return to a control diet, IgE and Th2 cytokines elevation induced by OVA-immunization in mice sera was inhibited. This result suggests that the suppressive effects on the IgE production and Th2 cytokines were maintained by the short-term feeding of the flavones. Diet therapy for allergic diseases has not been established except for avoidance of food allergens in cases when patients were sensitized to foods. We demonstrated collectively that a diet of either of the flavones may suppress IgE biosynthesis at least through the suppression of Th2 cytokines expression *in vivo*.

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