

Fisetin, a flavonol, inhibits T_H2-type cytokine production by activated human basophils

Shinji Higa, MD,^a Toru Hirano, MD,^a Mayumi Kotani, BSc,^b
Motonobu Matsumoto, BSc,^b Akihito Fujita, BSc,^b Masaki Suemura, MD, PhD,^c
Ichiro Kawase, MD, PhD,^a and Toshio Tanaka, MD, PhD^a Osaka, Japan

Background: Activation of mast cells and basophils through allergen stimulation releases chemical mediators and synthesizes cytokines. Among these cytokines, IL-4, IL-13, and IL-5 have major roles in allergic inflammation.

Objective: We sought to determine the potency of flavonoids (astragalol, fisetin, kaempferol, myricetin, quercetin, and rutin) for the inhibition of cytokine expression and synthesis by human basophils.

Methods: The inhibitory effect of flavonoids on cytokine expression by stimulated KU812 cells, a human basophilic cell line, and freshly purified peripheral blood basophils was measured by means of semiquantitative RT-PCR and ELISA assays. The effects of flavonoids on transcriptional activation of the nuclear factor of activated T cells were assessed by means of electrophoretic mobility shift assays.

Results: Fisetin suppressed the induction of IL-4, IL-13, and IL-5 mRNA expression by A23187-stimulated KU812 cells and basophils in response to cross-linkage of the IgE receptor. Fisetin reduced IL-4, IL-13, and IL-5 synthesis (inhibitory concentration of 50% [IC₅₀] = 19.4, 17.7, and 17.4 μmol/L, respectively) but not IL-6 and IL-8 production by KU812 cells. In addition, fisetin inhibited IL-4 and IL-13 synthesis by anti-IgE antibody-stimulated human basophils (IC₅₀ = 5.1 and 6.2 μmol/L, respectively) and IL-4 synthesis by allergen-stimulated basophils from allergic patients (IC₅₀ = 4.8 μmol/L). Among the flavonoids examined, kaempferol and quercetin showed substantial inhibitory activities in cytokine expression but less so than those of fisetin. Fisetin inhibited nuclear localization of nuclear factor of activated T cells c2 by A23187-stimulated KU812 cells.

Conclusion: These results provide evidence of a novel activity of the flavonoid fisetin that suppresses the expression of T_H2-type cytokines (IL-4, IL-13, and IL-5) by basophils. (J Allergy Clin Immunol 2003;111:1299-1306.)

Key words: Mast cells, basophils, cytokines, fisetin, nuclear factor of activated T cells

Abbreviations used

AP-1: Activator protein 1
DMSO: Dimethyl sulfoxide
EMSA: Electrophoretic mobility shift assay
IC₅₀: Inhibitory concentration of 50%
NFAT: Nuclear factor of activated T cells
PMA: Phorbol 12-myristate 13-acetate

Mast cells and basophils express FcεRI on their cell surface, and the cross-linking of the cell-bound IgE-FcεRI complex with specific antigens leads to the release of inflammatory mediators and cytokines.¹⁻⁵ Among cytokines produced by such cells, IL-4, IL-13, and IL-5 are key molecules related to IgE production, T_H2 differentiation, and allergic inflammation.⁶ Although it has long been thought that IL-4 is derived solely from CD4⁺ T cells, recent in vitro studies have clearly shown that human basophils account for most of the IL-4 and IL-13 generated in cultures by peripheral blood leukocytes.^{7,8} This is true not only for cultures activated with stimuli that are specific for basophils, such as anti-IgE antibody, but it is also evident in cultures stimulated with allergens, which additionally activate allergen-specific T lymphocytes.⁹⁻¹¹ The evidence that basophils produce more IL-4 and IL-13 than T cells suggests that strategies to interrupt their synthesis might be important for the successful treatment of allergic diseases.⁸ Several therapeutic agents that inhibit release of pharmacologically active chemical mediators from mast cells or basophils are now in clinical use.¹² However, there are few drugs that can suppress production of cytokines by these cells, except for steroids, cyclosporin A, FK506, and sodium cromoglycate.¹³⁻¹⁵ It is thus hoped that low-molecular-weight compounds with inhibitory activity on cytokine expression, as well as the release of chemical mediators by these cells, might constitute novel antiallergic drugs.

Flavonoids ubiquitously exist in plants, vegetables, fruits, and teas and thus can be taken orally.¹⁶ These compounds also have been demonstrated to possess a variety of anti-inflammatory effects.¹⁷ Some flavonoids, including quercetin, kaempferol, fisetin, and rutin, were previously shown to inhibit histamine release by human basophils and rat mast cells.^{18,19} We recently found that persimmon leaf extract and its major constituent of flavonoids, astragalol, inhibited histamine release by the human basophilic cell line KU812²⁰ and that oral admin-

From ^aDepartment III of Internal Medicine, Osaka University Medical School, Osaka; ^bthe Research & Development Center, Sunstar Incorporation, Osaka; and ^cNissay Hospital, Osaka.

Supported by the Ministry of Education, Science, and Culture of Japan; the Ministry of Health and Welfare of Japan; and the Pollution-related Health Damage Compensation and Prevention Association of Japan.

Received for publication August 5, 2002; revised January 23, 2003; accepted for publication February 3, 2003.

Reprint requests: Toshio Tanaka, MD, the Department III of Internal Medicine, Osaka University Medical School, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan.

© 2003 Mosby, Inc. All rights reserved.

0091-6749/2003 \$30.00 + 0

doi:10.1067/mai.2003.1456

istration of these substances markedly suppressed not only the development of dermatitis but also the serum IgE level increase in the atopic dermatitis-model mouse strain NC/Nga.^{21,22} In the study presented here, we have therefore investigated the inhibitory activities of 6 flavonoids (4 flavonols and 2 flavonol glycosides) that share structural similarities on the secretions of cytokines by a human basophilic leukemic cell line and freshly purified basophils.

METHODS

Culture medium

RPMI 1640 (Nacalai Tesque) supplemented with 10% FCS (Dainippon Pharmaceutical Co), L-glutamine (2 mmol/L), 2-mercaptoethanol (0.05 mmol/L), penicillin (100 U/mL), and streptomycin (100 µg/mL) was used as the culture medium.

Chemicals

Four flavonols (fisetin, kaempferol, myricetin, and quercetin) and 2 flavonol glycosides (astragaloside and rutin) were purchased from Extrasynthèse. A23187 and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Japan. Stock solutions of flavonoids were prepared in methanol, and A23187 and PMA were dissolved in dimethyl sulfoxide (DMSO). Control specimens were incubated with an equal concentration of methanol or DMSO. Cedar pollen extract-Cj (Japanese Cedar, *Cryptomeria japonica* pollen crude extract) was purchased from Cosmo Bio Co, Ltd. Anti-human IgE antibody was obtained from DAKO Japan.

Purification of basophils

Peripheral blood from patients with seasonal allergic rhinitis caused by Japanese cedar pollen or peripheral blood buffy coats from healthy transfusion donors were anticoagulated with 10 mmol/L EDTA and mixed with the same amount of PBS. The mixture was then layered onto Ficoll-Paque PLUS (Amersham Pharmacia Biotech) and centrifuged (400g for 20 minutes at 4°C). This was followed by negative selection with a MACS Basophil Isolation Kit (Miltenyi BioTech).²³ Cells were then applied to an LS cell-separation column mounted on a Midi MACS magnet (Miltenyi Corp). Basophils were collected as they passed through the column and washed. The number of total cells and their viability were determined by staining with Trypan Blue solution and counted with a hemocytometer. The purity of the basophils in this fraction was assessed by examining 1000 cells on cytospin preparations treated with May-Grünwald stain. The basophil purity was 43% to 93% in 5 different experiments, and the contaminating cells consisted mainly of small lymphocytes and a few monocytes. For convenience, these basophil-enriched fractions are henceforth simply referred to as basophils.

RT-PCR analysis of cytokine (IL-4, IL-5, IL-6, IL-8, IL-13, and IL-1β) mRNA expression

The expression of cytokines by KU812 (IL-4, IL-5, IL-6, IL-8, IL-13, and IL-1β) or by basophils (IL-4 and IL-13) was measured by using RT-PCR. KU812 cells²⁴ (5×10^5 cells/mL) or purified basophils (2×10^6 cells/mL) were first incubated without (equivalent amount of diluted methanol) or with flavonoids (30 µmol/L; fisetin, 8.58 µg/mL; kaempferol, 8.58 µg/mL; myricetin, 9.54 µg/mL; quercetin, 9.06 µg/mL; astragaloside, 13.44 µg/mL; and rutin, 18.33 µg/mL) for 30 minutes. Then A23187 (1 µmol/L) or an equivalent amount of diluted DMSO for KU812 cells or anti-IgE antibody (1 µg/mL) for basophils was added and further incubated for 3 hours. After washing, total RNA was extracted from the cell pel-

lets by using the RNeasy protocol (Tel-Test, Inc). Total RNA (800 or 300 ng) was then mixed with the RT mixture according to the manufacturer's protocol (Perkin-Elmer Cetus) and incubated at 37°C for 60 minutes, followed by boiling at 99°C for 5 minutes. After each RT, the product was distributed over 7 or 3 test tubes, and PCR amplification was performed with the following primers.

IL-4-specific primers were as follows: sense, 5' ATG-GGT-CTC-ACC-TCC-CAA-CTG-CT 3'; antisense, 5' CGA-ACA-CTT-TGA-ATA-TTT-CTC-TCT-CAT 3'. IL-5-specific primers were as follows: sense, 5' GCT-TCT-GCA-TTT-GAG-TTT-GCT-AGC-T 3'; antisense 5' TGG-CCG-TCA-ATG-TAT-TTC-TTT-ATT-AAG 3'. IL-6-specific primers were as follows: sense, 5' ATG-AAC-TCC-TTC-TCC-ACA-AGC-GC 3'; antisense, 5' GAA-GAG-CCC-TCA-GGC-TGG-ACT-G 3'. IL-8-specific primers were as follows: sense, 5' ATG-ACT-TCC-AAG-CTG-GCC-GTG-GCT 3'; antisense, 5' TCT-CAG-CCC-TCT-TCA-AAA-ACT-TCT-C 3'. IL-13-specific primers were as follows: sense, 5' CCA-CGG-TCA-TTG-CTC-TCA-CTT-GCC 3'; antisense, 5' CCT-TGT-GCG-GGC-AGA-ATC-CGC-TCA 3'. IL-1β-specific primers were as follows: sense, 5' ATG-GCA-GAA-GTA-CCT-AAG-CTC-GC 3'; antisense, 5' ACA-CAA-ATT-GCA-TGG-TGA-AGT-CAG-TT 3'. β-Actin-specific primers were as follows: sense, 5' GTG-GGG-CGC-CCC-AGG-CAC-CA 3'; antisense, 5' GTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC 3'.

The PCR process was performed with a Perkin-Elmer Cetus DNA Thermal Cycler, and the durations and temperatures were as follows: for IL-4 and IL-13 transcripts, 2 minutes at 94°C and then 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C for 38 cycles for KU812 or 24 cycles for basophils; for IL-5, 32 cycles; for IL-6, IL-8, and IL-1β, 30 cycles; and for β-actin, 22 cycles. The number of PCR cycles had been previously determined so that the assay was semiquantitative. The products were electrophoresed in 2% agarose gel, stained with ethidium bromide, and photographed.

Measurement of cytokine synthesis by KU812 cells and basophils

KU812 cells (10^6 to 3×10^6 cells/mL) or purified basophils (0.2 to 1×10^6 cells/mL) were incubated without (equivalent amount of diluted methanol) or with flavonoid at an indicated dose for 30 minutes and then stimulated with A23187 (1 µmol/L) plus PMA (10 ng/mL) for KU812 cells, which was the maximal concentration for the induction,²⁵ or allergen (cedar pollen extract-Cj, 10 ng/mL) or anti-IgE antibody (1 µg/mL) for basophils for 6 and 24 hours. The concentration of allergen or anti-IgE antibody was previously determined to induce maximal synthesis of IL-4. The supernatant was harvested, and the levels of IL-4, IL-5, IL-6, IL-8, and IL-13 in the culture supernatant or diluted supernatant were measured by means of ELISA according to the manufacturer's instructions. An ultrasensitive human IL-4 ELISA kit (Biosource International) was used to detect IL-4, a Pelikine Compact human ELISA kit (Central Laboratory, Netherlands Red Cross Blood Transfusion Service) was used to detect IL-6 and IL-13, and a Quantikine ELISA kit (R&D systems) was used to measure human IL-5 and IL-8 levels. These assays showed that the measurable range of cytokines was 65 to 25,000 fg/mL for IL-4, 1.5 to 1000 pg/mL for IL-13, 5 to 1000 pg/mL for IL-5, 15 to 3000 pg/mL for IL-6, and 10 to 3000 pg/mL for IL-8.

Nuclear extract and electrophoretic mobility shift assays

KU812 cells (1×10^7 cells) were first incubated without or with fisetin or myricetin (30 µmol/L) for 30 minutes and then stimulated with A23187 (1 µmol/L) for 2 hours at 37°C because the binding activity of nuclear factor of activated T cells (NFAT) was time dependent and reached its maximum after 1 to 2 hours' incubation.²⁶ Cells were washed in PBS 3 times, and nuclear extracts were

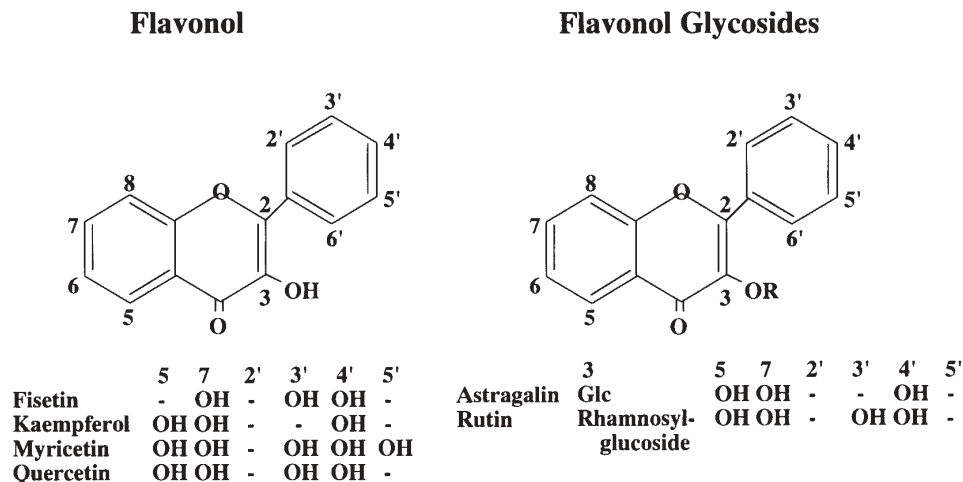


FIG 1. Structure of flavonoids. Four flavonols (fisetin, kaempferol, myricetin, and quercetin) and 2 flavonol glycosides (astragalin and rutin) were used in this study.

prepared as described in a previous report.²⁷ The protein concentrations of extracts were determined by means of BCA protein assay reagent (Pierce Chemical), and the extracts were then frozen at -80°C in aliquots containing 20% glycerol. NFAT and activator protein 1 (AP-1) consensus oligonucleotides were designed as previously reported.^{28,29} The unrelated oligonucleotide used in this study was the GATA-3 binding site in the IL-4 promoter.³⁰ Annealed binding sites were radiolabeled with T4 polynucleotide kinase (Toyobo Co, Ltd) and [$\gamma^{32}\text{P}$]ATP (Amersham Pharmacia Biotech, UK, Ltd). Radiolabeled oligonucleotides were purified with ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech, Inc), according to the manufacturer's protocol. Electrophoretic mobility shift assays (EMSAs) were performed as described previously, with some modifications.²⁷ Nuclear extracts (10 μg) were incubated with 3 μg of a poly-(dI-dC) carrier in a 20- μL reaction mix containing 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, and 5% glycerol for 30 minutes either at room temperature (to test antibody inhibition) or at 4°C (to test oligonucleotide competition). For antibody inhibition experiments, 4 μg of goat affinity-purified anti-NFATc1 (SC-1789X, Santa Cruz Biotechnology), goat anti-NFATc2 (SC-7295X), or goat anti-GATA-3 (C-18X) was included in the incubation. For oligonucleotide competition experiments, unlabeled oligonucleotides (250 fmol) were included in the incubation. Labeled binding site probes (5 fmol, approximately 5 to 6×10^4 cpm) were then added for an additional 20 minutes of incubation at 4°C . Samples were electrophoresed through a 4% polyacrylamide gel containing 45 mmol/L Tris-borate and 1 mmol/L EDTA. The gels were dried on Whatman no. 3 MM filter paper and autoradiographed.

Statistical analysis

Statistical analysis was performed by using the Student *t* test, and statistical significance was assumed at a *P* value of less than .05.

RESULTS

Fisetin suppresses $\text{T}_{\text{H}}2$ -type cytokine production (IL-4, IL-13, and IL-5) by KU812 cells

In the study presented here, we asked whether various kinds of flavonoids had an inhibitory effect on cytokine expression by human basophils. As shown in Fig 1, we

selected 6 flavonoids comprising 4 flavonols (fisetin, kaempferol, myricetin, and quercetin) and 2 flavonol glycosides (astragalin and rutin) that are similar in structure.^{16,17} With RT-PCR, we detected an upregulation of not only IL-4 and IL-5 but also IL-13, IL-6, and IL-8 mRNA expression by KU812 cells in response to A23187, whereas IL-1 β mRNA expression was not enhanced (Fig 2, A). Three hours after stimulation, the mRNA expression of these cytokines reached its maximum and then decreased gradually (data not shown). The presence of fisetin at a dose of 30 $\mu\text{mol/L}$ (but not at 3 $\mu\text{mol/L}$), as shown in Fig 2, A, inhibited IL-4, IL-5, and IL-13 mRNA expression, whereas IL-6, IL-8, and IL-1 β mRNA expression were not affected. Fisetin also showed an inhibition of IL-4, IL-5, and IL-13 mRNA expression by KU812 in response to A23187 plus PMA (10 ng/mL, data not shown). Fig 2, B, shows that quercetin and kaempferol at the same dose showed a marginal but substantial inhibition of both IL-4 and IL-5 mRNA expression, but 3 other flavonoids (myricetin, rutin, and astragalin) had little effect on this expression.

The cross-linkage of Fc ϵ RI of KU812 cells with mouse anti-human Fc ϵ RI α chain antibody (CRA-1) or anti-IgE antibody or the stimulation of KU812 cells with A23187 failed to produce detectable amounts of IL-4 and IL-13, as determined by means of ELISA, irrespective of the cell number, incubation period, and concentrations of these stimulants (data not shown). However, when KU812 cells were stimulated with A23187 plus PMA at a high density (3×10^6 cells/mL), measurable IL-4, IL-5, and IL-13 protein levels were produced in the culture supernatant. The synthesis of IL-6 and IL-8 was also detected, even at a lower density (10^6 cells/mL). The representative findings of 4 experiments are shown in Table I. Fisetin suppressed synthesis of these cytokines, but not of IL-6 or IL-8, at a dose of 30 $\mu\text{mol/L}$. Four independent experiments revealed that the inhibitory concentration of 50% (IC_{50}) of fisetin was 19.1 ± 4.9 $\mu\text{mol/L}$ for IL-4,

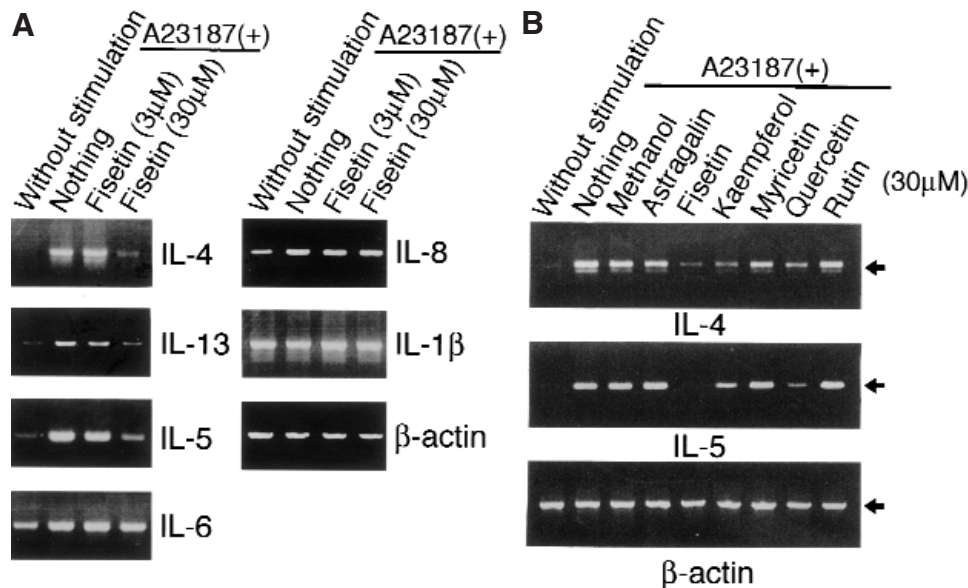


FIG 2. Fisetin inhibits the expression of IL-4, IL-13, and IL-5 mRNAs in KU812 cells. **A**, The cells (5×10^5 cells) were first incubated without (*nothing*) or with fisetin (3 or 30 $\mu\text{mol/L}$) for 30 minutes and stimulated with A23187 (1 $\mu\text{mol/L}$) or an equivalent amount of diluted DMSO (*without stimulation*) for 3 hours. RNAs were obtained, and the expression of IL-4, IL-13, IL-5, IL-6, IL-8, IL-1 β , and β -actin transcripts was examined by means of RT-PCR. **B**, Hierarchy of the inhibitory effect of flavonoids on IL-4 and IL-5 mRNA expression by KU812 cells. The cells (5×10^5 cells) were first incubated without (*nothing*) or with each of the flavonoids (30 $\mu\text{mol/L}$) or an equivalent amount of diluted methanol (*Methanol*) for 30 minutes and stimulated with A23187 (1 $\mu\text{mol/L}$) or an equivalent amount of diluted DMSO (*without stimulation*) for 3 hours. The expression of IL-4, IL-5, and β -actin mRNA was examined by means of RT-PCR.

TABLE I. Fisetin inhibits IL-4, IL-5, and IL-13 production by KU812 cells

	Cytokine production (pg/mL)				
	IL-4*	IL-5	IL-6	IL-8	IL-13
Stimulant					
Nothing	<65	<5	277 $\pm$ 38	320 $\pm$ 155	<1.5
A23187 + PMA	3298 $\pm$ 299	113 $\pm$ 15	21,170 $\pm$ 5998	12,951 $\pm$ 1707	16.5 $\pm$ 4.5
A23187 + PMA + fisetin (3 $\mu\text{mol/L}$)	2843 $\pm$ 1457	100 $\pm$ 1	21,199 $\pm$ 680	12,173 $\pm$ 1762	14.9 $\pm$ 1.7
A23187 + PMA + fisetin (30 $\mu\text{mol/L}$)	171 $\pm$ 126†	5.3 $\pm$ 0.4‡	21,592 $\pm$ 1837	14,520 $\pm$ 1051	2.0 $\pm$ 0.2
Cell density (cells/mL)	3×10^6	3×10^6	10^6	10^6	3×10^6

KU812 cells (10^6 cells/mL or 3×10^6 cells/mL) were incubated with the indicated reagent for 6 hours (IL-4 and IL-6) or 24 hours (IL-5, IL-8, and IL-13), and cytokine concentration in the culture supernatant was measured by means of ELISA. The data are shown as means $\pm$ SD.

*Femtograms per milliliter.

† $P < .05$.

‡ $P < .01$.

17.7 $\pm$ 8.1 $\mu\text{mol/L}$ for IL-5, and 17.4 $\pm$ 10.7 $\mu\text{mol/L}$ for IL-13 and that the IC_{50} of other flavonoids, including kaempferol and quercetin, was greater than 30 $\mu\text{mol/L}$.

Fisetin suppresses IL-4 and IL-13 synthesis by peripheral blood basophils

Peripheral blood basophils from patients sensitized with Japanese cedar or those from healthy blood donors were stimulated with allergen or anti-IgE antibody in the absence or presence of flavonoids for 6 or 24 hours to examine whether this inhibitory effect of fisetin might be found in normal basophils. As shown in Fig 3, A, at 6 and 24 hours' culture, the presence of fisetin significantly

reduced IL-4 production by human basophils through allergen stimulation ($\text{IC}_{50} = 4.8 \pm 0.4 \mu\text{mol/L}$; $n = 3$) or anti-IgE antibody stimulation ($\text{IC}_{50} = 5.1 \pm 2.1 \mu\text{mol/L}$; $n = 3$), confirming the inhibitory effects of fisetin on IL-4 production in KU812 cells. The presence of myricetin, however, showed a less inhibitory effect than fisetin ($\text{IC}_{50} > 30 \mu\text{mol/L}$; $n = 3$) but a substantial effect on IL-4 synthesis at a higher concentration. The synthesis of IL-13 was not detected in the supernatants from allergen-stimulated basophils at 6 and 24 hours' culture or from anti-IgE antibody-stimulated basophils at 6 hours' culture. However, the incubation of basophils with anti-IgE antibody for 24 hours caused production of detectable

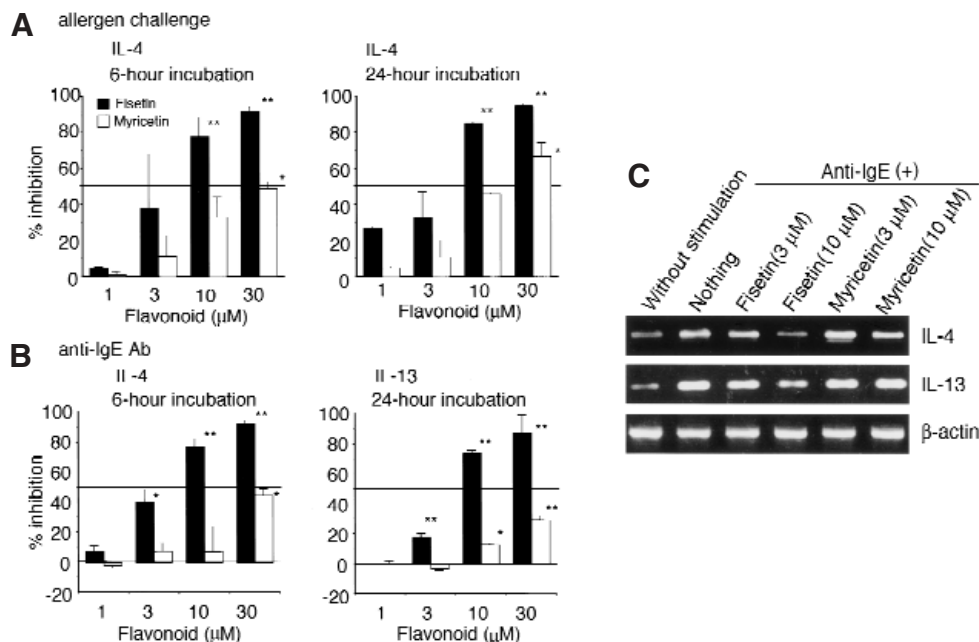


FIG 3. Fisetin inhibits IL-4 and IL-13 mRNA expression and synthesis by purified basophils. **A**, Purified human basophils (0.2 to 1×10^6 cells/mL) were first incubated with fisetin or myricetin at a dose of 1 to 30 $\mu\text{mol/L}$ or an equivalent amount of diluted methanol for 30 minutes and further stimulated with an allergen (cedar pollen extract-Cj; 10 ng/mL) for 6 and 24 hours. The concentration of IL-4 in the supernatant was measured by means of ELISA. **B**, Purified basophils (0.2 to 1×10^6 cells/mL) were first incubated with fisetin or myricetin at a dose of 1 to 30 $\mu\text{mol/L}$ or an equivalent amount of diluted methanol for 30 minutes and further stimulated with anti-IgE antibody (1 $\mu\text{g/mL}$) for 6 and 24 hours. The concentration of IL-4 and IL-13 in the supernatant was measured by means of ELISA. **C**, Purified basophils (2×10^6 cells/mL) were first incubated without or with fisetin or myricetin at a dose of 3 or 10 $\mu\text{mol/L}$ for 30 minutes and further stimulated with anti-IgE antibody (1 $\mu\text{g/mL}$) for 3 hours. RNAs were obtained, and the expression of IL-4, IL-13, and β -actin transcripts was examined by means of RT-PCR. The graph (Fig 3, A and B) represents the mean concentration $\pm$ SD from 3 different donors. * $P < .05$; ** $P < .01$.

IL-13 synthesis, and fisetin showed a similar inhibition on IL-13 production ($\text{IC}_{50} = 6.2 \pm 0.2$ $\mu\text{mol/L}$; $n = 3$). RT-PCR could be used to detect the inhibitory effect of fisetin at a dose of 10 $\mu\text{mol/L}$ on IL-4 and IL-13 mRNA expression by peripheral basophils in response to anti-IgE antibody (Fig 3, C).

Fisetin suppresses NFAT-DNA binding activity in activated KU812 cells

To investigate the mechanism of flavonoid inhibition in cytokine expression, we examined the NFAT-DNA binding activity by EMSAs. Because of the difficulty in obtaining enough normal purified basophils for this experiment, we used KU812 cells. A protein-DNA complex was formed with nuclear lysates from KU812 cells and NFAT consensus probe, with greater binding intensities detected in the lysates of activated cells (Fig 4, A). This induced binding activity was specific for the NFAT recognition motif because competition with nonradioactive NFAT oligonucleotide eliminated the DNA binding activity, whereas unrelated oligonucleotide did not, and an antibody to NFATc2 (NFAT1) supershifted the protein-DNA complex. The AP-1 consensus probe also

blocked the NFAT-DNA binding activity (Fig 4, B and C) because AP-1 forms part of the NFAT complex.^{26,31} In the presence of fisetin, the NFAT-DNA binding activity was markedly suppressed, whereas in the presence of myricetin, it was not. Under the same conditions, 1000 ng/mL cyclosporin A completely blocked nuclear NFAT induction (Fig 4, A). This result raises the possibility that fisetin inhibits IL-4, IL-13, and IL-5 synthesis through its suppression of NFAT activation.

DISCUSSION

In this study we have demonstrated a novel activity of fisetin in the inhibition of IL-4, IL-13, and IL-5 generation by both human basophils in response to cross-linkage of FcεRI and a basophilic cell line stimulated by A23187 (plus PMA). Kaempferol and quercetin had similar but less inhibitory effects on the expression of these cytokines by KU812 cells. This suppressive function of fisetin is potentially interesting because these cytokines produced from basophils (or mast cells) on activation are believed to be associated with the development of allergic inflammation, as well as IgE synthesis.^{1,3} Previous reports have

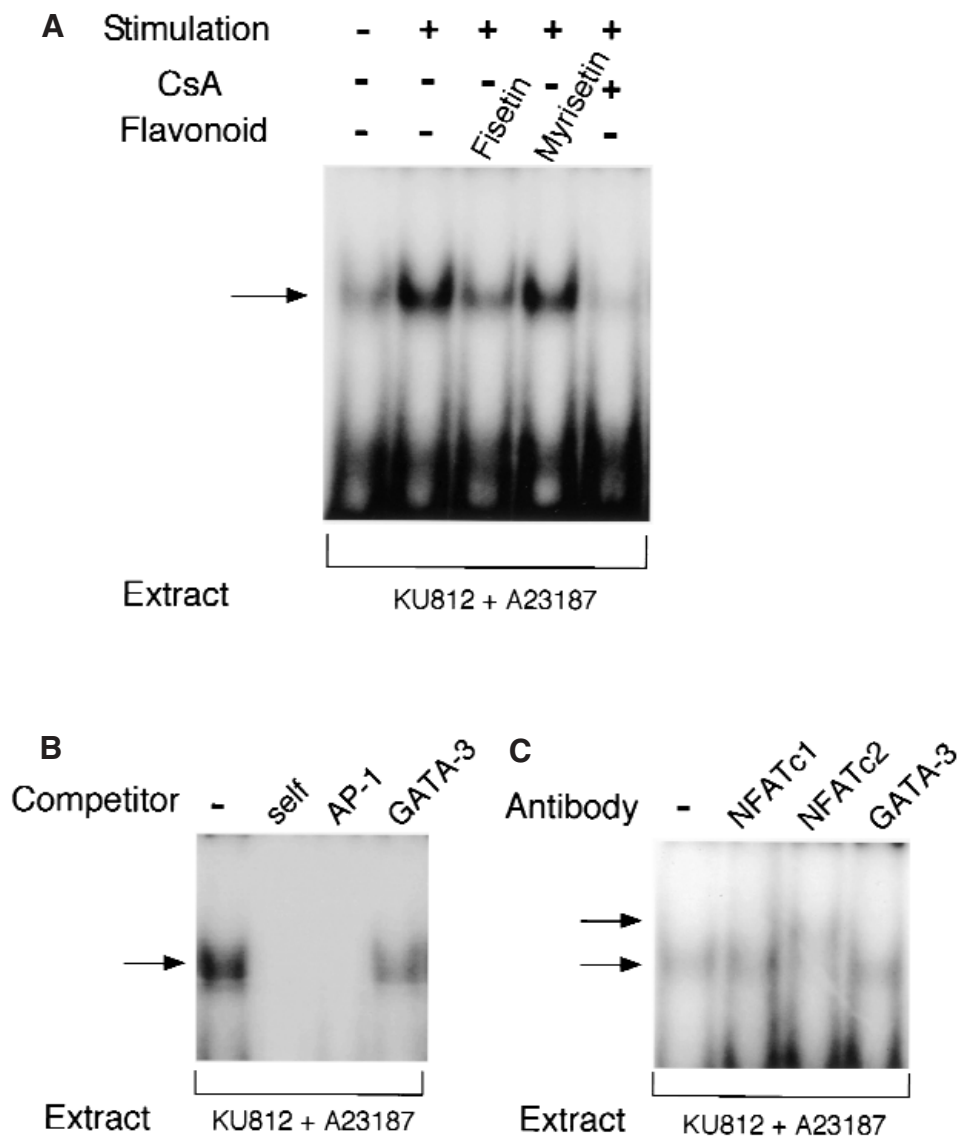


FIG 4. EMSA showing the inducibility of NFAT in KU812 cells by A23187 and the inhibitory effect of fisetin on NFAT-DNA binding activity. **A**, EMSA was performed with a ^{32}P -labeled NFAT probe and nuclear proteins (10 μg) from KU812 cells unstimulated or stimulated with A23187 for 2 hours in the presence of fisetin or myricetin or 1000 ng/mL cyclosporin A. **B**, Competition (50 times molar excess of the labeled oligonucleotide) was performed by adding the unlabeled NFAT probe or AP-1 probe or unrelated cold GATA-3 probe. **C**, Supershift EMSA was performed with A23187-stimulated nuclear proteins isolated from KU812 cells. Antibodies (4 μg) specific for NFATc1, NFATc2, and GATA-3 proteins were added to the reaction mixture and incubated for 30 minutes on ice before the addition of a labeled oligonucleotide. Data are representative of 3 independent experiments.

identified kaempferol, quercetin, rutin, and fisetin as inhibiting histamine release or hexosaminidase by human basophils or the rat basophilic cell line RBL.^{18,19} We confirmed that flavonoids used in this study substantially inhibited histamine release by peripheral blood basophils stimulated through Fc ϵ RI (data not shown). Its biologic properties of inhibiting these cytokines and chemical mediators make fisetin a potential candidate for a novel antiallergic substance targeting basophils.

Among the cytokines examined, fisetin specifically inhibited IL-4, IL-5, and IL-13 expression but not IL-6 and IL-8 by KU812 cells, suggesting that fisetin regulates the transcriptional activation of these cytokines. Cytokine gene transcription is regulated by cis elements and transcriptional DNA binding factors. The observations that fisetin reduces the expression of IL-4, IL-5, and IL-13 without any effect on IL-6 and IL-8 expression suggests that fisetin suppresses the activation of certain

common specific nuclear factors that positively regulate *IL4*, *IL5*, and *IL13* gene activation or induce repressor proteins. The NFAT family plays a key role in inducing many cytokine genes in T cells, including *IL2*, *IL4*, *IL5*, *IL13*, *IFNG*, *TNFA*, and *GMCSF*, and many studies have also identified NFAT-dependent cytokine gene expression in cells other than T cells, including B cells, hematopoietic cells, and rodent mast cells.^{31,32} With regard to cytokine production, recent observations that immunosuppressive drugs, such as cyclosporin A or FK506, inhibit both anti-IgE antibody and calcium ionophore-induced IL-4 and IL-13 production from basophils or mast cells suggest that these stimuli use a calcium-dependent calcineurin pathway for the generation of these cytokines.^{31,32} In our study we were able to show that, in fact, an NFAT-DNA complex was formed with nuclear lysates from KU812 cells and was detected with greater binding intensity in the lysates of activated cells. Fisetin suppressed the NFAT-DNA binding activity markedly, whereas myricetin did not, suggesting that fisetin might partly affect the calcium/calmodulin-calcineurin-NFAT pathway for the generation of these cytokines by KU812 cells. Nishino et al³³ reported that quercetin could interact directly with a calmodulin, the Ca²⁺-sensing protein, and we confirmed that fisetin could also bind with it (data not shown). Another explanation of the inhibitory effects of fisetin is that it might modulate the function of calmodulin and affect some Ca/calmodulin-dependent enzymes other than calcineurin, which regulate transcriptional activation of these cytokines in addition to NFAT inhibition. Schroeder et al³⁴ have recently demonstrated the involvement of NFAT in the generation of IL-4 by anti-IgE antibody-stimulated normal basophils. Among 3 isoforms of NFAT checked, NFAT2 (NFATc1) was abundantly involved in EMSA assay by human basophils. However, in our assay NFAT1 (NFATc2) appeared to a predominant isoform in KU812 cells. These results might come from the difference of cell type (cell line vs normal cell), but further study will be needed to clarify this point. In addition, whether flavonoids could affect other FcεRI-mediated signaling pathways or show similar inhibition on NFAT activation in normal basophils is yet to be determined.

Flavonoids are naturally occurring polyphenolic compounds with a wide distribution in the plant kingdom,^{16,17} but daily intake of flavonoids remains controversial. It is thought that the average daily US diet contains approximately 1 g of flavonoids. Quercetin is usually included at a high concentration in these materials, and it has been suggested that it is consumed at a rate of at least 50 mg/d.¹⁶ In contrast, the average intake of flavonoids was found to be only 23 mg/d in The Netherlands.³⁵ The fisetin content of most food items and its daily consumption, however, are still unknown as far as we know. Oral intake of substances, including higher amounts of fisetin or other flavonoids, might result in improvement of allergic symptoms or prevent development of allergic diseases.³⁶ We have, in fact, demonstrat-

ed that oral administration of astragaloside, which is absorbed after being converted into kaempferol, suppresses the onset of dermatitis in NC/Nga mice.^{21,22} More extensive screening of this class of compounds and preparation of synthetic analogues might thus yield molecules with increased potency and efficacy that could be useful for patients with allergic disease. We hope that the evidence presented here might contribute to the clinical application of these flavonoids as alternative and complementary therapeutic agents for allergic diseases.

REFERENCES

- Costa JJ, Weller PF, Galli SJ. The cells of the allergic response: mast cells, basophils and eosinophils. *JAMA* 1997;278:1815-22.
- Kinet JP, Garman SC, Jardetzky TS. On the high affinity IgE receptor (FcεRI): from physiology to pathology. *Annu Rev Immunol* 1999; 17:931-72.
- Marone G, Casolaro V, Patella V, Florio G, Triggiani M. Molecular and cellular biology of mast cells and basophils. *Int Arch Allergy Immunol* 1997;114:207-17.
- Schroeder JT, MacGlashan DW Jr, Lichtenstein LM. Human basophils: mediator release and cytokine production. *Adv Immunol* 2001;77:93-122.
- Falcone FH, Haas H, Gibbs BF. The human basophil: a new appreciation of its role in immune responses. *Blood* 2000;96:4028-38.
- Romagnani S. The role of lymphocytes in allergic disease. *J Allergy Clin Immunol* 2000;105:399-408.
- Schroeder JT, MacGlashan DW Jr, Kagey-Sobotka A, White JM, Lichtenstein LM. IgE-dependent IL-4 secretion by human basophils. The relationship between cytokine production and histamine release in mixed leukocyte cultures. *J Immunol* 1994;153:1808-17.
- Devouassoux G, Foster B, Scott LM, Metcalfe DD, Prussin C. Frequency and characterization of antigen-specific IL-4- and IL-13-producing basophils and T cells in peripheral blood of healthy and asthmatic subjects. *J Allergy Clin Immunol* 1999;104:811-9.
- Ochi H, Tanaka T, Katada Y, Naka T, Aitani M, Hashimoto S, et al. Peripheral blood T lymphocytes and basophils, freshly isolated from house-dust-mite-sensitive patients, produce interleukin-4 in response to allergen-specific stimulation. *Int Arch Allergy Immunol* 1996;111:253-61.
- Kasaian MT, Clay MJ, Happ MP, Garman RD, Hirani S, Luqman M. IL-4 production by allergen-stimulated primary cultures: identification of basophils as the major IL-4 producing cell type. *Int Immunol* 1996;8:1287-97.
- Shimizu Y, Shichijo M, Hiramatsu K, Takeuchi M, Nagai H, Takagi K. Mite antigen-induced IL-4 and IL-13 production by basophils derived from atopic asthma patients. *Clin Exp Allergy* 1998;28:497-503.
- Marone G, Spadaro G, De Marino V, Aliperta M, Triggiani M. Immunopharmacology of human mast cells and basophils. *Int J Clin Lab Res* 1998;28:12-22.
- Casolaro V, Spadaro G, Patella V, Marone G. In vivo characterization of the anti-inflammatory effect of cyclosporin A on human basophils. *J Immunol* 1993;151:5563-73.
- de Paulis A, Cirillo R, Ciccarelli A, Condorelli M, Marone G. FK-506, a potent novel inhibitor of the release of proinflammatory mediators from human FcεRI+ cells. *J Immunol* 1991;146:2374-81.
- Bissonnette EY, Enciso JA, Befus AD. Inhibition of tumour necrosis factor-α (TNF-α) release from mast cells by the anti-inflammatory drugs, sodium cromoglycate and nedocromil sodium. *Clin Exp Immunol* 1995;102:78-84.
- Formica JV, Regelson W. Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxic* 1995;33:1061-80.
- Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 2000;52:673-751.
- Middleton E Jr, Drzewiecki G. Flavonoid inhibition of human basophil histamine release stimulated by various agents. *Biochem Pharmacol* 1984;33:3333-8.
- Fewtrell CMS, Gomperts BD. Effect of flavone inhibitors of transport ATPases on histamine secretion from rat mast cells. *Nature* 1977;265:635-6.

20. Kotani M, Fujita A, Tanaka T. Inhibitory effects of persimmon leaf extract on allergic reaction in human basophilic leukemia cells and mice. *J Jpn Soc Nutr Food Sci* 1999;52:147-51.
21. Kotani M, Matsumoto M, Fujita A, Higa S, Wang W, Suemura M, et al. Persimmon leaf extract and astragaloside inhibit development of dermatitis and IgE elevation in NC/Nga mice. *J Allergy Clin Immunol* 2000;106:159-66.
22. Matsumoto M, Kotani M, Fujita A, Higa S, Kishimoto T, Suemura M, et al. Oral administration of persimmon leaf extract ameliorates skin symptoms and transepidermal water loss in atopic dermatitis model mice, NC/Nga. *Br J Dermatol* 2002;146:221-7.
23. Haisch K, Gibbs BF, Korber H, Ernst M, Grage-Griebenow E, Schlaak M, et al. Purification of morphologically and functionally intact human basophils to near homogeneity. *J Immunol Methods* 1999;226:129-37.
24. Kishi K. A new leukemia cell line with Philadelphia chromosome characterized as basophil precursors. *Leukoc Res* 1985;9:381-90.
25. Yanagihara Y, Kajiwara K, Basaki Y, Ikizawa K, Akiyama K, Saito H. Induction of human IgE synthesis in B cells by a basophilic cell line, KU812. *Clin Exp Immunol* 1997;108:295-301.
26. Hutchinson LE, McCloskey MA. FcεRI-mediated induction of nuclear factor of activated T-cells. *J Biol Chem* 1995;270:16333-8.
27. Ali H, Ahamed J, Hernandez-Munain C, Baron JL, Krangel MS, Patel DD. Chemokine production by G protein-coupled receptor activation in a human mast cell line: roles of extracellular signal-regulated kinase and NFAT. *J Immunol* 2000;165:7215-23.
28. Thomas MA, Mordvinov VA, Sanderson CJ. The activity of the human interleukin-5 conserved lymphokine element 0 is regulated by octamer factors in human cells. *Eur J Biochem* 1999;265:300-7.
29. Schwenger GT, Fournier R, Hall LM, Sanderson CJ, Mordvinov VA. Nuclear factor of activated T cells and YY1 combine to repress IL-5 expression in a human T-cell line. *J Allergy Clin Immunol* 1999;104:820-7.
30. Ranganath S, Ouyang W, Bhattacharya D, Sha WC, Grupe A, Peltz G, et al. GATA-3 dependent enhancer activity in IL-4 gene regulation. *J Immunol* 1998;161:3822-6.
31. Escoubet-Lozach L, Glass CK, Wasserman SI. The role of transcription factors in allergic inflammation. *J Allergy Clin Immunol* 2002;110:553-64.
32. Brown MA, Hural J. Functions of IL-4 and control of its expression. *Crit Rev Immunol* 1997;17:1-32.
33. Nishino H, Naito E, Iwashima A, Tanaka K, Matsuura T, Fujiki H, et al. Interaction between quercetin and Ca²⁺-calmodulin complex: possible mechanism for anti-tumor-promoting action of the flavonoid. *Gann* 1984;75:311-6.
34. Schroeder JT, Miura K, Kim HH, Sin A, Cianferoni A, Casolaro V. Selective expression of nuclear factor of activated T cells 2/c1 in human basophils: evidence for involvement in IgE-mediated IL-4 generation. *J Allergy Clin Immunol* 2002;109:507-13.
35. Hollman PCH, Hertog MGL, Katan MB. Analysis and health effects of flavonoids. *Food Chem* 1996;57:43-6.
36. Miller AL. The etiologies, pathophysiology, and alternative/complementary treatment of asthma. *Altern Med Rev* 2001;6:20-47.

Availability of Journal back issues

As a service to our subscribers, copies of back issues of *The Journal of Allergy and Clinical Immunology* for the preceding 5 years are maintained and are available for purchase until inventory is depleted. Please write to Mosby Subscription Customer Service, 6277 Sea Harbor Dr, Orlando, FL 32887, or call (800) 654-2452 or (407) 345-4000 for information on availability of particular issues and prices.