Flavonoids as Potential Anti-Allergic Substances

T. Tanaka[a]*, S. Higa[a], T. Hirano[a], M. Kotani[b], M. Matsumoto[b], A. Fujita[b] and I. Kawase[a]

[a]*Department III of Internal Medicine, Osaka University Medical School, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan* [b]*Research and Development Center, Sunstar Incorporation, 3-1 Asahi-machi, Takatuki City, Osaka 569-1134, Japan*

Abstract: Progress in the management of allergic diseases including asthma, pollen diseases and atopic dermatitis has recently improved patients' quality of life. In addition to steroids, which are powerful inhibitors of allergic inflammation, other anti-allergic compounds, humanized anti-IgE antibody and cytokine modulators have been developed. However, the world-wide prevalence of allergic diseases has been increased during the last two decades, making early intervention to prevent the development of such diseases essential. Through analysis of the clinical evaluation of one kind of traditional remedy in patients with atopic dermatitis, flavonoids were shown to possess significant anti-allergic activity. Among the twenty kinds of flavonoids examined, fisetin, luteolin, apigenin, quercetin and kaempferol inhibited not only the release of chemical mediators but also the production of T-helper (Th)-2 type cytokines (Interleukin (IL)-4, IL-5 and IL-13) by basophils. These flavonoids inhibit expression of these cytokines through their inhibitory effect on the activation of several calcium-calmodulin dependent kinases. Administration of either persimmon leaf extract, which is rich in flavonoids, or its major ingredient astragalin (a glycoside of kaempferol), in atopic dermatitis-model mice (NC/Nga) prevented the onset of dermatitis and showed a substantial ameliorative effect even in mice with open dermatitis. Moreover, a double-blind, randomized, placebo-controlled trial of flavonoid in patients with atopic dermatitis showed a significant effect. These results indicate the possibility that flavonoids, which are contained in vegetables, fruits and tea, may constitute a complementary and alternative medicine for allergic patients and may act as a prophylactic substance against the development of allergic diseases.

Keywords: flavonoid, allergy, mast cells/basophils, prevention, complementary and alternative medicine, IL-4/IL-5/IL-13

INTRODUCTION

Allergic diseases including asthma, atopic dermatitis and pollen diseases are among the most common chronic diseases and are characterized by the existence of allergic inflammation, hyper-production of IgE and hyper-reactivity of organs to various stimuli [1-3]. Accumulating evidence from both molecular biological studies of allergic inflammation and from clinical evidence has led to the establishment of guidelines for the management of allergic diseases [1, 4-8]. In addition to steroids, which are powerful inhibitors of allergic inflammation, a number of other anti-allergic drugs have been developed [9], and it is clear that the appropriate use of these drugs, depending on the severity of the diseases according to the guidelines, has recently improved patients' quality of life.

However, the worldwide prevalence of allergic diseases has increased during the last two decades [10-12] and it is assumed that one-third of the population of the developed countries is now suffering from at least one of these diseases. This has led not only to a significant increase in patient morbidity but also in the cost to the patients and their families and has placing a great burden on society [13-17]. The interaction between genetic and environmental factors is generally accepted as a key factor in the development of allergic diseases [1, 18-21] and it is believed that recent changes in the environment have contributed to the increase in such prevalence more significantly than the genetic factors, since it seems unlikely that genes would happen to change over two or three generations. Thus, it is essential in the future to reveal what environmental factor(s) cause such high prevalence and to find strategies to prevent their development [1, 22, 23]. In this article we will review the recent development of anti-allergic and preventative substances including flavonoids.

CURRENT UNDERSTANDING OF THE PATHOGENESIS OF ALLERGIC DISEASES

Three distinct features characterize allergic diseases [1-3]. One is atopy, the production of abnormal amounts of IgE antibodies in response to environmental allergens [24]. This characteristic appears to be determined genetically and is the strongest identifiable predisposing factor for developing these diseases. The second is hyper-reactivity of organs to various stimuli such as airway hyperresponsiveness in asthma or irritable skin in patients with atopic dermatitis. The third is the existence of allergic inflammation in localized organs. It has been shown that inflammatory cells such as mast cells, basophils, lymphocytes, and eosinophils are infiltrated, activated in the involved tissues and form an integrated allergy network through their production of soluble factors including chemical mediators, cytokines, chemokines, and tissue damaging proteins. This allergic

*Address correspondence to this author at the Department III of Internal Medicine, Osaka University Medical School, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan; Tel:+81-6-6879-3833; Fax: +81-6-6879-3839; E-mail: ttanak@imed3.med.osaka-u.ac.jp

1568-0142/03 $41.00+.00

inflammation not only induces clinical symptoms but also accelerates IgE synthesis and organ hyper-reactivity. Thus, the network is considered the fundamental basis for the development and continuation of such diseases [1-3]. However, it is yet to be determined what the primary cause(s) of triggering such inflammation are. There is evidence to implicate mast cells and eosinophils as the key effector cells of the inflammation through their capacity to secrete a wide range of chemical mediators and cytokines [25, 26]. On the other hand, recent analysis has placed T (Th2) cells as pivotal in orchestrating an inflammatory response through the release of multifunctional cytokines [27]. Similarly, cytokines such as IL-4, IL-5 or IL-13 have been demonstrated to be key the factors in inducing allergic inflammation, while chemical mediators including histamine and cysteinyl leukotrienes, eosinophil-derived tissue damaging proteins or chemokines have also been shown to relate to the pathogenesis of allergy [28]. In the near future, which cells and which molecules are the most important in forming allergic inflammation will no doubt be determined. Anti-allergic drugs such as anti-histamines and leukotriene modifiers have already been put to clinical use and other compounds or humanized antibodies designed to inhibit the function of the targeted molecule are now being developed [9]. The clinical effectiveness of such novel drugs will reveal the significance of the molecule in the pathogenesis of allergic diseases.

CURRENT DEVELOPMENT OF ANTI-ALLERGIC COMPOUNDS

The current concept is that a characteristic process of chronic inflammation causes the development of allergic diseases. Therefore, according to the guidelines for the management of allergic diseases, glucocorticosteroids are considered to be the most effective controller in inhibiting allergic inflammation [1, 29]. To maximize the effect, while minimizing adverse reactions, the route of administration is extremely important and modified steroids have been developed. Their efficacy is partly due to the inhibition of inflammatory cytokines and enzyme expression, mediated through the effect on glucocorticoid receptors of reversing the acetylation of core histones linked to the expression of inflammatory genes. Depending on the degree of severity, these drugs can be administered in different ways, including inhalation and oral, percutaneous, and parenteral (intravenous, subcutaneous or intramuscular) routes.

In addition to steroids, cromones (sodium cromoglycate and nedocromil sodium) [30], anti-allergic histamine antagonists (acrivastine, astemizole, azelastine, cetirizine, ebastine, ferofenadine, ketotifen, loratidine, mizolastine, and terferadine) [31, 32], leukotriene modifiers (cysteinyl leukotriene 1 receptor antagonists; montelukast, pranlukast, zafirlukast and a 5-lipoxygenase inhibitor; zileuton) [33], and an inhibitor of IL-4 production (suplatast tosylate) [34] are used clinically. For asthma, β2-agonists and methyxanthines are also used widely [35, 36]. It has been reported that the oral intake of cyclosporin A, which is a cytokine inhibitor through modulating calcineurin complex [37], is effective in patients with severe asthma and atopic dermatitis [38-40]. Recently, the novel topical immunosuppressant, tacrolimus (FK506), has been introduced to Japan and has also been approved in the United States [41].

As described earlier, in order to suppress the function of the targeted molecule, which plays an important role in the development of allergic diseases, novel compounds and (humanized) monoclonal antibodies have been developed. Humanized anti-IgE monoclonal antibody (Omalizumab) binds to free IgE, facilitating its removal, and binds to membrane-bound IgE on B cells inhibiting IgE production by B cells [42]. However, this antibody does not bind to IgE that is already bound to the IgE receptors on mast cells and basophils. It is able to attenuate the early and late airway responses, the increase in airway hyperresponsiveness, and the influx of eosinophils into the airway lumen that follows allergen challenge. Phase III trials for patients with allergic asthma showed significant effectiveness in improving asthma control [43].

As novel therapies for patients with asthma, cytokine modulators including humanized anti-cytokines, cytokine receptor antagonists and signal inhibitors have been developed and clinical trials of some of these novel compounds have been reported. For example, a single intravenous infusion of humanized anti-IL-5 antibody (Mepolizumab) markedly reduced blood eosinophils for several weeks and prevented eosinophil recruitment into the airways after allergen challenge but had no significant effect on the early and late response to allergen challenge [44]. Weekly nebulization of a soluble IL-4 receptor that is able to neutralize IL-4 activity is reported to have improved asthma control [45], while recent advances in a novel therapeutic approach for allergy have been reviewed [9, 46-48].

FLAVONOIDS ARE POTENTIAL ANTI-ALLERGIC SUBSTANCES

Changes in environmental factors may contribute to the increase in the prevalence of allergic diseases. As environmental factors that influence the susceptibility to the development of asthma in predisposed individuals, the Global Strategy for Asthma Management and Prevention lists indoor and outdoor allergens, occupational sensitizers, air pollution, respiratory infection, parasitic infections, socioeconomic status, family size and obesity [1]. Diet and drugs are also indicated as some of the risk factors. However the type of diet that is appropriate for allergic patients is limited. Whether omega-3 polyunsaturated fatty acids that are rich in fish oil are associated with a low prevalence of asthma is controversial [49-51]. The consumption of fruit rich in Vitamin C may reduce wheezing symptoms in childhood [52], while other nutrients including Vitamin B6, B12, magnesium and Zinc are also reported to be related to a reduction in asthma symptoms. However, no definitive conclusions have been drawn with regard to the supplementation of Vitamin B6, B12 and Zinc, or intravenous injection of magnesium sulfate in asthmatic patients [53].

Changes in life style, especially in diet over the last decade, have significantly affected the prevalence of other

diseases such as hyperlipidemia and atherosclerosis [54]. Similarly, if there are anti-allergic substances in foods or tea, dietary intake of such substances could ameliorate allergic symptoms and prevent the development of allergic diseases. We evaluated the clinical effect of one kind of traditional vegetarian diet on adult patients with atopic dermatitis seven years ago [55]. After a two-month period of treatment, the severity of dermatitis decreased from 49.9+18.6 to 27.4+16.8 based on a score of severity of the atopic dermatitis, the SCORAD index and on serological parameters including Lactate dehydrogenase (LDH)-5 activity and a number of peripheral eosinophils. Prostaglandin (PG)E2 production by peripheral blood mononuclear cells was also reduced but serum levels of IgE and the ratio of IL-4/Interferon (IFN)-γ synthesis by concanavalin-A-stimulated mononuclear cells were not suppressed. This diet treatment consisted of a low energy intake (1085 kcal), fresh vegetable juice (corresponding to 250g of fresh vegetables including Spinach, Komatsuna, Cabbage, Pak-Choi, and Garland Chrysanthemum) at breakfast, brown rice porridge (corresponding to 80g of brown rice) sprinkled with 5g of kelp powder, tofu (wet weight approximately 200g) and 10g of sesame paste for lunch and again for dinner. A daily requirement of 2.5g of non-refined salt was added to the diet. Instead of water, patients were given persimmon leaf tea (1-2 l/day). Subsequent studies showed that a low calorie diet caused a reduction in the number of peripheral blood neutrophils and the ratio of serum IL-4/IFN-γ and that the same vegetable juice given to adult healthy volunteers for 3 weeks resulted in a decrease in urinary secretion of 8-Hydroxy deoxyguanosine, an index of oxidative damage to DNA, and of the ratio of n-6/n-3 polyunsaturated fatty acids (Kotani *et al.* unpublished data). We then asked ourselves whether the extract of persimmon leaf possessed anti-allergic substances.

Persimmon leaf from Shi-chuan, China was purchased and was steeped in boiling distilled water for 5 min and was filtered through a nylon mesh. After centrifugation, the supernatant of the persimmon leaf was freeze-dried. HPLC analysis of the constituents from the extract showed that there were trace levels of Caffeine, Catechin including (-)-Epigallocatechin, (-)-Epicatechin gallate and (-)-Epigallocatechin gallate (EGCG) but the extract included 0.45-0.72% of astragalin, 0.32% of toripholin and 0.38-0.55% of isoquercitrin as flavonoids [56].

Flavonoids are comprised of a large group of low molecular weight polyphenolic secondary plant metabolites which are found in fruits, vegetables, nuts, seeds, stems, flowers, roots, bark, tea, wine and coffee and are thus common substances in our daily diet [57, 58]. Flavonoids have been recognized to exert antioxidant, anti-bacterial and anti-viral activity, anti-inflammatory, anti-angionic, analgestic, hepatoprotective, cytostatic, apoptotic, estrogenic or anti-estrogenic properties as well as anti-allergic effects [59]. Based on their skeleton, flavonoids are categorized into eight groups: flavans, flavanones, isoflavanones, flavones, isoflavones, anthocyanidines, chalcones and flavonolignans (Fig. **1**). Their skeleton is a heterocyclic hydrocarbon, chromane, and substitution of its ring C in position 2 or 3 with a phenyl group (ring B) results in flavans or isoflavans. An oxo-group in position 4 leads to flavanones and

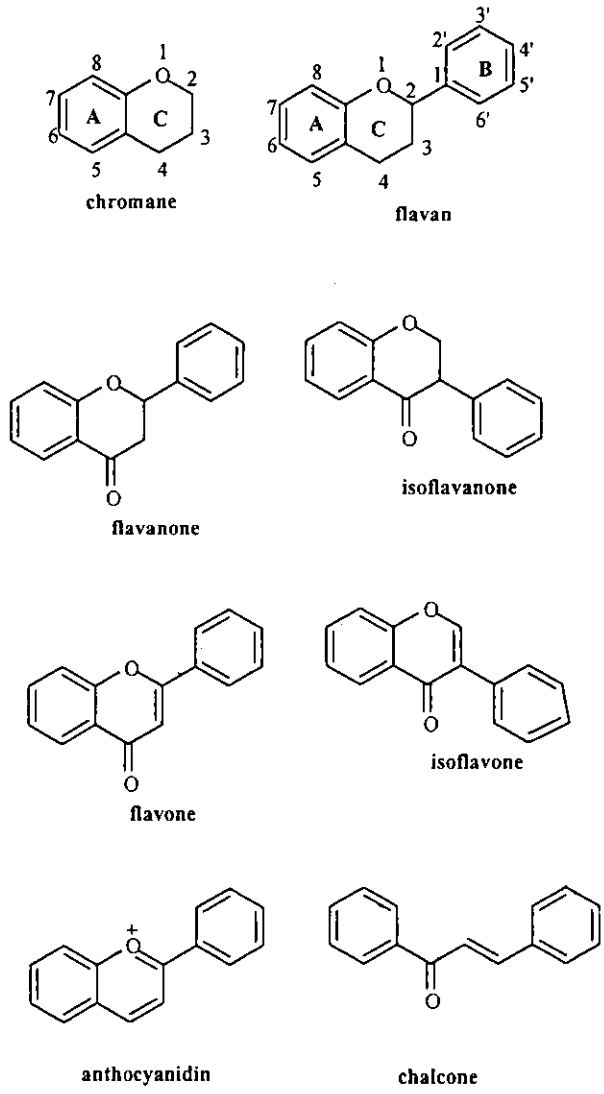


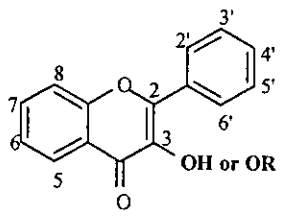
Fig. (1). Structures of basic flavonoid skeletons

isoflavanones. The presence of a double bond between C2 and C3 provides flavones and isoflavones. An additional double bond in between C1 and C2 makes these compounds colorful, designated as glycosides (e.g. glucosides, rhamnoglucosides, and rutinosides) and more than 8,000 different flavonoids have been identified so far [59].

Fewtress and Gomperts first demonstrated the anti-allergic effect of flavones inhibition of transport ATPase in histamine secretion from rat mast cells [60]. The effect of six flavonoids (fisetin, kaempferol, morin, quercetin, myricetin and rutin) on antigen-induced histamine secretion was measured. Fisetin, quercetin, myricetin and kaemferol inhibited histamine release while two other flavonoids showed little effect. Quercetin inhibition of allergen-stimulated human basophils was reported subsequently [61, 62]. Similarly, Cheong et al showed the structure activity relationship of flavonoids for anti-allergic actions through analyses of their inhibitory activity on hexosaminidase release from rat mast cells. Apigenin, luteolin, 3.6-dihydroxy flavones, fisetin, kaempferol, quercetin, and myricetin were found to inhibit such release with an IC50 value of less than

10 μM [63]. In addition to the inhibitory property of flavonoids on histamine release by mast cells or basophils, flavonoids have been shown to suppress cysteinyl leukotriene synthesis through an inhibition of phospholipase A2 (PLA2) and/ or 5-lipoxygenase (5LO). The inhibitory effect of several flavonoids on the PLA2 and 5LO was recently reviewed [64]. PLA2 releases arachidonic acid from membrane phospholipids and is metabolized by the 5LO pathway, leading to biosynthesis of cysteinyl leukotrienes, which are important mediators for pathogenesis of asthma. Quercetin was first demonstrated to be an inhibitor of PLA2 [65] and later it was reported that quercetagetin, kaempferol-3-O-galactoside, and scutellarein also possessed this activity with IC50 values ranging from 12.2 to 17.6 μM [66]. A number of flavonoids possess 5LO inhibitory activity [64, 67]. For instance, cirsiliol (3',4',5-trihydroxy-6,7-dimethoxy flavone) caused 97% inhibition of 5LO activity from rat basophilic cells and 99% suppression of release of cysteinyl leukotrienes from guinea pig lung [67].

Astragalin, a glycoside of kaempferol (Fig. 2), which is rich in persimmon leaf extract, was examined for its anti-allergic activity. Indeed, astragalin and the extract inhibited histamine release by the human basophilic cell line, KU812, in response to cross-linkage of high affinity IgE receptors as did other flavonoids described previously [68]. Mast cells or basophils have been shown to release chemical mediators and to produce cytokines [69]. Among the cytokines produced by these cells, IL-4, IL-13 and IL-5 are believed to be the key molecules related to IgE production, Th2 differentiation and allergic inflammation [28]. We therefore first screened the inhibitory activities of 6 kinds of flavonoids, including astragalin, which share structural similarities to the secretions of cytokines by KU812 (Fig. 2) (Higa *et al.* unpublished data). The presence of fisetin inhibited IL-4, IL-5 and IL-13 expression at a dose of 30 μM in A23187-stimulated KU812 cells but did not inhibit IL-1β, IL-6 and IL-8 expression by reverse-transcription (RT)-PCR



Flavonol	3	5	7	2'	3'	4'	5'
Fisetin	OH	-	OH	-	OH	OH	-
Kaempferol	OH	OH	OH	-	-	OH	-
Myricetin	OH	OH	OH	-	OH	OH	OH
Quercetin	OH	OH	OH	-	OH	OH	-

Flavonol Glycosides	3	5	7	2'	3'	4'	5'
Astragalin	Glc	OH	OH	-	-	OH	-
Rutin	Rhamnosyl-Glucoside	OH	OH	-	OH	OH	-

Fig. (2). Structures of flavonoids; four flavonols (fisetin, kaempferol, myricetin, and quercetin) and two flavone glycosides (astragalin and rutin)

analyses (Fig. 3). Similarly fisetin inhibited IL-4, IL-5 and IL-13 production in the supernatants of A23187+PMA-stimulated KU812 cells, but not IL-6 and IL-8 production. Kaempferol, a deglycosylated-astragalin and quercetin also showed substantial inhibitory activity but less than fisetin. Fisetin also suppressed IL-4 synthesis by allergen-stimulated purified peripheral basophils. The IC50 of fisetin for the inhibition of IL-4 expression by KU812 cells and normal basophils was 19.1+4.9 and 4.8+0.4 μM, respectively. Further screening of 20 different flavonoids assayed by IL-4 and IL-5 mRNA expression by means of RT-PCR, and IL-5 production by means of ELISA in KU812 cells, revealed that fisetin, apigenin, luteolin, kaempferol and quercetin showed such novel activity. A summary of this is shown in Fig. 4. The IC50 value in the inhibition of IL-5 synthesis is included. The basic structure of flavonoids for inhibitory activity on Th2 type cytokine expression by basophils is also shown in Fig. 5. For maximal inhibition, hydroxylation in positions 7 and 4' is essential and additionally the presence of OH in either position 3 or 5 is required. Since this hierarchy was examined directly by the addition of flavonoids into KU812 cells, the hierarchy cannot be applicable to clinical efficacy. This depends on the solubility, stability and efficacy of absorbance.

The mechanisms by which these flavonoids suppress Th2 type cytokine expression remains to be determined, however. The specific inhibitory activity of flavonoids on IL-4, IL-5 and IL-13 suggests that they suppress the activation of (a) certain common specific nuclear factor(s) that positively regulate IL-4, IL-5 and IL-13 gene activation or induce repressor protein(s). The nuclear factor of the activated T cell (NFAT) family plays a key role in inducing cytokine genes in T cells including IL-2, IL-4, IL-5, IL-13, IFN-γ, tumor necrosis factor (TNF)-α and granulocyte/macrophage colony stimulating factor (GM-CSF) [70]. The basophilic cell line KU812 expressed the NFAT family. The stimulation of KU812 cells with A23187 induced a greater binding intensity in the nuclear lysate, and the presence of fisetin, but did not control flavonoid, markedly inhibited the binding activity in the nuclear lysate, suggesting that fisetin can modulate NFAT activation. Although T cells seem to be more resistant to these flavonoids in the synthesis of IL-4 than basophils, other mechanisms may additionally contribute to such inhibition. The finding that flavonoids including fisetin, apigenin and luteolin did not reduce intracytoplasmic concentration of calcium in response to calcium ionophore, and the previous report that quercetin directly interacted with a calmodulin, the Ca^{2+}-sensing protein [71], suggest that flavonoids may modulate the function of calmodulin and affect some Ca/calmodulin dependent enzymes, which regulate transcriptional activation of these cytokines in addition to NFAT inhibition.

The *in vivo* effect of flavonoid was examined using an atopic dermatitis-model mouse, NC/Nga. NC/Nga mouse spontaneously develops severe eczema with aging under nonspecific pathogen free circumstances [72]. In addition, the histology of its dermatitis mimics human atopic dermatitis, and the serum level is significantly elevated after the onset of dermatitis. The mice were orally given persimmon leaf extract (250 mg/kg), astragalin (1.5 mg/kg) or a control diet [56]. Development of dermatitis was

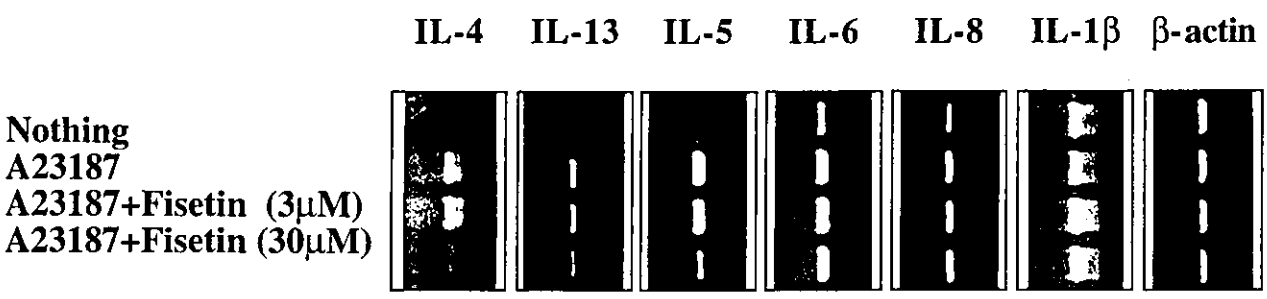
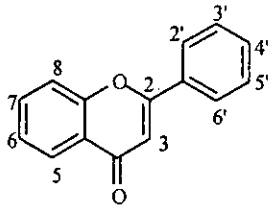
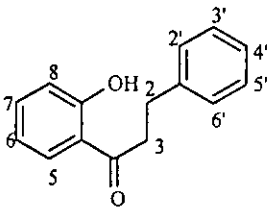


Fig. (3). Fisetin inhibits the expression of IL-4, IL-13 and IL-5 mRNAs in KU812 cells. KU812 cells were pre-incubated with fisetin (3 or 30 μM) or without for 15 min, and stimulated with A23187 (1 μM) for 3 hours. RNAs were obtained and the expression of IL-4, IL-13, IL-5, IL-6, IL-8, IL-1β and β-actin transcripts was examined by RT-PCR.

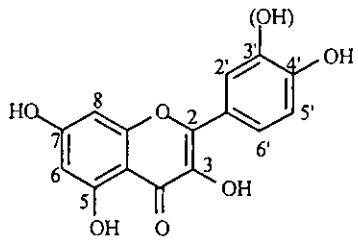


	3	5	6	7	8	2'	3'	4'	5'	IC50 (μM)
Apigenin	-	OH	-	OH	-	-	-	OH	-	11.6
Fisetin	OH	-	-	OH	-	-	OH	OH	-	17.1
Luteolin	-	OH	-	OH	-	-	OH	OH	-	18.4
Kaempferol	OH	OH	-	OH	-	-	-	OH	-	>30
Quercetin	OH	OH	-	OH	-	-	OH	OH	-	>30
Astragalin	O-glc	OH	-	OH	-	-	-	OH	-	>30
Myricetin	OH	OH	-	OH	-	-	OH	OH	OH	>30
Rutin	O-rutinose	OH	-	OH	-	-	OH	OH	-	>30
3-Hydroxyflavone	OH	-	-	-	-	-	-	-	-	>30
7-Hydroxyflavone	-	-	-	OH	-	-	-	-	-	>30
Galangin	OH	OH	-	OH	-	-	-	-	-	>30
Morin(dihydrate)	OH	OH	-	OH	-	OH	-	OH	-	>30
Gossypin	OH	OH	-	-	O-glc	-	OH	OH	-	>30
Fustin (2-3)	OH	-	-	OH	-	-	OH	OH	-	>30
Eriodictyol (2-3)	-	OH	-	OH	-	-	-	OH	OH	>30
Scutellarein (tetramethylether)	-	OH	OH	OH	-	-	-	OH	-	>30
Isoquercitrin	O-glc	OH	-	OH	-	-	OH	OH	-	>30
Myricitrin	O-rhamoside	OH	-	OH	-	-	OH	OH	OH	>30



	3	5	6	7	8	2'	3'	4'	5'	IC50 (μM)
Phloretin	-	OH	-	OH	-	-	-	OH	-	>30
Phloridzin (dihydrate)	-	O-glc	-	OH	-	-	-	OH	-	>30

Fig. (4). Structures of 20 flavonoids and their inhibitory activity on IL-5 synthesis by KU812 cells. Twenty kinds of representative flavonoids were tested for their inhibition of IL-5 synthesis by stimulated-KU812 cells. The underlined flavonoids (apigenin, fisetin, luteolin, kaempferol, and quercetin) substantially inhibited its synthesis.



	3	5	7	3'	4'	MW	IC50 (μM)
Apigenin	-	OH	OH	-	OH	270	11.6
Fisetin	OH	-	OH	OH	OH	286	17.1
Luteolin	-	OH	OH	OH	OH	286	18.4
Kaempferol	OH	OH	OH	-	OH	286	>30
Quercetin	OH	OH	OH	OH	OH	302	>30

(Each OH for 3 or 5 position)

Fig. (5). Basic structure of flavonoids for inhibitory activity on Th2 cytokine expression in basophils

observed in the control group with aging. Skin symptoms and histological features at the age of 13 weeks are shown in Fig. 6. Oral intake of persimmon leaf extract or astragalin remarkably inhibited the appearance of the skin symptoms. This preventative effect of persimmon leaf extract on the onset and development of eczema showed a dose-response effect [73]. Moreover, these substances significantly diminished the severity of dermatitis even after the onset without any adverse effects. The administration of persimmon leaf extract or astragalin inhibited scratching, transepidermal water loss and serum elevation of IgE [56, 73]. Thus, these analyses indicate that at least in an atopic dermatitis-model mouse, persimmon leaf extract and its major flavonoid, astragalin act as prophylactic substances against the development of atopic dermatitis and substantial anti-allergic substances. Since astragalin is absorbed after being converted into kaempferol due to intestine β-glucosidase, these effects were partly considered to be derived from the inhibition of basophil (and mast cell) activation.

Based on this evidence, we attempted to examine the clinical effect of persimmon leaf extract on adult patients with atopic dermatitis in randomized controlled trials (Takigawa *et al.* unpublished data). Patients were treated with placebo, 1g or 2g of persimmon leaf extract for 4 weeks without any change in medication, and the severity of their skin lesions was measured according to the SCORAD index. In addition, serological markers, including a number of peripheral eosinophils, and serum levels of IgE were also monitored. The intake of persimmon leaf extract at a dose of 1g and 2g/day significantly decreased the SCORAD index, which was accompanied by a decrease in peripheral eosinophils. However, serum levels of IgE were not suppressed. In order to verify that flavonoid is effective in patients with atopic dermatitis, further studies involving a large number of participants are required, but this preliminary data, we believe, seem to be promising as complementary and alternative medicine for atopic dermatitis

FLAVONOIDS AND HUMAN HEALTH

Flavonoids are naturally and ubiquitously included in plant foods and drinks and are therefore common components in our diet. Previously, it was estimated that the total intake of all flavonoids in the USA was 1 g/day [74]. However, other groups have demonstrated that an average Dutch intake of five selected flavonols and flavones was 23 mg/day, of which quercetin contributed 16 mg/day [75]. Hertog *et al.* examined the content of the representative flavonoids such as quercetin, kaempferol, myricetin, apigenin and luteolin in 28 vegetables and 9 fruits in the Netherlands after the extract was acid-hydrolyzed [76]. Quercetin levels in the edible parts of most vegetables were less than 10 mg/kg, except for onion (284-486 mg/kg), kale (110 mg/kg), broccoli (30 mg/kg), French beans (32-45 mg/kg), and slicing beans (28-30 mg/kg). Kaempferol could only be detected in kale (211 mg/kg), endive (15-91 mg/kg), leek (11-56 mg/kg), and turnip tops (31-64 mg/kg). In most fruits, the quercetin content averaged 15 mg/kg, except for

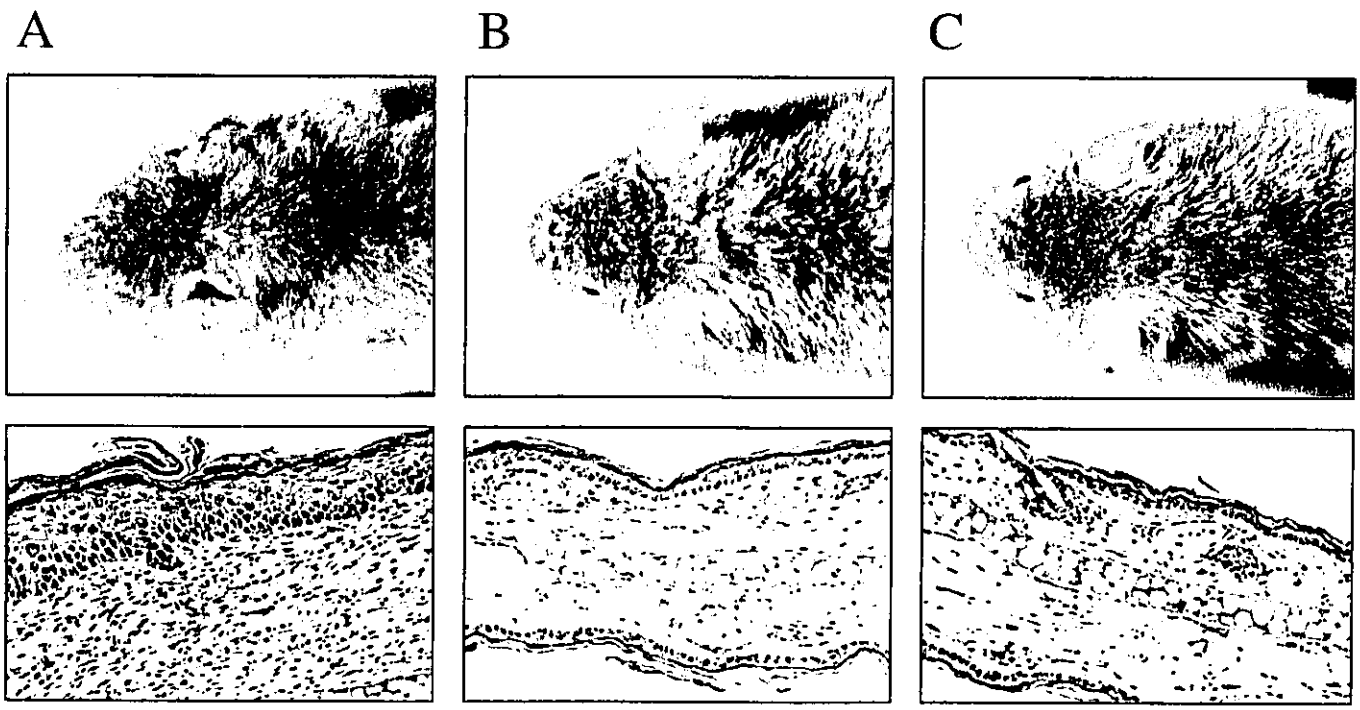


Fig. (6). Effect of persimmon leaf extract or astragalin on clinical and histologic features of NC/Nga skin. NC/Nga mice were provided with one of the following diets ad libitum: **A**, control diet; **B**, control diet plus persimmon leaf extract at 250mg/kg; or **C**, control diet plus astragalin at 1.5mg/kg from 5 to 13 weeks of age. The clinical features and Hematoxylin and eosin-stained sections of the ear at 13 weeks of age are shown.

different varieties of apple (21-72 mg/kg). The content of myricetin, luteolin and apigenin was less than 1 mg/kg, except for fresh broad beans (26 mg/kg myricetin) and red bell pepper (13-31 mg/kg luteolin). Celery included apigenin (108 mg/kg) and luteolin (22 mg/kg), both of which showed strong inhibitory activity on Th2 cytokine expression by basophils. The same authors also subsequently examined the content of flavonoids in tea, wines and fruit juices [77]. These data clearly show that significant amounts of flavonoids are present, mainly as glycosides, in vegetables especially in onion, kale or celery, fruits and drinks. The persimmon leaf extract as described above also contained a large amount of flavonoids (1%).

Is it evident that an excess (or appropriate) intake of flavonoids contributes to human health and decreases the prevalence of allergic diseases ? So far, epidemiological evidence does not appear to lead to a definitive conclusion that flavonoids have *in vivo* beneficial effects in cancer and cardiovascular diseases as well as in allergy [78]. However, some recent reports raise this possibility. EGCG in tea has the capacity of inhibiting urokinase that is a proteolytic enzyme for cancers to invade and form metastases [79]. EGCG is also shown to suppress angiogenesis, a key process of blood vessel growth required for tumor growth and metastasis and for retinopathy in patients with diabetes mellitus. Since a single cup of tea contains 150 mg of EGCG, these findings are potentially useful in the prevention of cancers [80]. Similarly, two studies have suggested that a high intake of fresh fruit [81] and vegetables [82] may protect against asthma, but neither could determine which specific foods or nutrients were responsible. But recently Shaheen *et al.* reported that by a population-based case-control study in South London, apple consumption or red wine intake were negatively associated with asthma prevalence or severity, respectively, perhaps due to a protective effect of flavonoids [83].

CONCLUSION

Allergy, one of the common diseases throughout the world, is of growing concern because of its increasing rate of prevalence. Allergic diseases, including asthma, atopic dermatitis and pollen diseases, impose a substantial social burden on both children and adults. At the present time, glucocorticosteroids are the key drugs for allergic patients, but other compounds, which inhibit, at a pinpoint, the functions of the targetted molecules are being developed for ameliorating symptoms and for improving quality of life. More importantly, to decrease such prevalence, intervention strategies for the primary prevention of allergic diseases are the ultimate goal. In order to achieve this aim, defining the interaction between genetic factors and environmental risk factors is a central issue. Recently, it was elegantly demonstrated that when Lactobacillus GG, a probiotic was administered prenatally to mothers and postnatally to their infants, it was effective in the prevention of early atopic eczema in children at high risk [84, 85]. Similarly, flavonoids, which possess anti-allergic activities, may act as preventative substances and as complementary and alternative medicines for allergic diseases [53, 86].

ABBREVIATIONS

Th	=	T-helper
IL	=	Interleukin
LDH	=	Lactate dehydrogenase
PG	=	Prostaglandin
IFN	=	Interferon
EGCG	=	Epigallocatechin gallate
PGA2	=	Phospholipase A2
5LO	=	5-lipoxygenase
RT	=	Reverse-transcription
NFAT	=	Nuclear factor of activated T cells
TNF	=	Tumor necrosis factor
GM-CSF	=	Granulocyte/macrophage colony stimulating factor

REFERENCES

[1] *Global Strategy for Asthma Management and Prevention*, National Institutes of Health, National Heart, Lung, and Blood Institute, Revised **2002**.

[2] Leung, D.Y.M. *J. Allergy Clin. Immunol.*, **2000**, *105*, 860.

[3] Baraniuk, J.N. *Curr. Allergy Asthma Rep.*, **2001**, *1*, 207.

[4] Guidelines of Care for Atopic Dermatitis. *American Academy of Dermatology, J. Am. Acad. Dermatol.*, **1992**, *26*, 485.

[5] Leung, D.Y.; Hanifin, J.M.; Charlesworth, E.N.; Li, J.T.; Bernstein, I.L.; Berger, W.E.; Blessing-Moore, J.; Fineman, S.; Lee, F.E.; Nicklas, R.A.; Spector, S.L. *Ann. Allergy Asthma Immunol.*, **1997**, *79*, 197.

[6] Furue, M. *Arerugi.*, **2000**, *49*, 324.

[7] Pawanker, R.; Fokkens, W. *Curr. Allergy Asthma Rep.*, **2001**, *1*, 218.

[8] Okuda, M. *Curr. Allergy Asthma Rep.*, **2001**, *1*, 235.

[9] Barnes, P.J. *Nature*, **1999**, *402*, 31.

[10] The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivities, and atopic eczema: ISAAC. *Lancet*, **1998**, *351*, 1225.

[11] Williams, H.; Robertson, C.; Stewart, A.; Ait-Khaled, N.; Anabwani, G.; Anderson, R.; Asher, I.; Beasley, R.; Bjorksten, B.; Burr, M.; Clayton, T.; Crane, J.; Ellwood, P.; Keil, U.; Lai, C.; Mallol, J.; Martinez, F.; Mitchell, E.; Montefort, S.; Pearce, N.; Shah, J.; Sibbald, B.; Strachan, D.; von Mutius, E.; Weiland, S.K. *J. Allergy Clin. Immunol.*, **1999**, *103*, 125.

[12] Holgate, S.T. *Nature*, **1999**, *402*, B2.

[13] Juniper, E.F. *Allergy*, **1997**, *52*, 971.

[14] Lenney, W. *Pediatri. Pulmonol. Suppl.*, **1997**, *15*, 13.

[15] Weiss, K.B.; Gergen, P.J.; Hodgson, T.A. *N. Engl. J. Med.*, **1992**, *326*, 862.

[16] Weiss, K.B.; Sullivan, S.D. *J. Allergy Clin. Immunol.*, **2001**, *107*, 3.

[17] Stempel, D.A.; Woolf, R. *Curr. Allergy Asthma Rep.*, **2002**, *2*, 223.

[18] Holgate, S.T. *J. Allergy Clin. Immunol.*, **1999**, *104*, 1139.

[19] Holt, P.G. *Environ. Health Perspect*, **1999**, *107 Suppl 3*, 485.

[20] Von Mutius, E. *J. Allergy Clin. Immunol.*, **2000**, *105*, 9.

[21] Strachan, D.P. *Thorax*, **2000**, *55 Suppl 1*, S2.

[22] Johansson, S.G.O.; Haahtela, T. *Allergy*, **2000**, *55*, 1.

[23] Bjorksten, B. *Curr. Opin. Allergy Clin. Immunol.*, **2001**, *6*, 545.

[24] Platts-Mills, T.A. *Am. J. Respir. Crit. Care Med.*, **2001**, *164*, S1.

[25] Eapen, S.S.; Busse, W.W. *Clin. Allergy Immunol.*, **2002**, *16*, 325.

[26] Makino, S.; Fukuda, T. *Allergy Proc.*, **1995**, *16*, 13.

[27] Umetsu, D.T.; McIntire, J.J.; Akbari, O.; Macaubas, C.; DeKruyff, R.H. *Nat. Immunol.*, **2002**, *3*, 715.

[28] Romagnani, S. *Mol. Immunol.*, **2002**, *38*, 881.

[29] Sidbury, R.; Hanifin, J.M. *Dermatol. Clin.*, **2000**, *18*, 1.

[30] Norris, A.A. *Clin. Exp. Allergy*, **1996**, *26 Suppl 4*, 5.

[31] Grant, J.A.; Nicodesmus, C.F.; Findlay, S.R.; Glovsky, M.M; Grossman, J.; Kaiser, H.; Meltzer, E.O.; Mitchell, D.Q.; Pearlman, D ; Selner, J. *J. Allergy Clin. Immunol.*, **1995**, *95*, 923.

[32] Howarth, P.H. *Clin. Exp. Allergy*, **1999**, *29 Suppl 3*, 87.

[33] Drazen, J.M.; Israel, E.; O'Byrne, P.M. *N. Engl. J. Med.*, **1999**, *340*, 197.

[34] Tamaki, J.; Kondo, M.; Sakai, N.; Aoshiba, K.; Tagaya, E.; Nakata, J.; Isono, N.; Nagai, A. *Lancet*, **2000**, *356*, 273.

[35] Barnes, P.J.; Pauwels, R.A. *Eur. Respir. J.*, **1994**, *7*, 579.

[36] Nelson, H.S. *N. Engl. J. Med.*, **1995**, *333*, 499.

[37] Faulds, D.; Goa, K.L.; Benfield, P. *Drugs*, **1993**, *45*, 953.

[38] Ho, S.; Clipstone, N.; Timmermann, L.; Northrop, J.; Graef, I.; Fiorentino, D.; Nourse, J.; Crabtree, G.R. *Clin. Immunol. Immnopathol.*, **1996**, *80*, S40.

[39] Lock, S.H.; Kay, A.B.; Barnes, N.C. *Am. J. Respir. Crit. Care Med.*, **1996**, *153*, 509.

[40] Korstanje, M.J.; van de Staak, W.J. *Acta Derm. Venereol.*, **1991**, *71*, 356.

[41] Bieber, T. *J. Allergy Clin. Immunol.*, **1998**, *102*, 555.

[42] Babu, K.S.; Arshad, S.H.; Holgate, S.T. *Allergy*, **2001**, *56*, 1121.

[43] Milgrom, H.; Fick, R.B.Jr.; Su, J.Q.; Reimann, J.D.; Bush, R.K.; Watrous, M.L.; Metzger, W.J. *N. Engl. J. Med.*, **1999**, *341*, 1966.

[44] Leckie, M.J.; Ten Brinke, A.; Khan, J.; Diamant, Z.; O'Connor, B.J.; Walls, C.M.; Mathur, A.K.; Cowley, H.C.; Fan Chung, K.; Djukanovie, R.; Hansel, T.T.; Holgate, S.T.; Sterk, P.J.; Barnes, P.J. *Lancet*, **2000**, *356*, 2144.

[45] Borish, L.C.; Nelson, H.S.; Lanz, M.J.; Claussen, L.; Whitmore, J.B.; Agosti, J.M.; Garrison, L. *Am. J. Crit. Care Med.*, **1999**, *160*, 1816.

[46] Hennekes, H.; Asadullah, K. *Current Medicinal Chemistry-Anti-Inflammatory & Anti-Allergy Agents*, **2002**, *1*, 41.

[47] Barnes, P.J. *Annu. Rev. Pharmacol. Toxicol.*, **2002**, *42*, 81.

[48] Ramshaw, H.S.; Woodcock, J.M.; Bagley, C.J.; McClure, C.J.; Hercus, T.R.; Lopez, A.F. *Immunol. Cell Biol.*, **2001**, *76*, 154.

[49] Weiland, S.K.; von Mutius, E.; Husing, A.; Asher, M.I.; *Lancet*, **1999**, *353*, 2040.

[50] Nagakura, T.; Matsuda, S.; Schichijyo, K.; Sugimoto, H.; Hata, K. *Eur. Respir. J.*, **2000**, *16*, 861.

[51] Woods, R.K.; Thien, F.C.; Abramson, M.J.; *Cochrane Database Syst. Rev.*, **2000**, *4*.

[52] Forastiere, F.; Pistelli, R.; Sestini, P.; Fortes, C.; Renzoni, E.; Rusconi, F.; Dell'Orco, V.; Ciccone, G.; Bisanti, L. *Thorax*, **2000**, *55*, 283.

[53] Miller, A.L. *Altern. Med. Rev.*, **2001**, *6*, 20.

[54] Keys, A. *Acta Med. Scand.*, **1980**, *207*, 153.

[55] Tanaka, T.; Kouda, K.; Kotani, M.; Takeuchi, A.; Tabei, T.; Masamoto, Y.; Nakamura, H.; Takigawa, M.; Suemura, M.; Takeuchi, H.; Kouda, M. *J. Physiol. Anthropol.*, **2001**, *20*, 353.

[56] Kotani, M.; Matsumoto, M.; Fujita, A.; Higa, S.; Wang, W.; Suemura, M.; Kishimoto, T.; Tanaka, T. *J. Allergy Clin. Immunol.*, **2000**, *106*, 159.

[57] Hollman, P.C.H.; Katan, M.B. *Free Rad. Res.*, **1999**, *31*, S75.

[58] Harborne, J.B.; Williams, C.A. *Phytochemistry*, **2000**, *55*, 481.

[59] Hodek, P.; Trefil, P.; Stiborova, M. *Chemico-Biological Interactions*, **2002**, *139*, 1.

[60] Fewtrell, C.M.S.; Gomperts, B.D. *Nature*, **1977**, *265*, 635.

[61] Middleton, E. Jr.; Drzewiecki, G.; Krishnarao, D. *J. Immunol.*, **1981**, *127*, 546.

[62] Middleton, E.; Kandaswami, C. *Biochem. Pharmacol.*, **1992**, *43*, 1167.

[63] Cheong, H.; Ryu, S.Y.; Oak, M.H.; Cheon, S.H.; Yoo, G.S.; Kim, K.M. *Arch. Pharm. Res.*, **1998**. *21*, 478.

[64] Middleton, E. Jr.; Kandaswami, C.; Theohardes, T.C. *Pharmacol. Rev.*, **2000**, *52*, 673.

[65] Lee, T-P.; Matteliano, M.L.; Middleton, E. *Life Sci.*, **1982**, *31*, 2765.

[66] Gil, B.; Sanz, M.J.; Terencio, M.; Ferrandiz, M.L.; Bustos, G.; Paya, M.; Gunasegaran, R.; Alcaraz, M.J. *Life Sci.*, **1994**, *54*, 333.

[67] Yoshimoto, T.; Furukawa, M.; Yamamoto, S.; Horie, T.; Watanabe-Kohno, S. *Biochem. Biophys. Res. Commun.*, **1983**, *116*, 612.

[68] Kotani, M.; Fujita, A.; Tanaka T. *J. Jpn. Soc. Nutr. Food Sci.*, **1999**, *52*, 147.

[69] Paul, W.E.; Seder, R.A.; Plaut, M. *Adv. Immunol.*, **1993**, *53*, 1.

[70] Rao, A.; Luo, C.; Hoga, P.G. *Annu. Rev. Immunol.*, **1997**, *15*, 707.

[71] Nishio, H.; Naitoh, E.; Iwashima, A.; Umezawa, K. *Experimentia*, **1984**, *40*, 184.

[72] Matsuda, H.; Watanabe, N.; Geba, G.P.; Tsudzuki, M.; Hiroi, J.; Matsumoto, M.; Ushio, H.; Saito, S.; Askenase, P.W.; Ra, C. *Int. Immunol.*, **1997**, *9*, 461.

[73] Matsumoto, M.; Kotani, M.; Fujita, A.; Higa, S.; Kishimoto, T.; Suemura, M.; Tanaka, T. *Brit. J. Dermatol.*, **2002**, *146*, 221.

[74] Kuhnan, J. *World Rev. Nutr. Diet*, **1976**, *24*, 117.

[75] Hertog, M.G.L.; Hollman, P.C.H.; Katan, M.B.; Kromhout, D. *Nutr. Cancer*, **1993**, *20*, 21.

[76] Hertog, M.G.L.; Hollman, P.C.H.; Katan, M.B. *J. Agric. Food Chem.*, **1992**, *40*, 2379.

[77] Hertog, M.G.L.; Hollman, P.C.H.; Putte, B. *J. Agric. Food Chem.*, **1993**, *41*, 1242.

[78] Lampe, J.W. *Am. J. Clin. Nutr.*, **1999**, *70*, 475S.

[79] Jankun, J.; Selman, S.H.; Swieroz, R.; Jankun, E.S. *Nature*, **1997**, *387*, 561.

[80] Cao, Y.; Cao, R. *Nature*, **1999**, *398*, 381.

[81] Butland, B.K.; Strachan, D.P.; Anderson, H.R. *Eur. Respir. J.*, **1999**, *13*, 744.

[82] La Vecchia, C.; Decarli, A.; Pagano, R. *Epidemiology*, **1998**, *9*, 208.

[83] Shaheen, S.O.; Sterne, J.A.C.; Thompson, R.L.; Songhurst, C.E.; Margetts, B.M.; Burney, P.G.J. *Am. J. Respir. Crit. Care Med.*, **2001**, *164*, 1823.

[84] Kalliomaki, M.; Salminen, S.; Arvilommi, H.; Kero, P.; Koskinen, P.; Isolauri, E. *Lancet*, **2001**, *357*, 1076.

[85] Kopp-Hoolihan, L. *J. Am. Diet Assoc.*, **2001**, *101*, 229.

[86] Ziment, I.; Tshkin, D.P. *J. Allergy Clin. Immunol.*, **2000**, *106*, 603.

Clin Exp Allergy 2003; 33:1097–1102

Association between interleukin-18 gene polymorphism 105A/C and asthma

S. Higa*, T. Hirano*, M. Mayumi†, M. Hiraoka†, Y. Ohshima†, M. Nambu‡, E. Yamaguchi§, N. Hizawa§, N. Kondo¶, E. Matsui¶, Y. Katada‖, A. Miyatake**, I. Kawase* and T. Tanaka*

*Department III of Internal Medicine, Osaka University Medical School, Osaka, †Department of Pediatrics, Fukui Medical University School of Medicine, Fukui, ‡Department of Pediatrics, Tenri Hospital, Nara, §First Department of Medicine, Hokkaido University School of Medicine, Sapporo, ¶Department of Pediatrics, Gifu University School of Medicine, Gifu, ‖Department of Allergy, Osaka Minami National Hospital, Osaka, and **Miyatake Asthma Clinic, Osaka, Japan

Summary

Background IL-18 has been shown to exert anti-allergic or allergy-promoting activities, but the existence of genetic polymorphisms in the coding regions of IL-18 gene has not been demonstrated.

Objective The aim of this study was to investigate whether polymorphism is present in the coding regions of the IL-18 gene and, if so, to further analyse the association between polymorphism and asthma in a case–control study.

Methods We screened the coding regions of the IL-18 gene for polymorphisms by using PCRsingle-stranded conformation polymorphism and direct sequencing of PCR products, followed by analysis of the association between polymorphism and asthma.

Results We identified one polymorphism (105A/C) in the coding regions. The frequency of the 105A allele was significantly higher in asthmatic patients than in controls ($P<0.01$; odds ratio (OR) = 1.83 (1.37–2.26)). Significant linkage disequilibrium was observed between the 105A/C and −137G/C polymorphisms in the 5′ flanking region of the IL-18 gene ($D=0.58$, $P<0.0001$). However, in asthmatic patients the 105A allele was not associated with either total serum IgE or IL-18 levels.

Conclusion The 105A/C polymorphism of the IL-18 gene may be associated with the pathogenesis of asthma.

Keywords asthma, IL-18, polymorphism

Submitted 5 August 2002; revised 15 January 2003; accepted 3 May 2003

Introduction

The IFN-γ-inducing cytokine IL-18 has been shown to exert innate as well as cellular immunity through both NK activation and Th1 induction by acting on the responding cell population [1–4]. It also has an inhibitory effect on IgE synthesis by directly acting on B cells when stimulated with IL-12 through the induction of endogenous IFN-γ [5]. In contrast, it was found that IL-18 directly stimulates IL-4 and histamine release from basophils [6], IL-13 production by NK and T cells [7], induces naïve T cells to differentiate into Th2 cells [8] and enhances IL-8 production by eosinophils [9]. These findings indicate that IL-18 has both anti-allergic and allergy-promoting effects [10].

Excess of IL-18 production was recently found in patients with allergic diseases, including asthma and atopic dermatitis [11–15]. Since IL-18 also increased in patients with other diseases in which a predominance of Th1 cells may play an important role [16–20], it remains to be clarified whether IL-18 has a positive or negative function at the onset or development of allergic diseases. In order to identify the genetic determinants for asthma, we examined whether or not polymorphisms occur in the coding regions of the IL-18 gene and, if so, what the relation is between polymorphism and asthma. In fact, one silent polymorphism (A/C) located at the 105th nucleotide initiating from ATG was identified in the coding regions of the IL-18 gene. The frequency of this polymorphism in controls significantly differed from that in patients with asthma, indicating an association of IL-18-105A/C with asthma.

Correspondence: Toshio Tanaka, Department III of Internal Medicine, Osaka University Medical School, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan. E-mail: ttanak@imed3.med.osaka-u.ac.jp

Materials and methods

Subjects

A total of 221 asthmatic children 1–18 years of age, 276 asthmatic adults 18–78 years old and 85 adult controls without any past or current allergic diseases, including asthma, atopic dermatitis and pollen's diseases, were enrolled in the study. Asthma was diagnosed on the basis of the American Thoracic Society guidelines. Classification of asthma severity was judged by global strategy for asthma management and prevention. Four hundred and seven asthmatic patients showed total IgE concentrations above 200 IU/mL or specific sensitization to major allergens such as

house dust mite, dog and cat, and classified into atopic type of asthma. A full verbal and written explanation of the study was given to the parents of the asthmatic children or asthmatic adults and written informed consent was obtained. The study was approved by the relevant ethical committees in accordance with the guidelines for the management of genetic studies.

Screening for polymorphisms in the coding regions of the IL-18 gene

Total RNA was extracted from peripheral blood mononuclear cells by using RNAzol (Biotecx, Houston, TX, USA) according to the manufacturer's instructions. The extracted RNA was then dissolved in DEPC water and its concentration was measured with a spectrometer. The coding region of the IL-18 gene spanning exons 3–7 was amplified by RT-PCR according to the manufacturer's protocol (Perkin-Elmer Cetus Co., Norwalk, CT, USA). The cDNA was amplified by PCR in the presence of 180 kBq (α-^{32}P)-dCTP (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK). PCR conditions were as follows: 94 °C, 1 min; 63 °C, 1 min; and 72 °C, 1 min for 36 cycles. The primers used for amplification of the coding region of IL-18 spanning exons 3–7 were: sense, ATG-GCT-GCT-GAA-CCA-GTA-GA; anti-sense, AGC-TAG-TCT-TCG-TTT-TGA-ACA-G. The product was then digested with the *Msp*I restriction enzyme (Toyobo Co., Osaka, Japan) at 37 °C for 4 h and examined for the presence of polymorphisms by single-stranded conformation polymorphism (SSCP) analysis [21].

DNA sequencing

PCR products were purified from the gel and DNA sequencing was performed according to the protocol for the dye terminator cycle sequencing FS ready reaction kit (Applied Biosystems, Osaka, Japan). Briefly, the amplification of the coding region of IL-18 cDNA for sequencing was performed in 25 cycles of denaturation for 10 s at 96 °C, annealing for 5 s at 50 °C and extension for 4 min at 60 °C by using the dye terminator cycle sequencing FS ready reaction kit (ABI) and the 2400 Thermal cycle and 377 sequencer (Perkin-Elmer Cetus).

Genotyping

Genomic DNA was extracted from peripheral blood leucocytes by means of the QIA prep Spin (Qiagen Gmbh, Hilden, Germany). For the PCR, 100 ng of DNA was used, and for the detection of IL-18-105A/C polymorphism, primers (sense, TGT-TTA-TTG-TAG-AAA-ACC-TGG-AAT-T; anti-sense, CCT-CTA-CAG-TCA-GAA-TCA-GT) were designed. The partial coding region of the IL-18 gene was then amplified by PCR according to the manufacturer's protocol. PCR conditions were as follows: 94 °C, 1 min; 50 °C, 1 min; and 72 °C, 1 min for 40 cycles. The PCR product was digested with *Taq*I restriction enzyme (Toyobo) at 65 °C for 2 h and electrophoresed on the PAGE gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). The gel was then stained with ethidium bromide and photographed. In order to examine the promoter regions, −607C/A and −137G/C for polymorphisms, previously reported methods and primers were employed [22]. The genotype of β2-adrenergic receptor (β2-ADR)-16 Arg/Gly was determined by using primers (sense, CGG-GAA-CGG-CAG-AGC-CTT-CTT-GCT-GGC-ACC-CAC-T; anti-sense, CCG-TCT-GCA-GAC-GCT-CGA-AC), and the PCR conditions were as follows: 94 °C, 1 min; 60 °C, 1 min; and 72 °C, 1 min for 40 cycles. The PCR product was digested with *Bbv*I (Toyobo) at 37 °C for 2 h.

Measurement of IL-18

Serum concentrations of IL-18 were measured by means of ELISA assays (IL-18 ELISA kit, MBL, Nagoya, Japan).

Statistical analysis

The frequencies of alleles were calculated by counting. Data were analysed with MedCalc and Arlequin software and the Hardy–Weinberg equilibrium was determined be means of the χ^2 goodness-of-fit test. Fisher's exact test was used to detect differences in allele distribution between the groups, and the exact-test-of-population differentiation was used to determine whether the genotypic composition of the groups was significantly heterogeneous. The odds ratio (OR) was calculated by means of logistic regression, and the confidence interval (CI) was calculated at the 95% level. The maximum likelihood estimates of relative linkage disequilibriums between 105A/C and −607C/A or −137G/C were calculated according to the methods of Thompson et al. [23]. Statistical significance was assumed for *P*-values less than 0.05.

Results

A novel polymorphism, 105A/C, in the coding regions of the IL-18 gene

It has been recently shown that IL-18 concentrations in the sera from patients with asthma and atopic dermatitis are elevated [11–15]. As shown in Fig. 1, we could confirm that the serum IL-18 levels for the patients with asthma were higher than for controls. Specifically, the average level of

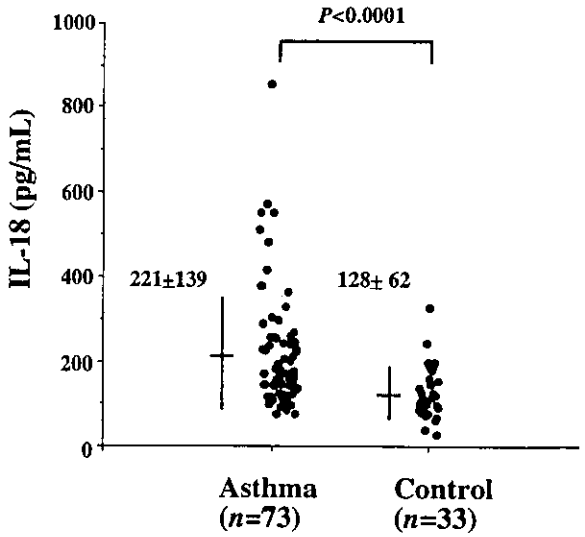


Fig. 1. Increased serum levels of IL-18 in patients with asthma.

serum IL-18 for patients with asthma was 221 pg/mL, which was significantly higher than for the controls (128 pg/mL) ($P<0.0001$). In order to determine whether this up-regulation of IL-18 levels in asthmatic patients was associated with genetic polymorphisms or whether IL-18 might play a positive or negative role at the onset of asthma, we examined the coding regions of the IL-18 gene for the existence of polymorphisms. The translational coding region spanning exons 3–7 was amplified through RT-PCR of 30 different samples. The existence of polymorphisms was then verified by SSCP analysis. Fig. 2 shows the results. A predominant band was obtained from most of the samples, as well as another, more slowly migrating band from some of them. The sequence analysis of the PCR products identified a novel polymorphism (A/C) at the 105th site counting from ATG, but this polymorphism was silent.

IL-18-105A allele was associated with asthma

Next, the frequency of this polymorphism was studied in 497 asthmatic patients and 85 controls by PCR as shown in Fig. 2. Table 1 demonstrates that the frequency of the A allele for patients with asthma was 0.891, which was significantly higher than that for controls (0.818) ($P<0.01$). The OR of carrying one of the two A alleles vs. none was calculated as 1.83. This OR increased substantially to 1.92 for the data from asthmatic children (Table 2). This result demonstrates that the IL-18-105A/C polymorphism is associated with asthma. In order to exclude that this significance is due to the chance of population admixture, allele frequencies of β2-ADR-16-Arg/Gly were measured. The frequency of Arg allele for patients with asthma was similar to that from controls. We also examined the association of IL-18-105A/C polymorphism with the current severity of asthma and with atopic status. However, we failed to observe an association between the genotype and severity or atopic status (Table 2).

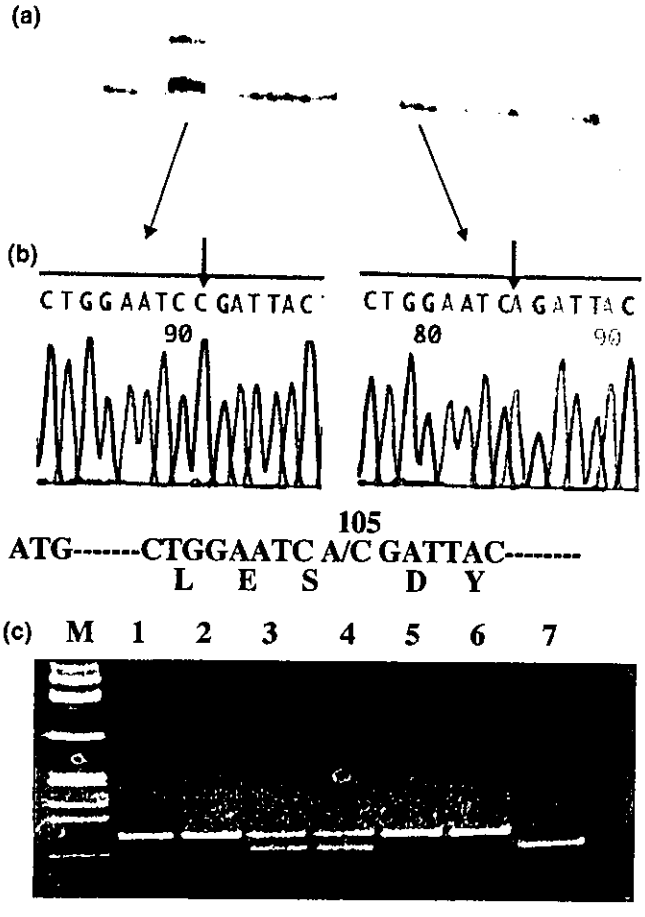


Fig. 2. IL-18-105A/C polymorphism RT-PCR-SSCP analysis of coding regions of the IL-18 gene. The coding region spanning exons 3–7 was amplified by RT-PCR and then digested with *MspI*. The products were then electrophoresed. Direct sequencing of the PCR products determined the existence of polymorphism located at the 105th site, A/C. Genotyping of IL-18-105A/C by RFLP-PCR.

Linkage disequilibrium between 105A/C and –137G/C promoter polymorphism

Polymorphisms in the promoter region of the IL-18 gene have recently been identified [22]. The changes from C to A at position –607 (– 607C/A) and from G to C at position –137 (– 137G/C) were found to affect the transcription of the IL-18 gene. We next tried to determine whether there was a linkage disequilibrium between the coding polymorphism 105A/C and – 607C/A or –137G/C. Table 3 shows the pairwise analysis of the polymorphisms, indicating a significant disequilibrium between 105A/C and –137G/C, and between 105A/C and –607C/A. A linkage disequilibrium between 105A/C and –137G/C ($D=0.575$, $P<0.0001$) was more significantly observed than that between 105A/C and –607C/A ($D=0.283$, $P=0.0034$). Finally, we verified the association of the genotype of 105A/C with serum IgE levels and IL-18 levels for asthmatic patients, but as shown in Table 4, no significant relation could be detected.

Discussion

In this study, we identified one polymorphism, 105A/C, in the coding regions of the IL-18 gene and demonstrated the association between this polymorphism and asthma. The OR was 1.83 for the asthmatic phenotype when the allele was A. Case–control studies are sometimes susceptible to the population admixture, but the frequency of Arg in patients with asthma was similar to that from controls. These results suggest that IL-18 may play a role in the pathogenesis of asthma, although it remains to be determined as to how the polymorphism influences the pathogenesis. The linkage disequilibrium between 105A and – 137G suggests that the 105A allele may be associated with the up-regulation of IL-18

Table 1. Allele and genotype frequencies of IL-18-105A/C polymorphisms in patients with asthma and controls

Allele	Asthma ($n=497$)	Control ($n=85$)	Genotype	Asthma ($n=497$)	Control ($n=85$)
A	0.891	0.818	AA	0.792	0.682
C	0.109	0.182	AC	0.197	0.271
$P=0.0062$			CC	0.010	0.047
			$P=0.0089$		

Table 2. Allele frequencies of IL-18-105A/C and β2-ADR-16-Arg/Gly in asthmatic adults and children (upper panel) and allele frequencies of IL-18-105A/C in asthmatic patients classified by severity and atopics (lower panel)

Allele	Control	Asthma	Adult	Children
IL-18-105A/C				
A	139	886	490	396
C	31	108	62	46
Odds ratio		1.83	1.76	1.92
(CI)		(1.37–2.26)	(1.10–2.82)	(1.18–3.10)
P-value		0.0062	0.0172	0.0089
β2-ADR-16				
Arg	71	484	269	215
Gly	81	506	283	223
Odds ratio		1.09	1.08	1.10
(CI)		(0.78–1.53)	(0.75–1.55)	(0.76–1.59)
P-value		0.6169	0.6588	0.6135

Table 2a

		Severity of asthma			Type of asthma	
Allele	Control	Intermittent	Mild to moderate	Severe	Atopics	Non-atopics
A	139	523	206	77	722	164
C	31	64	26	9	92	16
Odds ratio		1.82	1.76	1.91	1.75	2.29
(CI)		(1.43–2.31)	(1.01–3.08)	(0.87–4.18)	(1.11–2.75)	(1.20–4.37)
P-value		0.0110	0.0460	0.1058	0.0129	0.0104

Table 3. Combined genotype frequencies of IL-18-105A/C, IL-18-137G/C and IL-18-607C/A

	105A/C			
	A/A	A/C	C/C	
– 137G/C				
G/G	59	0	0	$P<0.0001$
G/C	1	17	1	$n=84$
C/C	0	0	6	$D=0.575$
– 607C/A				
C/C	16	0	0	$P=0.0034$
C/A	28	10	1	$n=83$
A/A	15	7	6	$D=0.283$

Linkage disequilibrium between 105A/C and – 137G/C was more significantly observed than that between 105A/C and – 607C/A.

Table 4. Serum levels of IgE and IL-18 from patients with asthma

	IgE (IU/mL)	IL-18 (pg/mL)
Genotype		
A/A	869+1852 ($n=360$)	220±148 ($n=57$)
A/C	1176±2973 ($n=87$)	224±102 ($n=16$)
C/C	1274±1176 ($n=4$)	ND

Serum levels of IgE and IL-18 were compared with the IL-18-105A/C genotype.
Results are indicated as means±SD. There was no significant difference.
ND = Not determined.

expression through the –137G allele, although we did not find an increase in the serum IL-18 levels of asthmatic patients with the AA haplotype of the 105 polymorphism. A previous report regarding polymorphisms in the promoter region showed that the –137G allele enhanced the transcriptional activity of the IL-18 gene when cells were stimulated with PMA plus ionomicin, but that there were no significant differences in promoter activity between –137G and –137C alleles in the absence of stimulants [22]. Alternatively, it is possible that 105A/C, although quiescent, directly affects the modulation of transcription of the gene or RNA stabilization [24] or that 105A/C is linked to another polymorphism, which alters the functional property of IL-18 or its gene regulation. Further studies are required to clarify these points.

Recently, several lines of *in vitro* and *in vivo* studies have generated contradictory results about the function of IL-18 in the pathophysiology of allergic diseases [10]. The activities of IL-18, including induction of IFN-γ synthesis by T cells or NK cells and of Th1 induction [1–3] as well as inhibition of IgE production by B cells when expressed together with IL-12 [5], show that this cytokine may be a negative regulator in view of the pathogenesis of allergic diseases. However, IL-18 has also been reported to induce the release of both histamine and IL-4 from basophils [6], and cause Th2 differentiation [8]. The effect of IL-18 on the asthma mouse model thus seems to be paradoxical. For instance, administration of IL-18 was found to enhance Ag-induced eosinophils recruitment into the trachea and bronchoalveolar lavage fluid of sensitized mice *in vivo* [25], and to increase Th2 cytokines and airway eosinophilia in a mouse model of allergic asthma [26]. In

contrast, coadministration of IL-12 and IL-18 was shown to inhibit antigen-induced airway hyper-responsiveness, eosinophilia and serum IgE levels [27], and IL-18 deficiency to cause enhancement of allergen-induced eosinophilia accumulation [28]. Although the reasons for such contradictory results remain to be determined, it is likely that they are related to the responding cells or to whether or not IL-18 together with IL-12 acts on the cells. On the other hand, changes in IL-18 properties or expression through genetic polymorphisms could lead to the exacerbation or suppression of allergic responses.

Polymorphisms in candidate genes have been identified and associated with asthma [29, 30]. The IL-18 gene is located at chromosomal region 11q23 [31]. Previous studies of genome-wide research for asthma have demonstrated that the region of 11q21–24 was linked to asthma phenotype, specific IgE to a group of common aeroallergens and positive skin test to house dust mite [32, 33]. Candidate genes in this region include matrix metalloproteinase genes 1, 3 and 8, and the δ, ε and γ subunits of CD3. Moreover, the newly identified IL-18-105A/C polymorphism and/or the previously identified IL-18-137G/C polymorphism can be considered to be one of such polymorphisms associated with asthma. We expect that our observations are going to contribute to an understanding of the pathophysiology of asthma and result in the establishment of predictive markers for asthma.

Acknowledgements

This investigation was supported by the Pollution-related Health Damage Compensation and Prevention Association of Japan, Ministry of Education, Science and Culture of Japan, the Ministry of Health and Welfare of Japan.

References

1 Okamura H, Tsutsui H, Komatsu T et al. Cloning of a new cytokine that induces INF-γ production by T cells. Nature 1995; 378:88–91.
2 Tsutsui H, Nakanishi K, Higashino K, Okamura H, Miyazawa Y, Kaneda K. IFN-γ-inducing factor upregulates Fas ligand-mediated cytotoxic activity of murine natural killer cell clones. J Immunol 1996; 156:3967–73.
3 Okamura H, Tsutsui H, Kashiwamura S, Yoshimoto T, Nakanishi K. Interleukin-18 (IL-18) a novel cytokine that augments both innate and acquired immunity. Adv Immunol 1998; 70:281–312.
4 Dinarello CA. IL-18: a TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. J Allergy Clin Immunol 1999; 103:11–24.
5 Yoshimoto T, Okamura H, Tagawa A, Nakanishi K. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-γ production from activated B cells. Proc Natl Acad Sci USA 1997; 94:3948–53.
6 Yoshimoto T, Tsutsui H, Tominaga K et al. IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. Proc Natl Acad Sci USA 1999; 96:13962–6.
7 Hoshino T, Wiltrout RH, Young HA. IL-18 is a potent coinducer of IL-13 in NK and T cells: a new potential role for IL-18 in modulating the immune response. J Immunol 1999; 162:5070–7.
8 Yoshimoto T, Mizutani H, Tsutsui H et al. IL-18 induction of IgE: dependence on $CD4^+$ T cells, IL-4 and STAT6. Nature Immunol 2000; 1:132–7.
9 Wang W, Tanaka T, Okamura H et al. Interleukin-18 enhances the production of interleukin-8 by eosinophils. Eur J Immunol 2001; 31:1010–6.
10 Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. Annu Rev Immunol 2001; 19:423–4.
11 Tanaka H, Miyazaki N, Oashi K et al. IL-18 might reflect disease activity in mild and moderate asthma exacerbation. J Allergy Clin Immunol 2001; 107:331–6.
12 Wong CK, Ho CY, Ko FWS et al. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-γ, IL-4, IL-10 and IL-13) in patients with allergic asthma. Clin Exp Immunol 2001; 125:177–83.
13 El-Mezzein REH, Matsumoto T, Nomiyama H, Miike T. Increased secretion of IL-18 *in vitro* by peripheral blood mononuclear cells of patients with bronchial asthma and atopic dermatitis. Clin Exp Immunol 2001; 126:193–8.
14 Tanaka T, Tsutsui H, Yoshimoto T et al. Interleukin-18 is elevated in the sera from patients with atopic dermatitis and from atopic dermatitis-model mice, NC/Nga. Int Arch Allergy Immunol 2001; 125:236–40.
15 Higashi N, Gesser B, Kawana S, Thestrup-Pedersen K. Expression of IL-18 mRNA and secretion of IL-18 are reduced in monocytes from patients with atopic dermatitis. J Allergy Clin Immunol 2001; 108:607–14.
16 Yamada G, Shijubo N, Shigehara K, Okamura H, Kurimoto M, Abe S. Increased levels of circulating interleukin-18 in patients with advanced tuberculosis. Am J Respir Crit Care Med 2000; 161:1786–9.
17 Shigehara K, Shijubo N, Ohmichi M et al. Increased levels of interleukin-18 in patients with pulmonary sarcoidosis. Am J Respir Crit Care Med 2000; 162:1979–82.
18 Kanai T, Watanabe M, Okazawa A et al. Interleukin 18 is a potent proliferative factor for intestinal mucosal lymphocyte in Crohn's disease. Gastroenterology 2000; 119:1514–23.
19 Nicoletti F, Di Marco R, Mangano K et al. Increased serum levels of interleukin-18 in patients with multiple sclerosis. Neurology 2001; 57:342–4.
20 Kawashima M, Yamamura M, Taniai M et al. Levels of interleukin-18 and its binding inhibitors in the blood circulation of patients with adult-onset Still's disease. Arthritis Rheum 2001; 44:550–60.
21 Hayashi K. PCR-SSCP: a method for detection of mutations. Genet Anal Tech Appl 1992; 9:73–9.
22 Giedraitis V, He B, Huang W-X, Hillert J. Cloning and mutation analysis of the human IL-18 promoter: a possible role of polymorphisms in expression regulation. J Neuroimmunol 2001; 112:146–52.
23 Thompson EA, Deeb S, Walker D, Motulsky AG. The detection of linkage disequilibrium between closely linked markers: RFLPs at the AI-CIII apolipoprotein genes. Am J Hum Genet 1988; 42: 113–24.
24 Du YZ, Dickerson C, Aylsworth AS, Schwartz CE. A silent mutation, C924T (G308G), in the L1CAM gene results in X linked hydrocephalus (HSAS). J Med Genet 1998; 35:456–62.
25 Kumano K, Nakao A, Nakajima H et al. Interleukin-18 enhances antigen-induced eosinophil recruitment into the mouse airways. Am J Respir Crit Care Med 1999; 160:873–8.
26 Wild JS, Sigounas A, Sur N et al. IFN-gamma-inducing factor (IL-18) increases allergic sensitization, serum IgE, Th2 cytokines, and airway eosinophilia in a mouse model of allergic asthma. J Immunol 2000; 164:2701–10.
27 Hofstra CL, Van Ark I, Hofman G, Kool M, Nijkamp FP, Van Oosterhout AJ. Prevention of Th2-like cell responses by coadministration of IL-12 and IL-18 is associated with inhibition of antigen-induced airway hyperresponsivesess, eosinophilia, and serum IgE levels. J Immunol 1999; 161:5054–60.

28 Kodama T, Matsuyama T, Kuribayashi K et al. IL-18 deficiency selectively enhances allergen-induced eosinophilia in mice. J Allergy Clin Immunol 2000; 105:45–53.
29 Cookson WOCC. Asthma genetics. Chest 2002; 121:7S–13S.
30 Palmer LJ, Cookson WOCM. Using single nucleotide polymorphisms as a means to understanding the pathophysiology of asthma. Respir Res 2001; 2:102–12.
31 Baysal BE, Willett-Brozick JE, Taschner PEM et al. A high-resolution integrated map spanning the SDHD gene at 11q23: a 1.1-Mb BAC contig, a partial transcript map and 15 new repeat polymorphisms in a tumour-suppressor region. Eur J Hum Genet 2001; 9:121–9.
32 Xu J, Meyers DA, Ober C et al. Genomewide screen and identification of gene–gene interactions for asthma-susceptibility loci in three U.S. populations: collaborative study on the genetics of asthma. Am J Hum Genet 2001; 68:1437–46.
33 Koppelman GH, Stine OC, Xu J et al. Genome-wide search for atopy susceptibility genes in Dutch families with asthma. J Allergy Clin Immunol 2002; 109:498–506.

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 (astragalin, 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% [IC_{50}] = 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 (IC_{50} = 5.1 and 6.2 μmol/L, respectively) and IL-4 synthesis by allergen-stimulated basophils from allergic patients (IC_{50} = 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
IC_{50}: 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, astragalin, inhibited histamine release by the human basophilic cell line KU812[20] and that oral admin-

From [a]Department III of Internal Medicine, Osaka University Medical School, Osaka; [b]the Research & Development Center, Sunstar Incorporation, Osaka; and [c]Nissay 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.

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 (astragalin and rutin) were purchased from Extrasynthese. 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 (400*g* 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 cells[24] (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; astragalin, 13.44 μg/L; 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 pellets by using the RNAzol 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′.

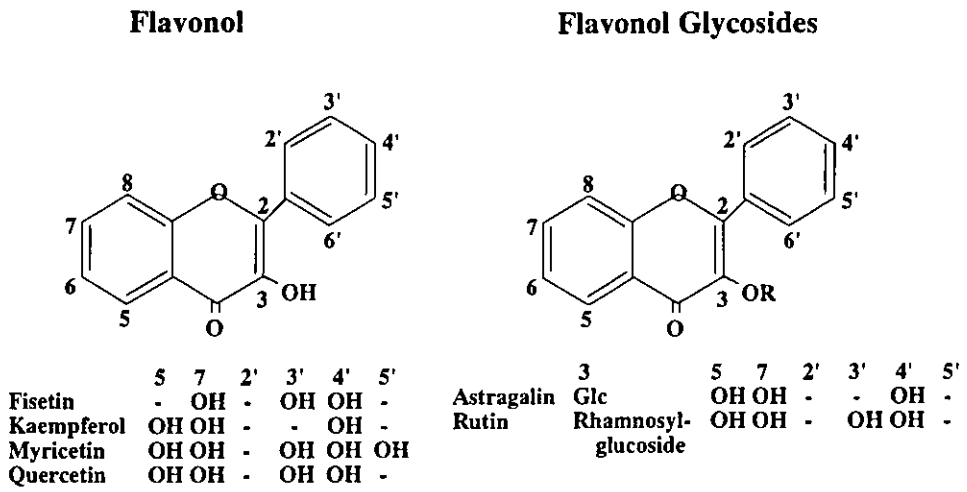
The PCR process was performed with a Perkin-Elmer Cetus DNA Thermal Cycler, and the durations and temperatures were as follows: for IL-4 and IL-13 transcripts, 2 minutes at 94°C and then 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C for 38 cycles for KU812 or 24 cycles for basophils; for IL-5, 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



	5	7	2'	3'	4'	5'
Fisetin	-	OH	-	OH	OH	-
Kaempferol	OH	OH	-	-	OH	-
Myricetin	OH	OH	-	OH	OH	OH
Quercetin	OH	OH	-	OH	OH	-

	3	5	7	2'	3'	4'	5'
Astragalin	Glc	OH	OH	-	-	OH	-
Rutin	Rhamnosyl-glucoside	OH	OH	-	OH	OH	-

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 [γ^{32}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 T_H2-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-1β 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 μmol/L (but not at 3 μmol/L), as shown in Fig 2, *A*, inhibited IL-4, IL-5, and IL-13 mRNA expression, whereas IL-6, IL-8, and IL-1β 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 FcεRI of KU812 cells with mouse anti-human FcεRIα 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 μmol/L. Four independent experiments revealed that the inhibitory concentration of 50% (IC_{50}) of fisetin was 19.1 ± 4.9 μmol/L for IL-4,

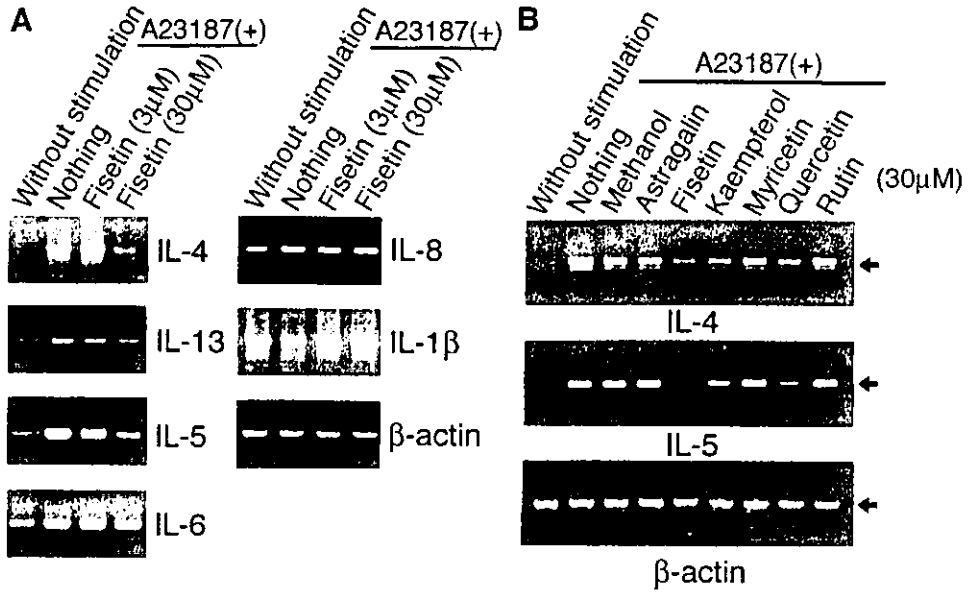


FIG 2. Fisetin inhibits the expression of IL-4, IL-13, and IL-5 mRNAs in KU812 cells. **A**, The cells (5×10^5 cells) were first incubated without *(nothing)* or with fisetin (3 or 30 μmol/L) for 30 minutes and stimulated with A23187 (1 μmol/L) or an equivalent amount of diluted DMSO *(without stimulation)* for 3 hours. RNAs were obtained, and the expression of IL-4, IL-13, IL-5, IL-6, IL-8, IL-1β, 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 μmol/L) or an equivalent amount of diluted methanol *(Methanol)* for 30 minutes and stimulated with A23187 (1 μmol/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 ± 38	320 ± 155	<1.5
A23187 + PMA	3298 ± 299	113 ± 15	21,170 ± 5998	12,951 ± 1707	16.5 ± 4.5
A23187 + PMA + fisetin (3 μmol/L)	2843 ± 1457	100 ± 1	21,199 ± 680	12,173 ± 1762	14.9 ± 1.7
A23187 + PMA + fisetin (30 μmol/L)	171 ± 126†	5.3 ± 0.4‡	21,592 ± 1837	14,520 ± 1051	2.0 ± 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 ± SD.

*Femtograms per milliliter.

†$P < .05$.

‡$P < .01$.

17.7 ± 8.1 μmol/L for IL-5, and 17.4 ± 10.7 μmol/L for IL-13 and that the IC_{50} of other flavonoids, including kaempferol and quercetin, was greater than 30 μmol/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 (IC_{50} = 4.8 ± 0.4 μmol/L; n = 3) or anti-IgE antibody stimulation (IC_{50} = 5.1 ± 2.1 μmol/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 (IC_{50} = >30 μmol/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

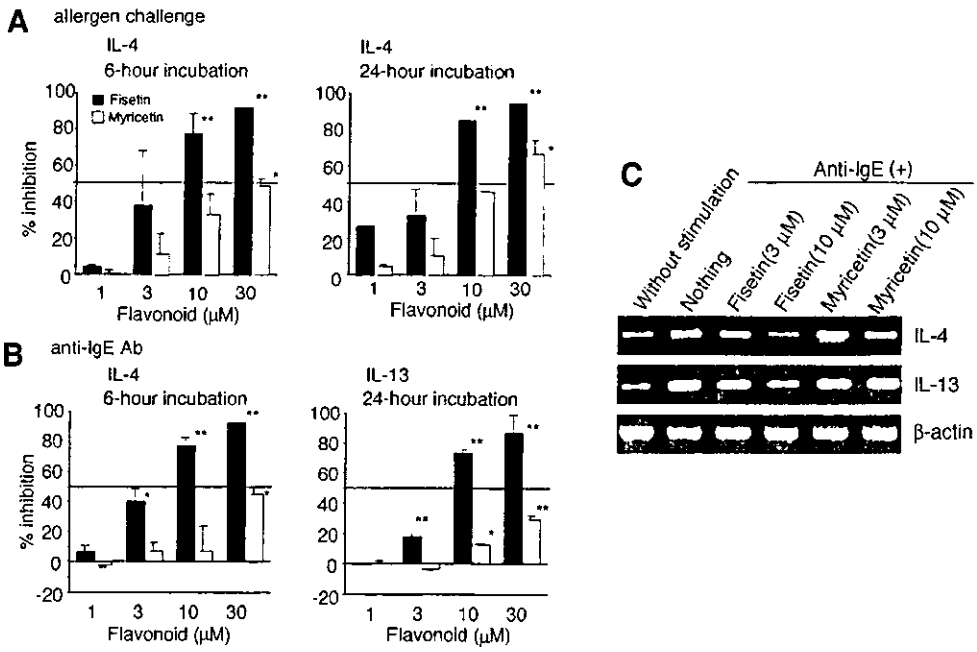


FIG 3. Fisetin inhibits IL-4 and IL-13 mRNA expression and synthesis by purified basophils. **A,** Purified human basophils (0.2 to 1 × 10^6 cells/mL) were first incubated with fisetin or myricetin at a dose of 1 to 30 μmol/L or an equivalent amount of diluted methanol for 30 minutes and further stimulated 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 μmol/L or an equivalent amount of diluted methanol for 30 minutes and further stimulated with anti-IgE antibody (1 μg/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 μmol/L for 30 minutes and further stimulated with anti-IgE antibody (1 μg/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 ± SD from 3 different donors. $*P < .05$; $**P < .01$.

IL-13 synthesis, and fisetin showed a similar inhibition on IL-13 production (IC_{50} = 6.2 ± 0.2 μmol/L; n = 3). RT-PCR could be used to detect the inhibitory effect of fisetin at a dose of 10 μmol/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