

NARIRUTIN INHIBITS AIRWAY INFLAMMATION IN AN ALLERGIC MOUSE MODEL

Norihiko Funaguchi,* Yasushi Ohno,* Bu Lin Bai La,* Toshihiro Asai,* Hideyuki Yuhgetsu,* Masahiro Sawada,* Genzou Takemura,* Shinya Minatoguchi,* Takako Fujiwara[†] and Hisayoshi Fujiwara*

*Second Department of Internal Medicine, Regeneration and Advanced Medical Science, Graduate School of Medicine, Gifu University, Gifu and [†]Department of Food Science, Kyoto Women's University, Kyoto, Japan

SUMMARY

1. Flavonoids are naturally occurring compounds that possess anti-allergic, anti-inflammatory, antiproliferative and anti-oxidant properties. In the present study, we investigated whether the flavonoid narirutin could reduce airway inflammation in ovalbumin (OVA)-sensitized/challenged NC/Nga mice, a model of allergic eosinophilic airway inflammation.

2. Mice were initially immunized intraperitoneally with OVA on Days 0 and 7 and then challenged with inhaled OVA on Days 14, 15 and 16. In addition, some mice received narirutin orally at doses of 0.1, 1 or 10 mg/kg bodyweight daily on Days 7–16.

3. At 10 mg/kg, but not 0.1 or 1 mg/kg, narirutin significantly diminished OVA-induced airway inflammation caused by infiltration of lung tissue with inflammatory and mucus-producing cells, as well as reduced eosinophil counts in the peripheral blood and bronchoalveolar lavage fluid (BALF), interleukin (IL)-4 levels in BALF and IgE levels in serum.

4. The mechanism of the anti-inflammatory effect of narirutin are likely to be associated with a reduction in the OVA-induced increases of IL-4 and IgE in a murine model of allergic eosinophilic airway inflammation. These findings suggest that narirutin may be an effective new tool in the treatment of bronchial asthma.

Key words: allergic eosinophilic airway inflammation, bronchial asthma, eosinophil, flavonoid, IgE, interleukin-4, narirutin.

INTRODUCTION

Bronchial asthma is a complex disease of the lung characterized by reversible airway obstruction, airway inflammation, excessive mucus production and airway hyperresponsiveness.¹ When an asthma sufferer inhales a specific allergen, a two-phase allergic response occurs. During the early phase of the response, activation of cells

bearing allergen-specific IgE, especially mast cells and macrophages within the airways, causes secretion of bronchoconstrictors such as histamine and cysteinyl leukotrienes. During the late phase of the response, activated airway cells release various cytokines, such as interleukin (IL)-4 and IL-5, and chemokines that stimulate infiltration by inflammatory leucocytes, especially eosinophils. To investigate this asthmatic response in detail, investigators frequently make use of mice subjected to ovalbumin (OVA)-induced allergic airway inflammation, which has proven to be good model of human bronchial asthma.²

Flavonoids are a group of phenolic compounds abundant in fruits, vegetables, nuts and seeds, as well as in beverages such as tea and wine.^{3,4} Differences in the chemical structures of flavonoids lead to variations in their bioavailability, distribution, metabolism and pharmacological properties and many flavonoids are now recognized to possess anti-allergic, anti-inflammatory, antiviral, antiproliferative, anti-oxidant and/or anticarcinogenic properties,^{5,6} but very little toxicity.^{7,8} In that regard, Knekt *et al.*⁹ reported that the incidence of asthma was inversely associated with a higher intake of the flavonoids quercetin, naringenin (aglycone of narirutin) and hesperetin, whereas Das *et al.*¹⁰ reported that some flavonoids (e.g. luteolin) have a therapeutic effect on allergic airway inflammation in the aforementioned murine model of bronchial asthma. In addition, narirutin (naringenin-7-O- β -D-rutinoside), which is abundant in citrus fruits, has anti-allergic properties *in vitro* and has been shown to exert an inhibitory effect on allergic skin reactions in a murine model of atopic dermatitis.¹¹ However, narirutin has not yet been demonstrated to have an inhibitory effect on allergic eosinophilic airway inflammation *in vivo*. With this background, the aim of the present study was to determine whether narirutin is able to inhibit airway inflammation in an allergic mouse model.

METHODS

Animals and experimental protocol

Specific pathogen-free female NC/Nga mice (6–8 weeks old) were purchased from Japan SLC (Sizuoka, Japan). This strain has been used previously as an animal model of human atopic dermatitis¹² and has proven to be a very suitable model for studying allergic asthma characterized by allergic inflammatory responses, including eosinophilic infiltration and allergen-specific IgE production.¹³ Mice were housed in separate cages according to the treatment protocol. Food and water were provided *ad libitum*. All experiments were performed following the *Guidelines for the Care and Use of Experimental Animals* formulated by the Japanese Association for Laboratory Animal Science in 1987 (<http://www.soc.nii.ac.jp/jalas/journal/notes.html>).

Correspondence: Hisayoshi Fujiwara, Second Department of Internal Medicine, Regeneration and Advanced Medical Science, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan. Email: gifuim-gif@umin.ac.jp

Received 9 August 2006; revision 9 January 2007; accepted 24 January 2007.

© 2007 The Authors

Journal compilation © 2007 Blackwell Publishing Asia Pty Ltd

Asthmatic responses were induced using the method of Padrid *et al.*¹⁴ Briefly, mice were immunized initially on Days 0 and 7 by intraperitoneal injection of 100 µg OVA (Grade V; Sigma Chemical, St Louis, MO, USA) plus 1.6 mg aluminium hydroxide in 200 µL of 0.9% sterile saline (Otsuka, Tokyo, Japan). Non-sensitized control mice were injected only with aluminium hydroxide in saline. Sensitized mice were then exposed to an aerosol of OVA (10 mg/mL) in 0.9% saline for 10 min each day on Days 14, 15 and 16. Non-sensitized mice were exposed only to saline. Mice were killed 24 h after the last aerosol challenge.

The OVA-sensitized mice were randomized into four groups ($n = 8$ in each). In the three narirutin groups, mice were administered narirutin (Extrasynthase, Genay, France) orally at doses of 0.1, 1 or 10 mg/kg bodyweight in sterile water (20 µL volume/mouse) from Day 7 to Day 16. The fourth group received only sterile water. In addition, non-sensitized control mice ($n = 8$) were treated with sterile water only.

Cell counts in peripheral blood and bronchoalveolar lavage fluid

Blood samples were collected from the orbital artery after mice were killed on Day 17. Total and differential cell counts were determined by counting cells in heparinized blood samples using a haemocytometer. Bronchoalveolar lavage was performed by instilling 0.5 mL saline into the lungs through a tracheal cannula and then gently aspirating the fluid. This was repeated three times. Total cell counts were determined by counting cells in samples of bronchoalveolar lavage fluid (BALF) using a haemocytometer. In addition, cytospin slides were prepared, fixed and then stained with Diff-Quick solution (American Scientific Products, McGaw Park, IL, USA), after which differential cell counts were obtained by counting a minimum of 300 cells/slide and using standard morphological criteria.

Measurement of serum IgE and BALF cytokines

Blood samples obtained from the orbital artery immediately after mice had been killed on Day 17 were centrifuged at 2000 g for 10 min at room temperature and the serum was separated and stored at -80°C until analysed. An enzyme-linked immunosorbent assay (ELISA) specific for total IgE was performed as described previously¹⁵ using a commercially available ELISA kit (Mouse IgE Quantitation Kit; Bethyl, Montgomery, TX, USA). The lower limit of detection for total IgE was 9.75 ng/mL. The BALF collected as described above was centrifuged at 3000 g for 10 min at 4°C , after which the supernatant was frozen immediately for later measurement of IL-4 and IL-5 using murine ELISA kits (Biosource, Camarillo, CA, USA). The lower limit of detection for both cytokines was 15.6 pg/mL.

Lung histology

After mice had been killed by anaesthesia with 30 mg/kg, i.v., sodium pentobarbital on Day 17, the lungs were fixed by injection of 10% buffered formalin into the trachea at a pressure of 25 cmH₂O. Lung tissues from the lower and upper lobes of the left lung were embedded in paraffin, cut into 4 µm sections using a microtome and stained with haematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). The degree of inflammation evident in the H&E-stained sections was scored as follows: 0, no signs of inflammation; 1, light or dispersed infiltrate in only a few areas of the section; 2, moderate infiltrate surrounding < 50% of the vessels and airways; and 3, heavy and focused infiltrate surrounding the majority of vessels and airways.¹⁶ Scoring for mucus production in the PAS-stained sections was as follows: 0, no signs of mucus or increased numbers of PAS-positive cells in the airways; 1, no mucus in the airways, but a slight increase in the numbers of PAS-positive cells in a few airways; 2, some mucus detectable in the airways and approximately 50% of airway epithelial cells PAS positive in multiple airways; and 3, several airways are plugged with mucus and most airway epithelial cells are PAS positive in multiple airways per section. Scoring was undertaken by two individuals blinded to the experimental protocol.

Statistical analysis

Data are presented as the mean $\pm$ SD. Multiple comparisons among groups were made using one-way ANOVA with post hoc Tukey's tests. $P < 0.05$ was considered significant.

RESULTS

Effect of narirutin on cell counts in peripheral blood and BALF

We found that eosinophil counts were significantly higher in peripheral blood and BALF in OVA-challenged mice than in saline-challenged control mice. Moreover, administration of 10 mg/kg narirutin significantly reduced eosinophil counts in peripheral blood (Fig. 1a,b) and BALF (Fig. 1c,d) in OVA-challenged mice. In contrast, in this experiment, as well as in those summarized below, 0.1 and 1 mg/kg narirutin had no significant effect on OVA-induced allergic responses.

Effect of narirutin on serum IgE and BALF cytokines

Serum IgE levels also were markedly higher in OVA-challenged mice than in saline-challenged control mice and again the OVA-induced response was significantly diminished following treatment with 10 mg/kg narirutin (Fig. 2a). Levels of IL-4 and IL-5 in BALF collected from saline-challenged mice were below the detectable limit (< 15.6 pg/mL) of our ELISA. In contrast, the levels of both cytokines were significantly elevated in OVA-challenged mice. Notably, administration of 10 mg/kg narirutin completely blocked the OVA-induced increase in IL-4 (Fig. 2b), but had no effect on the increase in IL-5 (Fig. 2c).

Effect of narirutin on lung histology

Histological analysis showed the number of inflammatory cells, comprised mainly of eosin-positive eosinophils located around the airways and vessels and PAS-positive mucus-producing cells within the airways, was markedly higher in OVA-challenged mice (Fig. 3b,g) than in control mice (Fig. 3a,f). Semiquantitative histological scores indicative of the degree of inflammation for the sections of OVA-challenged mice were 2.24 ± 0.23 in H&E-stained sections and 2.43 ± 0.53 in PAS-stained sections, both of which were significantly greater than the scores for the corresponding control sections (0.19 ± 0.12 and 0.03 ± 0.03 , respectively; Fig. 3k). As with the other asthma-like conditions tested, OVA-induced infiltration of the lung tissue by inflammatory cells was significantly attenuated by 10 mg/kg narirutin (Fig. 3e,j) and there was a concomitant reduction in the inflammation scores in H&E- and PAS-stained sections (1.49 ± 0.40 and 1.88 ± 0.48 , respectively; Fig. 3k).

DISCUSSION

Following sensitization, inhalation of OVA induces allergic inflammatory changes characterized by infiltration of inflammatory cells, mainly eosinophils, into regions around the airways and pulmonary blood vessels and by increasing in number of mucus-producing cells within the airways. The main finding in the present study was that the OVA-induced inflammatory changes in lung tissues, as well as the increases in eosinophil counts in both peripheral blood and

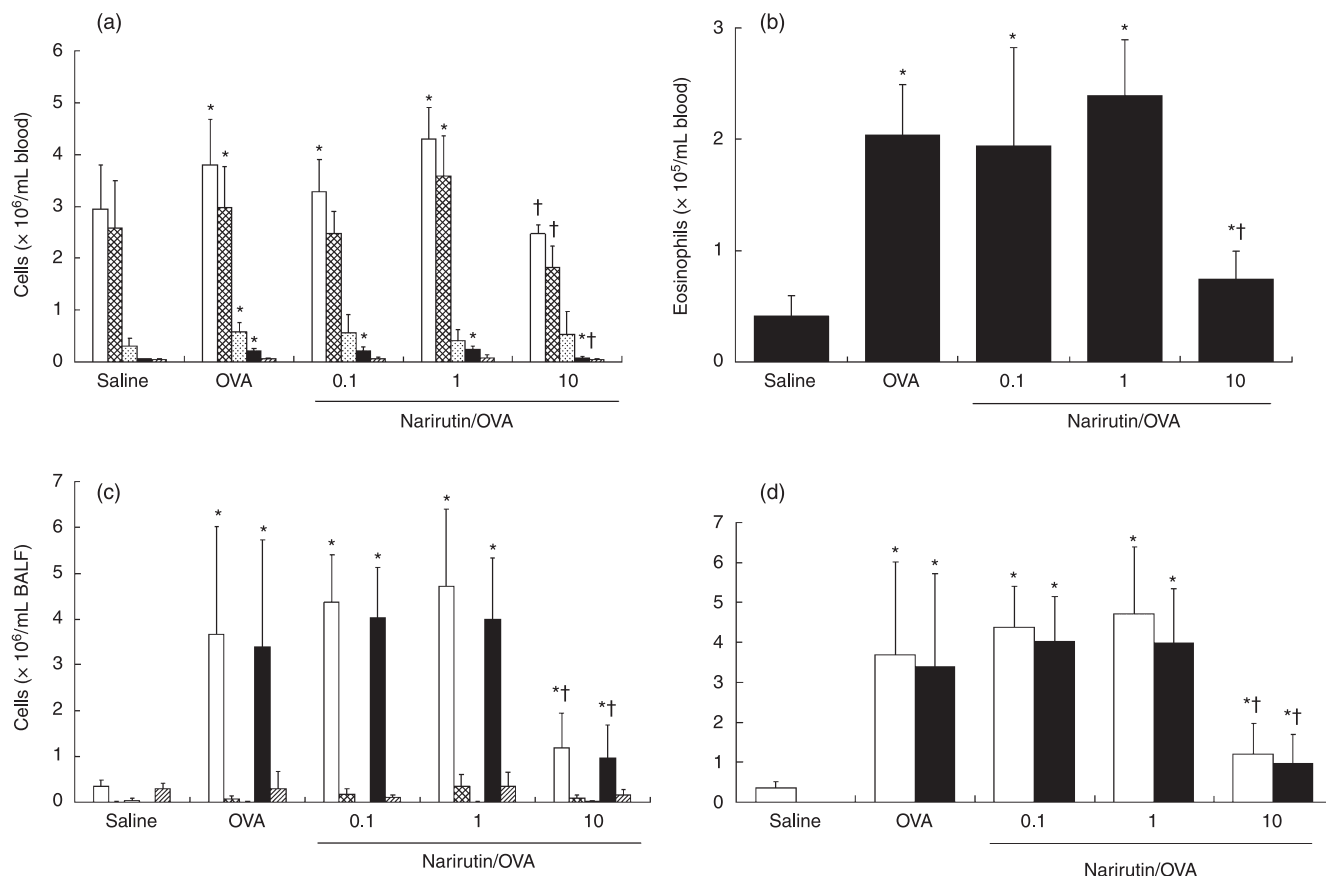


Fig. 1 Effect of treatment with narirutin on ovalbumin (OVA)-induced increases in cell counts in (a,b) peripheral blood and (c,d) bronchoalveolar lavage fluid (BALF). Twenty-four hours after the last aerosol challenge, blood samples and BALF were collected from saline-challenged control mice (Saline) and OVA-challenged mice either untreated (OVA) or treated with 0.1, 1 and 10 mg/kg narirutin (Narirutin/OVA). Total and differential cell counts in peripheral blood were determined using a haemocytometer. (a) Total and differential cell counts in peripheral blood samples. (□), total cells; (▨), lymphocytes; (▩), neutrophils; (■), eosinophils; (▤), monocytes. (b) Eosinophil counts in peripheral blood samples. Total cells were counted in samples of BALF using a haemocytometer. In addition, cytospin slides were prepared, fixed and stained with Diff-Quick solution (American Scientific Products, McGaw Park, IL, USA), after which differential cell counts were measured using standard morphological criteria. (c) Total and differential cell counts in BALF. (□), total cells; (▨), lymphocytes; (▩), neutrophils; (■), eosinophils; (▤), macrophages. (d) Eosinophil (■) and total cell (□) counts in BALF. Treatment with 10 mg/kg narirutin significantly reduced eosinophil counts in both peripheral blood and BALF in OVA-challenged mice. Data are the mean $\pm$ SD ($n = 8$ per group). * $P < 0.05$ compared with the saline-challenged control group; † $P < 0.05$ compared with the OVA-challenged group.

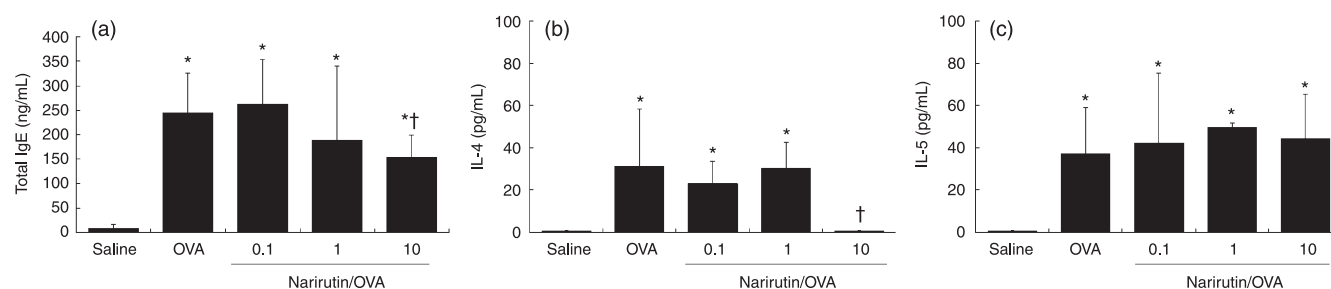


Fig. 2 Effect of treatment with narirutin on ovalbumin (OVA)-induced increases in serum IgE and cytokine levels in bronchoalveolar lavage fluid (BALF). (a) Using an ELISA, total IgE levels were measured in serum samples obtained from blood collected on Day 17. Treatment with 10 mg/kg narirutin (Narirutin/OVA) reduced the increases in total IgE levels in serum. (b,c) Using specific ELISAs, interleukin (IL)-4 (b) and IL-5 (c) levels were measured in samples of BALF collected on Day 17. Treatment with 10 mg/kg narirutin completely blocked the increases in IL-4 levels in BALF (b), but not IL-5 (c). Data are the mean $\pm$ SD ($n = 8$ per group). * $P < 0.05$ compared with the saline-challenged control group; † $P < 0.05$ compared with the OVA-challenged group.

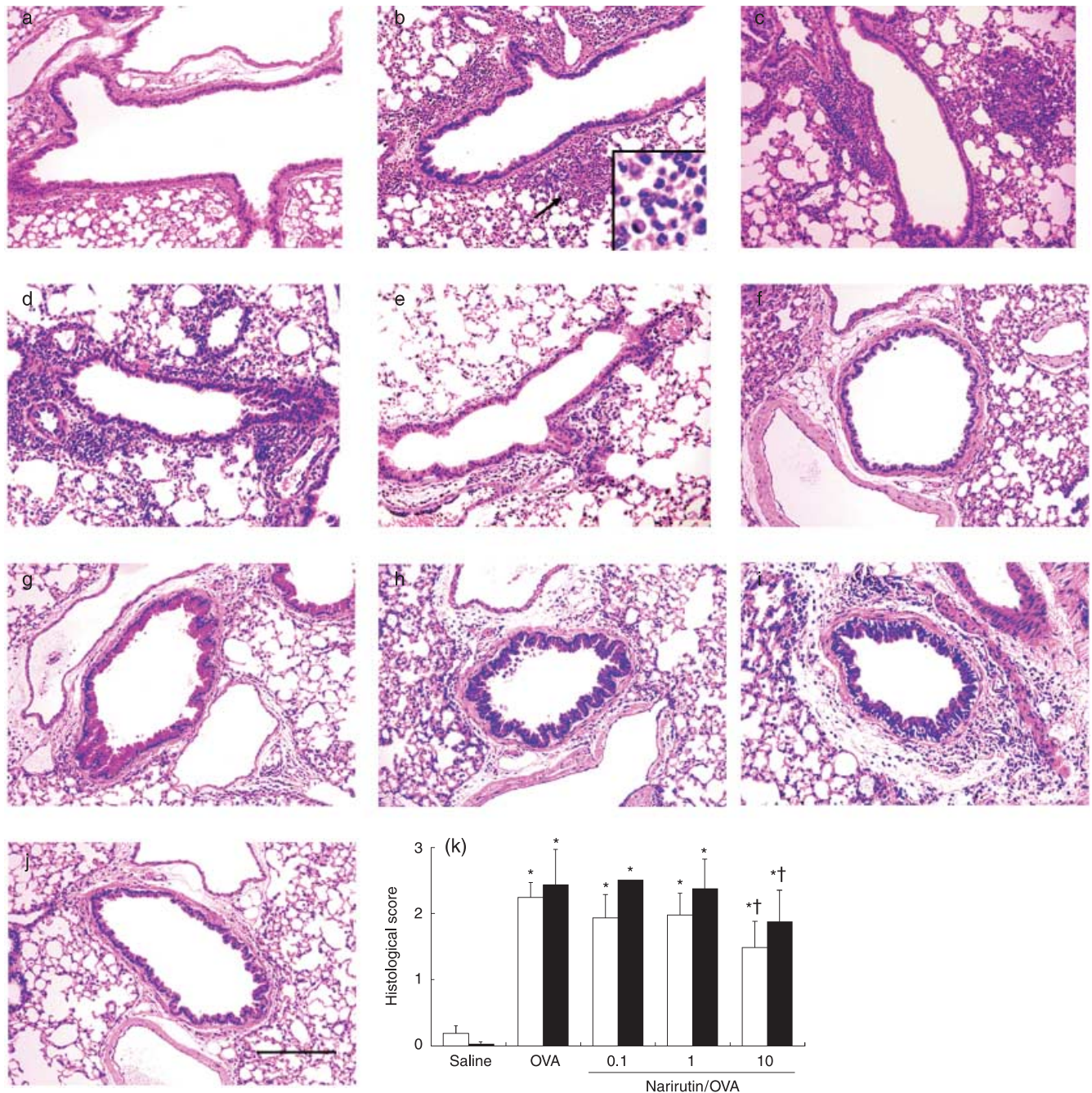


Fig. 3 Histological analysis of lung sections. The photomicrographs show (a–e) haematoxylin and eosin (H&E)- and (f–j) periodic acid-Schiff (PAS)-stained sections of lung tissue (original magnification $\times 200$). (a,f) Saline-challenged control mice (Saline). (b,g) Ovalbumin (OVA)-challenged mice untreated (OVA). (c–e,h–j) Ovalbumin-challenged mice treated with (c,h) 0.1 (d,i) 1 and (e,j) 10 mg/kg narirutin (Narirutin/OVA). Note the marked OVA-induced increases in inflammatory cells, mainly eosinophils around the airways and vessels, and PAS-positive mucus-producing cells within the airways. (b) The inset shows a higher magnification of the region indicated by the arrow, where numerous red-stained eosinophils are observed. Bar, 100 μ m. (k) At 10 mg/kg, narirutin reduces histological inflammation scores in lung tissue sections. Histological scores based on the cellular infiltration around the airways and vessels in the H&E-stained sections and numbers of PAS-positive cells in the airways were determined as described in the Methods. Data are the mean $\pm$ SD ($n = 8$ per group). * $P < 0.05$ compared with the saline-challenged control group; † $P < 0.05$ compared with the OVA-challenged group.

BALF, were significantly diminished following treatment with 10 mg/kg narirutin.

Inhalation of OVA has been reported to cause airway inflammation in large part by mediating increases in the levels of T helper (Th) 2 cytokines (e.g. IL-4 and IL-5) within lung tissue and increases

in the level of allergen-specific IgE in the circulation,^{17,18} findings that were confirmed in the present study. Interleukin-4 is a cytokine central to the allergic response, promoting isotype switching of B cells to IgE synthesis, directing T cells along the Th2 differentiation pathway, upregulating the expression of vascular cell adhesion

molecule (VCAM)-1 and controlling the level of expression of the high-affinity IgE Fc receptors, cytokine and chemokine receptors and leucocytes involved in the allergic cascade. Conversely, IL-5 is very specifically focused on the development, differentiation, recruitment, activation and survival of eosinophils.¹⁹ Allergic reactions in the airways are well known to be mediated via two pathways. The first pathway involves induction of IgE by B cells via IL-4 released from Th cells and mast cells. The other pathway involves recruitment of eosinophils via release of IL-5 from Th cells or mast cells.²⁰ In the present study, narirutin did not inhibit the OVA-induced increases in IL-5 levels in the airways, although it blocked the increases in IL-4, eosinophil numbers and serum IgE. This suggests that narirutin most likely exerts its beneficial effect by inhibiting the first pathway. Given that different flavonoid compounds exhibit different biological activities, the absence of an effect on IL-5 is likely a specific feature of narirutin and indicative of the specificity of the anti-inflammatory effects of narirutin, although that idea remains to be tested.

Narirutin, which is abundant in citrus fruits such as grapefruits and oranges, belongs to the class of flavonoids called flavanones. Narirutin has been shown to inhibit histamine release from rat peritoneal mast cells *in vitro* and to exert an inhibitory effect on allergic skin reactions in a murine model of atopic dermatitis.¹¹ However, studies into the mechanisms of the anti-allergic and anti-inflammatory effects of narirutin are rare. The effects of flavonoids on a variety of inflammatory processes and immune functions have been reviewed.²¹ Earlier studies showed that some of flavonoids were effective in reducing the release of histamine, leukotrienes, prostaglandins, cytokines and other mediators from effector cells *in vitro*.²² Flavonoids have been shown to protect tissues against free oxygen radicals and lipid peroxidation.²³ Some flavonoids (e.g. apigenin) block cytokine-induced upregulation in human endothelial cell adhesion molecules (e.g. VCAM-1).²⁴ Thus, the possibility that narirutin acts not only by inhibiting IL-4 and IgE, but also via poorly understood mechanisms cannot be eliminated in the present study.

Bronchial asthma is characterized by acute and chronic inflammation of the airways. Many of the features of the pulmonary inflammation observed in human asthma are reproduced in OVA-sensitized/challenged mice. Our finding that narirutin protected mice from OVA-induced allergic inflammation suggests that it may also exert a protective effect against human bronchial asthma and that further clinical analysis of narirutin is warranted.

In conclusion, narirutin dose-dependently inhibits OVA-induced airway inflammation in an allergic mouse model. The mechanism of the anti-inflammatory effect of narirutin is likely to be associated with a reduction in OVA-induced increases of IL-4 and IgE.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-Aid for Scientific Research Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

- Wills-Karp M. Immunologic basis of antigen-induced airway hyper-responsiveness. *Annu. Rev. Immunol.* 1999; **17**: 255–81.
- Hamelmann E, Gelfand EW. Animal models of airway sensitization. In: R Coico (ed.). *Current Protocols in Immunology*. John Wiley, New York. 1999; 15.18.1–15.18.13.
- Kuhnau J. The flavonoids: A class of semi-essential food components. Their role in human nutrition. *World Rev. Nutr. Diet* 1976; **24**: 117–91.
- Herrmann K. Flavonols and flavones in food plants: A review. *J. Food Technol.* 1976; **11**: 433–48.
- Gabor M. Anti-inflammatory substances of plant origin. In: Vane JR, Ferreira SH, eds. *Handbook of Experimental Pharmacology: Anti-Inflammatory Drugs*, Vol. 50. Springer, New York. 1979; 698–739.
- Kim CJ, Su SK, Joo JH, Cho SK. Pharmacological activities of flavonoids. II. Relationships of anti-inflammatory and anti-granulomatous actions. *Yakhak Hoeji* 1990; **34**: 407–14 (in Korean with an English abstract).
- Booth AN, Jones FT, Deeds F. Metabolic and glucosuria studies on naringin and phloridzin. *J. Biol. Chem.* 1958; **233**: 280–2.
- Hodgson JM, Puddey IB, Beilin LJ, Mori TA, Croft KD. Supplementation with isoflavonoid phytoestrogens does not alter serum lipid concentrations: A randomized controlled trial in humans. *J. Nutr.* 1998; **128**: 728–32.
- Knekt P, Kumpulainen J, Jarvinen R *et al.* Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr.* 2002; **76**: 560–8.
- Das M, Ram A, Ghosh B. Luteolin alleviates bronchoconstriction and airway hyperreactivity in ovalbumin sensitized mice. *Inflamm. Res.* 2003; **52**: 101–6.
- Kubo M, Fujita T, Nishimura S *et al.* Seasonal variation in anti-allergic activity of citrus fruits and flavanone glycoside content. *Nat. Med.* 2004; **58**: 284–94.
- Matsuda H, Watanabe N, Geba GP *et al.* Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int. Immunol.* 1997; **9**: 461–6.
- Iwasaki T, Tanaka A, Itakura A *et al.* Atopic NC/Nga mice as a model for allergic asthma: Severe allergic responses by single intranasal challenge with protein antigen. *J. Vet. Med. Sci.* 2001; **63**: 413–19.
- Padrid PA, Mathur M, Li X *et al.* CTLA4Ig inhibits airway eosinophilia and hyperresponsiveness by regulating the development of Th1/Th2 subsets in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* 1998; **18**: 453–62.
- Dohi M, Tsukamoto S, Nagahori T *et al.* Noninvasive system for evaluating the allergen-specific airway response in a murine model of asthma. *Lab. Invest.* 1999; **79**: 1559–71.
- Kelly-Welch AE, Melo MEF, Smith E *et al.* Complex role of the IL-4 receptor α in a murine model of airway inflammation: Expression of the IL-4 receptor α on nonlymphoid cells of bone marrow origin contributes to severity of inflammation. *J. Immunol.* 2004; **172**: 4545–55.
- Nagai H, Yamaguchi S, Tanaka S, Inagaki N. Effect of some immunosuppressors on allergic bronchial inflammation and airway hyper-responsiveness in mice. *Int. Arch. Allergy Immunol.* 1995; **108**: 189–95.
- Webb D, McKenzie A, Koskinen A, Yang M, Mattes J, Foster P. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J. Immunol.* 2000; **165**: 108–13.
- O'Byrne PM. Cytokines or their antagonists for the treatment of asthma. *Chest* 2006; **130**: 244–50.
- Sugita M, Kuribayashi K, Nakagomi T, Miyata S, Matsuyama T, Kitada O. Allergic bronchial asthma: Airway inflammation and hyperresponsiveness. *Int. Med.* 2003; **42**: 636–43.
- Middleton EJ, Kandaswami C. Effects of flavonoids on immune and inflammatory cell functions. *Biochem. Pharmacol.* 1992; **43**: 1167–79.
- Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalin on immunoglobulin E-mediated mediator release from human cultured mast cells. *Clin. Exp. Allergy* 2000; **30**: 501–8.
- Da Silva EL, Tsushida T, Terao J. Inhibition of mammalian 15-lipoxygenase-dependent lipid peroxidation in low-density lipoprotein by quercetin and quercetin monoglucosides. *Arch. Biochem. Biophys.* 1998; **349**: 313–20.
- Gerritsen ME, Carley WW, Ranges GE *et al.* Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *Am. J. Pathol.* 1995; **174**: 278–92.