Erythronium japonicum attenuates histopathological lung abnormalities in a mouse model of ovalbumin-induced asthma

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Abstract. Asthma is a chronic lung condition that can induce mucus hypersecretion and pulmonary obstruction and may even cause death, particularly in children and older individuals. Erythronium japonicum (E. japonicum) is a traditional herb used in Korea and East Asian countries that has been found to exert free radical scavenging activity and anti-proliferative effects in human colorectal carcinoma cells. In the present study, we evaluated the anti-asthmatic effects of an extract of E. japonicum in a mouse model of ovalbumin (OVA)-induced asthma. Female BALB/c mice were sensitized with an intraperitoneal injection of OVA and aluminum hydroxide hydrate on days 1 and 8 and then received the following treatments on days 21 to 25: i) control (no treatment), ii) sterilized tap water (given orally), iii) 1 mg/kg/day dexamethasone (administered orally), iv) 60 mg/kg/day E. japonicum extract, and v) 600 mg/kg/day E. japonicum extract. On the same days, all the mice except those in the control group were challenged 1 h later with nebulized 5% OVA for 30 min. We found that treatment with E. japonicum extract suppressed the OVA-induced increase in the number of white blood cells and decreased the IgE level in the bronchoalveolar lavage fluid samples obtained from the mice. Histopathological analysis of the lung tissues revealed that E. japonicum attenuated the asthma-related morphological changes in the mouse lung tissue, including the increased secretion of mucus in the bronchioles, eosinophil infiltration around the bronchioles and vessels, and goblet cell and epithelial cell hyperplasia. Immunohistochemical analysis revealed that treatment with E. japonicum extract suppressed the OVA-induced proliferation of T helper cells (CD4*) and B cells (CD19*) in the mouse lung tissue. Furthermore, treatment with E. japonicum extract modulated the expression of both Th helper 2 cell-related factors [GATA binding protein 3 (GATA-3), tumor necrosis factor-α (TNF-α), interleukin (IL)-4, IL-5, IL-6 and IL-13], as well as that of Th helper 1 cell-related factors [(interferon-γ (IFN-γ), IL-12p35 and IL-12p40]. These findings suggest that E. japonicum may potentially be used as an anti-asthmatic treatment.

Introduction

The World Health Organization estimates that 235 million individuals worldwide suffered from asthma in 2013 (1). In the United States, an estimated 25.9 million individuals, including almost 7.1 million children, suffered from asthma in 2013, and asthma was the third most common cause of hospitalization for children under 15 years of age (2). The symptoms of asthma are difficult to control (3), and its causes are diverse, including hereditary factors and external factors, such as pet dander, dust mites, cockroaches, viral infections, pollen, mold, fungi and tobacco smoke (1). The typical manifestations of asthma vary from a cough to obstructive apnea, which may arise due to the excessive production of mucus, goblet cell hyperplasia, epithelial cell shedding, basement membrane thickening, as well as eosinophil and lymphocyte infiltration (4,5).

Asthma is a chronic lung condition that involves an imbalance between Th helper (Th1) and Th2-related factors (4,5), including interleukin (IL)-12, interferon-γ (IFN-γ), IL-4, IL-5, IL-13, IL-6 and tumor necrosis factor-α (TNF-α). IL-12 modulates the induction of Th1-specific immune responses and the suppression of Th2-specific immune responses (6,7). IFN-γ is known to stimulate the Th1 transcription factor, T-bet, resulting in a positive feedback loop between IFN-γ and Th1 (8,9). The cytokines, IL-4 and IL-13, play important roles in asthma (10). IL-4 regulates the immunoglobulin class switching from IgG to IgE, attracts eosinophils into the interspace of pulmonary cells (11), and is involved in the induction of the Th2 transcription factor GATA-3 (12,13,15). IL-13 activates B cells and induces asthma-related changes, such as the excessive production of mucus, goblet cell hyperplasia, epithelial cell shedding, basement membrane thickening, and eosinophil and lymphocyte infiltration (14,16,17). Both IL-5 and IL-6 have been demonstrated to

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regulate the development, activation, migration and survival of eosinophils (6, 18). Furthermore, TNF-α is known to recruit granulocytes and to induce the proliferation of fibroblasts (19).

The drugs currently used to treat asthma include corticosteroids, bronchodilators, leukotriene modifiers, theophylline and anti-IgE agents; however, their therapeutic effects are not completely understood (20). Although inhaled corticosteroids are the most widely used therapy for suppressing the symptoms of asthma (21), they are associated with many adverse effects, including growth inhibition in children during the first year of treatment (22), cataracts and glaucoma, hypertension, hyperlipidemia, peptic ulcers, myopathy and immunosuppressive effects (23). Thus, the unwanted side-effects of corticosteroids have increased the need to develop anti-asthmatic drugs from natural products.

Erythronium japonicum (E. japonicum) is an indigenous herb in Korea and East Asian countries (24). It is distributed throughout Hokkaido in Japan, where the starch (‘katakuriko’ in Japanese) is obtained from the bulb after a long, cold winter. Although a limited number of studies have explored its potentially therapeutic effects, E. japonicum extract was found in one study to possess 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity and to exert anti-proliferative effects in human colorectal carcinoma cells (25). In the present study, we evaluated the effects of E. japonicum extract on ovalbumin (OVA)-induced asthma in mice.

Materials and methods

Plant material and preparation of 80% EtOH extract. During May 2013, E. japonicum was collected from a site situated close to Wolchul mountain in the southern part of Korea. A sample was deposited at the Jeonnam Biofood Technology Center (identification number: JBF-FRI-S-2013-0099). E. japonicum was dried in a dark, cool room. Dried E. japonicum (2 kg) was chopped and then extracted twice with 80% aqueous EtOH (30 liters) at room temperature for 24 h. The EtOH extracts of E. japonicum were concentrated and evaporated under a vacuum. E. japonicum extract was analyzed using high-performance liquid chromatography (HPLC).

Establishment of a mouse model of OVA-induced asthma. Female BALB/c mice (5 weeks old, n=80) were purchased from Samtako Korea (Osan, Korea) and randomly divided into the following 5 treatment groups: i) the control group (no treatment, no OVA challenge); ii) the group administered sterilized tap water and challenged with OVA; iii) the group administered 1 mg/kg/day dexamethasone followed by OVA challenge; iv) the group administered 60 mg/kg/day E. japonicum extract followed by OVA challenge; and v) the group administered 600 mg/kg/day E. japonicum extract followed by OVA challenge. On days 1 and 8, all mice were sensitized with an intraperitoneal injection of 20 μg OVA and 1 mg aluminum hydroxide hydrate (both from Sigma-Aldrich, St. Louis, MO, USA) in 500 μl saline. On days 21 to 25, all mice except those used as controls were challenged once daily with 5% OVA for 30 min using a nebulizer (3 ml/min, NE-U17; Omron Co. Ltd., Kyoto, Japan). During the same 5-day period, the mice in the treatment groups were also treated once daily with oral doses of either sterilized tap water, dexamethasone, or 60 or 600 mg/kg/day E. japonicum extract (hereafter referred to as E. japonicum) 1 h prior to the OVA challenge.

Ethics statement. E. japonicum was collected on private land with permission granted by the owner. All experiments were approved by the Institutional Animal Care and Use Committee at Dongshin University (approval no. 2014-08-04).

Analysis of bronchoalveolar lavage fluid (BALF). One day after the final treatment, the mice were anesthetized with intraperitoneal injections of 50 mg/kg Zoletin (Virbac, Fort Worth, TX, USA), and thereafter the tracheas were cannulated with disposable animal feeding needles. Lavages were performed with three 0.4 ml aliquots of cold phosphate-buffered saline (PBS). The BALF samples were collected and immediately centrifuged at 3,000 rpm for 5 min (Sorvall Legend Micro 17R; Thermo Fisher Scientific, Inc., Marietta, OH, USA). The cell pellets were resuspended in PBS in order to determine the total and differential white blood cell (WBC) counts. The numbers of total and differential cells were counted using the Hemavet Multispecies Hematology System (Drew Scientific, Inc., Waterbury, CT, USA) (n=8/group). The levels of IgE in the BALF were measured using a specific mouse IgE enzyme-linked immunosorbent assay kit (Abnova, Atlanta, GA, USA), according to the manufacturer’s instructions (n=8/group).

Histopathological analysis. One day after the final treatment, the mice were anesthetized with intraperitoneal injections of 50 mg/kg Zoletin and lung tissue was obtained. The mice were then sacrificed by exsanguination. The lung tissue was fixed in 10% (v/v) formaldehyde solution, dehydrated in a graded ethanol series (99.9, 90, 80 and 70%), and embedded in paraffin. The paraffin-embedded lung tissue was then sectioned (4-μm-thick sections) longitudinally and stained with hematoxylin and eosin. The sections were also stained with Periodic acid-Schiff for the semi-quantitative analysis of glycoproteins.

Immunohistochemical analysis. The deparaffinized tissue sections were treated with 3% hydrogen peroxide in methanol for 10 min to remove the endogenous peroxidase. Antigen retrieval was performed with sodium citrate buffer (0.1 M) using the microwave method. The slides were incubated with normal serum to block non-specific binding and then incubated overnight at 4°C with the following primary antibodies (diluted 1:100 to 1:200): rat anti-mouse CD4 monoclonal (14-9766; eBioscience, San Diego, CA, USA); rat anti-mouse CD8 monoclonal (sc-18913; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit anti-mouse CD19 polyclonal (bs-3599R; Bioss, Woburn, MA, USA); rabbit anti-mouse IL-4 monoclonal (sc-73318) and rabbit anti-mouse IL-5 poly (250585; Abbiotec, San Diego, CA, USA); rat anti-mouse Tbx21/T-bet polyclonal (bs-3599R; Bios, Woburn, MA, USA); goat anti-mouse GATA-3 (TA305795; OriGene, Rockville, MD, USA); rat anti-mouse IFN-γ monoclonal (sc-74104), goat anti-mouse IL-12p35 polyclonal (sc-9350) and rabbit anti-mouse IL-12p40 monoclonal (sc-57258) (all from Santa Cruz Biotechnology, Inc.); rabbit anti-mouse TNF-α polyclonal (3053R-100; BioVision, Milpitas, CA, USA); rat anti-mouse IL-4 monoclonal (sc-73318) and rabbit anti-mouse IL-5 polyclonal (sc-7887) (both from Santa Cruz Biotechnology, Inc.); rabbit anti-mouse IL-6 polyclonal (PAB16165; Abnova, Taipei City, Taiwan), and goat anti-mouse IL-13 polyclonal.
(sc-1776; Santa Cruz Biotechnology, Inc.) antibodies. Subsequently, the slides were incubated for 2 h with biotinylated secondary antibody (1:500; Dako, Carpinteria, CA, USA) and horseradish-peroxidase conjugated streptavidin. Signals were detected using 3,3-diaminobenzidine tetrahydrochloride substrate chromogen solution, and the cells were counterstained with Mayer’s hematoxylin. To determine the number of positively stained cells, we counted the cells in 5 randomly selected, non-overlapping fields (magnification, x200) from 3 separately immunostained lung sections per animal (n=8/group).

**HPLC analysis.** HPLC analysis was performed using an HPLC system (Agilent Technology, Santa Clara, CA, USA) according to the following parameters: column, Zorbax extend-C18 (4.6 mm inner diameter x 150 mm height, 5.0-µm particle diameter); mobile phase, 0.2% acetic acid and methanol used as A and B, respectively, with the following solvent gradient: 25% B, 0-5 min; 25-50% B, 5-10 min; and 50-25% B, 10-15 min; injection volume, 10 µl; flow rate, 1 ml/min; UV detection, 330 nm. All solvents were of extra-pure grade. Methanol and distilled water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetic acid, caffeic acid and chlorogenic acid were purchased from Sigma-Aldrich.

**Statistical analysis.** Data are shown as the means ± standard deviation (SD). Group differences were evaluated using one-way analysis of variance followed by Dunnett’s multiple comparison tests. A p-value of <0.05 or <0.001 was considered to indicate a statistically significant difference.

**Results**

Effects of *E. japonicum* on the total and differential WBC counts and on the IgE levels in the BALF samples. Compared with the BALF obtained from the control mice, the BALF collected from the OVA-challenged mice contained significantly higher numbers of total WBCs and differential WBCs (eosinophils, neutrophils, lymphocytes and monocytes), as well as significantly higher levels of IgE (Fig. 1). Treatment with both dexamethasone, a drug commonly used to suppress the symptoms of asthma, and *E. japonicum* significantly decreased the number of WBCs, as well as the IgE levels in the OVA-challenged mice.
**Effects of *E. japonicum* on lung tissue morphology.** The asthma-related histopathological changes in the mouse lung tissues were evaluated using hematoxylin and eosin staining and Periodic acid-Schiff staining (Fig. 2). Compared with the lung tissue isolated from the control mice, the lung tissue from the OVA-challenged mice exhibited typical asthma-related changes, including goblet cell and epithelial cell hyperplasia, eosinophil infiltration around the bronchioles and vessels, as well as the increased secretion of mucus in the bronchioles, and these changes were attenuated by treatment with dexamethasone. Treatment with *E. japonicum* also attenuated these histopathological changes in a dose-dependent manner; 600 mg/kg/day of *E. japonicum* appeared to be more effective at inhibiting the OVA-induced changes in the mouse lung tissue compared with the dose of 60 mg/kg/day.

**Effects of *E. japonicum* on the numbers of Th cells, T cells, and B cells.** The development of asthma involves an imbalance between Th1 and Th2 cells (4,5). In order to evaluate the effects of *E. japonicum* treatment on Th cells, T cells and B cells, we quantified the numbers of CD4⁺ Th cells, CD8⁺ cytotoxic T cells and CD19⁺ B cells in the mouse lung tissue by performing immunohistochemical analysis (Fig. 3). Compared with the control mice, the lung tissue isolated from the OVA-challenged mice had significantly higher numbers of all 3 types of cells, and these increased numbers were significantly reduced by treatment with both dexamethasone and *E. japonicum*. Furthermore, treatment with *E. japonicum* exerted a reducing effect on the numbers of CD4⁺ Th cells and CD19⁺ B cells in a dose-dependent manner, with greater effects observed at the dose of 600 mg/kg/day compared with 60 mg/kg/day.

**Effects of *E. japonicum* on the expression of T-bet and GATA-3.** To examine the effects of *E. japonicum* treatment on Th cell transcription factors, we examined the expression of T-bet (Th1 cell transcription factor) and GATA-3 (Th2 cell transcription factor) in the mouse lung tissue by perfoming immunohistochemical analysis (Fig. 4). Compared with the control mice, the lung tissue isolated from the OVA-challenged mice exhibited a significantly higher expression of T-bet and GATA-3, and these effects were significantly reduced by dexamethasone treatment. Treatment with *E. japonicum* exerted a reducing effect on GATA-3 expression in a dose-dependent manner, with the dose of 600 mg/kg/day significantly reducing the OVA-induced expression of GATA-3 to a greater extent compared with the dose of 60 mg/kg/day. However, the expression of T-bet in the *E. japonicum* treatment groups remained at higher levels compared to the control group, and there was no significant difference between the 2 doses used.
Figure 3. Effects of *E. japonicum* on the numbers of Th, T and B cells in mouse lung tissue. (A) Representative images of CD4+ Th cells. (B) Representative images of CD8+ cytotoxic T cells. (C) Representative images of CD19+ B cells. Immunopositive cells were counted in 5 randomly selected, non-overlapping fields (magnification, x200) from 3 separately immunostained lung sections per animal. Treatment groups: panel a, control (CON); panel b, ovalbumin (OVA); panel c, dexamethasone (DEX); panel d, 60 mg/kg/day *E. japonicum* (E.j); panel e, 600 mg/kg/day *E. japonicum*. (D) Quantitative analysis of positively-stained cells. *p<0.05 vs. CON; **p<0.001 vs. CON; $p<0.05$ vs. OVA; $$p<0.001$ vs. OVA; #p<0.05 vs. DEX; ##p<0.001 vs. DEX; $p<0.05$ vs. 60 mg/kg/day E.j; **p<0.001 vs. 60 mg/kg/day E.j.

Figure 4. Effects of *E. japonicum* on the expression of T-bet and GATA-3 in mouse lung tissue. (A) Representative images of T-bet+ cells. (B) Representative images of GATA-3+ cells. Immunopositive cells were counted in five randomly selected, non-overlapping fields (magnification, x200) from 3 separately immunostained lung sections per animal. Treatment groups: panel a, control (CON); panel b, ovalbumin (OVA); panel c, dexamethasone (DEX); panel d, 60 mg/kg/day *E. japonicum* (E.j); panel e, 600 mg/kg/day E.j. (C) Quantitative analysis of positively-stained cells. *p<0.05 vs. CON; **p<0.001 vs. CON; $p<0.05$ vs. OVA; $$p<0.001$ vs. OVA; #p<0.05 vs. DEX; ##p<0.001 vs. DEX; $p<0.05$ vs. 60 mg/kg/day E.j; **p<0.001 vs. 60 mg/kg/day E.j.
SEO et al: \textbf{E. japonicum} ATTENUATES ASTHMA SYMPTOMS BY MODULATING Th1-/Th2-RELATED CYTOKINES

Effects of \textit{E. japonicum} on the expression of Th1-related cytokines. The imbalance between Th1 and Th2 cells which occurs in asthma involves a decrease in Th1-related cytokine levels and an increase in Th2-related cytokine levels (4,5). In this study, to examine alterations in the levels of Th1-related cytokines, we measured the expression levels of IFN-$\gamma$, IL-12p35 and IL-12p40 in the mouse lung tissue (Fig. 5). Compared with the control mice, the lung tissue isolated from the OVA-challenged mice exhibited a significantly increased expression of IL-12p35 and IL-12p40, and these effects were significantly suppressed by dexamethasone treatment. \textit{E. japonicum} treatment exerted a reducing dose-dependent effect on the expression of IL-12p35 and IL-12p40, with the dose of 600 mg/kg/day (but not the dose of 60 mg/kg/day) significantly reducing the OVA-induced expression of IL-12p35 and IL-12p40. \textit{E. japonicum} treatment at the dose of 600 mg/kg/day increased the expression of IFN-$\gamma$.

Effect of \textit{E. japonicum} on the expression of Th2-related cytokines. To examine alterations in the levels of Th2-related cytokines, we measured the expression levels of TNF-$\alpha$, IL-4, IL-6 and IL-13 in the mouse lung tissue (Fig. 5). Compared with the control mice, the lung tissue isolated from the OVA-challenged mice exhibited a significantly increased expression of all the measured cytokines, and these changes were significantly reduced by dexamethasone treatment (Fig. 6). \textit{E. japonicum} treatment decreased the OVA-induced expression of most of these cytokines in a dose-dependent manner. Specifically, \textit{E. japonicum} treatment at the dose of 60 mg/kg/day significantly reduced the expression of IL-13, whereas treatment with \textit{E. japonicum} at 600 mg/kg/day significantly reduced the expression of TNF-$\alpha$, IL-4, IL-6, IL-5 and IL-13.

Discussion

In the present study, we found that treatment with \textit{E. japonicum} suppressed the OVA-induced increase in the number of WBCs, as well as the IgE level in BALF. Furthermore, treatment with \textit{E. japonicum} attenuated the asthma-related morphological changes which had occurred in the mouse lung tissue following exposure to OVA, including the increased secretion of mucus, eosinophil infiltration around the bronchioles and vessels, and goblet cell and epithelial cell hyperplasia. \textit{E. japonicum} treatment also suppressed the OVA-induced increase in the number of Th cells (CD4$^+$ cells), B cells (CD19$^+$ cells) and T cells (CD8$^+$ cytotoxic cells). Furthermore, we found that \textit{E. japonicum} treatment modulated the expression of not only Th2-related factors (GATA-3, TNF-$\alpha$, IL-4, IL-5, IL-6 and IL-13), but also that of Th1-related factors (IFN-$\gamma$, IL-12p35 and IL-12p40).

To identify the particular compounds in \textit{E. japonicum} that may exert anti-asthmatic effects, we conducted HPLC analysis. Although not all the compounds in \textit{E. japonicum} were isolated, we found that chlorogenic acid and caffeic acid were candidate compounds (Fig. 7). Chlorogenic acid is associated with lignin biosynthesis (26) and it has been demonstrated to exert several effects, including protective effects against oxidative stress-induced hepatotoxicity (27), anti-hypertensive effects (28) and the suppressive effects on several asthma-related factors in an animal model of ovalbumin-induced asthma (29). Furthermore, chlorogenic acid is an immunoregulatory molecule (30).
Asthma involves an imbalance between Th1 and Th2 cell-related factors (13), which results in airway hyperresponsiveness in asthma patients as well as morphological changes in lung tissues such as mucus hypersecretion in the bronchi.

Figure 6. Effect of E. japonicum on expression of Th2-related cytokines in lung tissue. (A) Representative images of tumor necrosis factor-α (TNF-α) + cells. (B) Representative images of interleukin-4 (IL-4) + cells. (C) Representative images of IL-5 + cells. (D) Representative images of IL-6 + cells. (E) Representative images of IL-13 + cells. Immunopositive cells were counted in 5 randomly selected, non-overlapping fields (magnification, x200) from 3 separately immunostained lung sections per animal. Treatment groups: panel a, control (CON); panel b, ovalbumin (OVA); panel c, dexamethasone (DEX); panel d, 60 mg/kg/day E. japonicum (E.j); panel e, 600 mg/kg/day E.j. (F) Quantitative analysis of positively-stained cells. *p<0.05 vs. CON; **p<0.001 vs. CON; *p<0.05 vs. OVA; ***p<0.001 vs. OVA; #p<0.05 vs. DEX; ##p<0.001 vs. DEX; $p<0.001 vs. 60 mg/kg/day E.j.

Figure 7. HPLC analysis of E. japonicum. HPLC analysis identified chlorogenic acid and caffeic acid as components of E. japonicum.

Asthma involves an imbalance between Th1 and Th2 cell-related factors (13), which results in airway hyperresponsiveness in asthma patients as well as morphological changes in lung tissues such as mucus hypersecretion in the bronchi-
oles, goblet cell and epithelial cell hyperplasia, and eosinophil infiltration near to the bronchioles and vessels (4). Depending on its severity, asthma may even be fatal (1). Asthma is associated with changes in the expression of Th2- and Th1-related cytokines (13), including the increased expression of IL-4, IL-5, IL-6, IL-13 and TNF-α and the decreased expression of IL-12 (31). In the present study, we found that E. japonicum treatment reversed many of the OVA-induced changes in Th1 and Th2 cell-related factors in a mouse model of asthma, suggesting that E. japonicum may ameliorate the symptoms of asthma by modulating the balance between Th1 and Th2 cells.

The most commonly prescribed drugs for asthma are steroids; however, these have many adverse effects, such as growth inhibition in children (22), cataracts and glaucoma, hypertension, hyperlipidemia, peptic ulcers and myopathy, as well as immunosuppressive effects (23). Moreover, steroids do not cure asthma (20). Therefore, much research has focused on identifying candidate pharmacological agents, including natural products, which are more effective and produce fewer unwanted side-effects than steroids (32,33). The present study suggests that E. japonicum is a promising candidate for the treatment of asthma, although additional studies are warranted in order to improve our understanding of the potential effects of E. japonicum, as well as the mechanisms responsible for these effects.

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