**Vitex rotundifolia** L. prevented airway eosinophilic inflammation and airway remodeling in an ovalbumin-induced asthma mouse model

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**Abstract**

**Vitex rotundifolia** L. (VR) as long been used in China and Korea in traditional medicine. This study was conducted to evaluate the ability of **Vitex rotundifolia** L. to prevent airway inflammation and remodeling in an ovalbumin (OVA)-induced murine asthma model. The total cell number and number of inflammatory cells in the bronchoalveolar lavage (BAL) fluid were counted. The levels of cytokines in the BAL fluid and serum IgE levels were measured using an ELISA. For histological analysis, hematoxylin and eosin staining, periodic acid-Schiff staining and immunohistochemistry were evaluated. The release of total cells into the BAL fluid was significantly inhibited in OVA-induced asthmatic mice treated with VR extract. In addition, eosinophilia and lymphocytosis were reduced significantly in mice that received VR extract. Furthermore, levels of the T\(_h\)2 cytokines IL-4 and IL-5 and pro-inflammatory cytokine TNF-\(\alpha\) in the BAL fluid and total IgE in serum were markedly suppressed by VR extract. OVA-specific IgE in the serum and IL-13 in the BAL fluid were decreased, but not significantly. The allergic effects of VR extract were accompanied by a reduction in airway hyperresponsiveness. Additionally, morphologic findings demonstrated that VR extract substantially inhibited OVA-induced eosinophilia, goblet cell hyperplasia and smooth muscle mass production. This finding suggests that VR extract may have pharmacological effects that would be useful for the treatment of asthma via the inhibition of the T\(_h\)2 response and airway remodeling.

**Keywords**: airway hyperresponsiveness, eosinophilia, goblet cell hyperplasia, smooth muscle actin, T\(_h\)2 response

**Introduction**

Asthma is an inflammatory disease characterized by the presence of increased numbers of leucocytes, especially eosinophils and lymphocytes, in the bronchial tissues. The clinical symptoms of asthma are wheezing, coughing, shortness of breath and chest discomfort (1). The initial stages of asthma are a result of airway inflammation in which leucocytes, especially eosinophils, play pathogenic roles (2). During an asthma attack, eosinophils selectively migrate into the airways, where they infiltrate and cause inflammation. The severity of asthma is determined by the degree of airway narrowing or airway hyper-responsiveness (AHFR) induced by activated lymphocytes in the airway. The severity of asthma is also associated with airway remodeling, which is characterized by goblet cell hyperplasia, pulmonary fibrosis and increased airway smooth muscle mass (3). Both the pro-inflammatory cytokine TNF-\(\alpha\) and the T\(_h\)2 cytokine interleukin IL-13 are well-known remodeling-associated cytokines. Asthma inflammation is also induced by cytokines released from T\(_h\)2 lymphocytes, which can act via positive feedback mechanisms to promote the production of more inflammatory mediators, including other cytokines (4). Among T\(_h\)2 cytokines, IL-4, IL-5 and IL-13 are directly linked with the immunopathogenesis of asthma (5). This is because these cytokines both regulate the main processes involved in IgE production and differentiate and activate eosinophils (4, 6).
Many natural products used in oriental medicine have been found to be effective agents for the treatment of asthma (7, 8). The results of our previous studies suggested that Moutan Cortex Radicis reduced eotaxin secretion (9) and that Bambusae Caulis in Taeniam reduced airway inflammation (10). However, the biomolecular activity of many natural products has yet to be confirmed by experimental testing (11). Additionally, despite their remarkable curative ethnopharmacological abilities, most natural products have not been widely used in western societies because their mode of action at the molecular level is not well understood. One such product, the dried fruit of Vitex rotundifolia L. (Vitex trifolia L. var. simplicifolia Cham.; VR), has long been used in China and Korea as a traditional medicine for treatment of various allergic diseases and upper respiratory infections (12). It contains the flavonoids such as artemetin, quercetagetin, 5,3'-dihydroxy-6,7,4'-trimethoxyflavanone and casticin, of which casticin is the primary active compound, and several studies have investigated the chemical constituents of the extracts of this fruit and the molecular mechanisms responsible for its effects (13–15).

In this study, we investigated the ability of VR extract to prevent airway eosinophilic inflammation and airway remodeling activity using an ovalbumin (OVA)-induced murine asthma model. Furthermore, we determined that VR substantially attenuates airway inflammation through the suppression of the Th2 response, and that this activity resulted in the abrogation of airway remodeling.

Methods

Preparation of the VR extract

VR-extract granules were purchased from Sun Ten Pharmaceutical (Taipei, Taiwan). The granules were weighed precisely to 100 mg and then dissolved in 10 ml of distilled water by stirring overnight at room temperature. Next, the solution was centrifuged for 10 min at 3000 r.p.m. (Eppendorf, Hamburg, Germany), after which the supernatant was sterilized by passing the solution through a 0.22 µm syringe filter. The stock of the VR extract was then diluted several times and used in the experiments. The stock (KHOP00210) was stored at 4°C until use.

Experimental animals

Animal studies were conducted using specific pathogen-free, 6-week-old male BALB/c mice (Charles River Technology Inc.) that were sensitized by intra-peritoneal (i.p.) injection of 100 µg of chicken OVA and 20 mg aluminum hydroxide (Sigma-Aldrich, MO, USA) on days 0 and 14. The OVA-challenged mice were exposed to 1.5 mg of OVA intra-nasally on days 15, 16 and 17. The positive control group of asthma-induced mice was then treated with 10 mg kg⁻¹ of dexamethasone (Dexa, Sigma-Aldrich), while the other group was treated with 100 mg kg⁻¹ of VR extract between days 15 and 17 via orogastric gavage. Two groups of mice were treated with Dexa and VR extract via an orogastric gavage route 1 h before the OVA challenge. The negative control group was sensitized and challenged with PBS. Mice were sacrificed on day 18 (Fig. 1).

Collection of bronchoalveolar lavage fluid and serum

Bronchoalveolar lavage (BAL) fluid was collected by lung lavage using 1 ml of PBS administered via the trachea. After three lavages, the BAL fluid was centrifuged for 10 min at 1300 r.p.m. The cell concentrations were then determined using a hemacytometer, and differential cell counts were conducted using slides prepared by cytocentrifugation and Diff-Quick staining. Approximately 500 cells were counted. The supernatant was stored at −80°C until subsequent cytokine measurement. Immediately prior to the first and final antigen challenges, blood was collected and sera were obtained by centrifugation and stored at −80°C until subsequent determination of total IgE and OVA-specific IgE.

Fig. 1. Treatment protocol of asthma model.
Measurement of levels of cytokines in BAL fluid and total IgE and OVA-specific IgE in the serum

The concentrations of IL-4, IL-5, IL-13, TNF-α, total IgE and OVA-specific IgE were measured by a sandwich ELISA using a Mouse CytoSet Kit (Invitrogen, CA, USA), OptEIA Set mouse IgE microtiter plate (BD Biosciences, CA, USA) and Mouse OVA-specific IgE ELISA assay kit (DS Pharma Biomedical Co., Ltd., Osaka, Japan). All assays were conducted in triplicate. The optical density was measured at 450 nm in a microplate reader.

Preparation of lung tissues and histology

Lung tissues were removed from the mice and fixed with 4% formaldehyde overnight at 4°C. The fixed tissues were then embedded in paraffin and cut into 4-µm sections with a microtome (Leica, Nussloch, Germany), after which the sections were placed on slide glasses, deparaffinized and stained with hematoxylin and eosin (Sigma-Aldrich) in order to examine the leucocytes that had infiltrated into the peribronchial connective tissues. In addition, the samples were stained in randomly selected sections with periodic acid-Schiff (PAS; IMEB Inc., CA, USA) to assess the goblet cell hyperplasia in connective tissues. In addition, the samples were stained in hematoxylin and eosin (Sigma-Aldrich) in order to examine the leucocytes that had infiltrated into the peribronchial connective tissues. Furthermore, the samples were stained in periodic acid-Schiff (PAS; IMEB Inc., CA, USA) to assess the goblet cell hyperplasia in connective tissues. In addition, the samples were stained in hematoxylin and eosin (Sigma-Aldrich) in order to examine the leucocytes that had infiltrated into the peribronchial connective tissues. Additionally, the slides were counterstained with Harris’s hematoxylin for 3 min and then mounted with Canada Balsam (Showa Chemical Co. Ltd, Tokyo, Japan).

Measurement of airway hyper responsiveness

The AHR of the mice was measured using a single-chamber, whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY) according to the manufacturer’s protocol. The plethysmograph and enhanced pause (Penh) variable was used to estimate airway resistance. Mice were serially exposed to increasing doses of nebulized methacholine (0, 25, 50 and 100 mg ml⁻¹) (Sigma-Aldrich) in PBS for 3 min, and Penh values were measured for 3 min following the end nebulization.

Quantitative chromatographic analysis

High performance liquid chromatography (HPLC) analysis was used for the quantification of casticin in VR extract and the serum of asthmatic mice treated with VR. The HPLC system consisted of a Nanospace SI-2/3001 pump, a 3004 column oven with a 10 µl fixed loop and a 3002 UV detector, was purchased from Shiseido (Tokyo, Japan). Chromatographic separation was performed using a Unison UK-C18 column (150 × 2.0mm ID, 3 µm; Imtakt, Kyoto, Japan). The mobile phase consisted of a 2% formic acid solution containing 5% acetonitrile, the flow rate was 0.2 ml min⁻¹, and the separation temperature was 30°C. The UV detector was set to 350 nm and was controlled by a computer running the Dschromn program supplied by Donam Instruments (Seoul, South Korea). All solvents were filtered and degassed prior to use.

Inhibitory effect of VR extract on leukocytes in BAL fluid

The number of total cells, eosinophils and lymphocytes in the BAL fluid from OVA-immunized and -challenged mice increased remarkably compared with normal mice that were sensitized and challenged with saline (**P < 0.01; Fig. 2A, B, and D). These results indicated that the allergic inflammation model of asthma was successfully established. However, oral pre-treatment with VR extract significantly inhibited the number of total cells when compared with the asthmatic controls (45.9%, **P < 0.01; Fig. 2D). The differential leukocyte counts show that oral administration of VR extract significantly inhibited eosinophilia when compared with the asthmatic control (86.0%, **P < 0.01; Fig. 2A). Additionally, lymphocytosis in asthmatic mice that were treated orally with VR extract was significantly reduced when compared with the asthmatic control groups (74.7%, **P < 0.01; Fig. 2B). Furthermore, the VR extract-treated group showed a 15.6% reduction in macrophage numbers, but this difference was not significant (Fig. 2C). Therefore, treatment of the induced asthma model with VR extract resulted in a significant reduction in the number of inflammatory cells that was as effective as dexamethasone.

Regulation of cytokines released into BAL fluid by VR extract

To investigate the possible inhibitory mechanisms of VR extract in airway inflammation and remodeling, the levels of IL-4, IL-5, IL-13 and TNF-α were examined using ELISA. In the asthmatic control mice, IL-4, IL-5, IL-13 and TNF-α levels were higher in BAL fluid compared with samples from normal mice.
Treatment with VR extract and dexamethasone significantly suppressed the elevated levels of IL-4 (*P < 0.05), IL-5 and TNF-α (**P < 0.01). The level of IL-13 was decreased, but not significantly, by VR extract treatment (Fig. 3).

**Decreases in the level of IgE in serum in mice treated with VR extract**

Because IgE is associated with the Th2-cell-mediated immune response in the pathogenesis of allergic inflammation, the effects of treatment with VR extract on the total IgE and OVA-specific IgE in the serum were investigated. In the asthmatic control mice, the total IgE and OVA-specific IgE levels in the serum were significantly up-regulated when compared with normal mice (**P < 0.01). Additionally, treatment with VR extract and dexamethasone led to significant down-regulation of the secretion of total IgE levels in the serum (*P < 0.05). However, OVA-specific IgE was not significantly decreased in the serum by the VR extract treatment (Fig. 4).

**Prevention of inflammation of lung tissue by VR extract in an asthmatic mouse model**

To evaluate the effect of VR extract on goblet cell hyperplasia, lung tissues were stained using PAS. The overproduction of goblet cell hyperplasia was detected in the bronchial airway epithelium of OVA-induced asthmatic mice, when compared with normal mice (**P < 0.01; Fig. 6A, B and E). Additionally, OVA-induced asthmatic mice that received VR extract showed a large reduction in the number of goblet cells in the bronchial epithelium compared with those that received 10 mg kg⁻¹ dexamethasone (**P < 0.01; Fig. 6C, D and E). These results demonstrate that VR extract may be effective in inducing airway remodeling in OVA-induced asthmatic mice.
Inhibition of increased airway smooth muscle actin by VR extract in an asthmatic mouse model

To investigate the effect of VR extract on airway SMA, lung tissues were subjected to immunohistochemistry analyses. Marked increases in the mass production of SMA-positive cells were observed in the lung tissues of OVA-induced asthmatic mice when compared with normal mice (\(** P < 0.01; \text{Fig. 7A, B and E} \)). All groups treated with VR showed decreased SMA immunoreactivity when compared with OVA-induced asthmatic mice (\(** P < 0.01; \text{Fig. 7B, D and E} \)), which was similar to the results seen in the dexamethasone-treated group (\(** P < 0.01; \text{Fig. 7B, C and E} \)). These results demonstrated that VR extract may be effective in inducing airway remodeling in OVA-induced asthmatic mice, producing results similar to dexamethasone.

Decline of AHR induced by VR extract

Penh values were measured to analyze the effect of VR extract in lung function. The OVA-induced asthmatic mice group had higher Penh values than the normal group. In the VR extract group, there was a marked decrease in Penh values with 50
and 100 mg ml⁻¹ doses of methacholine relative to the OVA-induced asthmatic mice group (**P < 0.01; Fig. 8).

Analysis of casticin in the VR extract and serum samples
Casticin was used as the standard for the quantitative analysis. The average casticin content in VR was 0.267 ± 0.012 mg g⁻¹ (n = 3) (data not shown), and the amount of casticin in serum samples was 1.8 ± 0.23 ng g⁻¹ (n = 3). Figure 9 shows the chromatogram of the casticin standard and serum samples.

Discussion
Asthma is characterized by chronic inflammation of the airways and airway remodeling as a result of overproduction of inflammatory cells (16). It is believed that production of the T₂ cytokines IL-4, IL-5 and IL-13 and secretion of the pro-inflammatory cytokine TNF-α are responsible for eosinophil recruitment, AHR and airway remodeling due to the fact that these cytokines are found in high concentrations in allergic sites (17). IL-4 allows for the recruitment of eosinophils, and also leads to the enhanced production of IgE and up-regulation of the IgE-mediated response to inflammatory cells within the airway, while IL-5 is involved in the maturation of eosinophils (17, 18). Eosinophils are the main modulators of airway remodeling factors (19), and eosinophil-mediated damage to airways is a major mechanism underlying the pathogenesis of asthma that occurs via both inflammation and remodeling (20). Murine models have also provided important evidence demonstrating that these eosinophils are crucial regulators of airway remodeling in that eosinophil-deficient mice are protected from the characteristic features of airway remodeling (21–23).

BALB/c mice are sensitive responders to OVA and are a well-established airway inflammatory disease model of human asthma (24). OVA sensitization and subsequent OVA challenge in a murine model induces the infiltration of immune...
cells, including eosinophils and lymphocytes in the BAL fluid and mucus-secreting goblet cell hyperplasia (20).

In recent years, VR extract was reported to have an inhibitory effect on anti-DNP IgE-induced TNF-α production (12) and to have inhibitory activity against T-lymphocyte proliferation (25). In this study, we investigated whether VR extract has an inhibitory effect against allergic inflammation in the OVA-induced asthmatic murine model. VR contains four flavonoids: cascin, artemetin, quercetagetin and 5,3′-dihydroxy-6,7,4′-trimethoxyflavanone (14). The quality of VR extracts was evaluated in terms of cascin, and the amount of cascin in the mouse serum samples treated with VR extracts was quantified. The amount of cascin in serum samples was very small, but could be quantitatively analyzed (Fig. 9).

Since the migration of inflammatory cells into the lungs is inevitable in allergic and asthmatic disorders, we tested whether VR extract inhibited the infiltration of inflammatory cells into the BAL fluid in OVA-challenged mice. The levels of each inflammatory cell type (eosinophils and lymphocytes) were reduced significantly (Fig. 2). We further examined changes in inflammatory cell recruitment in lung tissues of OVA-challenged mice using hematoxylin and eosin staining. Inflammatory cell recruitment into the lung tissue sections isolated from OVA-challenged mice was observed, and treatment with VR extract resulted in a marked decrease in inflammatory cell infiltration (Fig. 5). We also observed an increase in Th2 and pro-inflammatory cytokines following the allergen challenge that was prevented by treatment with VR extract (Fig. 3). The suppression of IL-4 and IL-5 secretions led to a reduction in total IgE levels in response to treatment with VR extract in the serum of the asthmatic mice (Fig. 4). These results suggest that the suppressive effects of VR extract on OVA-induced airway eosinophilia are due to modulation of the Th2 response. Because of the strong effects of VR extract on allergic responses, we measured the alteration in lung functions in OVA-induced asthmatic mice that were treated with VR extract. We confirmed that oral administration of VR extract resulted in a significant reduction in AHR (Fig. 8). Asthma is an inflammatory disorder that involves structural changes in the lungs. One such change is goblet cell hyperplasia, which leads to the obstruction of the airway through excessive mucus production. Another structural change is an increase in SMA-positive cell mass (20). Pulmonary fibrosis is increased by many pathologic agents, and SMA is localized near the fibrotic foci of the airway (26). In this study, the inhibition of both goblet cell hyperplasia and SMA mass was observed in response to treatment with VR extract when compared with OVA-induced asthmatic mice (Figures 6 and 7). We also detected an inhibitory effect of VR on IL-13 and TNF-α, both of which have a role in airway remodeling (Fig. 3C and D). These results suggested that treatment with VR extract can prevent the development of airway remodeling. Our results demonstrate that VR extract is a promising therapeutic candidate that is able to prevent the development of airway remodeling and interferes with eosinophilic inflammation and hyperplasia. These effects result from the inhibition of the Th2 response, indicating that VR extract may have pharmacological effects that would be useful in the treatment of asthma.
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