



Suppressive effect of *Petasites japonicus* extract on ovalbumin-induced airway inflammation in an asthmatic mouse model

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ABSTRACT

Aim of the study: Asthma is a disease marked by airway inflammation. *Petasites japonicus* (Pj) is known as an herb for treating asthma, oxidant stress and gastric ulcer in traditional Oriental medicine. In this study, the inhibitory effects of Pj extract on asthmatic responses were examined both *in vitro* and *in vivo*. **Materials and methods:** The Pj extract was acquired from whole plants of *Petasites japonicus* using 80% ethanol. Cytotoxicity of the Pj extract on Jurkat cells and THP-1 cells was determined using MTT assay. ELISA was performed to determine the expression levels of cytokines, chemokines, and IgE. BALB/c mice were used for an OVA-induced asthmatic mouse model. Reactive oxygen species (ROS) production was stained with 2',7'-dichlorofluorescein diacetate and measured by fluorescence-activated cell sorting analysis. The effects of the Pj extract on leukocyte infiltration and mucus production were determined using periodic acid-Schiff staining as well as hematoxylin and eosin staining.

Results: The Pj extract inhibits the increased release of interleukin (IL)-2, IL-4, IL-5, IL-13, and TNF- α due to house dust mite in Jurkat cells and blocks IL-6 expression in THP-1 cells without cytotoxicity. In the asthmatic mouse model, the Pj extract inhibits eosinophil infiltration, mucus hypersecretion, and IL-5 level in bronchoalveolar lavage (BAL) fluid, and it has a scavenging effect on ROS production of cells in BAL fluid.

Conclusion: The Pj extract has suppressive properties for the pathogenesis of airway inflammation and may be used as a potent agent for the treatment of asthma.

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1. Introduction

Allergic diseases including atopic dermatitis, atopic rhinitis, and asthma are chronic inflammatory diseases mediated by common environmental allergens (Holgate and Polosa, 2008). Asthma is an inflammatory disease of the airway. Its symptoms include wheezing, coughing, tightness of the chest, and shortness of breath induced by bronchial hyperresponsiveness and airway remodeling (Djukanović et al., 1990; Fireman, 2003). The increasing prevalence of asthma is a worldwide problem, and at present, there is a pressing need for the development of new agents for the treatment of this disease.

The pathogenesis of asthma is associated with increased infiltration of inflammatory cells and excessive mucus secretion into the airway (Busse and Rosenwasser, 2003). A variety of cell types such as the type 2 T-helper (Th2) cells, mast cells, and eosinophils are involved in this complex disease. Intake and presentation of allergens by antigen presenting cells induce the activation of Th2 cells. The activated Th2 cells release various cytokines, including interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, and IL-13, and induce the recruitment and mediator secretion of inflammatory cells into the airway (Holgate, 2008). IL-2 stimulates both proliferation and maturation of T cells. IL-4 and IL-13 play important roles in immunoglobulin E (IgE) switching in B cells, mucus hypersecretion, and eosinophil infiltration into the lung tissues (Kips, 2001). Eosinophils are also activated by IL-5, which induces the release of pro-inflammatory mediators and reactive oxygen species (ROS). In the lung tissues, monocytes and macrophages are an important source of lysosomal enzymes, ROS, and cytokines to mediate the infiltration of inflammatory cells and tissue damage (Holgate, 2008). Among inflammatory mediators secreted by macrophages, IL-6 is a pro-inflammatory cytokine and stimulates the growth and differentiation of various cell types in asthma (Hirano, 1998).

Abbreviations: Pj, *Petasites japonicus*; OVA, ovalbumin; BAL, bronchoalveolar lavage; IgE, immunoglobulin E; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species; Dex, dexamethasone.

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The extracts of numerous plants are reported as potent therapeutic materials in various diseases (Yuk et al., 2007; Yang et al., 2008). The extracts of *Petasites* species have also been reported as therapeutic agents for migraines, gastric ulcer, oxidant stress and asthma (Debrenner and Meier, 1998; Danesch, 2004; Sok et al., 2006). *Petasites japonicus* (Pj), known as bog rhubarb or giant butterbur, is an herbaceous perennial plant belonging to the *Asteraceae* family. Although it has been known that Pj extract has therapeutic effect on allergy and asthma in Korea and European countries, its effect on asthma, has not been fully understood (Choi, 2002; Sok et al., 2006).

In the present study, to investigate the inhibitory effects of Pj extract on asthmatic responses, its effects on cytokine release of inflammatory cells were determined *in vitro*. Ovalbumin-specific asthma was induced in a mouse in an *in vivo* study, and the effects of Pj extract on leukocyte infiltration, mucus secretion, and IgE level were determined using an asthmatic mouse model.

2. Materials and methods

2.1. Preparation of the Pj extract

Whole plants of *Petasites japonicus* were collected and standard extract were deposited at the Herbarium of the Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine, Daejeon, Korea and at the Division of Life Science, Daejeon University (TUT), Korea. The plants (100 g) were dried and powdered, and then extracted with 80% ethanol (3×0.5 L) for 2 days at room temperature. The combined liquid extracts were concentrated under reduced pressure.

2.2. Reagents

Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Life Technologies, Inc. (Gaithersburg, MD). Chicken ovalbumin (OVA), aluminum hydroxide, and dexamethasone (Dex) were obtained from Sigma (St. Louis, MO). The total extract of house dust mite, *Dermatophagoides pteronyssinus*, was supplied by Dr. Tai-Soon Yong (Yonsei University College of Medicine, Seoul, Korea). The cell proliferation kit was purchased from Roche (Penzberg, Germany). OptEIA Set human IL-2, IL-4, IL-5, IL-6, IL-8, IL-13, TNF- α , IFN- γ , and MCP-1 were obtained from BD biosciences (San Diego, CA). OptEIA Set mouse IL-5 and mouse IgE were obtained also from BD biosciences (San Diego, CA). 2',7'-Dichlorofluorescein diacetate (DCFDA) was purchased from Molecular Probes (Eugene, OR). Hematoxylin, eosin, and periodic acid-Schiff solution were purchased from Sigma (St. Louis, MO).

2.3. Cell culture

The human Jurkat cells and human monocytic THP-1 cells were purchased from the American Type Culture Collection (Rockville, MD). These cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 g/ml), and were incubated at 37 °C in 5% CO₂. To investigate the Pj extract-induced alterations of cytokine release in mite extract-stimulated cells, cell supernatant was collected and stored at –70 °C until cytokine analysis.

2.4. MTT assay

To determine cell viability, MTT assay was performed using cell proliferation kit. One hundred microliters (100 μ L) of the suspension of Jurkat cells and THP-1 cells with a concentration of 5×10^4 cells/100 μ L was plated in a 96-well plate. Pj extract was added to each individual well in a dose-dependent manner, and

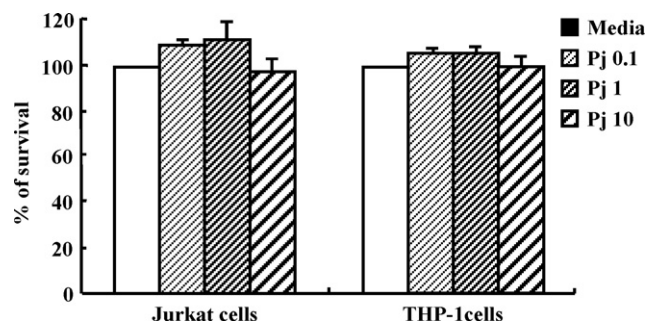


Fig. 1. Pj extract has no cytotoxic effect on Jurkat cells and THP-1 cells. Jurkat cells and THP-1 cells were seeded into 96-well plate at 5×10^4 cells/well and were treated with Pj extract (Pj) at the concentration of 0.1 μ g/ml (Pj 0.1), 1 μ g/ml (Pj 1), or 10 μ g/ml (Pj 10) for 24 h. The survival rate was measured by performing MTT assay as described in Section 2.5. The data are expressed as the relative ratio to the absorbance of the untreated cells, which was set at 100%. All data were expressed as the means $\pm$ S.E.M. of three independent experiments.

the plate was incubated for 24 h at 37 °C in a CO₂ incubator. After 10 μ L of MTT solution was added to each well, the plate was incubated 37 °C for 4 h. At the end of that period, 100 μ L of solubilization solution (10% SDS in 0.01 M HCl) was added to each well. After 24 h incubation, the absorbance at 550 nm was measured using an ELISA reader (Bio-Tek Instruments, Winooski, VT).

2.5. Cytokine measurement

To determine the concentrations of cytokines in the supernatant of Jurkat cells and THP-1 cells, a sandwich enzyme-linked immunosorbent assay (ELISA) was performed using OptEIA Set human IL-2, IL-4, IL-5, IL-6, IL-8, IL-13, TNF- α , and MCP-1 according to the manufacturer's instructions. In the BAL fluid or serum of mice, the concentrations of cytokines and the total IgE were measured using OptEIA Set mouse IL-5 and IgE according to the manufacturer's instructions. To measure OVA-specific IgE in the BAL fluid or serum of mice, a 96-well plate was coated by 100 μ g/ml of OVA at 4 °C overnight, and OVA-specific IgE was measured using OptEIA Set mouse IgE according to the manufacturer's instructions. All assays were performed in triplicate. The concentration of each protein was calculated from the standard curve.

2.6. Induction of asthma in BALB/c mice and drug administration

Six-week-old female BALB/c mice were obtained from Daehan Biolink Co. LTD (Seoul, Korea) and kept in an air-conditioned room at room temperature (about 22 ± 1 °C) and a humidity of about $55 \pm 10\%$. The mice were divided into six groups ($n = 10$), and the allergic lung inflammation of the mice in five groups was induced by OVA (Grade III) using the established protocol in a previous study (Yuk et al., 2007). Four groups of the asthma-induced mice were treated with oral injection of 50 mg/kg, 250 mg/kg, and 500 mg/kg of Pj extract in a dose-dependent manner and 3 mg/kg of Dex, respectively. The normal group was sensitized and challenged with PBS without OVA and drug treatment.

2.7. Collection of bronchoalveolar lavage (BAL) fluid

At day 28 from the first OVA sensitization, the mice were sacrificed, and BAL fluid was collected by lavage of the lung via the trachea with 1 ml of PBS. After three lavages, BAL fluid was collected, and the total cells were separated from the supernatant of BAL fluid. The cells in BAL fluid were used for the determination of total cells and differential counts, as well as the level of intracellular ROS. Total cell number was counted using a Neubauer hemocyt-

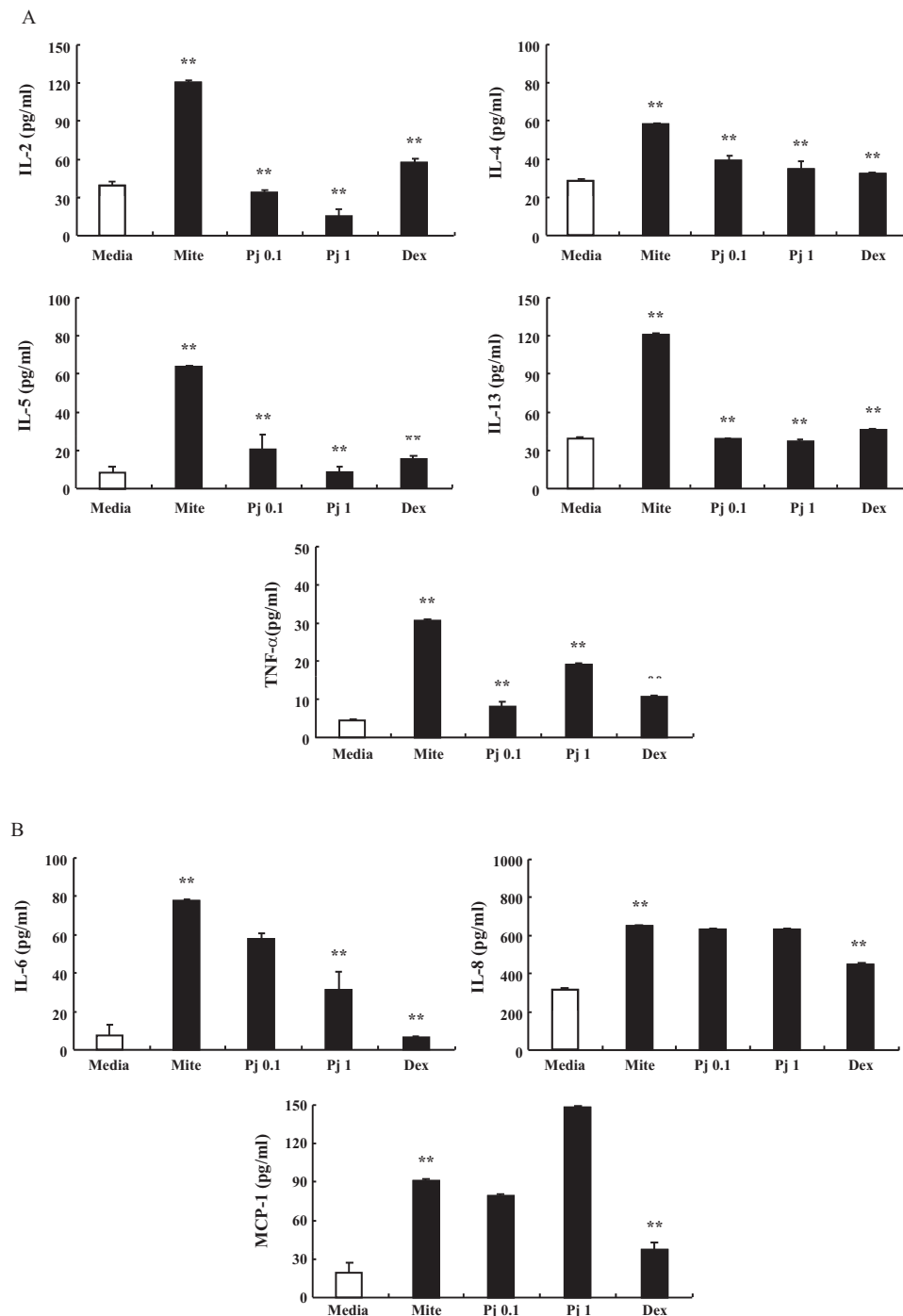


Fig. 2. Pj extract has inhibitory effects on cytokine release induced by mite extract in Jurkat cells and THP-1 cells. Jurkat cells (A) and THP-1 cells (B) were seeded into 24-well plate at 1×10^6 cells/ml, and were pre-treated in the absence (mite) or presence of the Pj extract (Pj) at the concentration of 0.1 $\mu\text{g/ml}$ (Pj 0.1) or 1 $\mu\text{g/ml}$ (Pj 1), or with 1 $\mu\text{g/ml}$ of dexamethasone (Dex) for 1 h. The cells were treated with 10 $\mu\text{g/ml}$ of mite extract for 24 h. The supernatants were collected and analyzed by ELISA as described in Section 2.5. All data were expressed as the means $\pm$ S.E.M. of three independent experiments. ** $p < 0.01$ was considered a significant difference between the untreated group and mite group or between the mite group and Pj-treated group.

tometer, and the cells were cytopspined and stained with Wright stain solution for differential counts. The cells in BAL fluid were divided into neutrophils, eosinophils, lymphocyte, and alveolar macrophage according to general leukocyte morphology and stain features. The percentage of each cell was determined by counting 300 cells per cytopspined slide. The supernatant was stored at -70°C until analysis of cytokines and immunoglobulin E (IgE).

2.8. Measurement of intracellular ROS

The cells in the BAL fluid were washed and resuspended with PBS, and 3.3 μM of DCFDA was added to label the intracellular ROS. The cells were then incubated for 10 min at room temperature. Labeled cells were immediately observed using fluorescence-activated cell sorting (FACS) analysis (BD Biosciences).

2.9. Analysis of histological alteration in airway inflammation

Lung tissues of mice were excised and fixed with 10% neutral formaldehyde solution for 24 h at room temperature. The fixed tissues were embedded in paraffin and cut into 5 μ m sections using a microtome (Leica, Nussloch, Germany). Hematoxylin and eosin stain was performed to evaluate the infiltration of inflammatory cells into the peribronchial connective tissues. Periodic acid-Schiff staining was performed to examine the mucus secretion around the peribronchial tissues. To estimate the severity of leukocyte infiltration, a peribronchial cell count based on a five-point scoring system was performed as previously described (Duan et al., 2004). The scoring system was as follows: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; and 4, a ring of cells greater than 4 cells deep. To evaluate the extent of mucus production, goblet cell hyperplasia in the airway epithelium was performed using a five-point scoring system as previously described (Kuperman et al., 2002). The scoring system was as follows: 0, no goblet cells; 1, lower than 25%; 2, 25–50%; 3, 50–75%; and 4, higher than 75%. The scoring of the leukocytes and goblet cells was examined in three independent fields of lung section from each mouse.

2.10. Statistical analysis

Data were expressed as the means $\pm$ S.E.M. The statistical significance of any difference was determined by one-way ANOVA followed by LSD and Turkey. The SPSS statistical software package (Version 10.0, Chicago, IL) was used for the statistical analysis. A significant value was defined as $p < 0.05$.

3. Results

3.1. Pj extract has no cytotoxic effect on Jurkat cells and THP-1 cells

The cytotoxic effect of Pj extract on Jurkat cells and THP-1 cells was determined. The survival rates of these cells were not altered by treatments with the Pj extract for 24 h in a dose-dependent manner (Fig. 1). These results indicate that the Pj extract is not cytotoxic to Jurkat cells and THP-1 cells.

3.2. Pj extract inhibits the release of mite extract-induced cytokines from Jurkat cells and THP-1 cells

Jurkat cells and THP-1 cells are reflected as human T cells and monocytic cells that act on the pathogenesis of asthma. To investigate the level of chemokines and cytokines associated with asthma, Jurkat cells and THP-1 cells were pre-treated with the Pj extract for 1 h in a dose-dependent manner and treated with 1 μ g/ml of mite extract for 24 h. The protein expressions of IL-2, IL-4, IL-5, IL-13, and TNF- α in Jurkat cells, and IL-6, IL-8, and MCP-1 in THP-1 cells were evaluated using ELISA. Pj extract considerably decreases the elevated levels of cytokines by mite extract in Jurkat cells (Fig. 2A). Although the expression levels of MCP-1 and IL-8 in THP-1 cells were not changed by the Pj extract, the increased IL-6 levels in THP-1 cells were significantly suppressed by the Pj extract (Fig. 2B). Dex was used as a positive control for its inhibitory effects. These results indicate that the Pj extract has anti-inflammatory effects by diminishing cytokine production in T cells and monocytic cells.

3.3. Pj extract has an inhibitory effect on the infiltration of eosinophils into the airway and on ROS production

To investigate the anti-asthmatic effect of the Pj extract *in vivo*, asthma was induced in BALB/c mice using OVA. The total

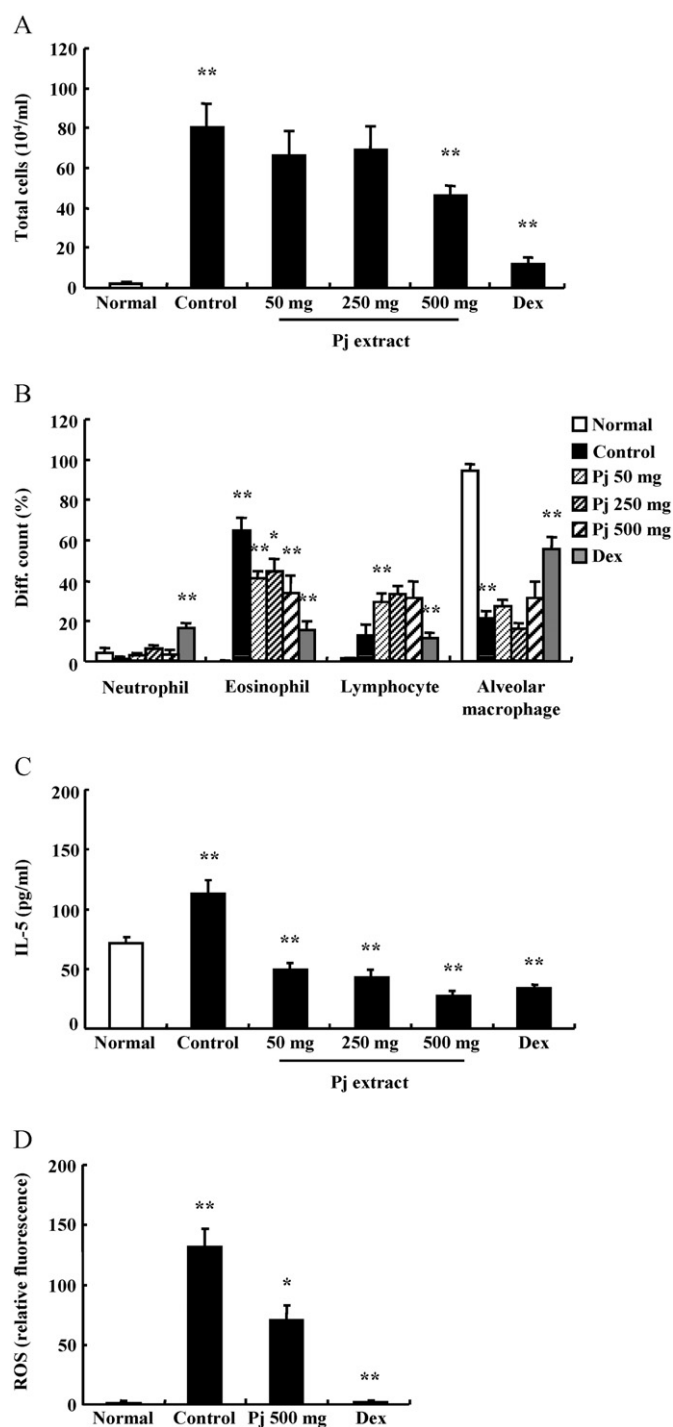


Fig. 3. Pj extract reduces the recruitment of inflammatory cells into BAL fluid and ROS production. The mouse model of OVA-induced asthma was divided into 5 treatment groups; untreated (control), the three Pj-treated groups (50 mg/kg, 250 mg/kg, and 500 mg/kg), and the dexamethasone-treated group (Dex). The negative control group (Normal) was sensitized and treated with PBS without drug administration. The recovered cells in BAL fluid were used for total cell counts (A) and used for differential counts after Wright's staining (B). The differential counts were performed as described in Section 2.7. The supernatant from the BAL fluid was used for determination of released IL-5 (C). Level of IL-5 was analyzed by ELISA as described in Section 2.5. To measure ROS production, the cells of BAL fluid were incubated for 10 min with PBS containing 3.3 μ M of 2',7'-DCFDA and then observed by FACS analysis (D). The results were expressed as the means $\pm$ S.E.M. of 10 independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate statistically significant differences between the normal group and asthma control group, or between the asthma control group and drug-treated group.

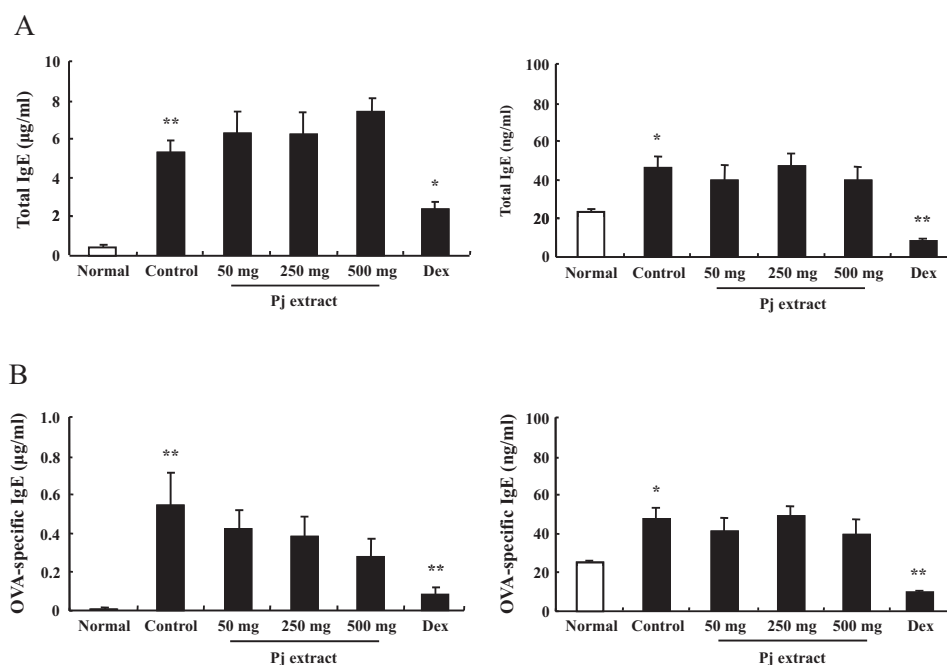


Fig. 4. Pj extract slightly decreases the level of OVA-specific IgE in serum. Both total IgE (A) and OVA-specific IgE (B) in the serum (left panel) and in the BAL fluid (right panel) were measured by ELISA as described in Section 2.5. The results were presented as the means $\pm$ S.E.M. of 10 independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate statistically significant differences between the normal group and asthma control group, or between the asthma control group and drug-treated group.

number of leukocytes and the alteration of the cellular components in BAL fluid of mice were evaluated. The total leukocytes in asthma-induced mice were remarkably increased as compared to the normal group, and leukocytosis was significantly inhibited by the administration of 500 mg/kg of Pj extract in the asthma-induced mice groups (Fig. 3A). Dex is one of the anti-inflammatory drugs used in the treatment of asthma and its action is associated with inhibition of pro-inflammatory transcription factors. Dex was used as a positive control for its inhibitory effects on asthmatic response. In addition, the alteration of cellular components, especially eosinophils, in the airway is an important step in the pathogenesis and treatment of asthma. To examine the number of leukocytes in BAL fluid, the cells were stained with Wright's stain, and differential counts of leukocytes were performed. The Pj extract significantly decreased eosinophilia as compared to the asthmatic control in a dose-dependent manner (Fig. 3B). Since the activation and recruitment of eosinophils are particularly modulated by IL-5 (Elsner and Kapp, 1999), the IL-5 expression in BAL fluid was evaluated. The IL-5 level increased after asthma induction, and the increased expression was remarkably inhibited by the Pj extract (Fig. 3C). These results indicate that the inhibitory effect of the Pj extract on eosinophilia is associated with the modulation of IL-5 release. The Pj extract also significantly decreased ROS production in the cells of BAL fluid (Fig. 3D).

3.4. Pj extract slightly decreases the release of OVA-specific IgE in serum

To determine the effect of the Pj extract on the levels of total IgE and OVA-specific IgE, the total IgE and OVA-specific IgE in the serum and BAL fluid were analyzed using ELISA. The total IgE and OVA-specific IgE in the serum and BAL fluid were considerably increased in the asthmatic control as compared to the normal group (Fig. 4). Although both levels of IgE in the serum and BAL fluid were not significantly suppressed by the Pj extract, it slightly diminished the level of OVA-specific IgE in the serum in a dose-dependent manner (Fig. 4B).

3.5. Pj extract reduces the pathological changes of the lung tissue in airway inflammation

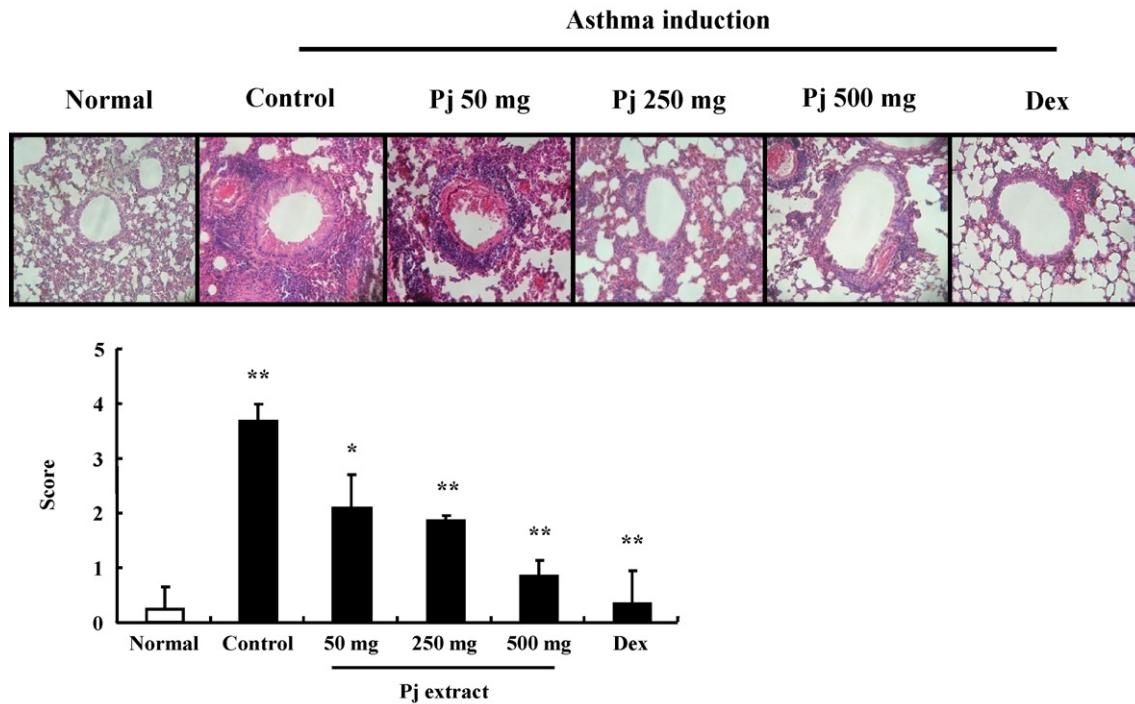
To investigate the effects of the Pj extract on the pathological changes of the lung tissue in an asthmatic mouse model, the lung tissues were stained with hematoxylin and eosin and periodic acid-Schiff staining. The asthmatic control showed increased leukocytosis in the peribronchial tissues (Fig. 5A) and mucus hypersecretion by goblet cells into the bronchi (Fig. 5B) compared with normal lung tissues. The Pj extract, however, significantly inhibited these pathological changes in a dose-dependent manner (Fig. 5). Both inflammatory and mucus scores represent the suppressive effects of Pj extraction on airway inflammation with statistical significance. This is in agreement with the results shown in Fig. 3A and B, which suggest that the Pj extract decreases histological alteration in the lung tissues in asthma.

4. Discussion

In this study, the possibility of herbal extract from Pj as an anti-asthmatic drug was examined using human inflammatory cell line and an OVA-induced asthmatic mouse model. The results show that the Pj extract decreased cytokine release from Jurkat cells and THP-1 cells without cytotoxicity. In an *in vivo* study, the Pj extract suppressed eosinophil infiltration and excessive mucus secretion into the airway. It also significantly inhibited IL-5 release and ROS production in the lung tissues.

As shown in Figs. 3 and 5, the number of leukocytes, particularly eosinophils, in the peribronchial tissues was increased in the asthmatic group, and the infiltration was diminished by the Pj extract. The activation and migration of inflammatory cells into the inflammatory site are modulated by various cytokines and chemokines. Among these cytokines, Th2 cytokines such as, IL-3, IL-4, IL-5, IL-6, and IL-13 are closely associated with the pathogenesis of asthma. Th1 cytokine such as IL-2 activates T cell proliferation. IL-5 plays a major factor in the maturation and recruitment of eosinophils into the airway (Robinson et al., 1999). IL-6 regulates the proliferation,

A



B

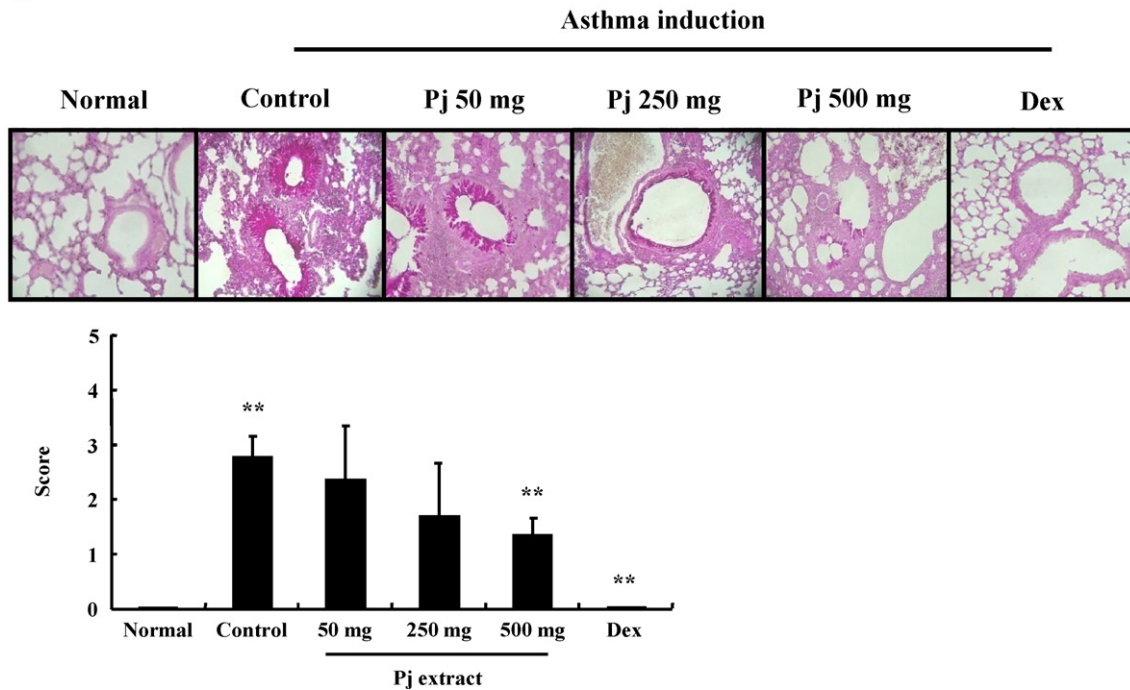


Fig. 5. Pj extract reduces the pathologic changes of the lung tissue in airway inflammation. The cell infiltration in the lung tissues was confirmed by hematoxylin and eosin staining (A). To identify the mucus hypersecretion in the lung tissues, we performed periodic acid-Schiff staining (B). Magnification 200 $\times$. The scored the cell infiltration (lower panel of A) and mucus production (lower panel of B) in the lung tissues were calculated as described in Section 2.9. The results were presented as the means $\pm$ S.E.M. of 10 independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate statistically significant differences between the normal group and asthma control group, or between the asthma control group and drug-treated group.

activation, and survival of various cell types, including eosinophils (Lucey et al., 1996; Hirano, 1998). In the *in vivo* results, the Pj extract inhibited IL-5 expression in BAL fluid. It also inhibited the increased levels of IL-2, IL-4, IL-5, IL-13, and TNF- α due to mite extract in Jurkat T cells and blocked the increased IL-6 release from THP-1

cells (Fig. 2). House dust mite acts as a common allergen in asthmatic patients and increases several cytokine levels from various cells during asthmatic response (Arlian and Platts-Mills, 2001; Lee et al., 2008). These results indicate that the Pj extract has suppressive properties on the pathogenesis of asthma associated with mite

allergen. The increased secretion of mucus and the hyperplasia of goblet cells in the lung tissue were significantly decreased by the Pj extract (Fig. 5B). However, the IgE level was slightly inhibited without statistical significance. Therefore, these results indicate that the suppressive effect of the Pj extract on leukocytosis and mucus hypersecretion into the airway may be associated with the inhibitory effect of the Pj extract on the secretion of cytokines.

Pj consists of flavonoid glycoside, fukinolic acid, and petasiformin A (Hirono et al., 1977; Hasa and Tazaki, 2004; Sok et al., 2006). In various compounds of *Petasites japonicus*, flavonoid glycosides and other natural flavonoids are also isolated from plants. This compound shows allergy-preventive effects and suppressive effects on the production of nitric oxide synthase and cytokines induced by inflammatory responses (Comalada et al., 2005; Choi et al., 2008; Iwaoka et al., 2009). Fukinolic acid has biological activities including anti-inflammatory and anti-oxidant properties. The degranulation of IgE-sensitized RBL-2H3 mast cells and the activation of neutrophils elastase are inhibited by fukinolic acid (Löser et al., 2000; Shimoda et al., 2006). Petasiformin A has a scavenging effect on ROS production and protects against cellular DNA and tissue damage (Lin et al., 2004). At present, the exact compound of the Pj extract as an anti-asthmatic material is not yet known, but it is assumed that it contains anti-inflammatory materials, such as the above compounds.

5. Conclusion

In conclusion, the Pj extract blocks leukocytosis, excessive mucus secretion by goblet cells, increased cytokines, and ROS production in an asthmatic mouse model. Although the inhibitory mechanism of the Pj extract against asthmatic response is not fully understood, it may have anti-inflammatory properties for airway inflammation and may be beneficial for the treatment of asthma.

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