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Inhibitory effects of *Duchesnea chrysantha* extract on ovalbumin-induced lung inflammation in a mouse model of asthma

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ABSTRACT

Ethnopharmacological relevance: Duchesnea chrysantha (*D. chrysantha*) is a herb with anti-oxidative, anti-inflammatory and immune-enhancing properties.
Aim of the study: Asthma is an inflammatory disease of the lungs, and the hallmarks of the disease are increased inflammatory cell infiltration into the airways and poor respiratory function. Although there is the possibility that *D. chrysantha* may have an inhibitory effect on lung inflammation, the effects of *D. chrysantha* on asthma have not been fully investigated. In the present study, we investigated the anti-inflammatory activity of *D. chrysantha* extract (Dc extract) on lung inflammation in a murine model of ovalbumin-induced asthma.
Materials and methods: Dc extract was obtained from dried and powdered whole plants of *D. chrysantha* using 80% ethanol. BALB/c mice induced by ovalbumin sensitization and nebulization were used as a mouse model of asthma. RT-PCR and ELISA were performed to measure mRNA and protein expression of cytokines. We examined the effects of Dc extract on leukocyte infiltration and mucus secretion using periodic acid-Schiff staining as well as hematoxylin and eosin staining.
Results: Dc extract significantly inhibited leukocytosis and eosinophilia in the bronchoalveolar lavage (BAL) fluid ($p < 0.01$). Dc extract significantly reduced the elevated infiltration of inflammatory cells ($p < 0.05$) and inhibited the increased mucus secretion, despite the absence of significant value. Although Dc extract weakly inhibited the mRNA expression of IL-4, IL-5, IL-13, and eotaxin, it strongly inhibited the protein expression of IL-5 ($p < 0.05$) and eotaxin ($p < 0.01$) in BAL fluid. Ovalbumin-specific IgE levels in the serum and BAL fluid were blocked by Dc extract ($p < 0.05$).
Conclusions: These results suggest the possibility that Dc extract can exert suppressive effects on asthma and may provide evidence that Dc extract is a useful agent for the treatment of allergic airway disease.

1. Introduction

Duchesnea chrysantha (*D. chrysantha*), which belongs to the *Rosaceae* family, is a perennial plant, and has been traditionally known as an herb with anti-oxidative, anti-inflammatory and immune-enhancing properties. *D. chrysantha* extract (Dc extract) has been used for the prevention and treatment of inflammatory diseases and cancers in traditional Asian medicine throughout East Asia. Although it has been recently reported that the phenolic compounds of *D. chrysantha* have cytotoxic activities and inhibit the survival of human cancer cells, the effect of *D. chrysantha* on inflammatory diseases, such as asthma, has not been fully understood (Lee and Yang, 1994; Kim et al., 2007).

Asthma is an inflammatory disease of the lungs characterized by increased infiltration of leukocytes, especially eosinophils, into the airways and reduced respiratory function. The inflammation leads to bronchoconstriction, increased airway hyperresponsiveness, and mucus production (Djukanovic et al., 1990). The prevalence of asthma is rapidly increasing around the world, especially in young children, and it has become a significant cause of morbidity and mortality in developed countries (Braman, 2006).

Both eosinophils and T helper (Th) 2 lymphocytes play pathogenic roles in asthma (Busse and Rosenwasser, 2003).

Abbreviations: *D. chrysantha*, *Duchesnea chrysantha*; Dc extract, *D. chrysantha* extract; BAL, bronchoalveolar lavage; Th, T helper; IgE, immunoglobulin *E*; i.p., intraperitoneal; ELISA, Enzyme-linked immunosorbent assay.

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Eosinophils are commonly associated with allergic inflammation, and act as effector cells in the pathogenesis of this disease by releasing cytotoxic granule proteins (Kay, 2001). Eotaxin is a potent chemoattractant for eosinophils and it is generally elevated after asthma induction. An imbalance between Th1 and Th2 leads to the clinical expression of allergic disease. Th2 cytokines, including IL-4, IL-5, and IL-13, typically increase in allergic diseases and have important effects on airway infiltration, eosinophil activation, induction of immunoglobulin E (IgE) production, mucus secretion and the release of a variety of inflammatory mediators (Woodfolk, 2006).

In the present study, we investigated whether Dc extract has an anti-inflammatory effect on the inhibition of leukocyte infiltration into the airways, mucus production, and the expression of Th2 cytokines and IgE in a murine model of ovalbumin-induced asthma. The inhibitory effects of Dc extract on asthma were compared with those of dexamethasone, an attractive anti-inflammatory drug (Caramori and Adcock, 2005).

2. Materials and methods

2.1. Preparation of the D. chrysantha extract

Whole plants of *D. chrysantha* Miq (Rosaceae) were collected in Okchon, Chungbuk, Korea, in October 2004 and were identified by Professor J.-H. Kim. A voucher specimen (number 86) and standard extract have been deposited at the Herbarium of the Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine, Daejeon, Korea and Division of Life Science, Daejeon University (TUT), Korea. The dried and powdered whole plants of *D. chrysantha* (36 g) were extracted with 80% ethanol (3 × 0.5 l) for 2 days at room temperature. The combined liquid extracts (4.84 g) were concentrated under reduced pressure.

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

Six-week-old female BALB/c mice were purchased from Daehan Biolink Co. Ltd. (Seoul, Korea) and maintained in an air-conditioned room at room temperature (about 22 ± 1 °C) and a humidity of about 55 ± 10%. The mice were divided into four groups (n = 10), and airway inflammation was induced by ovalbumin (Grade III; Sigma–Aldrich, Korea) in three groups using the method described by Yuk et al. (2007). Briefly, each mouse was immunized by intraperitoneal (i.p.) injection of 20 μg of chicken ovalbumin (Grade III; Sigma–Aldrich) with 1 mg aluminum hydroxide (Sigma–Aldrich) on days 1 and 14. The ovalbumin-challenged mice were exposed to inhalation with 5% ovalbumin solution aerosolized using an ultrasonic nebulizer (ME-U12, Omrom, Tokyo, Japan) for 1 h per day from days 21 to 27 after the second sensitization. Two groups of asthma-induced mice were treated with oral injection of 50 mg/kg of *D. chrysantha* extract or with i.p. injection of 1 mg/kg of dexamethasone (Sigma–Aldrich) between days 14 and 27, respectively. *D. chrysantha* extract and dexamethasone are dissolved in ethanol or DMSO, respectively and then was diluted to less than 1/100 with PBS. The normal group was sensitized and challenged with PBS without drug treatment.

2.3. Collection of bronchoalveolar lavage (BAL) fluid

BAL fluid was collected by lavaging the lung via the trachea with 1 ml of PBS. After five lavages, approximately 0.7 ml of BAL fluid was recovered and centrifuged (400 × *g*, 4 °C, 5 min). The supernatant was stored at −70 °C for the measurement of cytokines. The cells in the BAL fluid were resuspended in 100 μl of PBS for total cell and differential counts. After the measurement of total cell number using a Neubauer hemocytometer, the cells suspended in PBS were applied to a slide by cytospinning and stained with Wright stain solution. The percentage of each leukocyte was determined, and the absolute leukocyte count was calculated by multiplying each leukocyte percentage by the total cell count.

2.4. Histological analysis

Lung tissues were detached from the mice and fixed with Carnoy's solution overnight at 4 °C. The fixed tissues were embedded in paraffin and cut into 4-μm sections with a microtome (Leica, Nussloch, Germany). The sections were placed on slide glasses, deparaffinized, and stained with hematoxylin and eosin (Sigma, Korea) in order to examine the cells that had infiltrated into the peribronchial connective tissues or with periodic acid-Schiff stain (Sigma) to evaluate mucus production. A peribronchial cell count based on a five point scoring system was performed as previously described to estimate the severity of leukocyte infiltration (Duan et al., 2004). The scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cell layers deep; and 4, a ring of cells more than 4 cell layers deep. Mucus hypersecretion by goblet cells in the airway epithelium was analyzed using a five-point scoring system as previously described (Kuperman et al., 2002). The scoring system was: 0, no goblet cells; 1, less than 25%; 2, 25–50%; 3, 50–75%; and 4, more than 75%. The leukocyte and goblet cell scoring was examined in three independent fields of lung section from each mouse.

2.5. Semi-quantitative RT-PCR

RT-PCR was performed to measure the relative mRNA quantities of IL-4, IL-5, IL-13, and eotaxin in the lung. The lung samples collected from the mice were homogenized, and the total RNA was extracted with Trizol reagent according to the manufacturer's instructions. Reverse transcription was carried out using the first-strand cDNA synthesis kit (Promega, Madison, WI). The primer sequences were as follows: mouse IL-4, 5′-TCT CTA GAT CAT GGG CAT TTT GAA CGA GGT C-3′and 5′-TGC ATG ATG CTC TTT AGG CTT TCC-3′; mouse IL-5, 5′-ATG ACT GTG CCT CTG TGC CTG GAG C-3′ and 5′-CTG TTT TTC CTG GAG TAA ACT GGG G-3′; mouse IL-13, 5′-CTC CCT CTG ACC CTT AAG GAG-3′ and 5′-GAA GGG GCC GTG GCG AAA CAG-3′, mouse eotaxin; 5'-AGA GGC TGA GAT CCA AGC AG-3′ and 5′-CAG ATC TCT TTG CCC AAC CT-3′; mouse β-actin, 5′-AGG CTG TGC TGT CCC TGT ATG C-3′ and 5′-ACC CAA GAA GGA AGG CTG GAA A-3′. The cDNA products were denatured at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. β-actin was used as an internal control for each PCR reaction. The PCR products were analyzed via 1% agarose gel electrophoresis.

2.6. Measurement of cytokine, aspartate aminotrasferase (AST) and alanine aminotransferase (ALT)

The concentrations of IL-4, IL-5, IL-13, eotaxin and IgE in the BAL fluid or serum were measured by a sandwich ELISA using OptEIA Set mouse IL-4, IL-5 and IgE (BD Biosciences, San Diego, CA) and Quantikine kit IL-13 and eotaxin (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All assays were performed in triplicate. The concentration of each protein was calculated from the standard curve. Both AST and ALT kits (Shinyang, Seoul, Korea) were used for the measurement of AST and ALT in serum, according to the manufacturer's manual. The data are expressed as the relative ratio to the normal group, which was set at 100%.

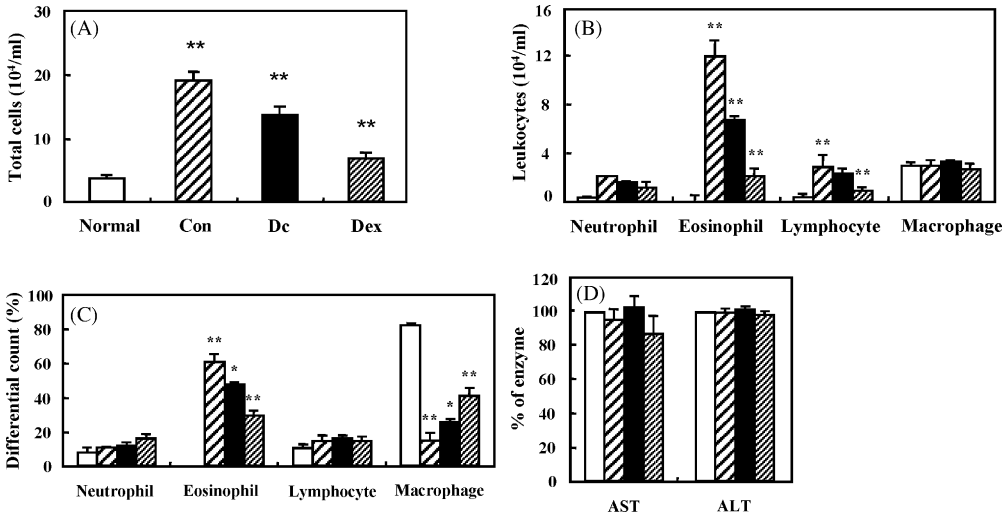

Fig. 1. Effect of *D. chrysantha* extract on the recruitment of inflammatory cells in BAL fluid obtained from OVA-induced asthma model mice. Ovalbumin-induced asthma model mice were divided into 3 treatment groups: untreated (Con; thick hatched bar), treated with 50 mg/kg of Dc extract (Dc; closed bar), or treated with 1 mg/kg of dexamethasone (Dex; thin batched bar) between the sensitization and inhalation periods. The negative control group (Normal; open bar) was sensitized and treated with PBS without drug administration. The number of cells in the fluid was totally and differentially counted after Wright's staining (A, B and C). The levels of AST and ALT were measured as described in Section 2 (D). The results were expressed as the means ± S.E.M. of ten independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$ were considered indicative of statistically significant differences between the normal group and asthma control group or between the asthma control group and drug-treated group.

Statistical analysis

The statistical significance of any difference was determined by one-way ANOVA. The data are expressed as the means ± S.E.M. The SPSS statistical software package (Version 10.0, Chicago, IL) was used for the statistical analysis. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Dc extract inhibits the infiltration of total leukocytes and eosinophils into the airways

To examine the inhibitory effect of *D. chrysantha* in asthma, we induced asthma in BALB/c mice using ovalbumin and evaluated the number of total leukocytes and eosinophils in the BAL fluid. Dc extract significantly inhibited leukocytosis (30%) and eosinophilia (40%) ($p < 0.01$) (Fig. 1A and B). Differential leukocyte counting showed that the Dc extracts decreased the number of eosinophils and increased the number of macrophages in comparison with the control group ($p < 0.05$) (Fig. 1C). In addition, Dc extract has no effect on alteration of AST and ALT level as compared to normal group (Fig. 1D). These results indicate that Dc extract reduces lung inflammation in an asthma model.

3.2. Dc extract reduces the degree of pathological alteration of lung tissues in a mouse model of asthma

Hematoxylin and eosin and periodic acid-Schiff staining were performed on the lung tissues to analyze the effects

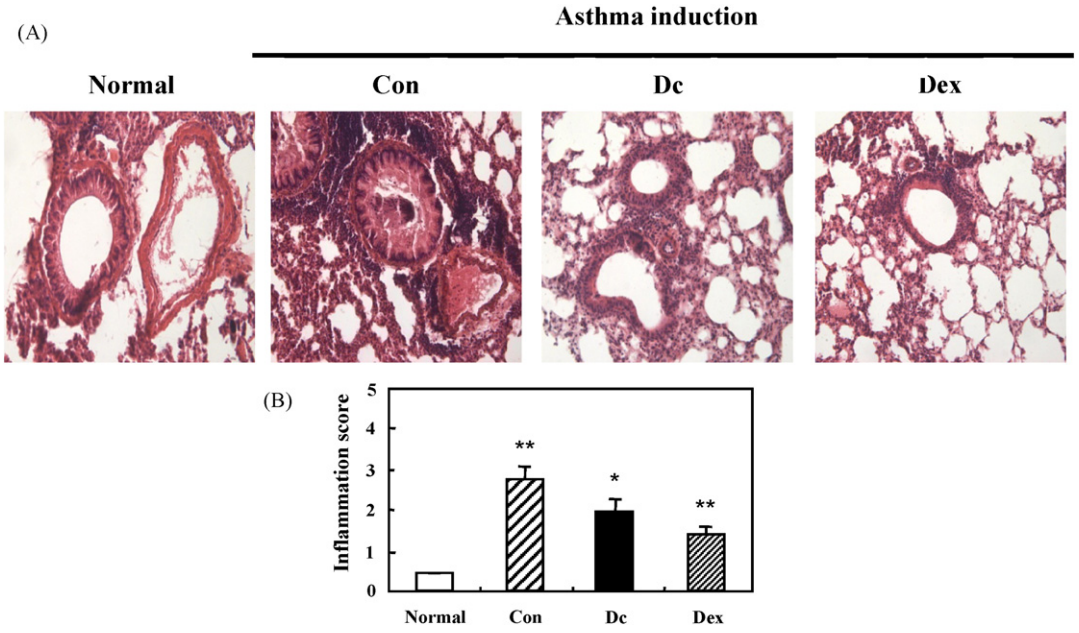

Fig. 2. Effect of *D. chrysantha* extract on airway inflammation caused by cell infiltration in lung tissues. To confirm the pathologic changes in lung tissues, we performed hematoxylin and eosin staining (A) and scored the inflammatory cell infiltration in the lung tissues (B) as described in Section 2. Magnification ×200.

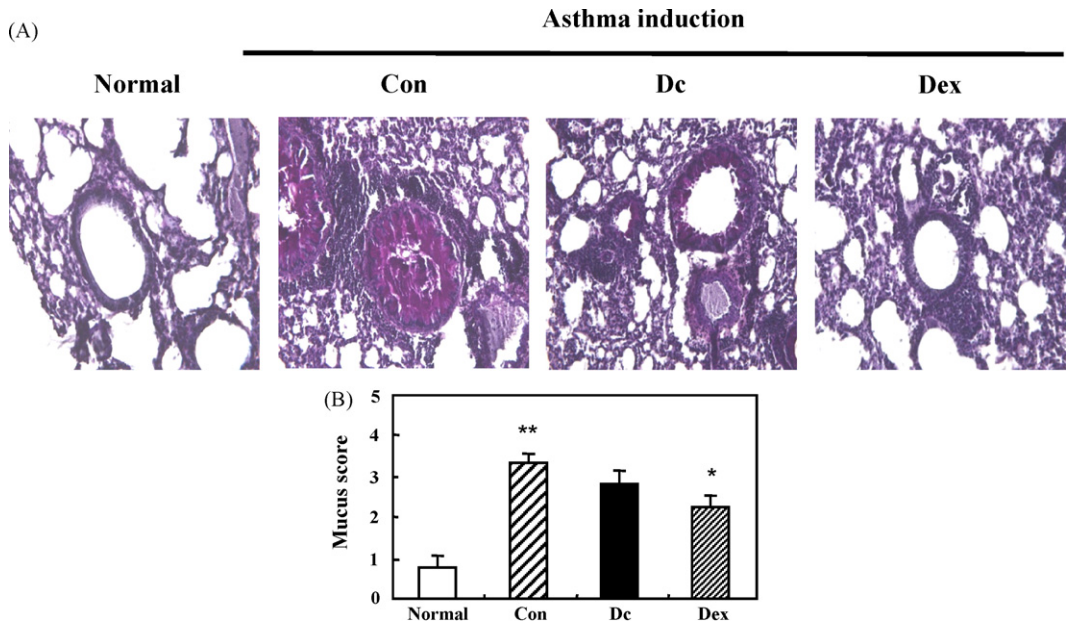

Fig. 3. Effect of *D. chrysantha* extract on mucus hypersecretion by lung tissues. To confirm the pathologic changes in lung tissues, we performed periodic acid-Schiff staining (A) and scored mucus production in the lung tissues (B) as described in Section 2. Magnification ×200.

of Dc extract on histological feature of asthma. The lung tissue obtained from ovalbumin-induced asthmatic mice was characterized by dense peribronchial inflammation due to leukocyte infiltration and mucus hyperproduction by goblet cells within the bronchi when compared with the normal tissue. This inflammation resulted in the narrowing of the bronchi (Fig. 2). Dc extract significantly reduced the degree of inflammatory cell infiltration ($p < 0.05$). Dc extract also decreased the degree of mucus secretion, but this decrease was not significant (Fig. 3). These results indicate that Dc extract reduces the degree of pathological inflammation in lung tissues in asthma ($p < 0.05$).

3.3. Dc extract decreases cytokine expression in lung tissues from asthma model mice

Because Dc extract reduced inflammation in the lung tissues of ovalbumin-induced asthma model mice, we examined the mRNA and protein expression of cytokines and chemokines associated with asthma in lung tissue. Dc extract weakly inhibited the increased mRNA expression of IL-4 and IL-5 following the induction of asthma and significantly inhibited the increase in the protein level of IL-5 ($p < 0.05$) (Fig. 4). The mRNA expression of IL-13 was elevated following the induction of asthma and reduced by Dc extract, but the protein expression of IL-13 remained constant between experimental groups. Dc extract significantly decreased the increased protein level of eotaxin in BAL fluid by 50%, which is similar to the effect of dexamethasone ($p < 0.01$). These results are in agreement with the data shown in Figs. 1–3.

3.4. Dc extract regulates both total IgE and ovalbumin-specific IgE levels in serum and BAL fluid

Since IgE is associated with the pathogenesis of allergic asthma, we investigated whether Dc extract modulates the concentration of total IgE and ovalbumin-specific IgE in serum and BAL fluid. Total and ovalbumin-specific IgE in serum significantly increased following the induction of asthma ($p < 0.01$) (Fig. 5A and C). Dc extract weakly inhibited the total IgE level but strongly inhibited the ovalbumin-specific IgE level in the serum and BAL fluid ($p < 0.05$) (Fig. 5B and D). These results indicate that Dc extract uniquely blocks ovalbumin-specific IgE in both serum and BAL fluid.

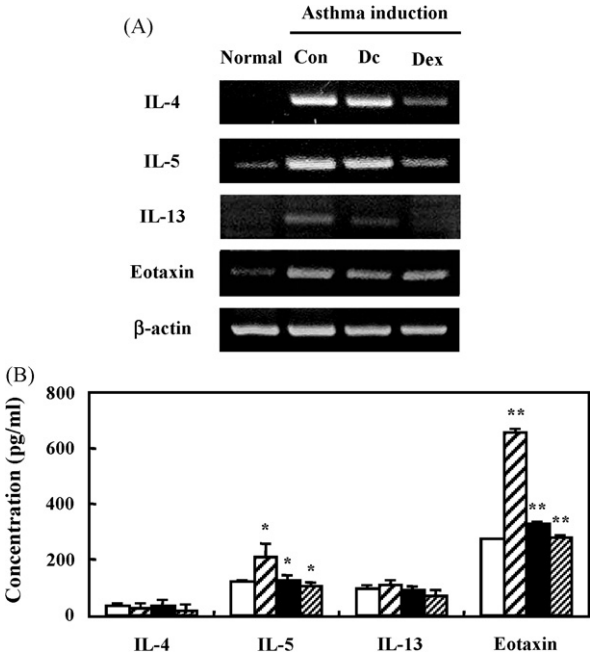

Fig. 4. Effect of *D. chrysantha* extract on the expression of IL-4, IL-5, IL-13 and eotaxin in lung tissues and BAL fluid. (A) The total RNA was extracted from each lung tissue, and the mRNA expression of IL-4, IL-5, IL-13 and eotaxin were analyzed by RT-PCR as described in Section 2. β-actin was used as an internal control for each PCR reaction. The data are expressed as representative of ten independent experiments. (B) The BAL fluid was collected the cells in the fluid were eliminated. The protein expression of IL-4, IL-5, IL-13 and eotaxin in BAL fluid was analyzed by ELISA. The data are expressed as the means ± S.E.M. of ten independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered indicative of significant differences between the normal group and asthma control group or between the asthma control group and drug-treated group.

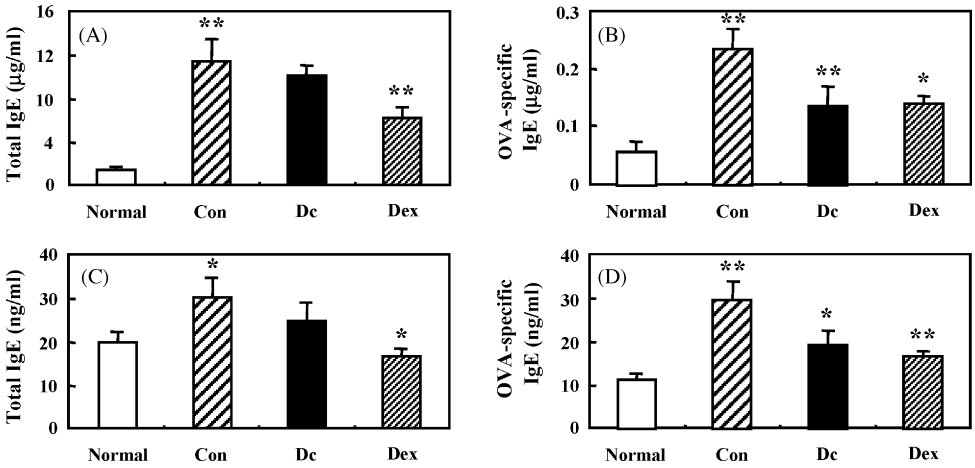

Fig. 5. Effect of *D. chrysantha* extract on the total IgE and ovalbumin-specific IgE levels in the serum and BAL fluid. The total and ovalbumin (OVA)-specific IgE levels in the serum (A and B) and BAL fluid (C and D) were analyzed by ELISA as described in Section 2. The data are expressed as the means ± S.E.M. of ten independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered to be indicative of significant differences between the normal group and asthma control group or between the asthma control group and drug-treated group.

4. Discussion and conclusion

Asthma is a chronic inflammatory disease characterized by airway inflammation and remodeling, bronchial hyperresponsiveness, variable airflow obstruction, and mucus hypersecretion (Busse and Rosenwasser, 2003). *D. chrysantha* is a perennial plant that belongs to the *Rosaceae* family. The inhibitory effects of various plant extracts on respiratory inflammation have been recently reported (Lee et al., 2005, 2006; Kim et al., 2006; Maatta et al., 2007). Although Dc extract may have anti-allergic effects in asthma, these effects are not fully understood. In this study, we focused on the possibility of Dc extract as an additional therapeutic drug for successful asthma therapy using a mouse model of asthma. In the present study, we demonstrated that: (1) Dc extract significantly inhibits total leukocytosis and eosinophilia in BAL fluid; (2) Dc extract reduces the inflammatory response and mucus hypersecretion in the airways; (3) Dc extract decreases protein expression of IL-5 and eotaxin in the lung tissue and BAL fluid; and (4) Dc extract reduces the level of ovalbumin-specific IgE in serum and BAL fluid.

Before investigating the effects of Dc extract in a mouse model of asthma, we investigated the alteration of AST and ALT in serum to confirm the liver toxicity of Dc extract. Both ALT and AST, which are known indicators of hepatocellular damage, were not altered by the oral administration of Dc extract as in the other experimental groups (Fig. 1D). These data indicate that the concentration of Dc extract used in the present study had no cytotoxic effect on the mice.

The migration of inflammatory cells, including eosinophils, mast cells and Th2 lymphocytes, into lung tissue is a pivotal process for the pathogenesis of asthma (Elsner and Kapp, 1999). Eosinophils, in particular, act as effector cells in allergic diseases by releasing cytotoxic granule proteins (Helene et al., 2007; Palmqvist et al., 2007). The total cell number and degree of eosinophilia in the BAL fluid were increased after the induction of asthma and significantly decreased by Dc extract (Fig. 1A–C). Because Dc extract shows the suppressive effect on eosinophil migration, we examined whether the inhibitory effect of Dc extract on chemotaxis in a human mast cell line (HMC-1). Mast cells play important roles in the development of asthma. After migrating into lung tissue, mast cells secrete pro-inflammatory mediators, such as histamine, cytokines and prostaglandin (Palmqvist et al., 2007). Stem cell factor has also been known as an essential chemoattractant in mast cell migration (Nilsson et al., 1994; Okumura et al., 1996). The results of our experiment demonstrated that treatment with 10 μg/ml Dc extract for 24 h inhibited up to 62% of HMC-1 cell migration in response to SCF (data not shown). This result indicates that Dc extract may reduce the infiltration of various inflammatory cells, including eosinophils and mast cells. We are currently investigating this activity in an attempt to unveil the complex suppression mechanism of Dc extract on lung inflammation.

Dc extract markedly reduced the infiltration of inflammatory cells within the peribronchiolar and perivascular regions, which is in agreement with the results shown in Fig. 1 (Fig. 2). Mucus hypersecretion accompanied by airway obstruction is an important histopathological feature in the development of asthma (Woodruff and Fahy, 2002). As shown in Fig. 3, Dc extract weakly inhibited both goblet cell hypertrophy and mucus production, despite the absence of significant value and these results indicate that Dc extract may have an inhibitory effect in the development of asthmatic responses.

Th2 lymphocytes play an important role in the initiation and progression of allergic diseases, including asthma, by releasing IL-4, IL-5 and IL-13 (Brightling et al., 2002). These Th2 cytokines induce inflammatory responses, such as airway infiltration and eosinophil activation, immunoglobulin E (IgE) production, and mucus secretion (Kips, 2001; Wills-Karp, 2004; Ngoc et al., 2005). IL-5 is a specific stimulator of eosinophil activation and is synthesized by Th2 lymphocytes, mast cells and eosinophils (Helene et al., 2007). In the examination of the alteration of the expression of these important cytokines, the Dc extract strongly inhibited the elevated protein expression of IL-5 up to the secretion level observed in the normal group (Fig. 4). Since Dc extract blocked eosinophil recruitment into lung, as shown in Figs. 1 and 2, we evaluated the protein level of eotaxin. The results of this investigation showed that Dc extract inhibited the production of eotaxin at a level comparable to that observed in the dexamethasone-treated group (Fig. 4B). Eotaxin is produced by epithelial cells in response to IL-4 and IL-13 and synergistically promotes the function of eosinophil recruitment into the lung with IL-5 (Blanchard et al., 2005). Therefore, the results of the present study indicate that the inhibition of eosinophilia in lung tissue is associated with decreased IL-5 and eotaxin expression. Although Dc extract decreases IL-13 mRNA, the protein expression of IL-13 is not affected and this data show that Dc extract may have different regulation on the mRNA and protein expression of cytokine, depending on a type of cytokine. The elevated IgE in the early asthmatic response induces the degran-

ulation of mast cells by cross-linkage of allergen-specific IgE, and this process is an important step in the development of asthmatic responses (Owen, 2007). The total and ovalbumin-specific IgE in serum and BAL fluid increased after the induction of asthma. The total antibody was not blocked by Dc extract, but the ovalbumin-specific IgE was significantly suppressed by Dc extract in the serum and BAL fluid (Fig. 5). Although the exact mechanism for this activity is unclear, these results suggest that Dc extract blocks specific IgE induction by certain allergens.

In conclusion, we found that Dc extract inhibited the expression of IL-5, eotaxin, and eosinophilia in lung tissue and that it finally alleviated the pathogenesis of lung tissue damage in a mouse model of asthma. These results indicate that Dc extract may have inhibitory effects on asthma, but the exact mechanism of anti-asthmatic processes by Dc extract is not fully understood. Nevertheless, the study results show that Dc extract may provide a useful therapeutic approach for the treatment of allergic airway diseases.

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