



Panax ginseng ameliorates airway inflammation in an ovalbumin-sensitized mouse allergic asthma model

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ABSTRACT

Ethnopharmacological relevance: *Panax ginseng* (PG) is a medicinal herb that has been used to treat various immune diseases including asthma and COPD. In this study, we investigated the inhibitory mechanism of PG on asthma parameters in mice.

Materials and methods: BALB/c mice were sensitized with 20 µg/200 µl OVA adsorbed on 1.0 mg/50 µl aluminum hydroxide gel adjuvant by i.p. injection on days 0 and 14. Mice were then challenged with 5% OVA in PBS to the nose for 30 min once a day for 3 days, from day 20 until day 22, using a nebulizer. PG (20 mg/kg) or vehicle was administered by i.p. injection once a day 10 min before every OVA challenge for 3 days. The recruitment of inflammatory cells into bronchoalveolar lavage fluid or lung tissues was measured. The expression of EMBP, Muc5ac, CD40, and CD40 ligand (CD40L) in lung tissues was investigated. In addition, the cytokines and mitogen activated protein (MAP) kinases were measured by RT-PCR and Western blot.

Results and conclusions: PG restored the expression of EMBP, Muc5ac, CD40, and CD40L, as well as the mRNA and protein levels of interleukin (IL)-1β, IL-4, IL-5, and tumor necrosis factor (TNF)-α. In addition, PG inhibited the numbers of goblet cells and further small G proteins and MAP kinases in bronchoalveolar lavage cells and lung tissues increased in ovalbumin-induced allergic asthma in mice. These results suggest that PG may be used as a therapeutic agent in asthma, based on reductions of various allergic responses.

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1. Introduction

Allergic asthma is a chronic, complex respiratory disease caused by exacerbated responses to inhaled allergens and characterized by reversible airway obstruction, increased mucus production, infiltration of eosinophils, and nonspecific airway hyperresponsiveness (Corrigan and Kay, 1991). The development of asthma is mediated by the over-expression of Th2-mediated or Th1-mediated cytokines, including interleukin (IL)-4, IL-5, IL-8, and tumor necrosis factor (TNF)-α (Corry, 2002). Despite the increasing prevalence of this disease, its pathophysiological mechanism is still unclear, and current treatments are not satisfactory.

Abbreviations: PG, *Panax ginseng*; GL, mice group sensitized with ovalbumin; CD40L, CD40 ligand; MAP, mitogen activated protein; IL, interleukin; TNF, tumor necrosis factor; OVA, ovalbumin; EtBr, ethidium bromide; EMBP, eosinophil major basic protein; JNK, Jun N-terminal kinases; BCR, B cell receptor; ERK, extracellular signal-regulated kinases.

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Asthma is a serious public health problem affecting people of all ages, throughout the world (Agarwal and Gupta, 2011). However, patients with severe asthma are difficult to treat, with current therapies incompletely controlling symptoms and even intensive treatment having little effect (Moore et al., 2007). Consequently, a major effort has been made to identify new molecules, preferably of natural origin, for mitigating these disorders.

Panax ginseng (PG), one of the most well-known medicinal herbs, has been used in traditional Korean medicine as an herbal remedy for various disorders (Ji et al., 2005). PG contains various pharmaceutical components including ginsenosides, polyacetylenes, polyphenolic compounds, and acidic polysaccharides (Kim et al., 2005). Recently, a number of studies have demonstrated the effects of PG on allergic asthma (Babayigit et al., 2008; Kim et al., 2008; Lim et al., 2009). Despite such positive findings in various experimental models, the molecular mechanism underlying the anti-asthmatic effects of PG is not fully elucidated.

In this study, we evaluated the effects of PG on measures of allergen-induced airway inflammation including EMBP level, Muc5ac, CD40/CD40L, cytokines, and MAP kinase expression.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice, 6–8 weeks old, were obtained from ORIENT BIO (Seongnam Co., Korea) and maintained in specific pathogen-free conditions. All animals were housed in accordance with guidelines from the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), and all protocols were approved by the institutional review board of our institution and conducted in the Laboratory Animal Research Center of Sungkyunkwan University.

2.2. Sensitization, challenge and experimental protocol

C57BL/6 mice were divided into three groups (8 mice/group): C (control), mice sensitized and nebulized with PBS; GL, mice sensitized and nebulized with ovalbumin (OVA, Grade V; Sigma–Aldrich, St. Louis, MO); and PG, OVA sensitized mice and PG treated 10 min before each OVA challenge. Mice were sensitized with 20 µg/200 µl OVA adsorbed on 1.0 mg/50 µl aluminum hydroxide gel adjuvant (Superfos Biosector, Vedbaek, Denmark) by i.p. injection on days 0 and 14. Mice were then challenged with 5% OVA in PBS to the nose for 30 min once a day for 3 days, from day 20 until day 22, using a nebulizer (Mega Medical, Seoul, Korea). PG (20 mg/kg) was administered by i.p. injection once a day 10 min before every OVA challenge for 3 days (from day 20 to day 22). All mice were sacrificed on day 25.

2.3. Preparation of the extract of PG

Panax ginseng C.A. Meyer was purchased from Wonkwang Herb (Seoul, Korea). A 300 g sample of dried ginseng was boiled with 6 L water at 100 °C for 2 h, and the suspension was filtered. The filtrate was concentrated under reduced pressure and lyophilized. The yield was 26% (dry weight 76.60 g), which was stored at 4 °C. A voucher specimen (PG001) has been deposited at the Department of Prescriptionology at Kyung Hee University. Before each experiment, PG was dissolved in PBS and vortexed for 2 min at room temperature. PG was standardized based on the content of ginsenoside Rg1, and determined using reverse-phase high-performance liquid chromatography–evaporative light scattering detection (HPLC–ELSD). The HPLC system was consisted of a chromatographic pump (Quat pump G1311A), an automated sample injector (ALS G1329A), a thermostatted column compartment equipped with ELSD (Agilent Technology 1200 Series). The output signal was detected using G1315D Diode Array Detector (DAD). Chromatographic separation was achieved on a Eclipse XDB-C18 (5 µm, 4.6 mm × 150 mm); the column temperature was set at 27 °C. The eluent consisted of water and acetonitrile at a flow rate of 1.0 ml/min. Detection wavelength was 203 nm. Ginsenoside Rg 1 was detected at around 13 min in this system. The content of ginsenoside Rg 1 in water extract of PG was 2.4 mg/g.

2.4. Lung tissue histology

Lung tissues were perfused with 5 ml PBS via the right ventricle to eliminate blood in the tissues. Left lung tissues were removed from the chest cavity, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 µm. Tissue sections were subjected to H&E (Sigma–Aldrich, St. Louis, MO) staining to examine changes in general histology and PAS staining (Sigma–Aldrich, St. Louis, MO) to examine goblet cell hyperplasia in the bronchial epithelium (Kim et al., 2007). Right lung tissues were removed and stored at –70 °C for assays of activities and amounts of various proteins.

2.5. Western blot analysis

Immunoblottings were performed as described previously (Stelts et al., 1998). Lung tissues (50 mg/50 l) were suspended in a low-salt lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 (g/ml aprotinin, 2.0 (g/ml leupeptin)] and allowed to swell on ice for 30 min. The cells or tissues were then homogenized using a Polytron (Kinematica, Littau, Switzerland). After centrifugation, supernatants obtained from cells or tissue extracts (20 (g) were subjected to 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were washed with PBS containing 0.1% Tween 20 (PBST), and then blocked for 1 h in 5% skim milk in PBST. After the membranes were washed with PBST, they were incubated for 60 min at room temperature with primary antibodies against actin, EMBP, Muc5ac, ERK, p-ERK, JNK, p-JNK, p38, and p-p38 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted with PBST (1:1000). Membranes were washed with PBST and treated with HRP-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG (diluted to 1:5000–1:10,000) in PBST for 60 min. After washing, the protein bands were visualized by enhanced chemiluminescence using a chemiluminometer (Amersham Biosciences, Buckinghamshire, UK).

2.6. RT-PCR

Total cellular RNA was isolated from lung tissues (50 mg/50 µl) using Trizol reagent. RT-PCR was performed in a final volume of 50 l using an amfiRivert one-step RT-PCR kit (GenDEPOT, Barker, TX) in an automated thermal cycler (BIOER Technology, Hangzhou, China). PCR assays were performed for 35 cycles. Each cycle consisted of the following steps: denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min. PCR products were analyzed using 1.0% agarose gel containing ethidium bromide (EtBr).

The primer sequences used were as follows: Muc5ac sense, 5'-AAA GAC ACC AGT AGT CACTCA GCAA-3'; Muc5ac anti-sense, 5'-CTG GGA AGT CAG TGT CAA ACC A-3'; IL-1β sense, 5'-TGA AGG GCT GCT TCC AAA CCT TTG ACC-3'; IL-1β anti-sense, 5'-TGT CCA TTG AGG TGG AGA GCT TTC AGG-3'; IL-4 sense, 5'-TCG GCA TTT TGA ACG AGG TC-3'; IL-4 anti-sense, 5'-GAA AAG CCC GAA AGA GTC TC-3'; IL-5 sense, 5'-ATG GAG ATT CCC ATG AGC AC-3'; IL-5 anti-sense, 5'-GTC TCT CCT CGC CAC ACT TC-3'; TNFα sense, 5'-TTA TCT CTC AGC TCC ACG CC-3'; TNFα anti-sense, 5'-TGC GCA CTG AAA GCA TGA TC-3'; GAPDH sense, 5'-GAT GCA GGG ATG ATG TTC TG-3'; GAPDH anti-sense, 5'-GTG AAG GTC GGT AAC GG-3'.

2.7. Immunohistochemistry for CD40 or CD40L

Briefly, the paraffin-embedded sections of right lung lobes were deparaffinized with xylene and then rehydrated. Section slides were incubated at 4 °C with 3% hydrogen peroxide for 5 min and then in 1% BSA in PBS (blocking solution) for 1 h, and then incubated with anti-CD40 or anti-CD40L antibody (1:50 dilution) in blocking solution for 24 h. After washing with PBS, the slides were treated with biotinylated secondary antibody for 10 min, streptavidin-HRP (horseradish peroxidase) for 10 min, and 3,3'-diaminobenzidine (chromogen) for 5–10 min (DAKO, Carpinteria, CA).

2.8. Statistical analysis

Experimental data are presented as mean ± S.E.M. of $n=8$. Multiple group comparisons were performed using a one-way analysis of variance (ANOVA) followed by the Scheffe's post hoc test using SPSS (SPSS Inc., Chicago, IL). P values < 0.05 were regarded

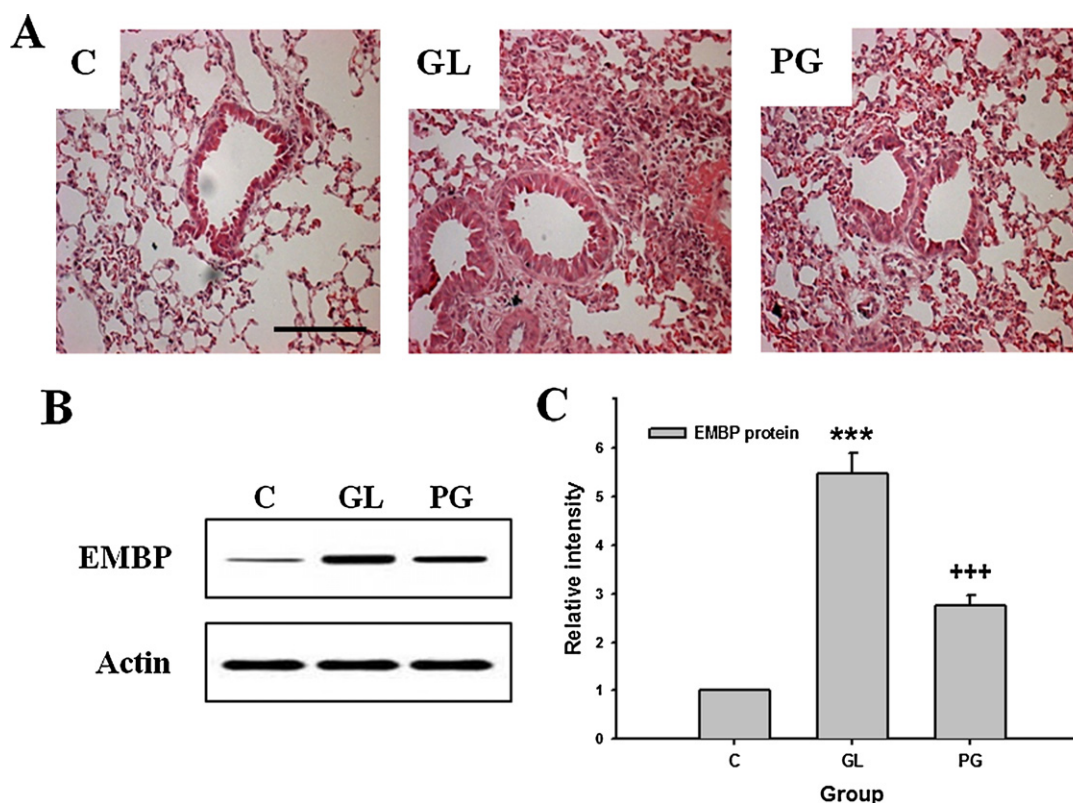


Fig. 1. Effect of PG on the recruitment of inflammatory cells in lung tissues from OVA-challenged allergic asthma in mice. C57BL/6 mice were sensitized and challenged with OVA. Experimental details were performed as described in Section 2. PG (20 mg/kg) was administered by i.p. injection once a day 10 min before every OVA challenge for 3 days. C (negative control), mice sensitized and nebulized with PBS; GL (OVA mice), mice sensitized and challenged with OVA; PG (20 mg/kg), OVA mice treated with each dose of PG. (A) Lung tissues stained with hematoxylin and eosin (H&E) (magnification: 200 $\times$). (B) Band image for EMBP expression. Six animals were assigned to each group. (C) Histogram for expression of EMBP. Histogram was indicated as mean $\pm$ S.E.M. ($n=6$) for values yielded by densitometry analysis. Bar in control group (C) indicates 100 μ m.

as significant. The densitometry analysis of immunoblots and PCR was performed with Quantity One version 4.6.3 (BIO-RAD, Hercules, CA). Histogram for densitometry analysis was indicated by mean $\pm$ S.E.M. ($n=4$) obtained from four independent experiments.

3. Results

3.1. Effect of PG on inflammatory cell recruitment

We examined the effects of PG on the infiltration of inflammatory cells into lung tissues in the OVA-induced mice. GL mice showed inflammatory cell infiltration in the peribronchial and perivascular areas (Fig. 1A). PG reduced inflammatory cell infiltration in these areas (Fig. 1A). We examined the effect of PG on EMBP, a constituent of eosinophil secondary granules that is elevated in biological fluids from patients with asthma and other eosinophil-associated diseases. PG treatment reduced the increased EMBP expression in lung tissues (Fig. 1B) of OVA mice.

3.2. Effect of PG on histopathologic changes

OVA challenge increased goblet cells (pink dots) containing mucus, and PG decreased goblet cells (Fig. 2A) (For interpretation of the references to color in this text, the reader is referred to the web version of the article.). Airway mucus hypersecretion originates from hyperplastic goblet cells that typically express Muc5ac (Ordonez et al., 2001). PG pretreatment reduced the expression of Muc5ac mRNA and protein (Fig. 2B), which was increased in lung tissues of OVA mice.

3.3. Expression of CD40 or CD40L in lung tissues

Using immunohistochemistry, we examined changes in the expression of CD40 and CD40L (co-stimulatory and adhesion molecules, respectively), which play important roles in isotype switching (production of IgE) and in the migration of inflammatory cells into inflamed sites (Snapper et al., 1995). PG reduced expression of CD40 (Fig. 3A) and CD40L (Fig. 3B), which was higher in GL mice (brown color) (For interpretation of the references to color in this text, the reader is referred to the web version of the article.).

3.4. Effect of PG on expression of cytokines

Allergic asthmatic inflammation and airway remodeling are associated with the secretion of a series of inflammatory cytokines, particularly IL-1 β , IL-4, IL-5, and tumor necrosis factor (TNF)- α (Zhu and Gilmour, 2009). The GL mice had increased cytokine expression in lung tissues, and PG inhibited this increase (Fig. 4A). The inhibitory effect of cytokine mRNA expression by PG showed a similar pattern.

3.5. Effect of PG on activity of mitogen-activated protein (MAP) kinases

The MAP kinase pathway has been reported to play a major role in biological responses, including cytokine expression. Therefore, we examined whether the phosphorylation of the three MAP kinases in OVA-induced allergic mouse lung tissues would be reduced by PG. PG inhibited the increase in the level of phosphorylated MAP kinases (Fig. 4B).

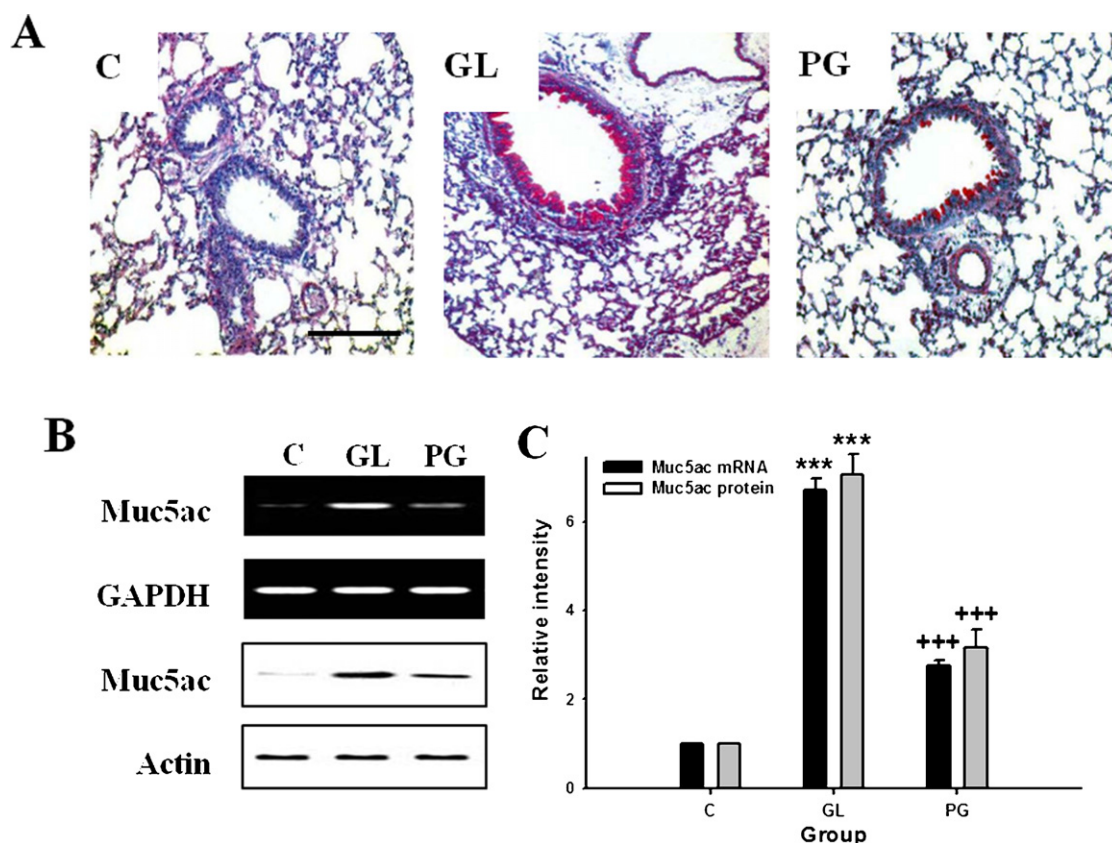


Fig. 2. Effects of PG on the histopathologic changes in lung tissues from OVA-challenged allergic asthma in mice. The symbols used are described in Fig. 1. (A) Lung tissues stained with periodic acid-Schiff (PAS) (magnification: 200 $\times$). (B) Band images for expressions of Muc5ac mRNA and protein. Six animals were assigned to each group. (C) Histogram for expression of Muc5ac mRNA or protein. Histogram was indicated as mean $\pm$ S.E.M. ($n=6$) for values yielded by densitometry analysis. Bar in control group (C) indicates 100 μ m.

4. Discussion and conclusion

Eosinophil major basic protein (EMBP) is the most abundant constituent of the eosinophil secondary granule, forming its crystalloid core. The level of EMBP is elevated in biological fluids from

patients with asthma and other eosinophil-associated diseases (Wassom et al., 1981). Detection of EMBP provides a means of determining eosinophil participation in the pathogenic process. Eosinophilia in asthmatic bronchoalveolar lavage fluid is associated with the production of IL-5, which plays a critical role in the

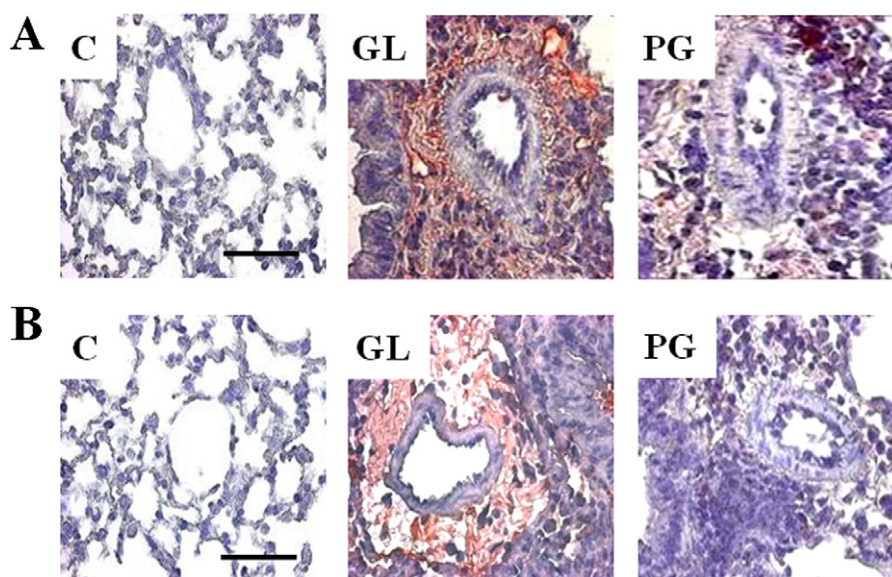


Fig. 3. Effects of PG on CD40 or CD40L expressions in the lung tissues of OVA-challenged allergic asthma in mice. C57BL/6 mice were sensitized and challenged with ovalbumin as described in Section 2. The symbols used are described in Fig. 1. Immunohistochemistry was carried out using paraffin-embedded lung tissues and primary anti-CD40 or anti-CD40L antibody (diluted 1:25) and the DAB system. Magnification of each immunohistochemical changes was 200 times. Six animals were assigned to each group. Bar in control group (C) indicates 100 μ m.

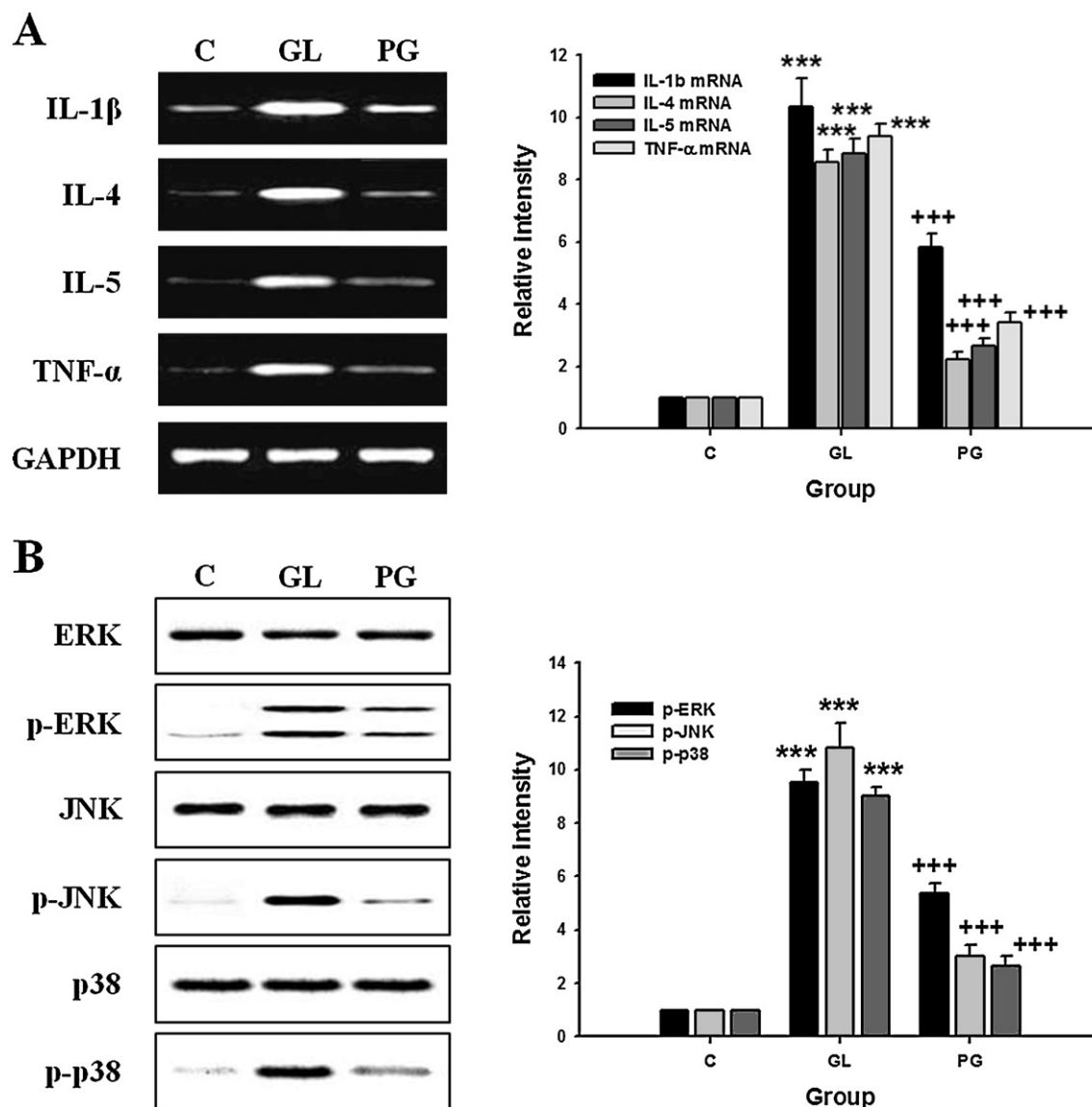


Fig. 4. Effect of PG on cytokine expression or MAP kinase activity in lung tissues from OVA-challenged allergic asthma in mice. Experimental details was performed as described in Section 2. The symbols used are described in Fig. 1. mRNA expressions of cytokines from homogenized lung tissues (50 mg/50 μ l) were determined by RT-PCR. Activity of MAP kinases was determined by Western blot. (A) Band images for expression of cytokines in lung tissues. (B) Band images for activity of MAP kinases. Six animals were assigned to each group. Right panel, histogram for expression of cytokines or MAP kinases. Histogram was indicated as mean $\pm$ S.E.M. ($n=6$) for values yielded by densitometry analysis.

differentiation, infiltration, and activation of pulmonary eosinophils (Wills-Karp and Karp, 2004). In our data, PG treatment reduced EMBP expression and IL-5 level, showing an inhibition of OVA-induced changes (Figs. 1 and 4). Therefore, our data support our findings that changes in the EMBP level by PG may be directly caused by changes in IL-5 expression.

Respiratory diseases such as COPD and asthma are characterized by increased numbers of goblet cells and excessive mucus production. In asthma and COPD, the major mucin components of airway mucus secretions are Muc5ac and Muc5b, which contribute to the viscoelastic properties of the mucus (Rogers, 2003). Muc5ac production is mediated by a diverse range of factors (Turner and Jones, 2009). These factors include the cytokines such as IL-4, IL-1 β and TNF- α (Dabbagh et al., 1999; Song et al., 2003; Gray et al., 2004). In the model tested, PG restored Muc5ac-expressing goblet cells and also reduced Muc5ac mRNA and protein expression in lung tissues (Fig. 2) demonstrating PF extract may restore increases in mucus-

expressing goblet cells via regulating the expression of IL-4, IL-1 β and TNF- α (Fig. 4).

CD40 delivers key input at various stages in B cell activation and differentiation. The CD 40 ligand (CD40L) is expressed on activated T cells. *In vitro* stimulation of B cells by CD40L and appropriate cytokines, such as IL-4, induced proliferation and up-regulation of activation-induced cytidine deamination followed by Ig class switching recombination (Dedeoglu et al., 2004) and it also plays a central role in the induction of allergen mediated airway immune responses (Burgess et al., 2005). According to our data, PG inhibited both signals such as IL-4 and CD40/CD40L ligation demonstrating inhibition of these effector functions of helper T cells, leading to suppression of immune function during this allergic condition.

MAP kinases regulate the expression of inflammatory cytokines (Venuprasad et al., 2006; Ray et al., 2007). In addition, it has been reported that CD40 ligation strongly activates Jun N-terminal kinases (JNK) and p38, whereas the B cell receptor (BCR) activates primarily extracellular signal-regulated kinases (ERK)-2 in B cell

activation *in vitro*, and that the MAP kinases are activated by different upstream kinases (G proteins, Rac, cdc42 for CD40; Ras for BCR), resulting in the activities of different transcription factors (Sutherland et al., 1996; Jabara and Geha, 2005). Our data, the activities of MAP kinases increased by ovalbumin-challenge, are consistent with the finding in the previous reports, although we did not observe separately the results caused by T and B cells. In this study, PG inhibited these signal pathways such as MAP kinase, CD40 ligation, and cytolines. These data suggest that inhibition of MAP kinases regulated via CD40 ligation by PG treatment may inhibit the production of inflammatory cytokines and serves as a mechanism of its anti-allergic and anti-asthmatic activity.

In conclusion, PG may effectively suppress airway hyper-responsiveness and tissue remodeling with various allergic responses by regulating CD40 engagement of lymphocytes, cytokines, and MAP kinase signaling. We have demonstrated that PG could regulate CD40 ligation and MAP kinase signaling resulting in anti-allergic and anti-inflammatory effects such as EMBP and Muc5ac mainly regulated cytokines. However, as most medicinal herbs have multiple components, it is unclear whether one component acts alone or multiple compounds act with multiple functions. Further studies are needed to clarify the molecular mechanisms underlying these effects based on their multi-function and multi-target characteristics.

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