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Anti-allergic effects of PG102, a water-soluble extract prepared from *Actinidia arguta*, in a murine ovalbumin-induced asthma model

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Clinical and Experimental Allergy

Summary

Background Asthma is a chronic inflammatory disease of the lung and its incidence has been increasing around the world. We previously reported that oral administration of a water-soluble extract prepared from *Actinidia arguta*, code-named PG102, could modulate the level of Th1 and Th2 cytokines and suppress the production of immunoglobulin E (IgE) in the ovalbumin (OVA)-immunized murine model as well as in the *in vitro* cell culture system, and furthermore could significantly improve dermatitis conditions in the NC/Nga murine model. These data suggested that PG102 might have therapeutic effects in a broad range of allergic diseases.

Objective To assess the possible anti-allergic effects of PG102 in the OVA-induced murine asthma model.

Methods The quality of PG102 was standardized, using its effects on the production of IgE, IL-5, and IL-13, in *in vitro* cell culture systems. To test effects on asthma, BALB/c mice were orally administrated with PG102, followed by OVA sensitization and challenge to induce asthmatic symptoms. Airway hyperresponsiveness (AHR), bronchoalveolar lavage fluid, serum, and lung tissue were analysed by using various methods.

Results PG102 could decrease the level of IgE, IL-5, and IL-13 in *in vitro* cell culture systems with IC₅₀ being 1.12–1.43 mg/mL. PG102 could ameliorate asthmatic symptoms, including AHR and eosinophilia in the lungs. Such improvement of asthmatic symptoms by PG102 was accompanied by the down-regulation of IL-5 and IgE. In PG102-treated mice, high level expression of heme oxygenase-1, a potent anti-inflammatory enzyme, was observed in alveolar inflammatory cells, while the mRNA levels of foxp3, TGF-β1, and IL-10, important markers for regulatory T cells, were also up-regulated in the lung tissue.

Conclusions PG102 may have potential as a safe and effective reagent for the prevention or treatment of asthma.

Keywords *Actinidia arguta*, AHR, airway hyperresponsiveness, asthma, eosinophilia, heme oxygenase-1, HO-1, IgE, IL-5, PG102

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Introduction

Asthma is a complex inflammatory disease of the lung characterized by airflow obstruction, bronchial eosinophilic inflammation, and airway hyperresponsiveness (AHR) [1–3]. During the early response of asthma, allergens processed by antigen presenting cells activate T helper (Th) cells, resulting in the unregulated over-expression of various Th2 cytokines, such as interleukin (IL)-5 and IL-13 [2, 4, 5]. Among these cytokines, IL-13 plays an

important role in the isotype switching to immunoglobulin E (IgE) production in B cells [2, 6]. Circulating IgE is able to bind to FcεRI on mast cells and basophils in the blood and the tissue. The activation of these cells subsequently releases proinflammatory molecules, such as histamine, prostaglandins, leukotrienes, as well as various cytokines and chemokines [2, 3]. These molecules cause the enhancement of AHR, the recruitment of inflammatory cells, and the production of mucus. During the late-stage of asthma, inflammatory cells produce various

mediators, inducing excessive inflammation and resulting in structural changes known as airway remodeling [4]. Eosinophils are predominant cells in this process [1, 2, 4]. One key cytokine regulating eosinophils is IL-5, which controls the differentiation of eosinophils in the bone marrow, promotes the migration of eosinophils from blood into tissue, prolongs the survival of eosinophils in the tissue, and causes eosinophils to release cytotoxic products [1–3, 7].

We previously found that in the ovalbumin (OVA)-sensitized murine model, PG102, isolated from *Actinidia arguta*, commonly called hardy kiwifruit, significantly decreased the level of IgE, IgG1, and Th2 cytokines, such as IL-5 and IL-13, whereas it increased the level of IgG2a and IFN- γ [8]. It was subsequently shown that oral administration of PG102 could improve dermatitis conditions in the NC/Nga murine model by modulating the level of Th1 and Th2 cytokines, and immunoglobulins [9]. Based on these data, it was hypothesized that PG102 might be able to control the fundamental aspects of allergy pathogenesis and have preventive and/or therapeutic effects on various allergic diseases. In this study, we used a murine asthma model to examine the efficacy of PG102. Oral administration of PG102 suppressed AHR, and decreased the level of IgE and the number of eosinophils, probably by regulating IL-5. Immunohistological analysis of the lung tissue from the PG102-treated animals revealed that heme oxygenase-1 (HO-1) was highly expressed in various cells that infiltrated into the bronchial tissue. Consistent with this result, PG102 increased the level of HO-1 mRNA in a dose- and time-dependent manner in rat mast cell line, RBL-2H3. Furthermore, the RNA levels of foxp3, TGF- β 1, and IL-10 mRNA, which are expressed by regulatory T (Treg) cells [10–12], were also up-regulated. These results indicated that the one mechanism for anti-allergic effects of PG102 might be the control of HO-1 and subsequent regulation of Treg cells. Our data suggest that oral intake of PG102 may be a useful treatment for asthma.

Materials and methods

Preparation of PG102

The hardy kiwifruits used in this study were purchased from a company specializing in this fruit (Vital Berry Marketing S.A., Santiago, Chile). PG102 were prepared from these hardy kiwifruits as described by Park et al. [8]. Briefly, the dried fruits were extracted by boiling in distilled water (DW) for 3 h. The extract was filtered with Whatman filter paper (No. 2, 110 nm), and concentrated by using a rotary evaporator, followed by a freeze-drying process. Powdered PG102 was dissolved in DW at a concentration of 200 mg/mL and stored at -80°C until use. All the PG102 preparations contained undetectable

levels of endotoxin, as determined by the Limulus Amebocytes Lysate assay (Cambrex, Walkersville, MD, USA) which is commonly used for the detection of gram-negative bacterial endotoxin. PG102 was also tested to be negative for heavy metals, residual pesticides, and micro-organisms.

Bioassay in U266B1 cells, EL4 cells, and RBL-2H3 cells

The IgE-inhibitory effects of PG102 in lipopolysaccharide (LPS)-stimulated U266B1, a human B lymphoblastoma line, were measured, as described by Park et al. [8]. To measure the IL-5-inhibitory activity of PG102, EL4 cells, a mouse T lymphoma line, were grown on 24-well culture plates (5×10^4 cells/well) using 0.5 mL of DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37°C under 5% CO_2 . Various concentrations of PG102 were added to the culture media, and 30 min later, EL4 cells were then stimulated by PMA (10 ng/mL) and cAMP (1 mM) (Sigma). After 12 h, the supernatants were collected to measure the level of IL-5 by ELISA (Pierce, Rockford, IL, USA). To measure the effect on IL-13, RBL-2H3 cells, a rat mucosa mast cell line, were plated at 2×10^5 cells/well in the 24-well plates and cultured in 0.5 mL of minimum essential media (Sigma) containing 15% FBS for 6 h. The cells were incubated in the absence or the presence of PG102 for 30 min before stimulation with 1 mM of A23187 (Sigma). After 12 h, the cultured media were taken to determine the level of IL-13 by ELISA (Biosource, Grand Island, NY, USA).

Murine asthma model

Female BALB/c mice (6 weeks old) were obtained from Orientbio Inc. (Seongnam, Korea), kept in an air-conditioned room, and acclimated for at least 1 week before use. Six to eight mice per group were used in all experiments. All the experimental procedures were performed in accordance with the guidelines set by the University Animal Care and Use Committee at Seoul National University. One group (OVA/PG102) of mice was administrated with PG102 at 300 mg/kg/day by gavage from day 0 to day 25, while another group (OVA/water) of mice was fed with 200 μL of water. As another control, normal mice were maintained by being intragastrically fed with 200 μL of water without OVA (Fraction V; Sigma) and PG102. The first two groups of mice were primed by one intraperitoneal injection with 100 μg of OVA emulsified in aluminum hydroxide (2.25 mg) (Pierce) on day 0. After this systemic priming, the mice were repeatedly challenged with gas of 1% of OVA in phosphate-buffered saline (PBS) for 30 min on days 14, 21, 22, and 23. The evaporation of OVA was generated by an ultrasonic nebulizer NE-U12 (Omron, Tokyo, Japan). On day 24, 1 day after the last OVA challenge, AHR was measured. On day 25, the mice were

killed, and sera, bronchoalveolar lavage fluids (BALFs), and lungs were obtained [13].

Measurement of airway hyperresponsiveness

AHR was measured in the mice by recording respiratory pressure curves using a whole body plethysmograph (All Medicus, Anyang, Korea) in response to inhaled methacholine (Sigma) at 0, 20, 40, and 60 mg/mL concentrations for 3 min. Measured respiratory curves were converted into values of enhanced pause (Penh), which was shown to correlate with a pulmonary obstruction, a resistance, and an intrapleural pressure [13–16].

Analysis of bronchoalveolar lavage fluid

Immediately subsequent to killing of the animals, bronchoalveolar lavage was performed with three 1 mL aliquots of PBS. The washed fluid (BALF) was centrifuged to separate infiltrating cells and supernatants. BALF supernatant was used to measure the level of cytokines and chemokines. Remaining cell pellets were treated with RBC lysis buffer (Sigma), cytospun onto microscope slides, and stained with Diff-Quick solution (DADE Behring, Dudingen, Switzerland). According to their microscopic morphology, differential cell counts were performed by counting at least 300 cells per sample [13].

Measurement of the level of immunoglobulin E, cytokines, and chemokines

The total level of IgE was determined, using a mouse IgE detection kit (Shibayagi, Gunma, Japan). The level of OVA-specific IgE, IgG1, IgG2a, and IgG2b was measured by the sandwich ELISA method as described by Hirano et al. (BD Sciences, San Jose, CA, USA and Bethyl Laboratories, Montgomery, TX, USA) [8, 9, 17]. The levels of IFN- γ , IL-5, IL-13, MCP-1, eotaxin, and thymus and activation-regulated chemokine (TARC) in BALF were measured by ELISA kits (purchased from Pierce and R&D Systems, Minneapolis, MN, USA).

Histological analysis of lung

Lungs were fixed in 4% neutral formaldehyde. Fixed lungs were embedded in paraffin wax and sectioned at 5 mm thickness. After deparaffinization and dehydration, the paraffin blocks were stained with hematoxylin and eosin for microscopic examination. For immunohistological analysis, the endogenous peroxidase activity was first blocked with 3% H₂O₂, followed by incubation with an anti-HO-1 polyclonal antibody (Stressgen, Ann Arbor, MI, USA) at 37 °C for 3 h. The peroxidase binding sites were detected by staining with 3,3'-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). Finally the slides

were counterstained with Meyer's hematoxylin [18]. Normal goat IgG was used to access non-specific reactions.

Analysis of heme oxygenase-1 expression using Western blot assay and Northern blot assay

For analysing the level of HO-1 protein in *in vitro* cells, the cells were washed once with PBS and lysed with RIPA lysis buffer. Whole cell lysates were subjected to 8% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Immunoblotting was performed with an anti-HO-1 polyclonal antibody or an anti- β -actin monoclonal antibody (Stressgen and Sigma). Blots were visualized by chemiluminescence, using a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody or an HRP-conjugated anti-mouse IgG antibody, respectively (Pierce).

To confirm the expression of HO-1 mRNA, total RNAs were extracted from the cells using Trizol reagent according to the manufacturer's manual (Invitrogen, Carlsbad, CA, USA). Twenty mg of total RNA was subjected to 1% formaldehyde agarose gel electrophoresis, blotted to a nylon membrane (Hybond-N; Amersham, Piscataway, NJ, USA), and hybridized with ³²P-labelled HO-1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

Messenger ribonucleic acid analysis using real-time quantitative polymerase chain reaction

Whole lung tissue was pooled according to respective experimental groups and subjected for mechanical disruption under liquid nitrogen by using a mortar and pestle. Total RNAs were extracted using Trizol reagent according to the manufacturer's recommendation (Invitrogen). Total RNA quantity and purity were determined by optical density at 260 and 280 nm wavelengths using a spectrophotometer (Amersham Biosciences, Uppsala, Sweden). Five hundred ng of total RNA was used for a reverse transcription reaction with oligo(dt)_{12–18} primer and Avian Myeloblastosis Virus reverse transcriptase (Roche Applied Science, Mannheim, Germany). The resulting single-stranded cDNA was then used for a real-time PCR with primer sets and SYBR[®] Premix Ex Taq[™] (Takara Bio, Shiga, Japan) according to the manufacturer's instruction by using the Thermal Cycler Dice Real Time System (Takara Bio, Shiga, Japan). Real-time PCR primers were synthesized (COSMO co. Ltd, Seoul, Korea) as follows: IL-10 (5'-CAGAGCCACATGCTCCTA-3'; 5'-GGAGTCGGTTAGCAGTATG-3'), TGF- β (5'-CACTGATACGCCTGAGTG-3'; 5'-GTGAGCGCTGAATCGAAA-3'), foxP3 (5'-CCCAGGAAAGACAGCAACCTT-3'; 5'-TTCTCACAACCAGGCCACTTG3'), and GAPDH (5'-AGCCTCGTCCCGTAGACAA-3'; 5'-AATCTCCACTTTGCCACTGC-3'). The PCR conditions were 95 °C for 10 s, followed by 45 cycles with

denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 34 s. Cycle threshold (Ct) of respective samples were normalized internally using the average Ct value of GAPDH. The difference in mRNA levels of each gene between OVA immunized groups and the normal group was calculated from the Ct value as described [19, 20].

Statistics

Data are expressed as the means $\pm$ SEM or SD. Statistical differences between mean values were analysed by using the Student's *t*-test. *P*-values of less than 0.05 or 0.01, calculated as 1-tailed *P*-values, were considered to be statistically significant.

Results

Suppression of the production of immunoglobulin E and three Th2 cytokines in in vitro cell culture systems

PG102 is a water-soluble extract prepared from *A. arguta*. Because it is a mixture, the quality of PG102 reagents has been standardized as described by Park et al. [8]. The particular batch used in the murine asthma model experiment has the following features. First, PG102 used in this study inhibited the production of IgE from LPS-stimulated U266B1 B cells in a dose-dependent manner (Fig. 1a). The IC₅₀ value was 1.12 mg/mL. Second, PG102 also revealed suppressive activity on the production of IL-5 in the mouse EL4 T cells. PMA- and cAMP-stimulated EL4 cells overproduced IL-5, but PG102 inhibited the production of IL-5, displaying its IC₅₀ value at 1.43 mg/mL (Fig. 1b). Third, PG102 could also down-regulate IL-13 cytokine in rat RBL-2H3 mast cells. Treatment of RBL-2H3 cells with A23187, calcium ionophore, induced the expression of IL-13. When cells were treated with PG102, the supernatant

level of this cytokine was decreased in a dose-dependent manner, with its IC₅₀ value being 1.21 mg/mL (Fig. 1c). PG102 did not have any cytotoxic effects in any of the concentrations used in these experiments (data not shown). In summary, the IC₅₀ value of PG102 was remarkably similar, ranging from 1.12 to 1.5 mg/mL, even though three different cell lines and four different protein markers were used for the assay. These data indicated that these bioassay systems could be used to control the quality of PG102 as a research reagent and also that PG102 might contain bioactivities useful for the control of various allergic responses.

Attenuation of airway hyperresponsiveness by PG102

Effects of PG102 were investigated in a murine asthma model. Mice were sensitized once on day 0, inhaled four times with OVA, and orally administrated with PG102 or a vehicle (water) alone as indicated in Fig. 2. On day 24, AHR was examined by the inhalation of a serially increased dose of methacholine. In the water-fed mice, AHR, as indicated in the Penh value, was sharply increased with an increase in the amount of methacholine. When animals were orally administrated with PG102, however, AHR was significantly suppressed at high concentrations of methacholine (Fig. 3). In a non-immunized and water-fed normal mouse group, the Penh value was low at the whole range of methacholine concentrations. This result suggested that oral administration with PG102 might lower the hyperresponsiveness of airways in this animal model.

Suppression of eosinophil infiltration

Infiltration of eosinophils in the airway results in the abnormal production of inflammatory proteins and cytokines, such as eosinophil cationic protein, major basic

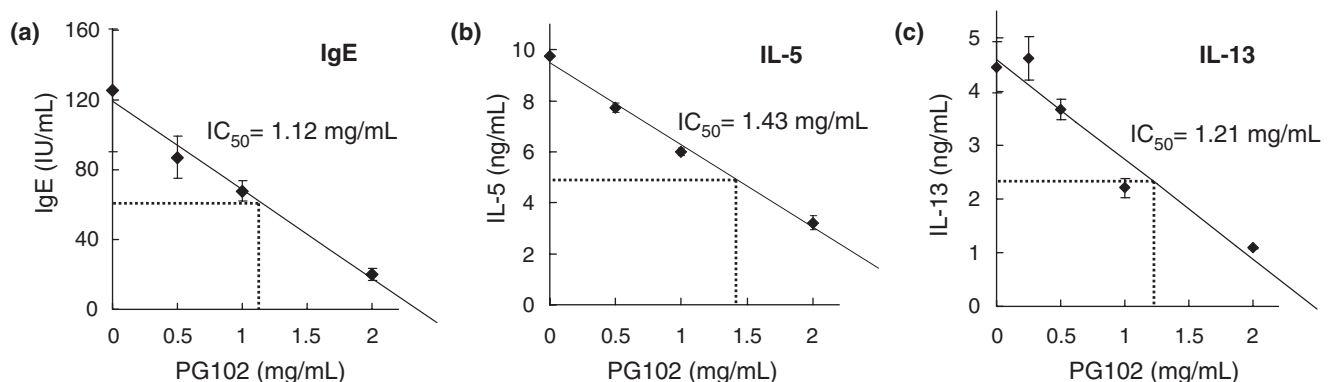


Fig. 1. Effects of PG102 in the *in vitro* cell culture system: (a) Human U266B1 B cells were stimulated with lipopolysaccharide (2 µg/mL) and PG102 (0, 0.5, 1, 2 mg/mL). After 7 days, the level of secreted IgE was analysed with an ELISA kit. (b) Mouse EL4 cells were treated with PG102 and then stimulated with 10 ng/mL PMA and 1 mM cAMP. After 12 h, the supernatant level of IL-5 was measured by ELISA kit. (c) Rat RBL-2H3 cells were cultured with 1 mM A23187 and various concentrations of PG102 for 12 h. The supernatant level of IL-13 was determined by ELISA kit. Each value represents means $\pm$ SD measured in the duplicate microplate wells. More than three independent sets of experiments were performed in each case.

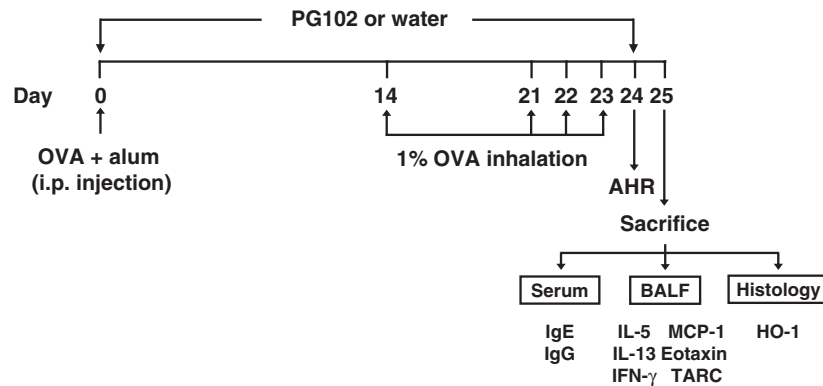


Fig. 2. Protocols for sensitization, challenge, and PG102 administration: Allergic mice were prepared by intraperitoneal injection with ovalbumin (OVA) plus alum on days 0 (sensitization) followed by repeated inhalation with OVA (challenge) on days 14, 21–23. The PG102-treated group was orally administered with 300 mg/kg PG102 from day 0 to day 24 on a daily basis. Normal and OVA immunized mice were fed with water only as indicated. On day 24, airway hyperresponsiveness (AHR) was measured. On day 25, mice were killed, and samples were collected.

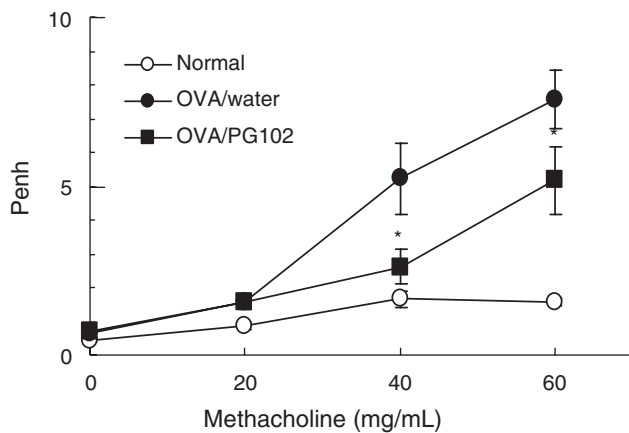


Fig. 3. Effects of PG102 in allergic asthma: Airway hyperresponsiveness (AHR) was measured after inhalation with the indicated concentrations of methacholine. Each group consisted of six to eight mice. Penh was used to measure the degree of AHR in conscious mice. Normal, naïve mouse; ovalbumin (OVA)/water, animals immunized with OVA and fed with water only; OVA/PG102, immunized with OVA and orally administered with PG102. The results are shown as means $\pm$ SEM. * P < 0.05 vs. OVA/water group (Students t -test).

protein, leukotrienes, IL-4, IL-5, IL-6, IL-13, and TGF- β [1, 2, 4]. It has previously been shown that in the OVA-immunized asthma model, AHR is closely associated with eosinophilia [2, 14], as evidenced by an increase in the number of eosinophils in BALF. Therefore, the effects of PG102 on various cell types present in BALF were investigated. The total number of infiltrating cells was low in the normal mouse group. In the OVA-immunized animals, the number was increased by approximately 57-fold. When mice were orally fed with PG102, it was decreased by 27% (Fig. 4a). Different cell types were counted based on their morphological characteristics visualized by Diff-Quick staining. The number of eosinophils in PG102-treated animals was found to be only a half of that in

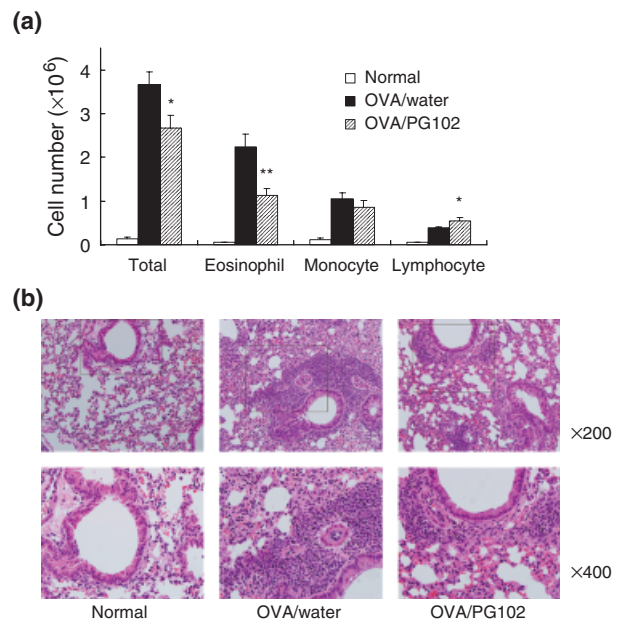


Fig. 4. Effect of PG102 on various cell types in BALF: (a) The number of total cell (Total), eosinophils, monocyte/macrophage, and lymphocytes in BALF was counted according to stained morphological characteristics. Values are shown as means $\pm$ SEM. * P < 0.05 and ** P < 0.01 vs. ovalbumin (OVA)/water group (Students t -test). (b) Lung tissue from animals of respective groups was stained with hematoxylin and eosin, and inflammatory cells were studied by light microscopy. A black arrow indicates infiltrating eosinophils. The original microscope magnification is 200 $\times$ or 400 $\times$.

OVA-immunized and water-fed mice (Fig. 4a). The number of monocytes between the two groups was comparable, while PG102 administration slightly increased the number of lymphocytes. Histological analysis revealed that the number of inflammatory cells, especially eosinophils, was significantly increased around perivascular and peri-bronchiolar regions in OVA-immunized and water-fed

mice, but decreased in PG102-treated animals (Fig. 4b). The infiltration of inflammatory cells was virtually absent in normal mice. These data suggested that PG102 might suppress AHR by inhibiting eosinophilia.

Effects of PG102 on cytokines and chemokines in bronchoalveolar lavage fluid

To understand the biochemical basis of the above data, the level of various cytokines and chemokines in BALF was measured. OVA-immunization increased the level of IL-5 and IL-13, while simultaneously decreasing that of IFN- γ ,

Table 1. Effects of PG102 on the level of variable cytokines and chemokines in bronchoalveolar lavage fluid (BALF)

Cytokines (pg/mL)	IL-5	IL-13	IFN- γ
Normal	3.2 $\pm$ 0.2*	10.4 $\pm$ 0.3	490 $\pm$ 114
OVA/water	22.0 $\pm$ 1.2 ^{††}	20.4 $\pm$ 2.3 ^{††}	224 $\pm$ 35 [†]
OVA/PG102	8.4 $\pm$ 0.8 ^{**‡}	19.1 $\pm$ 3.9	341 $\pm$ 83
Chemokines (pg/mL)	Eotaxin (CCL-11)	MCP-1 (CCL-2)	TARC (CCL-17)
Normal	20.6 $\pm$ 17.7	8.7 $\pm$ 3.1 [‡]	ND
OVA/water	41.9 $\pm$ 17.6 ^{††}	23.8 $\pm$ 6.3 ^{††}	267 $\pm$ 43 ^{††}
OVA/PG102	35.2 $\pm$ 23.4	20.4 $\pm$ 5.8	230 $\pm$ 94

^{**} $P < 0.01$, vs. OVA/water mice (Student's *t*-test).

^{††} $P < 0.01$.

[†] $P < 0.05$, vs. normal mice (Student's *t*-test).

*The lower limit of detection for IL-5 is for < 5 pg/mL. Therefore, this value is close to 0 pg/mL.

[‡]These two points were not within the standard range of ELISA assay, but above the lower limit of detection. Except for these values, all other concentrations were measured within the standard range provided in the manufacturer's manual.

ND, not detectable.

Values are expressed as means $\pm$ SEM for six to eight animals.

OVA, ovalbumin; TARC, thymus and activation-regulated chemokine.

all in a statistically significant manner, as compared with the normal mouse group (Table 1). PG102 down-regulated an OVA-mediated increase in the level of IL-5 by more than 2.5-fold, while exerting little influence on IL-13. On the contrary, the mean value of IFN- γ level was increased by 52% when mice were administrated with PG102 (Table 1). The level of Eotaxin, MCP-1, and TARC was also sharply increased in the OVA-immunized animals. These are chemokines involved in the pathogenesis of asthma by recruiting various immune cells, such as eosinophils, monocytes, and T cells, to the site of allergic reactions [2, 4, 21]. Oral administration with PG102 lowered the amount of these chemokines, although the difference was not statistically significant (Table 1). These results demonstrated that IL-5 might be a key target of PG102, thereby suppressing eosinophilia and the overproduction of pathologic chemokines.

Reduction of serum level of immunoglobulin E by PG102

IgE plays an essential role in allergic responses by the stimulation of mast cells and basophils, which lead to the synthesis and the release of various inflammatory mediators and cytokines [2, 3, 22]. We examined the effect of PG102 on the serum level of IgE. Normal mice produced approximately 360 ng/mL of IgE. When mice were immunized with OVA, the level of IgE was increased by 18-fold. However, treatment with PG102 decreased the serum level of IgE by more than twofold (Fig. 5a). To be certain, the level of IgE specific to OVA was also measured. The level of OVA-specific IgE was also significantly lower in the serum samples from PG102-treated animals (Fig. 5b). In contrast, the level of IgG1, IgG2a, and IgG2b specific to OVA was comparable between PG102-treated and control groups (data not shown). These results indicated that oral intake of PG102 could systemically down-regulate the level of total and OVA-specific IgE in the OVA-immunized mouse model.

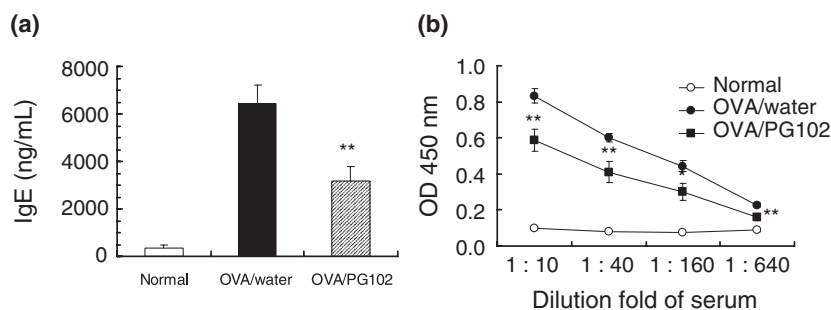


Fig. 5. Effect of PG102 on total and ovalbumin (OVA)-specific IgE in serum: (a) The serum level of total IgE was determined using an ELISA kit. (b) Because OVA-specific IgE antibody was unavailable, the relative level of OVA-specific IgE was represented as absorbance at O.D.450 nm using a manual ELISA method. Values are shown as means $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$ vs. OVA/water mice (Student *t*-test).

Enhanced expression of heme oxygenase-1, foxp3, transforming growth factor- β 1, and interleukin-10 in the lung by PG102

It has previously been shown that HO-1 could increase the percentage and suppressive function of CD4⁺CD25^{hi} Treg cells, coinciding with a decrease in the level of IgE and eosinophil infiltration in BALF [23, 24]. Because our previous microarray analysis indicated that PG102 could increase the RNA level of HO-1 (unpublished data), it was tested whether PG102 administration has any effect on the expression of HO-1 in this murine asthma model [25–27]. The lung tissue was taken after the mice were killed and examined by an immunohistochemistry using a specific antibody to HO-1. HO-1 was detected in some cells, which appeared to be infiltrating cells, in the normal mice (Fig. 6a). The expression level of HO-1 did not seem to change noticeably in the animals immunized with OVA (Fig. 6b). When immunized animals were treated with PG102, however, the high level expression of HO-1 was observed in a number of cells. They appeared to be cells that infiltrated to the lung (Fig. 6c).

To examine whether the observed increase of HO-1 expression could indeed induce the activity of Treg cells, we examined the mRNA level of three important marker genes for Treg cells (foxp3, TGF- β 1, and IL-10) by using

real-time quantitative PCR. Foxp3 is a transcription factor, specifically expressed in Treg cells that plays a key role in the differentiation and function of Treg cells [10, 28]. The RNA level of foxp3 in PG102-treated animals was increased by 4.8-fold, as compared with that in the normal and OVA/water groups (Fig. 7a). TGF- β 1 and IL-10 are the cytokines that not only regulate the activities of Treg cells but also promote the differentiation of precursors to Treg cells [11, 12]. The RNA level of both cytokines was significantly higher in PG102-treated mice than two other control groups (Fig. 7b and c). These data indicated that PG102 might affect the expression of HO-1 and subsequently inhibit the inflammatory activities of infiltrating cells by regulating Treg cells.

Regulation of heme oxygenase-1 messenger ribonucleic acid and protein in rat RBL-2H3 mast cell line by PG102

To understand how PG102 increased the expression of HO-1, effects of PG102 on HO-1 expression were studied in the rat mast cell line, RBL-2H3. RBL-2H3 cells were treated with various concentrations of PG102 in the presence of calcium ionophore, A23187, for 3 h. (In the absence of A23187, the overall gene expression level was too low to analyse various markers.) Total RNAs were

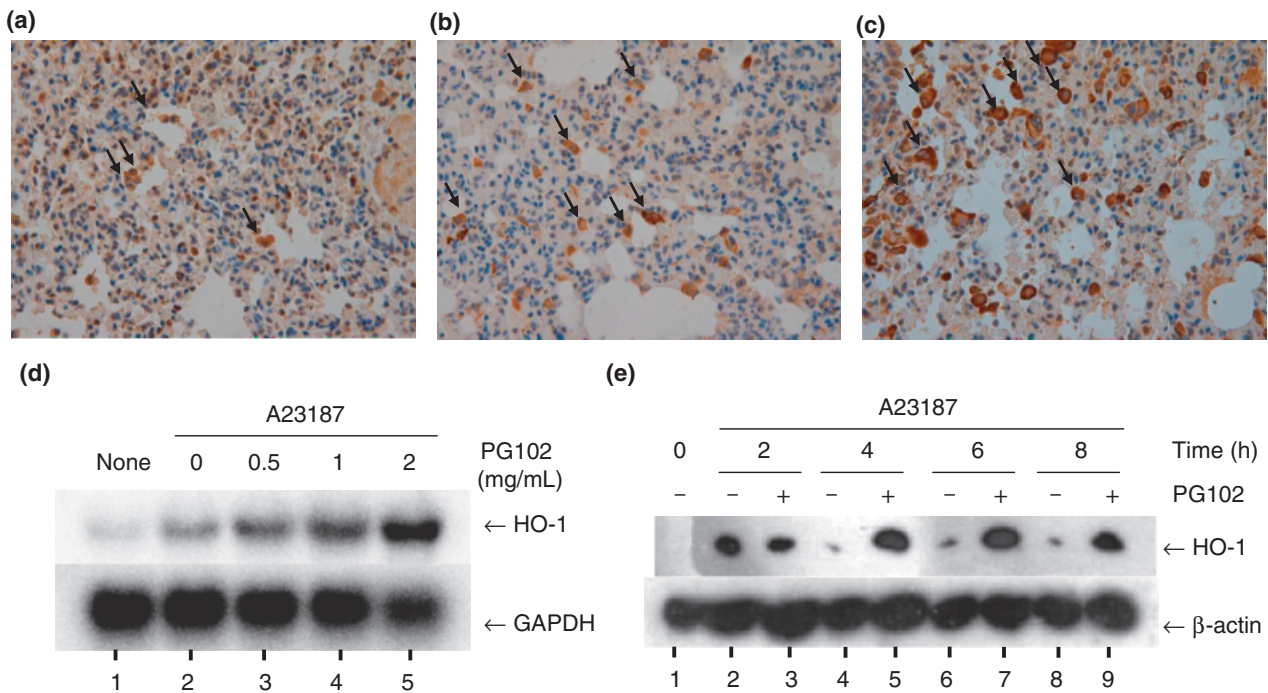


Fig. 6. Expression of heme oxygenase-1 (HO-1) in cells of lung tissue and in rat mast cell line, RBL-2H3: Lung biopsies of normal mice (a), ovalbumin (OVA)/water mice (b) and OVA/PG102 mice (c) were immunostained with a specific antibody to HO-1. Black arrow indicated cells expressing HO-1. The microscope original magnification is 400 $\times$. (d) RBL-2H3 cells were treated with various concentrations of PG102 and total RNAs were prepared, followed by Northern blot hybridization analysis. GAPDH mRNA was used as a loading control. (e) RBL-2H3 cells were treated with 2 mg/mL of PG102 in the presence of A23187, and total proteins were prepared at various time-points, followed by Western blot analysis using a specific antibody for HO-1 or β -actin. β -actin was used as a loading control.

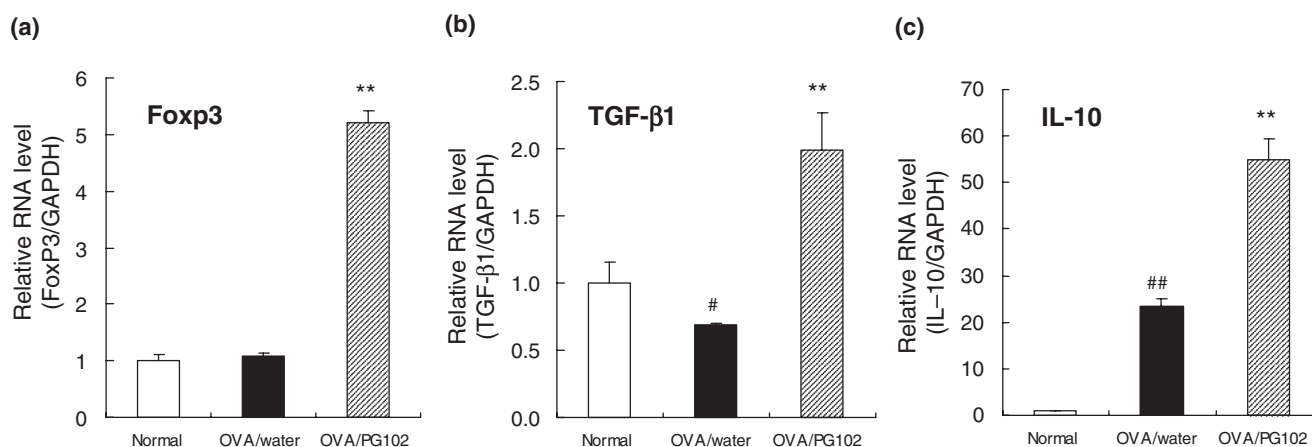


Fig. 7 Effect of PG102 on IL-10, TGF- β 1, and foxp3 mRNA in the lung tissue: The RNA levels of foxp3 (a), TGF- β 1 (b), and IL-10 (c) were analysed by using real-time quantitative PCR. Total RNAs were prepared from lung tissue, followed by real-time quantitative PCR. The data for these three markers were normalized with GAPDH level. Values are shown as means $\pm$ SD measured in triplicate reactions. ** P < 0.01 vs. ovalbumin (OVA)/water mice and # P < 0.05 and ## P < 0.01 vs. normal mice (Student t -test).

prepared, followed by Northern blot analysis. The level of HO-1 mRNA was increased in a dose-dependent manner (Fig. 6d; compared from lane 2 to lane 5). The effect of PG102 on HO-1 expression was more prominent when the protein was measured. RBL-2H3 cells were treated with 2 mg/mL of PG102, in the presence of A23187, and total proteins were prepared at various time-points. In the absence of PG102, the effect of calcium ionophore on HO-1 was very transient. The level of HO-1 protein was dramatically increased at 2 h, but sharply dropped at 4 h (Fig. 7e; compared lanes 1, 2, and 4). However, in the presence of PG102, a high level of HO-1 protein was maintained throughout the experiment (Fig. 7e). These data indicated that PG102 might control the expression of HO-1 at the transcriptional level, resulting in the high level maintenance of this important anti-oxidative and anti-inflammatory protein even when cells were in the excited state, such as by the use of calcium ionophore.

Discussion

Asthma is one of the most common chronic disorders of the airways that affects adults and children of all ages [1, 2, 29]. It is estimated that world-wide, approximately 300 million people currently suffer from asthma, and about 180 000 deaths are associated with asthma each year [29, 30]. Many asthma deaths are preventable, if treated with optimal medical care. Current standard medications are combination therapies, including inhaled corticosteroids, β_2 -agonists, leukotriene receptor antagonists, and others [30, 31]. However, these therapies produce potential side effects, such as retardation of growth, loss of bone mass, induction of insulin resistance, and suppression of immune system, and do not consistently ameliorate airway inflammation in many asthmatic individuals [1, 32, 33].

Therefore, there has long been a strong need for the development of safe and efficacious medicine [34, 35].

In this study, we investigated the effects of oral administration with PG102, extracted from *A. arguta*, in the murine asthma model. This investigation was based on our previous works showing that PG102 regulates the expression of IgE and Th1 and Th2 cytokines involved in an allergy pathogenesis in the OVA-sensitized mouse and NC/Nga dermatitis models as well as in the *in vitro* cell culture systems [8, 9]. Our data indicated that PG102 alleviated asthma symptoms, namely AHR, by suppressing eosinophilia, presumably through the control of IgE and IL-5.

The increase of eosinophilic inflammation and enhancement of AHR in the asthmatic individuals are regarded as the cardinal features of asthma [2, 4]. The increased number of eosinophils in the airways is thought to contribute to the development of AHR by injuring epithelial layers and releasing inflammatory mediators, resulting in the thickening of the airway wall [1, 2, 4]. IL-5 could induce the terminal differentiation of immature eosinophils, stimulate the release of these cells into the airway, and maintain the existence of eosinophils [1, 2, 4]. Indeed, it has been shown that a local concentration of IL-5 directly correlates with the degree of eosinophilia in the airway [1]. Our result showed that the level of IL-5 in BALF was sharply decreased in PG102-treated animals. Therefore, it is reasonable to assume that the improvements of AHR and suppression of eosinophilia observed in PG102-treated animals might be the consequence of decreased amount of IL-5 at the local site.

IgE is one of the most important factors responsible for the progression of allergic reactions [2, 3, 36]. IgE has been a key target in developing anti-asthma strategies. Clinical trials involving the neutralization of IgE have

generated satisfactory outcomes in treating patients with asthma [1, 36]. PG102 down-regulated the serum level of total and OVA-specific IgE, which further strengthens the potential of PG102, as an anti-asthma reagent.

HO-1 is an inducible isoform of the enzymes that convert heme into biliverdin, carbon monoxide and iron. Biliverdin is subsequently converted to bilirubin by biliverdin reductase [25, 26]. According to recent *in vivo* reports, the induction of HO-1 expression could improve airway inflammation, mucus hypersecretion, oxidative stress, and hyperresponsiveness in an asthma guinea pig model and exogenous bilirubin or low-dose CO has been also shown to ameliorate airway inflammation in a murine asthma model by inhibiting the migration of leucocytes, the production of cellular ROS, and the production of IL-5 [37–40]. In another murine model, induction of HO-1 could regulate CD4⁺CD25^{hi} Treg cells by increasing the level of foxp3, IL-10, and membrane-bound TGF- β 1, and these modulation correlated with decrease of OVA-specific IgE level and eosinophil infiltration in BALF [23, 24]. PG102 induced the expression of HO-1 in the alveolar inflammatory cells of the lung. The data from RNA and protein analysis of the PG102-treated rat mast cell line RBL-2H3 indicated that the HO-1 expression was controlled at the transcriptional level, and that the presence of PG102 helped the expression of HO-1 maintained at a high level, and also that the RNA level of foxp3, TGF- β 1, and IL-10 was increased in the lung tissue from PG102-treated animals, as compared with those from the control groups. Therefore, it is tantalizing to postulate that the increased expression of HO-1 might have contributed to the amelioration of allergic inflammation by regulating Treg cells in our animal model experiments. Various experiments are underway to unveil the molecular and biochemical details of the anti-allergic activities of PG102.

An active compound(s) of PG102 has not yet been identified. Because PG102 shows multiple functions, it is reasonable to assume that the bioactivities of PG102 observed in our experimental systems have resulted from several different compounds. Although PG102 is a complex mixture, its biological effects are remarkably reproducible and consistent in various animal models and cell culture systems [8, 9]. The key features include the suppression of expression of IgE, selective Th2 cytokines, and chemokines. Therapeutic or preventive effects of PG102 in the animal models are also highly consistent with the data from cell culture systems involving U266B1, EL4, RBL-2H3, and primary splenocytes. Our extensive safety data indicated that PG102 is also a very safe agent (data not shown). Taken together, PG102 demonstrates great potential as a preventive and/or therapeutic agent for asthma, and further investigation into the identification of active compounds and the unraveling of underlying mechanisms are warranted.

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