DA-9601, *Artemisia Asiatica* Herbal Extract, Ameliorates Airway Inflammation of Allergic Asthma in Mice

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We previously reported that DA-9601, ethanol herbal extract of *Artemisia asiatica*, inhibited histamine and leukotriene releases in guinea pig lung mast cells activated with specific antigen/antibody reaction. This study aimed to evaluate the inhibitory effect of DA-9601 on the OVA-induced airway inflammation in allergic asthma mouse model. BALB/c mice were sensitized and challenged with OVA. DA-9601 was administered orally 1 h before every local OVA-challenge. OVA-specific serum IgE was measured by ELISA, recruitment of inflammatory cells in BAL fluids and lung tissues by Diff-Quik and H&E staining, respectively, the expressions of CD40, CD40L and VCAM-1 by immunohistochemistry, goblet cell hyperplasia by PAS staining, activities of MMPs by gelatin zymography, expressions of mRNA and proteins of cytokines by RT-PCR and ELISA, activities of MAP kinases by western blot, and activity of NF-κB by EMSA. DA-9601 reduced IgE level, recruitment of inflammatory cells into the BAL fluid and lung tissues, expressions of CD40, CD40L and VCAM-1 molecules, goblet cell hyperplasia, MMPs activity, expressions of mRNA and productions of various cytokines, activities of MAP kinases and NK-κB increased from OVA-challenged mice. These data suggest that DA-9601 may be developed as a clinical therapeutic agent in allergic diseases due to suppressing the airway allergic inflammation via regulation of various cellular molecules expressed by MAP kinases/NF-κB pathway.

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Introduction

There are difficulties in the drug development process because of the limited understanding of molecular pathogenesis of various diseases. Therefore, this has produced a trend toward newly discovered compounds that are used in clinic rather than to develop new compounds. DA-9601 has been used to treat gastric ulcer (Hahm *et al*., 2001; Huh *et al*., 2003) as newly discovered compound. DA-9601 is the formulated ethanol extract of *Artemisia asiatica Nakai* (*Asteraceae*) and it contains the pharmacologically active flavonoid, eupatilin (Seo and Surh, 2001). Moreover, eupatilin has been reported to have anti-oxidative and antiinflammatory effects on experimentally induced gastrointestinal, hepatic and pancreatic damage (Lee *et al*., 2001). Our laboratory previously reported that eupatilin blocks multi-signal pathways and Ca^{2+} influx in the mast cells activated by specific antigen/antibody reaction (Kim *et al*., 2005).

Allergic asthma is a chronic and complex inflammatory disease of the lung characterized by reversible obstruction of airway, hyper-responsiveness (AHR), infiltration of inflammatory cells into lung tissues, mucus overproduction (Kon and Kay, 1999), the overexpressions of Th2-mediated cytokines including IL-4, IL-5, IL-13 and TNF-α, and chemokines such as eotaxin and RANTES in the airways of allergic asthmatics (Kon and Kay, 1999; Renauld, 2001;

Zimmermann *et al.*, 2003). The recruitment of inflammatory cells requires the expression of adhesion molecules such as ICAM-1 and VCAM-1 (Ohkawara *et al.*, 1995; Wills-Karp and Karp, 2004). The release of Th2-mediated cytokines also causes persistent inflammatory cell recruitment and induces structural changes in airway walls, such as, increased basement membrane thickness, increased collagen deposition, smooth muscle hypertrophy, and goblet cell hyperplasia (Chiapara *et al.*, 2001). Moreover, these clinical features are accompanied by an underlying inflammatory pathology and airway remodeling, and these pathological changes are known to contribute to the clinical symptoms of allergic diseases.

Based on the described above, a potent anti-allergic agent is needed with a pronounced anti-inflammatory effect and minimal toxicity to treat allergic diseases. And, oral administration may reduce its toxicity and risk in utility, compared to routes of other administration. Therefore, we examined for the first time whether DA-9601 newly developed and administrated orally suppresses airway inflammation in OVA-induced allergic asthma mice.

Materials and Methods

Sensitization and antigen challenge protocol Specific pathogen free female BALB/c mice, 6–8 week old, were divided by six groups (8 mice/group). Con (negative control), mice sensitized and challenged with phosphate buffered saline (PBS); G, mice sensitized with OVA and challenged with PBS (general sensitization); OVA, mice sensitized and challenged with OVA (OVA-challenged mice); GL30, mice treated with 30 mg/kg DA-9601 1 h before every OVA challenge (nebulization); GL100, mice treated with 100 mg/kg DA-9601 1 h before every OVA challenge. Mice were sensitized with 10 μg ovalbumin (OVA, Grade V; Sigma-Aldrich, USA) adsorbed in 250 μg/200 μl of alum (aluminum hydroxide, 2% Alhydrogel; Superfos Biosector, Denmark) by intraperitoneal (i.p.) injection on days 0, 5, 14, 21, and 28 (general sensitization). One week (day 35) after the final injection, mice were challenged with 2% OVA for 10min once daily for 7 consecutive days until day 41 (local challenge). On day 49 mice were challenged with 2% OVA for 10 min (OVA-challenged mice), and mice were sacrificed on day 50.

Treatment protocol DA-9601 (30 or 100 mg/kg) (Dong-A Pharmaceutical Co. Ltd., Korea) was dissolved in 3% hydroxypropylmethylcellulose (HPMC) (Sigma-Aldrich, USA) solution (5 ml) and administered orally for 7 consecutive day 1h before every local challenge (from day 35 to day 41). In order to examine the effect of HPMC solution (the DA-9601 solvent), HPMC was orally administrated instead of DA-9601 1 h before every local challenge. DA-9601 was administrated 1 h before every local challenge because it has a half-life ($t_{1/2}$) of 1 h *in vivo*. All animal experiments were conducted in the Laboratory Animal Research Center of Sungkyunkwan University and were approved by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Serum collection and analysis of serum OVA-specific IgE antibody levels After anesthesia, blood was collected by cardiac puncture. Blood was allowed to clot for 30 min, centrifuged at $900 \times g$ for 30 min, and then analyzed for OVA-specific IgE antibody level by ELISA (Lee *et al.*, 2004a). For OVA-specific IgE, microplates were coated with purified rat anti-mouse IgE, and then treated with mouse sera followed by biotinylated OVA prepared using a peroxidase-conjugated goat anti-biotin antibody, which was used as secondary antibody. Reactions were read using an ELISA plate reader at 415 nm. Mouse IgE (BD Biosciences, USA) was used for calibrating the assay.

Collection and analysis of bronchoalveolar lavage (BAL) fluid cells After blood collection, tracheas were exposed by cannulating upper tracheas and BAL fluid was collected by lavaging twice with 1 and 0.8 ml of PBS (85–90% of the input volumes were recovered). Collected lavage fluid was centrifuged at $400 \times g$ for 5 min at 4°C. Briefly, after centrifugation, lavage supernatants were used for matrix matalloproteinases (MMPs) activities and total viable cell numbers in pellets were determined by trypan blue exclusion using a hematocytometer. BAL cells in pellets were adjusted to 5×10^5 cells/ml in PBS for different cell counting. Cytospin preparations were made using a Cytospin III (Shandon, USA), and then stained with Diff-Quik (International Reagents Corp., Japan). Differential cell counting was performed using standard morphological criteria (McKay *et al.*, 2004).

Zymography MMPs activities in BAL fluid supernatant was performed as described previously (Kumagai *et al.*, 1999; McMillan *et al.*, 2004). The first BAL fluid samples (35 μl) collected were loaded onto 7.5% polyacrylamide gels containing 10 mg/ml gelatin (Sigma-Aldrich, USA), in the presence of SDS under nonreducing conditions. After electrophoresis, gels were washed three times for each 15 min in 20 mM Tris/HCl (pH 7.8) containing 2.5% Triton X-100, and then repeatedly washed twice for 15 min in developing buffer [20 mM Tris/HCl (pH 7.8), 1.0% Triton X-100, 10 mM $CaCl_2$, and 5 μM $ZnCl_2$]. After washing, gels were incubated in developing buffer at 37°C for 18 h, stained with 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad, USA), destained in a solution of 10% acetic acid containing 40% methanol, dried, and analyzed with a LAS1000 Image Reader (Fuji film, Japan). Gelatinolytic activity was detected as a clear band against a blue background. Human MMP-2 and MMP-9 (Chemicon, USA) were used as gelatinase zymography standard markers.

Lung histology After BAL, lungs were perfused with 5 ml PBS via the right ventricle. Exsanguinated left lungs were then removed from the chest cavity and fixed in 4% paraformaldehyde. Lobes were isolated, embedded in paraffin, and sectioned at 3 μm. Tissue sections were stained with H & E for general morphology (McKay *et al.*, 2004) and with periodic acid-Schiff (PAS) reaction for goblet

cell identification in airway epithelium (Edwan *et al*., 2004). Numbers of goblet cell hyperplasia were quantified in percentage of PAS-positive cells/total cells in 50 μm × 50 μm area by microscopy. Removed right lung tissues were stored at –70°C for determinations of cytokine mRNA and protein expressions.

Immunohistochemistry for CD40, CD40L or VCAM-1 expressions Immunohistochemistry was conducted using a modification of the method described previously (Burgess *et al*., 2005; Ohkawara *et al*., 1995). Sections (μm) in paraffin-embedded sections were deparaffinized with xylene and then rehydrated. Slides were incubated in 3% hydrogen peroxide for 5min to quench endogenous peroxidase activity, blocked with PBS containing 1% BSA for 1 h, and then incubated with anti-CD40 or anti-CD40L antibody (1:25 dilution, Lab Vision, USA) or anti-VCAM-1 (1:50 dilution, Santa Cruz biotechnology, USA) in blocking solution at 4°C for 24 h. After washing in PBS, slides were treated with biotinylated secondary antibody for 10 min, streptavidin-HRP (horse radish peroxidase) for 10 min, DAB chromogen substrate for 5–10 min (DAKO, USA) and counterstained with hematoxylin (Sigma-Aldrich, USA), and finally mounted using aqueous mounting medium.

Cytokine levels in lung tissues The levels of IL-4, IL-5, IL-13, and TNF-α in supernatants isolated from lung tissue homogenates were determined by ELISA (McMillan *et al*., 2004). Standard curves were generated using different concentrations of recombinant cytokines. The limit of detection was at least 7.8 pg/ml.

Quantification of cytokine mRNAs in lung tissues by RT-PCR Cellular RNAs were extracted using TRIZOL reagent (Invitrogen, USA). Briefly, TRIZOL reagents (1 ml) were added to 50 mg of lung tissues, and homogenized using a Polytron (Kinematica, Switzerland). Two hundred microliters of chloroform were added to the homogenates, and these were then incubated for 10 min at room temperature. After centrifugation at $10,600 \times g$ for 15 min, an equal volume of isopropyl alcohol was added. The samples were then incubated for 10 min at room temperature and centrifuged at $10,600 \times g$ for 10 min. RNA pellets were washed with 75% ethanol and dried. RT-PCR was performed in a final volume of 25 μl using a SuperScript One-step RT-PCR kit (Invitrogen, USA) in automated thermal cycler (GeneAmp PCR system 9700, Perkin Elmer, USA). PCR assays were performed for 35 cycles. Each cycle consisted of the following step: 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 in 1.7% agarose gel containing ethidium bromide (EtBR) (Picado *et al*., 2003).

Assay of MAP kinases by Western blot Lung tissues (30 mg/50 μl lysis buffer) were homogenized in 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 μg/ml aprotinin, 2.0 μg/ml leupeptin] using a Polytron (Kinematica, Switzerland), and allowed to swell on ice for 10 min. Cell lysates (μg) were subjected to 10% SDS-PAGE and transferred to PVDF membrane (Schleicher & Schuell, Germany). Membranes were washed with TBS containing 0.1% Tween 20 (TBST), and then blocked for 1 h in TBST containing 5% skim milk. After washing the membranes with TBST, they were treated with antibodies against ERK, JNK, p38 (Santa Cruz Biotechnology, Inc., USA) and p-ERK, p-JNK, p-p38 (Cell Signaling, USA) diluted with TBST (1:1,000) and incubated for 60 min at RT. Membranes were washed with TBST, and treated with HRP-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG (diluted to 1:5,000–1:10,000) (Zymed Laboratory Inc., USA) in TBST for 60min. After washing, the protein bands were visualized using ECL solution (Amersham Biosciences UK Limited, little Chalfont Buckinghamshire, UK).

Preparation of nuclear extract The activity of NF-κB in supernatants isolated from lung tissue homogenates were determined by EMSA (Desmet *et al*., 2004; Picado *et al*., 2003). Lung tissues (30 mg/500 μl lysis buffer) were homogenized in 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 μg/ml aprotinin, 2.0 μg/ml leupeptin] using a Polytron (Kinematica, Switzerland), and allowed to swell on ice for 10 min. Ten percent of Nonidet P-40 was then added and nuclei were pelleted by centrifugation at $20,800 \times g$ for 1 min. Nuclear pellets were lysed in 40 μl of nuclear lysis buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM $MgCl_2$, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin], and incubated on ice for 30 min. The nuclear extract (supernatant) was retained after centrifuging at $15,300 \times g$ for 10 min at 4°C.

Electromobility shift assay The consensus oligonucleotide of NF-κB (3.5 pmol) (Promega, USA) was radiolabeled using 10 μCi of [γ-^{32}P] ATP. Labeled probes were then separated from unincorporated isotope by size exclusion chromatog-raphy using Microspin G-25 Columns (Amersham Biosciences, USA). Nuclear extracts (5 μg) were mixed with binding buffer (3 μl) and incubated for 10 min at room temperature (final volume 15 μl). The mixture was then added a labeled probe (1 μl, 300,000 cpm) and incubated for 20 min at room temperature. Ten microliters of binding reaction mixture were loaded onto 6% polyacrylamide gel and run at 150 V for 30 min. Gels were dried and NF-κB bands were analyzed by phosphoimaging using FLA-2000 (Fuji Film, Japan).

Statistical analysis Experimental data are presented as means ± S.E.M. Data were analyzed by two-way analysis of variance (ANOVA) with multiple comparisons. *P* values of < 0.05 were regarded as significant.

Results

Inhibition of serum OVA-specific IgE production by DA-9601 In order to examine whether DA-9601 influenced

Table 1. Effects of DA-9601 on serum IgE antibody levels in OVA-challenged allergic asthma mouse model[a].

	Serum IgE Ab (ng/ml)
Control	0.5 ± 1.34
G	135.1 ± 5.42***
OVA	192.2 ± 15.13***,#
OVA with HPMC	182.1 ± 13.31***,#
GL30	139.5 ± 4.04 (25.4)++
GL100	123.2 ± 9.64 (34.2)+++

[a] BALB/c mice were sensitized and challenged with OVA as described in **Materials and Methods**. Control, mice sensitized and nebulized with PBS; G, mice sensitized with OVA and challenged with PBS; OVA, mice sensitized and challenged OVA (OVA-challenged group); OVA with HPMC, OVA-challenge plus HPMC mice (positive control); GL30 or GL100, mice treated with 30 mg/kg or 100 mg/kg DA-9601 1 h before every local challenge. Parentheses were expressed as a decreasing percentage evoked by DA-9601 administration, versus OVA-challenged mice. Eight animals were assigned to each group. *** $P <$ 0.001 versus the control; # $P <$ 0.05 versus general sensitization (G); ++ $P <$ 0.01; +++ $P <$ 0.001 versus OVA-challenged mice (OVA-challenged group) or OVA with HPMC.

OVA-specific IgE production in allergic mouse model, OVA-specific IgE antibody levels were measured by ELISA (Table 1). DA-9601 administration reduced serum OVA-specific IgE level increased in OVA-induced allergic mice, but did not completely. HPMC administrated mice had no effect in OVA-challenged mice.

Reduction in the recruitment of inflammatory cells in BAL fluids by DA-9601 We examined the effect of DA-9601 on the infiltration of inflammatory cells into BAL fluids in OVA-challenged mice. The OVA-challenged mice showed a significant increase in total cell numbers, as compared with PBS-treated control. The order of increase of inflammatory cells was: eosinophils, lymphocytes, neutrophils, and macrophages. The administration of DA-9601 significantly reduced the number of total cells, eosinophils, neutrophils, and lymphocytes recruited in dose-dependent manner (Table 2). These results indicate that DA-9601 blocks the migration of inflammatory cells into the bronchial lumens of OVA-challenged mice. OVA-challenged mice with HPMC (solvent of DA-9601) produced similar results in OVA-challenged mice without HPMC.

Histopathologic examinations of lung tissues treated with DA-9601 We investigated inflammatory cell recruitment into the lung tissues of OVA-challenged allergic mice. In these mice we observed inflammatory cell recruitment into the surrounding peribronchial and perivascular areas, and epithelial thickening (Fig. 1A-c). Although 30 mg/kg of DA-9601 reduced inflammatory cell

recruitment and epithelial thickening (Fig. 1A-e), a high dose of DA-9601 (100 mg/kg) completely reduced (Fig. 1A-f). And, no significant changes were observed in OVA-challenged mice with HPMC (Fig. 1A-d).

In addition, many goblet cells (pink color dot) containing mucus using Periodic acid-Schiff (PAS) stain were increased in OVA-challenged mice (Fig. 1B-c). The numbers of goblet cells, which were quantified in percentage of PAS-positive cells/total cells in 50 μm × 50 μm area by microscopy, were significantly lower in DA-9601-administrated mice (55.7 ± 1.60% for OVA-challenged mice; 55.9 ± 1.54% for OVA with HPMC treated mice; 21.3 ± 3.01% for 30 mg/kg DA9601; 6.2 ± 3.03% for 100 mg/kg DA9601). That is, DA-9601 (30 or 100 mg/kg) decreased up to 61.8% and 88.9%, respectively.

Expression of CD40, CD40L or VCAM-1 in lung tissues We further examined the expressions of CD40 and CD40L, which are involved in B and T cell activation and are major costimulatory molecules in B cells and antigen presenting cells (APCs) (Bancereau *et al.*, 1994; Kawabe, 1994), and also examined VCAM-1 expression, which directs the extravasations of different cell populations of leukocytes recruited to inflammatory sites (Ohkawara *et al.*, 1995). DA-9601 treatment reduced CD40, CD40L (brown color; Figs. 2A and 2B) or VCAM-1 (brown color; Fig. 2C) expressions in OVA-challenged mice. OVA-challenged mice with HPMC did not affect the expressions of these costimulatory and adhesion molecules, compared to OVA-challenged mice.

Expressions of cytokines mRNA and proteins in the lung tissues We also examined whether mRNAs and protein levels of individual inflammatory cytokines were changed in DA-9601-administrated mice. DA-9601 reduced the up-regulations of IL-4, IL-5, IL-13, and TNF-α mRNA in the lung tissues of OVA-challenged mice (Fig. 3 upper panel). The protein expressions of cytokines produced (Fig. 3 lower panel) behaved in the same manner as their mRNA expressions. The changes of mRNA and protein expressions of these inflammatory cytokines were not observed in OVA-challenged mice with HPMC, compared to OVA-challenged mice.

Activities of matrix metalloproteinases (MMPs) in BAL fluid or lung tissues by DA-9601 MMPs, well known extracellular matrix (ECM)-degrading enzymes, are up-regulated in area showing inflammatory cell accumulation and area showing lung tissue remodeling (Kumagai *et al.*, 1999; Nagase and Woessner, 1999). Therefore, we examined the levels of constitutive MMP-2 and inducible MMP-9 in the BAL fluids of OVA-induced mice. The gelatinolytic activities of MMP-2 and MMP-9 in BAL fluids were elevated in OVA-challenged mice. Both MMPs activities were reduced in dose-dependent manner (Fig. 4).

Table 2. Effect of DA-9601 on the recruitment of inflammatory cells into BAL in OVA-induced allergic asthmatic mice[a].

	Total cell ($\times 10^4$)	Macrophage ($\times 10^4$)	Lymphocyte ($\times 10^4$)	Neutrophil ($\times 10^4$)	Eosinophil ($\times 10^4$)
Control	2.9 ± 2.80	1.6 ± 0.17	1.4 ± 0.07	0.0 ± 0.00	0.0 ± 0.00
G	3.0 ± 4.26	1.6 ± 0.17	1.4 ± 0.17	0.0 ± 0.00	0.0 ± 0.00
OVA	15.3 ± 6.12	2.8 ± 0.09***	5.1 ± 0.13***	3.5 ± 0.24***	3.8 ± 0.2***
OVA[b]	15.4 ± 3.00	3.0 ± 0.36***	5.3 ± 0.32***	3.4 ± 0.05***	3.7 ± 0.70***
GL30	11.7 ± 5.00	2.6 ± 0.32*[+]	3.2 ± 0.12***[+]	2.8 ± 0.11***[+]	2.2 ± 0.12***[++]
GL100	7.4 ± 3.00	1.8 ± 0.14[++]	1.9 ± 0.15[+++]	1.3 ± 0.14***[+++]	1.3 ± 0.13***[+++]

[a] BALB/c mice were sensitized and challenged with OVA, and then BAL fluids were collected as described in **Materials and Methods**. BAL cells were separated using a Cytospin, and then stained with Diff-Quik. Differential cell counting was performed using standard morphological criteria. The symbols used are described in Table 1. Eight animals were assigned to each group.

[b] OVA with HPMC. * $P < 0.05$; *** $P < 0.001$ versus the control or OVA mice; [+] $P < 0.05$; [++] $P < 0.01$; [+++] $P < 0.001$ versus the OVA-challenged mice or OVA with HPMC.
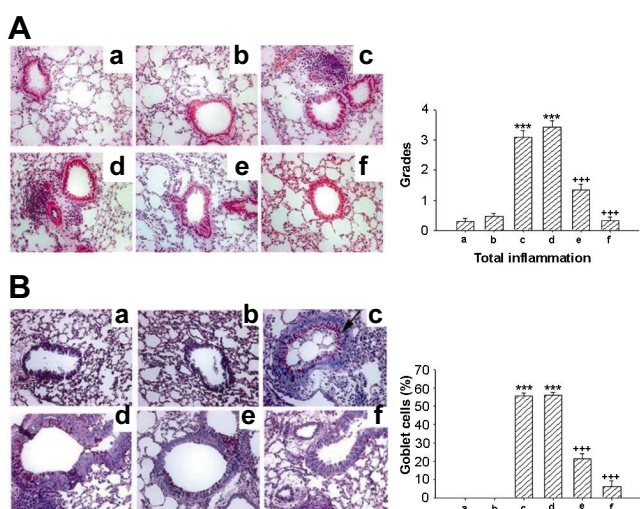

Fig. 1. Effect of DA-9601 on histological changes in the lung tissues of OVA-induced allergic mice. BALB/c mice were sensitized and challenged with OVA as described in **Materials and Methods**. Lung tissues fixed with 10% formalin were sectioned and stained with haematoxylin and eosin (H&E) (A) or periodic acid-Schiff (PAS) (B). a (Con), mice sensitized and challenged with PBS alone; b, mice sensitized with OVA and challenged with PBS; c, mice sensitized and challenged with OVA (OVA-challenged mice); d, mice treated with HPMC alone 1 h before every local challenge; e (GL30), f (GL100), mice treated with 30 mg/kg (e) or 100 mg/kg (f) DA-9601 1 h before every local challenge. Histological appearances were scored for the presence of peribronchial and perivascular inflammation, and these scores were added together to give a total lung inflammation score (Histogram). Numbers of goblet cell hyperplasia were quantified in percentage of PAS-positive cells/total cells in 50 μm × 50 μm area by microscopy. Arrow in lower panel indicates goblet cells. Magnification of each histological change was 400 times. *** $P < 0.001$ versus the control or general sensitized mice; [+++] $P < 0.001$ versus OVA-challenged or OVA with HPMC mice. Eight animals were assigned to each group.
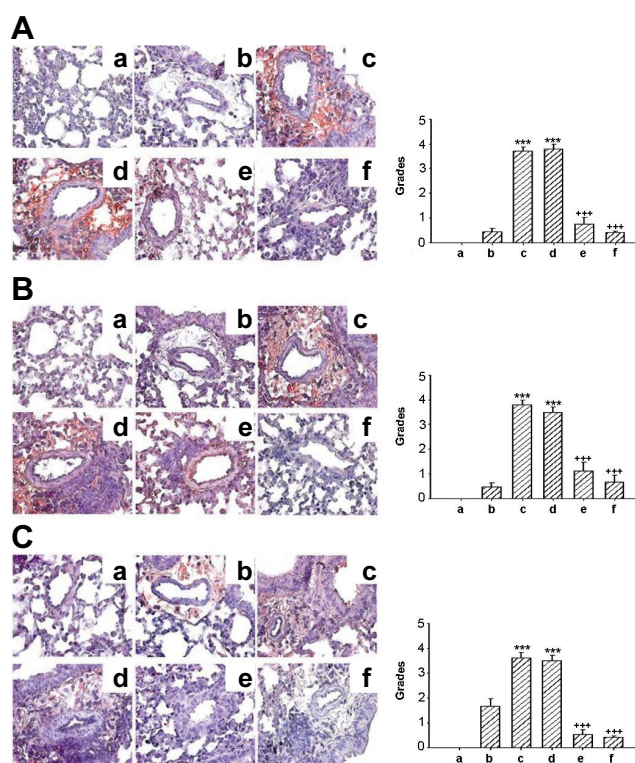

Fig. 2. Effect of DA-9601 on CD40, CD40L, or VCAM-1 expressions in the lung tissues of OVA-induced allergic mice. BALB/c mice were sensitized and challenged with OVA as described in **Materials and Methods**. Immunohistochemistry was carried out using paraffin-embedded lung tissues and primary anti-CD40 antibody, anti-CD40L antibody (diluted 1:25) or anti-VCAM-1 antibody (diluted 1:50) and the DAB system. (A) CD40 molecule, (B) CD40L molecule, and (C) VCAM-1 molecule. The other symbols used are described in Fig. 1. Immunohistochemical appearances were scored for the presence of molecules (Histogram). Magnification of each immunohistochemical changes was 200 times. *** $P < 0.001$ versus the control or general sensitized mice; [+++] $P < 0.001$ versus OVA-challenged or OVA with HPMC mice. Eight animals were assigned to each group.
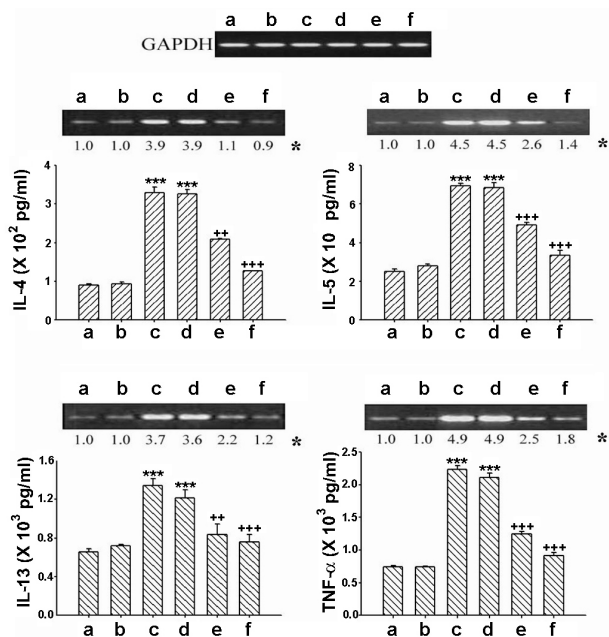
Fig. 3. Effect of DA-9601 on cytokine mRNA expressions and proteins in the lung tissues of OVA-induced allergic mice. BALB/c mice were sensitized and challenged with OVA as described in **Materials and Methods**. Each mRNA expression was determined using RT-PCR analysis (upper panel for each cytokine). Individual cytokine levels in lung tissues were determined by ELISA (lower panel). The symbols used are described in Fig. 1. * Relative intensity versus the control and GAPDH mRNA. *** *P* < 0.001 versus the control or general sensitized mice; [++] *P* < 0.01, [+++] *P* < 0.001 versus OVA-challenged or OVA with HPMC mice. Eight animals were assigned to each group.

As the gelatinolytic activities of MMPs showed changes in BAL fluids (Fig. 4), the mRNA expressions of MMPs and tissue inhibitors of metalloproteinases (TIMPs) were examined in lung tissues. The mRNA levels of these species showed no change in all groups (data not shown). This result may have been obtained because changes of MMPs and TIMPs were undetectable due to the mRNA of various lung tissues. However, these issues require further study. These data are inconsistent with the results of the zymography study on BAL fluids.

Activities of MAP kinases by DA-9601 In a previous data, we found that DA-9601 reduces the expressions of cytokines in OVA-challenged mice. MAP kianse pathway has been reported to play a major role in biological responses including cytokine expressions. Therefore, we examined whether the phosphorylations of the three MAP kinases in the OVA-induced allergic mice lung tissues were reduced by DA-9601. DA-9601 inhibited the increased level of phosphorylated MAP kinases (Fig. 5A).

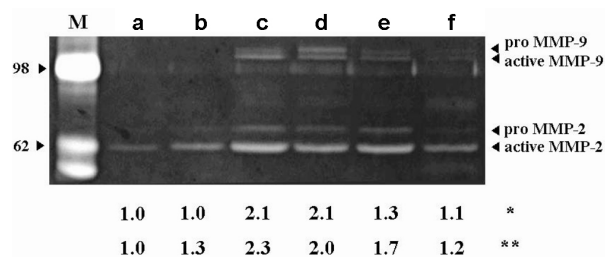**DNA binding activity of nuclear factor-kappa B (NF-**

Fig. 4. Effect of DA-9601 on the gelatinolytic activities of matrix metalloproteinases (MMPs) in the BAL fluids of OVA-induced asthmatic mice. MMPs in BAL fluids collected from mice as described in **Materials and Methods** were analyzed by gelatin zymography. Gelatinolytic activity was detected as clear bands against a blue background. Human MMP-2 and MMP-9 were used as gelatinase zymography standard markers. M, Marker. All symbols used are as described in Fig. 1. MMP-2, -9, matrix metalloproteinase-2, -9. * Relative intensity for MMP-9 versus the control; ** Relative intensity for MMP-2 versus the control. Eight animals were assigned to each group.

κB) and AP-1 by DA-9601 We further examined whether DA-9601 administration influences NF-κB and AP-1 activities in inflammatory cells of lung tissues. Nuclear extracts from OVA-induced allergic mice lung tissues showed increased NF-κB DNA binding activity, and DA-9601 inhibited this activity (Fig. 5B). However, AP-1 transcription factor activity was not detected in any experimental mice (data not shown).

Discussion

Allergic asthma is one of the most common disorders encountered in clinic, and the mortality associated with allergic asthma has increased worldwide over the last two decades (Renauld, 2001). Therefore, it is important to understand the pathogenesis of allergic asthma and to develop novel therapeutic agents. In our previous study, we observed that eupatilin (an active component of DA-9601) inhibited histamine and leukotriene releases in guinea pig lung mast cell activation (Kim *et al*., 2005). Therefore, it is essential to examine whether DA-9601 reduces airway inflammatory responses in OVA-induced allergic mouse model. In the present study using an allergic mouse model, we found that DA-9601 administrated by oral, the formulated ethanol extract of *Artemisia asiatica*, is potentially of use as a therapeutic agent for the treatment of allergic diseases such as asthma.

Allergen-induced airway diseases are caused by allergen-specific Th2-mediated cytokines, which induce the development of airway inflammation, e.g., Th2 cell activation, inflammatory cells, and T/B cell involvement in Ig isotype switching (Kon and Kay, 1999; Renauld, 2001; Romagnani, 2001; Steinke and Borish, 2001; Zimmermann
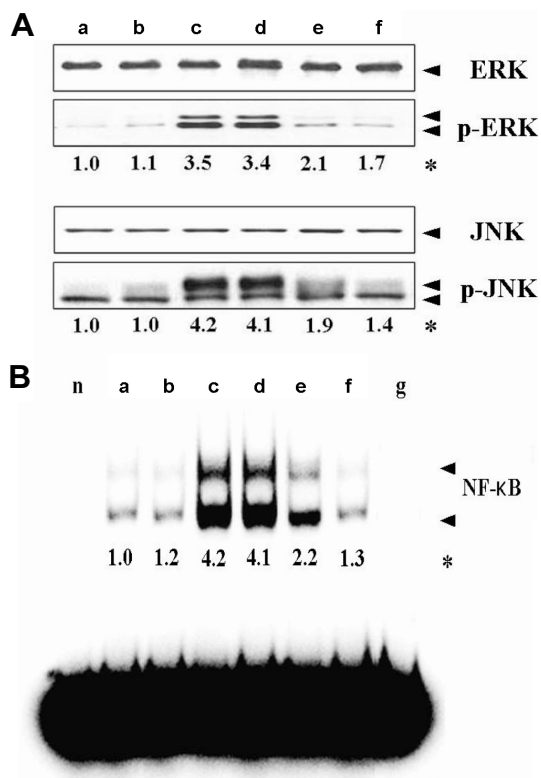
A

B

Fig. 5. Effect of DA-9601 on MAP kinases or NF-κB activity in the lung tissues of OVA-induced allergic mice. MAP kinases or NF-κB activity were determined in protein and nuclear extracts, respectively, prepared from sensitized and challenged mouse lung tissues as described in **Materials and Methods**. The symbols used are described in Fig. 1. **A.** MAP kinase activity. **B.** NF-κB activity. n, negative control; g, competition assay. * Relative intensity versus the control. Eight animals were assigned to each group.

et al., 2003). Table 2 showed that DA-9601 reduced lymphocyte and granulocyte numbers, and thus DA-9601 administration might induce a reduction in Th2 lymphocyte response. This reduced responses elicited by DA-9601 may affect the production and secretion of various Th2 cytokines (IL-4, IL-5, IL-13, and TNF-α). In our data, DA-9601 administration reduced cytokine mRNA (Fig. 3 upper panel) and protein productions (Fig. 3 lower panel), and inflammatory cell infiltration into BAL fluids (Table 2) and lung tissues (Fig. 1A).

The interaction of CD40 on B cells with CD40L-expressing cells, mainly T cells or mast cells, is important for Ig isotype class switching (Banchereau *et al.*, 1994; Burgess *et al.*, 2005; Kawabe, 1994) and plays a central role in the induction of allergen-mediated airway immune responses (Burgess *et al.*, 2005; Ryzhov *et al.*, 2004). IL-4 and IL-13 reductions are also known to suppress IgE production by B cells (Renauld, 2001; Steinke and Borish, 2001; Zimmermann *et al.*, 2003). According to our data, DA-9601 treatment reduced expressions of CD40 and

CD40L molecules (Fig. 2), and productions of IL-4 and IL-13 (Fig. 3) increased in lung tissues of OVA-induced mice. Therefore, the data suggest that DA-9601 reduces the production of IgE antibody via probably suppressing Ig isotype class switching, which had been caused by inhibiting inflammatory cytokine production and by inhibiting co-stimulatory molecules, CD40 and CD40L expressions.

It is known that one of the predominant inflammatory cells recruited into asthmatic lung tissues is eosinophils, but neutrophils and macrophages have also been found to be elevated in the BAL fluids and lung tissues (McKay *et al.*, 2004). Eosinophilia in asthmatic BAL fluids is associated with production of IL-5, which plays a critical role in the differentiation, infiltration, and activation of pulmonary eosinophils (Wills-Karp and Karp, 2004). DA-9601 also reduced the recruited eosinophils into BAL fluids (Table 2) and inflammatory cells into lung tissues (Fig. 1A), mRNA and protein expression of IL-5 (Fig. 3) in OVA-induced lung tissues. Infiltration of inflammatory cells into airway tissues is also associated with expressions of specific adhesion molecules (ICAM-1 and VCAM-1) enhanced by Th2-mediated cytokines such as IL-4 and TNF-α (Lim *et al.*, 2003; Ohkawara *et al.*, 1995; Steinke and Borish, 2001; Wills-Karp and Karp, 2004). Our data (Fig. 2C) are in agreement with these reports previously. Therefore, our data suggest that changes in the numbers of eosinophils in BAL fluids by DA-9601 may be directly caused by changes in IL-5 production, and by inhibiting the expression of VCAM-1 molecule.

IL-13 induces goblet cell hyperplasia (Finkelman *et al.*, 2005), and TNF-α increases inflammatory cell infiltration (Banchereau *et al.*, 1994; Ryzhov *et al.*, 2004). DA-9601 suppressed goblet cell hyperplasia (Fig. 1B), and also suppressed the mRNA and protein expressions of these cytokines (Fig. 3). These wide-ranging actions of DA-9601 may result in relief from the symptoms of allergic asthma. Therefore, our data suggest that the inhibitory effect of DA-9601 shown in allergic mice may in part be mediated through its suppression of the production of cytokines and interaction of T/B cells, although we did not separate subtypes of T and B cells.

Tissue remodeling is characterized by collagen deposition, airway smooth muscle hypertrophy, and goblet cell hyperplasia (Wills-Karp and Karp, 2004), and by MMPs produced via various factors such as TNF-α or IL-1β (Bond *et al.*, 1998; Johnson *et al.*, 2004). MMPs are major proteolytic enzymes, and are actively involved in extra cellular matrix (ECM) turnover, due to their ability to cleave all proteins that contribute to the ECM. Recently, it was reported that alveolar macrophage-derived metalloproteinases and proteinases in sputum lead to tissue remodeling in asthma (Bruce and Thomas, 2005; Ko *et al.*, 2005). According to our data, DA-9601 inhibited inducible MMP-9 and constitutive MMP-2 activities (Fig. 4) and TNF-α production (Fig. 3) produced by inflammatory cells as well

as goblet cell hyperplasia (Fig. 1B). Thereby, DA-9601 reduces tissue remodeling due to inhibiting MMPs regulated by TNF-α and goblet cell hyperplasia caused by IL-5.

Previous studies have shown that the productions of many inflammatory cytokines are dependent on MAP kinase pathway (Lee *et al*., 2004b). Our data (Fig. 5A) suggest that DA-9601 inhibits the production of inflammatory cytokines via MAP kinase pathway in OVA-induced allergic mouse lung tissues.

The pleiotropic transcription factor NF-κB plays important roles in the regulations of multiple genes. Allergic inflammation enhances NF-κB activity in human and animal lung tissues (Bureau *et al*., 2000; Desmet *et al*., 2004; Yang *et al*., 1998). The expressions of inflammatory proteins (IL-4, IL-13, and TNF-α etc.), costimulatory and adhesion molecules, or extracellular molecules (MMPs and TIMPs etc.) are up-regulated in allergic airway inflammation. Moreover, the genes of these proteins are regulated by NF-κB. In the present study, DA-9601 was also found to inhibit up-regulation of NF-κB activity in OVA-challenged mice (Fig. 5B). Therefore, the data suggest that DA-9601 inhibits productions of various cellular molecules as well as cytokine productions by regulating NF-κB pathway.

In conclusion, it is suggested that DA-9601 may be developed as an oral therapeutic agent for allergic diseases due to suppressing the airway allergic inflammation via regulating MAP kinases/NF-κB pathway.

References

Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J. P., *et al.* (1994) The CD40 antigen and its ligand. *Annu. Rev. Immunol.* **12**, 881−922.

Bond, M., Fabunmi, R. P., Baker, A. H., and Newby, A. C. (1998) Synergistic up-regulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-κB. *FEBS Lett.* **435**, 29−34.

Bruce, C. and Thomas, P. S. (2005) The effect of marimastat, a metalloprotease inhibitor, on allergen-induced asthmatic hyper-reactivity. *Toxicol. Appl. Pharmacol.* **205**, 126−132.

Bureau, F., Bonizzi, G., Kirschvink, N., Delhalle, S., Desmecht, D., *et al.* (2000) Correlation between nuclear factor-κB activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. *Am. J. Respi.r Crit. Care Med.* **161**, 1314−1321.

Burgess, J. K., Blake, A. E., Boustany, S., Johnson, P. R., Armour, C. L., *et al.* (2005) CD40 and OX40 ligand are increased on stimulated asthmatic airway smooth muscle. *J. Allergy Clin. Immunol*. **115**, 302−308.

Chiappara, G., Gagliardo, R., Siena, A., Bonsignore, M. R., Bousquet, J., *et al.* (2001) Airway remodeling in the pathogenesis of asthma. *Curr. Opin. Allergy Clin. Immunol*. **1**, 85−93.

Desmet, C., Gosset, P., Pajak, B., Cataldo, D., Bentires-Alj, M., *et al.* (2004) Selective blockade of NF-κB activity in airway immune cells inhibits the effector phase of experimental asthma. *J. Immunol*. **173**, 5766−5775.

Edwan, J. H., Perry, G., Talmadge, J. E., and Agrawal, D. K. (2004) Fit-3 ligand reverses late allergic response and airway hyper-responsiveness in a mouse model of allergic inflammation. *J. Immunol*. **172**, 5016−5023.

Finkelman, F. D., Yang, M., Perkins, C., Schleifer, K., Sproles, A., *et al.* (2005) Suppressive effect of IL-4 on IL-13-induced genes in mouse lung. *J. Immunol*. **174**, 4630−4638.

Hahm, K. B., Oh, T. Y., Lee, J. S. Ahn, B. O., Lee, K. J., *et al.* (2001) Efficacy of combination treatment of antioxidant and acid suppression in the treatment of gastroesophageal reflux disease: Implication for the complete healing. *Gut* **49**, 364−371.

Huh, K., Kwon, T. H., Shin, U. S., Kim, W. B., Ahn, B. O., *et al.* (2003) Inhibitory effect of DA-9601 on ethanol-induced gastrohemorrhagic lesion and gastric xanthine oxidase activity in rats. *J. Ethnopharmacol*. **88**, 269−273.

Johnson, P. R., Burgess, J. K., Underwood, P. A., Au, W., Poniris, M. H., *et al.* (2004) Extracellular matrix proteins modulate asthmatic airway smooth muscle cell proliferation via an autocrine mechanism. *J. Allergy Clin. Immunol*. **113**, 690−696.

Kawabe, T. (1994) The immune response in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity* **1**, 167−178.

Kim, J. Y., Kwon, E. Y., Lee, Y. S., Kim, W. B., and Ro, J. Y. (2005) Eupatilin blocks mediator release via tyrosine kinase inhibition in activated guinea pig lung mast cells. *J. Toxicol. Environ. Health* **68**, 2060−2077.

Ko, F. W., Diba, C., Roth, M., McKay, K., Johnson, P. R., *et al.* (2005) A comparison of airway and serum matrix metalloproteinase-9 activity among normal subjects, asthmatic patients, and patients with asthmatic mucus hypersecretion. *Chest* **127**, 1919−1927.

Kon, O. M. and Kay, A. B. (1999) T cells and chronic asthma. *Int. Arch. Allergy Immunol*. **118**, 133−135.

Kumagai, K., Ohno, I., Okada, S., Ohkawara, Y., Suzuki, K., *et al.* (1999) Inhibition of matrix metalloproteinase prevents allergen-induced airway inflammation in a murine model of asthma. *J. Immunol*. **162**, 4212−4219.

Lee, J. S., Oh, T. Y., Ahn, B. O., Kim, W. B., Kim, Y. B., *et al.* (2001) Involvement of oxidative stress in experimentally induced reflux esophagitis and Barrett's esophagus: clue for chemoprevention by antioxidant treatment. *Mutat. Res*. **480−481**, 189−200.

Lee, B. K., Yoo, J. E., Jang, Y. S., Kim, J. Y., Hong, C. S., *et al.* (2004a) Allergen-specific immunosuppression by ovalbumin fused with diphtheria toxin in mice sensitized with albumins of different origin. *Clin. Exp. Allergy* **34**, 1642−1648.

Lee, Y. N., Tuckerman, J., Nechshtan, H., Schutz, G., Razin, E., *et al.* (2004b) c-Fos as a regulator of degranulation and cytokine production in Fcepsilon RI-activated mast cells. *J. Immumol*. **173**, 2571−2577.

Lim, Y.-C., Garcia-Cardena, G., Allport, J. R., Zervoglos, M.,

Connolly, A. J., *et al.* (2003) Heterogeneity of endothelial cells from different organ sites in T-cell subset recruitment. *Am. J. Pathol.* **162**, 1591–1601.

McKay, A., Leung, B. P., McInnes, I. B., Thomson, N. C., and Liew, F. Y. (2004) A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. *J. Immunol.* **172**, 2903–2908.

McMillan, S. J., Kearley, J., Campbell, J. D., Zhu, X-W., Narbi, K. Y., *et al.* (2004) Matrix metalloproteinase-9 deficiency results in enhanced allergen-induced airway inflammation. *J. Immunol.* **172**, 2586–2594.

Nagase, H. and Woessner, J. F. Jr. (1999) Matrix metalloproteinases. *J. Biol. Chem.* **274**, 21491–21494.

Ohkawara, Y., Yamauchi, K., Maruyama, N., Hoshi, H., Ohno, I., *et al.* (1995) *In situ* expression of the cell adhesion molecules in bronchial tissues from asthmatics with air flow limitation: *in vivo* evidences VCAM-1/VLA-4 interaction in selective eosinophil infiltration. *Am. J. Respir. Cell. Mol. Biol.* **12**, 4–12.

Picado, C., Bioque, G., Roca-Ferrer, J., Pujols, L., Mullol, J., *et al.* (2003) Nuclear factor-κB activity is down-regulated in nasal polyps from aspirin-sensitive asthmatics. *Allergy* **58**, 122–126.

Renauld, J. C. (2001) New insights into the role of cytokines in asthma. *J. Clin. Pathol.* **54**, 577–589.

Romagnani, S. (2001) Cytokines and chemoattractants in allergic inflammation. *Mol. Immunol.* **38**, 881–885.

Ryzhov, S., Goldstein, A. E., Matafonov, A., Zeng, D., Biaggioni, I., *et al.* (2004) Adenosine-induce IgE synthesis by B lymphocytes: an A2B-medisted process involving Th2 cytokines IL-4 and IL-13 with implications for asthma. *J. Immunol.* **172**, 7726–7733.

Seo, H. J. and Surh, Y. J. (2001) Eupatilin, a pharmacologically active flavone derived from *Artemisia* plants, induces apoptosis in human promyelocytic leukemia cells. *Mutat. Res.* **496**, 191–198.

Steinke, J. W. and Borish, L. (2001) Th2 cytokines and asthma: Interleukin-4-its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir. Res.* **2**, 66–70.

Wills-Karp, M. and Karp, C. L. (2004) Eosinophils in asthma: remodeling a tangled tale. *Science* **305**, 1726–1729.

Yang, L., Cohn, L., Zhang, D. H., Homer, R., Ray, A., *et al.* (1998) Essential role of nuclear factor κB in the induction of eosinophilia in allergic airway inflammation. *J. Exp. Med.* **188**, 1739–1750.

Zimmermann, N., Hershey, G. K., Foster, P. S., and Rothenberg, M. E. (2003) Chemokines in asthma: Cooperative interaction between chemokines and IL-13. *J. Allergy Clin. Immunol.* **111**, 227–242.