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

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(Received July 10, 2006; Accepted July 14, 2006)

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 NF-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.

Keywords: Allergic Asthma; CD40; CD40L; Goblet Cells; IgE; MAP Kinases; Matrix Metalloproteinases (MMPs); NF-κB; VCAM-1.

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 Ca2+ 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 (AIR), 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; Renaudl, 2001; Abbreviations: BAL, bronchial alveolar lavage; ERK, extracellular signal-regulated kinase; HPMC, hydroxypropylmethyl-cellulose; JNK, c-Jun NH2-terminal kinase; NF-κB, nuclear factor-kappa B; OVA, chicken ovalbumin.

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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-inflammatory 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 10 min 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 days until day 41 (local challenge). 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 (t1/2) 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 × 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 × g for 5 min at 4°C. Briefly, after centrifugation, lavage supernatants were used for matrix metalloproteinases (MMPs) activities and total viable cell numbers in pellets were determined by trypsin blue exclusion using a hematocytometer. BAL cells in pellets were adjusted to 5 × 105 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 CaCl2, and 5 μM ZnCl2]. 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 5 min 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). Cytokine levels in lung tissues 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 × 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 × 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 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 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, 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 60 min. After washing, the protein bands were visualized using ECL solution (Amersham Biosciences UK Limited, little Charton 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 × 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 MgCl2, 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 × 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-32P] ATP. Labeled probes were then separated from unincorporated isotope by size exclusion chromatography 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.

<table>
<thead>
<tr>
<th>Serum IgE Ab (ng/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.5 ± 1.34</td>
</tr>
<tr>
<td>G</td>
<td>135.1 ± 5.42***</td>
</tr>
<tr>
<td>OVA</td>
<td>192.2 ± 15.13***</td>
</tr>
<tr>
<td>OVA with HPMC</td>
<td>182.1 ± 13.31***</td>
</tr>
<tr>
<td>GL30</td>
<td>139.5 ± 4.04 (25.4)**</td>
</tr>
<tr>
<td>GL100</td>
<td>123.2 ± 9.64 (34.2)***</td>
</tr>
</tbody>
</table>

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 decrease of inflammatory cells was: eosinophils, lymphocytes, neutrophils, and macrophages. The administration of DA-9601 significantly reduced the number of total cells, neutrophils, and macrophages. The administration of DA-9601 significantly reduced the number of total cells, eosinophils, neutrophils, and macrophages. The administration of DA-9601 significantly reduced the number of total cells, eosinophils, neutrophils, and macrophages 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.

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) (Banchereau 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-administered 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.

<table>
<thead>
<tr>
<th></th>
<th>Total cell (× 10⁴)</th>
<th>Macrophage (× 10⁴)</th>
<th>Lymphocyte (× 10⁴)</th>
<th>Neutrophil (× 10⁴)</th>
<th>Eosinophil (× 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.9 ± 2.89</td>
<td>1.6 ± 0.17</td>
<td>1.4 ± 0.07</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>G</td>
<td>3.0 ± 4.26</td>
<td>1.6 ± 0.17</td>
<td>1.4 ± 0.17</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>OVA</td>
<td>15.3 ± 6.12</td>
<td>2.8 ± 0.09***</td>
<td>5.1 ± 0.13***</td>
<td>3.5 ± 0.24***</td>
<td>3.8 ± 0.2***</td>
</tr>
<tr>
<td>OVA b</td>
<td>15.4 ± 3.00</td>
<td>3.0 ± 0.36***</td>
<td>5.3 ± 0.32***</td>
<td>3.4 ± 0.05***</td>
<td>3.7 ± 0.70***</td>
</tr>
<tr>
<td>GL30</td>
<td>11.7 ± 5.00</td>
<td>2.6 ± 0.32*</td>
<td>3.2 ± 0.12***</td>
<td>2.8 ± 0.11***</td>
<td>2.2 ± 0.12***</td>
</tr>
<tr>
<td>GL100</td>
<td>7.4 ± 3.00</td>
<td>1.8 ± 0.14**</td>
<td>1.9 ± 0.15**</td>
<td>1.3 ± 0.14***</td>
<td>1.3 ± 0.13***</td>
</tr>
</tbody>
</table>

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 cell. 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.
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 kinase 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-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 (Renaud, 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; Renaud, 2001; Romagnani, 2001; Steinke and Borish, 2001; Zimmermann
For Ig isotype class switching (Banchereau expressing cells, mainly T cells or mast cells, is important and lung tissues (Fig. 1A). and inflammatory cell infiltration into BAL fluids (Table 2) and protein productions (Fig. 3 lower panel), 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, which had been caused by inhibiting inflammatory cytokine production and by inhibiting costimulatory 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; Obikawa 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.

Acknowledgments This work was supported by the Postdoctoral Research Program of Sungkyunkwan University (2004). We thank for Dong-A Pharmaceutical Co. Ltd. (Korea) for supplying DA-9601.

References


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