

Effect of *Oryza sativa* Extract on the Progression of Airway Inflammation and Remodeling in an Experimental Animal Model of Asthma

Abstract

Airway inflammation and remodeling in chronic asthma are characterized by airway eosinophilia, hyperplasia of smooth muscle and goblet cells, and subepithelial fibrosis. The present study was undertaken to evaluate the effects of DA-9201, an ethanolic extract of black rice (*Oryza sativa* L.), on airway inflammation and remodeling in a murine model of chronic asthma. BALB/c mice sensitized to ovalbumin (OVA) were chronically challenged with aerosolized OVA for 6 weeks. DA-9201 (30, 100, or 300 mg/kg) or dexamethasone (3 mg/kg) was orally administered during the last 4 and 2 weeks, respectively. Airway inflammation, lung pathology by histomorphometry and immunohistochemistry, IgE level and Th2 cytokines were evaluated. The OVA-treated mice showed extensive eosinophilia, chronic inflammatory responses and characteristics of airway remodeling including subepithelial fibrosis, smooth muscle hypertrophy, and goblet cell hyperplasia. As compared to the OVA-treated control group, treatment with DA-9201 resulted in significant reductions in the accumulation of eosinophils in peribronchial areas, chronic pulmonary inflammation and progression of airway remodeling. Furthermore, DA-9201 significantly reduced total serum and BALF IgE levels and Th2 cytokines. These results indicate that DA-9201 may play an important role in attenuating the progressing of airway inflammation and remodeling and

suggest the potential benefits of DA-9201 in prevention or treatment of asthma.

Key words

Chronic asthma · pulmonary inflammation · airway remodeling · *Oryza sativa*

Abbreviations

OVA:	ovalbumin
BALF:	bronchoalveolar lavage fluid
PAS:	periodic acid – Schiff
α -SMA:	α -smooth muscle actin
BSA:	bovine serum albumin
PBS:	phosphate buffered saline
EDTA:	ethylenediaminetetraacetic acid
H & E:	hematoxylin and eosin
ELISA:	enzyme linked immunosorbent assay
IL:	interleukin
Penh:	enhanced pause
IgE:	immunoglobulin E
MT:	masson's trichrome

Supporting information available online at
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Received May 31, 2005 · Accepted October 6, 2005

Bibliography

Planta Med 2006; 72: 405–410 © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2005-916238 · Published online February 17, 2006
ISSN 0032-0943

Introduction

Asthma is a chronic inflammatory disease characterized by airway inflammation, increased mucus production, intermittent airway obstruction, hyperresponsiveness and airway remodeling. Airway remodeling, in particular, is characterized by structural and morphometric changes including airway wall thickening, subepithelial fibrosis and hyperplasia of mucus glands, myofibroblasts and smooth muscle [1], [2]. These structural changes are considered to play important roles in the pathophysiology of bronchial hyperresponsiveness, the functional hallmark of asthma [3]. The remodeling process that produces these changes is thought to result from the combination of chronic, repetitive injuries to the airway wall and the ensuing tissue repair process. A variety of proinflammatory mediators, enzymes, cytokines and growth factors have been implicated in the pathophysiology of airway remodeling [4]; however, both the pathogenesis and the functional consequences of such remodeling must be further elucidated.

Over the last decade, investigations into the pathogenesis and therapy of chronic asthma have focused on the Th1/Th2 paradigm and anti-inflammatory therapy, respectively [5]. However, anti-inflammatory therapy, the established asthma treatment during the recent past decades, has been shown to have no effect on airway remodeling [6]. Furthermore, chronic asthma patients on long-term use with these drugs may develop serious side effects [7]. Consequently, up until now, there has been no therapeutic agent showing high efficacy with a low toxicity in preventing airway remodeling. Therefore, the development of therapeutic agents that can suppress airway tissue remodeling has the potential to fulfill a significant unmet medical need in the treatment of asthma.

Oryza sativa L. var *japonica* [Poaceae (syn: Gramineae)], which is commonly known as 'Heuk-Mi' (black rice) in South Korea, is a dark purple-colored seed and a major rice crop in south Asia and China. It is broadly known as enriched rice and has been used as a traditional medicine for various allergic disorders such as chronic dermatitis and bronchitis [8], [9]. While there are few articles on the safety, previous reports showed that administration of black rice extract to rats induced no physiological differences up to 10 g/kg, which means that black rice extract has low toxicological potential [8], [9].

In this study, we evaluated the effect of DA-9201, an ethanolic extract of the black rice, on airway remodeling in a murine model of chronic asthma.

Materials and Methods

Mice

Female BALB/c mice (5 weeks old) were purchased from Charles River Japan (Japan) and kept under the standard animal facility conditions at the institutes of Dong-A Pharmaceutical company [temperature $23 \pm 2^\circ\text{C}$, humidity range 40–70%, 12-h light/dark cycle (lighting: 7:00–19:00)]. Food and UV-sterilized tap water were provided ad libitum. This study was performed in compliance with the institutional "Standard operating procedure for

animal care and experiments" of the Dong-A Pharmaceutical Co Ltd. and with the "Principles of laboratory animal care" established by the National Institute of Health.

Preparation of DA-9201

The black rice (*Oryza sativa* L. var *japonica*) was purchased from the National Agricultural Cooperative Federation (Geochang, S. Korea). The black rice (8.00 kg) was processed by using a household rice-polishing machine (Bio SangSang, Korea) to obtain the rice bran (1.348 kg). Then, the rice bran was extracted with 70% ethanol (6.74 L) for 24 hours at room temperature with agitating. The extract was filtered through filter paper (Advantec, Japan), and the filtrate (5.13 L) was concentrated at 60°C under vacuum using an evaporative system and lyophilized at -40°C to dryness; 77.80 g (yield: 5.77%) of extract powder were obtained. The dried extract, DA-9201, was suspended in a 1% hydroxypropylmethylcellulose (HPMC) solution for oral administration.

HPLC analysis of DA-9201

The dried ethanol extract, 10 mg of DA-9201, was dissolved in 1 mL of mobile phase followed by filtration using a $0.45\ \mu\text{m}$ syringe filter (PVDF, Waters, Milford, USA). Standards of several anthocyanins including cyanidine 3-O- β -glucopyranoside, peonidine 3-O- β -glucopyranoside, delphinidine 3-O- β -glucopyranoside and pelargonidine 3-O- β -glucopyranoside were obtained from Polyphenols Laboratories (Hanabryggene Technology Centre, Norway). Standards of phenolic acids such as protocatechuic acid and ferulic acid were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The chromatographic systems consisted of a Dionex equipped with a P580 pump, ASI-100 automated sample injector and UV detector, and were monitored and controlled by using the Chromeleon computer program (Dionex). Wave lengths used for the identification of protocatechuic acid and anthocyanins were 254 nm and 520 nm, respectively. To quantify the ferulic acid, the HPLC-MS analyses were carried out by using a Waters Alliance 2975 LC system (Waters Co., USA) coupled to a mass spectrometer.

Antigen sensitization, challenge and treatment

The protocol for sensitization and inhalational challenge has previously been described in detail [10], [11], [12]. Mice were sensitized on days 0 and 7 by intraperitoneal injection of 50 μg chicken egg ovalbumin (OVA, grade V, Sigma, USA) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical, USA) in a total volume of 100 μL . Two weeks after the last sensitization, mice were exposed to aerosolized 1% OVA for 30 min/day, 3 days/week for 6 weeks. OVA challenge was performed by placing mice in a Plexiglas box ($29 \times 22 \times 18\ \text{cm}$) and aerosolizing OVA using an ultrasonic nebulizer (NE-U12; Omron, Japan) driven by compressed air for 30 min. During the last 4 weeks, DA-9201 (30, 100, 300 mg/kg, each $n = 8$) was orally administered for 5 days a week. These experimental conditions were obtained based on several preliminary studies to determine the optimal experimental conditions, which were performed over a broad range of dose levels (3, 10, 30, 100, 300, 1000 mg/kg) and treatment period (1, 2, 4 weeks). Dexamethasone (3 mg/kg, $n = 8$) was also administered orally during the last 2 weeks. Negative control mice were intragastrically administered HPMC. All animals were sacrificed 24 h after the last exposure.

Bronchoalveolar lavage fluid (BALF) collection and differential count

BALF was collected for differential cell count and measurement of cytokines [10], [11], [12]. The differential BAL cells were counted by microscopic evaluation after cytospin preparations and Giemsa staining.

Histological examination

Lungs were dissected, fixed in 10% buffered formalin, embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin (H&E) to assess the inflammatory cell infiltrates, Masson's trichrome (MT) to determine collagen deposition in the lung, periodic acid-Schiff (PAS) to quantify airway goblet cells and Congo red to evaluate the peribronchial infiltration with eosinophils.

Immunohistochemistry and morphometry

Immunohistochemistry against α -smooth muscle actin (α -SMA) was performed as previously described [13]. The volumetric densities of the α -SMA, fibrotic tissues and mucin, the relative volumes which these structures occupy in the tissue, were determined using the color analysis software. All the analyses were performed with a microscope (Zeiss, Germany) connected to a digitalized camera (Cool SNAP, USA), which transferred the captured images to a microcomputer. From each animal, 5 randomly selected sections stained with either anti- α -SMA, Masson's trichrome or PAS were used, and five fields per slide, totaling 25 fields per animals were obtained. The photomicrographs were taken from these fields, scanned and quantified for the areas occupied by fast red (red color) stained α -SMA, aniline blue (blue color) stained collagen and periodic acid (purple color) stained mucin using a computerized image analysis system, Imaga-Pro® Plus (Media Cybernetics, USA). Image analysis calculated the percentage of the stained areas relative to the total tissue area. Quantification of peribronchial eosinophilia in airway tissue was achieved by counting the numbers of these cells in five fields per slide in a Congo red stained section.

Cytokine and immunoglobulin assay

Plasma and BALF samples from each animal were analyzed for total IgE. Complementary capture and detection antibody pairs for mouse IgE antibody were purchased from PharMingen (San Diego, CA), and the IgE enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions. IL-4, IL-5 and IL-13 levels in the BALF were also determined by ELISA according to the manufacturer's instructions (R&D Systems).

Statistical analysis

All statistical analyses were performed using SigmaStatR for Windows 2.0 software (Jandel corporation, USA). An ANOVA test was used to examine the comparisons between the experimental groups and within each test group. All the data are expressed as a mean $\pm$ standard error of the mean (S.E.M.). The comparison between the group means was conducted using a Dunnett multiple range test with a significance level of $p = 0.05$.

Results

DA-9201, an ethanolic extract of black rice, was a black to purple colored powder. The amounts of the standard materials con-

tained in DA-9201 are shown in Supporting Information Table S1. Cyanidine 3-*O*- β -glucopyranoside, peonidine 3-*O*- β -glucopyranoside, pelargonidine 3-*O*- β -glucopyranoside, protocathechuic acid and ferulic acid were present at 1.49%, 0.30%, 0.30%, 0.47% and 0.05%, respectively.

A differential cell count revealed a significantly increased percentage of eosinophils in the OVA-exposed animals, whereas a significantly decreased percentage of eosinophils was observed in mice treated with DA-9201 in a dose-dependent manner (Fig. 1A). The total cell numbers of the BAL were 2.07 ± 0.14 (negative control, $\times 10^6$), 3.35 ± 0.72 (ovalbumin), 2.81 ± 0.58 (DA-9201, 30 mg), 1.81 ± 0.90 (DA-9201, 100 mg), 2.03 ± 0.71 (DA-9201, 300 mg), and 1.73 ± 0.50 cells (dexamethasone), respectively. Infiltrating eosinophils around the peribronchial area were evaluated by Congo red staining. The number of eosinophils in the peribronchial area in OVA-exposed animals was significantly increased as compared to the negative control group. In groups treated with DA-9201, 100 mg or 300 mg/kg, the number of eosinophils was significantly lower than that in the OVA control group (Fig. 1B). Tissue cellular infiltration was also assessed based on H&E stained lung sections. Figure S1 (see Supporting Information) shows a representative lung section from each treatment group. Chronic OVA challenge induced dramatic perivascular and peribronchial infiltration of various types of cells, and DA-9201 treatment markedly reduced infiltration of inflammatory cells.

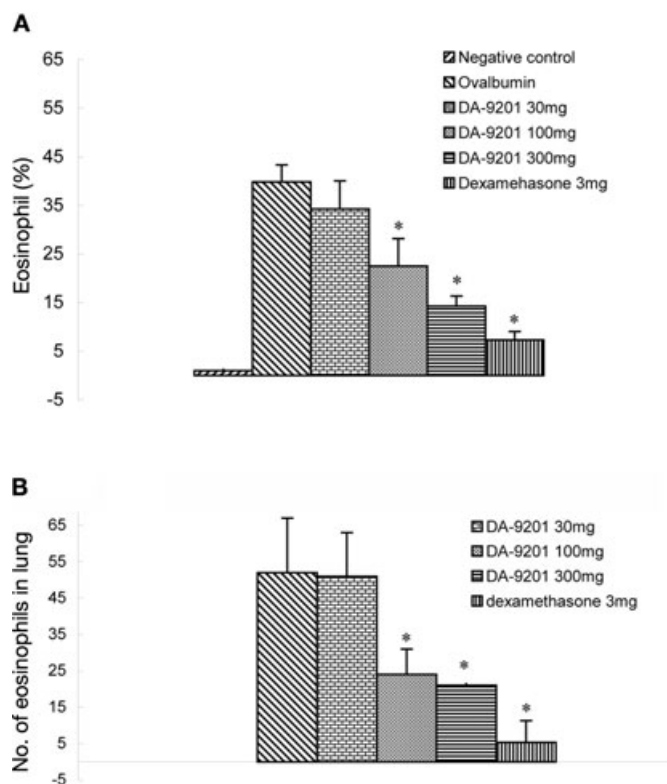


Fig. 1 DA-9201 inhibited eosinophil infiltration in the airways of OVA sensitized/challenged mice. Different cell types in the BAL were analyzed (A). Their lungs were fixed, and tissue sections were stained with Congo red (B). The eosinophil in normal mice was not detectable. Each bar represents the mean $\pm$ SEM ($n = 8$). * Significantly different from the OVA control group ($p < 0.05$).

Total IgE levels in serum and BALF as well as cytokines in BLAF were also measured using an ELISA. IgE levels in serum and BALF were elevated in sensitized mice challenged with OVA as compared to the negative controls. These parameters were significantly reduced by the treatment with DA-9201 (see Supporting Information Fig. S2). Levels of Th2 cytokines were also increased in the chronically inflamed lungs, whereas DA-9201 significantly suppressed all of these cytokines (Supporting Information Table S2).

The number of goblet cells, as identified by PAS staining, was significantly increased as compared to the negative control (40.9 ± 2.4 in OVA control and 0 ± 0 in negative control, respectively). Treatment with DA-9201, 100 mg or 300 mg/kg, significantly reduced the number of goblet cells. There was a significant reduction in the proportion of goblet cells in the airway of mice administered DA-9201, 30 mg/kg, or dexamethasone [34.1 ± 1.4 and 32.0 ± 1.3 , respectively (Fig. 2A)]. Moreover, morphometric analysis in the epithelial portion of the airway wall also revealed that chronic allergen exposure increased the substantial amount of mucin present in comparison with the negative control group, which was significantly decreased by treatment with DA-9201 (Fig. 2B).

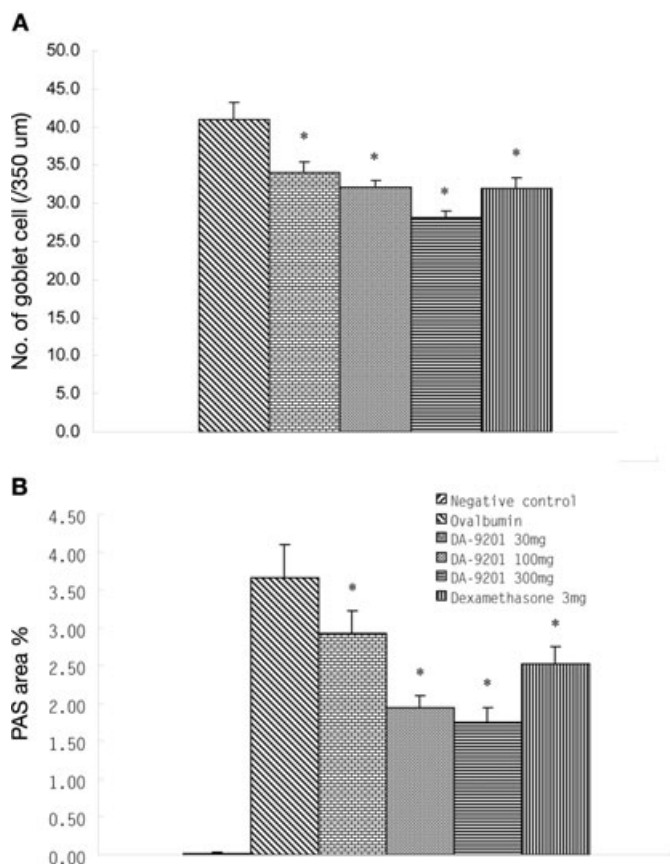


Fig. 2 The number of goblet cells in airway after 6 weeks of exposure to OVA or concomitant treatment with DA-9201 (A). In normal mice, goblet cells were not detectable. Morphometric analysis in the epithelial portion of the airway wall was performed after visualizing with periodic acid-Schiff (PAS) staining (B). Results are expressed as mean $\pm$ SEM ($n = 8$). * Significantly different from the OVA sensitized control group ($p < 0.05$).

As shown in Figure S3 (see Supporting Information Fig. S3), the thickness of the smooth muscle layer surrounding the airways was markedly increased in mice chronically exposed to OVA as compared with the negative control group. In morphometric analysis, the percentage of airway smooth muscle content was $45.0 \pm 1.1\%$ in OVA-treated mice, which was significantly increased when compared to the negative control group ($22.8 \pm 1.5\%$). Treatment with dexamethasone had little effect on the thickening of airway smooth muscle, but there was a significant reduction in the proportion of smooth muscle content in the airway of mice receiving DA-9201, 30 mg, 100 mg or 300 mg (Fig. 3).

In OVA-sensitized and challenged mice, dense collagen deposition/fibrosis was seen throughout the lung interstitium surrounding the airways and in blood vessels by Masson's trichrome staining. To analyze collagen deposition in a more quantitative manner, subepithelial collagen deposition within the bronchioles was measured using the Image-Pro Plus system and presented as a percentage of area. As shown in Fig. 4, the mean percentage of airway fibrosis in OVA-treated mice was significantly increased with $4.97 \pm 0.75\%$ as compared to the HPMC-treated control ($1.77 \pm 0.67\%$). Administration of DA-9201 resulted in a significant reduction in the collagen deposition in the lung interstitium including the perivascular collagen deposition. The mean percentages of airway collagen in mice treated with DA-9201, 100 mg or 300 mg, were $2.62 \pm 0.24\%$ and $2.60 \pm 0.38\%$, respectively, which were significantly different from the OVA-treated control.

Discussion

Human asthma is a chronic inflammatory disorder with significant airway structural changes or airway remodeling [14]. However, most of the currently developed animal models of asthma have been limited to acute airway inflammatory changes following short periods of allergen exposure. Although these models represent the phenotypes of acute asthma attacks or exacerbations, the chronic nature of human asthma and airway remodeling are not well reflected. Chronic asthma models, particularly

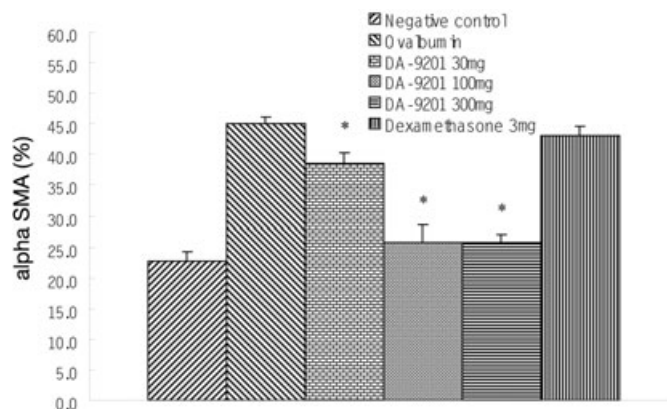


Fig. 3 DA-9201 inhibits airway smooth muscle thickening. Morphometric analysis in the epithelial portion of the airway wall for smooth muscle content was performed. Results are expressed as mean $\pm$ SEM ($n = 8$). * Significantly different from the OVA-sensitized control group ($p < 0.05$).

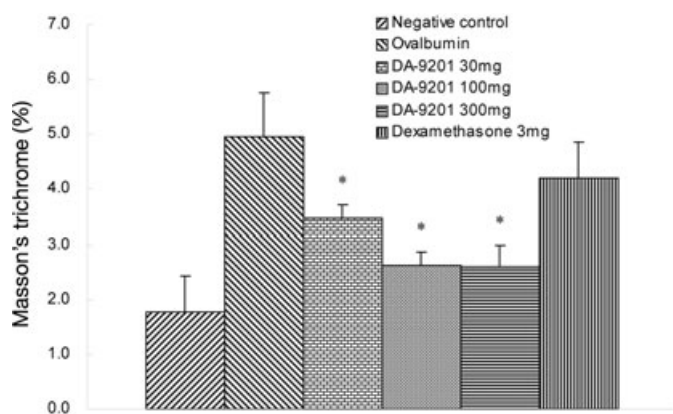


Fig. 4 DA-9201 significantly inhibited subepithelial fibrosis. Airway walls from mice treated with DA-9201 were stained with Masson's trichrome (MT) to visualize collagen deposition. The MT positive subepithelial area for each bronchiole was calculated using the Image-Pro Plus system and expressed as the ratio to the total bronchiole area. Each bar represents the mean $\pm$ SEM of bronchioles from 8 mice in each group. * $p < 0.05$ compared to the OVA control group.

those involving airway remodeling, would be more relevant to human asthma and the development of a novel asthma therapy. In the present study, we employed an animal model of prolonged OVA exposure on the airways of sensitized mice. When repeatedly exposed to aerosolized OVA for 6 weeks the sensitized mice developed OVA-specific IgE, persistent eosinophilic airway infiltration and greater structural airway changes such as thickening of the airway basement membrane and subepithelial collagen deposition, smooth muscle mass increase and mucus hypersecretion [10], [11], [12].

In acute and chronic animal models, repeated OVA challenge was shown to induce significant increases in serum and BALF IgE levels. IgE mediates the allergic response in humans as well as in animal models of allergic disease by triggering mast cell degranulation. Furthermore, elevated IgE levels and eosinophilia may correlate with the incidence or severity of asthma [15]. In this study, we demonstrated that administration of DA-9201 significantly reduced total serum and BALF IgE levels. We also observed that eosinophilia in BALF and peribronchial tissues was significantly reduced in DA-9201-treated mice. Furthermore, lung histological examination revealed that DA-9201 reduced inflammatory cell infiltration in the lungs. These findings provide experimental evidence that this herbal extract exhibits an immunoregulatory and anti-inflammatory effect, especially on the infiltration of eosinophils that may be favorable for the treatment of allergic diseases. As expected, dexamethasone, one of the most potent corticosteroids, markedly suppressed antigen-induced eosinophilic inflammation. However, unlike DA-9201, dexamethasone had little effect on the reduction of IgE levels.

Airway smooth muscle thickening as a result of both smooth muscle cell hyperplasia and hypertrophy is one of the main characteristics of lung remodeling in asthma [2]. After chronic exposure to an allergen, a significant increase in the amount of α -SMA was observed, which is consistent with a previous study in allergen sensitized and challenged mice [16]. An allergen-induced increase in airway smooth muscle was significantly reduced by

treatment with DA-9201. These results indicate that chronic DA-9201 treatment played an important role in the progression of airway remodeling and may be an effective therapy against lung remodeling in human asthma.

Mucus gland metaplasia and mucus plugging are well-documented features of chronic asthma and the major causes of asthma-associated mortalities [17]. In this study, DA-9201 treatment significantly and dose-dependently decreased the mucus production and goblet cell hypertrophy. Goblet cells, normally absent in small airways [18], were observed in chronic airway inflammatory conditions and attributed to bronchial obstruction [19]. Taken together, the reduction in PAS-stained goblet cells observed in this study further demonstrated that DA-9201 was effective in controlling excessive mucus production. Inhibition of mucous cell hyperplasia and metaplasia by dexamethasone was also observed, corresponding with previous studies with animals [20], [21].

Another index of airway remodeling enhanced by prolonged allergen exposure is increased deposition of collagen. It is known as a key feature of airway remodeling in human asthma and is associated with the severity of the disease and correlated with a decline in pulmonary function [22]. To access the potential effect of DA-9201 on fibrosis in the chronic asthma model, tissue collagen deposition was visualized with MT staining. Our data clearly demonstrated that repeated OVA exposure induced significant subepithelial collagen deposition and the treatment with DA-9201 significantly suppressed collagen deposition in the connective tissue surrounding the blood vessels and alveolar interstitium. These results suggest that DA-9201 may be effective in preventing or halting collagen deposition/lung fibrosis in this model of chronic asthmatic responses.

The pharmacologically active components and action mechanism of DA-9201 have not yet been characterized. However, in this study, using a mouse model of chronic asthma, we found that DA-9201 suppressed the pulmonary inflammation, accumulation of eosinophils in peribronchial areas, and progression of airway remodeling such as goblet cell hyperplasia, smooth muscle hypertrophy and subepithelial collagen deposition. These results suggest that DA-9201 could be of value in the treatment of asthma either as an alternative or as a complementary therapy to conventional therapies.

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