



Effect of fudosteine, a cysteine derivative, on airway hyperresponsiveness, inflammation, and remodeling in a murine model of asthma

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ABSTRACT

Aims: Fudosteine is a cysteine derivative that is used as an expectorant in chronic bronchial inflammatory disorders. It has been shown to decrease the number of goblet cells in an animal model. This study examined the effects of fudosteine on airway inflammation and remodeling in a murine model of chronic asthma.

Main methods: BALB/c mice were sensitized by an intraperitoneal injection of ovalbumin (OVA), and subsequently challenged with nebulized ovalbumin three days a week for four weeks. Seventy-two hours after the fourth challenge, airway hyperresponsiveness (AHR) and the cell composition of bronchoalveolar lavage (BAL) fluid were assessed. Fudosteine was administered orally at 10 mg/kg or 100 mg/kg body weight from the first to the fourth challenge.

Key findings: We investigated the effects of fudosteine on the development of allergic airway inflammation and airway hyperresponsiveness after chronic allergen challenges. The administration of fudosteine during the challenge with ovalbumin prevented the development of airway hyperresponsiveness and accumulation of lymphocytes in the airways. Eotaxin, IL-4, and TGF- β levels and the relative intensity of matrix metalloproteinase-2 and matrix metalloproteinase-9 (MMP-2 and MMP-9) in BAL fluid were reduced by the fudosteine treatment; however, the number of eosinophils in BAL fluid and serum IgE levels did not change. The expression of TGF- β , the development of goblet cell hyperplasia, subepithelial collagenization, and basement membrane thickening were also reduced by the fudosteine treatment.

Significance: These results indicate that fudosteine is effective in reducing airway hyperresponsiveness, airway inflammation, and airway remodeling in a murine model of chronic asthma.

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Introduction

Bronchial asthma is characterized by reversible airway obstructions and increased airway hyperresponsiveness (AHR), which is recognized as an allergic inflammation. The airway inflammation associated with asthma is accompanied by the accumulation of eosinophils, lymphocytes, neutrophils, macrophages, and natural killer cells in the airways or bronchial walls (Azzawi et al., 1990; Larche et al., 2003; O'Donnell et al., 2006). These cells secrete several chemical mediators, such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), lipid eicosanoids, elastase, and Th2 cytokines, such as IL-4, IL-5, and IL-13 (Minty et al., 1997). The persistent airway inflammation associated with chronic asthma causes the hypertrophy of airway smooth muscle (ASM) cells and mucus glands, thickening of airway walls, fibrosis of the basement

membrane, and mucus hypersecretion by goblet cells in the airways (Hogaboam et al., 2000; Kuwano et al., 1993). These responses have been referred to as airway remodeling, which is thought to occur as a result of an imbalance in the mechanism of airway regeneration and repair. AHR is defined as excessive airway narrowing in response to various chemical and physical stimuli and is the result of a complex array of factors. In general, there are two different components of AHR. One is airway inflammation, such as a large number of inflammatory cells, cytokines, chemokines, and chemical mediators. The other is airway remodeling, such as subepithelial collagen deposition and ASM hypertrophy/hyperplasia (Bromley et al., 2006; Brusasco and Pellegrino, 2003).

Fudosteine ((-)-(R)-2-amino-3-(3-hydroxypropylthio) propionic acid) is a derivative of L-cysteine that is used as an expectorant for the treatment of bronchial asthma and chronic bronchitis. It inhibits increases in the number of goblet cells associated with lipopolysaccharide-induced lung injury in the rat (Takahashi et al., 1998a,b). In endotoxin- and antigen-induced airway inflammation in this animal model, fudosteine significantly inhibited the number of chemokines, neutrophils in bronchoalveolar lavage (BAL) fluid, and goblet cells and also eosinophil

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infiltration (Komatsu et al., 2005). The expression of MUC5AC, the major respiratory mucin in goblet cell secretions, was reduced by fudosteine through the inhibition of extracellular signal-related kinase (ERK) and p38 mitogen activated-protein kinase (MAPK) (Rhee et al., 2008). Fudosteine suppressed increased tracheal blood flow, and contributed to anti-inflammatory activities through scavenging of the superoxide anion (Takahashi et al., 2001). Furthermore, fudosteine reduced peroxynitrite, a powerful oxidant/nitrosant, and the release of IL-8 in airway epithelial cells (Osoata et al., 2009). These reports suggest that fudosteine has anti-inflammatory effects at the sites of inflammation such as asthma. We hypothesized that fudosteine may reduce chronic inflammation and inhibit the remodeling associated with asthma. We used a murine model of chronic asthma to evaluate the effects of fudosteine on airway remodeling.

Materials and methods

Animals

BALB/c mice (females, 6–8 weeks of age) were purchased from Charles River Japan, Inc. (Yokohama, Japan). All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of Okayama University Medical School.

Sensitization and airway challenge

Mice were divided into 4 groups ($n = 10$ per group). Saline (0.1 ml) was intraperitoneally administered to a non-sensitized and non-challenged group on Days 0 and 14. The other groups were sensitized and challenged with ovalbumin (OVA). Briefly, mice were sensitized by an intraperitoneal injection of 20 μ g OVA (Albumin Chicken egg, Grade V; Sigma Chemical Co., St. Louis, IL, USA) emulsified in 2.25 mg of aluminum hydroxide (Imject Alum, Pierce, Rockford, IL) in a total volume of 0.1 ml on Days 0 and 14. Mice were challenged via the airways with aerosolized 1% OVA for 20 min, 3 days per week, from Days 28 to 51 by an ultrasonic nebulizer (Omron Healthcare, Kyoto, Japan) (Fig. 1). Non-sensitized and non-challenged mice receiving aerosolized saline were treated in the same fashion. AHR

was assessed 72 h after the last challenge, and tissues and cells were obtained for further assays.

Administration of fudosteine

OVA-sensitized and OVA-challenged mice receiving a low dose of fudosteine (FL) 10 mg/kg or a high dose of fudosteine (FH) 100 mg/kg orally in water 5 days per week from Days 28 to 53 (Fig. 1). Fudosteine was provided from SS Pharmaceutical Co., Ltd. (Tokyo, Japan). Control groups (vehicle and OVA-sensitized/OVA-challenged groups) received water in the same manner.

Determination of AHR

AHR was assessed by a change in airway function after the challenge with aerosolized MCh (methacholine, Wako Pure Chemical Industries, Ltd., Osaka, Japan) using barometric plethysmography (Buxco Electronics Inc., Troy, NY) as previously described (Hirano et al., 2006; Ito et al., 2005). Briefly, pressure differences were measured between the main chamber of the plethysmograph, which contained conscious, spontaneously breathing animals, and the reference chamber (box pressure signal). Mice were challenged with aerosolized saline for baseline measurements or with MCh (6.3–50 mg/ml) for 3 min, and readings were taken and averaged for 3 min after each nebulization. Data were expressed using the dimensionless parameter, enhanced pause (Penh).

BAL fluid and blood

After the assessment of Penh, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium. The lungs were lavaged via the tracheal tube with saline (1 ml $\times$ 2, 37 °C). The volume of collected BAL fluid was measured in each sample, and the number of cells in BAL fluid was counted. BAL fluid was centrifuged at 1800 rpm for 15 min at 4 °C. After centrifugation, the supernatant was stored at -80 °C. The sediment was resuspended in 1 ml of phosphate buffered saline (PBS), and smear preparations were prepared by an automatic cytocentrifuge system (1000 rpm, 1 min). Preparations were stained with Giemsa, and differentiated in a blinded fashion by

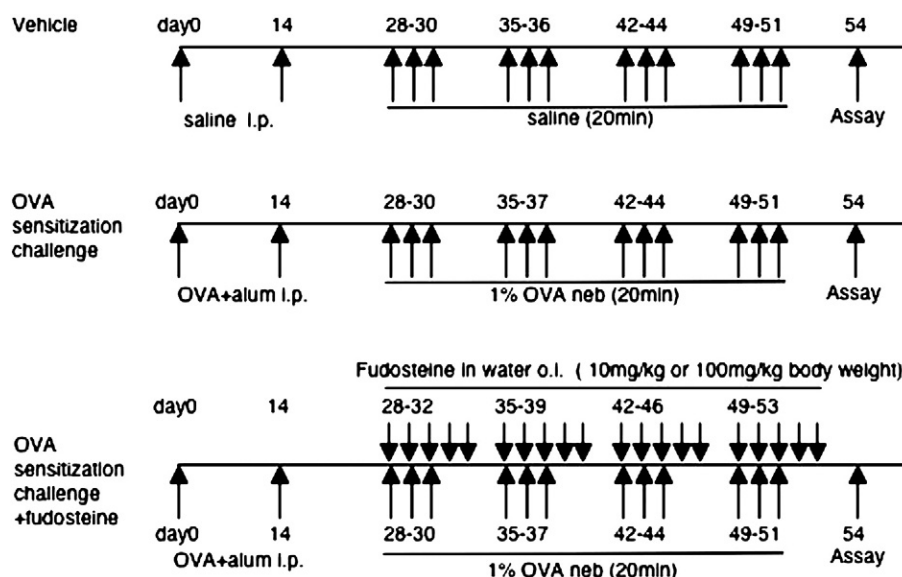


Fig. 1. Experimental protocols. Mice were divided into 4 groups; vehicle, OVA, OVA + FL, and OVA + FH. In the OVA-sensitized and OVA-challenged receiving water (OVA) group, mice were sensitized by two intraperitoneal injections of OVA/alum and then received three consecutive days of an aerosolized OVA challenge from Days 28–51 for 4 weeks. To evaluate the effect of fudosteine, OVA-sensitized and OVA-challenged mice received 5 consecutive days of an orally administered low-dose of 10 mg/kg (OVA-FL) or high-dose of 100 mg/kg (OVA + FH) fudosteine from Days 28 to 53 for 4 weeks. The OVA + FL group receiving fudosteine at a dose of 10 mg/kg daily, and the OVA + FH group received 100 mg/kg of fudosteine daily. Non-sensitized and non-challenged mice receiving water represented the vehicle. Values are means $\pm$ SEM. * $p < 0.001$, $\$p < 0.02$.

counting at least 200 cells under light microscopy. Blood samples were collected from the abdominal space by excising the vena cava. Blood was centrifuged at 3000 rpm for 20 min. After centrifugation, the serum was stored at -80°C . Finally, the lungs were removed and fixed in 10% formalin.

Zymographic analysis of MMPs

Using gelatin zymography, MMPs were detected in BAL fluid by their capacity to degrade gelatin. A total of 20 μl BAL fluid underwent electrophoresis in 7.5% polyacrylamide gels containing 2% gelatin (DIFCO), in the presence of SDS (Katayama Chemical, Osaka, Japan) under non-reducing conditions. After electrophoresis, gels were washed twice in 2.5% Triton X-100 (Sigma) and 50 mM Tris-HCl (Wako) (pH 7.5) containing 2.5% Triton X-100, 150 mM NaCl (Sigma), and 10 mM CaCl_2 (Nacalai Tesque, Kyoto, Japan), and were then incubated in 50 mM Tris-HCl (pH 7.5) containing 0.02% Triton X-100, 150 mM NaCl, and 10 mM CaCl_2 at 37°C for 20 h. Following incubation, gels were stained with Coomassie brilliant blue R250 (Nacalai Tesque) and destained in a solution of 10% acetic acid with 40% methanol. Gelatinolytic activity was detected as clear bands against a blue background. Images of gels were taken with a digital camera and the intensities of the bands were measured using a Basic Quantifier (Bio Image Systems Inc., Jackson, MI, USA). Results were expressed as relative activity using human MMP-2 and MMP-9 (CHEMICON, Temecula, CA, USA) loaded onto each gel.

Measurement of serum anti-OVA antibody

Anti-OVA IgE antibody levels in the serum were measured by ELISA. Each well of a 96-well micro-titer plate (Nunc, Roskilde, Denmark) was coated with 100 μl OVA (50 $\mu\text{g}/\text{ml}$ in PBS) and kept overnight at 4°C . After a subsequent wash, standard serum and samples diluted 50-fold (50 μl) were added. After a subsequent wash, the plate was incubated with 50 μl biotin-conjugated anti mouse-IgE antibody (Cosmo Bio Co., Ltd., Tokyo Japan). The plate was again washed and incubated with 50 μl horseradish-peroxidase (HRP)-conjugated streptavidin (Cosmo Bio). Enzyme substrate (ABTS; Sigma, St. Louis, MO, USA) with 0.05% H_2O_2 solution was added to yield a total volume of 100 μl . Absorbance was read at 405 nm on a microplate reader. The antibody titer of samples was related to pooled standards that were generated in the laboratory and expressed as ELISA units per milliliter (EU/ml).

Measurement of BAL fluid cytokines

Cytokine concentrations in BAL fluid were measured by ELISA according to the manufacturer's instructions. IL-4 and eotaxin levels in BAL fluid were assayed using an IL-4 and eotaxin ELISA kit (Quantikine M, R&D Systems, Minneapolis, MN, USA). TGF- β levels were measured using a TGF- β Emax ImmunoAssay System (Promega KK, Tokyo, Japan).

Histological studies

After BAL fluid was obtained, the right lungs were inflated through the tracheal tube with 2 ml air and fixed in 10% formalin. Blocks of lung tissue were cut around the main bronchus and embedded in paraffin blocks. Tissue sections 4 μm thick were affixed to microscope slides and deparaffinized. The slides were stained with hematoxylin-eosin and periodic acid Schiff (PAS) for the identification of mucus-containing cells, and were examined under light microscopy. In hematoxylin and eosin-stained lung sections, the thicknesses of basement membranes were analyzed at 5 points in each bronchiole using the NIH Image Analysis system (National Institutes of Health, Bethesda, MD). More than 10 bronchioles in a minimum of 10 high-power fields per lung tissue were randomly examined in a blinded fashion. The number of mucus-containing cells (goblet cells) was counted in more

than 10 bronchioles in 10 high-power fields per animal by measuring the length of the epithelium defined along the basement membrane and luminal area using the NIH Image Analysis system.

To visualize the expression of TGF- β , rabbit IgG against TGF- β (1:200; Promega) was applied to the dewaxed sections for the primary reactions, followed by an avidin-biotin coupling immunoperoxidase technique (EnVision + System – HRP Labelled Polymer Anti-Rabbit, Dako, Carpinteria, CA, USA). The area of Masson's trichrome-positive peribronchiolar collagen layer was measured using the NIH Image Analysis system. All bronchioles of the size and shape defined by this system were selected. The NIH Image Program allows for the manual outlining of the trichrome-stained collagen layer and computes the area within the outlined ring of tissue. The perimeter is the airway basement membrane circumference. More than 10 bronchioles in a minimum of 10 high power fields per lung tissue were randomly examined in a blinded fashion.

Quantitative measurement of TGF- β mRNA by real-time PCR

RNA from lungs was isolated using an RNeasy Mini kit (Qiagen Japan, Tokyo, Japan), and RT reactions were performed on a LightCycler (Roche Japan, Tokyo, Japan) using a LightCycler primer and probe (TaqMan) for mouse TGF- β 1 (NIHON GENE RESEARCH LABORATORIES Inc., Sendai, Japan). Results were expressed as the number of TGF- β 1 gene copies normalized to the number of glyceraldehyde-3-phosphate dehydrogenase gene copies (GAPDH, housekeeping gene).

Statistical analysis

All results were expressed as the mean $\pm$ SEM. An ANOVA was used to determine the levels of difference among all groups followed by Fisher's PLSD post hoc test after the determination of significance. Significance was set at $p < 0.05$.

Results

Treatment with fudosteine attenuates AHR and lymphocyte cell accumulation in BAL fluid

To determine the effects of fudosteine on the development of altered airway function in a chronic asthma murine model, mice were orally administrated fudosteine (10 mg/kg or 100 mg/kg body weight) or water from days 28 to 53 and AHR was assessed on Day 54 (Fig. 1). In OVA-sensitized and OVA-challenged mice receiving water (OVA), AHR significantly increased in an MCh dose-dependent manner relative to non-sensitized and non-challenged mice receiving water (vehicle). The administration of a high dose (OVA + FH) and low dose (OVA + FL) of fudosteine significantly suppressed AHR ($p < 0.001$) relative to OVA-sensitized and OVA-challenged mice receiving water in an MCh dose-dependent manner (Fig. 2a). However, no dose-dependency was observed between the low-dose and high-dose fudosteine treatments.

The number and type of inflammatory cells in the airways were determined in BAL fluid 72 h after the last OVA challenge. In non-sensitized and non-challenged mice (vehicle), $76.59 \pm 3.51\%$ of detected cells were macrophages. In OVA-sensitized and OVA-challenged mice receiving water (OVA), a marked increase in the number of lymphocytes ($5.31 \pm 1.23 \times 10^4/\text{ml}$), neutrophils ($0.61 \pm 0.21 \times 10^4/\text{ml}$), and eosinophils ($2.19 \pm 0.47 \times 10^4/\text{ml}$) was measured in BAL fluid. The number of lymphocytes in OVA-sensitized and OVA-challenged mice receiving a high dose of fudosteine (OVA + FH) was significantly lower ($1.65 \pm 0.36 \times 10^4/\text{ml}$, $p < 0.02$) than that in the OVA group; however, it did not alter the number of neutrophils ($0.48 \pm 0.16 \times 10^4/\text{ml}$) or eosinophils ($1.68 \pm 0.30 \times 10^4/\text{ml}$) (Fig. 2b). Interestingly, the number of macrophages in OVA-sensitized and OVA-challenged mice receiving a low dose of fudosteine (OVA + FL) was significantly higher ($9.74 \pm$

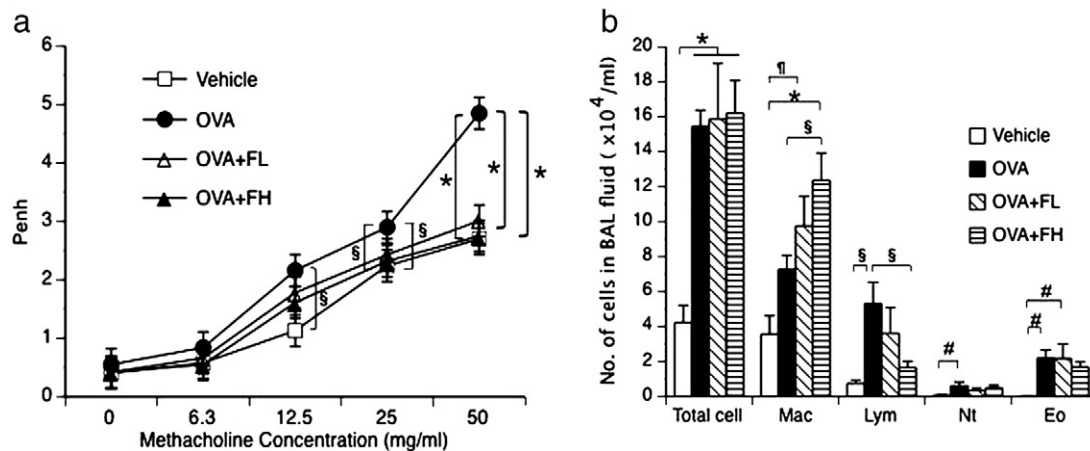


Fig. 2. Treatment with fudosteine prevents the development of AHR (a) and inflammatory cell accumulation in BAL fluid (b). Penh values for increasing concentrations of inhaled MCh and the cell composition of BAL fluid were measured in the 4 groups, non-sensitized and non-challenged mice receiving water (Vehicle), OVA-sensitized and OVA-challenged mice receiving water (OVA), OVA-sensitized and OVA-challenged mice receiving 10 mg/kg of a low dose of fudosteine (OVA + FL), and OVA-sensitized and OVA-challenged mice receiving 100 mg/kg of a high dose of fudosteine (OVA + FH), 72 h after the last challenge. Mac: macrophage; Lym: lymphocyte; Nt: neutrophil; Eo: eosinophil. Values are means $\pm$ SEM. * $p < 0.001$, $\eta p < 0.01$, $\$p < 0.02$, # $p < 0.05$.

$1.70 \times 10^4/\text{ml}$, $p < 0.01$) than that in the vehicle group ($3.56 \pm 1.05 \times 10^4/\text{ml}$). The number of macrophages in the OVA + FH group with the fudosteine treatment ($12.36 \pm 1.56 \times 10^4/\text{ml}$) was significantly higher than that in the vehicle group ($p < 0.001$) and OVA group ($7.27 \pm 0.79 \times 10^4/\text{ml}$, $p < 0.02$).

Treatment with fudosteine does not affect serum anti-OVA IgE antibody levels

Serum anti-OVA IgE levels were significantly higher in OVA-sensitized and OVA-challenged mice receiving water (OVA) (1059.75 ± 398.97 EU/ml, $p < 0.02$) than in non-sensitized and non-challenged mice receiving water (23.97 ± 6.03 EU/ml). Anti-OVA IgE levels in OVA-sensitized and OVA-challenged mice receiving the high dose of fudosteine (OVA + FH) or low dose of fudosteine (OVA + FL) (984.73 ± 189.95 and 1231.73 ± 326.47 EU/ml, respectively) were not different from the OVA group (not significant) (Fig. 3).

Treatment with fudosteine suppresses cytokine and growth factor levels in BAL fluid

The concentrations of cytokines (eotaxin and IL-4) and growth factor (TGF- β) in BAL fluid were measured by ELISA. Eotaxin (45.43 ± 3.68 pg/ml, $p < 0.001$), IL-4 (62.88 ± 4.35 pg/ml, $p < 0.001$), and TGF- β (123.69 ± 6.57 pg/ml, $p < 0.001$) levels in BAL fluid were significantly higher in OVA-sensitized and OVA-challenged mice receiving water (OVA) than in non-sensitized and non-challenged mice receiving water (vehicle). Eotaxin (19.84 ± 1.47 and 24.77 ± 3.71 pg/ml, $p < 0.001$, respectively), IL-4 (49.59 ± 2.17 and 52.7 ± 1.8 pg/ml, $p < 0.05$, respectively), and TGF- β (94.05 ± 4.04 and 99.88 ± 5.40 pg/ml, $p < 0.05$, respectively) levels in BAL fluids were significantly lower in OVA-sensitized and OVA-challenged mice receiving high- and low-dose fudosteine treatments (OVA + FH and OVA + FL) than in the OVA group (Fig. 4a–c). However, the high-dose fudosteine treatment did not decrease eotaxin to the vehicle level ($p < 0.05$).

Treatment with fudosteine suppresses airway remodeling after chronic allergen exposure

Lung sections were stained with PAS to identify mucus-containing cells in airway epithelia. The number of PAS positive goblet cells and PAS positive areas found in OVA-sensitized and OVA-challenged mice receiving water (OVA) (76.0 ± 20.0 cells/mm BM and $60.00 \pm 4.86 \mu\text{m}^2/\text{mm BM}$, $p < 0.001$, respectively) were significantly higher

than those of non-sensitized and non-challenged mice receiving water (vehicle) (3.2 ± 1.0 cells/mm BM and $14.00 \pm 2.00 \mu\text{m}^2/\text{mm BM}$, respectively). The number of PAS positive goblet cells and PAS positive areas were significantly reduced in OVA-sensitized and OVA-challenged mice receiving a high dose of fudosteine (OVA + FH) (29.0 ± 5.0 cells/mm BM, $p < 0.05$ and $36.00 \pm 3.04 \mu\text{m}^2/\text{mm BM}$, $p < 0.001$ respectively) and the PAS positive area was significantly lower in OVA-sensitized and OVA-challenged mice receiving a low dose of fudosteine (OVA + FL) ($45.0 \pm 3.22 \mu\text{m}^2/\text{mm BM}$, $p < 0.01$) than in the OVA group (Fig. 5e–h, m, n). The thickness of the basement membrane, including the smooth muscle layer, and area of Masson's trichrome-positive peribronchiolar collagen layer was significantly higher in the OVA group ($9.96 \pm 0.37 \mu\text{m}$ and $1700 \pm 178 \mu\text{m}^2/\text{mm BM}$, $p < 0.001$, respectively) than in the vehicle group ($3.25 \pm 0.19 \mu\text{m}$ and $580 \pm 277 \mu\text{m}^2/\text{mm BM}$, respectively). Treatment with a high dose or low dose of fudosteine significantly suppressed increases in thickness including that of the basement membrane (Fig. 5a–d) (7.89 ± 0.21 and $7.14 \pm 0.23 \mu\text{m}$, $p < 0.001$, respectively). Treatment with a high dose of fudosteine significantly suppressed increases in

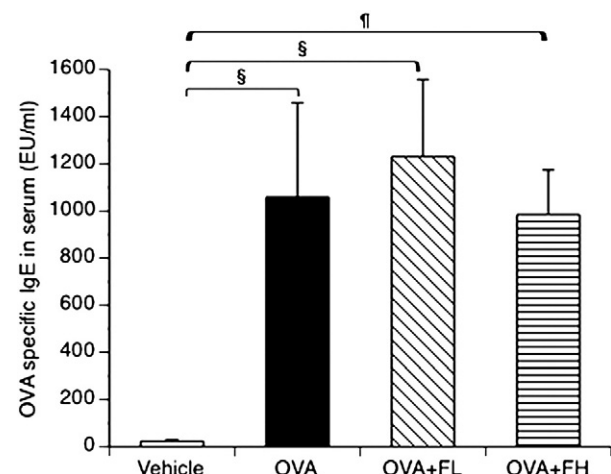


Fig. 3. OVA-specific IgE levels. OVA-specific IgE levels in the serum were measured in the 4 groups; non-sensitized and non-challenged mice receiving water (Vehicle), OVA-sensitized and OVA-challenged mice receiving water (OVA), OVA-sensitized and OVA-challenged mice receiving 10 mg/kg of a low dose of fudosteine (OVA + FL), and OVA-sensitized and OVA-challenged mice receiving 100 mg/kg of a high dose of fudosteine (OVA + FH), 72 h after the last challenge. Values are means $\pm$ SEM. $\eta p < 0.01$, $\$p < 0.02$.

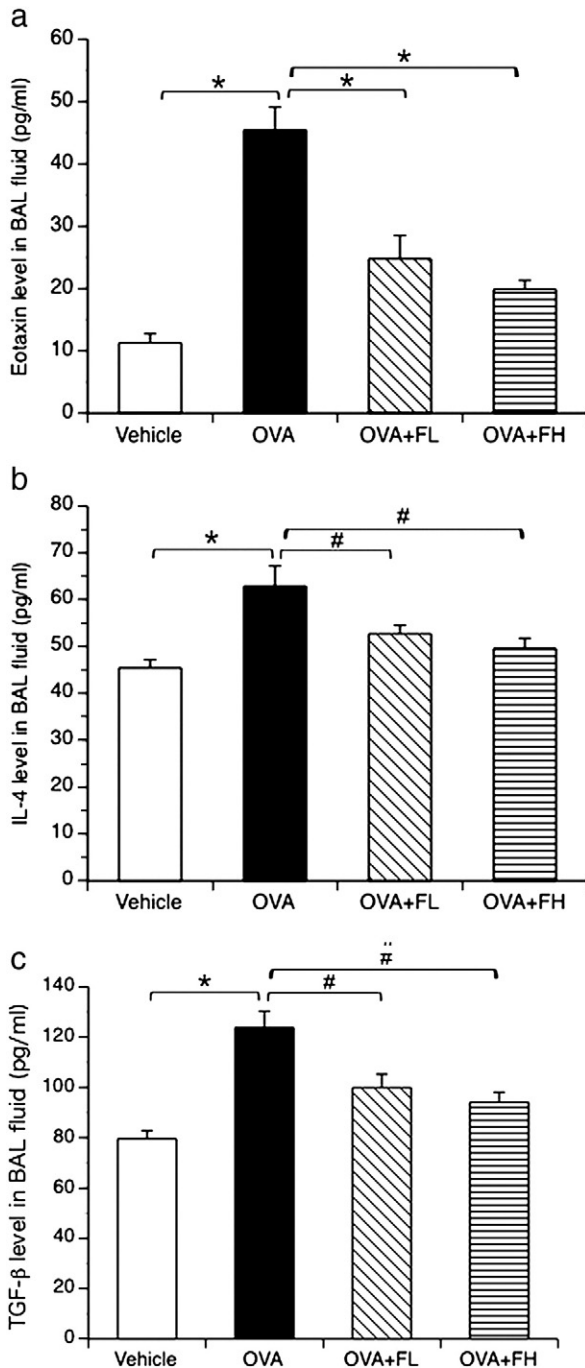


Fig. 4. The treatment with fudosteine alters cytokine and growth factor levels in BAL fluid. (a) Eotaxin, (b) IL-4, and (c) TGF-β levels in BAL fluid were measured in the 4 groups; non-sensitized and non-challenged mice receiving water (Vehicle), OVA-sensitized and OVA-challenged mice receiving water (OVA), OVA-sensitized and OVA-challenged mice receiving 10 mg/kg of a low dose of fudosteine (OVA + FL), and OVA-sensitized and OVA-challenged mice receiving 100 mg/kg of a high dose of fudosteine (OVA + FH), 72 h after the last challenge. Values are means $\pm$ SEM. * p < 0.001, # p < 0.05.

subepithelial collagenization (Fig. 5i–l, o, p) ($1181 \pm 162 \mu\text{m}^2/\text{mm BM}$, p < 0.05). However, the thickness of the basement membrane was not suppressed to the vehicle level by the fudosteine treatment (p < 0.001).

Localization and mRNA expression of TGF-β in lung tissue

Significant increases in TGF-β staining were observed in OVA-sensitized and OVA-challenged mice receiving water (OVA) (349 ± 41 cells/mm BM, Fig. 6b), whereas only slightly positive immunolabeling

for TGF-β was detected in the airway epithelia of non-sensitized and non-challenged mice receiving water (vehicle) (42 ± 6.2 cells/mm BM, Fig. 6a). Positive immunolabeling for TGF-β was detected in eosinophils and mononuclear cells (data not shown) as well as the airway epithelia of peribronchial and perivascular areas in the OVA group. OVA-sensitized and OVA-challenged mice receiving a high dose of fudosteine (OVA + FH) or a low dose of fudosteine (OVA + FL) resulted in marked decreases in the number of TGF-β positively stained cells (174 ± 6 and 245 ± 12 cells/mm BM) (Fig. 6c–e). The expression level of TGF-β mRNA in the lung tissue increased in the OVA group (6040.5 ± 968.23 copies) (Fig. 6f). Fudosteine treatment suppressed the increased expression of TGF-β mRNA in the OVA + FH and OVA + FL groups (1386.8 ± 271.92 and 1664.75 ± 360.42 copies, p < 0.01, respectively). However, the fudosteine treatment did not reduce the number of TGF-β positive cells to that of the vehicle.

Treatment with fudosteine inhibits the production of pro-MMP-2 and pro-MMP-9

MMP-2 and MMP-9 activities in BAL fluid were measured by gelatin zymography. OVA-sensitized and OVA-challenged mice receiving water (OVA) had higher pro-MMP-2 and pro-MMP-9 activities in BAL fluid than those of non-sensitized and non-challenged mice receiving water (vehicle). The increase in pro-MMP-2 and pro-MMP-9 activity was significantly less in the OVA + FH group than in the OVA group (p < 0.02) (Fig. 7a, b). Pro-MMP-2 activity in BAL fluid (p < 0.05) was significantly less in the OVA + FL group than in the OVA group. However, the fudosteine treatment did not inhibit the level of MMPs to that of the vehicle. The active forms of MMP-2 and MMP-9 could not be detected.

Discussion

Fudosteine is used as an expectorant in patients with bronchial asthma and chronic bronchitis. In our study, fudosteine suppressed increases in AHR by inhibiting the production of mucin from goblet cells and airway remodeling. The pathophysiology of AHR is controversial. We used enhanced pause (Penh) to assess AHR, although it is considered to be a nonspecific parameter of airway resistance (Bates et al., 2004, 2009). We could not detect the dose dependency of fudosteine in the Penh results. However, the reduction in AHR by the fudosteine treatment correlated well with histology results, cytokine levels and MMP expressions. We regarded Penh as a parameter of airway resistance in this study.

Although fudosteine suppressed eotaxin and IL-4, it could not inhibit the infiltration of eosinophils into the airways. On the other hand, fudosteine inhibited the infiltration of lymphocytes. Epithelial cells produce eotaxin by the stimulation of IL-4 (Stellato et al., 1999). Eotaxin levels were lower in OVA-sensitized and OVA-challenged mice receiving fudosteine than in OVA-sensitized and OVA-challenged mice receiving water. However, fudosteine did not suppress eotaxin levels to that of the non-sensitized and non-challenged mice receiving water (vehicle). Since the inhibition of eotaxin by fudosteine was partial, eosinophil infiltration may not have been inhibited by the fudosteine treatment. IL-5 is a strong chemotactic cytokine of eosinophils. We did not measure IL-5 levels in BAL fluid in this experiment. IL-5 may have also been inhibited by fudosteine, similar to IL-4. We assume that the infiltration of eosinophils was not inhibited by fudosteine because of the presence of IL-5 and the relatively high levels of eotaxin. Komatsu et al. reported that fudosteine inhibited OVA-induced eosinophil infiltration into BAL fluids in an acute asthma model. However, the mechanism of eosinophil infiltration inhibition was unknown because they did not measure cytokines in the BAL fluids of rats. Furthermore, our murine model was a chronic asthma model characterized by remodeling, which differed from that of an acute asthma model due to the terms of inflammation. The fudosteine treatment increased the number of macrophages in

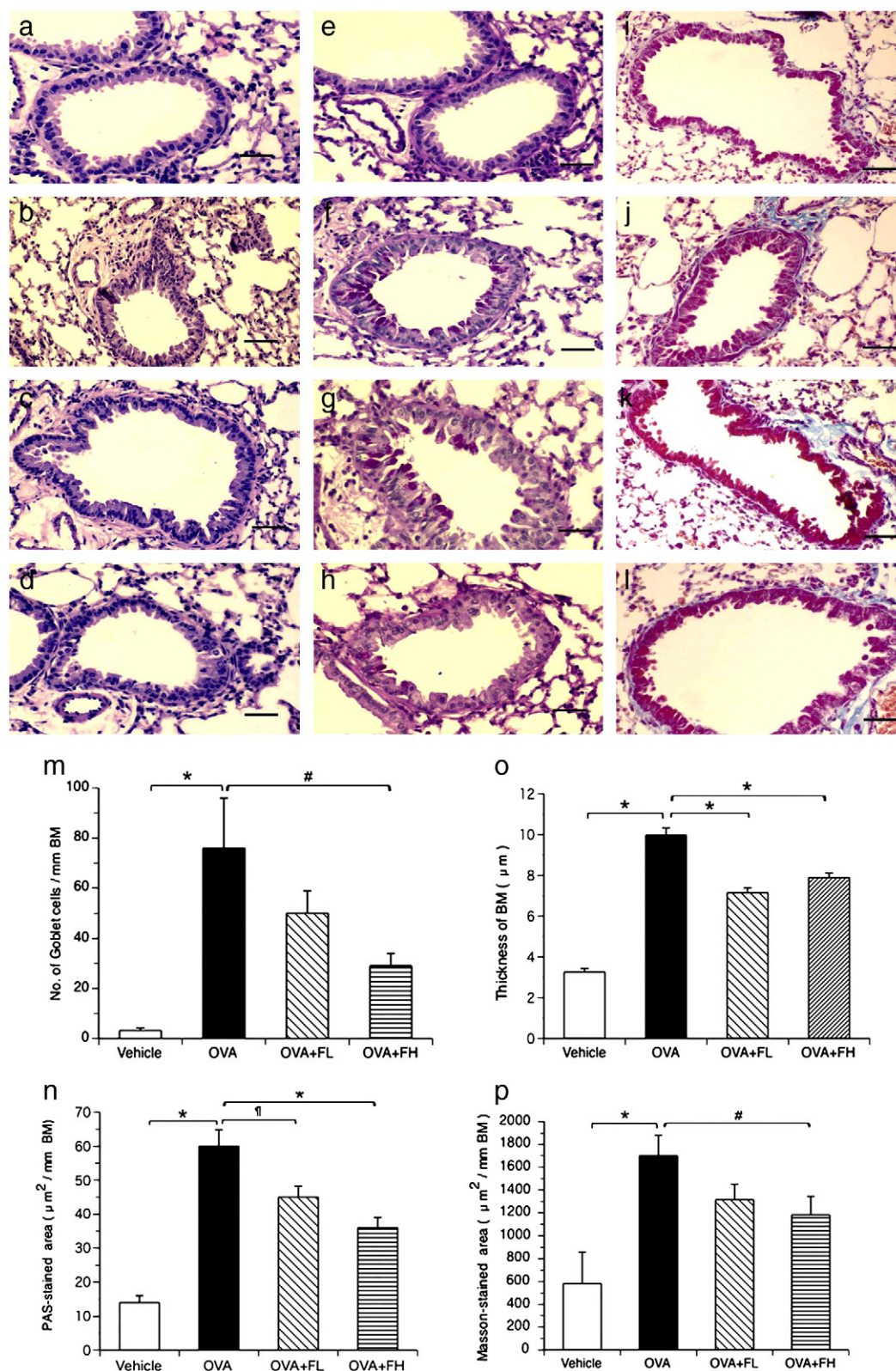


Fig. 5. Treatment of fudosteine inhibits airway remodeling. Hematoxylin & eosin (a–d), PAS staining (e–h), and Masson's trichrome (i–l) in lung tissue from the 4 groups; non-sensitized and non-challenged mice receiving water (Vehicle) (a, e, i), OVA-sensitized and OVA-challenged mice receiving water (b, f, j), OVA-sensitized and OVA-challenged mice receiving 10 mg/kg of a low dose of fudosteine (OVA + FL) (c, g, k), and OVA-sensitized and OVA-challenged mice receiving 100 mg/kg of a high dose of fudosteine (OVA + FH) (d, h, l). The number of mucus-positive cells per millimeter basement membrane (m) and PAS-stained area per millimeter basement membrane (n) were measured in PAS-stained sections as described in the [Materials and methods](#). The thicknesses of the basement membranes including the smooth muscle layer (o) in H&E staining sections and Masson's trichrome positive collagenization area (p) in Masson's trichrome-stained sections were measured as described in the [Materials and methods](#). BM: basement membrane. Values are means $\pm$ SEM. — 50 μ m. * $p < 0.001$, # $p < 0.01$, # $p < 0.05$.

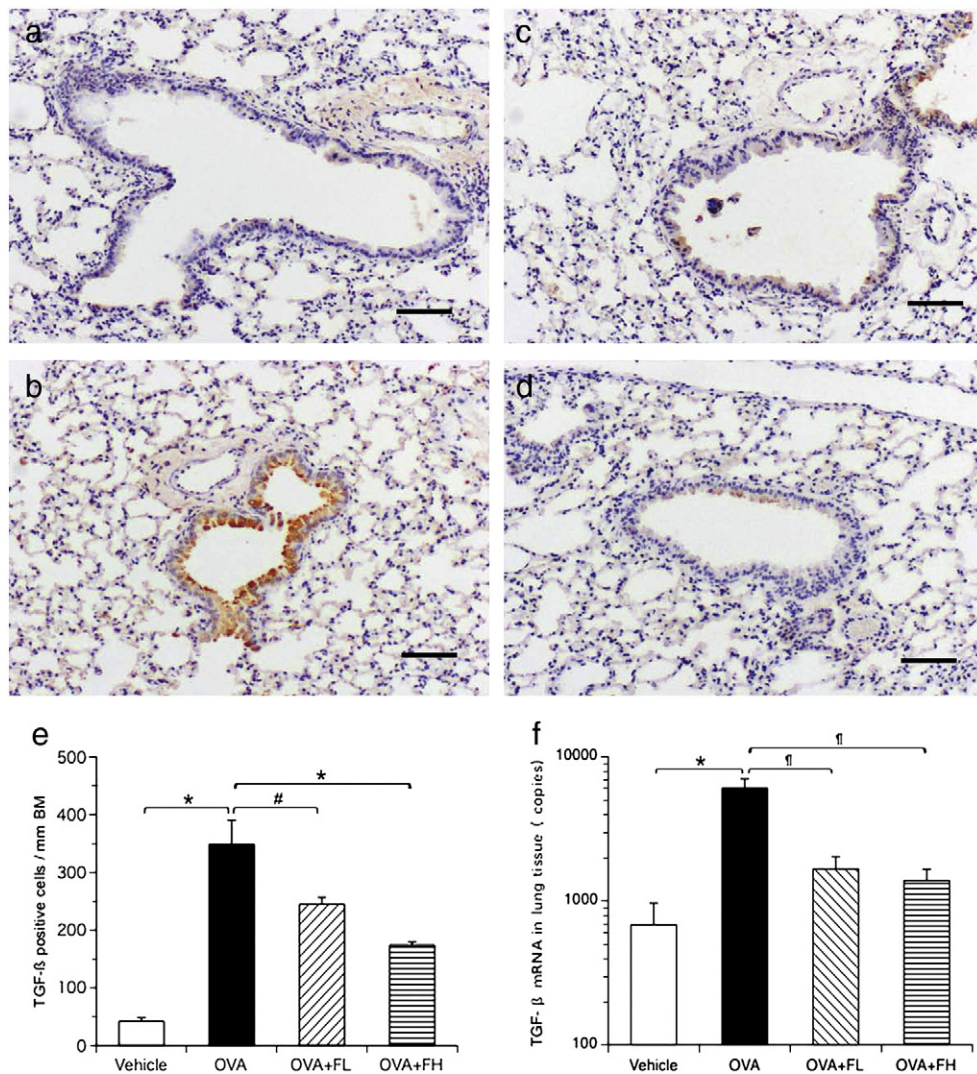


Fig. 6. Treatment of fudosteine inhibits TGF- β expression. Immunohistochemical staining of TGF- β (a–d) in lung tissue from the 4 groups; non-sensitized and non-challenged mice receiving water (Vehicle) (a), OVA-sensitized and OVA-challenged mice receiving water (OVA) (b), OVA-sensitized and OVA-challenged mice receiving 10 mg/kg of a low dose of fudosteine (OVA + FL) (c), and OVA-sensitized and OVA-challenged mice receiving 100 mg/kg of a high dose of fudosteine (OVA + FH) (d). The number of TGF- β positive cells was measured in the epithelia per millimeter basement membrane (e). TGF- β 1 gene expression in lung tissue was measured by real-time RT-PCR, and results were expressed as the number of TGF- β 1 gene copies normalized to the number of glyceraldehyde-3-phosphate dehydrogenase gene copies (housekeeping gene). BM: basement membrane. Values are means $\pm$ SEM. — 50 μ m. * p < 0.001, $\dagger p$ < 0.01, # p < 0.05.

BAL fluid. Although we did not assess non-sensitized and non-challenged mice receiving fudosteine, these results may be attributable to the effects of fudosteine.

Activated CD4 + T cells turn into Th2 cells, secrete IL-4, IL-5, and IL-13, and induce eosinophil accumulation and B-cell activation. T cells attach to airway smooth muscle (ASM) cells and induce the

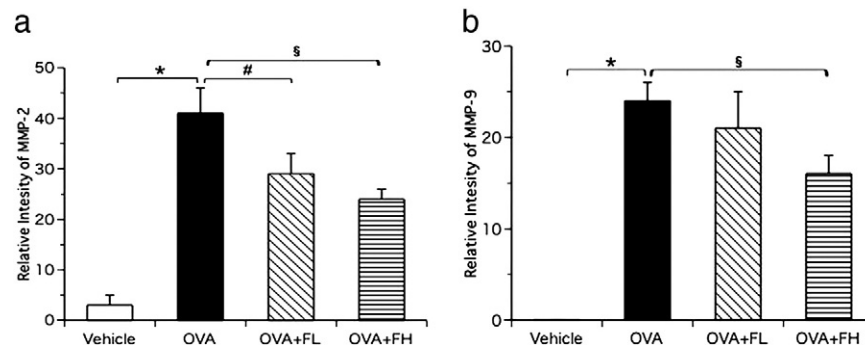


Fig. 7. Treatment of fudosteine inhibits MMPs production. The activity of matrix metalloproteinases (MMPs) was measured by gelatin zymography in BAL fluid from the 4 groups; non-sensitized and non-challenged mice receiving water (Vehicle), OVA-sensitized and OVA-challenged mice receiving water (OVA), OVA-sensitized and OVA-challenged mice receiving 10 mg/kg of a low dose of fudosteine (OVA + FL), and OVA-sensitized and OVA-challenged mice receiving 100 mg/kg of a high dose of fudosteine (OVA + FH). Gelatinolytic bands were estimated by the intensity of gel images. Results were expressed as relative activity using MMP-2 (a), MMP-9 (b). Values are means $\pm$ SEM. * p < 0.001, $\S p$ < 0.02, # p < 0.05.

proliferation of these cells (Al Heialy et al., 2011; Ramos-Barbon et al., 2005). Fudosteine has shown to suppress the number of lymphocytes in BAL fluid. RANTES has been shown to be secreted by airway epithelial cells (Stellato et al., 1995). Fudosteine inhibited TNF- α induced MUC5AC and RANTES expression by inhibiting p38 activation (Hashimoto et al., 2000; Rhee et al., 2008). Decreased RANTES production may inhibit T cell accumulation into the ASM layer and BAL fluid. TGF- β , which is thought to be the master regulator of airway remodeling, is secreted by Th2 cells (McMillan et al., 2005) and the structural cells of airways such as fibroblasts (Kelley et al., 1991), endothelial cells (Coker et al., 1996), vascular smooth muscle cells (de Boer et al., 1998), and ASM cells (Lee et al., 2006). It induces the differentiation of fibroblasts to myofibroblast cells and the proliferation of ASM cells (Morishima et al., 2001). Thus, fudosteine appeared to inhibit T cell attachment to ASM cells and caused decreased TGF- β levels in BAL fluid as well as TGF- β mRNA expression in lung tissues, which then decreased the thickness of the basement membrane.

TGF- β was shown to induce the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) (Ito et al., 2009). MMPs are known to be extracellular proteinases that contribute to tissue remodeling and cancer metastasis (Atkinson and Senior, 2003; Lokeshwar, 1999; Powell et al., 1993). MMPs also play a crucial role in the remodeling process of airways, such as accelerating the turnover of the extracellular matrix (Khandoga et al., 2006), ASM cell proliferation, and the deposition of collagen type 1, type 3, and fibronectin in the reticular layer of the basement membrane (Boulet et al., 1997; Chakir et al., 1996). In the pathogenesis of inflammation associated with asthma, increases in MMP-2 and MMP-9 were observed in BAL fluid after antigen exposure in OVA-sensitized mice (Kumagai et al., 1999). MMP-9 is produced by the stimulation of inflammatory cytokines, such as IL-1 β and TNF- α , through p38 and AP-1 activations (Kim et al., 2008; Liang et al., 2007). It may be suppressed by fudosteine through p38 inhibition because it scavenges the superoxide anion (Takahashi et al., 2001) and inhibits the activation of p38 by reactive oxygen species (Rhee et al., 2008). MMP-2 is secreted by bronchial and alveolar epithelial cells (Nishihara-Fujihara et al., 2010) and also by smooth muscle cells; it induces cell proliferation, and accelerates the turnover of the extracellular matrix (Khandoga et al., 2006). MMP-2 levels were decreased by the fudosteine treatment in our experiment. Fudosteine may possibly inhibit ASM cell proliferation and collagenization. The fudosteine treatment did not suppress MMP-2 and MMP-9 levels to vehicle levels. Because of the TGF- β positive cells remaining after the fudosteine treatment, TGF- β might stimulate the secretion of MMPs from smooth muscle cells.

Conclusions

We investigated the effects of fudosteine on airway hyperresponsiveness, airway inflammation, and airway remodeling in a murine model of chronic asthma. The administration of fudosteine induced reductions in AHR and the number of lymphocytes, IL-4, eotaxin, and TGF- β cytokine levels in BAL fluid. It also reduced the number of goblet cells, the collagenization area, the thickness of the basement membrane, and the activities of MMP-2 and MMP-9.

Conflict of interest statement

The authors declare that they have no competing interests.

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