

ORIGINAL ARTICLE

Effect of tiotropium bromide on airway inflammation and remodelling in a mouse model of asthma

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Summary

Background Tiotropium bromide, a long acting muscarinic receptor inhibitor, is a potent agent for patients with bronchial asthma as well as chronic obstructive pulmonary disease.

Objective The aim of this study was to evaluate whether tiotropium bromide can inhibit allergen-induced acute and chronic airway inflammation, T helper (Th)2 cytokine production, and airway remodelling in a murine model of asthma.

Methods Balb/c mice were sensitized and challenged acutely or chronically to ovalbumin (OVA). The impact of tiotropium bromide was assessed using these mice models by histologic, morphometric, and molecular techniques. Moreover, the effect of tiotropium bromide on Th2 cytokine production from purified human peripheral blood mononuclear cells (PBMCs) was assessed.

Results Treatment with tiotropium bromide significantly reduced airway inflammation and the Th2 cytokine production in bronchoalveolar lavage fluid (BALF) in both acute and chronic models of asthma. The levels of TGF- β 1 were also reduced by tiotropium bromide in BALF in a chronic model. The goblet cell metaplasia, thickness of airway smooth muscle, and airway fibrosis were all significantly decreased in tiotropium bromide-treated mice. Moreover, airway hyperresponsiveness (AHR) to serotonin was significantly abrogated by tiotropium bromide in a chronic model. Th2 cytokine production from spleen cells isolated from OVA-sensitized mice was also significantly inhibited by tiotropium bromide and 4-diphenylacetoxy-N-methylpiperidine methiodide, which is a selective antagonist to the M3 receptor. Finally, treatment with tiotropium bromide inhibited the Th2 cytokine production from PBMCs.

Conclusion These results indicate that tiotropium bromide can inhibit Th2 cytokine production and airway inflammation, and thus may reduce airway remodelling and AHR in a murine model of asthma.

Keywords asthma, cytokine, inflammation, muscarinic receptor, tiotropium bromide

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Introduction

Episodic airflow obstruction, airway inflammation, and airway hyperresponsiveness (AHR) are characteristic features of asthma [1–3]. Lymphocytes, mast cells, and eosinophils play important roles in airway inflammation [4–7] and two studies using targeted deletion approaches have demonstrated that eosinophils are necessary for ovalbumin (OVA)-induced AHR and airway remodelling in mice [8, 9]. Airway eosinophilia is highly regulated by

the T helper (Th)2 cytokines IL-4, IL-5, and IL-13 produced by T lymphocytes [10–12]. Conversely, eosinophils can also produce cytokines, chemokines, lipid mediators, and growth factors that may lead to the airway smooth muscle (ASM) hypertrophy, mucus production, and subepithelial fibrosis [13, 14]. Airway remodelling that includes non-reversible structural change such as increases in smooth muscle mass, mucus hyperplasia, and subepithelial fibrosis might explain the progressive loss of lung function in patients with asthma [15]. Thus, cytokines and mediators

produced in chronic airway inflammation may account for the lack of efficacy of current asthma treatments [16–19], such as inhaled corticosteroids, β_2 -agonists, antileukotrienes, and theophylline [20, 21] to impact on airway remodelling in established asthma.

The ineffectiveness of established therapies on airway remodelling in asthma has focused attention on anticholinergic interventions. Although anticholinergics are commonly used for the treatment in chronic obstructive pulmonary disease and in asthma primarily as a bronchodilator, recent studies showed the ability of tiotropium bromide to inhibit airway remodelling in a guinea-pig model of asthma [22, 23]. However, the underlying mechanisms for the inhibitory effect of tiotropium bromide on the suppression of airway remodelling were not precisely addressed.

The purpose of the current study was to examine the effect of anticholinergic treatment on acute and chronic allergic airway inflammation in mice. We assessed *in vivo* the effects of tiotropium bromide on airway inflammation and Th2 cytokine responses in the acute and chronic OVA-sensitized model, and on airway remodelling and hyperresponsiveness in the chronic OVA-sensitized model. We also assessed *in vitro* the direct effects of tiotropium bromide and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), which is a selective antagonist to the M3 receptor, on Th2 cytokine production from spleen cells. We hypothesized that anticholinergic treatment reduces airway inflammation, remodelling, and hyperresponsiveness, partly associated with a direct inhibition of Th2 cytokine production in a murine model of asthma.

Methods

Animals

BALB/c mice (female, 6–8 weeks old, weight range 19–24 g) were obtained from the Saitama Experimental Animals Supply Co. Ltd (Saitama, Japan). All animal experiments were performed under Animal Care and Use Committee approval and conformed to institutional guidelines.

Sensitization and challenge for the establishment of acute and chronic airway inflammation

OVA sensitization was performed by an intraperitoneal injection of OVA (Sigma-Aldrich, St Louis, MO, USA) and then challenged by inhalation as described in Fig. 1. For anticholinergic treatment, animals received tiotropium bromide in saline (0.1 mM, 3 min) via nebulization. Control animals received only saline. Tiotropium bromide was kindly donated by Boehringer Ingelheim Co. Ltd (Ingelheim, Germany). No previous paper has shown the

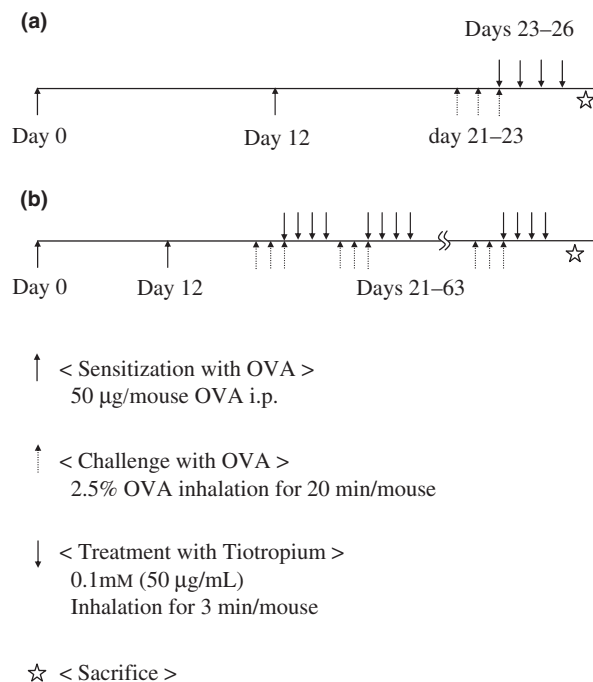


Fig. 1. Ovalbumin (OVA)-sensitization, challenge, and treatment protocols in acute (a) and chronic (b) models of asthma.

concentration of tiotropium bromide in human bronchoalveolar lavage fluid (BALF) after inhalation, and a paper showed 0.1 mM of tiotropium bromide attenuated ASM remodelling in a guinea-pig model [22]. Therefore, we chose this concentration of tiotropium bromide to investigate the effects of tiotropium bromide in mice models of asthma. Therapeutic treatment with tiotropium bromide were conducted on days 23–26 in an acute model, and 4 straight days weekly from days 21 to 63 in a chronic model.

Bronchoalveolar lavage and cellular analysis

Mice were killed 24 h after the last treatment in the acute model or 48 h after the last treatment in the chronic model. The trachea was exteriorized via blunt dissection, and a small calibre needle was inserted and secured in the airway. The successive three time-wash of 0.35 mL of saline was instilled and gently aspirated. The recovered BALF was kept on ice until centrifugation. Each BALF was centrifuged (at 300 g, 7 min, 4 °C). Cells were washed and resuspended in 100 µL of saline. Total number of the cells was calculated using a haemocytometre with Turk solution (Wako, Osaka, Japan). Differential cell counts were performed on cytopsin cell preparations (Cytospin 3; Shandon, Pittsburgh, PA, USA) and stained with Diff-Quick (Dade Behring Inc., Newark, DE, USA). A differential count of 400 cells was performed using standard morphological criteria.

Measurement of cytokines

Levels of cytokines including IL-4, IL-5, IL-13, IFN- γ , and TGF- β 1 in BALF and cell culture supernatants, were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Analysis of lung histopathology

After performing BAL, lungs were dissected from the chest and inflated with 500 μ L of 10% formalin in phosphate-buffered saline (PBS) (pH 7.4) for 24 h inflated at 10 cm H₂O pressure. Lung tissues were dehydrated in ethanol, embedded in paraffin, sectioned (5 μ m), and stained with periodic acid-Schiff (PAS) staining system (Sigma-Aldrich). An optical microscope was used with $\times$ 100 magnification in order to analyse the lung histopathology. For analysis of goblet cells, four lung sections were examined per mouse ($n=8$), and the percentage of PAS-positive cells were counted in four airways from one section. For analysis of smooth muscle hypertrophy, the airways with diameter from 100 to 300 μ m were chosen in a random manner. The mass of ASM was estimated from the measurement of the area of smooth muscle-specific α -smooth muscle actin immunoreactivity by FLOVEL Filing System (Flovel, Tokyo, Japan), and the data were divided by the diameter of the airway. Averages of four airway walls per animal were calculated ($n=8$). Masson trichrome staining was used to determine collagen deposition in four airways from four different sections per mouse ($n=8$) by classification on a 0–4+ scale. Semiquantitative analyses were undertaken by two inter-observers. Lung and spleen sections were stained with a rabbit anti-mACh R M3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Control normal goat IgG was used as negative control. After washing with PBS, a secondary goat anti-rabbit biotinylated antibody was added, then M3 receptor was detected by HRP-Dab Staining kit (R&D Systems).

Measurement of airway responsiveness

To assess the airway responsiveness, we followed the previous protocol [24]. The mice were tracheostomized, and connected to a Harvard ventilator under anaesthesia with pentobarbital. Then, mice were placed in the plethysmograph chamber (Buxco Electronics Inc., Troy, NY, USA) to measure airway resistance (Raw). Ten milligram per millilitre of serotonin, of which concentration was led by the previous studies [25, 26], was administered using an in-line aerosol system for 3 min. Data are expressed as percent Raw, which is defined by the formula: ((Raw after inhalation of serotonin/Raw before inhalation) $\times$ 100 (%)).

Cytokine production by spleen cells

Spleen cells isolated from OVA-sensitized BALB/c mice were suspended in RPMI 1640 with 10% fetal bovine serum and antibiotics (penicillin, 100 μ g/mL; streptomycin, 100 U/mL) at a concentration of 4.0×10^6 cells/mL. Cells were aliquoted into 48-well plates and stimulated with OVA (100 μ g/mL) at 37 °C in 5% CO₂ in the presence or absence of tiotropium bromide (50 μ g/mL). For analysis of cytokine production, cell culture supernatants were obtained following 72 h of stimulation with OVA. Moreover, the inhibitory effect of another anticholinergic agent, 4-DAMP (Sigma-Aldrich), was also investigated.

Cytokine production by human peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated from heparinized whole blood of asthmatic subjects with density gradient centrifugation using Lymphocyte Separation Solution (Nacalai Tesque Inc., Kyoto, Japan). PBMCs (2×10^6 cells/mL) were cultured in 24-well culture plates and stimulated with PHA (1 μ g/mL) after pre-treatment with tiotropium bromide for an hour. Concentrations of IL-5 and IL-13 in the culture supernatants were measured after 48 h by mean of ELISA kits (R&D Systems). We chose these durations of stimulation following our previous experiments [27].

Statistical analysis

Differences between two groups were investigated by an unpaired *t* test. The significance of the differences between three groups was determined by ANOVA with Bonferroni correction. Data are expressed as mean $\pm$ SEM, and $P < 0.05$ was considered to indicate significance.

Results

Treatment with tiotropium bromide reduces acute and chronic airway inflammation

We first investigated the effects of tiotropium bromide on airway inflammation. Tiotropium bromide treatment was effective in preventing the acute inflammatory reaction observed after an allergen challenge. The number of total cells, macrophages, and eosinophils in BALF were significantly decreased by tiotropium bromide (Fig. 2a). Short-term exposure of OVA to mice elicits an inflammatory response, which is usually restricted to proximal airways and not associated with airway remodelling, while chronic exposure exhibits a different phenotype of airway inflammation compared with acute exposure [28]. Thus, the effects of tiotropium bromide on chronic airway inflammation in mice were also investigated. In a chronic model of asthma, the number of total cells, lymphocytes, and

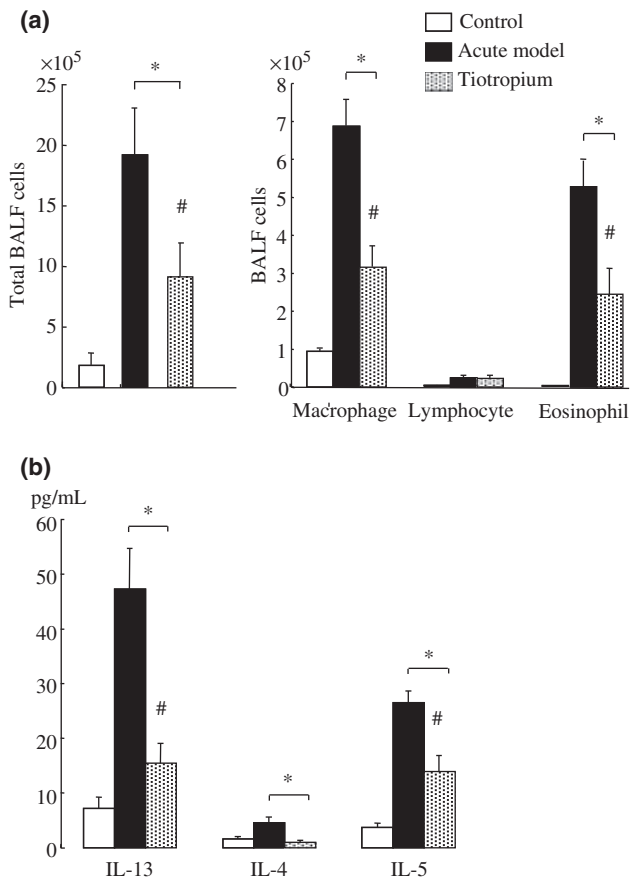


Fig. 2. Analysis of inflammatory cells and cytokines in bronchoalveolar lavage fluid (BALF) in an acute model of asthma. (a) Total number of inflammatory cells and differential count of the inflammatory cells in the BALF. (b) Cytokine levels in the BALF. Results are expressed as mean SEM ($n = 8$ for all groups). * $P < 0.05$ compared with saline-treated and ovalbumin-challenged mice. # $P < 0.05$ compared with control mice.

eosinophils in BALF were significantly decreased by tiotropium bromide (Fig. 3a). These results suggest that tiotropium bromide has an efficacy to suppress airway inflammation in both acute and chronic murine models of asthma.

Treatment with tiotropium bromide reduced cytokine levels in bronchoalveolar lavage fluid

The elevated levels of Th2 cytokines IL-4, IL-5, and IL-13 in BALF in an acute model of asthma, were significantly decreased by tiotropium bromide (Fig. 2b). In a chronic model of asthma, Th2 cytokines IL-4, IL-5, and IL-13 in BALF were also significantly reduced by tiotropium bromide (Fig. 3b). In addition, treatment with tiotropium bromide significantly suppressed the levels of TGF- β 1 in BALF after a chronic allergen challenge (Fig. 3c). Intriguingly, plasma levels of OVA-specific IgE were not significantly affected in either the acute or the chronic model of asthma by tiotropium bromide (data not shown).

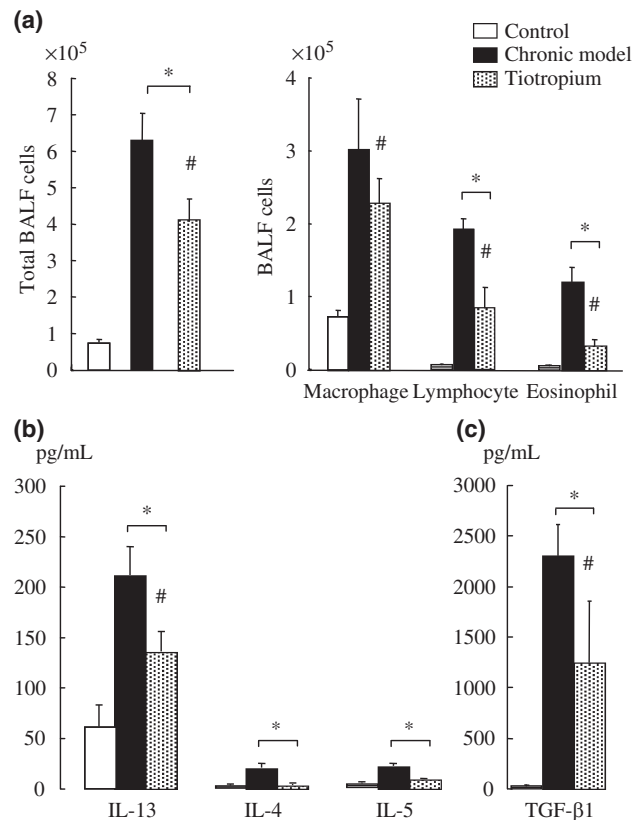


Fig. 3. Analysis of inflammatory cells and cytokines in bronchoalveolar lavage fluid (BALF) in a chronic model of asthma. (a) Total number of inflammatory cells and differential count of the inflammatory cells in the BALF. (b and c) Cytokine levels in the BALF. Results are expressed as mean SEM ($n = 8$ for all groups). * $P < 0.05$ compared with saline-treated and ovalbumin-challenged mice. # $P < 0.05$ compared with control mice.

Treatment with tiotropium bromide reduces airway remodelling

Goblet cell hyperplasia was quantified by counting the percentage of PAS-positive airway epithelial cells. The OVA-sensitized and challenged mice had an increase in the percentage of PAS-positive airway epithelial cells compared with control mice (Fig. 4a). Treatment with tiotropium bromide significantly reduced the increase in the percentage of PAS-positive airway epithelial cells induced by a chronic allergen challenge (Fig. 4a). Sensitization and repeated allergen challenge led to a more ASM-specific α -actin staining in lung sections than that observed in mice that were saline-challenged (Fig. 4b). The lungs of mice that were treated with tiotropium bromide showed less α -actin immunoreactivity than those from the untreated animals (Fig. 4b). Airway fibrosis was also studied by the staining of the tissue with Masson trichrome, and evaluated by the score system. The increase in airway fibrosis by repeated allergen challenge was significantly inhibited by treatment with tiotropium

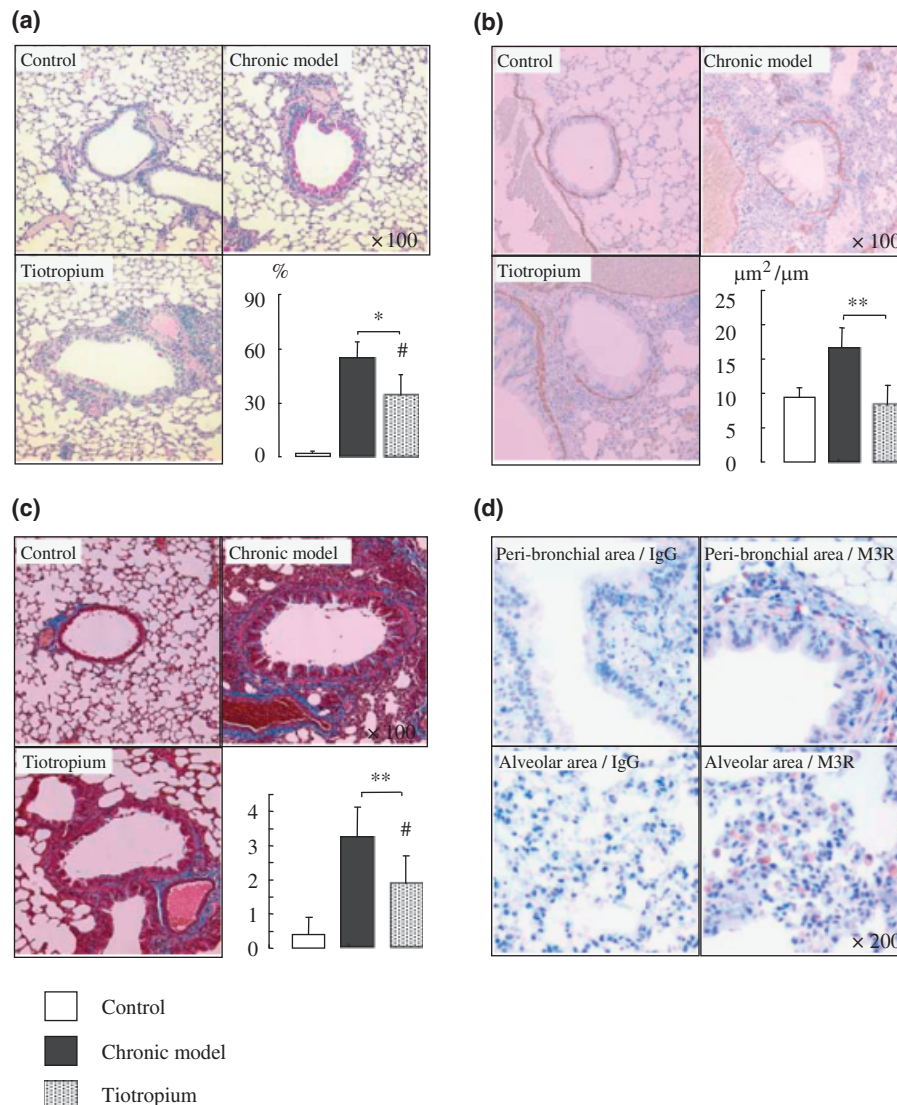


Fig. 4. Representative lung sections showing periodic acid-Schiff (PAS) staining (a), airway smooth muscle-specific α -actin staining (b), and Masson trichrome staining (c) from ovalbumin (OVA)-sensitized animals chronically challenged with saline (control), OVA (chronic model), or OVA plus tiotropium bromide treatment (tiotropium). Percentage of goblet cell metaplasia (a), the average of smooth muscle-specific α -actin (b), and scoring of airway fibrosis (c) were determined as described in methods ($n=8$ for all groups). * $P<0.05$ and ** $P<0.01$ compared with saline-treated and OVA-challenged mice. # $P<0.05$ compared with control mice. Representative lung sections showing M3 receptor immunoreactivity in mouse (d). IgG, goat IgG; M3R, M3 receptor.

bromide (Fig. 4c). Moreover, we verified that the M3 receptors were present on ASMs, inflammatory cells, goblet cells, and airway epithelium in lung section and on lymphocytes in the spleen section of mouse (Fig. 4d).

Treatment with tiotropium bromide prevents airway hyperresponsiveness

Airway responsiveness to serotonin was assessed in a chronic model of asthma. No difference was shown in Raw in between non-treated mice and saline-exposed mice (data not shown). OVA-sensitized and challenged mice showed AHR to serotonin compared with control

mice (Fig. 5). Treatment with tiotropium bromide significantly inhibited the AHR to serotonin induced by a repeated allergen challenge (Fig. 5).

Treatment with tiotropium bromide inhibits cytokine production from spleen cells

Because cytokine levels in BALF in both acute and chronic model of asthma were significantly inhibited by tiotropium bromide, we investigated the direct effects of tiotropium bromide on T cells *in vitro*. Production of Th2 cytokines IL-5 and IL-13, and Th1 cytokine IFN- γ by spleen cells isolated from OVA-sensitized mice was

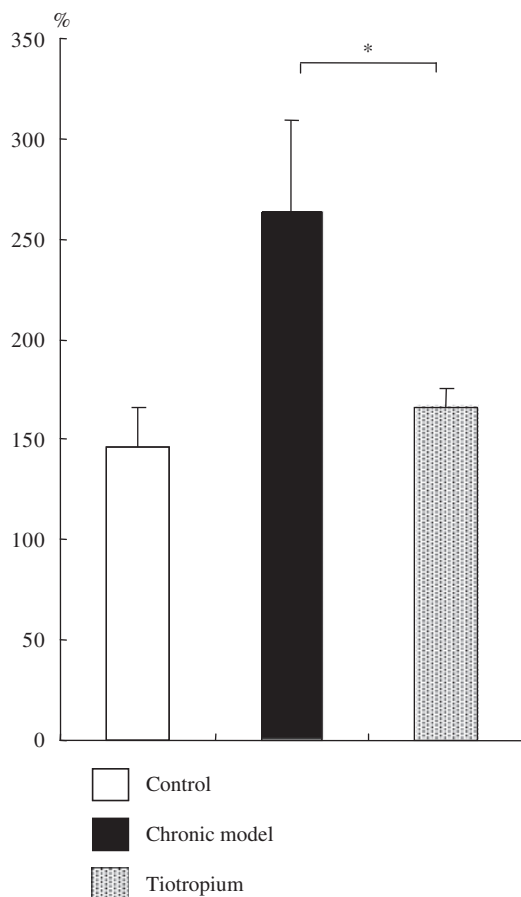


Fig. 5. Airway responsiveness to serotonin in ovalbumin (OVA)-sensitized animals chronically challenged with saline (control), OVA (chronic model), or OVA plus tiotropium bromide treatment (tiotropium) ($n = 6$ for all groups). * $P < 0.05$ compared with saline-treated and OVA-challenged mice.

significantly inhibited by tiotropium bromide after stimulation with OVA (Fig. 6a). Moreover, 4-DAMP, which is a specific antagonist to the M3 receptor, also significantly inhibited the production of these cytokines in a dose-dependent manner (Fig. 6b).

Treatment with tiotropium bromide inhibits cytokine production from human peripheral blood mononuclear cells

We finally investigate the effect of tiotropium bromide on PBMCs purified from patients with asthma. PHA stimulation increased the levels of IL-5 (Fig. 7a) and IL-13 (Fig. 7b) by PBMCs, and these increments were significantly inhibited by the treatment with tiotropium bromide in a dose-dependent manner (Figs 7a and b).

Discussion

In the present study, we observed that the anticholinergic agent tiotropium bromide inhibited the production of

Th2-type cytokines, and attenuated acute and chronic inflammation of the airway in OVA-sensitized mice. In addition, treatment with tiotropium bromide significantly inhibited ASM thickness, goblet cell metaplasia, and lung fibrosis, leading to the decrease in AHR to serotonin. Moreover, Th2 cytokine production by spleen cells was significantly and directly inhibited by tiotropium bromide and 4-DAMP, which is a specific antagonist to the M3 receptor. These results suggest that tiotropium bromide can inhibit both acute and chronic airway inflammation, and thus may attenuate airway remodelling in part by suppressing Th2 cytokine production by T cells.

According to the delayed dissociation of the tiotropium-receptor complexes, M3 receptor may play an important role in pharmacological effects of tiotropium [29–31]. We, therefore, examined the receptor expression in lung tissues and spleen cells using anti-M3 receptor antibodies. We verified that the M3 receptor was present on ASMs, inflammatory cells, goblet cells, and airway epithelium in our mouse model of asthma. Based on the distribution of M3 receptors, tiotropium bromide may exhibit a wide variety of actions to inhibit airway inflammation [32], prompting us to study initially the effect of tiotropium bromide in a mouse model of asthma. There are only a few published studies on the therapeutic effect of tiotropium bromide in animal models of asthma [22, 23]. In the acute model, we found for the first time that the number of eosinophils and levels of IL-4, IL-5, and IL-13 were significantly inhibited by tiotropium bromide. Th2 responses and eosinophils observed in the acute inflammation have been implicated in the development of chronic airway remodelling [9, 10]. In mice, chronic allergen exposure to low-dose allergen has been shown to reproduce some of the hallmarks of human asthma, such as airway remodelling and persistent AHR. In the present study, we also demonstrated that tiotropium bromide decreased the number of lymphocytes and eosinophils, in addition to Th2 cytokines including IL-4, IL-5, and IL-13 in BALF of chronic asthma model. These results suggest that tiotropium bromide has anti-inflammatory effects on allergic airway inflammation. Intriguingly, an elevation of plasma IgE levels by exposure of OVA was not inhibited by tiotropium bromide, suggesting that the impact of tiotropium bromide on inflammation was mainly acting locally in the airway.

To further investigate the mechanisms of the inhibition of Th2 cytokine production, spleen cells from OVA-sensitized mice were stimulated with OVA *in vitro*. Tiotropium bromide significantly inhibited the production of IL-5, IL-13, and IFN- γ but not IL-4 after OVA stimulation. These results highlight the discrepancy that tiotropium did inhibit IL-4 production *in vivo* but not *in vitro*. These results indicate that the inhibitory effect of tiotropium bromide on IL-4 production might be indirect in a mouse model of asthma. However, intimate mechanisms of this

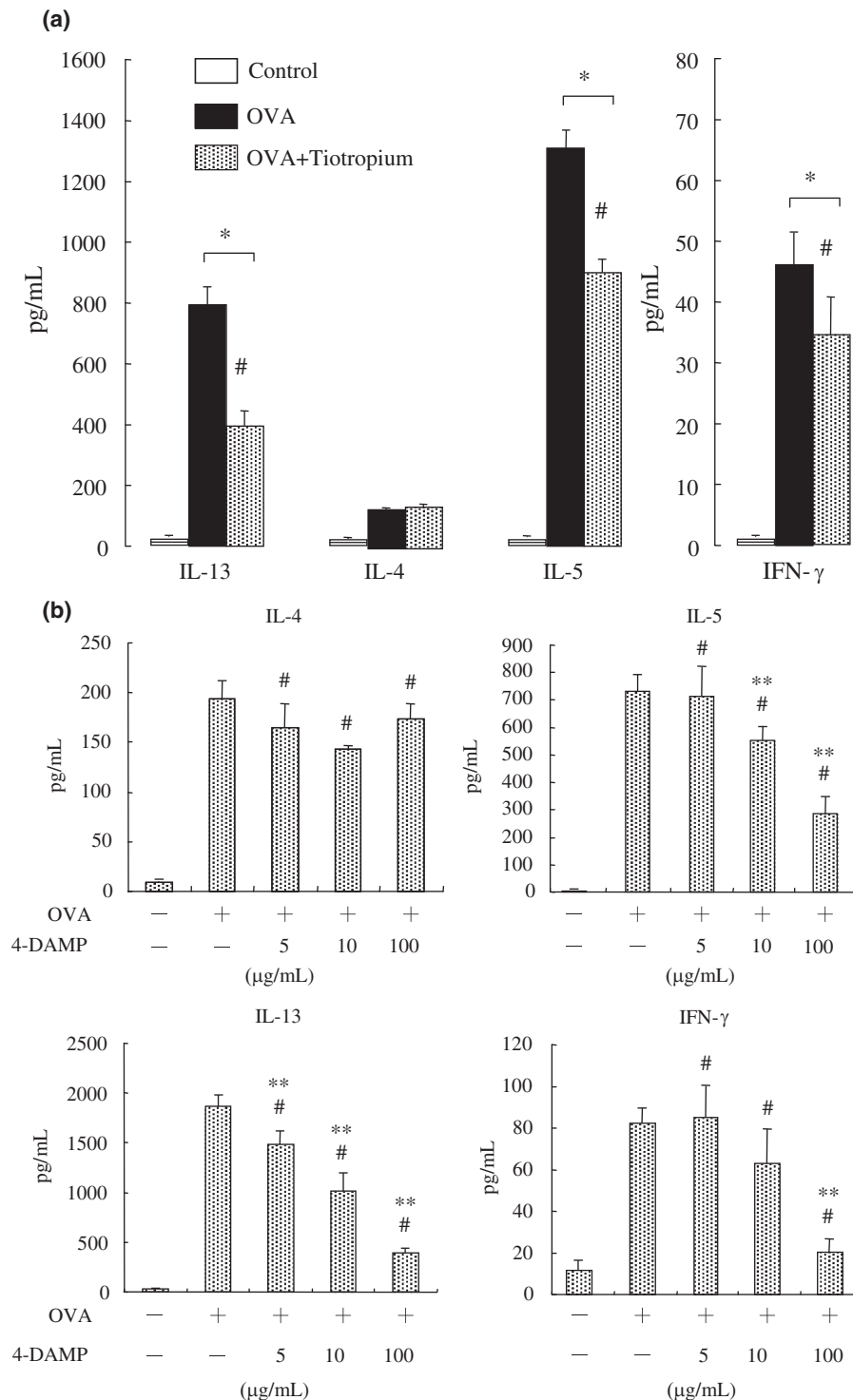


Fig. 6. Analysis of cytokines in the supernatant after stimulation of spleen cells with ovalbumin (OVA) in the presence or absence of anticholinergic treatment. Murine spleen cells were stimulated by OVA in the presence or absence of tiotropium bromide (50 μ g/mL) (a) or 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (b). After 72 h of culture, supernatants were collected and analysed for IL-13, IL-4, IL-5, and IFN- γ ($n = 8$). * $P < 0.05$ and ** $P < 0.01$ compared with the group treated with OVA alone. # $P < 0.05$ compared with control or non-treated cells.

remain unclear. Acetylcholine has been reported to directly promote cytokine production from human T lymphocytes [33]. However, we could not detect the levels of acetylcholine in the supernatant in which pur-

ified murine spleen cells were cultured in the presence or absence of OVA (data not shown). Moreover, the fact that tiotropium bromide can suppress cytokine production by T cells *in vitro* in the absence of exogenous cholinergic

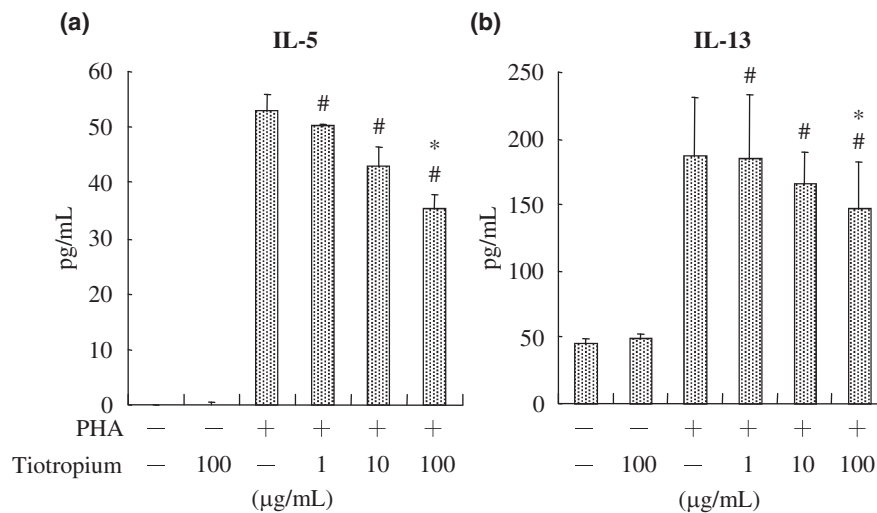


Fig. 7. Analysis of cytokines in the supernatant after stimulation of peripheral blood mononuclear cells (PBMCs) with PHA in the presence or absence of anticholinergic treatment. PBMCs were stimulated by PHA in the presence or absence of tiotropium bromide for 48 h, and supernatants were collected and analysed for IL-5 and IL-13 ($n = 8$). * $P < 0.05$ compared with the group treated with PHA alone. # $P < 0.05$ compared with non-treated PBMCs.

stimuli suggests that the inhibition of signal transduction in T cells after allergen stimulation may not simply be dependent on competitive antagonism of muscarinic receptors. However, to reveal the involvement of intracellular acetylcholine in the effect of tiotropium bromide, further studies are required.

In a previous study, Gosens *et al.* [22] showed the inhibitory effects of tiotropium bromide on ASM remodelling characterized by an increase in ASM mass and pulmonary contractile protein expression. Likewise, in the present study, we observed that tiotropium bromide attenuated thickening of ASM in a murine model of asthma. In addition, we observed that tiotropium bromide inhibited an increase in the positive area of PAS staining or of Masson trichrome staining, suggesting the protection from goblet cell metaplasia and the deposition of extracellular matrix, respectively. Considering the evidences that Th2 cytokines directly induce goblet cell metaplasia and that IL-13 but not IL-4 can increase the contractile response to muscarinic agonist in smooth muscle cells [34–38], down-regulation of Th2 cytokines by tiotropium bromide may in part correspond to an inhibition of airway remodelling. Here, we also found that treatment with tiotropium bromide significantly suppressed the levels of TGF- β 1 in BALF. TGF- β induces proliferation of fibroblasts [39] and their differentiation into myofibroblasts, extracellular matrix protein synthesis [40], and mucin formation. Thus, it is possible that the inhibitory effects of tiotropium bromide on airway remodelling may be due to the reduction of TGF- β 1 production. Tiotropium bromide has been reported previously to have no effects on extracellular matrix deposition in the airway wall in a guinea-pig model of chronic asthma [23]. However, in the present study, we found significant decreases in the

deposition of extracellular matrix by tiotropium bromide as determined by the Masson trichrome staining. This discrepancy may, in part, result from the differences of an animal and the procedure to establish an asthma model. Previous study has shown that muscarinic receptor stimulation induces cell proliferation of primary cultured pulmonary fibroblasts [41]. Thus, inhibitory effects of tiotropium bromide on extracellular matrix deposition may be in part mediated by a direct inhibition of lung fibroblasts.

The non-neuronal cholinergic system is widely expressed in the airway because choline acetyltransferase and/or acetylcholine are located in epithelial cells, submucosal glands, smooth muscle cells, and a variety of immune cells including lymphocytes, macrophages, and mast cells [32]. Moreover, these cells express nicotinic and muscarinic receptors. In this study, we have confirmed the expression of M3 receptors on these cells, suggesting that endogenous acetylcholine may play a broad role in chronic airway inflammation and remodelling. Although there is evidence that the content of acetylcholine is increased 15-fold in the skin of the patients with atopic dermatitis [42], there is no clear evidence indicating the importance of a non-neuronal cholinergic system in patients with asthma. However, intriguingly, we showed tiotropium bromide decreased Th2 cytokine production from human lymphocytes, suggesting the possibility that the down-regulation of Th2 response might contribute to the effect of inhaled tiotropium bromide in patients with asthma.

Our data demonstrate that tiotropium bromide can reduce allergic inflammation and airway remodelling in a mouse model of asthma, indicating the involvement of cholinergic pathway via M3 receptor in T cell activation.

However, it should be noted that the inhibition of allergic inflammation and airway remodelling are not total. Instead, other factors such as JAK-STAT pathway and NF- κ B, are likely to be more critical for the regulation of allergic inflammation and airway remodelling than the cholinergic pathway. In fact, inhaled corticosteroid is currently the standard treatment for patients with asthma.

In conclusion, we have shown that tiotropium bromide inhibits cytokine production by T cells and can decrease airway inflammation, airway remodelling, and AHR in a murine model of asthma. Therefore, tiotropium bromide may be beneficial therapeutically not only as a bronchodilator but also as an anti-inflammatory agent.

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