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Mechanism of Airway Remodeling Induced by Repeated Inhalation of Methacholine in a Mouse Model of Asthma

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SUMMARY

Background：Increased severity of asthma is contributed by airway tissue remodeling, which may be associated with chronic allergic inflammation. A recent study revealed the potential capacity of repeated bronchoconstriction, e.g. induced by a muscarine agonist, methacholine（Mch）challenge, to involve in airway remodeling, even though allergic inflammation is not implicated. We have evaluated the influence of repeated bronchoconstriction induced by Mch inhalation on airway remodeling in a murine model of asthma and have examined its machanisms.

Methods：Mice were immunized with ovalbumin（OVA）, and consequently, challenged by either daily OVA inhalation（the OVA group；a model of asthma with allergic inflammation）or daily Mch inhalation（the Mch group；a model of asthma without allergic inflammation）. Lung tissues were obtained and were evaluated histologically after 5, 10, and 15 consecutive inhalation challenges of both OVA and Mch.

Results：Eosinophilia in the airway observed only on the OVA group. Subepithelial collagen-band thickness increased also in the OVA group（$p<0.01$）after 15 challenges, but not in the Mch group. Significant increase in thickness of airway smooth muscle layer and the number of goblet cells were revealed in both the OVA and Mch group after 10（$p<0.05$ and $p<0.01$, $p<0.01$ and $p<0.05$, respectively, for the comparison of the two challenge groups with the control group）and 15 challenges（$p<0.05$ and $p<0.01$, both $p<0.01$, respectively, for the comparison with control）, further, all these measurements were greater in the OVA group than in the Mch group after both 10 and 15 challenges（both $p<0.05$ and $p<0.01$, respectively）. An increase in mast cell counts within the airway wall was shown in the OVA group after 10 challenges（$p<0.01$ compared with control）, not in the Mch group at all. Epithelia expression of transforming growth factor β（TGF-β）increased in both challenge groups after 15 challenges（both $p<0.05$ compared with control）, and was higher than in Mch（$p<0.05$）.

Conclusion：Repeated Mch inhalation may induce airway remodeling, while comparatively mild, potentially resulting in progressive severity of asthma. The results implicate that the potential risk associated with Mch challenge should be considered.
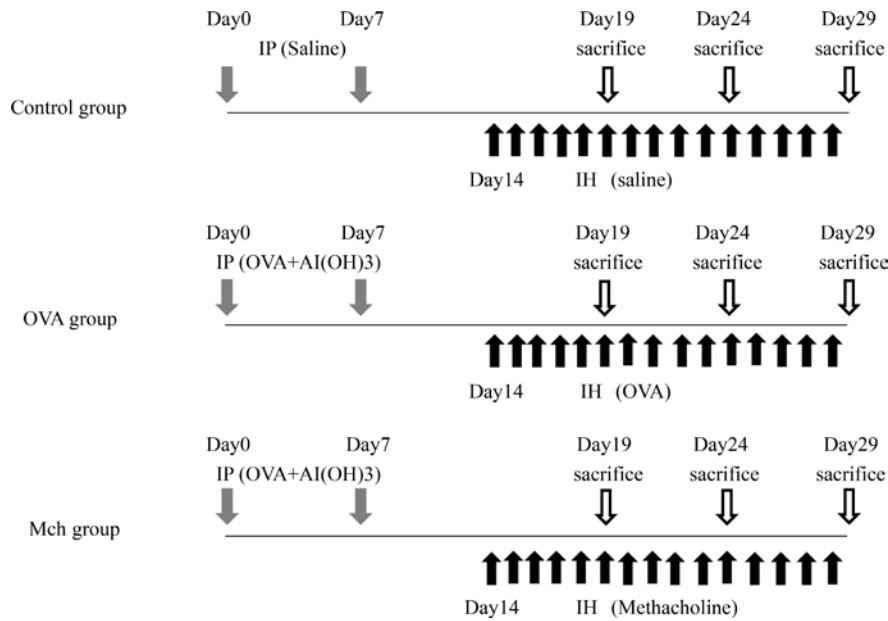
Figure 1 Protocols for sensitization and challenge

Closed arrows indicate inhalation (IH), gray arrows indicate intraperitoneal administration, and open arrows indicate sacrifice. N = 21 for each group and n = 7 for each measurement.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways, characterized by airway hyperresponsiveness (AHR) and reversible airflow limitation caused by bronchoconstriction and is associated with proliferative structural changes of the airway walls, referred to as airway remodeling[1~4]. Whereas, these structural changes contribute to the AHR and the limitation of reversible airflow obstruction, and consequently lead to an increase in severity of the disease in asthmatic patients. Airway remodeling represents structural changes of the airways, such as subepithelial fibrosis, hypertrophy and hyperplasia of airway smooth muscle mass, and goblet cell proliferation, and has been considered a consequence of eosinophilic inflammation, i.e. allergic inflammation, in the airways, induced by inhaled-allergen challenge in atopic asthma[5].

Recently a study has demonstrated that repeated inhalation of methacholine (Mch), a muscarine agonist, which causes bronchoconstriction without eosinophilic inflammation, possibly induce the similar structural changes of the airways[6]. To develop our understanding of the pathophysiology of the airway remodeling in asthma should be most effective measures for investigating potential targets for therapeutic intervention.

Accordingly, we examined the influence of repeated inhalation of Mch on pathology and pathogenesis of the airway disease by means of employing a mouse model of asthma. The results have clarified whether repeated Mch inhalation, namely recurrent bronchoconstriction without non-allergic inflammation, can lead the similar structural modification of the airways, i.e. airway remodeling, in asthma, and therefore discussed the potential mechanisms of forming that airway remodeling.

MATERIAL and METHODS

Animals and Study design

Mouse models of asthma were established from OVA-sensitized mice according to methods reported previously with some modifications[7~10]. Male BALB/c mice (Clea, Japan) of 8 weeks of age were immunized by intraperitoneal (IP) injected with $8\,\mu g$ ovalbumin (OVA ; Sigma-Aldrich, St. Louis, MO) in $4\,mg$ Al $(OH)_3$ gel (alum ; Sigma-Aldrich) on days 0 and 7 (Figure 1), and was consecutively challenged by 20-min daily inhalation of $1\,mg/ml$ OVA for 5, 10, or 15 consecutive days (OVA group). To induce bronchoconstriction, as mechanical stress alone, without allergic inflammation, mice were challenged by 15-min daily
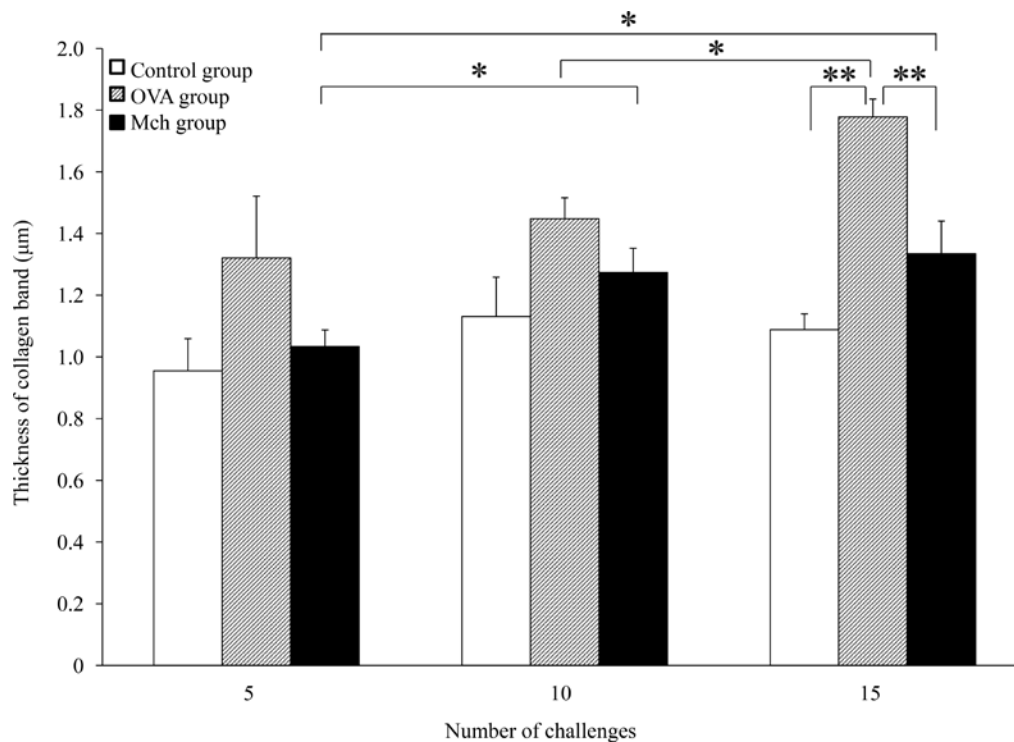
Figure 2 Expansion of the airway collagen band in the OVA group but not in the Mch group
Change in collagen band thickness in OVA-injected/OVA-boosted (OVA group) mice, OVA-injected/methacholine (Mch)-challenged mice (Mch group), and saline-injected control group mice after 5, 10, or 15 challenges. The allergic inflammation model (OVA group) showed greater collagen band thickness after 15 challenges than either the Mch or control groups. **p<0.01 and *p<0.05. Significant differences by number of challenges were observed between 10 and 15 challenges in the OVA group (p<0.05), between 5 and 10 challenges in the Mch group (p<0.05), and between 5 and 15 challenges in the Mch group (p<0.05). N=21 for each group, and n =7 for each measurement. Data expressed as mean ± SEM.

inhalation of acetyl-β-methylcholine chloride, methacholine (Sigma-Aldrich) (Mch group) to establish an inflammation-independent mechanical stress asthma model. The control group received intraperitoneal saline injections instead of OVA-alum on days 0 and 7, and inhaled saline daily for 20 min for 5, 10, or 15 consecutive days. For inhalation, mice were placed in sealed chambers and drugs were aerosolized by an ultrasonic nebulizer (Omron, Kyoto, Japan). On days 19, 24, and 29 (after 5, 10, and 15 inhalation treatments, respectively), lung tissues were collected for histological analyses. The above protocol was approved by the Committee of Laboratory Animal Research Center at Dokkyo Medical University (#02-261).

Histological preparation

Lungs were removed, fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 3-μm thick sections. Sections were stained histochemically with Hansel (Eosinostain, Torii Pharmaceutical, Tokyo, Japan) Elastica-Masson, and alcian-blue and periodicacid-Schiff (PAS) for the detection of eosinophils, the collagen band, and goblet cells, respectively, and were stained immunohistochemically with the use of anti-mouse mast cell tryptase mAb (Abcam, Cambridge, UK) and with the use of anti-mouse actin and α-smooth muscle mAb (Sigma-Aldrich), each of which combined with N-Histofine MOUSESTAIN KIT® (Nichirei, Tokyo, Japan) for the detection of mast cells and smooth muscle mass, respectively. To evaluate the expression of TGF-β, tissues also were stained with anti-mouse TGF-β mAb (Abcam), combined with the VECTASTAIN Elite ABC Kit® (Vector Laboratories, CA), and N-Histofine Simple Stain DAB solution® (Nichirei)
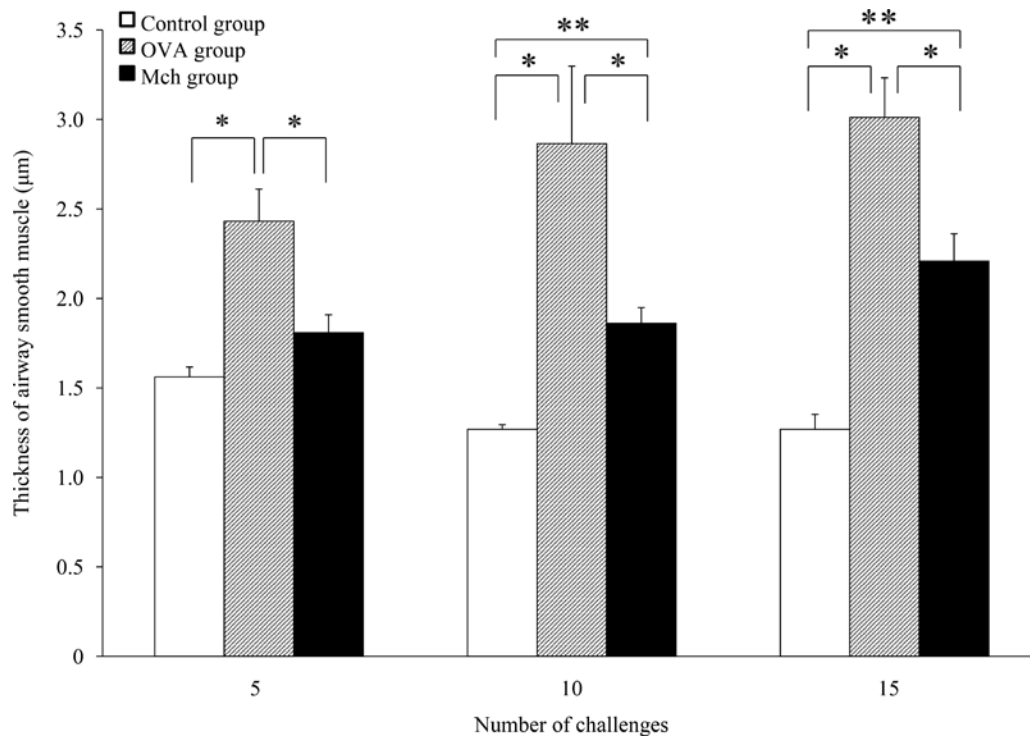
Figure 3 Expansion of the airway smooth muscle layer in both the OVA and Mch group
**p<0.01 and *p<0.05. Both the OVA and Mch groups exhibited smooth muscle layer expansion compared to the control group. Expansion was greater in the OVA mice, indicating more substantial remodeling. No significant differences by number of challenges were observed in any of the groups. N=21 for each group and n=7 for each measurement. Data expressed as mean ± SEM.

Measurement of airway thickness, collagen band thickness, infiltrating cell numbers, and TGF-β expression

Airway and collagen band thicknesses were estimated along the main bronchus. The number of goblet cells in the main bronchus was also counted during estimation of airway and collagen band thicknesses. The number of infiltrating eosinophils in the main bronchus was estimated by counting Hansel-stained cells and expressed per one millimeter square[11]. Similarly, the number of infiltrating mast cells was estimated by counting the number of tryptase-positive cells. The expression of TGF-β in trachea was estimated by integration of the immunostaining signal.

Statistical Analysis

Statistical analyses were performed using Microsoft Excel® or JMP® (for the Wilcoxon signed rank test). A *P* value<0.05 was considered significant. Results are expressed as mean±standard error of the mean (SEM).

RESULTS

Airway remodeling by methacholine

Collagen band thickness, which is a marker of inflammatory fibrosis, increased progressively as a frequency of OVA challenge (Fig. 2). Collagen band thickness increased significantly in the OVA group between 10 and 15 challenges (1.45±0.07 μm vs. 1.78±0.06 μm, p<0.05), while collagen band thickness in Mch group increased significantly between the five and ten challenge (1.03±0.05 μm vs. 1.27±0.08 μm, p<0.05) and between the five and fifteen (p<0.05), but not between the ten and fifteen (1.27±0.08 μm vs. 1.33±0.11 μm). The collagen band was significantly thicker in the OVA group after 15 daily OVA challenges than in either the Mch group after the same number of challenges (p<0.01) or in the control group (p<0.01). In contrast, the collagen band thickness was no greater in the Mch group compared to the control group (p>0.05). Thus, Mch-induced remodeling was
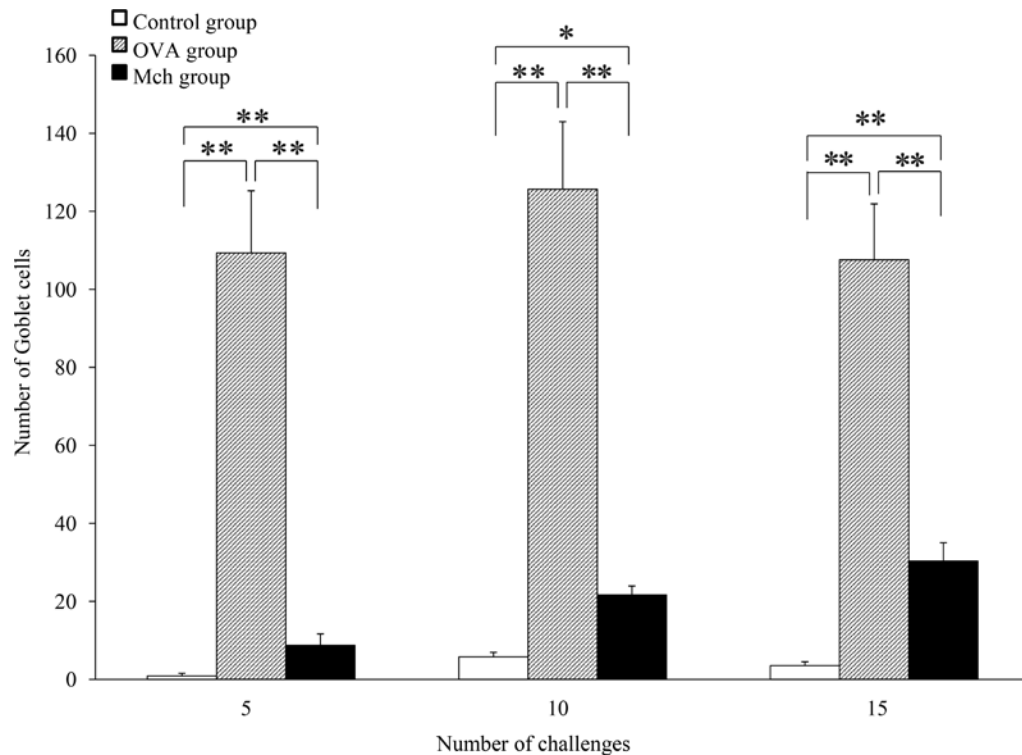
Figure 4　Goblet cell hyperplasia in both the OVA and Mch group

The OVA group showed substantial goblet cell hyperplasia. The Mch group showed more modest goblet cells hyperplasia. **p<0.01 and *p<0.05. No significant differences by number of challenges were observed in the OVA group, but there were significant differences between 5 and 10 challenges（p<0.01）and between 5 and 15 challenges（p<0.01）in the Mch group. N＝ 21 for each group and n＝7 for each measurement. Data expressed as mean±SEM.

not associated with fibrosis.

Airway smooth muscle thickness increased progressively with the number of challenges in both the OVA and Mch groups（Fig. 3）, although the differences among the three measurements were not significant. At all measurements, however, airway smooth muscle thickness was greater in the OVA group compared to both the Mch group（p<0.05）and control group（p< 0.05）. After 10 and 15 challenges, smooth muscle thickness was also greater in the Mch group compared to the control group（p<0.01）. Thus, Mch-induced remodeling appears to include smooth muscle hyperplasia and/or hypertrophy.

The OVA group exhibited a significantly higher number of goblet cells（Fig. 4）, although these numbers did not increase significantly with the number of challenges. In contrast, the number of goblet cells did increase progressively with the number of challenges in the Mch group（five vs. ten p<0.01）. At all measurements, goblet cell number was significantly greater

in the OVA group than the Mch and control groups （both p<0.01）and greater in the Mch group compared to the control group（p<0.01 or p<0.05）. Thus, Mch-induced remodeling includes mild goblet cell hyperplasia.

Immune cell infiltration

The numbers of eosinophils infiltrated into the airway was significantly higher in the OVA group at all measurements（Fig. 5；p<0.01 vs. Mch and control groups）, but there was no effect of challenge number. In contrast, no significant eosinophil infiltration was observed in the Mch group as cell numbers were no higher than in the control group. The numbers of mast cells infiltrated into the airway（Fig. 6）was also significantly higher in the OVA group compared to the Mch group after 5 and 10 challenges（5 challenges：4.0 ±1.0 cells vs. 1.3±0.4 cells, p<0.05；10 challenges：8.7 ±0.7 cells vs. 4.5±1.0 cells, p<0.05）and compared to the control group after both 5 and 10 challenges（p<
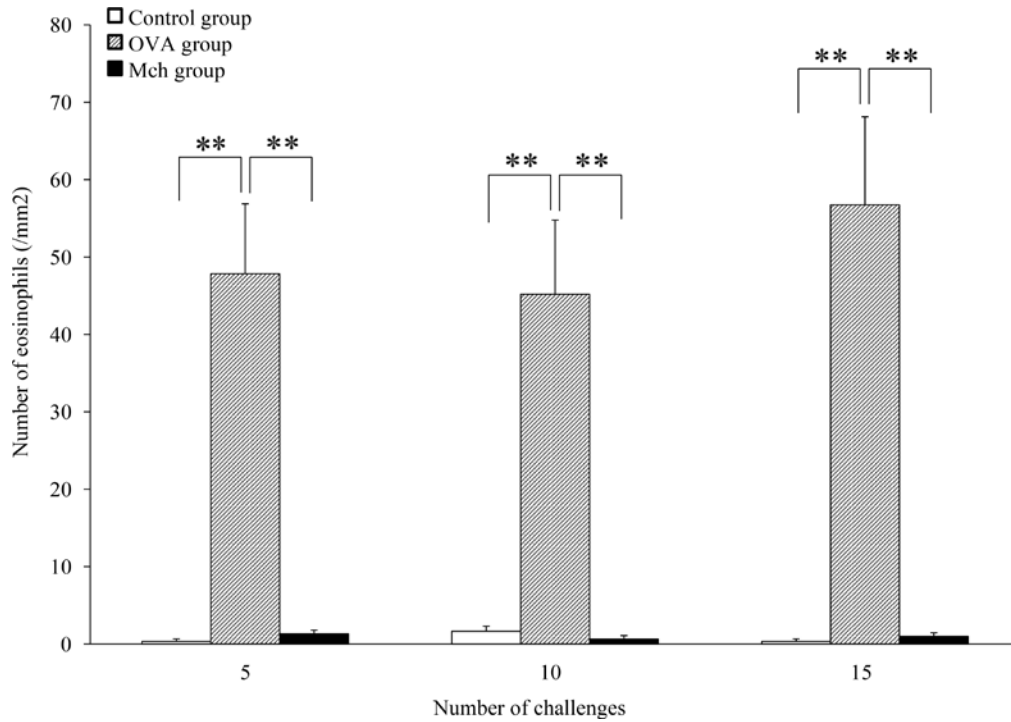
Figure 5   Eosinophilia in the OVA but not Mch group

The OVA group showed substantial eosinophil infiltration into the airway, while the Mch group did not. $**$p <0.01. No significant differences by number of challenges were observed in any of the three groups. N＝21 for each group and n＝7 for each measurement. Data expressed as mean±SEM.
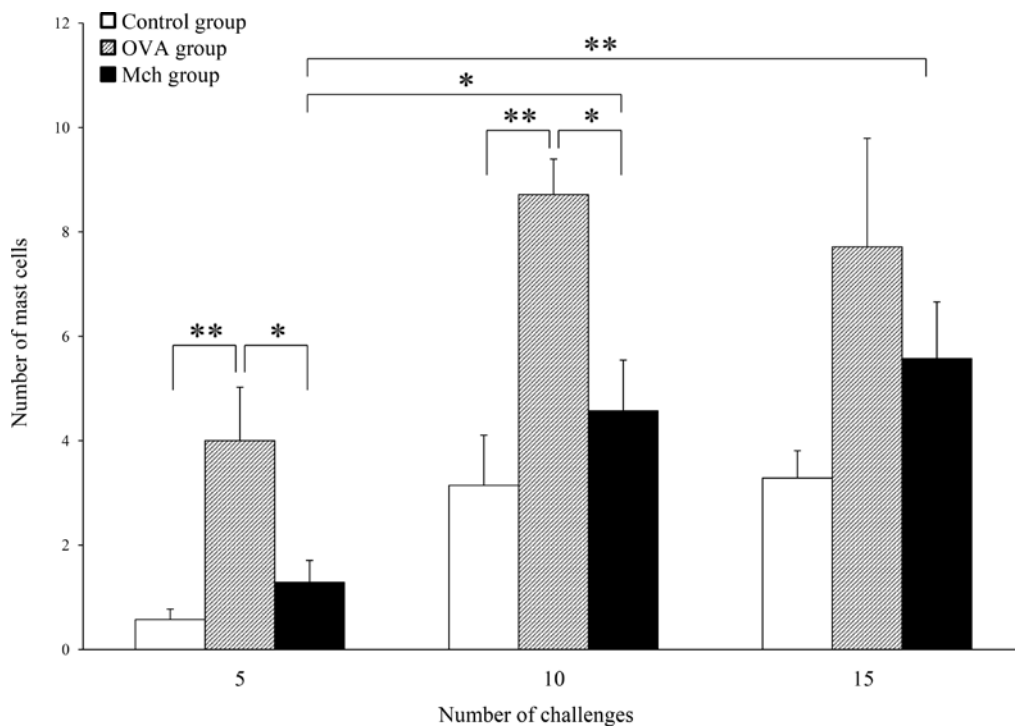

Figure 6   Mast cell infiltration

The numbers of mast cells increased in the airway of the OVA group compared to the Mch and control groups after 5 and 10 challenges. $**$p<0.01 and $*$p<0.05. No significant differences by number of challenges were observed in the OVA group. However, significant differences were observed in the Mch group（5 vs. 10 challenges；p<0.05, 5 vs. 15 challenges；p<0.01）and in the control group（5 vs. 10 challenges；p<0.05, 5 vs. 15 challenges；p<0.01）. N＝21 for each group and n＝7 for each measurement. Data expressed as mean ± SEM.
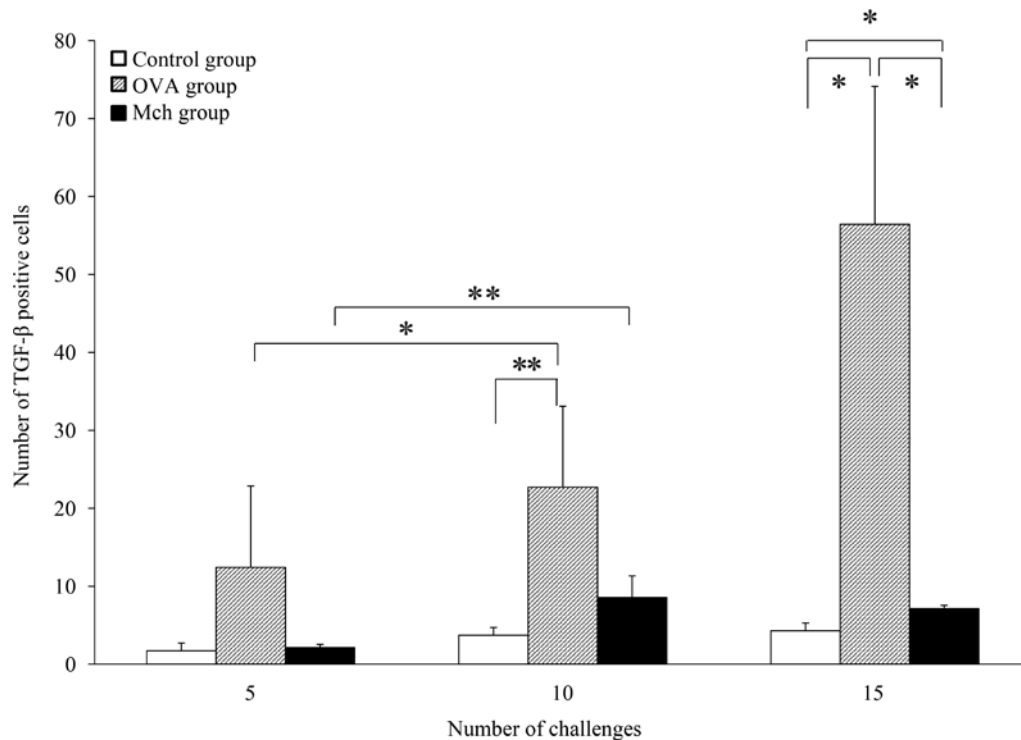
Figure 7 Substantial overexpression of TGF-β by the OVA group and more modest overexpression by Mch group

The OVA group expressed substantially higher levels of TGF-β than the control group after 10 and 15 challenges and higher levels than the Mch group after 15 challenges. The Mch group expressed higher levels of TGF-β than the control group after 15 challenges. $^{**}p<0.01$ and $^*p<0.05$. Significant differences by number of challenges were observed between 5 and 10 challenges (p< 0.05) and between 5 and 15 challenges (p<0.05) in the OVA group and between 5 and 15 challenges (p<0.01) in the Mch group. N=21 for each group and n=7 for each measurement. Data expressed as mean ± SEM.

0.01), but not after 15 challenges (7.7±2.1 cells vs. 5.6 ±1.1 cells vs. 3.3±0.5). Expression of TGF-β (Fig. 7) was significantly higher in the OVA group compared to the control group after 10 challenges (22.7±10.4 vs. 3.7±1.1, p<0.01) and compared to both the Mch group and control groups after 15 challenges (56.4± 17.7 vs. 7.1±0.4 vs. 4.3±0.9; p<0.05 vs. both). TGF-β expression was significantly higher in the Mch group compared to the control group after 15 challenges (p< 0.05). Unlike OVA-associated remodeling, Mch-induced remodeling does not include eosinophilia, but does include mild mast cell infiltration and TGF-β expression.

DISCUSSION

Both the subepithelial collagen band and airway smooth muscle layer were expanded significantly by OVA immunization and subsequent booster sensitiza-

tion, verifying successful induction of airway remodeling. However, frequent inhalation of the non-inflammatory agent MC also induced airway remodeling, albeit more restricted than that induced by OVA. These findings support results from patients with asthma and suggest that overuse of Mch may exacerbate asthma severity[6].

Airway remodeling induced by Mch may result from two pathogenic pathways, bronchoconstriction-dependent stress and Mch-induced inflammation. Airway smooth muscles may become hypertrophied by frequent Mch-induced bronchoconstriction even if it were without airway inflammation (stress-induced hypertrophy). For example, arterial smooth muscles were hypertrophied and synthesis of matrix components stimulated by mechanical pressure[12,13]. Actually, the effects of frequent mechanical stress alone have not been considered in airway, because it is difficult to separate

these effects from those of airway inflammation[2~5]. In the present study, airway smooth muscles treated with Mch were significantly hypertrophied. In contrast to the OVA group, however, collagen band thickness was not significantly expanded by Mch, indicating that airway inflammation induces more uniform remodeling than frequent mechanical bronchoconstriction. Nonetheless, frequent mechanical bronchoconstriction can induce uneven airway remodeling that may impede respiration.

These results have two major implications for the diagnosis and treatment of asthma. First, examination of airway hyperresponsiveness using Mch or acetylcholine should be limited to use. Namely the airway hyperresponsiveness examination should be performed only for the initial diagnosis of asthma. Second, bronchodilators like LABA may contribute to inhibit remodeling. The US-FDA recommendation against regular use except by patients not successfully treated by ICS is based on the view that inhibition of airway inflammation is the most important treatment goal for asthma medication, and we agre[14]. Nonetheless, regular use of LABA may lead to prevention airway remodeling caused by frequent bronchoconstriction.

The other possible Mch-induced remodeling pathway is mimic allergic inflammation. In one study, Mch increased Ki67-positive cell population in both the epithelium and the submucosa of asthma patients[6]. The authors suggested that Mch could damage the airway epithelium and that upregulation of the proliferation regulator, Ki67 is a reparative response. If Mch does cause mimic allergic inflammation, it likely occurs without eosinophil infiltration as neither the aforementioned clinical study[6] nor our study found a significant increase in tissue eosinophil number. In contrast, we did not evaluate the number of Ki67-positive cells. We cannot eliminate the possibility of Mch-induced cellular damage leading inflammation as no study has yet investigated Mch toxicity on isolated airway epithelial cells. It has also been reported that chronic intermittent mechanical stress from bronchoconstriction can increase mucin protein expression and goblet cell proliferation[15], consistent with our observation of increased numbers of alcian-blue PAS-positive cells. The other possibility is that Mch or mechanical stress on the airway may induce mimic allergic inflammation

though a pathway not involving activation of eosinophils. In such a case, mast cells could be the principal mediator cell, because Mch enhanced mast cells infiltration and infiltration increased with the number of challenges[16]. Mast cells express receptors for IL-3, IL-5, and granulocyte-macrophage-colony stimulating factor (GM-CSF), and they regulate allergic airway inflammation together with T-cells[17]. Also, mast cells activate fibroblasts to produce fibrogenic cytokines such as TGF-β[18], thereby contributing to pulmonary fibrosis. Epithelial TGF-β was increased by Mch in both a previous study on asthma patients[6] and in our study, and again expression increased with Mch challenge number. Therefore, it appears possible that Mch caused mimic allergic inflammation without eosinophilia. However, Mch caused no significant expansion of the collagen band. Further studies are required to distinguish Mch-induced remodeling via inflammation from remodeling via mechanical stress.

Eosinophilic inflammation induced by OVA had the strongest effect on airway remodeling, but frequent inhalation of Mch also induced several remodeling responses without airway eosinophilia. Eosinophilia is important in the pathogenesis of asthma, but may not be essential key factor because mepolizumab, a humanized monoclonal antibody against IL-5 that inhibits airway eosinophilia, did not improve airway hyperresponsiveness[19]. Subsequent studies reported conflicting results on the efficacy of mepolizumab for asthma treatment[20~25]. A more recent study reported that anti-IL-5 receptor a mAb, not anti-IL-5 mAb, inhibited airway hyperresponsiveness in a mouse asthma model[10]. Airway smooth muscles express IL-5 receptor-a, and IL-5 contributes to airway remodeling[26]. Thus, IL-5 may be an important signaling factor for airway remodeling, although the expression of IL-5 was not measured in our study.

In summary, frequent inhalation of Mch may be a risk factor for airway remodeling. Airway remodeling induced by Mch may arise from frequent mechanical bronchoconstriction or mast cell-dependent, eosinophil-independent mimic allergic inflammation. We recommend that frequent inhalation of Mch should be limited to use. This test should be performed for initial asthma diagnosis but not for regular treatment monitoring.

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