

Mast cells play a partial role in allergen-induced subepithelial fibrosis in a murine model of allergic asthma

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Summary

Background Role of mast cells in the development of allergen-induced airway remodelling has not been fully investigated *in vivo*.

Objective To clarify the possible role of mast cells in the development of allergen-induced airway remodelling, we compared their responses of genetically mast cell-deficient mice, WBB6F1-W/W^v (c-kit mutant) and Sl/Sl^d (c-kit ligand mutant) mice with those of congenic normal mice in a murine model of allergic asthma.

Methods Mice were sensitized to ovalbumin (OA) with alum, and exposed daily for 3 weeks to aerosolized OA. Twenty-four hours after the last inhalation, bronchial responsiveness to acetylcholine (Ach) was measured, and bronchoalveolar lavage (BAL), and biochemical and histological examinations were performed.

Results In both sensitized mast cell-deficient mice, the degree of bronchial hyper-responsiveness to Ach, the number of inflammatory cells and the level of transforming growth factor- β 1 in BAL fluid, IgE response and goblet cell hyperplasia in the epithelium after repeated allergen provocation were not significantly different from those of congenic mice. In contrast, subepithelial fibrosis, evaluated in the fibrotic area around the airways, observed in congenic mice after repeated allergen challenge was partially attenuated in both mast cell-deficient mice. In addition, the amount of hydroxyproline in the lung of mast cell-deficient mice was significantly lower than that of congenic mice. Furthermore, the decreased fibrotic area and amount of hydroxyproline in W/W^v mice was completely recovered by reconstitution of tissue mast cells with bone marrow-derived mast cells of congenic mice.

Conclusion These findings suggest that mast cells play a partial role in the development of allergen-induced subepithelial fibrosis, although airway inflammation, epithelial remodelling and BHR caused by repeated allergen challenge are independent of mast cells, at least in this model.

Keywords airway inflammation, airway remodelling, bronchial hyper-responsiveness, goblet cell, mast cell, subepithelial fibrosis, TGF- β 1

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Introduction

Bronchial asthma is a chronic inflammatory disorder of the airways characterized by variable airway obstruction, airway eosinophilic inflammation and bronchial hyper-responsiveness (BHR) [1]. Airway inflammation is associated with the infiltration of eosinophils, lymphocytes and mast cells into the airways and increased numbers of these cells have been found in the bronchoalveolar lavage fluid (BALF) and bronchial biopsies from asthmatics [2]. However, some patients with asthma have been shown to have irreversible changes in lung function [3], despite appropriate and aggressive anti-inflammatory therapy. This is thought to be the result of structural changes referred to as 'airway remodelling', which are characterized by

goblet cell hyperplasia/hypertrophy, subepithelial fibrosis and smooth muscle cell hyperplasia/hypertrophy [4, 5]. Although airway remodelling is thought to be an injury-repair response driven by variable molecules derived from inflammatory cells, the mechanisms of the development of airway remodelling are still obscure.

Mast cells are the main effector cells of especially the early phase of the allergic reaction and immediate asthmatic response. Through antigen cross-linking to high affinity IgE receptors on the cell surface, mast cells discharge a group of mediators [6], including histamine. It has also been reported that mast cell-derived lipid mediators including leukotrienes and prostaglandins play an important role in the late asthmatic response and BHR [7, 8]. Furthermore, mast cells can secrete inflammatory cytokines such as IL-4, IL-5, IL-6, IL-13 and TNF- α , which have a potential role in cellular infiltrates and airway inflammation [9].

Moreover, some recent studies *in vitro* demonstrated that mast cells produce various mediators such as transforming

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growth factor (TGF)- β 1 [10] and matrix metalloproteinases (MMPs) [11], which may contribute to the development of airway remodelling; however, the role of mast cells in the development of airway remodelling has been never investigated *in vivo*.

In the present study, to clarify the possible role of mast cells in the development of antigen-induced airway remodelling *in vivo*, we investigated the responses of two strains of mast cell-deficient mice, W/W^v (c-kit mutant [12]) and Sl/Sl^d (c-kit ligand mutant [13]) mice, in a murine model of allergic asthma [14].

Materials and methods

Animals

Seven-week-old female WBB6F₁-+/+, WBB6F₁-W/W^v and WBB6F₁-Sl/Sl^d mice were purchased from Japan SLC (Shizuoka, Japan). The animals were housed in plastic cages in an air-conditioned room at 22 ± 1 °C with a relative humidity of 60 ± 1%, fed a standard laboratory diet and given water *ad libitum*. Experiments were performed following the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animals Science in 1987.

Agents

The following drugs and chemicals were purchased commercially and used: ovalbumin (OA) (Seikagaku Kogyo, Tokyo, Japan), acetylcholine chloride (Ach, Nacalai Tesque Inc., Kyoto, Japan), bovine serum albumin (BSA, Seikagaku Kogyo), Türk solution (Wako Pure Chemical Industries Ltd, Osaka, Japan), pancuronium bromide (Sigma, St Louis, MO, USA), sodium pentobarbitone (Abbott Laboratory, Chicago, IL, USA), disodium ethylenediaminetetraacetic acid (EDTA-2Na, Nacalai Tesque), Diff-Quick solution (International Reagent Corp. Ltd, Kobe, Japan), monoclonal rat anti-mouse IgE antibody (LO-ME-3, Serotec Co. Ltd, Oxford, UK), peroxidase-conjugated streptavidin (Dakopatts a/s, Glostrup, Denmark) and hydroxy-L-proline (Nacalai Tesque).

Sensitization and antigen challenge

Mice were actively sensitized by intraperitoneal injections of 50 µg OA with 1 mg alum on days 0 and 12 as described previously [14, 15]. Starting on day 22, they were exposed to OA (1% w/v diluted in sterile physiological saline) for 30 min every day for three consecutive weeks. Control animals were injected with saline or sensitized with OA, and exposed to saline in a similar manner. The aerosol (particle size 2.0 to 6.0 µm) was generated by a nebulizer (Ultrasonic nebulizer UN-701, Azwell Co. Ltd, Osaka, Japan) driven by filling a perspex cylinder chamber (diameter 5.5 cm, height 12 cm) with a nebulized solution.

Measurement of airway function

Measurement of bronchial responsiveness to intravenous acetylcholine (Ach) was performed as previously described [14, 15]. Briefly, to measure airway responsiveness to Ach, mice were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and the jugular vein was cannulated for intravenous injection of Ach. Mice were injected with pancuronium bromide (0.1 mg/kg, i.v.) to stop spontaneous respiration, and animals were ventilated with a rodent ventilator (New England Medical Instruments Inc., Medway, MA, USA) with oxygen-

supplemented air at 60 strokes/min, at a stroke volume of 0.6 mL/animal. Bronchoconstriction was measured according to the overflow method, using a bronchospasm transducer (Ugo Basil 7020, Milan, Italy) connected to the tracheal cannula. To measure bronchial responsiveness to Ach, changes in respiratory overflow volume were measured using increasing dose of Ach. The increase in respiratory overflow volume induced by Ach was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. The bronchial responsiveness to Ach was evaluated as a provocative dose of Ach required to cause a 40% bronchoconstriction (PD₄₀), which was calculated from each dose-response curve for Ach.

Bronchoalveolar lavage study

To evaluate airway inflammation, we examined the accumulation of inflammatory cells in BALF. Experiments were performed according to a method previously described [14]. Animals were sacrificed with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg). The trachea was cannulated and the left bronchi were tied for histological examination. Then, the right air lumen was washed four times with 0.5 mL calcium- and magnesium-free phosphate-buffered saline (PBS) containing 0.1% BSA and 0.05 mM EDTA-2Na. This procedure was repeated three times (total volume 1.3 mL, recovery > 85%). BALF from each animal was pooled in a plastic tube, cooled on ice and centrifuged (150 g) at 4 °C for 10 min. Cell pellets were resuspended in the same buffer (0.5 mL). BALF was stained with Türk solution and the number of nucleated cells was counted in a Burkner chamber. A differential count was made on a smear prepared with a cytocentrifuge (Cytospin II, Shandon, Cheshire, England) and stained with Diff-Quick solution (based on standard morphological criteria) of at least 300 cells (magnification × 500). The supernatant of BALF was stored at -30 °C for determination of transforming growth factor (TGF)- β 1.

Reconstitution of mast cells

Reconstitution of mast cells in W/W^v mice was carried out using the method described by Kung et al. [16] with a slight modification [17]. Suspended bone marrow cells from +/+ normal mice were cultured in RPMI 1640 medium (Life Technologies Inc. (Gibco BRL), Rockville, MD, USA) supplemented with 10 ng/mL recombinant murine IL-3 (Peprotech Inc., Rocky Hill, NJ, USA), 10% FCS, 2 mM glutamine, 50 U/mL penicillin, 100 mg/mL streptomycin at a concentration of 5×10^5 cells/mL and cultured at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Culture medium was replaced every 7 days. After 5 weeks culture, cells were harvested and stained with alcian blue to confirm mast cells. The staining indicated that more than 99% cells were viable mast cells. Then, 2×10^7 bone marrow-derived mast cells were injected i.v. into each W/W^v mice via a tail vein. Four weeks later, these reconstituted mice were immunized with OA and alum as described above.

Measurement of serum antigen-specific IgE

Immediately prior to the last antigen challenge, blood was collected and sera were obtained by centrifugation and stored

at -80°C . Antigen-specific IgE in the mouse serum was measured using the enzyme-linking immunosorbent assay (ELISA) as previously described [15]. Briefly, microtiter plates were coated with monoclonal rat anti-mouse IgE antibody (LOME-3) at a concentration of $50\text{ }\mu\text{g/mL}$. After blocking with 1% BSA, serum dilutions were incubated for 1 h followed by biotinylated-OA and peroxidase-conjugated streptavidin. Sequentially diluted monoclonal anti-OA IgE (Donated by Kuniwa, Taiho Pharmaceutical Co. Ltd) was used as a standard.

Optical densities of the enzymatic reactions were read using an automatic ELISA plate reader (Multiscan MS, ver 8, Lab-systems Oy, Helsinki, Finland) at 450 nm (reference 690 nm) and analysed using Deltasoft 3 (Biometallics Inc., Princeton, NJ, USA). The detection limit was 1 ng/mL .

TGF- β 1 levels in BALF

The amount of TGF- β 1 in the supernatant of BALF was measured using ELISA (Genzyme Tecne., Minneapolis, MN, USA), which can detect mouse TGF- β 1 protein, because of the high homology of TGF- β 1 across species. The assay detects only the active form of TGF- β 1. Each sample was activated by acidification before measuring, according to the manufacturer's recommendations. The detection limit was 7 pg/mL .

Measurement of hydroxyproline content in right lung

Whole collagen content of the right lung was evaluated by determining hydroxyproline content [14, 18]. Briefly, after recovery of BALF, the right lung lobes were removed and cut into sections (1 mm thick). The chopped lungs were dried with acetone. Then, the dried lung samples were hydrolysed with 2 mL of 6 N HCl at 120°C for 24 h in sealed glass tubes. The amount of hydroxyproline in the hydrolysate was measured according to Kivirikko et al. [19]. Authentic hydroxyproline (hydroxy-L-proline) was used to establish a standard curve.

Histological study

Mice were sacrificed 24 h after repeated antigen inhalation daily for three consecutive weeks. The left lungs were distended by injection of 10% buffered formalin via the trachea, excised and immersed in the same fixative with the trachea clamped for 24 h. Tissues were sliced and embedded in paraffin, and $6\text{ }\mu\text{m}$ sections were stained with haematoxylin and eosin (H&E) for general morphology, periodic acid-Schiff (PAS) for identification of goblet cells in the epithelium, van Gieson elastica (vGE) for connective tissue, and toluidine blue for mast cells, respectively.

Examination of goblet cell hyperplasia was carried out to a method previously described by Padrid et al. [20] with a slight modification [14] using a Leica image analysis system (Leica, Cambridge, UK). Briefly, two to four specimens of the PAS-stained histological preparations of the left lobe, in which the total length of the epithelial basement membrane of the bronchioles were 1.0 to 2.5 mm, were selected and the pathological changes were evaluated according to the modified five-point scoring system [14], using a Leica microscope ($\times 20$ objectives) and an Olympus Video Meter system (Olympus, Tokyo, Japan). The preparations, in which the maximum internal diameter of the bronchioles was two times as large as the minimum internal diameter or exceeded two times, were not used for analysis. The

hyperplasia of the goblet cells in the epithelial lining was expressed by a score according to the percentage of the goblet cells in the epithelial cells. To minimize sampling errors, the five-point scoring system (grade 0 to 4) [20] was adopted: grade 0 (no goblet cells); grade 1, $< 25\%$; grade 2, 25 to 50%; grade 3, 50 to 75%; grade 4, $= 75\%$. The mean score of the total epithelial cells in two to four preparations of one mouse was counted. The mean scores of hyperplasia of the goblet cells were calculated in six to seven animals.

vGE-stained sections were used for assessment of subepithelial fibrosis using a Leica image analysis system (Leica). As described above, two to four specimens of the vGE-stained histological preparations of the left lobe, in which the total length of the epithelial basement membrane of the bronchioles was 1.0 to 2.5 mm, were selected and the fibrotic area (stained in red) beneath the basement membrane at $20\text{-}\mu\text{m}$ depth was measured. The mean value of the fibrotic area divided by basement membrane length in two to four preparations of one mouse was calculated, then the mean values of subepithelial fibrosis were calculated in five to six animals.

Statistical analysis

Values are presented as the mean $\pm$ SEM. Statistical significance between two groups was estimated using the two-tailed Student's *t*-test or the Aspin-Welch test after the variances of the data were evaluated with *F*-test. For morphometric semi-quantitative analysis (goblet cell hyperplasia), the Mann-Whitney rank sum test was used. Spearman's correlation test was used to analyse for correlation between the amount of hydroxyproline and the fibrotic area around the airways. *P*-values less than 0.05 were considered to be significant.

Results

Inflammatory cells in BALF and antigen-specific IgE in serum

To clarify whether the deficiency of mast cells influenced the development of airway remodelling, we examined the accumulation of inflammatory cells in BALF of mast cell-deficient mice compared with those of congenic litter-mates. In $+/+$ and mast cell-deficient mice, the number of total leucocytes, eosinophils and lymphocytes in the OA-exposed mice were significantly increased after 3 weeks of aeroallergen exposure compared with those in the saline-exposed mice (Table 1). However, the increases in the numbers of total leucocytes, eosinophils and lymphocytes of mast cell-deficient mice were comparable in extent to those observed in $+/+$ mice.

All sensitized animals produced detectable levels of antigen-specific IgE antibody in serum, immediately prior to the first inhalation, at day 21. Significant differences were not found between $+/+$ and mast cell-deficient mice at day 21 (data not shown). In $+/+$ and mast cell-deficient mice, the levels of antigen-specific IgE were elevated or tended to elevate compared with those in the saline-exposed mice immediately prior to the last inhalation (Table 1). The levels of antigen-specific IgE in mast cell-deficient mice didn't significantly differ from those in $+/+$ mice. In contrast, there were no detectable antigen-specific IgE in serum in non-sensitized and saline-exposed animals (Table 1).

Fibrogenic responses

To investigate possible roles of mast cells in the fibrogenic responses, we examined the production of a fibrogenic cytokine, TGF- β 1, in BALF, the amount of hydroxyproline, which is an amino acid specifically consisting of collagen, in the right lung tissues, and the fibrotic area around the airways. In $+/+$ and mast cell-deficient mice, the levels of active form TGF- β 1 in BALF of the OA-exposed mice were significantly increased after repeated antigen inhalation compared with those in the saline-exposed mice, although significant differences were not found between $+/+$ and mast cell-deficient mice (Table 2). In contrast, the amount of hydroxyproline in the right lungs and the fibrotic area in the OA-exposed mice were significantly increased in $+/+$ and mast cell-deficient mice compared with those in the saline-exposed mice; however, these parameters in mast cell-deficient mice were significantly lower than those in $+/+$ mice (Table 2).

Histological analyses

To clarify the deficiency of mast cells in the development of airway remodelling, the left lungs were histologically examined 24 h after the final antigen challenge. Histological analyses of lungs from saline-exposed sensitized mice showed normal lung

histology (Fig. 1a–c). In contrast, similar to the BAL study, histological sections of lung tissue from OA-exposed mice exhibited airway inflammation. Infiltrating eosinophils and mononuclear cells were mainly observed in the peribronchial and perivascular regions of the lung. Significant differences were not found between $+/+$ and mast cell-deficient mice (Fig. 1d–f).

As for the number of mast cells, we analysed the sections stained with toluidine blue. None could be identified in any of the sections examined from mast cell-deficient mice (Fig. 1h, i, k, l). In $+/+$ mice, mast cells were identified (arrow heads); however, significant differences were not found between saline-exposed and OA-exposed mice (Fig. 1g vs. j).

Figure 2 also shows the histological examination with PAS staining for detection of goblet cells (a–f) and with van Gieson elastica for detection of fibrotic area (g–l). In $+/+$ and mast cell-deficient mice, all of OA-exposed mice but none of saline-exposed animals demonstrated severe goblet cell hyperplasia (Fig. 2d–f, demonstrated qualitatively in Table 2). The number of goblet cells in the epithelium was increased after antigen challenge in all of the OA-exposed animals. Significant differences were not found between $+/+$ and mast cell-deficient mice (Table 2). In contrast, marked subepithelial fibrosis was observed beneath the basement membrane of the bronchi in

Table 1. Antigen-induced increases in the number of eosinophils and lymphocytes in BALF, and serum OA-specific IgE in mice

Strain	Sensitization	Inhalation	Total leucocytes in BAL fluid ($\times 10^6$)	Eosinophils ($\times 10^5$)	Lymphocytes ($\times 10^5$)	OA-specific IgE (ng/mL)
$+/+$	SAL	SAL	0.77 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	ND
$+/+$	OA	SAL	0.84 ± 0.12	ND	0.03 ± 0.03	61.5 ± 6.8
$+/+$	OA	OA	$8.35 \pm 0.95^{***}$	3.51 ± 0.38	$1.55 \pm 0.33^{**}$	$210.0 \pm 17.5^{***}$
W/W ^v	SAL	SAL	0.76 ± 0.15	ND	0.01 ± 0.00	ND
W/W ^v	OA	SAL	1.06 ± 0.12	0.00 ± 0.00	0.04 ± 0.02	73.5 ± 6.0
W/W ^v	OA	OA	$9.60 \pm 2.61^*$	$3.27 \pm 1.84^{***}$	$2.44 \pm 0.54^{**}$	$175.6 \pm 37.1^*$
$+/+$	OA	SAL	1.06 ± 0.23	0.04 ± 0.03	0.04 ± 0.02	102.2 ± 9.6
$+/+$	OA	OA	$8.46 \pm 2.52^*$	$4.20 \pm 1.97^*$	$1.69 \pm 0.53^*$	184.3 ± 50.8
SI/SI ^d	OA	SAL	1.69 ± 0.15	0.03 ± 0.03	0.13 ± 0.08	104.7 ± 13.0
SI/SI ^d	OA	OA	$7.65 \pm 1.49^*$	$2.62 \pm 0.62^{**}$	$2.06 \pm 0.75^*$	177.6 ± 33.8

Values represent the mean $\pm$ SEM of six to seven animals in each group. SAL = saline; OA = ovalbumin; ND = not detected. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (vs. OA/SAL group).

Table 2. Antigen-induced increases in the number of goblet cells in the epithelium, TGF- β 1 level in BALF, the amount of hydroxyproline in the right lungs and the fibrotic area around the airways in mice

Strain	Sensitization	Inhalation	Goblet cell hyperplasia (scores)	TGF- β 1 in BALF (pg/mL)	Hydroxyproline content (g/right lung)	Fibrotic area (area/BM)
$+/+$	SAL	SAL	0.21 ± 0.13	44.9 ± 6.1	158.5 ± 2.7	0.87 ± 0.13
$+/+$	OA	SAL	0.08 ± 0.08	41.8 ± 6.5	152.2 ± 4.0	0.92 ± 0.11
$+/+$	OA	OA	$2.58 \pm 0.15^{**}$	$583.6 \pm 25.0^{***}$	$249.8 \pm 6.3^{***}$	$2.59 \pm 0.15^{***}$
W/W ^v	SAL	SAL	0.24 ± 0.14	38.4 ± 2.3	141.9 ± 8.1	0.98 ± 0.12
W/W ^v	OA	SAL	0.17 ± 0.11	45.3 ± 5.1	160.2 ± 5.1	0.87 ± 0.10
W/W ^v	OA	OA	$2.25 \pm 0.17^{**}$	$555.8 \pm 60.3^{***}$	$220.8 \pm 6.1^{***\dagger\dagger}$	$1.86 \pm 0.13^{***\dagger\dagger}$
$+/+$	OA	SAL	0.21 ± 0.13	96.0 ± 7.0	198.8 ± 8.0	1.21 ± 0.11
$+/+$	OA	OA	$2.20 \pm 0.46^{**}$	$438.2 \pm 128.3^*$	$266.9 \pm 13.6^{**}$	$2.56 \pm 0.15^{**}$
SI/SI ^d	OA	SAL	0.40 ± 0.29	102.4 ± 14.2	184.6 ± 8.9	1.13 ± 0.09
SI/SI ^d	OA	OA	$2.20 \pm 0.46^*$	$464.6 \pm 121.3^*$	$223.7 \pm 12.3^{\dagger}$	$1.75 \pm 0.12^{**\dagger\dagger}$

Values represent the mean $\pm$ SEM of six to seven animals in each group. SAL = saline; OA = ovalbumin; ND = not detected; BM = basement membrane. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (vs. OA/SAL group); $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ (vs. $+/+$ mice).

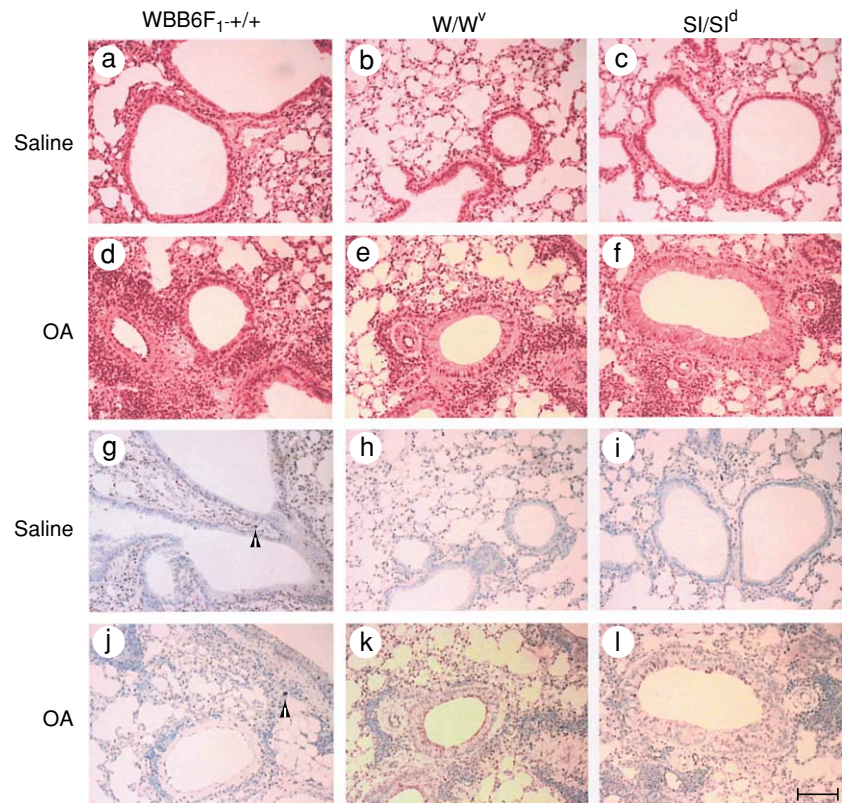


Fig. 1. Histological analyses of lung sections from $+/+$ mice (a, d, g and j), W/W^v mice (b, e, h and k) and SI/SI^d mice (c, f, i and l) 24 h after the final antigen challenge. The sections were stained with haematoxylin and eosin (a–f) and toluidine blue (g–l), respectively ($\times 50$). Scale bar 100 μ m.

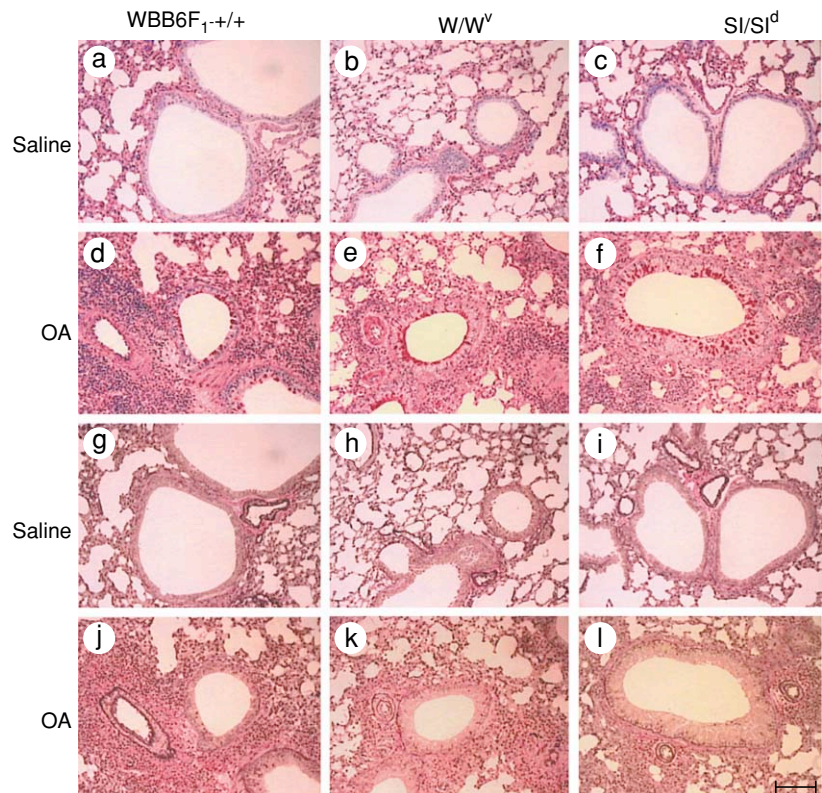


Fig. 2. Histological analyses of lung sections from $+/+$ mice (a, d, g and j), W/W^v mice (b, e, h and k) and SI/SI^d mice (c, f, i and l) 24 h after the final antigen challenge. The sections were stained with periodic acid-Schiff (a–f) and van-Gieson elastica (g–l), respectively ($\times 50$). Scale bar 100 μ m.

congenic litter-mates (Fig. 2j). However, the degree of subepithelial fibrosis in mast cell-deficient mice was slight compared with that in $+/+$ mice (Fig. 2k or l vs. j).

Bronchial hyper-responsiveness

To investigate whether the deficiency of mast cell influenced the development of antigen-induced BHR, we measured bronchial responsiveness to Ach, as estimated using PD40, a provocative dose of Ach required to cause a 40% bronchoconstriction, after repetitive allergen exposure. In both $+/+$ and mast cell-deficient mice, the PD40 in the OA-exposed mice was significantly decreased compared with that in the saline-exposed mice, whereas significant differences were not found in PD40 between $+/+$ and mast cell-deficient mice (Fig. 3).

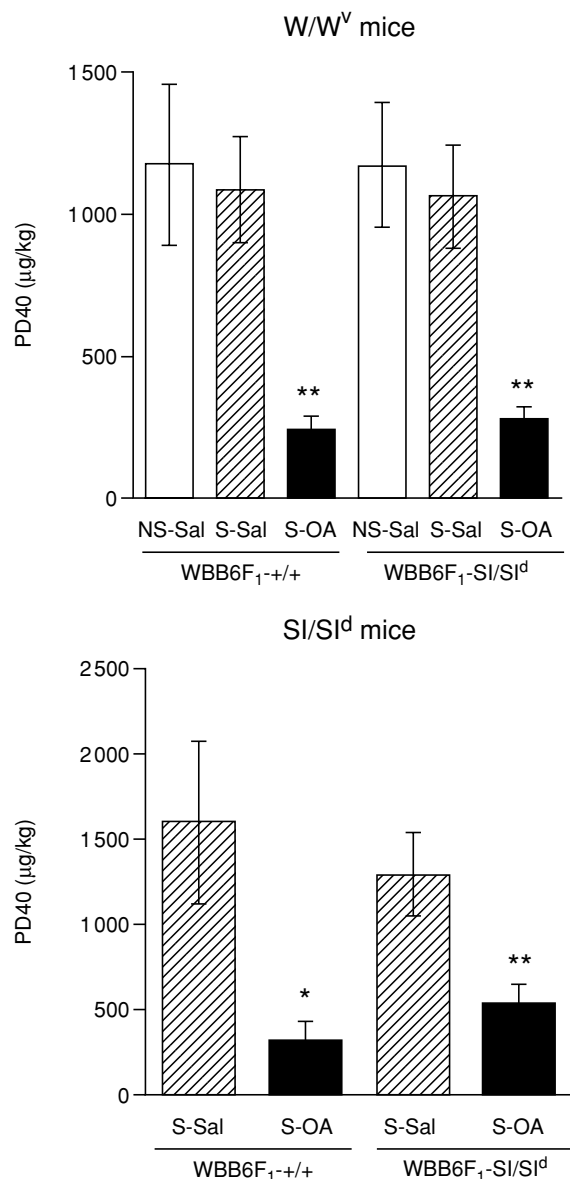


Fig. 3. Bronchial responsiveness to Ach 24 h after repeated antigen challenge in $+/+$ mice and mast cell-deficient mice. Values represent the mean $\pm$ SEM of six to seven animals in each group. PD40, a provocative dose to induce a 40% bronchoconstriction; NS, non-sensitized; S, sensitized; Sal, saline-exposed; OA, OA-exposed. * $P < 0.05$, ** $P < 0.01$ (vs. S-Sal).

Effect of reconstitution of mast cells into W/W^v mice on the decreased collagen content in the right lung tissues after repeated allergen exposure

To confirm whether the decreased collagen content in the right lung tissues of W/W^v mice was due to the deficiency of mast cells, we reconstituted BMMC derived from congenic mice into W/W^v mice and examined the hydroxyproline content after repeated allergen challenge. Lung tissue mast cells were observed in W/W^v mice reconstituted with BMMC, and the number of mast cells was comparable with that in congenic mice (data not shown). As described above, the collagen content estimated by the amount of hydroxyproline and the fibrotic area in W/W^v mice were significantly lower than those in congenic animals; however, the reconstitution of the BMMC into these mice completely recovered these parameters, which were comparable in extent to those in congenic litter-mates (Fig. 4). Moreover, there was a significant correlation between the amount of hydroxyproline and the fibrotic area among all groups ($n = 33$; $r(\rho) = 0.783$, $P < 0.001$).

Discussion

In the present study, we investigated the role of mast cells in the development of airway remodelling using two kinds of mast cell-deficient, W/W^v mice and SI/SI^d mice. Our results suggest that mast cells play a partial role in the development of allergen-induced subepithelial fibrosis but not of epithelial changes, airway eosinophilic inflammation or BHR in a murine model of allergic asthma.

The main question to be addressed is whether mast cells are involved in the development of airway remodelling. In the present study, we examined goblet cell hyperplasia in the epithelium and subepithelial fibrosis after repeated allergen challenge in mast cell-deficient mice. As a result, OA-challenged mast cell-deficient mice exhibited comparable epithelial changes as observed in the congenic litter-mates. The mechanisms of goblet cell hyperplasia after allergen challenge are still obscure; however, cytokines [21, 22], growth factors [23] and mediators [24, 25] have been reported as the candidates. Among these functional molecules, IL-13 is reported to induce goblet cell hyperplasia *in vitro* [22] and *in vivo* [26]. In the present study, allergen challenge induced the increase in IL-13 production in BALF of all sensitized animals, and there are no significant differences in the production between mast cell-deficient mice and control mice (data not shown). Moreover, reconstitution of mast cells into W/W^v mice, which were sensitized and challenged, exhibited comparable epithelial changes compared with W/W^v mice or control mice (data not shown), suggesting that mast cells are not essential for allergen-induced epithelial changes, such as goblet cell hyperplasia, in this model.

As for fibrotic changes, the responses of subepithelial fibrosis and the amounts of hydroxyproline in their mutant mice were significantly lower than in congenic mice. The mechanism of the development of subepithelial fibrosis is also not fully elucidated. Thus, we focused on a cytokine, TGF- β 1, which was reported to be an important factor in the pathogenesis of fibrosis, because it stimulates production of extracellular matrix and inhibits the formation of extracellular proteases [27]. In fact, large amounts of TGF- β 1 are found to be present in clinical specimens

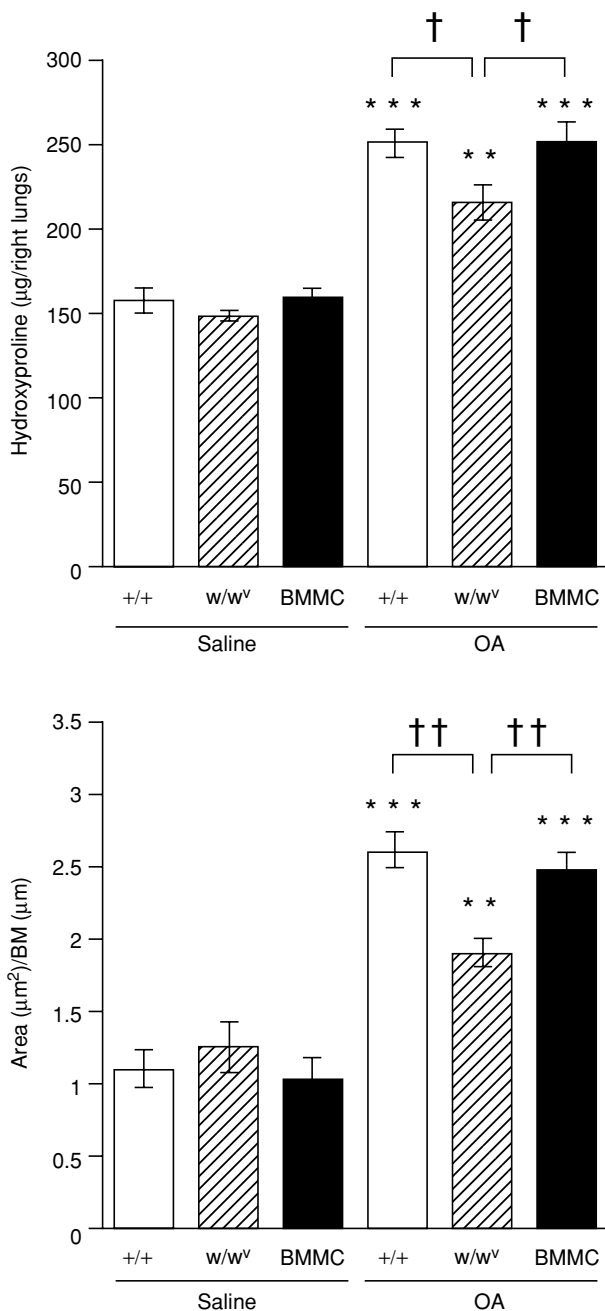


Fig. 4. The amount of hydroxyproline in the right lung tissues and the fibrotic area around the airways 24 h after the final antigen challenge in +/+ mice, W/W^v mice and those reconstituted with bone marrow-derived mast cells (BMMC). Values represent the mean $\pm$ SEM of six to seven animals in each group. Saline, sensitized and saline-exposed; OA, sensitized and OA-exposed; BM, basement membrane. *** P < 0.01, *** P < 0.001 (vs. saline in corresponding group), † P < 0.05, †† P < 0.01 (vs. W/W^v mice).

obtained from fibrotic diseases, such as early skin lesions from keloid, scleroderma and lung fibrosis [27]. Furthermore, it has been reported that inhibition of TGF- β 1 prevents liver and lung fibrosis in animal models [28, 29]. TGF- β 1 is also thought to play an important role in asthma. Increased expression of TGF- β 1 in BALF and biopsy or specimens obtained from asthmatics [30, 31], especially patients with severe asthma, has been reported [31]. Furthermore, TGF- β 1 expression was found to

correlate with the degree of subepithelial fibrosis [32]. In the present study, we have found that no significant difference between W/W^v and +/+ mice in the level of TGF- β 1 in BALF. These results suggest that mast cells are not the main source of TGF- β 1, at least in this model. Moreover, we have examined the effect of airway inflammation induced by different concentrations of OA (0.01, 0.1 and 1%) on airway remodelling [14]. Because the level of TGF- β 1 in BALF was significantly correlated with the number of eosinophils in BALF and the increased amount of hydroxyproline in right lungs, we suspect that the main source cell of TGF- β 1 may be eosinophils in this model. However, eosinophils, T lymphocytes, epithelial cells, macrophages and fibroblasts may produce TGF- β 1 [33–36]; further experiments will be needed to determine the source of the production induced by allergen challenge.

In the present study, we have found that the amount of hydroxyproline and the fibrotic area around the airways were significantly increased after antigen inhalation compared with saline-exposed groups of mast cell-deficient mice, although the degree in OA-exposed animals was significantly lower than that in congenic mice. In addition, the decreased amounts of hydroxyproline and fibrotic area were completely restored by the reconstitution of bone marrow-derived mast cells of congenic mice. Moreover, in +/+ mice, the number of mast cells in lung were not significantly different between saline-exposed and OA-exposed mice in our model. Gruber et al. [37] have reported that human mast cells stimulated fibroblast chemotaxis and procollagen messenger RNA synthesis in a coculture system of mast cell and fibroblasts. Furthermore, mast cells produce not only histamine, tryptase, chymase, leukotrienes, IL-4 and IL-13 that stimulate fibroblasts to activate or proliferate and increase collagen synthesis [9], but also MMPs that have the ability to degrade extracellular matrix [11]. Taken together, mast cells could be partially involved in the development of subepithelial fibrosis, probably through the production of these functional molecules.

Animal models, especially mice, are often used for studying human diseases; however, clinical trials based on these studies are sometimes disappointing, because the results obtained in animal studies cannot be reproduced. For example, anti-IL-5 antibody completely inhibited allergen-induced BHR in a mouse model of allergic asthma [38], whereas therapeutic approach of humanized anti-IL-5 antibody did not affect the BHR in asthmatics [39]. The reason for this discrepancy could be explained by, at least, three possibilities. First, the mechanism of BHR could be different between animals and man. Regarding the involvement of mast cells in BHR and airway remodelling, we recently examined the expression of > 10 000 genes in human and mouse mast cells using a microarray to find molecules similarly regulated and expressed by the two mast cell types [40]. As a result, the Fc ϵ receptor I-induced expression of CC chemokines (CCL-1, -3 and -4) and some of costimulatory molecules (CD137: 4-1BB) are highly conserved in both species, while some molecules, e.g. major basic protein (expressed in humans but not in mice), are differently regulated. Therefore, we should pay more attention to whether molecules examined are similarly regulated and conserved in both species. Secondly, mouse asthma models are quite different from each other. For instance, the involvement of eosinophils (IL-5) and mast cells in the development of BHR is not constant [17, 38, 41, 42]. The differences may be due to the protocol used: the frequency and

concentration of allergen challenge and/or sensitization protocol as reported [17, 42]. Finally, the therapeutic approach is also different. There are few studies that examined the curative effects of drugs (administered when inflammation is already established) (e.g. for IL-5) [41] compared with the prophylactic effects (administration before an allergen challenge) as discussed between Gelfand and Persson [42, 43]. Taken together, we encountered such a problem using animal models as described above. However, asthma is a heterogeneous disease in humans, but not in mice. It is well known that inbred mice are controlled genetically and environmentally. We think that *in vivo* animal models like the present study might still provide important information on this point, although our murine model did not demonstrate the complete features of the asthmatic airway remodelling.

In conclusion, we have demonstrated that mast cell-deficient mice showed almost the same degree of airway hyper-responsiveness, antigen-specific IgE levels, the number of inflammatory cells and TGF- β 1 levels in BALF and goblet cell hyperplasia to those in $+/+$ mice after the repeated antigen challenge. Furthermore, the amount of hydroxyproline in the lung and subepithelial fibrosis in mast cell-deficient mice was increased, but was significantly lower than that in $+/+$ mice, and the decreased amount of hydroxyproline was completely restored by the reconstitution of bone marrow-derived mast cells of congenic mice. These findings suggest that mast cells play a partial role in the development of subepithelial fibrosis, although antigen-induced airway inflammation and AHR are independent of mast cells, at least in this murine model.

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