ORIGINAL ARTICLE

Effects of imatinib mesylate on pulmonary allergic vasculitis in a murine model

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Abstract

Objectives: Imatinib mesylate (IM) is a potent and specific tyrosine inhibitor and has been reported to inhibit mesenchymal cell proliferation in pulmonary fibrosis. In the present study, we examine the effects of IM on vascular remodeling in a murine model of allergic vasculitis with eosinophil infiltration.

Methods: C57BL/6 mice were sensitized with ovalbumin (OVA) and alum. The positive controls were exposed to aerosolized OVA daily for 7 days. IM treated mice with exposure to OVA were administered IM in parallel with daily exposure to aerosolized OVA for 7 days. On the 7th day, bronchoalveolar lavage (BAL) was performed and the lungs were excised for pathological analysis. Cell differentials were determined and the concentrations of cytokines in the BAL fluid (BALF) were measured. Semi-quantitative analysis of pathological changes in the pulmonary arteries was evaluated according to the criteria of severity of vasculitis. Immunohistochemistry for Ki-67 to detect proliferating cells was performed.

Results: The number of eosinophils in BALF was reduced significantly in the IM-treated group compared to the positive control. There was no significant difference in the concentrations of interleukin (IL)-2, IL-4, IL-5, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, tumor growth factor (TGF)-β or platelet-derived growth factor in the BAL fluid between the positive control and the IM-treated group. The pathological scores of vasculitis and the ratio of Ki-67-positive intra-luminal cells were reduced significantly in the IM-treated group compared to the control group after OVA exposure.

Conclusion: IM-suppressed pulmonary vascular remodeling in a murine model of allergic vasculitis with eosinophil infiltration.

Key words: disease aetiology and pathogenesis – Human, drug treatment, vasculitides, vasculitides.

INTRODUCTION

Allergic granulomatous angitis (AGA) is characterized by bronchial asthma, eosinophilia and systemic necrotizing vasculitis involving medium and small-sized vessels with or without granulomas.[1–3] AGA causes serious organ damages, including skin, nerves, digestive canals, lungs and so on. To date, an effective therapy has not been established despite many clinical trials.

The mechanism of AGA is not completely understood. Eosinophils are the most dominant cells in the blood and extravascular tissues in AGA, and are known to release cytotoxic products such as major basic proteins, eosinophil-derived neurotoxins and oxygen radicals.[4,5] In this regard, endothelial cell injury triggered by eosinophils has been considered to be the initial step toward the vasculitis of AGA.[5]

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We previously reported a murine model of pulmonary allergic vasculitis, which was induced by repeated inhalation of ovalbumin (OVA) in C57BL/6 mice sensitized with OVA.[6] We observed that small pulmonary arteries were occluded with accumulated myofibroblasts and collagen deposition on the seventh day.

Imatinib mesylate (IM) is a potent and specific tyrosine kinase inhibitor against the tyrosine kinases *c-ABL*, *BCR-ABL* and *c-KIT*. IM has been demonstrated to be highly active in chronic myeloid leukemia and gastrointestinal stromal tumors.[7–10] The reported data regarding the specificity of IM for various tyrosine kinases show that IM also specifically inhibits platelet-derived growth factor receptor (PDGFR) tyrosine kinase.[11] It is known that PDGF acts as a chemotactic factor and growth factors for vascular smooth muscle and fibroblasts.[12,13]

In this regard, we examined the effects of IM on the histological changes of allergic vasculitis in this model. The result of this study may contribute to finding a therapy for allergic vasculitis.

METHODS AND MATERIALS

Animals

Female C57/BL6 mice (6–8 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed under specific pathogen-free conditions following a 12-h light–dark cycle, fed a standard laboratory diet and given water *ad libitum*. All experiments described in this study were performed according to the guidelines for the care and use of experimental animals as determined by the Japanese Association for Laboratory Animal Science in 1987.

Administration of IM

IM powder was dissolved in distilled water (Otsuka Pharmaceutical Co., Tokushima, Japan). IM (150 mg/kg) or water was administered orally by a flexible tube once daily during the 1 week of OVA inhalation.

Immunization and aerosolization protocol

The mice were sensitized according to the methods described in a previous paper.[14] In brief, mice were sensitized at days 0 and 5 of the protocol by an intraperitoneal injection of 0.5 mL aluminum hydroxide-precipitated antigen containing 8 μg OVA (Sigma Chemical Co., St. Louis, MO, USA) adsorbed overnight at 4°C to 4 mg of aluminium hydroxide (Wako Chemical Co., Tokyo Japan) in phosphate-buffered saline (PBS). Twelve days after the second immunization, mice were divided into four groups (A, B, C, D). A and

B groups of mice (*n* = 6) were placed in a plastic chamber (10 × 15 × 25 cm) and exposed to aerosolized 0.9% saline. On the other hand, the C and D groups of mice (*n* = 12) were exposed to aerosolized OVA (5 mg/mL in 0.9% saline) for 1 h daily until the seventh day. The aerosolized OVA and saline were produced by a Pulmo-Aide Compressor/Nebulizer (Devilbiss) (Sunrise Medical HHG, Inc. Somerset, PA, USA) at a flow rate of 5–7 L/min. The B and D groups of mice were treated with IM as described above. On the other hand, the A and C groups of mice were provided saline orally instead of IM.

Collection and measurement of specimens

After being exposed to aerosolized saline or OVA every day over 1 week, the mice were killed by cutting the femoral artery on the seventh day, 24 h after the final inhalation, and blood, bronchoalveolar lavage fluid (BALF) and lung tissues were collected. To collect BALF, the lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD, USA). The lungs were lavaged twice with 0.5 mL phosphate-buffered saline (PBS), and ~ 0.8 mL of the instilled fluid was consistently recovered. The recovered fluid was centrifuged (300 × *g* for 6 min) and the cells were resuspended in 0.5 mL PBS. The total number of cells was counted using an improved Neubauer hemocytometer chamber. An air-dried slide preparation was made of each sample containing 10 000 cells by cytospin (Cytocentrifuge; Sakura Seiki, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain. Differential counts of at least 500 cells were made according to standard morphologic criteria. The numbers of cells recovered per mouse were then expressed as the mean and standard deviation (SD) for each treatment group.

After centrifugation, the supernatants were stored at −80°C for measurement of the cytokines. After harvesting BALF, lungs were fixed with 10% neutral buffered formalin and embedded in paraffin. These 3-μm-thick sections were stained with hematoxylin and eosin (HE) and Elastica Masson's trichrome (EM). The cell differentials in BALF were determined under microscopy with Giemsa staining and the concentrations of interleukin (IL)-2, IL-4, IL-5, interferon (IFN)-γ and tumor necrosis factor (TNF)-α in BALF were measured.

Immunohistochemical staining of Ki-67

We adopted the biotin–streptavidin system using a Histofine Kit (Nichirei, Tokyo, Japan) for the immunohistochemical staining. The sections were deparaffinized

and treated with 0.3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity. The sections were incubated with 10% normal rabbit serum for 30 min at room temperature to block the non-specific antibody reaction. We used anti-antigen Ki-67 (Dako, UK). The sections were incubated for 60 min at room temperature with 1 : 50 fold antibody followed by the biotin–streptavidine system, then 3′3-diaminobenzidine (DAB) was used as the chromogenic substrate.

Counting Ki-67-positive cells

Ki-67-positive cells, those with brown-colored nuclei, were counted under microscopy. Intra-luminally accumulated cells in the pulmonary arteries were counted. The ratio of Ki-67-positive cells was calculated as the number of brown nuclei/number of whole cells stained with hematoxylin (blue nuclei).

Semi-quantitative analysis of pathological changes in the pulmonary arteries

The extent of histological changes in pulmonary arteries was assessed as previously described.[6] In brief, the tissue was cut into sections 3 m thick, stained with hematoxylin and eosin and evaluated by light microscopy. Histological scores were determined according to the following criteria: 0 = no abnormality; 1 = minimum, 2 = mild: shedding of endothelial cells, no change of the vascular smooth muscle layer and mild perivascular cell infiltration were observed; 3 = moderate: shedding of endothelial cells, thickening of the vascular smooth muscle layer and moderate perivascular cell infiltration were observed; and 4 = severe: disruption of internal elastic laminae, proliferation of mesenchymal cells in the intraluminal space in pulmonary arteries, and moderate to severe perivascular cell infiltration. The severity was judged by the extent of endothelial injury, vascular smooth muscle cell proliferation, loss of vascular wall integrity and peri-vascular cell infiltration. We scored five vessels in which diameters ranged from 20 to 50 μm in each of the lung tissue sections and the average was determined as the histological index of one mouse.

Cytokine measurement

BALF were used for the measurement of IL-2, IL-4, IL-5, TNF-α and IFN-γ using a cytokine bead kit (Becton Dickinson). Concentrations of TGF-β1 and PDGF-AA were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine; R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Mann–Whitney U-test was used in the analysis of results. All values are expressed as means ± SEM. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effects of IM on cell numbers in BALF

OVA inhalation induced a marked increase of the total cells, including alveolar macrophages, lymphocytes and eosinophils in both mousee groups with and without IM treatment (groups C and D as describe in Methods) compared to those exposed to saline (groups A and B as describe in Methods). The number of eosinophils in BALF of OVA-exposed mice with IM treatment (group D) decreased significantly compared to those without IM treatment (group C) (Fig. 1). The numbers of total cells, alveolar macrophages, lymphocytes and neutrophils were not significantly different between these groups.

Cytokine concentration in BALF

We measured the concentrations of IL-2, IL-4, IL-5, IFN-γ, TNF-α, TGF-β and PDGF in BALF after the seventh day of exposure to saline or OVA and compared them between OVA-exposed mice and those treated with IM as described in Methods. As shown in Figure 2a,b, the concentrations of TNF-α, IL-4, IL-5, TGF-β1 and PDGF-AA in the BALF were significantly increased in groups C and D. The concentrations of IL-2, IL-4, IL-5, IFN-γ, TNF-α, TGF-β1 and PDGF-AA in BALF were not significantly different between the
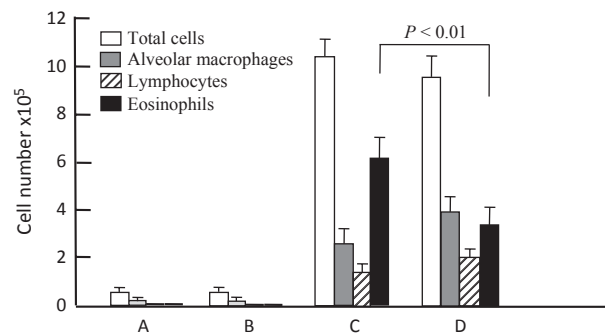
Figure 1 Cell number and cell differentials in bronchoalveolar lavage fluid (BALF). (a) Ovalbumin (OVA)-sensitized mice exposed to saline ($n = 6$), (b) those exposed to saline and treated with imatinib mesylate (IM) ($n = 6$), (c) those exposed to OVA as described in Methods section ($n = 12$), (d) those exposed to OVA and treated with IM as described in Methods ($n = 12$). Data are means ± SD.
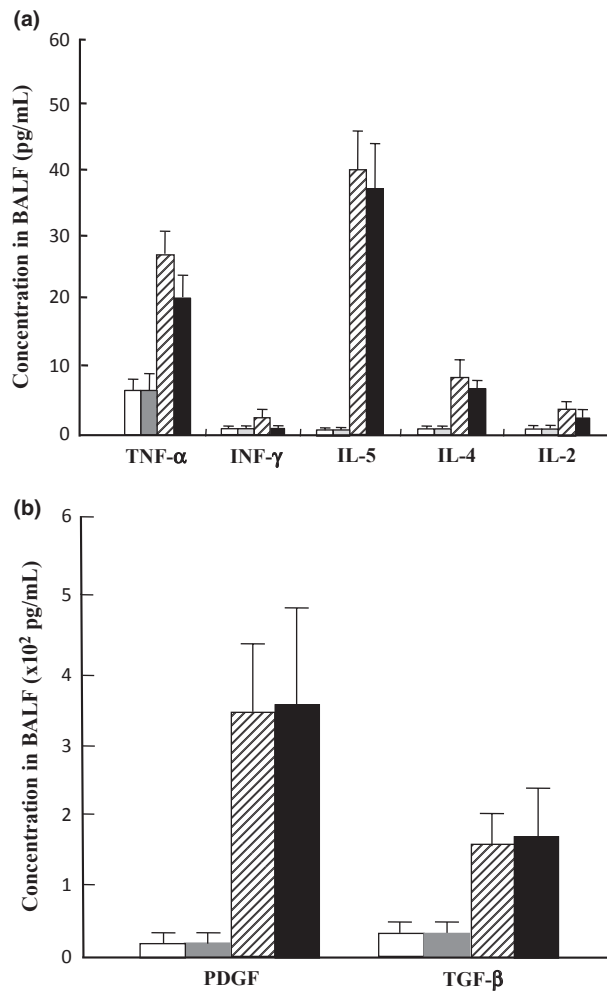
**Figure 2** Cytokine concentration in bronchoalveolar lavage fluid (BALF). (a) concentrations of tumor necrosis factor (TNF)-α, interferon (INF)-β, interleukin (IL)-5, IL-4 and IL-2 in BALF. Open column: ovalbumin (OVA)-sensitized mice exposed to saline (group A); gray column, those exposed to saline and treated with imatinib mesylate (IM) (group B); hatched column, those exposed to OVA (group C); black column, those exposed to OVA and treated with IM (group D). (b) concentrations of platelet-derived growth factor (PDGF) and tumor growth factor (TGF)-β in BALF. The four columns are as described in panel a.

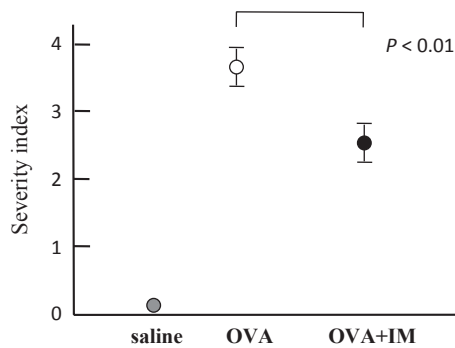OVA-exposed mice treated with IM (group D) and those not treated with IM (group C).

Effects of IM on the histological changes in pulmonary arteries

Almost all small pulmonary arteries were highly obstructed due to the accumulation of cellular components in OVA-exposed mice (group C) (Fig. 3a).
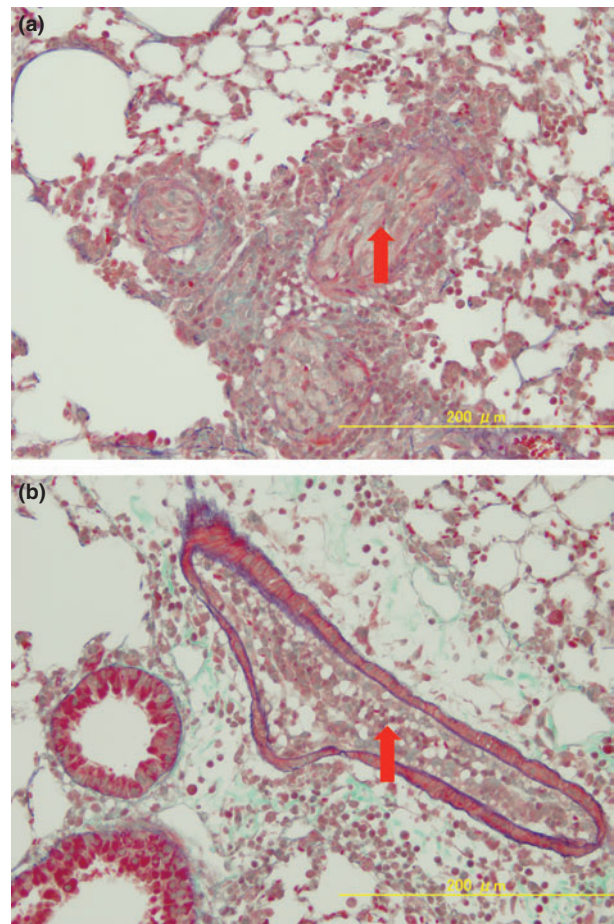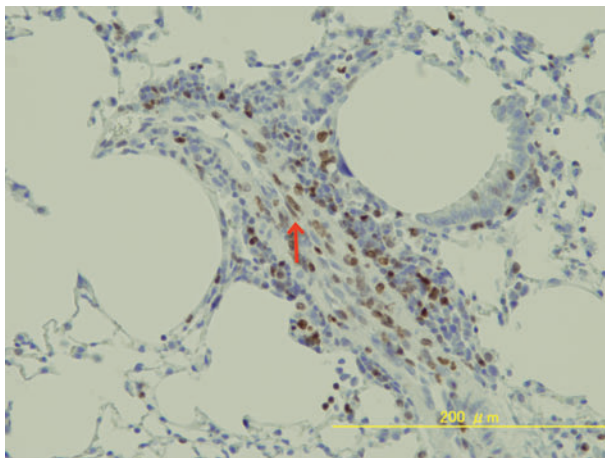
Figure 3 Effects of imatinib mesylate (IM) on allergic pulmonary vascular remodeling. (a) Totally occluded pulmonary artery by intra-luminal myofibroblasts in group C. Intra-luminal accumulation of myofibroblasts with collagenous fibers (green) indicated by a red arrow (Elastica-Masson staining) in group C. The structure of the laminae elastica interna was destroyed. (b) Intraluminal myofibroblast accumulation was not observed in group D (a red arrow). Thickening of the vascular smooth muscle cells in the vascular wall was also reduced. The structure of the laminae elastica interna was maintained (Elastica-Masson staining) in group D.

Intraluminally accumulated cells were positively stained by anti-actin antibody (data not shown), suggesting characteristics of myofibroblasts in group C. In contrast, the histological changes in IM-treated mice (group D) were markedly reduced (Fig. 3b). Semiquantitative analysis of the histological vascular changes in the mice after the seventh day of OVA-exposure and in those treated with IM was performed as described in Methods in terms of the severity index. The severity index in group D was significantly lower than that in group C

Figure 4 Effects of imatinib mesylate (IM) on severity of vascular changes. White circle: histological scores of the ovalbumin (OVA)-sensitized mice after the seventh day of exposure to OVA ($n = 12$) (group C); black circle, the OVA-sensitized mice after the seventh day of exposure to OVA and treated with IM ($n = 12$) (group D); gray circle, group A. Data are given as mean ± SD and $P < 0.01$.

Figure 6 The effect of imatinib mesylate on the ratio of Ki-67 positive cells. Open circle: the ratio of Ki-67 positive cells in the intra-luminal space in group C ($n = 12$); closed circle: group D ($n = 12$). Data are given as mean ± SD and $P < 0.01$.

Ki-67-expressing cells in the intraluminal space of the pulmonary artery in OVA-exposed mice treated with IM was decreased significantly compared to that in the mice not treated with IM (Fig. 6).

DISCUSSION

The present study demonstrated that IM suppressed the histological changes of allergic vasculitis in the pulmonary arteries of OVA-exposed mice and reduced the number of eosinophils in BALF without changing IL-4 or IL-5 concentrations in the BALF.

IM is a potent and specific tyrosine kinase inhibitor against Abl, Bcr/Abl, Kit, and PDGF receptor-α (PDGFRA) and -β (PDGFRB) tyrosine kinases. Recently, IM has been demonstrated to be highly effective for the treatment of a subgroup of patients with hypereosinophilic syndrome (HES) or clonal eosinophilia, including systemic mast cell disease (SMCD).[16–20]

In the present study, we used the murine model of pulmonary allergic vasculitis which we previously reported as an animal model of AGA.[6] The following histopathological changes in our murine model resembled allergic granulomatous angiitis in humans: (i) infiltration of mononuclear cells and eosinophils, and granuloma with multinuclear giant cells in the arterial wall; (ii) disruption of internal elastic layer; and (iii) obliteration of pulmonary arteries by mesenchymal cells. On the other hand, the fibrinoid degeneration of arterial walls observed in the human cases was not found in the murine model. As described above, the histopathological features of this model mouse are not
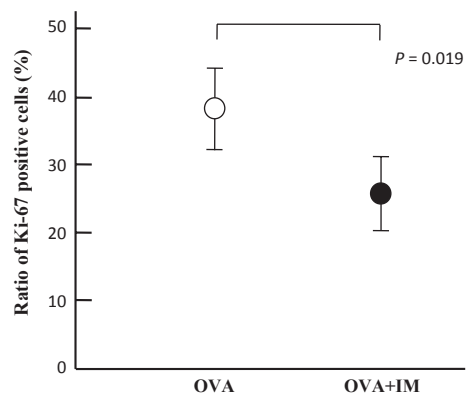

Figure 5 Ki-67-positive cells in vascular remodeling. Ki-67-positive cells were seen among the myofibroblasts that accumulated in the intraluminal space of pulmonary arteries (a red arrow) in group C.

(Fig. 4). Histological changes were minimal in groups A and B exposed to saline.

Effects of IM on the ratio of Ki-67-positive cells

Immunohistochemistry for Ki-67 was performed to detect the proliferating cells in pulmonary vasculitis.[15] Ki-67 was expressed in intraluminal myofibroblasts and cells in the vascular wall in OVA-exposed mice (Fig. 5). In contrast, Ki-67-expressing cells were very sparse in the pulmonary vascular tissue of the mice treated with IM (data not shown). The ratio of the number of

completely the same as those of human AGA. However, our AGA mouse model is thought to be a useful animal model for analyzing human disease on the basis of its granulomatous pulmonary vasculitis accompanied by eosinophils infiltration with eosinophilia.

In order to elucidate the pathogenesis of vasculitis in this model, we tried to detect myeloperoxidase – antineutrophil cytoplasmic antibodies (MPO-ANCA) in the serum of our murine model against the recombinant murine MPO,[21] but we could not detect an antibody against the recombinant murine MPO in the serum of the murine model. Therefore, to date, there is no evidence of ANCA-associated vasculitis in our murine model.

The proliferation and differentiation of eosinophils are known to be regulated by IL-5.[22,23] As shown in the results, OVA exposure to sensitized mice induced marked increases in the number of eosinophils and IL-5 concentration in BALF, suggesting that IL-5 was a major inducer of eosinophil accumulation in BALF in our murine model of pulmonary allergic vasculitis. On the other hand, activation of c-*kit* also induces eosinophil activation and degranulation[24] and proliferation that may be synergistic with IL-3, granulocyte macrophage–colony-stimulating factor (GM-CSF) and IL-5,[25] and increased adhesion that could contribute to tissue localization.[26] In addition, platelet-derived growth factor activates eosinophils.[27] The present study demonstrated that the number of eosinophils in BALF was significantly reduced in OVA-exposed mice treated with IM compared to those not treated with IM. These results suggest that PDGF-A or B might have played a role in the pulmonary accumulation of eosinophils in our murine model of pulmonary allergic vasculitis. However, the reduction of the eosinophil number in BALF by IM was limited. This result suggested that eosinophilia was caused mostly by IL-5 and PDGF might have played a partial role in eosinophil accumulation in the lung in the present murine model. The pathways of PDGF and IL-5 were independent of each other. Therefore, IM is thought to have a limited role in suppressing the IL-5 pathway.

Although it has been known that neutrophils were thought to play a critical role in AGA,[21] we could find very few neutrophils in the bronchoalveolar lavage fluid in our murine model. In addition, IM did not significantly suppress the number of alveolar macrophages. We believe the PDGF pathway was not strongly involved in the increase of alveolar macrophages in our murine model. Further study to elucidate the role of neutrophils and alveolar macrophages in this murine model is needed.

Drastic obstructive remodeling of small-sized pulmonary arteries was observed on the seventh day in the OVA-sensitized mice stimulated with repetitive OVA inhalation. The intraluminally accumulated cells were myofibroblasts which are spindle-shaped cells, and were positively stained with anti-actin antibody.[6] These cells expressed Ki-67, suggesting that they were proliferating cells. Several growth factors have been reported to be involved in vascular smooth muscle cell proliferation. Among them, PDGF plays a critical role in chemotaxis and proliferation of vascular smooth muscle cells and myofibroblasts.[28–31] As shown in the results, OVA exposure to sensitized mice induced an increase of the PDGF concentration in the BALF, suggesting that the increased PDGF might be involved in the intraluminal myofibroblast proliferation. Danal *et al.*[32] demonstrated that IM exerted suppressive effects on vascular smooth muscle cell proliferation in hypoxia-induced pulmonary hypertension in mice. In this case, IM was thought to inhibit tyrosine phosphorylation of the PDGF receptor, resulting in attenuation of the vascular smooth muscle cell proliferation.

IM has been also reported to be a possible therapeutic molecule for pulmonary fibrosis.[13] Abdollahi *et al.* reported the increased PDGF molecules (PDGF-A, B, C, D) induced exaggerated fibroblast proliferation in radiation-induced pulmonary fibrosis. They also demonstrated that SU9518, as a PDGF receptor tyrosine kinase inhibitor, inhibited radiation-induced pulmonary fibrosis and reduced PDGF-β receptor phosphorylation.[33]

The effects of IM were also evaluated in bleomycin-induced pulmonary fibrosis in mice. Aono *et al.* demonstrated that IM attenuated bleomycin-induced pulmonary fibrosis on days 7 and 14 without affecting the number of inflammatory cells in the BALF.[34] They suggested that IM prevented the proliferation of mesenchymal cells, including murine lung fibroblasts, by inhibiting the autophosphorylation of PDGFR-β induced by PDGF. As shown in our results, a higher percentage of intraluminal myofibroblasts were positive for Ki-67 in our murine model of pulmonary allergic vasculitis and the treatment with IM reduced the percentage of Ki-67-positive cells which indicated proliferating cells.

TGF-β is also an important molecule that is involved in pulmonary fibrosis. In the present murine model of pulmonary allergic vasculitis, the concentration of TGF-β in BALF was strikingly high in OVA-exposed mice regardless of IM treatment. In this regard, TGF-β might also have played a role in the vascular remodeling in the present model. Concerning myofibroblast

proliferation, Daniels *et al.* reported that fibroblasts respond to TGF-β by stimulating *c-ABL* kinase activity independently of Smad2/3 phosphorylation or PDGFR activation, and that inhibition of *c-ABL* by IM prevented TGF-β -induced ECM gene expression, morphologic transformation, and cell proliferation independently of any effect on Smad signaling.[35] These findings suggest that in the present study, the inhibition of *c-ABL* by IM might have been involved in the inhibition of vascular remodeling in the mice treated with IM.

In conclusion, IM suppressed the vascular remodeling in a murine model of pulmonary allergic vasculitis by inhibiting the proliferation of vascular myofibroblasts.

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DECLARATION OF INTEREST

The authors report no conflicts of interest.

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