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ORIGINAL ARTICLE

Rapamycin Attenuates Pulmonary Allergic Vasculitis in Murine Model by Reducing TGF-β Production in the Lung

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ABSTRACT

Background: Rapamycin has been reported to inhibit mesenchymal cell proliferation in a murine model of pulmonary fibrosis. In the present study, we examined the effects of rapamycin on vascular remodeling including intraluminal myofibroblast proliferation 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. The other group of mice was administered with rapamycin (1 mg/kg) intraperitoneally, in parallel with daily exposure to aerosolized OVA for 7 days. On the 3rd and 7th day, bronchoalveolar lavage (BAL) was performed and the lungs were excised for pathological analysis. Cell differentials were determined and concentrations of IL-4, IL-5, IL-13 and TGF-β in the BAL fluid (BALF) were measured. Semi-quantitative analysis of pathological changes in the pulmonary arteries was evaluated according to the severity of vasculitis.

Results: The number of eosinophils in BALF was reduced significantly in the mice treated with rapamycin compared to the positive control. There was a significant decrease in the TGF-β concentration of the BALF in the rapamycin-treated group compared to that of the positive control. The pathological scores were reduced significantly in the rapamycin-treated group compared to the positive control group. Intraluminal myofibroblasts in pulmonary arteries were reduced dramatically in the rapamycin-treated group compared to the positive control group.

Conclusions: Rapamycin suppressed pulmonary vascular remodeling in a murine model of allergic vasculitis with eosinophil infiltration through reducing eosinophil infiltration and TGF-β production in the lung and inhibition against biological action of TGF-β.

KEY WORDS

allergic vasculitis, C57BL/6, murine model, rapamycin, TGF-β

ABBREVIATIONS

IL-4, interleukin-4; IL-5, interleukin-5; IL-13, interleukin-13; IFN-γ, interferon γ; IgE, serum immunoglobulin E; TGF-β, transforming factor β.

INTRODUCTION

Eosinophilic granulomatous polyangitis (EGPA), a disease of vasculitis the main lesion of which is located in medium and small-sized vessels, is characterized by bronchial asthma, eosinophilia, and systemic necrotizing vasculitis with or without granulomas.[1-3] EGPA causes serious damages to the skins, nerves,

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digestive canals, lung and other organs. To date, an effective therapy has not been established despite many clinical trials.

The mechanism of EGPA is not completely understood. Eosinophils are the most dominant cells in the blood and extravascular tissues in EGPA, and are known to release cytotoxic products such as major basic protein, eosinophil-derived neurotoxin, and cytokines including TGF-β.[4,5]

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 the small pulmonary arteries of the C57BL/6 mice were occluded with accumulated myofibroblasts and collagen deposition on the 7th day.

The mammalian target of rapamycin (mTOR) is a multifunctional protein involved in the regulation of cell growth, proliferation, and differentiation. Rapamycin is a 31-membered macrolide immunosuppressant which exerts potent antiproliferative effects on lymphoid and nonlymphoid cells by inhibiting cytokine and growth-factor mediated cell signalling.[7]

The antiproliferative effects of rapamycine on mesenchymal cell growth including lung, kidney, liver etc have been reported.[8-11] Especially, the inhibition of mTOR by rapamycin suppressed TGF-β-induced pulmonary fibrosis by reducing collagen synthesis.[12]

In this regard, we examined the effects of rapamycin on the histological changes in our model of allergic pulmonary vasculitis. This study may contribute to finding a therapy for allergic vasculitis.

METHODS

ANIMALS

Female C57/BL6 mice (6-8 wk old) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed under specific pathogen-free conditions following a 12-hour light-dark cycle and 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 Animals Science in 1987.

ADMINISTRATION OF RAPAMYCIN

Rapamaycin (RAPACAN™1) was purchased from Biocon (Bangalore, India). The rapamycin powder was dissolved once in dimethyl sulfoxide (DMSO) at 2.5 mg/ml. Finally rapamycin was prepared at 125 μg/ml in a solution containing 5% polyethylene glycol, 5% Tween 80 and 5% DMSO in distilled water. Rapamycin in the solution above was administered at 1 mg/kg intraperitoneally by injection once daily during 1 week of OVA inhalation in the rapamycin-treated group mice. We performed preliminary experiments concerning the relationship between the doses of rapamycin (0.1-5 mg/kg) and their effects on the severity of vasculitis. In addition, taking the lethal rate into consideration, we decided to perform the current experiment at 1 mg/kg rapamycin. In the positive control group mice, distilled water containing 5% polyethylene glycol, 5% Tween 80 and 5% DMSO without rapamycin was injected intraperitoneally once daily during 1 week of OVA inhalation.

IMMUNIZATION AND AEROSOLIZATION PROTOCOL

The mice were sensitized according to the methods described in a previous paper.[13] 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, St. Louis, MO, USA) adsorbed overnight at 4℃ to 4 mg of aluminium hydroxide (Wako Chemical, Tokyo, Japan) in phosphate-buffered saline (PBS). 12 days after the second immunization, mice were divided into 2 groups consisting of 12 animals each. Each mouse of the positive control group was placed in a plastic chamber (10 cm × 15 cm × 25 cm) and exposed to aerosolized OVA (5 mg/ml in 0.9% saline) for 1 h daily until the 7th day as shown in Figure 1. The aerosolized OVA was produced by a Pulmo-Aide Compressor/Nebulizer (Devilbiss) (Sunrise Medical HHG, Inc. Somerset, PA, USA) at a flow rate of 5-7 liter/min. The rapamycin-treated group of mice were exposed to aerosolized OVA for 1 h daily until the 7th day and in parallel, administered rapamycin as described above (Fig. 1).

COLLECTION AND MEASUREMENT OF SPECIMENS

After being exposed to aerosolized OVA every day over one week, the mice were killed by cutting the femoral artery on the 3rd and 7th day, 24 hours after the final inhalation, and blood, BALF and lung tissue were collected as shown in Figure 1. As a control, after being exposed to aerosolized saline instead of OVA every day over one week, samples 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 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.

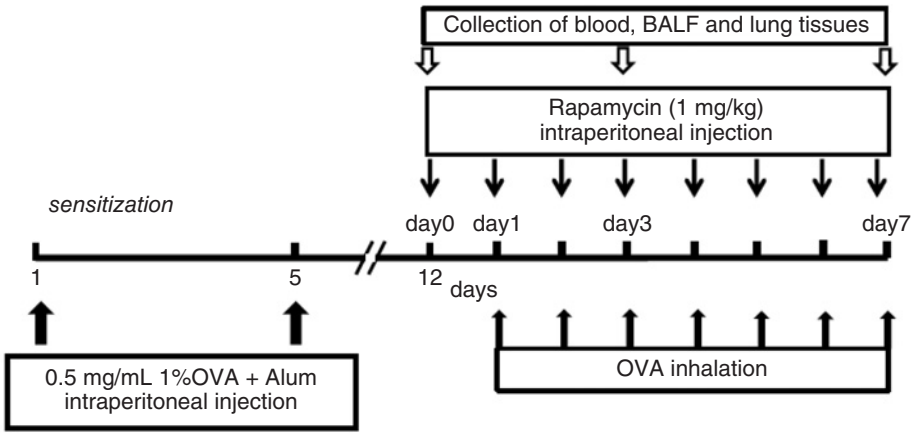

Fig. 1 Experimental protocol. Mice were sensitized at days 0 and 5 of the protocol by an intraperitoneal injection of 0.5 ml aluminum hydroxide-precipitated antigen containing 8 μg OVA. Mice were exposed daily to the aerosolized OVA produced by a Pulmo-Aide Compressor /Nebulizer at a flow rate of 5-7 liter/min in the plastic chamber as described in "Methods". Rapamycin was administered as 1 mg/kg intraperitoneally by injection once daily during 1 week as described in "Methods".

After centrifugation, supernatants were stored at -80°C for measurement of the cytokines. After harvesting the BALF, the lungs were fixed with 10% neutral buffered formalin and embedded in paraffin. These 3-μm-thick sections were stained with hematoxylin eosin (HE). The white blood cells and the cell differentials in BALF were determined under microscopy with Giemsa staining, and the concentrations of IL-4, IL-5, IL-13 and TGF-β in BALF were measured.

IMMUNOHISTOCHEMICAL STAINING FOR TGF-β, α SMOOTH MUSCLE ACTIN AND 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 minutes to block endogenous peroxidase activity. The sections were incubated with 10% normal rabbit serum for 30 minutes at room temperature to block the non-specific antibody reaction. We used anti-TGF-β1 (Novus Biologicals, Littleton, CO, USA), anti-α smooth muscle actin (α-SMA) (Spring Bioscience, Fremont, CA, USA) and anti-mouse Ki-67 (Biolegend, San Diego, CA, USA). The sections were incubated 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.

SEMI-QUANTITATIVE ANALYSIS OF PATHOLOGICAL CHANGES IN THE PULMONARY ARTERIES

The extent of histological changes in pulmonary arteries was assessed as described previously.[6] In brief, the tissue was cut into sections 3 μm thick, stained with hematoxylin-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 perivascular cell infiltration. We scored 5 vessels whose diameters ranged from 20 to 50 μm in each of the lung tissue sections and its average was determined as the histological index of one mouse.

COUNTING Ki-67 POSITIVE CELLS

Ki-67 positive cells, those with brown-colored nuclei, were counted under microscopy. Intraluminally 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) in the five arteries of each mouse.

CYTOKINE MEASUREMENT

BALF was used for the measurement of IL-4, IL-5, IFN-γ and TGF-β1 concentration using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

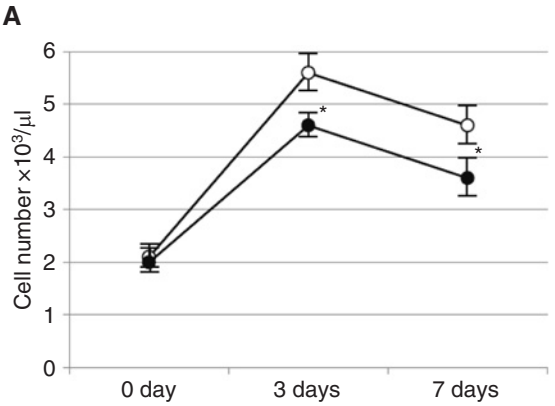

Fig. 2A The effect of rapamycin on total white blood cell counts in peripheral blood. Open circles: OVA-sensitized mice with exposure to OVA (positive control), Closed circles: OVA-sensitized mice with exposure to OVA and treated with rapamycin as described in "Methods". Samples on the 0 day were collected before OVA exposure. Samples on 3rd and 7th day were collected after OVA exposure. Data are means ± SD. *$p < 0.01$.

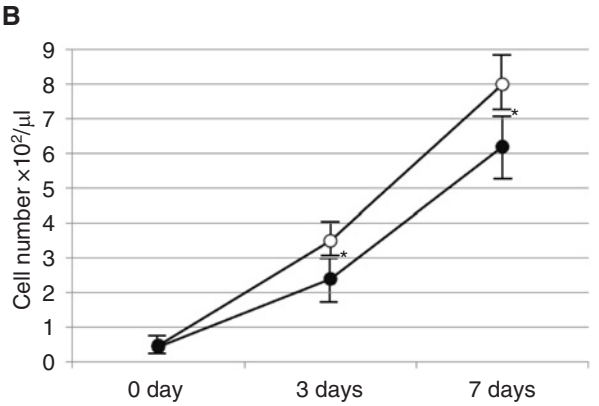

Fig. 2B The effect of rapamycin on eosinophil counts in peripheral blood. Open circles: OVA-sensitized mice with exposure to OVA (positive control), Closed circles: OVA-sensitized mice with exposure to OVA and treated with rapamycin.

MEASUREMENT OF IgE

The IgE levels in serum were determined with a commercially available enzyme-linkedimmunosorbent assay (ELISA) kit (Yamasa, Chiba, Japan).

STATISTICAL ANALYSIS

Mann-Whitney U Test was used in the analysis of results. All values are expressed as means ± SD. Values of $p < 0.05$ were considered statistically significant.

RESULTS

EFFECTS OF RAPAMYCIN ON CELL NUMBERS IN PERIPHERAL BLOOD AND IN BALF

After repetitive exposure to OVA, the total white blood cell number in peripheral blood increased to 2.6-fold of the control level on the 3rd day in the positive control mice (Fig. 2A). In contrast, the total white blood cell numbers in the rapamycin-treated mice were significantly lower on the 3rd and 7th day than those in the positive control (Fig. 2A). The blood eosinophil counts in the positive control increased after OVA inhalation. The blood eosinophil counts in the rapamycin-treated mice were significantly lower on the 3rd and 7th day than those in the positive control (Fig. 2B). The total cell number and the number of eosinophils in BALF on the 3rd day increased markedly compared with those before OVA inhalation. The number of eosinophils in BALF on the 3rd day was decreased significantly in the rapamycin-treated mice compared with those in control positive mice. The number of eosinophils in BALF in the rapamycin-treated group on the 7th day also decreased significantly compared to that in the positive control group (Fig. 3). In contrast, the numbers of alveolar macrophages in BALF in the rapamycin-treated group increased significantly compared to that in the positive control group.

CYTOKINE CONCENTRATION IN BALF

We measured the concentrations of IL-4, IL-5, IL-13, IFN-γ and TGF-β in BALF on the 3rd and 7th day in the positive control group mice and in the rapamycin-treated group mice as described in Methods. As shown in Figure 4A, B, C respectively, concentrations of IL-4, IL-5 and IL-13 in BALF of the positive control peaked on the 3rd day during 7 days and these concentrations in the BALF of the rapamycin-treated group mice were decreased significantly compared with those in the positive control. Among these cytokines, the IL-4 concentration in BALF on the 7th day was rather higher in the rapamycin-treated group compared with the positive control. IFN-γ concentration in BALF increased on the 3rd day after OVA inhalation in both the positive control mice and the rapamycin-treated mice. The IFN-γ concentrations in BALF on the 3rd day and 7th day were not significantly different between these two groups (Fig. 4D). The concentrations of TGF-β in the BALF of the positive control group increased until the 7th day after OVA inhalation and the concentrations of TGF-β in BALF of the rapamycin-treated group decreased significantly on the 7th day compared with those of the positive control (Fig. 4E).

IgE CONCENTRATION IN SERUM

The IgE concentrations in serum increased on the 3rd and 7th day after OVA inhalation in both the positive control mice and the rapamycin-treated mice. The IgE concentrations in serum on the 7th day were significantly lower in rapamycin-treated mice compared to the positive control mice (Fig. 4F).

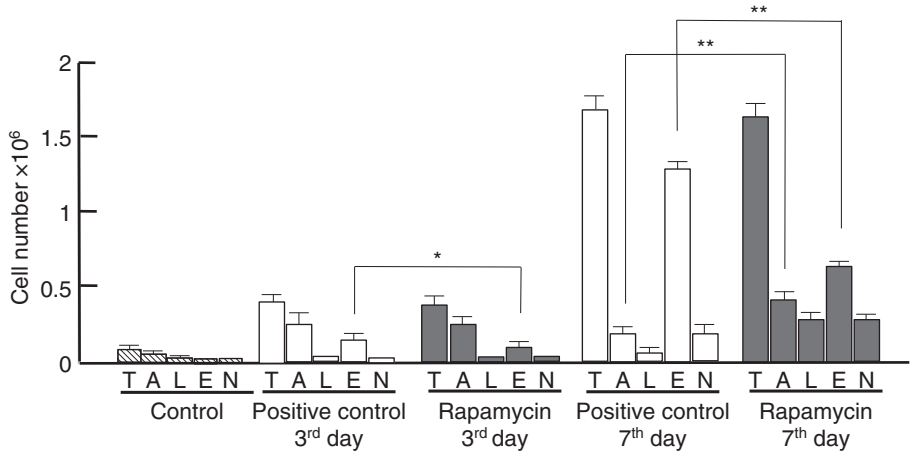

Fig. 3 The effect of rapamycin on cell differentials in BALF. Hatched columns: OVA-sensitized mice (n = 6) with exposure to saline (control). Open columns: OVA-sensitized mice (n = 6) with exposure to OVA (positive control). Closed columns: OVA-sensitized mice (n = 6) with exposure to OVA and treated with rapamycin. T, total cells; A, alveolar macrophages; L, lymphocytes; E, eosinophils; N, neutrophils. Data are means ± SD. *p < 0.05, **p < 0.01.

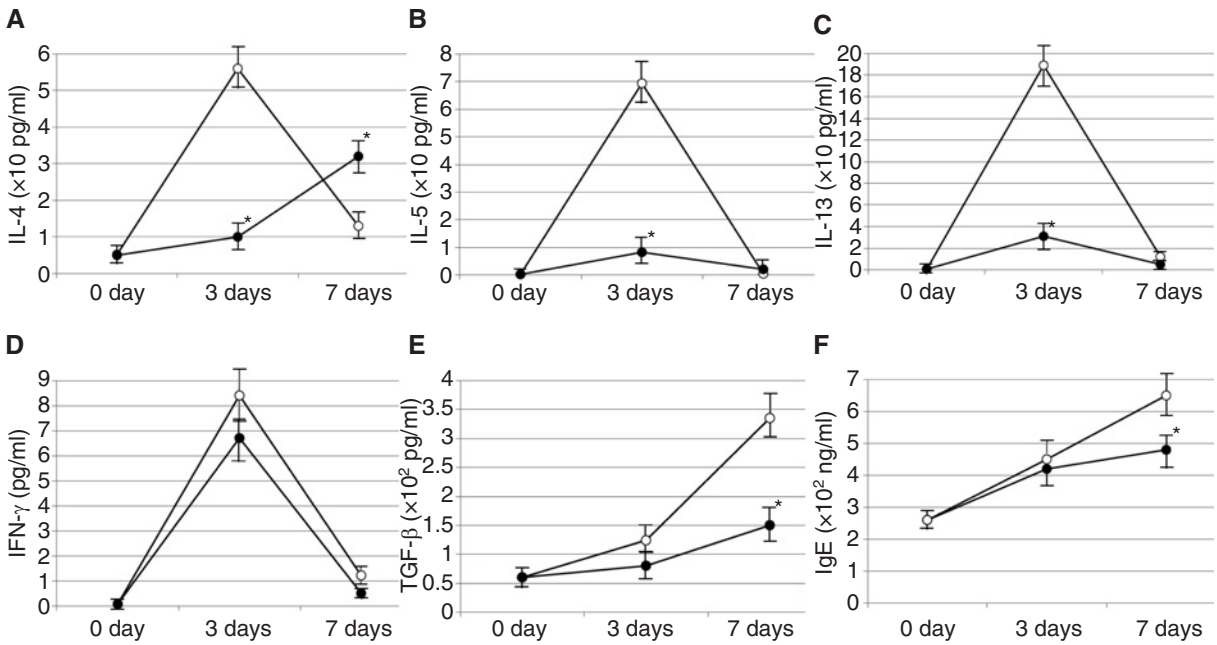

Fig. 4 Cytokine concentration in BALF and IgE concentration in serum Concentrations of IL-4, IL-5, IL-13, INF-γ and TGF-β in BALF, and IgE concentration in serum were expressed. **A**, IL-4; **B**, IL-5; **C**, IL-13; **D**, INF-γ; **E**, TGF-β; **F**, IgE. Open circles: OVA-sensitized mice with exposure to OVA (positive control). Closed circles: OVA-sensitized mice with exposure to OVA and treated with rapamycin. Data are means ± SD. *p < 0.01.

EFFECTS OF RAPAMYCIN ON THE HISTOLOGICAL CHANGES IN PULMONARY ARTERIES

In the positive control group mice, almost all small pulmonary arteries were highly obstructed due to the accumulation of cellular components (Fig. 5A, panel a). In contrast, the histological changes of the small pulmonary arteries in the rapamycin-treated mice were markedly reduced (Fig. 5A, panel b). Semiquantitative analysis of the histological vascular changes on the 7th day in the positive controls and the rapamycin-treated mice was performed as described in "Methods" according to severity index. The sever-

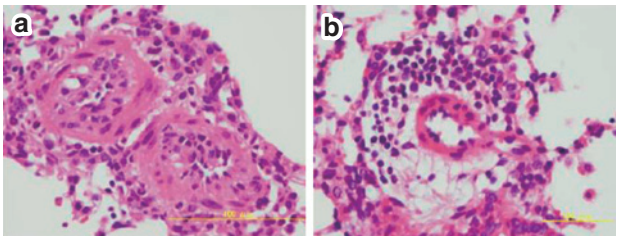

Fig. 5A Effects of rapamycin on allergic pulmonary vascular remodeling. **a**: Totally occluded pulmonary artery by intraluminal myofibroblasts (a yellow arrow) in the OVA-sensitized mice with exposure to OVA in 7th day (hematoxylin-eosin staining). **b**: Intraluminal myofibroblast accumulation was not observed in the OVA-sensitized mice with exposure to OVA and treated with rapamycin in 7th day (hematoxylin-eosin staining). Thickening of vascular smooth muscle cells in vascular wall was also reduced by rapamycin treatment.

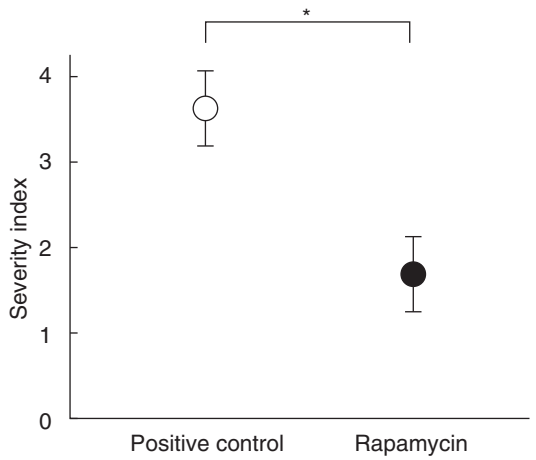

Fig. 5B Effects of rapamycin on severity of vascular changes. Open circles: histological scores of the OVA-sensitized mice with exposure to OVA in 7th day (positive control, n = 6); closed circles: the OVA-sensitized mice with exposure to OVA and treated with rapamycin in 7th day (n = 6). Data are given as mean ± SD. *p < 0.01.

ity index in the rapamycin-treated group mice was significantly reduced compared to that in the positive controls (Fig. 5B). The histological changes were minimal in the control group mice as previously reported (data were not shown).

IMMUNOSTAINING FOR α-SMA ACTIN Ki-67 AND TGF-β1

The myofibroblasts in the intraluminal space of vasculitis were strongly stained with anti-α-SMA in the positive control group mice (a yellow arrow) (Fig. 6A, panel a). On the other hand, positive cells for α-SMA were sparse in the intraluminal space of the pulmonary artery in the rapamycin-treated group mice (Fig.

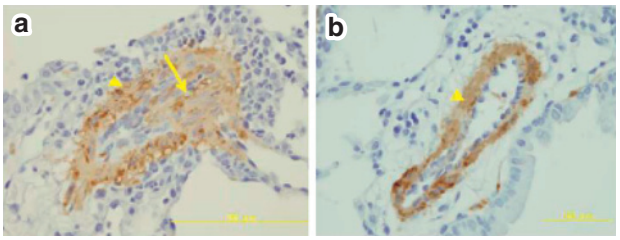

Fig. 6A Immunostaining for α smooth muscle actin (α-SMA). **a**: The intraluminal myofibroblasts in the OVA-sensitized mice with exposure to OVA in 7th day were positively stained with anti-α-SMA (a yellow arrow). Smooth muscle cells in vascular wall were also positive (a yellow arrow head) **b**: Intraluminal myofibroblast accumulation was not observed in the OVA-sensitized mice with exposure to OVA and treated with rapamycin. Only smooth muscle cells in vascular wall were positive (a yellow arrow head).

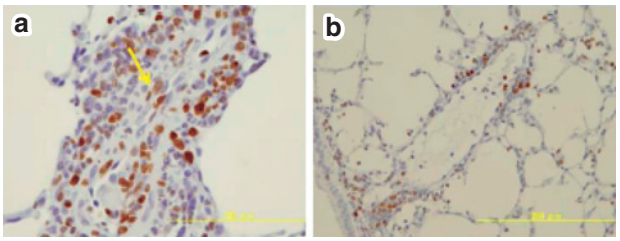

Fig. 6B Immunostaining for Ki-67. **a**: Ki-67 positive cells were seen in the myofibroblasts accumulated intraluminal space of pulmonary artery in the OVA-sensitized mice with exposure to OVA in 7th day (a yellow arrow). **b**: Ki-67 positive cells were very few in the intraluminal space of the OVA-sensitized mice with exposure to OVA and treated with rapamycin on the 7th day.

6A, panel b). The vascular smooth muscle cells were strongly stained for α-SMA in both groups (a yellow arrow head).

Immunohistochemistry for Ki-67 was performed to detect proliferating cells in pulmonary vasculitis.[14] Ki-67 was expressed in intraluminal myofibroblasts (a yellow arrow) and cells in the vascular wall in the positive controls (Fig. 6B, panel a). In contrast, Ki-67 expressing cells were very sparse in the pulmonary vascular tissue of the rapamycin-treated group mice (Fig. 6B, panel b). Semi-quantitative analysis revealed that the intraluminal Ki-67 positive cells on the 7th day were significantly reduced in the rapamycin-treated mice compared with those the positive control mice (Fig. 6C).

Immunostaining for TGF-β revealed that TGF-β was expressed in smooth muscle cells in the vascular wall of pulmonary arteries (a yellow arrow head) and myofibroblasts in the intraluminal space of vasculitis (a yellow arrow) in the positive control (Fig. 6D,

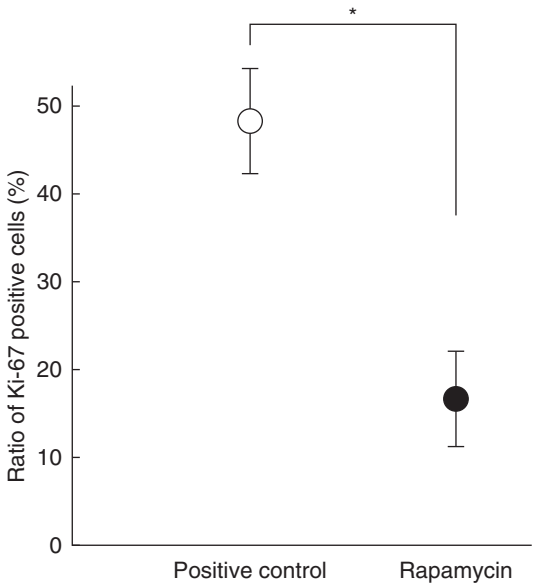

Fig. 6C Effects of rapamycin on the number of Ki-67 positive cells. Open circles: intraluminal Ki-67 positive cells of the OVA-sensitized mice with exposure to OVA in 7th day (positive control, $n = 6$); closed circles: intraluminal Ki-67 positive cells of the OVA-sensitized mice with exposure to OVA and treated with rapamycin in 7th day ($n = 6$). Data are given as mean ± SD. *$p < 0.01$.

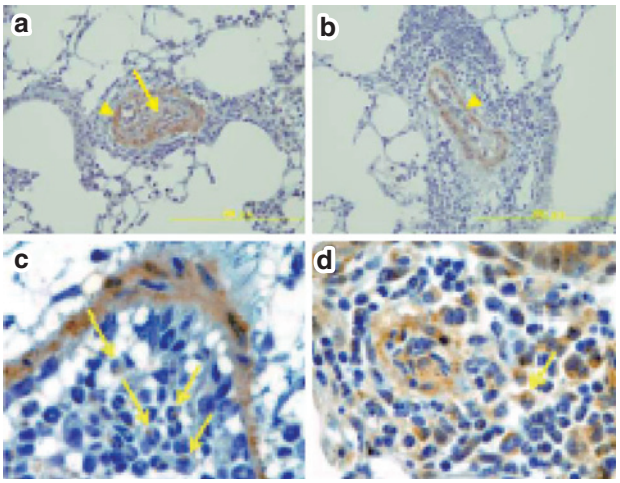

Fig. 6D Immunostaining for TGF-β. **a**: Myofibroblasts occupied intraluminal space of pulmonary artery in the OVA-sensitized mice with exposure to OVA in 7th day and were positively stained with anti-TGF-β 1 (a yellow arrow). Smooth muscle cells in vascular wall were also positive for TGF-β (a yellow arrow head) **b**: Only smooth muscle cells in vascular wall were positive (a yellow arrow head). Intraluminal myofibroblast accumulation was not observed in the OVA-sensitized mice with exposure to OVA and treated with rapamycin. **c**: Eosinophils with horse shoe shaped nuclei in the vessel were not strongly but definitely brown colored (yellow arrows). **d**: Eosinophils with horse shoe shaped nuclei in the perivascular space were stained brown for TGF-β (a yellow arrow). Mononuclear cells in the perivascular space were also stained for TGF-β.

panel a). In contrast, TGF-β positive cells were only smooth muscle cells and not detected in the intraluminal space in the rapamycin-treated group mice (Fig. 6D, panel b). Mesenchymal cells such as myofibroblasts and smooth muscle cells were strongly stained. On the other hand, the inflammatory cells were weakly stained for TGF-β compared with the mesenchymal cells. However, eosinophils with horse shoe shaped nuclei in the vessel were not strongly but definitely brown colorrd (yellow arrows) (Fig. 6D, panel c). In addition, eosinophils with horse shoe shaped nuclei in the perivascular space were stained brown for TGF-β (a yellow arrow) (Fig. 6D, panel d). Mononuclear cells in the perivascular space were also stained for TGF-β (Fig. 6D, panel d).

DISCUSSION

The present study demonstrated that rapamycin ameliorated the histological changes of vasculitis in the pulmonary arteries of the OVA-exposed mouse model of AGA and reduced the number of eosinophils in BALF on the 7th day after OVA inhalation. Rapamycin reduced the TGF-β concentration in the BALF of OVA-sensitized mice after OVA inhalation and the number of TGF-β positive cells in the lesions of vasculitis in the pulmonary arteries.

As previously reported, mTOR is a multifunctional protein involved in the regulation of cell growth, proliferation, and differentiation.

mTOR has been recognized as an essential molecule for the maturation and differentiation of multiple immune cell lineages.[15] Fujitani *et al*. reported that SDR 943, a rapamycin analogue (1 mg/kg) suppressed the eosinophil counts in BALF of BALB/C mice sensitized with OVA after a single inhalation of OVA.[16] Fredriksson *et al*. reported the effects of papamycin on the eosinophil counts in BALF differed depending on the timing of administration in an experimental house dust mite (HDM)-induced asthma model of BALB/c mice.[17] They demonstrated the suppressive effects of rapamycin on eosinophil counts in BALF of mice sensitized with HDM after HDM exposure when rapamycin was administered during the induction period of sensitization. In contrast, 6 weeks after sensitization with HDM, rapamycin did not suppress but rather increased the eosinophil counts in BALF of the sensitized mice after re-exposure to HDM. Thus, consistent with the context-dependent effects of rapamycin on inflammation, they suggested that the timing of mTOR inhibition might be an important determinant of the efficacy and toxicity in HDM-induced asthma. Furthermore, Mushaben *et al*.

reported the effects of rapamycin on allergic asthma model of mice sensitized with HDM.[18] In their report, rapamycin suppressed IgE, goblet cells and total $CD4^+$ T cells in the lung tissue, with no effect on the total inflammatory cell numbers in the BALF, although the IL-4 and eotaxin 1 levels were augmented. Their murine asthma model was a similar to the model in our study except for the murine species and sensitizing allergen used. Total cell numbers in BALF on the 7th day after OVA exposure were not affected by rapamycin in our study, the same as in the report of Mushaben *et al*. In addition, in our study the IL-4 concentration in BALF on the 7th day was not suppressed by rapamycin and was rather higher in the rapamycin-treated group compared with the positive control. Mushaben *et al*. also reported similar results in their murine model. These results suggested that the effects of rapamycin were time-dependent and that some refractory mechanism was involved in the long-term.[18] However we found a significant reduction of eosinophils in BALF on the 7th day after OVA exposure by rapamycin treatment. We speculated that these differences in the eosinophil counts in BALF between the study of Mushaben *et al*. and ours might be, in part, due to the difference in the allergen, the way of sensitization and mouse species. In addition, the time course of the produced cytokines involved in eosinophilopoiesis, eosinophil recruitment and eosinophil activation were thought to play key roles in the accumulation of eosinophils in the BALF.

IL-4, IL-5 and IL-13 are thought to play critical roles in the eosinophilopoiesis, and chemotaxis, survival and activation of eosinophils. Especially, the proliferation and differentiation of eosinophils are known to be regulated by IL-5.[19-25] The IFN-γ concentrations in BALF on the 3rd and 7th day were not influenced by the rapamycin treatment. Therefore, the decrease in the eosinophil number in BALF was thought to be caused mainly by the decreases in Th2 cytokines such as IL-5 and IL-13 by rapamycin. Rapamycin treatment reduced the IgE concentration on the 7th day in our murine model. The reduction in the IgE level might be involved in the suppression of vasculitis by rapamycin. We studied possible Th1 and Th2 cytokines influencing the effects of rapamycin, but IL-17 was not detected in the BALF. Since Th17 is thought to be an important T lymphocyte involved in allergic inflammation and vasculitis, Th17 cytokines need to be studied in the next step.

In our experiments, the concentrations of IL-4, IL-5 and IL-13 in the BALF of the rapamycin-treated mice on the 3rd day after OVA inhalation were markedly reduced compared with those of OVA-exposed mice without rapamicin. In this regard, the decrease of eosinophil counts in the BALF of the rapamycin-treated mice on the 7th day after OVA inhalation was thought to be due to the marked decrease of the concentrations of IL-4, IL-5 and IL-13 in the BALF on the 3rd day after OVA inhalation. These results suggested that rapamycin might suppress the synthesis of IL-4, IL-5 and IL-13 from $CD4^+$ T lymphocytes sensitized with OVA.[26,27]

Eosinophils have been thought to be a major source of TGF-β in allergic airway diseases.[28,29] Several reports demonstrated that the TGF-β produced by eosinophils played an important role in airway remodeling including increased synthesis and deposition of extracellular matrix proteins such as collagens.[30,31] In our study, the eosinophils in the pulmonary vessels and perivascular space were positively stained for TGF-β. In this regard, it was suggested that the decreased number of eosinophils caused the reduction of TGF-β concentration in the lung tissue and BALF of the rapamycin-treated mice with OVA exposure.

TGF-β has been known to be a key molecule involved in tissue fibrosis including subepithelial fibrosis in asthma and pulmonary fibrosis.[29,32,33] In our experimental model, we previously reported the marked increase of TGF-β concentration in the BALF of the OVA sensitized mice after OVA exposure.[34] Present study demonstrated that rapamicin decreased TGF-β concentration in the BALF of the OVA sensitized mice after OVA exposure. Simler NR *et al*. reported rapamycin analogue suppressed bleomycin-induced pulmonary fibrosis.[35] In their reports, rapamycin analogue SDZ RAD reduced collagen accumulation in the lung of rats with pulmonary fibrosis. Concerning the mechanism of collagen reduction by mTOR inhibitor, rapamycin decreased steady-state levels of collagen mRNA *via* phosphatidylinositol 3-kinase-independent pathway.[36]

In this regard, we speculated the mechanism of inhibitory effects of rapamycin on allergic pulmonary vasculitis as following: 1) Rapamcin suppressed production of Th2 cytokines such as IL-4, IL-5 and IL-13 from $CD4^+$ T lymphocytes sensitized with OVA in our experimental murine model. 2) The reduced production of Th2 cytokines especially IL-5 caused the decrease of eosinophils recruited into lung tissue. 3) The decreased number of eosinophil in the lung of the rapamycin-treated mice caused lower level of TGF-β concentration compared with OVA-exposed mice without rapamicin. 4) The vascular remodeling including myofibroblast proliferation and ECM deposition did not progress because of the reduced level of TGF-β concentration in the lung tissue.

In addition, rapamycin inhibited TGF-β induced fibroblast proliferation.[12]

In conclusion, rapamicin attenuated vascular remodeling of murine model with allergic pulmonary vasculitis by reduction of TGF-β production in the lung and inhibition against biological action of TGF-β.

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