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Remodeling of Airway Walls in Fatal Asthmatics Decreases Lymphatic Distribution; Beyond Thickening of Airway Smooth Muscle Layers

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ABSTRACT

Background: We previously reported the phenotypic distribution patterns of airway smooth muscles in fatal asthmatics; Type I asthmatics with smooth muscle bundle thickening only in large airways and Type II in whole airways. We hypothesized that increased smooth muscle bundles in the airway walls would disrupt airway lymphatics to impair airway clearance in these fatal asthmatics.

Methods: The autopsy lungs of seven fatal asthmatics (three Type I, four Type II asthmatics) and five controls were examined by immunohistochemistry to reveal the lymphatics distributed in the airway walls. The total area of lymphatics around each cross-sectioned airway was measured and its airway radius was calculated using an image analyzer system. Finally, the distribution areas of lymphatics in the same level of airways of bronchial trees were compared among Type I, Type II asthmatics and controls.

Results: The total area of airway lymphatics in each lung was found to be positively correlated with the airway radius (R). The distribution areas of lymphatics in larger airways ($1.5 < R < 2.0$ mm) of both types of asthmatics were significantly decreased than controls, and Type I asthmatics contained much less lymphatics than Type II asthmatics in these airways. The lymphatics around smaller airways ($0.5 < R < 1.0$ mm) were also reduced in both phenotypes of asthmatics without statistic difference between them. The airway lymphatics of these fatal asthmatics were observed to be interrupted by thickened muscle bundle layers, and by fibrotic tissues developed around these airways as well.

Conclusions: These results indicate that distribution of lymphatics were decreased in the airway walls of fatal asthmatics which contained muscle bundles and fibro-connective tissues both of which were augmented in these airway walls to disrupt lymphatics, impair airway clearance and accelerate mucosal edema which would cause refractory status of these patients.

KEY WORDS

airway-remodeling, bronchial asthma, lymphangiogenesis, lymphatic capillary, mucosal edema

INTRODUCTION

Despite the emergence of novel therapeutic strategies for patients with bronchial asthma, there remain severe asthmatics who suffer from continuous severe asthmatic attacks and chronic dyspnea. To understand the pathogenesis of refractory conditions of these patients, there is a need to analyze the pathogenesis of the airway remodeling in fatal asthmatics. We previously reported morphometric analysis of thickened airway smooth muscle layers of patients who died of asthma in comparison with normal control lungs,[1] differentiated two distribution patterns of thickening in the airway smooth muscle layers[2] and

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Table 1 The lungs of patients examined; their clinical background and pathologic findings

Clinical background				Pathologic findings			
Patient No.	Age	Gender	Duration of asthma	Muscular thickening		Peri-airway fibrosis	
				Large airways	Small airways	Large airways	Small airways
Type I asthmatics							
1	58 yr	F	10 yr	+++	+/−	++	++
2	63 yr	F	14 yr	+++	−	++	+++
3	71 yr	M	11 yr	+++	+/−	++	++
Type II asthmatics							
4	21 yr	M	7 yr	+++	+++	+	+
5	61 yr	M	5 yr	+++	+++	+	+
6	68 yr	M	6 yr	+++	++	++	+
7	64 yr	F	13 yr	+++	++	++	++

defined the muscular hypertrophy and/or hyperplasia which contributed to the muscular thickening in these phenotypes of fatal asthmatics.[3] These phenotypes of fatal asthmatics consist of Type I asthmatics whose muscle layers were increased only in the larger airways, and Type II, who exhibited thickened muscular layers in the entire airways in bronchial trees, from the central to peripheral regions.[2] These increased muscle layers surrounding the airways are thought to reflect hypersensitive airway constrictions in these patients, which would contribute to airway narrowing by constricting the airways more powerfully and more frequently, making the airway walls become rigid with the excessive airway folding, and causing inflammation by cytokine-secretion.[4] The important role of muscular thickening of the airways in severe asthmatics may be in the process of being re-recognized because of recent reports of successful asthma control by bronchial thermoplasty targeting airway smooth muscles,[5] even though the mechanism underlying the therapeutic effect in asthma has not been fully established yet.

Mucosal edema is also a notorious factor for accelerating severe airway narrowing in bronchial asthma as well as sub-epithelial fibrosis, deposition of matrix around the airways, and mucus plugging with increased tall columnar airway-epithelial cells. Recent studies using mouse models of chronic respiratory tract infection showed that blood vessel remodeling and lymphangiogenesis were both robust in infected airways, and that lack of lymphatic growth exaggerated mucosal edema. These findings suggest that when lymphangiogenesis is impaired airway inflammation may lead to bronchial edema and exaggerated airflow obstruction.[6] As is broadly acknowledged, the lungs possess a highly developed network of lymphatics, consisting of a "superficial" or pleural plexus and a "deep" or peribronchovascular plexus, located in the connective tissue surrounding the airways, pulmonary arteries, and veins.[7] Although the disruption of lymphatics would possibly be an important pathogenesis of mucosal edema in bronchial asthma, no information has been reported on the remodeling of lymphatics in the airway walls of these patients. In this context, the morphological changes in the airways of the fatal asthmatics analyzed in our previous studies[1-3] were re-examined and the remodeling of lymphatics in these airways was evaluated.

METHODS

THE LUNG TISSUES EXAMINED

The lung tissues examined in this study were obtained from the postmortem lungs of seven fatal asthmatics who died of asthma, three Type 1 asthmatics whose muscle layers were increased only in the larger airways and four Type 2 asthmatics who exhibited thickened muscular layers in the entire airways in bronchial trees, both of these phenotypes of fatal asthmatics were distinguished in our previous studies.[2] The clinical backgrounds of these seven fatal asthmatics were shown in Table 1. These lungs were selected from the samples used in the previous studies[2,3] because of the reliable immunoreactivity required for this current study. Five autopsy lungs without chronic lung diseases or cancer were also examined. All of these lung tissues were obtained at Tohoku University Hospital and the approval of the ethics committee of Tohoku University was obtained to use these clinical samples.

IMMUNOHISTOCHEMISTRY

For our pilot study on lymphatic endothelial cells, we employed anti-human podoplanin monoclonal antibody (AngioBio), D2-40 (Nichirei), a rabbit polyclonal antibody against human LYVE-1 (Research Diagnostic), and an antihuman VEGFR-3 goat antibody (R &D). We chose podoplanin for further study for its specific and intense staining. The other monoclonal antibodies used in this study were the antibodies against human CD34 (Nichirei) and von Willebrand factor (vWF) (Nichirei). A rabbit polyclonal antibody against human VEGF-C (IBL) and a goat polyclonal antibody against human VEGF-D (R&D) were also used. The antigen-antibody complex was visualized

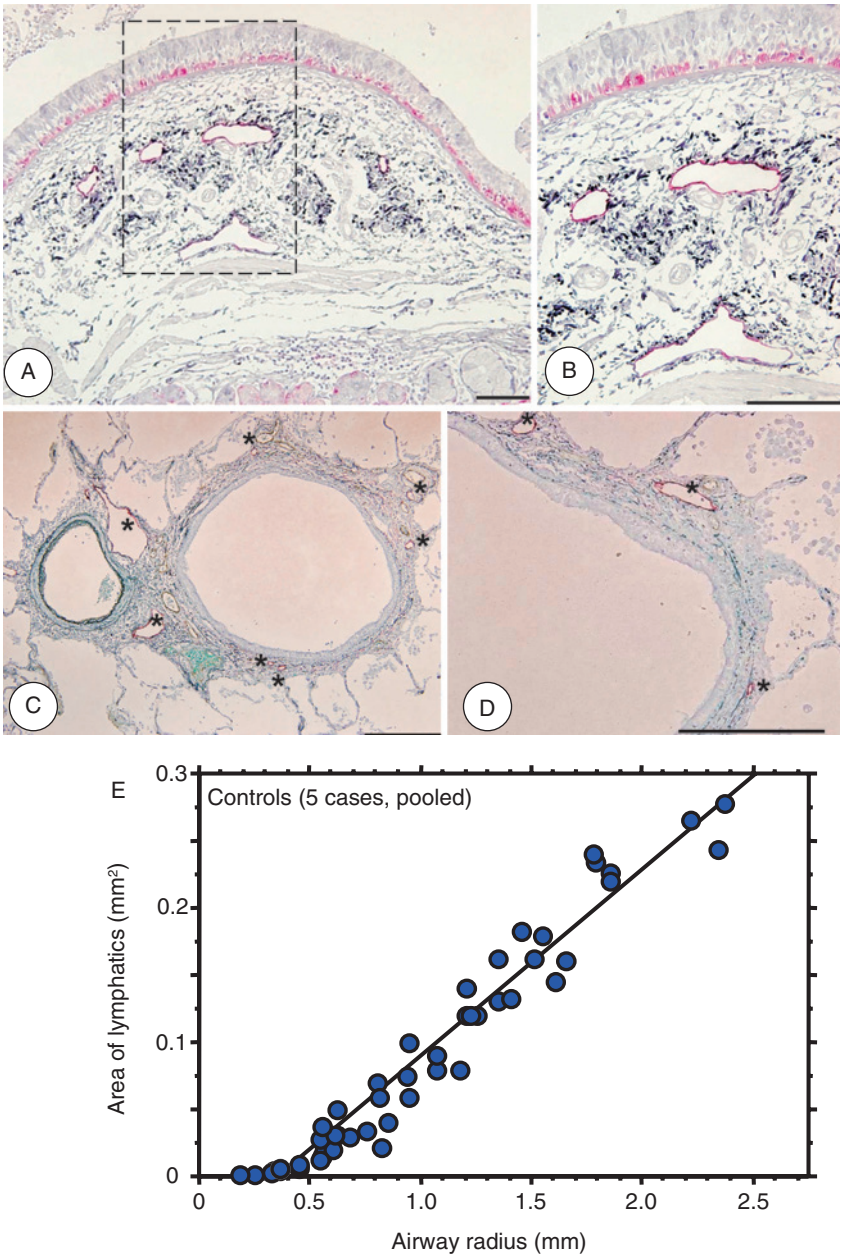

Fig. 1 Lymphatics in the airway walls in control lungs. **A&B**: In the bronchial walls of control lungs, lymphatic capillaries (immunostained in red) are distributed in the sub-mucosal tissues where airway smooth muscles distribute thinly and in the bottom of airway epithelial cells. The boxed area by broken lines in **A** is enlarged in **B** (bars = 100 μm, back stained by E－G). **C&D**: Double immunostaining of lymphatic (in red, marked by*) and vWF-positive capillary endothelial cells (in brown) reveals dense distribution of both capillaries in the walls of a small airway of a control, the radius of which is approximately 0.15 mm (bars = 100 μm, back stained by E－G). **E**: The data of total areas of lymphatics around each airway of five control lungs, 52 airways in total, were pooled.There is a significant correlation between them with a coefficient of $r = 0.952$ ($p < 0.001$).

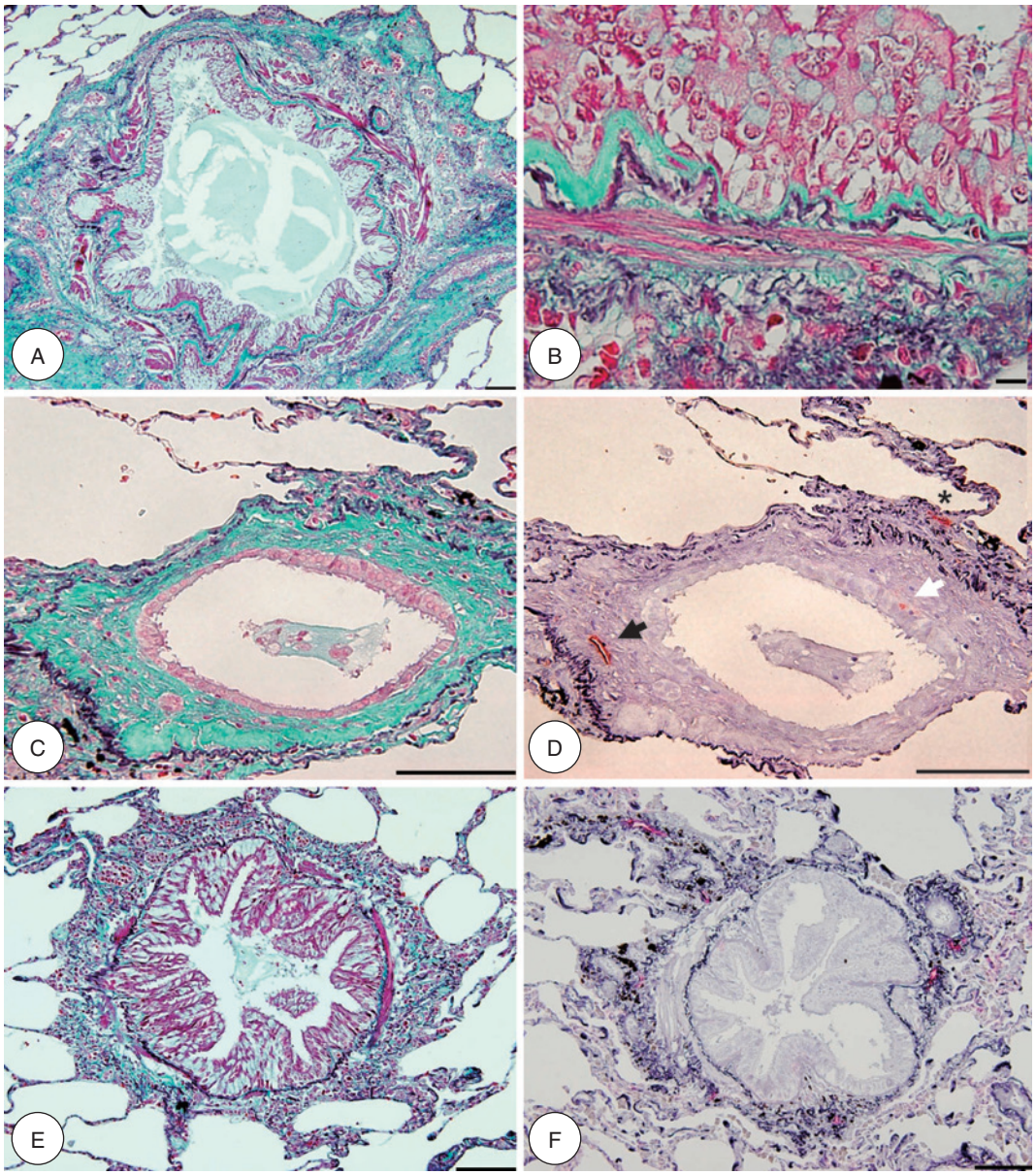

Fig. 2 Lymphatics in the airway walls in Type 1 asthmatics (bars = 100 μm except **B**). **A&B**: A small airway of Type I asthmatics (Case 1) is surrounded by thick fibrotic tissues with atrophic small muscle bundles (**A**, EM staining) with fibrotic changes in part (**B**, E－G staining, bar = 10 μm). **C&D**: A small airway of Type I asthmatics (Case 2) surrounded by thick fibrotic tissues where no airway smooth muscles are observed (**C**, E－G staining). In the consecutive section (**D**), double immunostaining of lymphatic (in red, marked by＊) and vWF-positive capillary endothelial cells (in brown, indicated by arrows) shows scarce distribution of lymphatics, though vWF ＋ blood vessels penetrated the fibrotic tissues into epithelial cells (indicated by a white arrow). **E&F**: A small airway of Type I asthmatics (Case 3) were also surrounded by thick fibrotic tissues without muscle layers (**E**, E－G stain) and few lymphatics (in red) were distributed in the airway walls (**F**).

by Vector Red (Vector Laboratories) and/or diaminobenzidine (DAB), and counterstained by elastica-Goldner (E-G) stain, modification elastica-Mason stain.[1]

MORPHOMETRIC EVALUATION

The total distribution areas of the lymphatics in airway walls and the perimeter of each airway were measured by a digital image analyzer (Lumina Vision, Mitani Corporation, Fukui) at the final magnification

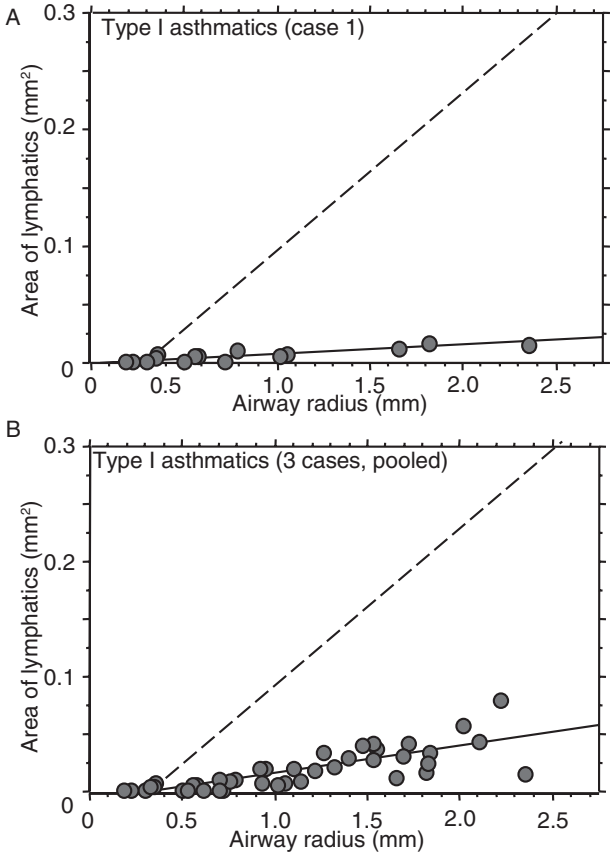

Fig. 3 The distribution of airway lymphatics in Type I asthmatics. **A**: The data of total areas of lymphatics around each airway of Type I asthmatics (Case 1), 19 airways in total, were plotted ($r = 0.752$, $p < 0.001$). In comparison with the regression line of 5 control lungs, revealed as a broken line, the lymphatic distribution in the airway walls of this case is significantly decreased. **B**: The pooled data of the lymphatics around each airway of 3 cases of Type I asthmatics, 44 airways in total, were plotted. Although the data are dispersed, there is a positive correlation between them ($r = 0.656$, $p < 0.001$). Note again the lymphatic distribution around airways of these patients is decreased in comparison with the regression line in controls (a broken line).

of 40,000. The radius of each airway was calculated from its perimeter.

STATISTICAL ANALYSIS

For analysis of two unpaired samples, the nonparametric Mann-Whitney U test was used (Stat View ver.5; Abacus Concepts Inc., Berkley, California). Significant difference was defined as $p < 0.05$ and no significant difference was determined by Student T test. All values were represented as the means ± SEM.

RESULTS

AIRWAY LYMPHATICS IN CONTROLS

We examined first to show the distribution of lymphatic capillary endothelial cells in airway walls of control lungs (Fig. 1). From large to small airways, lymphatics are diffusely distributed in the airway walls of these control lungs. In larger airways, the lymphatics are dilated and observed clearly in the submucosal tissues and also in the bottom of airway epithelial layers (Figs. 1A, B). Even in the small airways, they are distributed close to airway lumen (Figs. 1C, D). The regression line ($y = -0.05 + 0.139x$) was obtained from the pooled data of 52 airways and there was found to be a close positive correlation between the total areas of airway lymphatics and the radius of each airway in these control lungs ($r = 0.952$, $p < 0.001$) (Fig. 1E).

AIRWAY LYMPHATICS IN TYPE I ASTHMATICS

Type I asthmatics were revealed to have thickening of airway smooth muscles chiefly in larger airways by our previous study.[1] The autopsy lungs of three patients of Type I asthmatics (Table 1) were examined. Although the airway smooth muscle bundles in the peripheral airways were not thick, the peri-airway fibrosis was found to be increased in these lungs (Fig. 2). In the lung tissues of Case 1, muscle bundles were observe to be atrophic in the thick fibrotic tissues and changed in part into fibrosis (Figs. 2A, B). The airway walls in the lung tissues of Case 2 were completely changed to fibrosis where only small blood vessels, but not lymphatics, penetrated into airway epithelial layers (Figs. 2C, D). The small airways of Case 3 of Type I asthmatics were also changed to fibrosis, and few lymphatics were recognized surrounding the airways (Figs. 2E, F). Figure 3A shows relation between lymphatic areas and each airway radius in Case 1 of Type I asthmatics. The airway lymphatics are remarkably decreased in Type I asthmatics in comparison with controls. This tendency was also confirmed by the regression line ($y = -0.007 + 0.023x$) from the pooled data of three cases of Type I asthmatics (Fig. 3B).

AIRWAY LYMPHATICS IN TYPE II ASTHMATICS

In the large bronchi of Type II asthmatics (Table 1), where the airway smooth muscle layers are thick (Fig. 4A), the distribution of lymphatics in the airway walls are attenuated in comparison with control lungs (Figs. 4B–D). Lymphatics with penetrating basement membranes into epithelial layers are rarely observed and were interrupted by thick muscle layers surrounding the constricted airways (Figs. 4B–D). In contrast, the blood capillaries were densely distributed even in the protruded fold of the airway walls with thick connective tissues and elastic fibers and these blood capillaries extended over sub-epithelial fi-

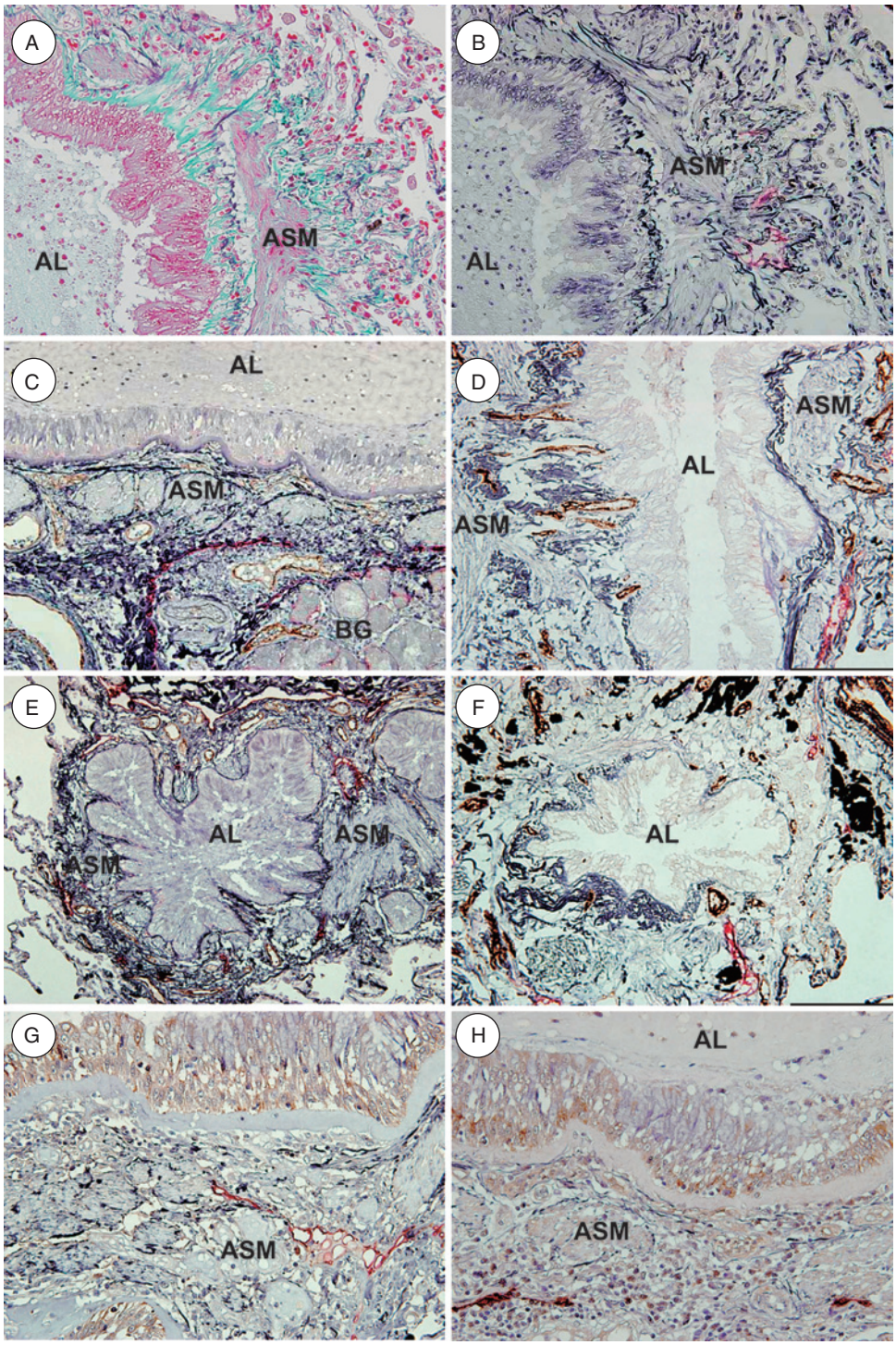

Fig. 4 The distribution of airway lymphatics in Type II asthmatics. **A**－**B**: The airway walls in controls stained by EM are followed by a serial section immunochemically stained for lymphatics (in red) in **B**. **C**－**F**: The airway walls in bronchi (**C**－**D**) and bronchioles (**E**－**F**) in double immunohistochemistry stain for podoplanin with lymphatic capillary endothelial cells appeared in red and blood vessels with vWF-positive (in brown) endothelial cells. Note the root of lymphatics toward airway lumen are blocked and changed by thick muscular mass (**B**－**D**). **G**－**H**: Double immunohistochemistry stain for lymphatic growth factors VEGF-C (**G**) or VEGF-D (**H**) in brown and podoplanin-positive lymphatic capillary endothelial cells in red (ASM, airway smooth muscles; AL, airway lumen; BG, bronchial glands).

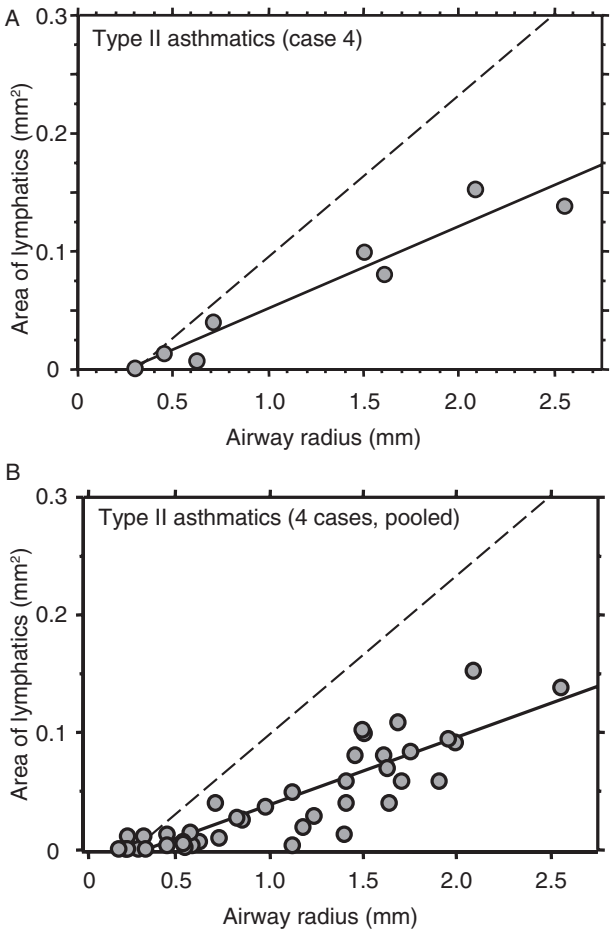

Fig. 5 The distribution of airway lymphatics in Type II asthmatics. **A**: The data of total areas of lymphatics around each airway of Type II asthmatics (Case 4), 8 airways in total, were plotted ($r = 0.928$, $p < 0.001$). In comparison with the regression line of 5 control lungs, revealed as a broken line, the lymphatic distribution in the airway walls of this case is decreased. **B**: The pooled data of the lymphatics around each airway of 4 cases of Type II asthmatics, 42 airways in total, were plotted. Although the data are dispersed among the cases, there is again a positive correlation between them ($r = 0.768$, $p < 0.001$). The lymphatic distribution around airways of these patients is decreased in comparison with the regression line in controls (a broken line).

brosis and were distributed within the airway epithelial layers (Figs. 4D, E). This different distribution patterns between lymphatic and blood capillaries are also observed in the small airways of Type II asthmatics, whereas only Type II asthmatics contained thick muscle layers (Figs. 4E, F). The airway epithelial cells of these fatal asthmatics, from both the large to the small airways, were intensely immunoreactive for the lymphatic growth factors VEGF-C and VEGF-D (Figs. 4G, H). However, the extension of new lymphatics into these airway epithelial layers seemed to be interrupted by thick muscle layers and connective tissues, including sub-epithelial fibrosis (Figs. 4G, H).

Relation between lymphatic areas and each airway radius in Case 4 of Type II asthmatics shows airway lymphatics were less distributed than controls but more than Type I asthmatics (Fig. 5A), as confirmed by the regression line ($y = -0.019 + 0.058x$) of the pooled data of four cases of Type II asthmatics (Fig. 5B).

STATISTIC ANALYSIS OF LYMPHATIC DISTRIBUTIONS IN THE AIRWAY WALLS

Figure 6A shows all the pooled data of lymphatic areas in the airway walls of Type I (44 airways in 3 cases), Type II asthmatics (42 airways in 4 cases), and controls (52 airways in 5 cases) plotted together. The mean value of lymphatic areas of each case in smaller airways ($0.5 \text{ mm} < R < 1 \text{ mm}$) and in larger airways ($1.5 \text{ mm} < R < 2.0 \text{ mm}$) were calculated (Fig. 6B). The airway lymphatics in both Type I ($0.008 \pm 0.004 \text{ mm}^2$ in smaller airways, $0.028 \pm 0.007 \text{ mm}^2$ in larger ways) and Type II asthmatics ($0.016 \pm 0.005 \text{ mm}^2$ in smaller airways, $0.080 \pm 0.004 \text{ mm}^2$ in larger ways) were decreased in the whole airways, in comparison with controls ($0.042 \pm 0.004 \text{ mm}^2$ in smaller airways, $0.191 \pm 0.017 \text{ mm}^2$ in larger ways), and especially Type 1 asthmatics decreased lymphatics more than Type II asthmatics in larger airways.

DISCUSSION

Although airway hyper-reactivity is one of the most characteristic features of bronchial asthma, irreversible and chronic constrictions of the airway are thought to advance mild asthma to refractory status. In this sense, the postmortem lungs of fatal asthmatics have provided important information on airway remodeling in these patients, much of which is different from the pathological features observed in the biopsied materials of ordinary, mild asthmatics. Because of the diversity in prognosis among the phenotypes of these asthmatics, it is important to determine the pathogenesis which differentiates these phenotypes in a range from mild to fatal, and invent new strategies for early diagnosis and subsequently successful treatment of refractory asthmatics. We should identify the genetic predisposition and susceptibility of these patients, which are now profoundly believed to underlie the pathogenesis of bronchial asthma, as in various other diseases.

Among the structural changes reported in the airways of asthmatics are included epithelial injury, goblet cell hyperplasia, enlarged submucosal mucus glands, angiogenesis, increased deposition of extracellular matrix in the airway wall, and increased airway smooth muscle layer. The importance of airway smooth muscle involvement should be re-evaluated because of the recent report on successful asthma control by bronchial thermoplasy targeting the air-

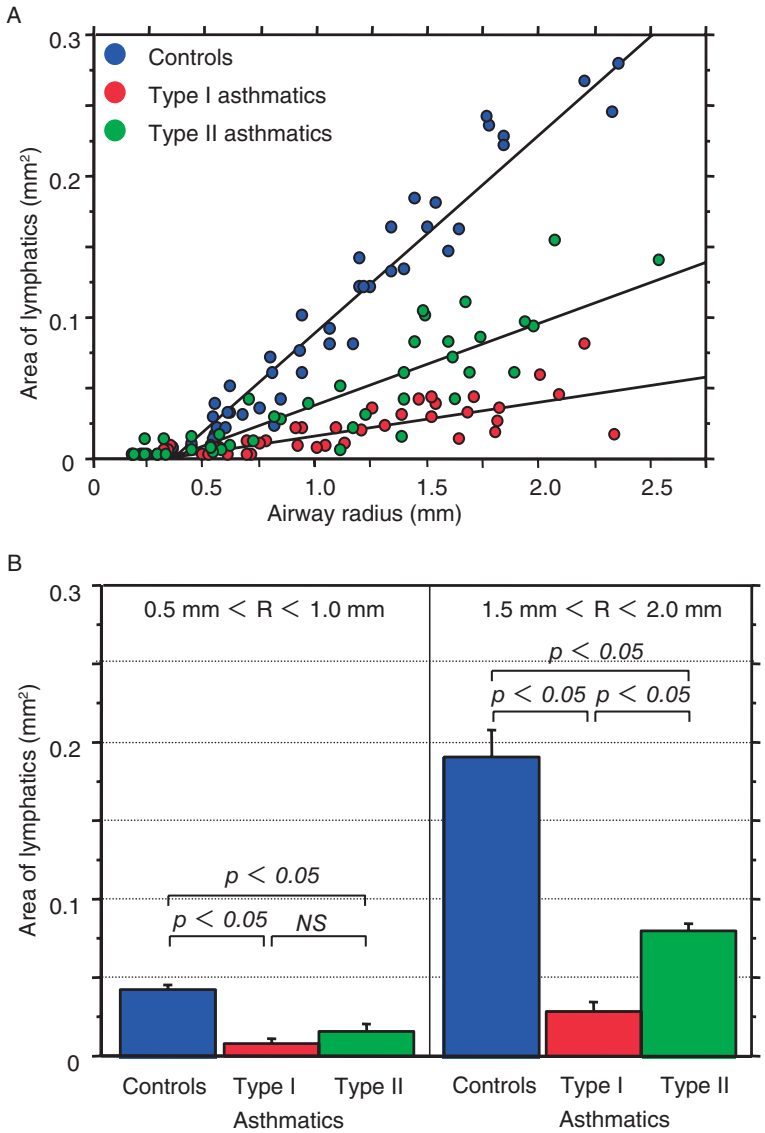

Fig. 6 Comparison of lymphatic distributions in the airway walls among Type I, Type II asthmatics, and controls. **A**: All of the pooled data of lymphatic areas in the airway walls of Type I (3 cases), Type II asthmatics (4 cases), and controls (5 cases) were plotted together. The regression lines of these three groups were also compared. **B**: For statistic analysis, the mean value of lymphatic areas of each case in smaller airways (0.5 mm < R < 1 mm) and in larger airways (1 mm < R < 1.5 mm) were calculated and the means ± SEM of each group in these rages of airways were plotted. The airway lymphatics in both Type I and Type II asthmatics were decreased in the whole airways, and especially Type 1 asthmatics decreased lymphatics more than Type II asthmatics in larger airways.

way smooth muscle,[5] although the mechanism underlying this effect in asthma as not been fully established yet.

Our results in this study indicate the reduced distribution of lymphatics in the airway walls of fatal asthmatics where the thick muscle layer and/or fibroconnective tissue develop. In contrast, the vascular densities are augmented in these airway walls, even within the epithelial layers. Considering that the mucosal edema induces airway narrowed, the reduced distribution in lymphatics in the airway walls of fatal asthmatics are thought to cause impairment of lym-

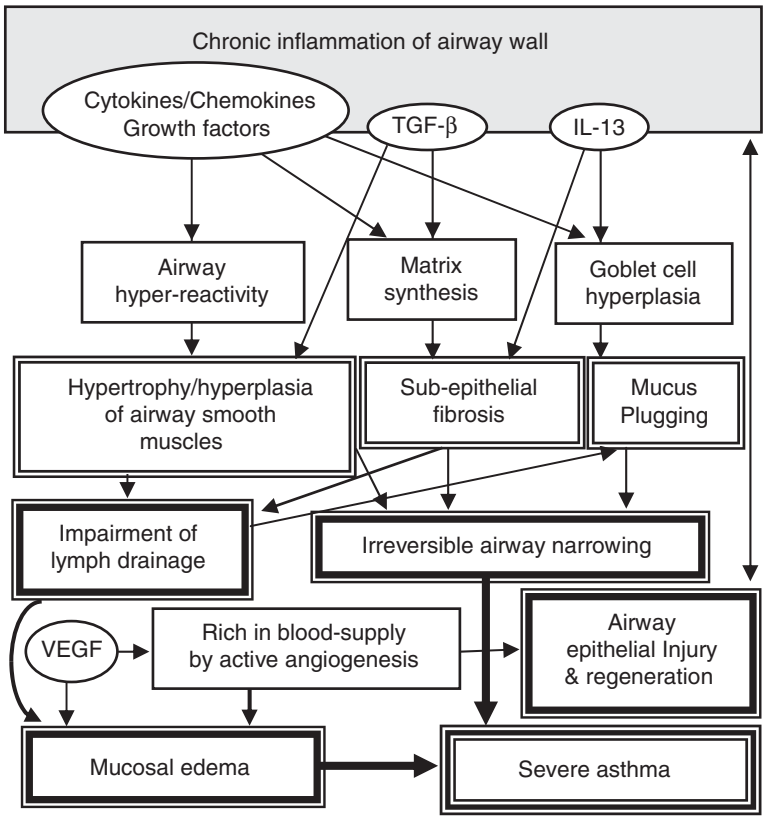

Fig. 7 Scheme of hypothetic roles of lymphatic disruption in the pathogenesis of refractory asthma

phatic drainage of the airway epithelial layers in these lungs. The increased blood capillaries in these lesions may be required for maintaining the airway remodeling, which is composed of hyperplasia of goblet and airway epithelial cells, along with muscular mass, but these thick airway walls with sub-epithelial fibrosis prevent the development of lymphatic capillaries by blocking the permeation of lymphatic growth factors, VEGF-C and/or D, which are produced by airway epithelial cells.[6] The hypothesis of reduced lymphatics in the airway walls of fatal asthmatics and the influence on the pathogenesis of this refractory disease should be clarified in future study.

The hypothetical roles of disruption of airway lymphatics in the pathogenesis of refractory asthma are shown schematically in Figure 7. The airway epithelial injury and apoptosis induced by chronic inflammation with and without allergic reaction induce type 2 cytokine-dominant inflammation, which induces hypertrophy and hyperplasia of airway smooth muscle by recurrent contraction and the activity of growth factors. IL-13, one of the important cytokines for airway remodeling, differentiates ciliated epithelial cells to goblet cells,[8] which comprise the mucus plugs in the airways.[9] TGF-β is produced as an anti-inflammatory factor against excessive inflammation in the tissues,[10] which differentiates fibroblasts into myofibroblasts which produce matrix protein around the airways and thus generate sub-epithelial fibrosis. These thick airway walls composed of muscular bundle layer and fibro-connective tissues interrupt the extension of lymphangiogenesis to the epithelial layers, where extensive angiogenesis takes place to support the growth of goblet cell hyperplasia and mucus secretion. The unbalanced distribution of lymphatics and blood capillaries, with reduced lymphatics and increased blood capillaries, prolongs the mucosal and peri-airway edema which accelerates airway obstruction and ultimately leads to fatality in severe asthmatics.

In conclusion, the impairment of airway lymphatics in the fatal asthmatics discovered first in this study should be a novel target of therapeutic strategies against this refractory airway disease.

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REFERENCES

1. Ebina M, Yaegashi H, Takahashi T, Motomiya M, Tane-

mura M. Distribution of smooth muscles along the bronchial tree. A morphometric study of ordinary autopsy lungs. *Am. Rev. Respir. Dis.* 1990;**141**:1322-1326.
2. Ebina M, Yaegashi H, Chiba R, Takahashi T, Motomiya M, Tanemura M. Hyperreactive site in the airway tree of asthmatic patients revealed by thickening of bronchial muscles. A morphometric study. *Am. Rev. Respir. Dis.* 1990;**141**:1327-1332.
3. Ebina M, Takahashi T, Chiba T, Motomiya M. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am. Rev. Respir. Dis.* 1993;**148**:720-726.
4. Panettieri RA Jr. Airway smooth muscle: immunomodulatory cells? *Allergy Asthma Proc.* 2004;**25**:381-386.
5. Cox G, Thomson NC, Rubin AS *et al*. Asthma Control during the Year after Bronchial Thermoplasty. *New Eng. J. Med.* 2007;**356**:1327-1337.
6. Baluk P, Tammela T, Ator E *et al*. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J. Clin. Invest.* 2005;**115**:247-257.
7. Lauweryns JM, Baert JH. Alveolar clearance and the role of the pulmonary lymphatics. *Am. Rev. Respir. Dis.* 1977; **115**:625-683.
8. Tyner JW, Kim EY, Ide K *et al*. Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 transdifferentiation signals. *J. Clin. Invest.* 2006;**116**: 309-321.
9. Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 1992;**101**:916-921.
10. Ebina M. TGF-β in pulmonary fibrosis. In: Jakowlew SB (ed.). *TGF-β in Cancer Therapy. Part III TGF-β in inflammation and fibrosis*. New Jersey: Humana Press, 2007;569-578.