

ORIGINAL ARTICLE

Remodelling of nasal mucosa in mild and severe persistent allergic rhinitis with special reference to the distribution of collagen, proteoglycans, and lymphatic vessels

T. H. Kim¹, J. Y. Lee², H. M. Lee¹, S. H. Lee¹, W. S. Cho³, Y. H. Ju¹, E. H. Park¹, K. W. Kim¹ and S. H. Lee¹

¹Department of Otorhinolaryngology-Head & Neck Surgery, College of Medicine, Korea University, Seoul, South Korea, ²Department of Otorhinolaryngology-Head & Neck Surgery, College of Medicine, Soonchunhyang University, Bucheon Hospital, Geonggi-do, South Korea and ³Department of Otorhinolaryngology-Head & Neck Surgery, College of Medicine, Bundang JeSaeng Hospital, Geonggi-do, South Korea

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Summary

Background Small leucine-rich repeat proteoglycans (decorin, biglycan, and lumican), collagen, and lymphangiogenesis are involved in tissue remodelling of various organs with inflammatory diseases.

Objective We determined the expression level and the distribution pattern of small leucine-rich repeat proteoglycans (decorin, biglycan, and lumican), collagen and lymphatic vessels in healthy, mild, and severe persistent allergic nasal mucosa.

Methods The distribution pattern of collagen, proteoglycans, and lymphatic vessels in healthy, mild, and severe persistent allergic nasal mucosa was evaluated by the van Gieson staining, immunohistochemistry, RT-PCR, and Western blotting. Quantitative analyses of collagen deposition were calculated as the median of the total percentage area in the tissue specimen. For the evaluation of proteoglycans, the percentage area stained and median optical density were measured for each image. Lymphatic vessels were identified by D2-40 antibody and calculated using the lymphatic vessel density and endothelial length density in tissue specimens. The expression of MMP 2 and 9, TIMP1 and 2 was evaluated with RT-PCR and Western blotting.

Results In mild and severe persistent allergic nasal mucosa, compared with healthy nasal mucosa, collagen showed more intense staining in the superficial and submucosal layer. In healthy and allergic nasal mucosa, decorin was lightly stained without significant differences in the percentage area and optical density of staining. However, lumican and biglycan showed strong immunoreactivity in mild and severe persistent allergic nasal mucosa, which was verified by Western blotting. The number and endothelial length density of lymphatic vessels were increased in mild and severe persistent allergic nasal mucosa compared with healthy nasal mucosa. The expression of MMP 9 was increased in severe persistent allergic rhinitis.

Conclusion and Clinical Relevance These results suggest that the altered distribution pattern of collagen, proteoglycans, and lymphatic vessels could potentially modulate the remodelling of nasal mucosa in mild and severe persistent allergic nasal mucosa.

Keywords allergic nasal mucosa, biglycan, collagen, D2-40, decorin, lumican, lymphangiogenesis, MMP, TIMP

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Correspondence:

Sang Hag Lee, Department of Otorhinolaryngology-Head & Neck Surgery, College of Medicine, Korea University, 126-1, 5 Ka, Anam-Dong, SungBuk-Ku, Seoul, 136-705, South Korea. E-mail: sanghag@kumc.or.kr

Introduction

Allergic rhinitis is a chronic inflammatory disease of the nasal airways and is characterized by typical nasal symptoms, which include itching, sneezing, rhinorrhoea, and

obstruction. In particular, nasal obstruction constitutes the most common symptom resulting from allergic inflammation, and may be considered the key symptom in patients with allergic rhinitis [1]. Recent studies have demonstrated that the reversibility of the decongestion

test is lower in patients with allergy to perennial allergens, and tends to become stiff because of nasal airway remodelling that causes an impaired response to decongestive drug [2, 3].

It is known that allergic nasal obstruction caused by turbinate hypertrophy is produced by inflamed and swollen mucosa, with engorgement of sinusoidal capacitance vessels, rhinorrhoea, and increased mucus production [4]. However, some recent reviews have analysed the features of airway remodelling in allergic rhinitis and concluded that airway remodelling exists in rhinitis, but it seems to be far less extensive than in asthma [5, 6]. These results suggested that turbinate hypertrophy in allergic rhinitis may be caused by remodelling of nasal mucosa [7]. Nevertheless, greater attention has not been given to the evaluation of structural changes within the upper airways in allergic rhinitis.

Airway remodelling is a dynamic process of extracellular matrix (ECM) deposition and degradation in response to an insult, leading to the reconstruction of damaged tissue [8]. ECM tissue embodies the following two families of macromolecules: (1) those that provide a fibrous scaffolding of the lamina propria, namely collagen and elastins; and (2) those that provide the filling around the fibrous scaffolding. This latter family, in turn, contains two macromolecular groups: the proteoglycans and the structural glycoproteins. Proteoglycans are composed of a protein core and glycosaminoglycans side chains that have been implicated in the maintenance of the biophysical properties of many tissues, in the assembly of collagen fibrils, and in tissue regulation of water balance. They influence cell migration, act as growth supporters, and influence vessel permeability. Different subclasses of proteoglycans have been described, including small leucine-rich proteoglycans, which are composed of decorin, biglycan, and lumican. All members of these small proteoglycan families interact with fibrillar collagen and may influence the interaction of collagen fibrils with other components of the ECM that participate in its maintenance [9]. The expression of these proteoglycans was described in several human tissues and their levels of expression were altered under various pathological conditions, such as asthma [10–13]. Further, matrix metalloproteinases (MMPs) are known to be important in normal remodelling processes. Overexpression and activation of MMPs or an imbalance of active MMPs and tissue inhibitors of metalloproteinases (TIMPs) has been linked with a number of specific disease states associated with the breakdown and remodelling of the ECM [14, 15]. In this regard, it can be postulated that the expression level of these small proteoglycans associated with an imbalance of MMPs and TIMPs are involved in the remodelling of nasal mucosa of patients with allergic rhinitis. However, little attention has been paid to the expression of these proteoglycans in nasal mucosa of allergic rhinitis.

In addition, it is known that an increased vascularity due to angiogenesis is an important part of remodelling in a variety of pathological process, including asthma [16–18]. Recently, an immunohistochemical evaluation of the nasal mucosa of patients with allergic rhinitis also revealed an abundance of blood vessels as well as an increased expression of angiogenic factors, and suggested that increased angiogenesis may play an important role in the remodelling of nasal mucosa of subjects with allergic rhinitis [19, 20]. On the other hand, a recent immunohistochemical study indicated the reduced distribution of lymphatics in the airway walls of fatal asthmatics [21]. Therefore, to understand the pathogenesis of allergic rhinitis, there is a need to analyse the pattern of distribution of lymphatics in nasal mucosa of patients with allergic rhinitis.

In this study, in order to elucidate the role of these small proteoglycan families and lymphangiogenesis in the remodelling of nasal mucosa in allergic rhinitis, we determined the level of expression and pattern of distribution of collagen, small-proteoglycan families (decorin, lumican, and biglycan), and lymphatics in healthy, mild, and severe persistent allergic nasal mucosa.

Methods

Subjects

Thirty patients with mild persistent allergic rhinitis, 30 patients with severe persistent allergic rhinitis, and 30 healthy control subjects were enrolled in this study. Patients undergoing augmentation rhinoplasty were considered healthy control subjects and were excluded if they had a history of nasal infection or allergy, smoking, or ongoing drug treatment. All patients with allergic rhinitis had typical symptoms of perennial nasal allergy, nasal eosinophilia, and a positive skin test or positive RAST to house dust mites, as well as positive reactions on provocation tests. In addition, serum total IgE (IU/mL) was determined by ELISA method (CAP system). The detectable range of the assay for total IgE is 2–2000 IU/mL. Allergic patients with exacerbation of upper respiratory infection during the 6 weeks preceding the study were not included in the study. Other exclusion criteria included current smoking, a medical history of asthma and recurrent infections of the respiratory tract, and use of topical or oral corticosteroids, leukotriene modifiers, and antihistamines during the 8 weeks that preceded the study.

Thirty pieces of normal nasal mucosa were obtained from the inferior turbinate of 30 healthy control subjects (22 men and eight women; age range, 20–44 years) who had normal nasal mucosa and were admitted for augmentation rhinoplasty. A mucosal biopsy sample was largely taken about 2 cm posterior to the anterior edge of the

inferior turbinate using a Blakesley forceps with a cup diameter of 4×6 mm.

Sixty patients with persistent allergic rhinitis were classified according to the disease severity and the severity of rhinitis was classified according to the allergic rhinitis and its impact on asthma report as mild or moderate–severe [22]. Patients with moderate–severe rhinitis had one or more of the following symptoms: sleep disturbance, impairment of daily activities, leisure and/or sports, and impairment of school or work, or troublesome symptoms. Patients with mild rhinitis had none of these symptoms.

Nasal biopsy specimens were also obtained from the inferior turbinate of all patients with mild (22 men and eight women; age range, 24–47 years) and severe persistent allergic rhinitis (20 men and 10 women; age range, 22–45 years) using a Blakesley forceps with a cup diameter of 4×6 mm under local anaesthesia. A mucosal biopsy sample was also taken about 2 cm posterior to the anterior edge of the inferior turbinate. Before obtaining the tissue specimens, the protocols and informed consent form were approved by the institutional review boards for human beings at our institution, and all participants gave written informed consent.

All tissue samples were divided into two portions. One portion was frozen in liquid nitrogen, and stored at –70 °C for RNA and protein isolation. For immunohistochemistry, another sample was immersed overnight in a freshly prepared fixative containing 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. All samples were then dehydrated in a graded series of ethanol to xylene and embedded in paraffin wax.

Total RNA isolation, reverse transcriptase-polymerase chain reaction and semiquantitative reverse transcriptase-polymerase chain reaction

Total RNA (1 µg) from each sample was reverse transcribed in 20 µL of a reaction mixture containing 2.5 U of Moloney murine leukaemia virus reverse transcriptase (RT; Gibco BRL, Grand Island, NY, USA) and 50 pmol of random hexanucleotides at 42 °C for 60 min. RNA integrity and the success of the reverse transcription reaction were monitored by PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. Negative controls consisted of the omission of the RT enzyme from the cDNA synthesis for each specimen. The primer sequences of each gene used in the study are listed in Table 1. Amplified PCR products were resolved in 2% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light. The identity of each PCR product was confirmed by sequencing and found to be identical with the mRNA sequence of each gene as deposited in the National Center for Biotechnology Information database.

Table 1. Sequences of PCR primers

Primer	Size (bp)	Sequence
MMP 2	225	S: 5'-AGATCTTCTTCTCAAGGACCGGTT-3' AS: 5'-GGCTGGTCAGTGGCTTGGGGTA-3'
MMP 9	209	S: 5'-GCGGAGATTGGGAACCACTGTA-3' AS: 5'-GACGCGCTGTGTACACCCACA-3'
TIMP 1	168	S: 5'-CATCTGTGTGTGTGTGGCTGAT-3' AS: 5'-GTCATCTTGATCTCATAACGCTGG-3'
TIMP 2	155	S: 5'-CTCGTGGACGTTGGAGGAAAGAA-3' AS: 5'-AGCCCATCTGTTACCTGTGGTTCA-3'
Decorin	455	S: 5'-CTGTGCTCA CTGTATTGTG G-3' AS: 5'-ATTAGGTGGCACTGTCTCTC-3'
Lumican	482	S: 5'-TAAACCACAACAACCTGACA-3' AS: 5'-ACCTCCAGGTAATAGTT-3'
Biglycan	357	S: 5'-ACACAC CGGACAGATA GA-3' AS: 5'-CTCTTTGGGACAGACTT-3'
GAPDH	502	S: 5'-ATCTTCCAGGAGCGA GATCC-3' AS: 5'-ACCACTGACACGTTGGCAGT-3'

PCR, polymerase chain reaction; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Semi-quantitative RT-PCR was performed on the samples to assess the differential expression of each gene mRNA in normal and allergic nasal mucosa. The optimum number of PCR cycles for each cDNA species was determined by plotting the PCR product yield of different cycles on a semilogarithmic graph and the cycle number representing exponential amplification was chosen for the final amplification. For quantification of the PCR products of all samples, the expected bands were analysed by densitometry and the data expressed as the ratio of each gene to the corresponding GAPDH cDNA.

Collagen staining and analysis

Collagen was stained with van Gieson stain using a modification of the technique. In brief, 4-µm-thick, paraffin-embedded sections were dewaxed and rehydrated in PBS. The nuclei were attained with 5 min in Weigert's iron haematoxylin and for another 5 min in van Gieson stain. The sections were then dehydrated in alcohol gradients and mounted. Using this technique, collagen fibres could be identified by their characteristic red staining.

Collagen deposition was measured in the total submucosal area, which included the subepithelial basement membrane and deeper submucosal areas. Two sections and four random fields per section were analysed for each subject, by two independent observers blinded to the clinical status of the samples. For the quantification of collagen deposition, free software (Image J 1.30; National Institutes of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov>) was used to estimate the volume fraction of deposited collagen within the tissue sample. Images were converted from red, green, and blue (RGB) colour to grey

scale RGB stack, then differentiated into three grey scale images representing red, green, and blue. The grey scale is 0 (black) to 255 (white). For analysis, green–grey scale images were used. The threshold function of Image J automatically and selectively identified the collagen staining at a predetermined density. This option allows the visualization of selected grey level values within the region of interest between 0, which represents white, and 255, which represents pure black. The threshold for each image was determined separately and uniformly to allow the complete selection of collagen deposition while leaving the non-labelled area unselected. The submucosal area was outlined by the threshold function. The absolute area of collagen and the corresponding submucosal area were then measured and saved in the computer for each measurement. Collagen deposition in the total submucosal area was expressed as the total submucosal collagen area/total submucosal area, which was expressed as a percentage area. Quantitative analyses of collagen deposition for each patient were calculated as the median of the total percentage area in the tissue specimen.

Immunohistochemical analysis for proteoglycans

Immunohistochemical staining was performed using a peroxidase-labelled streptavidin–biotin technique. Briefly, paraffin sections (4 µm) were cut and placed on poly-L-lysine-coated glass slides. Sections were treated with 0.03% H₂O₂ in methanol for 15 min to quench endogenous peroxidase activity. After washing in PBS, sections were then incubated overnight at 4 °C with a 1 : 100 dilution of anti-decorin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), a 1 : 250 dilution of anti-lumican antibody (Santa Cruz Biotechnology Inc.), and a 1 : 200 dilution of anti-biglycan antibody (Santa Cruz Biotechnology Inc.). The colour was developed using 3,3'-diaminobenzidine.

Areas of nasal mucosa showing the immunopositivity were evaluated using a computerized scoring method. Two sections and four random fields per section were also analysed for each subject, by two independent observers blinded to the clinical status of the samples. Quantification of immunoreactivity was performed on digitally captured colour images saved as tiff files and analysed using ImageJ 1.37 (<http://rsb.info.nih.gov/ij>). After subtracting the non-white background colour, the haematoxylin component was separated from the diaminobenzidine tetrachloride (DAB) component using colour deconvolution, as described previously [23]. Calibration of optical density was performed using a Kodak photographic 21-step grey-scale tablet with a density range of 0.00–2.60. For each specimen, the median optical density (MOD) values measured from eight manually identified, positively stained cells selected from the DAB component was used to set the threshold manually for whole sample

staining density quantification. The percentage area stained and MOD were measured for each image and were calculated as the median of the total score in each specimen.

Immunohistochemical analysis for lymphatic vessels

In all cases, 4-µm-thick, paraffin-embedded sections of formalin-fixed tissues were used. Sections were deparaffinized and dehydrated through a series of graded ethanol solutions, then treated with 0.03% H₂O₂ in methanol for 15 min to quench endogenous peroxidase activity. The vascular endothelial cells were detected with a 1 : 200 dilution of anti-D2-40 antibody (Signet Lab, Dedham, MA, USA).

Microvessel density was assessed by light microscopy based on the criteria of Weidner *et al.* [24]. Two sections were analysed for each subject, by two independent observers blinded to the clinical status of the samples. In each section, five microscopic fields (mean area, 0.4 mm²) were randomly selected for quantitative analysis. The number of blood vessels was counted and the total length of blood vessels lined by the endothelial cells was measured with Image J software (<http://rsb.info.nih.gov/ij>). Then, the lymphatic vessel density (vessel number per mm²) and the endothelial length density (mm per mm²) were calculated for each section and were expressed as the median.

Western blot analysis

Samples frozen in liquid nitrogen were crushed into pieces and vigorously vortexed in a buffer solution containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and a proteinase inhibitor cocktail. A total of 50 µg extracted protein, which was digested with chondroitinase ABC (500 mU/250 µL, Sigma Aldrich Corp., St Louis, MO, USA) and keratanase-1 (50 mU/50 µL, Sigma-Aldrich Corp.) at 37 °C, was resuspended in SDS sample buffer and boiled for 5 min. Equal amounts of total protein were separated on 12% sodium dodecyl sulphate–polyacrylamide gels and transferred to immobilon (Millipore, Bedford, MA, USA).

The blots were reacted with the anti-decorin, anti-lumican, or anti-biglycan antibodies in Tris-buffered saline [20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Triton X-100, and 0.5% skim milk] for 2 h at 4 °C. Antibody reactions were detected by using the ECL detection kit (Amersham Bioscience, Piscataway, NJ, USA), followed by detection of chemiluminescence on radiographs. As an internal control, β-actin expression was analysed in parallel blots using with a 1 : 1000 dilution of the β-actin antibody (Sigma Aldrich Corp.). The intensity of detected bands was quantified by using scion image Beta 4.0.2 (Scion Corporation, Frederick, MD, USA). The

relative intensity of each protein signal was obtained by dividing the intensity of each protein signal by the β -actin signals.

Statistical analysis

Statistical analyses were carried out using SPSS for windows, version 16.0.0 (SPSS Inc., Chicago, IL, USA). The age differences in the three groups were calculated by the one-way ANOVA, and the Bonferroni *post hoc* correction was applied. The duration of rhinitis between the mild and severe persistent rhinitis groups was statistically evaluated by the Mann-Whitney *U*-test. Kruskal-Wallis test was performed to establish statistically significant differences in values obtained from the nasal mucosa of healthy controls, the patients with mild or severe persistent allergic rhinitis, including the data evaluated by semi-quantitative RT-PCR, the total percentage area of collagen deposition, the stained percentage area and the median optical density of decorin, lumican, and biglycan evaluated by immunohistochemistry. The level of significance was set at $P < 0.05$. The Mann-Whitney *post hoc* tests were carried out for comparing differences between different groups.

The lymphatic vessels density and the endothelial length density among the three groups were also statistically evaluated by the Kruskal-Wallis test. The Mann-Whitney *post hoc* tests were also carried out for comparing differences between different groups. The level of significance was set at $P < 0.05$.

Results

The characteristics of healthy controls and patients with mild or severe persistent allergic rhinitis are shown in Table 2. Age was similarly distributed among the healthy controls and the patients with mild and severe persistent allergic rhinitis (Table 2). The duration of rhinitis was not significantly different between mild and severe persistent allergic rhinitis (Table 2). However, serum total IgE levels were increased in mild and severe persistent allergic rhinitis compared with healthy nasal mucosa (Table 2, $P < 0.05$).

The expression of MMP2, MMP9, TIMP1, TIMP2, decorin, lumican, biglycan, and GAPDH mRNA was determined by RT-PCR in total RNA preparations isolated from healthy controls and patients with mild or severe persistent allergic rhinitis. As shown in Fig. 1a, all mRNAs were expressed in healthy controls and patients with mild or severe persistent allergic rhinitis and showed 100% sequence homology with the published sequences. Negative control reactions without RT in the cDNA synthesis process showed no bands, indicating that there was no contamination by genomic DNA in the RNA samples (data not shown). The relative abundance of MMP9 with respect

Table 2. Clinical characteristics of patients

	Healthy nasal mucosa	Mild persistent allergic rhinitis	Severe persistent allergic rhinitis
Characteristics	(n = 30)	(n = 30)	(n = 30)
Female/Male	8/22	8/22	10/20
Age (year)	33.7 $\pm$ 6.2	35.4 $\pm$ 7.9	33.9 $\pm$ 7.8
Rhinitis duration (year)	NA	11.78 $\pm$ 1.3	12.43 $\pm$ 1.73
Serum total IgE (IU/mL)	100.34 $\pm$ 98.20	505.23 $\pm$ 202.85	528.04 $\pm$ 250.76

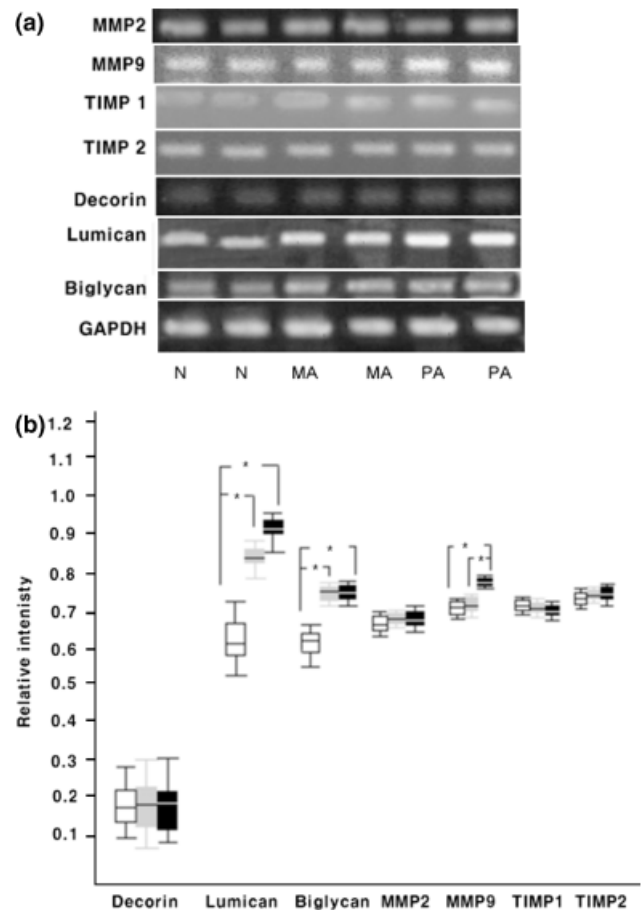


Fig. 1. (a) Semiquantitative RT-PCR analysis of decorin, lumican, biglycan, MMP2, MMP9, TIMP1, and TIMP2 mRNA level in healthy (N), mild (MA), and severe persistent allergic rhinitis (PA). (b) The relative amount of each gene in healthy (white column) and mild (grey column), and severe persistent allergic nasal mucosa (black column) was calculated by dividing by the internal control (GAPDH). The bottom and top of the box are the lower and upper quartiles, respectively, and the band near the middle of the box is the median. * $P < 0.05$.

to GAPDH was significantly increased in nasal mucosa of patients with severe persistent allergic rhinitis compared with healthy nasal mucosa and mild persistent allergic

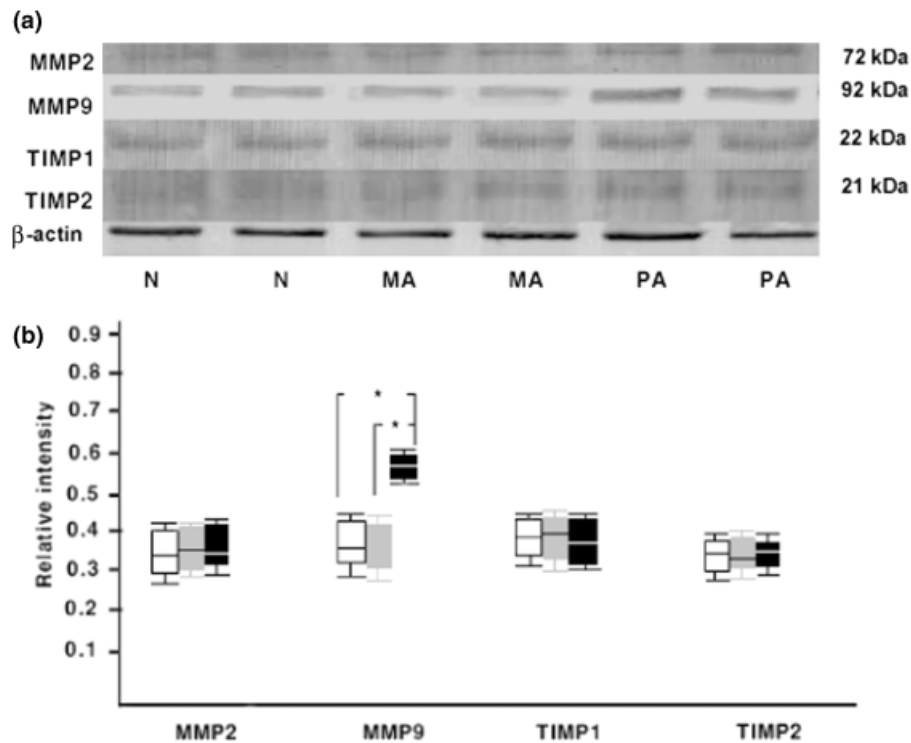


Fig. 2. (a) Western blot analysis of MMP2, MMP9, TIMP1 and 2 in healthy (N) and mild (MA), and severe persistent allergic nasal mucosa (PA). (b) The relative amount of each protein in healthy (white column) and mild (grey column), and severe persistent allergic nasal mucosa (black column) was calculated by dividing by the internal control, β -actin. The bottom and top of the box are the lower and upper quartiles respectively, and the band near the middle of the box is the median. * $P < 0.05$.

nasal mucosa (Fig. 1). The levels of lumican and biglycan mRNA expression were also increased in mild and severe persistent allergic rhinitis in comparison with healthy nasal mucosa (Fig. 1). However, MMP2, TIMP1, TIMP2, and decorin mRNA were not significantly different in their expression levels among three groups (Fig. 1). Western blot analysis also detected the MMP2, MMP9, TIMP1, and TIMP 2 proteins in healthy, mild, and severe persistent allergic turbinate mucosae (Fig. 2). The relative abundance of MMP9 with respect to β -actin was significantly increased in persistent allergic turbinate mucosa compared with healthy and mild persistent allergic nasal mucosa, demonstrating that the levels of expression of MMP9 were increased in severe persistent allergic turbinate mucosa (Fig. 2). However, the expression levels of decorin, MMP 2, TIMP1, and TIMP2 were not significantly different among three groups (Fig. 2).

Microscopic analysis at low magnification of the nasal mucosa of healthy controls evaluated by the van Gieson staining method showed overall similarities in the distribution of collagen fibres through the lamina propria, with a superficial band of collagen fibres immediately below the epithelium. In the deep region of the lamina propria and submucosal layer, staining for collagen was relatively sparse in the nasal mucosa of healthy controls. In mild and severe persistent allergic nasal mucosa,

compared with healthy nasal mucosa, more intense staining was localized in the superficial and submucosal layer where a band-like staining was localized most abundantly below the epithelial basement membrane zone and around the submucosal gland. Typical examples of nasal mucosa in healthy controls and patients with mild and severe persistent allergic nasal mucosa identifying collagen fibres are shown in Fig. 3. Using image analysis techniques, collagen deposition was determined as shown in Fig. 3. Deposition of collagen was significantly greater in nasal mucosa of patients with mild and severe persistent allergic nasal mucosa than healthy controls. (Fig. 3). However, there were no differences in deposition of collagen between mild and severe persistent allergic rhinitis (Fig. 3).

Positive staining for decorin, lumican, and biglycan was identified in all nasal mucosa of both healthy controls and patients with mild, and severe persistent allergic rhinitis (Fig. 4). Positive staining for decorin, lumican, and biglycan was present in both the subepithelial and deep submucosal layer. However, the pattern of staining for lumican and biglycan differed between healthy and both types of allergic rhinitis while the staining pattern for decorin was not different (Fig. 4). In the healthy controls, as well as patients with mild, and severe persistent allergic rhinitis, decorin was lightly stained in the subepithelial basement membrane zone and deep submucosa

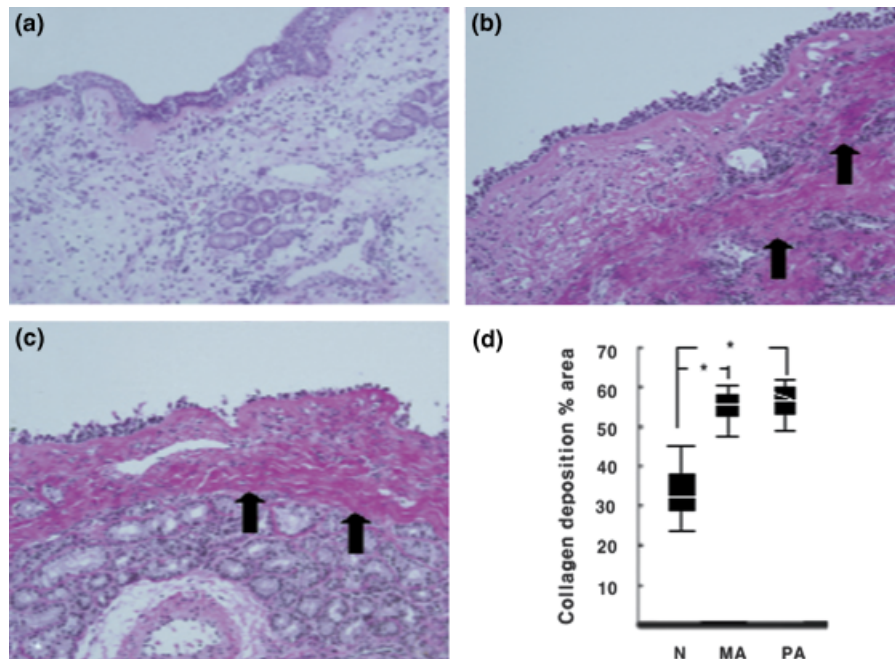


Fig. 3. Collagen deposition in nasal mucosa of healthy controls (a) and patients with mild (b), and severe persistent allergic rhinitis (c) was evaluated by van Gieson staining method. Positive areas are stained red (arrow). Healthy nasal mucosa shows faint staining, whereas mild and severe persistent allergic rhinitis shows prominent staining intensity. Original magnification $\times 200$ (d). Comparison of collagen deposition percentage area between nasal mucosa of healthy controls and patients with mild and severe persistent allergic rhinitis. $*P < 0.05$.

surrounding the submucosal glands. However, a light, distinct line of decorin staining is found at the epithelial basement membrane zone, whereas epithelium does not show any specific staining. The image analysis showed that there are no significant differences in the percentage area of staining and optical density of decorin between the healthy controls and the patients with mild and severe persistent allergic nasal mucosa. The presence of decorin in healthy and allergic nasal mucosa was verified by Western blot analysis, which showed no significant differences in the levels of decorin expression between healthy controls and patients with mild persistent allergic rhinitis, and between healthy controls and patients with mild and severe persistent allergic rhinitis (Fig. 5).

The staining for biglycan and lumican showed a similar pattern and was prominent in the subepithelial and deep submucosal layers in mild and severe persistent allergic nasal mucosa, although it was observed in healthy nasal mucosa. The staining patterns for biglycan and lumican in mild and severe persistent allergic nasal mucosa were variable, but usually showed strong reactivity in the subepithelial layer and prominent localization within the ECM around submucosal glands. Unlike decorin, the staining for lumican and biglycan was observed in epithelial cells and submucosal glandular cells (Fig. 4). Quantification of the area of positive staining (percentage area), as well as the median optical density in the subepithelial layer and deep submucosal area are shown in Fig. 4. In the mild and severe persistent allergic nasal mucosa, com-

pared with healthy nasal mucosa, Western blot showed that there was significant greater expression of biglycan and lumican (Fig. 5). However, there were no differences in their expression levels between mild and severe persistent allergic rhinitis (Fig. 5).

Lymphatic vessels in healthy and allergic nasal mucosa were identified by D2-40 staining in all samples (Fig. 6). In healthy and both types of allergic nasal mucosa, lymphatic vessels were seen in the lamina propria and in the submucosal layer. However, most lymphatic vessels were found in the lamina propria beneath the epithelial layer. Morphometric examination using tissue section was performed at the light microscopic level by measuring the number and endothelial length of vessels in each specimen. There was statistically significant difference in the number and endothelial length density of lymphatic vessels between the healthy controls and patients with mild persistent allergic rhinitis, and between healthy controls and patients with severe persistent allergic rhinitis. Figure 6 shows data from morphometric analysis of the lymphatic vessels.

Discussion

The results of the present study show that the deposition of collagen in nasal mucosa differs between healthy controls and patients with mild persistent allergic rhinitis, and between healthy controls and severe persistent allergic rhinitis. While the deposition of decorin, one of the

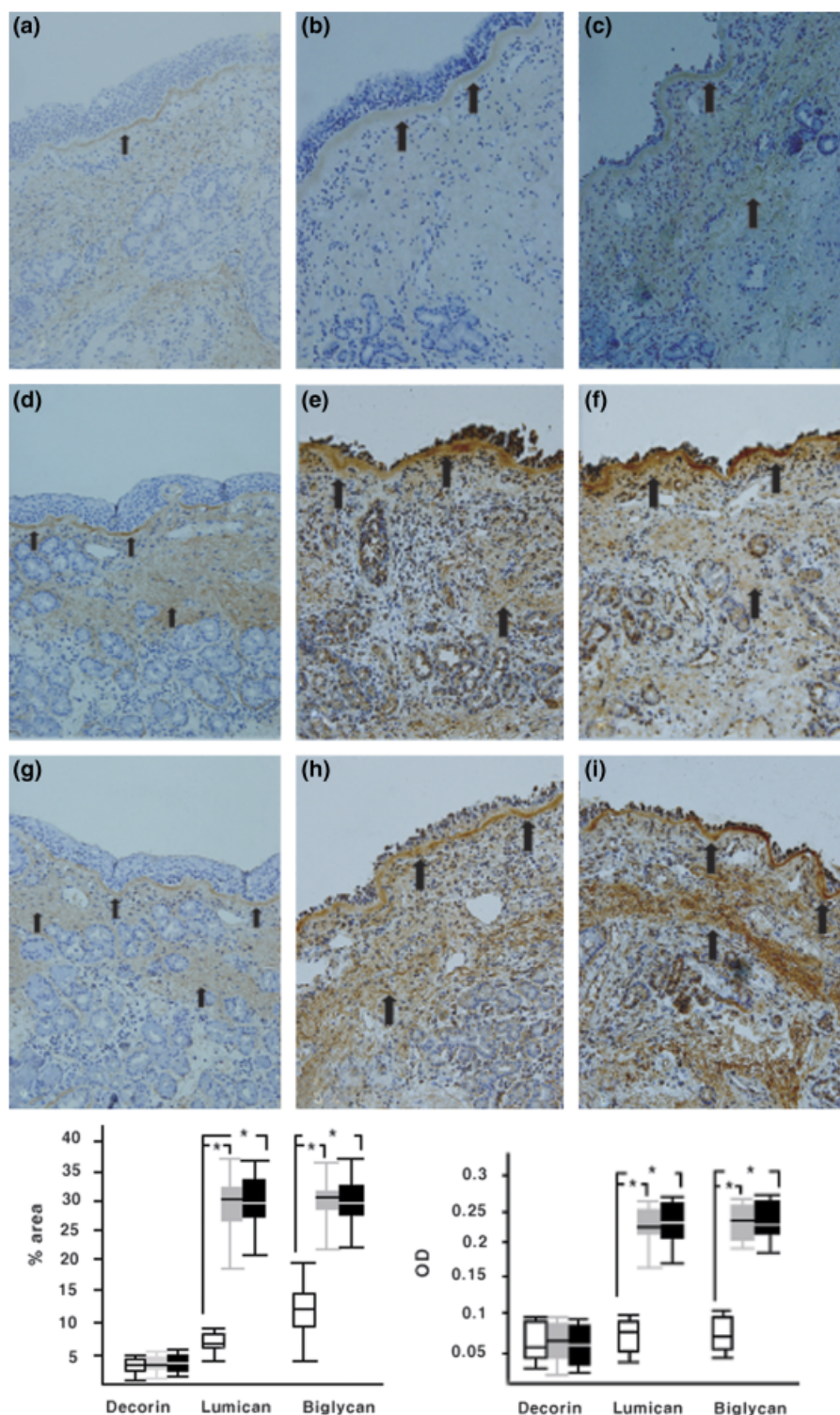


Fig. 4. Immunohistochemical analysis of distribution pattern of decorin (a, b, c), lumican (d, e, f), and biglycan (g, h, i) in nasal mucosa of healthy controls (a, d, g) and patients with mild (b, e, h) and severe persistent allergic rhinitis (c, f, i). Original magnification $\times 200$. The graph shows deposition percentage area (% area) and optical density (OD) of decorin, lumican, and biglycan in healthy (white column) and mild (grey column), and severe persistent allergic nasal mucosa (black column). Arrow indicates the deposition of decorin, lumican and biglycan. * $P < 0.05$.

proteoglycans, was similar among the three groups, patients with mild or severe persistent allergic rhinitis had significantly greater deposition of lumican and biglycan

in nasal mucosa than healthy controls. In addition, immunohistochemistry using D2-40 antibody revealed that the number of lymphatic vessels in nasal mucosa

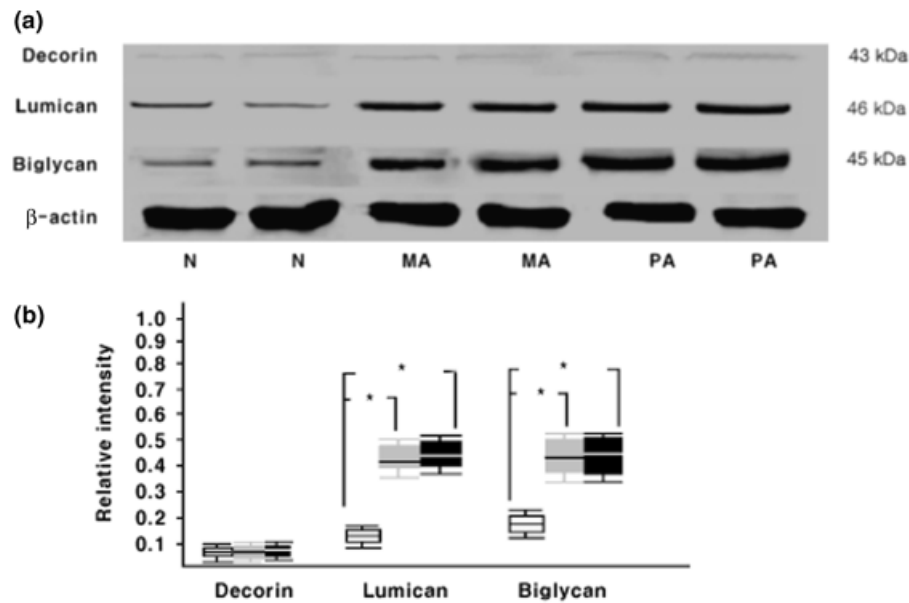


Fig. 5. (a) Western blot analysis of decorin, lumican, and biglycan in healthy (N), and mild (MA) and severe persistent allergic nasal mucosa (PA). (b) The relative amount of each protein in healthy (white column) and mild (grey column), and severe persistent allergic nasal mucosa (black column) was calculated by dividing by the internal control, β -actin. The bottom and top of the box are the lower and upper quartiles, respectively, and the band near the middle of the box is the median. * $P < 0.05$.

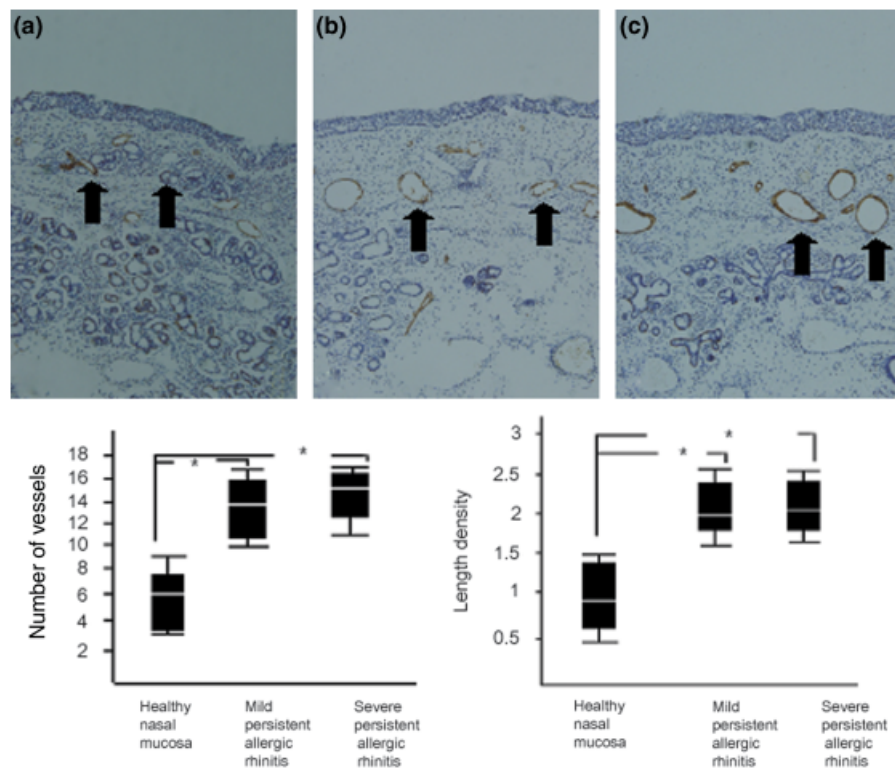


Fig. 6. Immunohistochemical analysis of lymphatic vessels analysed by using D2-40 antibody in healthy (a), mild (b), and severe persistent allergic nasal mucosa (c). Original magnification $\times 200$. The graph shows the number and endothelial length density of lymphatic vessels in healthy, mild, and severe persistent allergic nasal mucosa. Arrow indicates lymphatic vessels. * $P < 0.05$.

was increased in mild and severe persistent allergic rhinitis compared with healthy controls. Taken together, these results suggest that the altered distribution of collagen, proteoglycans, and lymphatic vessels could potentially modulate the remodelling of nasal mucosa in mild or severe persistent allergic rhinitis. Indeed, this is the first study to show significant differences in the pattern of distribution and content of collagen, proteoglycans, and lymphatic vessels between healthy controls and patients with mild or severe persistent allergic rhinitis.

Collagen is a major component of the ECM in tissue. It provides the structural framework for tissue and is involved in the process of tissue remodelling after injuries or inflammation. Among the structural changes reported in the nasal mucosa of patients with allergic rhinitis are included epithelial injury, goblet cell hyperplasia, enlarged submucosal mucus glands, and angiogenesis [5, 8]. Thus far, the details of collagen deposition in healthy and allergic nasal mucosa are unclear because there have been few studies concerning the remodelling of nasal mucosa. The results of the present study showed that in mild and severe persistent allergic nasal mucosa, compared with healthy nasal mucosa, more intense staining was localized in the superficial layer where band-like staining was localized most abundantly below the epithelial basement membrane zone. The quantitative data such as percentage area, showed the increased expression of collagen content in mild and severe persistent allergic rhinitis compared with healthy nasal mucosa. Although few studies have focused on collagen changes in the nasal mucosa of patients with allergic rhinitis, our results were in accordance with the results showing that the thickness of the basement membrane zone underlying the epithelia was statistically significantly greater in allergic subjects than in non-allergic subjects [25]. The collagen deposition in the submucosal area was confirmed in the murine nasal allergic rhinitis model [26]. Taken together, these results suggested that activation of the collagen-producing system in the submucosal layer of allergic nasal mucosa is involved in tissue remodelling, indicating that the remodelling response in the submucosal layer is actively processed in allergic rhinitis, at least in mild and severe persistent allergic rhinitis. However, Sanai *et al.* [25] did not report any difference in the total amount of collagen in nasal biopsies from allergic rhinitis and non-allergic rhinitis, although extensive immunoreactivity for type I and III collagen was observed in allergic subjects at the subepithelial basement zone. Because we did not measure the total amount of collagen in nasal mucosa, we cannot conclude that collagen content of the submucosal layer may increase in allergic rhinitis. Indeed, conflicting results has been reported for asthma. Wilson *et al.* [27], and Chakir *et al.* [28], showed that airway fibrosis in patients with asthma involves the submucosa. However, Chu *et al.*

[29] did not find significant differences in submucosal collagen content between asthmatic patients and control subjects. Another study using collagen analysis revealed greater collagen I and less collagen III content in the small airways of patient with fatal asthma, with no differences in collagen content observed in the large airways between the groups [30]. More studies are required to fully appreciate remodelling in rhinitis.

The current study showed relatively greater amounts of submucosal deposition of lumican and biglycan in the nasal mucosa of patients with mild and severe persistent allergic rhinitis, whereas no significant change was found with respect to decorin deposition between subjects with mild or severe persistent rhinitis and healthy controls. Therefore, the present results suggest that the deposition of proteoglycan, at least lumican and biglycan, as well as collagen occurred as remodelling in bronchial mucosa may cause the thickening of the basement membrane zone underlying the epithelia in mild and severe persistent allergic rhinitis. However, the functional role of the altered expression of these proteoglycans in nasal mucosa is unclear. What could be the explanation for our present findings that patients with mild and severe persistent allergic rhinitis had higher lumican and biglycan densities in the subepithelial layer as compared with normal controls? It is becoming increasingly obvious that small proteoglycans are not only involved in matrix formation and cell adhesion, but also in the modulation of cytokine activity and in direct cell signalling [31]. Decorin and biglycan have been shown to play important regulatory roles on TGF- β action, which stimulates synthesis of biglycan while inhibiting synthesis of decorin by lung fibroblasts [32, 33]. Furthermore, it is known that TGF- β is directly related to the amount of fibrosis in the lungs [34]. By contrast, the lack of biglycan gene expression is known to be associated with the failure of the fibrotic response [33, 35]. On the other hand, evidence has been provided of enhanced epithelial TGF- β immunoreactivity in allergic rhinitis, including correlation with intraepithelial mast cell numbers, and co-localization of TGF- β receptors to mast cells, suggesting that the epithelial expression of TGF- β may represent an important biological process in allergic rhinitis [36]. Taken together, it is expected that the increased biglycan content without altered expression levels of decorin in allergic nasal mucosa would reflect a higher pro-fibrotic TGF- β level, which would lead to collagen deposition and airway remodelling. Lumican is also distributed in interstitial collagenous matrices in various organs, including lungs, and is considered a key regulator of collagen fibrillogenesis, regulating the assembly and diameter of collagen fibres [37, 38]. These lines of evidence suggest that an increased expression of lumican in allergic rhinitis may play a role in the deposition of collagen and fibrosis of nasal mucosa in patients with mild and severe persistent allergic rhinitis [37, 38].

It is generally known that nasal obstruction is a common symptom of allergic rhinitis and is created by oedema and swelling of nasal mucosa. Based on our current findings, deposition of matrix protein as well as subepithelial fibrosis may also be notorious factors for accelerating severe nasal obstruction in severe persistent allergic rhinitis. In addition, our results indicate the increased distribution of lymphatics in the nasal mucosa of patients with mild and severe persistent allergic rhinitis, and suggest that lymphangiogenesis might be a characteristic of remodelling of nasal mucosa in patients with mild and severe persistent allergic rhinitis, contributing to the nasal mucosa thickening. On the other hand, Mori et al. [19] reported that the microvessel count in the lamina propria of allergic mucosa was significantly higher than that of control mucosa, and suggested that hyper-vascularity in the nasal mucosa may be involved in the pathogenesis of allergic rhinitis. In contrast to our findings, a reduced distribution of lymphatics was noted in the airway walls of fatal asthmatics, although the vascular densities are augmented in these airway walls, even within the epithelial layers. Their results suggested that the reduced distribution in lymphatics in the airway walls of fatal asthmatics is thought to cause impairment of lymphatic drainage of the airway epithelial layer in these lungs [21]. Considering the facts that lymphangiogenesis is seen in chronic states of inflammation, such as persistent airway infection and angiogenic markers such as vascular endothelial growth factor induce angiogenesis as well as lymphangiogenesis [20, 39, 40], our observation suggests that the presence of lymphangiogenesis and related remodelling cannot be denied in patients with mild and severe persistent allergic rhinitis. However, because there are no previous studies in the medical literature that have investigated the presence of lymphangiogenesis in allergic rhinitis, a more detailed evaluation of lymphangiogenesis and the influence of lymphangiogenesis on the pathogenesis of allergic rhinitis should be clarified in future study.

The cell signalling that leads to ECM synthesis and degradation in the airway is mediated by a variety of pathways and was found to be complex. Small leucine-rich proteoglycans composed of decorin, biglycan, and lumican are present within the ECM of connective tissues, acting as structurally and functionally related molecules and interacting with a variety of other proteins. Foremost among these interactions are those with TGF- β , which has been implicated in airway remodelling, acting as chemoattractant and proliferation factor for fibroblasts and induces fibroblasts to synthesize ECM proteins [41]. Actually, TGF- β signalling is activated in allergic rhinitis and contributes to the development of ECM synthesis including goblet cell hyperplasia and the epithelial expression of TGF- β may represent an important biological process in allergic rhinitis [36]. Therefore, based on our results and

published literature [41], TGF- β may induce the deposition of proteoglycans including lumican and biglycan in allergic rhinitis. As well, MMPs are involved in remodelling of ECM of various organs through their broad proteolytic capability and the activity of MMP is inhibited reversibly by TIMP [14, 15]. Therefore, the major endogenous factor leading to ECM remodelling in nasal mucosa in allergic rhinitis may be regarded as an imbalance between the activity of MMP and TIMP. However, in the current study, increases in MMP 9 was detected, but not in MMP2, or in TIMP1 or 2 in the nasal mucosa in cases of severe persistent allergic rhinitis compared with healthy nasal mucosa. The functional significance of the detection of increased levels of expression of MMP 9 in nasal mucosa in severe persistent allergic rhinitis is at present unknown. Considering the facts that tissue eosinophilia and eosinophil degranulation have been associated with allergic rhinitis, and the cells are a source of several molecules implicated in tissue remodelling processes such as TGF- β , and moreover, degradation of ECM by MMP 9 can lead to release and activate inflammatory mediators, growth factors, and chemokines sequestered in ECM [42, 43], MMP 9 is thought to contribute to the pathogenesis of allergic rhinitis by influencing the migration of inflammatory cells such as eosinophils across the basement membranes of blood vessels, as well as by influencing ECM deposition and matrix degradation. On the other hand, those results of a few data available on MMP expression in the allergic rhinitis were inconsistent. Shaida et al. [44] demonstrated the presence of large amounts of TIMP1 and 1 mRNA and proteins in nasal mucosa and showed that there is no upregulation of MMP or changes in TIMP expression in the nasal mucosa of patients with allergic rhinitis. In another study, the level of MMP 9 in nasal lavage fluid has been shown to increase immediately in response to nasal challenge with allergen extract [45]. Our results were in accordance with the results of study showing that the MMP 9 levels in nasal mucosa extracts in the allergic rhinitis patients were significantly higher than in the controls. In fatal asthma, increases in MMP 9 were detected, but not MMP 2 or TIMP 1 or 2 in airway smooth muscles bundles [46]. Recently, it has been proved that long-term allergen challenge in the murine model of allergic rhinitis can result in some feature of airway remodelling in the nasal mucosa, including subepithelial fibrosis, and an increased expression of MMP 9 and TGF- β [47]. Taken together with these results, one might expect that the interaction between small leucine-rich proteoglycans and the growth factor like TGF- β may have a role in modifying the composition of submucosal ECM to facilitate the process of airway remodelling in allergic rhinitis, preventing ECM degradation by an increased MMP 9 expression. Indeed, small leucine-rich proteoglycans protect the connective tissue matrix from excessive damage by MMP during remodelling [48]. In

addition, ECM is also a reservoir for many biologically active molecules, such as cytokines and proangiogenic factors. Therefore, MMP promote angiogenesis by not only degrading ECM but also by regulating matrix-bound growth factors such as VEGF, which induces endothelial cell proliferation [49]. These mechanisms may play an important role on the lymphangiogenesis in allergic rhinitis.

In conclusion, the present results demonstrated altered deposition of collagen, proteoglycan, and lymphangiogenesis in the nasal mucosa of patients with mild and severe persistent allergic rhinitis, compared with healthy nasal mucosa, and suggests that these distinctive histological changes may play an important role in the remodeling process in allergic rhinitis and may also be notorious factors for accelerating nasal obstruction in mild and severe persistent allergic rhinitis.

Clinical relevance

We have found distinctive staining patterns for collagen, proteoglycan, and lymphatic vessels in normal and allergic nasal mucosa. These findings provide the basis for further studies on the role of collagen and proteoglycan deposition, and lymphangiogenesis on nasal stuffiness of patient with allergic rhinitis.

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