

Increased expression of YKL-40 in mild and moderate/severe persistent allergic rhinitis and its possible contribution to remodeling of nasal mucosa

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ABSTRACT

Background: Prominent expression of YKL-40 has been associated with pathological conditions characterized by tissue remodeling. We determined the expression level and distribution pattern of YKL-40 in allergic nasal mucosa and evaluated the effect of YKL-40 on the proliferation and migration of fibroblasts, the production of the mediators related to tissue remodeling, and collagen production. Additionally, the cytokine-driven regulation of YKL-40 expression was evaluated in cultured epithelial cells.

Methods: The expression of YKL-40 in normal, mild, and moderate/severe allergic nasal mucosa was evaluated using real-time polymerase chain reaction (PCR), Western blot, and immunohistochemistry. Fibroblast migration was observed using a scratch wound method, and proliferation was determined by the MTT methods. Expression of proteoglycans, transforming growth factor (TGF) β 1, MMP2, MMP9, TIMP1, TIMP2, and collagen concentration were analyzed in fibroblasts treated with YKL-40. The expression levels of YKL-40 in cultured epithelial cells were examined after stimulation with mediators including Th2 cytokines, interferon (IFN) γ , and TNF- α with real-time PCR and ELISA.

Results: The expression of YKL-40 was up-regulated in allergic rhinitis and distributed in superficial epithelium, submucosal glands, and vascular endothelium, in addition to infiltrating cells. TGF- β 1, TIMP1, MMP9, and biglycan were up-regulated in fibroblasts on stimulation with YKL-40, accompanying increased proliferation and migration, and collagen production. IL-13, IFN- γ , and TNF- α induced the increased production of YKL-40 in cultured epithelial cells.

Conclusion: YKL-40 is up-regulated in mild and moderate/severe persistent allergic rhinitis, and its expression can be regulated differentially by different cytokines, possibly contributing to the remodeling of nasal mucosa in allergic rhinitis.

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Remodeling represents a dynamic process of extracellular matrix (ECM) deposition and degradation, leading to the reconstruction of damaged tissue in reaction to inflammation.¹ Although the presence of nasal mucosa remodeling as a feature of allergic rhinitis is still controversial, there is growing evidence that remodeling of nasal mucosa occurs in allergic rhinitis, but to a lesser extent than asthma.^{2,3} Biopsy specimens taken during allergen exposure have shown that epithelial damage is associated with conspicuous intercellular edema and epithelial shedding.⁴ Epithelial metaplasia and pseudothickening of reticular basement membrane with increased deposition of collagen types I and III were observed in perennial allergic rhinitis.⁵ Previously, we reported that the distribution pattern of collagen, proteoglycans, and lymphatic vessels is altered in mild and moderate/severe persistent allergic rhinitis mucosa.⁶

The cell signaling that leads to these remodeling processes was found to be complex. Remodeling, deposition of collagen and other ECM components, may be at least partially attributed to an increased fibroblast number and activity. A variety of mediators are increased in allergic rhinitis, which may be released by local inflammatory cells to activate the fibroblasts, and have been reported to induce these pathological changes.^{2,7–9} Among the various factors that are involved in tissue remodeling, transforming growth factor (TGF) β has been

implicated in the development of allergic airway remodeling.² In the murine model of allergic rhinitis, subepithelial fibrosis and gland hypertrophy were associated with increased expression of MMP9 and TIMP1.¹⁰ Recently, an imbalance between MMPs and their natural inhibitor was reported in allergic rhinitis.⁸ Furthermore, other factors such as angiogenic factors and fibrinolytic components may be involved in remodeling of nasal mucosa in allergic rhinitis.^{9,11} Nevertheless, the inflammatory mechanisms that drive progression of allergic rhinitis to remodeling are still incompletely understood. Therefore, another compound may be involved in the remodeling of nasal mucosa in allergic rhinitis.

YKL-40, also known as human cartilage glycoprotein 39 and chitinase-3-like-1, is a glycoprotein that belongs to the chitinase family.¹² It is induced specifically during the course of inflammation in various chronic inflammatory diseases.¹² Although the detailed physiological function of YKL-40 is unclear, it has been reported to potentially stimulate the growth of human fibroblasts derived from the fetal lung and adult skin and the migration of vascular endothelial cells.^{13,14} A recent study indicated that the serum levels of YKL-40 were increased in patients with severe asthma and its expression levels were correlated positively with the thickness of the subepithelial basement membrane.¹⁵ In idiopathic pulmonary fibrosis, an abundant expression of YKL-40 is involved in tissue remodeling and fibrosis.¹⁶ These data suggest that YKL-40 is involved in tissue remodeling in various human diseases. In this respect, we hypothesized that YKL-40 is associated with tissue remodeling of nasal mucosa in patients with allergic rhinitis.

In the present study, we determined the expression levels and distribution pattern of YKL-40 in normal nasal mucosa of healthy controls and in mild and moderate/severe persistent allergic nasal mucosa to evaluate the possible effects of YKL-40 on the remodeling of nasal mucosa in allergic rhinitis. Using fibroblast cultures, we investigated whether the YKL-40 could induce the synthesis of proteoglycans, such as decorin, lumican, and biglycan, as well as TGF- β 1,

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MMP2, MMP9, TIMP1, and TIMP2, and regulate vital cell processes, such as growth and migration, in nasal tissues fibroblasts. In addition, we also determined the effects of cytokines related to the pathogenesis of allergic rhinitis, on the expression of YKL-40, using an epithelial culture system.

METHODS

Subjects

Healthy nasal mucosa (*n* = 30) were obtained from the inferior turbinate of 30 patients who had normal nasal mucosa and were admitted for augmentation rhinoplasty. The patients had no history of nasal infection or allergy, smoking, or ongoing drug treatment. Allergic nasal mucosa were obtained from the inferior turbinate of patients with mild (*n* = 30) and moderate/severe persistent allergic rhinitis (*n* = 30). They had typical allergy symptoms such as nasal stuffiness, rhinorrhea, sneezing, nasal eosinophilia, and a positive skin test or positive radioallergosorbent test to house-dust mites. Patients with allergic rhinitis were classified as mild or moderate/severe persistent according to the Allergic Rhinitis and Its Impact on Asthma criteria.¹⁷ Exclusion criteria included current smoking, medical history of asthma, and recurrent infections of the respiratory tract and use of antiallergic drugs during the 8 weeks that preceded the study. Before obtaining the tissue specimens, the Institutional Review Board at our institution approved the protocols and informed consent form.

All tissue samples were divided into three portions. The first and second portions were frozen in liquid nitrogen and stored at -70°C for RNA and protein isolation. For immunohistochemistry, the third sample was immersed in a fixative containing 4% paraformaldehyde and was embedded in paraffin wax. A part of normal, mild, and moderate/severe allergic nasal mucosa was used to isolate and culture the fibroblasts and the epithelial cells.

Isolation and Culture of Fibroblasts

Normal and allergic nasal mucosa harvested from healthy subjects and patients with mild and moderate allergic rhinitis were cut into small pieces (2–3 mm²). Diced tissues were plated in DMEM containing 10% FCS at 37°C in a humidified atmosphere containing CO₂. When a confluent monolayer of fibroblasts had formed, the explanted tissues were removed. The cells were replated at a concentration of 5 × 10⁵ cells/mL. The medium was changed every 3–4 days for 2–3 weeks until confluence was attained. The cells were characterized with antibodies against vimentin, cytokeratin, and fibronectin; fibroblast purity exceeded 99%. Fibroblasts passaged six times were washed with DMEM—FCS, and fresh medium containing YKL-40 (5, 15, or 30 ng/mL) was added into each well in a total volume of 3 mL. After 12 and 24 hours, fibroblasts were collected for real-time polymerase chain reaction (PCR) and Western blot to evaluate the expression levels of small leucine-rich proteoglycans (decorin, lumican, and biglycan), TGF-β1, MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA and protein. Supernatants were collected and used for total collagen production using the Sircol collagen assay kit (Biocolor, Northern Ireland, U.K.).

Fibroblast Proliferation Assay

Nasal fibroblasts were counted with a Neubauer counting chamber, seeded at 10,000 cells/well in 96-well plates, and incubated until they reached subconfluence. Thereafter, cells were washed with PBS, and then were cultured for 12 and 24 hours in serum-free medium in the presence or absence of YKL-40 (5, 15, or 30 ng/mL; Quidel, San Diego, CA) at 37°C in a humidified atmosphere containing CO₂. At each time point, fibroblast proliferation was analyzed by using MTT proliferation assay as follows. MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide; 5 mg/mL; Sigma-Aldrich, St. Louis,

Table 1 Sequences of PCR primers

Primer	Size (bp)	Sequence
YKL-40	158	S: 5'- ATACGACGACCAGGAAAGCGTCAA-3' AS: 5'- GTGCATCCTTGATGGCATTGGTGA-3'
TGF-β1	135	S: 5'- ACAATTCCTGGCGATACCTCAGCA-3' AS: 5'- CGCTAAGGCGAAAGCCCTCAATTT -3'
Decorin	94	S: 5'- TGAGGGAGCTTCACTTGGACAACA-3' AS: 5'- AAGCAGTGGCTTTGCATTACCTGG-3'
Lumican	116	S: 5'- TGGCATTGATTGGTGGTACCAGTG-3' AS: 5'- TGGGTAGCTTTTCAGGGCAGTTACA-3'
Biglycan	166	S: 5'- TTGGACAACAACAAGTTGGCCAGG-3' AS: 5'- TGAAGAGGCTGATGCCGTTGTAGT-3'
MMP2	125	S: 5'- AGAAGGATGGCAAGTACGGCTTCT-3' AS: 5'- AGTGGTGCAGCTGTCATAGGATGT-3'
MMP9	193	S: 5'- TACCACCTCGAAGCTTTGACAGCGA-3' AS: 5'- GCCATTACGTCGCTCTTATGCAA-3'
TIMP1	133	S: 5'- AGCGAGGAGTTTCTCATTGCTGGA-3' AS: 5'- AGCCAACAGTGTAGGTCTTGGTGA-3'
TIMP2	90	S: 5'- TGCAGATGTAGTGATCAGGGCCAA-3' AS: 5'- TGGATCCTCTTGATAGGGTTGCCA-3'
GAPDH	122	S: 5'-ATCATCCCTGCCTCTACTGG-3' AS: 5'-GTCAGGTCCACCACTGACAC-3'

AS = antisense; S = sense; PCR = polymerase chain reaction; SOCS = suppressor of cytokine signaling; TGF = transforming growth factor.

Table 2 Clinical characteristics of patients

Characteristics	Healthy Nasal Mucosa (n = 30)	Mild Allergic Rhinitis (n = 30)	Moderate/Severe Allergic Rhinitis (n = 30)
Female/male	18/12	16/14	17/13
Age (yr)	32.7 ± 3.2	36.4 ± 6.9	35.9 ± 7.8
Rhinitis duration (yr)	NA	10.78 ± 3.3	10.42 ± 6.73
Serum total IgE (IU/mL)	25.34 ± 16.20	305.23 ± 102.85	377.04 ± 130.76

MO) was added to each well. After the removal of the medium and the addition of DMSO to the culture plates, the absorbance at 570 nm was measured using a spectrophotometer.

Fibroblast Migration Assay

Nasal fibroblasts were seeded at 100,000 cells/well in serum-free MEM and allowed to grow until confluence for 7 days. A linear defect was produced in a confluent cell monolayer as previously reported.¹⁸ In brief, the monolayer was wounded by pressing a 200-μL pipette tip onto the well, making a sharp visible demarcation at the wound edge. The wounded layers were incubated with YKL-40 (5, 15, or 30 ng/mL) at 37°C in a humidified atmosphere containing CO₂ for 24 hours. For reference, the wound area was marked with two or three dots on the external surface of the bottom of the plate with a black marker pen. The area marked by the reference dots was examined with the use of a phase contrast microscope and photographed, 12 and 24 hours after wounding, to determine the extent of cell migration into the wound area. The distance from the wound's margin to the leading edge of the migrating cells was measured.

Sircol Collagen Assay

The total soluble collagen assay was measured in supernatants collected after stimulation of cultured fibroblasts with YKL-40 (5, 15,

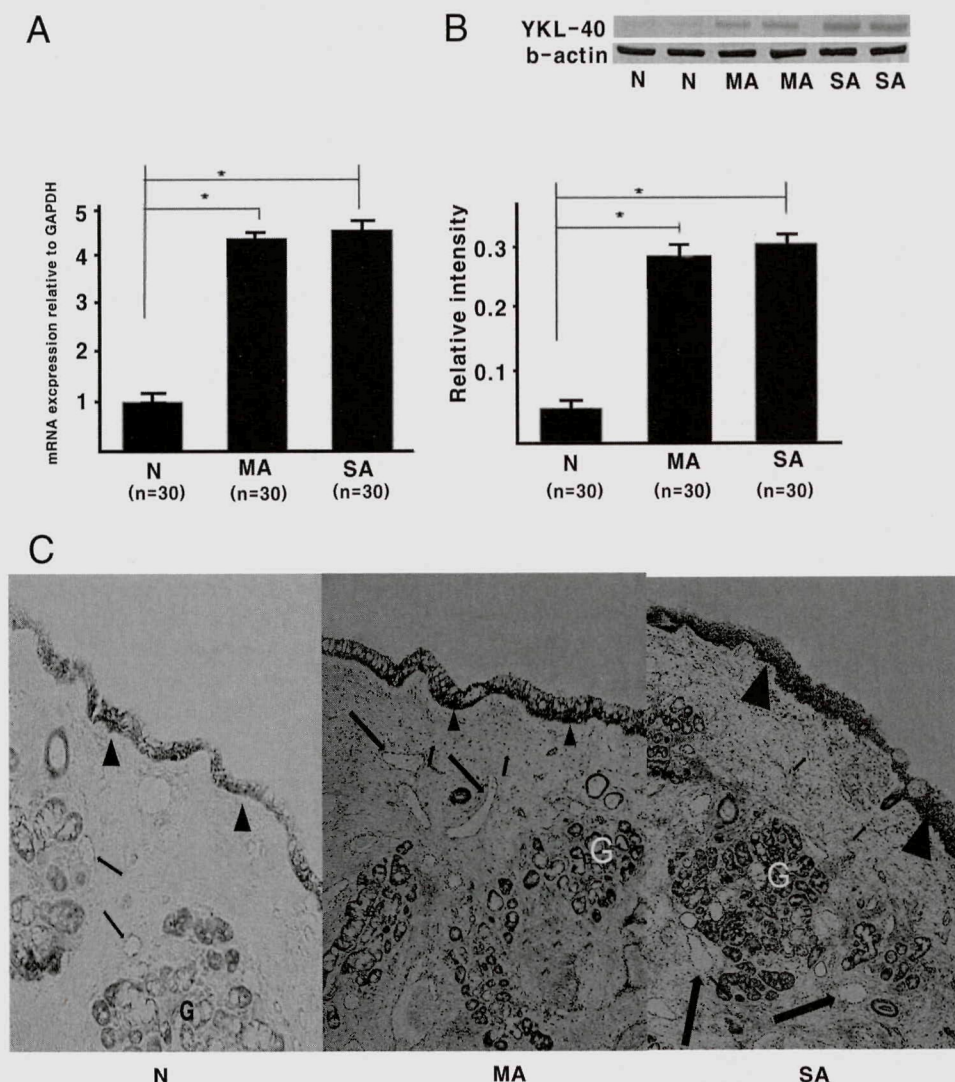


Figure 1. (A) Real-time polymerase chain reaction (PCR) and (B) Western blot analysis of YKL-40 expression levels in healthy, mild, and moderate/severe persistent allergic nasal mucosa. (C) Immunohistochemical localization of YKL-40 (* $p < 0.05$). (C) In healthy, mild, and moderate/severe persistent allergic nasal mucosa, YKL-40 was detected in the surface epithelium (arrow head), submucosal glands (G), vascular endothelium (large arrow), and infiltrating cells (small arrow; original magnification, $\times 100$). N, healthy nasal mucosa; MA, mild persistent allergic nasal mucosa; SA, moderate/severe persistent allergic nasal mucosa.

or 30 ng/mL) using the Sircol collagen assay kit according to the manufacturer's instruction. Briefly, 1 ml of Sircol dye reagent was added to an equal volume of test sample in triplicate and mixed for 30 minutes at room temperature. The collagen-dye complex was precipitated by centrifugation and dissolved in 250 μ L of alkali reagent. The absorbance was measured by a microplate reader at a wavelength of 570 nm.

Isolation and Culture of Epithelial Cells

Normal, mild, and moderate severe allergic nasal mucosa were treated with 0.5% Dispase (Sigma-Aldrich) in a 1:1 mixture of DMEM and Ham's nutrient F12 (Gibco BRL, Grand Island, NY) supplemented with penicillin G sodium (50 IU/mL) and streptomycin sulfate (50 μ g/mL) overnight at 4°C. Thereafter, nasal epithelial cells were freed from nasal mucosa and collected into a conical tube. The cells were cultured in serum-free bronchial epithelial growth medium (Lonza Walkersville, Inc., Walkersville, MD). Cultured cells were routinely 99–100% positive for cytokeratin staining at the time of harvest and were used for all *in vitro* studies at second passage only. When cells reached 80% confluence, they were stimulated with cytokines and mediators, respectively (10 and 20 ng/mL of IL-4, IL-5, IL-13, TGF- β 1, TNF- α , and interferon [IFN] γ ; Peprotech, Rocky Hill, NJ) for 12 and 24 hours. Cultured cells and supernatants after stim-

ulation were harvested and used for real-time PCR or ELISA to analyze the expression levels of YKL-40.

Real-Time Reverse Transcription PCR Analysis

Total RNA (1 μ g) obtained from frozen nasal mucosa, cultured fibroblast, and epithelial cells was reverse transcribed in 20 μ L of a reaction mixture containing 2.5 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The primer sequences of each gene used in the study are listed in Table 1. Real-time PCR was performed using an iCycler (BioRad Laboratories, Hercules, CA). Each PCR reaction included 50 ng of cDNA, 12.5 μ L of iTAQ SYBR supermix, and 200 nM of primers. PCR conditions for 40 cycles were 95°C denaturation for 30 seconds, 59°C annealing for 30 seconds, and 72°C extension for 30 seconds. GAPDH was used as a control housekeeping gene. All PCR reactions were performed in duplicate. Target gene expression was expressed as the fold increase and decrease relative to the expression of GAPDH. The mean value of the replicates for each sample was calculated and expressed as the cycle threshold (Ct). The amount of gene expression was then calculated as the difference (Ct) between the Ct value obtained for the target gene and the Ct value obtained for GAPDH. Fold change in target gene mRNA expression were determined as $2^{-\Delta\Delta Ct}$.

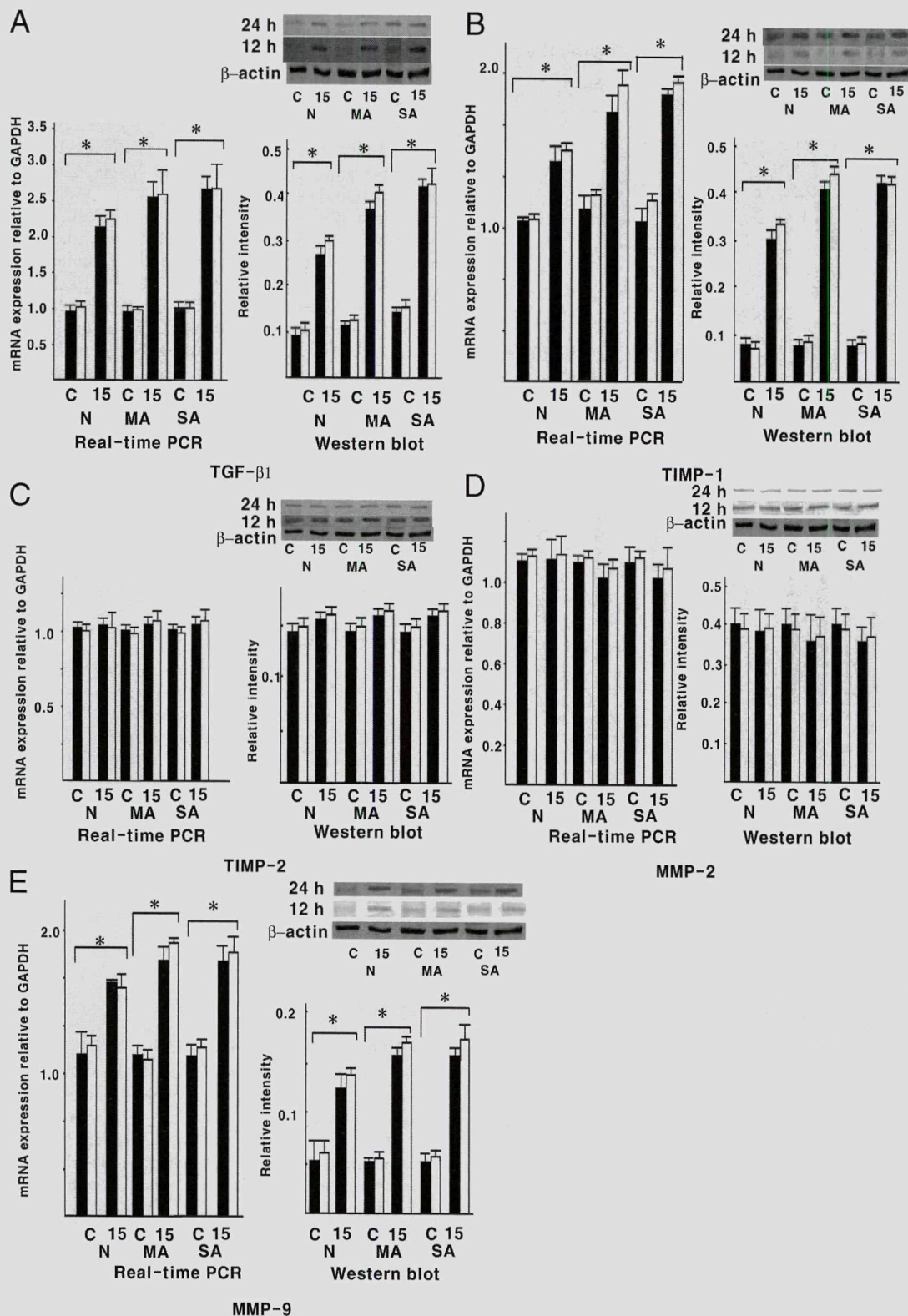


Figure 2. Real-time polymerase chain reaction (PCR) and Western blot analysis of (A) transforming growth factor (TGF) β 1, (B) TIMP-1, (C) TIMP-2, (D) MMP-2, (E) MMP-9 expression levels in cultured fibroblasts derived from normal (N), mild (MA), and moderate/severe persistent allergic nasal mucosa (SA) after stimulation with YKL-40 (15 ng/mL). *Statistical significance among the expression levels of target molecules after stimulation with YKL-40, compared with nonstimulated fibroblast. Black bar, the expression levels of target molecules after stimulation for 12 hours ($n = 6$); white bars, stimulation for 24 hours ($n = 6$). (C) Nonstimulated fibroblasts ($n = 6$; $*p < 0.05$).

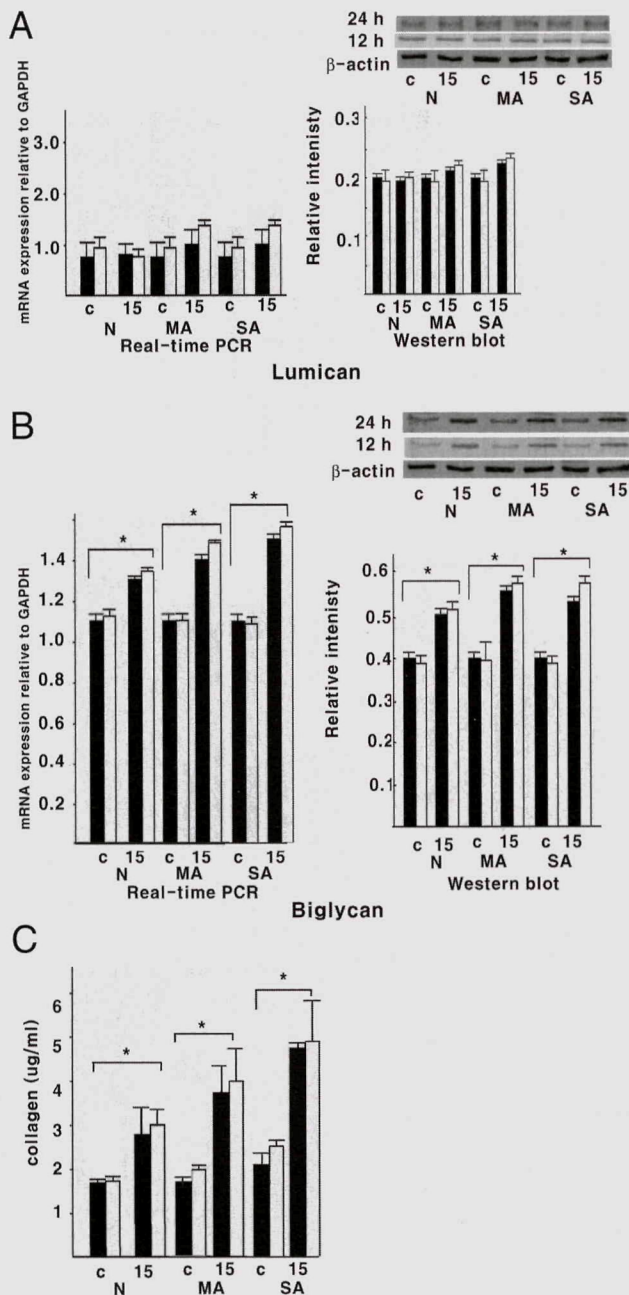


Figure 3. Real-time polymerase chain reaction (PCR) and Western blot analysis of (A) lumican and (B) biglycan, (C) concentration of collagen in cultured fibroblasts derived from normal (N), mild (MA), and moderate/severe persistent allergic nasal mucosa (SA) after stimulation with YKL-40 (15 ng/mL). *Statistical significance among the expression levels of target molecules ($P < 0.05$). Black bar, the expression levels of target molecules after stimulation for 12 hours ($n = 6$); white bars, stimulation for 24 hours ($n = 6$). (C) nonstimulated fibroblasts ($n = 6$).

Immunohistochemical and Western Blot Analysis

Immunohistochemical staining was performed using a peroxidase-labeled streptavidin biotin technique. Briefly, paraffin sections were cut and placed on poly-L-lysine-coated glass slides. Sections were incubated overnight at 4°C with a 1:500 dilution of anti-YKL-40 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The color was developed using 3,3'-diaminobenzidine. The negative immunohistochemical control procedure included omission of the primary

antibodies and replacement of the primary antibodies with normal goat IgG in appropriate concentrations.

For Western blot analysis, total protein (50 µg) extracted from frozen tissue, cultured epithelial cells, or fibroblasts was resuspended in SDS sample buffer and was separated on 12% SDS-PAGE and transferred to Immobilon (Millipore, Bedford, MA). The blots were blocked for 1 hour at room temperature with PBS-T containing 1% skim milk and then incubated with a 1:1000 dilution of anti-YKL-40 antibody (Quidel), anti-TGF-β1 antibody, anti-decorin antibody, anti-biglycan antibody, anti-lumican antibody, anti-MMP2, anti-MMP9, anti-TIMP1, or anti-TIMP2 antibody (Santa Cruz Biotechnology, Inc.) in PBS-T overnight at room temperature. Antibody reactions were detected using the ECL detection kit (GE Health Care Amersham Biosciences, Piscataway, NJ), followed by detection of chemiluminescence on x-ray film. As an internal control, the expression of β-actin was analyzed in parallel blots using the β-actin antibody (Santa Cruz Biotechnology, Inc.). The intensity of detected bands was quantified using Scion image Beta 4.0.2 (Scion Corp., Frederick, MD). The relative intensities of each protein signal were obtained by dividing the intensities of each protein signals by the β-actin signals.

Enzyme-Linked Immunosorbent Assay

The concentration of YKL-40 in cell culture supernatants were measured ELISA kits (Quidel) according to the manufacturer's instructions. Experiments were performed in triplicate experiments for each sample.

Statistical Analyses

Statistical analyses were performed using SPSS for Windows (Version 16.0.0; SPSS, Chicago, IL). The age and total IgE differences in the three groups were calculated by one-way ANOVA, and the Bonferroni *post hoc* correction was applied. The duration of rhinitis between the mild and moderate/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 and the patients with mild or moderate/severe persistent allergic rhinitis, including the data evaluated by real-time PCR, Western blot analysis, and ELISA. The level of significance was set at $p < 0.5$. Mann-Whitney's *post hoc* tests were performed for comparing differences between different groups.

RESULTS

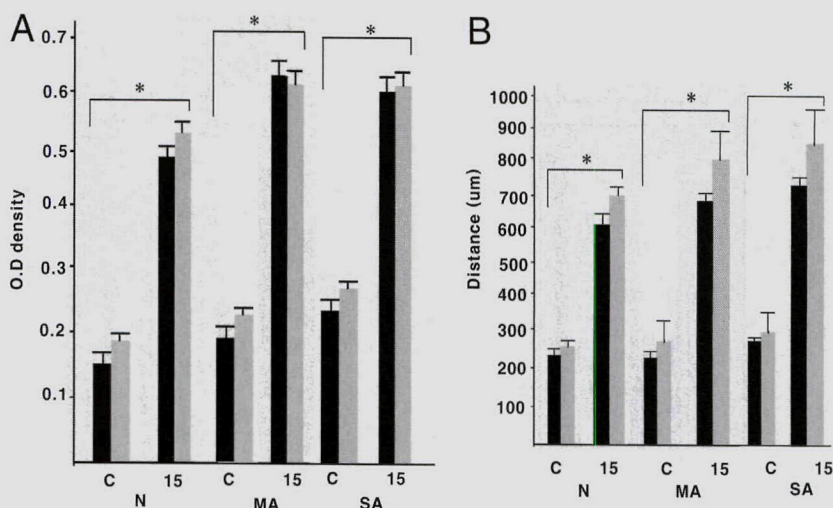
Clinical Characteristics of the Subjects

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

Expression Levels and Distribution Pattern of YKL-40 in Nasal Mucosa

Levels of YKL-40 mRNA and protein were determined in healthy, mild, and moderate/severe persistent allergic nasal mucosa using real-time PCR and Western blot (Fig. 1, A and B). The expression levels of YKL-40 mRNA and protein were significantly increased in nasal mucosa of patients with mild and moderate/severe persistent allergic nasal mucosa compared with healthy nasal mucosa. However, there was no significant difference between mild and moderate/severe persistent allergic nasal mucosa (Fig. 1, A and B).

Figure 4. (A) Effect of YKL-40¹⁵ on fibroblasts proliferation derived from normal (N), mild (MA), and moderate/severe persistent allergic nasal mucosa (SA) after stimulation with YKL-40 (15 ng/mL) after 12 and 24 hours. (B) Effect of YKL-40 (15 ng/mL) on fibroblast migration after 12 and 24 hours. Black bars indicate optical density and migration after stimulation for 12 hours (n = 6); gray bars indicate stimulation for 24 hours (n = 6). *Statistical significance ($p < 0.05$) versus control group.



Immunohistochemical staining showed a homologous pattern in healthy, mild, and moderate/severe persistent allergic nasal mucosa (Fig. 1 C). In healthy and allergic nasal mucosa, YKL-40 was exclusively localized to the superficial epithelium, submucosal glands, and vascular endothelium. In addition, many YKL-40⁺ infiltrating cells were found in both types of allergic nasal mucosa (Fig. 1 C). These findings were completely inhibited in the negative immunohistochemical control procedure including omission of the primary antibodies and replacement of the primary antibodies by normal goat IgG in appropriate concentrations (data not shown).

Production of TGF- β 1, MMP2, MMP9, TIMP1, TIMP2, Small Leucine-Rich Proteoglycans, and Collagen from Fibroblast in Response to YKL-40 Stimulation *In Vitro*

To evaluate the possible effects of YKL-40 on the remodeling of nasal mucosa in allergic rhinitis, the effects of YKL-40 stimulation on the ability of fibroblasts to produce TGF- β 1, MMP2, MMP9, TIMP1, TIMP2, and small-leucine-rich proteoglycans *in vitro* were examined. Unstimulated cultured fibroblasts expressed TGF- β 1, MMP2, MMP9, TIMP1, and TIMP2 mRNA and protein at baseline levels (Fig. 2). Concerning the expression levels of MMP2, MMP9, TIMP1, and TIMP2 in unstimulated fibroblasts, MMP2 level was expressed with the greatest intensity. TIMP1 and TIMP2 levels were lower than MMP2, and MMP9 was least expressed. Fibroblasts stimulated with YKL-40 contained higher levels of TGF- β 1, TIMP1, and MMP9 when compared with unstimulated controls. However, MMP2 and TIMP2 levels were not changed after stimulation (Fig. 2). These results were similarly observed in cultured fibroblasts derived from normal, mild, and moderate/severe persistent allergic rhinitis mucosa (Fig. 2).

The expressions of decorin mRNA and proteins out of small leucine-rich proteoglycans were not expressed in stimulated or unstimulated fibroblasts, whereas lumican and biglycan were detected in unstimulated fibroblasts. After treatment of fibroblasts with YKL-40, the expression levels of lumican were unchanged, but biglycan was up-regulated in comparison with unstimulated fibroblast (Fig. 3, A and B). These results were similarly found in cultured fibroblasts derived from normal, mild, and moderate/severe persistent allergic rhinitis mucosa (Fig. 3, A and B). Furthermore, the production of collagen measured in supernatants after stimulation with YKL-40 was, respectively, increased in normal, mild, and moderate/severe persistent allergic nasal mucosa, compared with unstimulated fibroblasts (Fig. 3 C).

Effect of YKL-40 on Proliferation and Migration of Fibroblast

MTT assay revealed that optical density was increased in groups stimulated with 5, 15, and 30 ng/mL of YKL-40 for 12 and 24 hours, respectively, compared with untreated control groups. The groups incubated with 5, 15, and 30 ng/mL of YKL-40, respectively, showed increased migration ability compared with untreated controls. These results were similarly observed in cultured fibroblasts derived from normal, mild, and moderate/severe persistent allergic rhinitis mucosa. Figure 4 represents the results stimulated with 15 ng/mL of YKL-40.

Regulation of YKL-40 Expression by Cytokine Stimulation in Cultured Epithelial Cells

We examined the expression of YKL-40 after stimulation with inflammatory cytokines in human nasal epithelial cell cultures derived from normal, mild, and moderate/severe persistent allergic nasal mucosa using real-time PCR and ELISA. Expression of YKL-40 was induced after stimulation with IL-13, IFN- γ , or TNF- α compared with nontreated control (Fig. 5). Dose-response analysis showed that IL-13, IFN- γ , or TNF- α could induce YKL-40 expression after 12–24 hours at the concentrations of 10 ng/mL. However, other cytokines did not induce substantial YKL-40 expression compared with non-stimulated control (Fig. 5). These results were similarly observed in cultured epithelial cells derived from normal, mild, and moderate/severe persistent allergic rhinitis mucosa (Fig. 5).

DISCUSSION

The expression of YKL-40 in serum is strongly associated with disease severity and is regarded as a useful prognostic or diagnostic marker and therapeutic targets in various diseases.¹² Indeed, serum YKL-40 levels in patients with allergic rhinitis positively correlate with symptom severity.¹⁹ However, the cellular source and functional role of YKL-40 in allergic nasal mucosa and molecular processes controlling the induction of YKL-40 remain poorly understood. In the present study, we characterized the expression levels and distribution of YKL-40 in normal, mild, and moderate/severe persistent allergic nasal mucosa. The expression of YKL-40 was up-regulated in mild and moderate/severe persistent allergic nasal mucosa, where it was localized to the superficial epithelium, submucosal glands, and vascular endothelium. Fibroblasts stimulated with YKL-40 showed increased proliferation and migration and produced TGF- β 1, TIMP1, MMP9, the small leucine-rich proteoglycans, particularly biglycan,

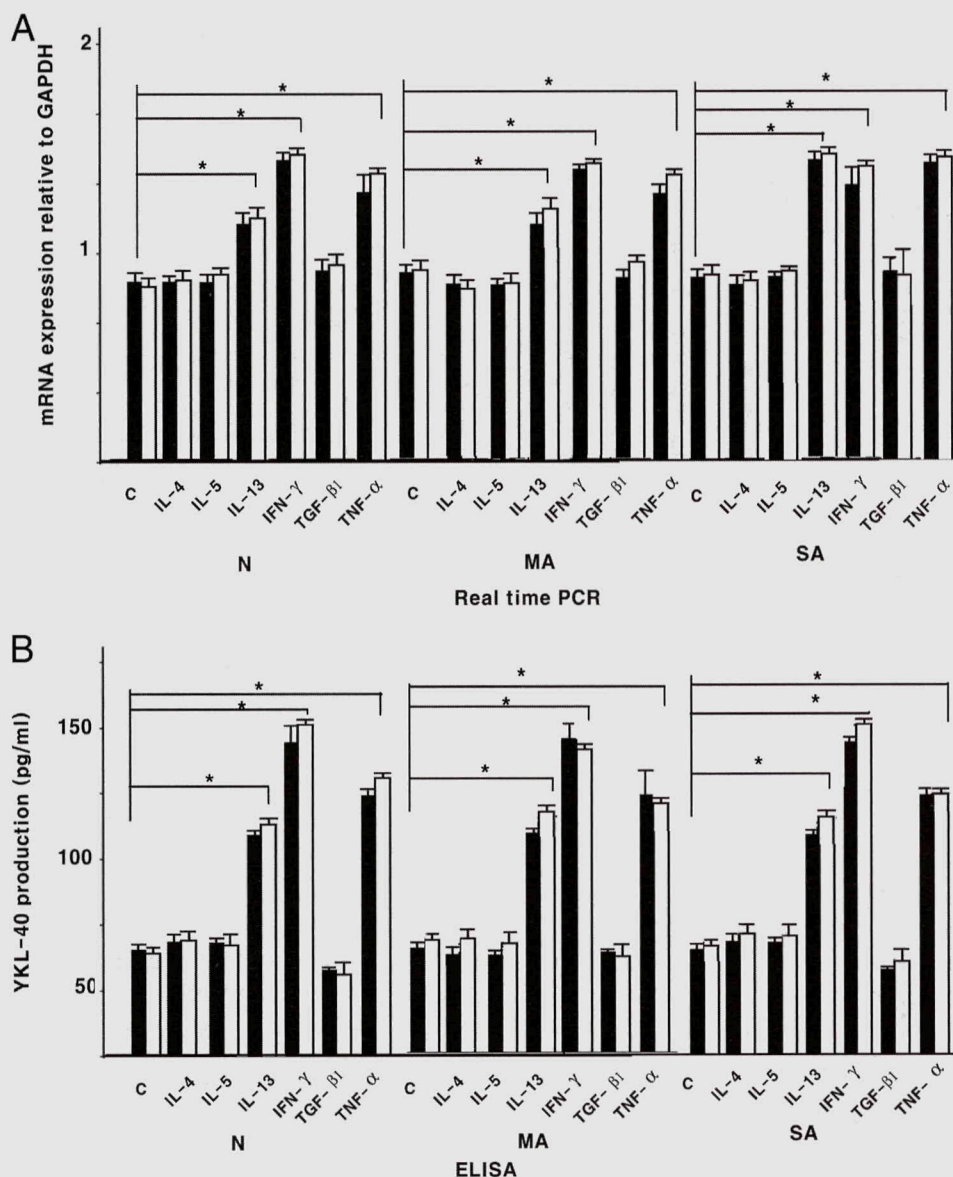


Figure 5. Real-time polymerase chain reaction (PCR) and ELISA analysis of YKL-40 production in cultured epithelial cells derived from normal (N), mild (MA), and moderate/severe persistent allergic nasal mucosa (SA), which were treated with indicated cytokines for 12 or 24 hours. Black bar, the expression levels of target molecules after stimulation for 12 hours ($n = 6$); white bars, stimulation for 24 hours ($n = 6$; $*p < 0.05$).

and collagen. Furthermore, the expression of YKL-40 in nasal mucosa may be regulated by cytokines relevant to the pathogenesis of allergic rhinitis. Collectively, these data suggest that YKL-40 is up-regulated in allergic rhinitis, possibly contributing to the remodeling of nasal mucosa.

Although mammals can not synthesize or metabolize chitin, a number of chitinolytic enzymes such as chitotriosidase and acidic mammalian chitinases or chitin-binding proteins including YKL-40 have been identified in mammals and have been reported to be a family of mediators increasingly associated with infection and T-cell-mediated inflammation.^{12,20,21} YKL-40 is known to a chitinase-like protein because it can bind to chitin but is unable to degrade it.¹² Increased secretion of chitinases and YKL-40 is crucial in murine models of allergic inflammation and is closely associated with Th2-dominated pathological conditions including infection and asthma.^{12,15} We previously showed that AMCase and chitotriosidase are up-regulated in allergic rhinitis where they are expressed in the superficial epithelium, submucosal glands, and inflammatory cells infiltrating into the nasal mucosa.²² Presently, similar to AMCase and chitotriosidase, the chitinase-like protein, YKL-40 was up-regulated in mild and moderate/severe persistent

allergic nasal mucosa. Enhanced expression of YKL-40 was found in surface epithelium, submucosal gland, vascular endothelium, and various inflammatory cells infiltrating into the nasal mucosa. These results are consistent with the findings that a variety of inflammatory cells and structural cells such as neutrophils, monocytes, epithelial cells, and endothelial cells endogenously express this protein.^{23–25} Serum YKL-40 levels have been correlated with symptom severity in patients with allergic rhinitis, suggesting the role of YKL-40 in the pathogenesis of allergic rhinitis.¹⁹ Collectively, these data suggest that chitinase and YKL-40 may act as a family of mediators in human allergic rhinitis. However, further studies are needed to clarify the role of YKL-40 in allergic rhinitis.

YKL-40 potentially promotes the growth of fibroblasts, stimulating its fibril formation with the inhibition of collagen degradation, and has been associated with pathological conditions characterized by tissue inflammation and remodeling, such as chronic obstructive pulmonary disease and asthma.^{13,16,25–27} In the present study, the increased proliferation and migration of nasal fibroblasts were induced by YKL-40 stimulation, supporting the other studies that YKL-40 stimulates the proliferation of connective tissue cells (fibroblasts, chondrocytes, and synovial cells) in a dose-dependent manner.^{13,28} On the

other hand, fibroblasts are the major and exclusive producers of ECM in airways. They secrete collagen as well as glycoproteins. TGF- β is a pleiotropic cytokine that has been implicated in the remodeling process in patients with inflammatory diseases.^{29,30} We have previously reported that the altered distribution pattern of collagen and proteoglycans could potentially modulate the remodeling of nasal mucosa in mild and moderate/severe persistent allergic rhinitis.⁶ In this respect, we speculated that once generated within the nasal mucosa, YKL-40 may promote the generation of factors involved in the remodeling of nasal mucosa in allergic rhinitis. Therefore, we tested the possibility that the stimulation of fibroblasts with YKL-40 could induce the production of TGF- β 1, MMP, and TIMP and proteoglycans. The current results show that YKL-40 is involved in the production of TGF- β 1, TIMP1, and MMP9 but has no effects on the induction of TIMP2 and MMP2 in cultured fibroblasts.

The overexpression and activation of MMPs and TIMPs can induce ECM deposition and degradation in the respiratory tract.^{31,32} Nevertheless, results from a few studies that have examined the expression of MMPs and TIMPs in nasal mucosa of allergic rhinitis have been inconsistent. In one study, the presence of large amounts of TIMP1 and TIMP2 in nasal mucosa and small amounts of MMP1, -2, -3, and -9 in the same samples were reported, and their expression levels, compared with normal nasal mucosa, were not up-regulated in allergic rhinitis.⁷ Another reported that the MMP9 levels were much higher than in the control group.³³ Several studies using an inhalation challenge with allergens also showed inconsistent results, with increased expression of TIMP2 and MMP9 expression after inhalation challenge.^{34,35} The reasons for the discrepancy among these data are not clear at present, and the role of MMPs and TIMPs in prediction of remodeling of nasal mucosa in allergic rhinitis remain impossible to interpret with accuracy. Nevertheless, the current study indicates that TIMP1 and MMP9 levels are increased in fibroblasts treated with YKL-40, compared with control, although the expression levels of TIMP2 and MMP2 are not changed after stimulation. Therefore, our data suggest that YKL-40 may contribute to the remodeling of nasal mucosa through the activation of fibroblasts and production of TIMP1 and MMP9. In addition, the expression levels of TIMP1 in nonstimulated and stimulated fibroblasts are higher than those of MMP9. Therefore, a high concentration of TIMP1 can lead to an increased deposition of ECM in allergic rhinitis. Our data are consistent with results showing that YKL-40 stimulates alveolar macrophages and fibroblasts to release inflammatory and fibrogenic mediators such as MMP9.²⁹

Small leucine-rich proteoglycans, which are composed of decorin, lumican, and biglycan, contribute to tissue remodeling of various organs.^{36,37} In our previous study, collagen, lumican, and biglycan levels were increased in the nasal mucosa of patients with allergic rhinitis, showing prominent deposition in the subepithelial layers and around the submucosal glands.⁶ Based on these data, we tried in the present study to evaluate whether fibroblasts treated with YKL-40 produce small leucine-rich proteoglycans such as decorin, lumican, and biglycan and collagen. We found that the stimulation of fibroblasts with YKL-40 results in increased expression of biglycan and collagen, suggesting that YKL-40 may regulate the production of biglycan and collagen, at least in fibroblast cells, contributing to the remodeling of nasal mucosa. YKL-40 can stimulate total proteoglycan synthesis by chondrocytes in a dose-dependent manner.²⁸ Taken together with these data, the present observations provide more evidence that YKL-40 may contribute to the remodeling of nasal mucosa in allergic rhinitis, playing an important role in the deposition of ECM.

The regulation of YKL-40 expression has been previously studied in a variety of cell types but remains somewhat controversial. Recent studies conducted on freshly isolated chondrocytes showed that IL-6 up-regulates TIMPs and stimulates the YKL-40 production.³⁸ Another study indicated that IL-6 is able to stimulate YKL-40 release, providing evidence of a link between inflammation and increased levels of

YKL-40.³⁹ In allergic rhinitis, numerous cytokines, including proinflammatory cytokines such as TNF- α , and Th1 cytokine such as IFN- γ , are characteristically produced by activated cells present in target cells, including T cells and mast cells, and eosinophils. Moreover, allergic inflammation is associated with a shift in the balance between cytokines produced by Th1 and Th2 cells toward Th2 predominance. Th2 cytokines, such as IL-4, IL-5, and IL-13, induce the production of IgE and eosinophilia.⁴⁰ Therefore, we speculated that the elevated levels of cytokines in individuals with allergic rhinitis may result in induced elevation of YKL-40. Of the mediators presently tested, IL-13, IFN- γ , and TNF- α induced the YKL-40 expression in the epithelial culture model. IL-4, IL-5, and TGF- β 1 had no significant effects on YKL-40 production by cultured epithelial cells. Therefore, the current results suggest that YKL-40 expression in allergic rhinitis can be regulated differentially by different cytokines. TNF- α stimulates YKL-40 synthesis in alveolar macrophage from smokers with chronic obstructive pulmonary disease.²⁷ However, in cartilage, TNF- α inhibits YKL-40 production.³⁸ In an animal model, the ability of IL-13 to induce tissue inflammation was markedly diminished in the absence of YKL-40.²⁵ Taken together, the dichotomous results concerning the effects of cytokines on YKL-40 expression may be caused by differences in tissue condition between the various studies in the literature. In addition, we found that inflammatory cells infiltrating into the allergic nasal mucosa show the immunoreactivities against YKL-40, compared with normal nasal mucosa. Therefore, it is possible that increased expression levels of YKL-40 in inflammatory sinus mucosa are the result of inflammatory cells influx. This suggestion is supported by the results that after allergen provocation in atopic asthma, total inflammatory cells, and eosinophils increased in induced sputum, where increased levels of YKL-40 were observed.⁴¹ Additional studies to elucidate the molecular mechanisms linking to YKL-40 expression will be needed for further our understanding of the regulatory molecules.

In conclusion, the expression levels of YKL-40 are increased in mild and moderate/severe persistent allergic nasal mucosa, compared with normal nasal mucosa. Stimulation of fibroblasts with YKL-40 induces the production of TGF- β 1, TIMP1, MMP9, and biglycan, increasing the proliferation and migration. Furthermore, IL-13, IFN- γ , and TNF- α induce the increased expression of YKL-40 in epithelial cells. The data provide evidence of the potential role of YKL-40 in the remodeling of nasal mucosa in allergic rhinitis that contributes to further understanding of its pathophysiology.

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