

ORIGINAL ARTICLE

# YKL-40 in Induced Sputum After Allergen Bronchial Provocation in Atopic Asthma

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## ■ Abstract

**Background:** Serum chitinase-like proteins such as YKL-40 in asthmatic patients are known to positively correlate with disease severity but controversy remains regarding their role. The allergen bronchial provocation test (ABPT) can induce allergic airway inflammation in individuals with atopic asthma.

**Objective:** To evaluate the induction and kinetics of YKL-40 during allergen-induced airway inflammation in atopic asthmatics.

**Methods:** Thirteen patients were enrolled from May to November 2008. They all underwent ABPT with *Dermatophagoides farinae* crude extract. Induced sputums (IS) and serum were collected 3 times: 7 days before ABPT (baseline), 7 hours after ABPT, and 24 hours after ABPT. We examined the cytology of induced sputum (IS) and measured levels of YKL-40, interleukin (IL) 4, IL-5, IL-13, IL-33, tumor necrosis factor (TNF)  $\alpha$ , and eosinophilic cationic protein (ECP) in IS and/or serum.

**Results:** Following ABPT, total inflammatory cells, eosinophils, and neutrophils increased in a time-dependent manner in IS. YKL-40 levels were increased in IS but not in serum at 7 or 24 hours after ABPT ( $P=.011$  and  $P=.006$ , respectively). Similarly to YKL-40, IL-5 and ECP levels were also increased in IS at 7 and 24 hours after ABPT ( $P=.011$  for IL-5 and  $P=.006$  for ECP). Overall, YKL-40 levels were well correlated with ECP levels in IS ( $\rho=0.576$ ,  $P<.001$ ).

**Conclusions:** YKL-40 levels increased immediately in IS but not in the serum of atopic asthmatics. The correlation between YKL-40 levels and ECP in IS suggests that YKL-40 may play a pathophysiologic role in human atopic asthma.

**Key words:** Chitinase-like protein. Allergen bronchial provocation test. Asthma. Allergic inflammation. Induced sputum.

## ■ Resumen

**Antecedentes y objetivo:** Las proteínas séricas "chitinase-like" como YKL-40 podrían correlacionarse en pacientes asmáticos con la severidad de la enfermedad. El objetivo de este estudio es evaluar la producción de YKL-40 y su cinética tras la provocación bronquial con alérgeno (PBA), una prueba que induce una potente respuesta inflamatoria en sujetos afectados de asma bronquial alérgica.

**Metodos:** Se incluyeron en el estudio un total de 13 pacientes asmáticos en un periodo comprendido entre mayo y noviembre del 2008. a todos ellos se les realizó la PBA utilizando un extracto de *Dermatophagoides farinae*. Se obtuvieron muestras de suero y de esputo inducido (EI) en tres ocasiones — 7 días antes de la PBA (situación basal), y 7 y 24 horas tras la PBA. Se realizó un análisis citológico de las muestras de EI, y en el sobrenadante se cuantificaron los niveles de YKL-40, interleuquina 4 (IL) 4, IL-5, IL-13, IL-33, factor de necrosis tumoral (TNF)  $\alpha$ , y de la proteína catiónica del eosinófilo (ECP). Estas determinaciones también se realizaron en las muestras de suero.

**Resultados:** tras la PBA se observó un incremento marcado en función del tiempo en el total de células inflamatorias, eosinófilos y neutrófilos en las muestras de EI. Los niveles de YKL-40 también se incrementaron en el EI a las 7 y 24 horas de la PBA ( $p=0,011$  y  $p=0,006$ , respectivamente), mientras que no se modificaron los niveles séricos. Los niveles de IL-5 y ECP en EI también se incrementaron significativamente ( $p=0,011$  para la IL-5 y  $p=0,006$  para la ECP) tanto a las 7 como a las 24 horas de la PBA. Se constató una correlación entre los niveles YKL-40 y ECP en EI ( $\rho=0,576$ ,  $p<0,001$ ).

**Conclusiones:** Los niveles de YKL-40 se incrementan de forma inmediata tras la provocación en EI de pacientes asmáticos alérgicos, y no así en el suero. La correlación presente entre los niveles de ECP e YKL-40 en esputo, sugeriría un posible papel de YKL-40 en la fisiopatología del asma alérgica.

**Palabras clave:** YKL-40. Proteínas "chitinase-like". Provocación bronquial con alérgeno. Asma. Inflamación alérgica. Esputo inducido.

## Introduction

YKL-40, also called human cartilage glycoprotein 39 (HcGP-39), is a chitinase-like protein that was first discovered in synovial fluid; its murine homolog is known as BRP-39 [1,2]. YKL-40 is known to be associated with a number of human inflammatory diseases, such as rheumatoid arthritis [3], osteoarthritis [4], sarcoidosis [5], inflammatory bowel disease [6], hepatic fibrosis [7], and several malignant tumors [8]. YKL-40 levels were recently shown to be related to endocrine diseases including obesity [9], diabetes mellitus, and atherosclerosis [10]. In addition, elevated YKL-40 levels have been reported in the serum, sputum [11], and bronchoalveolar lavage (BAL) fluid [12] of patients with chronic obstructive pulmonary disease (COPD). The relationship between YKL-40 and various human diseases suggests that this protein could be a useful diagnostic and prognostic biomarker.

The activation of type 2 helper (T<sub>H</sub>2) lymphocytes and their subsequent secretion of interleukin (IL) 13 are well-established key responses in the pathogenesis of allergic airway inflammation [13]. Induction of IL-13 secretion results in the alternative activation of alveolar macrophages, and these activated macrophages have been reported to play a role in the propagation of inflammation [14]. Respiratory epithelial cells, alveolar macrophages, and neutrophils are known to secrete YKL-40 in human airways, and hence, YKL-40 has been attributed a role in airway inflammation [15]. Furthermore, an animal model study has shown that YKL-40 may play a critical role in various steps of asthma pathogenesis by increasing allergic sensitization, propagating T<sub>H</sub>2 inflammation, and regulating inflammatory cell apoptosis [16].

In this study, we evaluated dynamic changes in YKL-40 levels in induced sputum (IS) following an allergen bronchial provocation test (ABPT) with house dust mite extract (*Dermatophagoides farinae* [DF]) in the hope that our findings would shed light on the mechanisms underlying atopic asthma [17].

## Methods

### Patients

Thirteen patients who visited Severance Hospital to have their allergic asthma presumptively diagnosed by a physician were enrolled between May and November 2008. The Institutional Review Board of Yonsei University approved the study (IRB no. 4-2012-0002) and informed consent was obtained from all patients. According to international guidelines [18], the diagnosis of allergic asthma was based on clinical history, symptoms and signs, the methacholine bronchial provocation test, eosinophil count in IS, and specific immunoglobulin (Ig) E measurements by skin prick testing (Allergopharma) and/or the ImmunoCAP test (Pharmacia). DF-specific IgE was documented in all patients. All participants underwent ABPT with DF extract. IS and serum were collected 3 times: 7 days before ABPT (baseline) and at 7 and 24 hours after the first dose of allergen administration (Table 1).

### Allergen Bronchial Provocation Test

DF whole body extract (1:100 w/v, containing 3.4 µg/mL of *Der f* 1) cultured at the Institute of Allergy, Yonsei University was used for ABPT. To prepare the inhalation material, a 1:100 extract of DF in phosphate buffered saline (PBS) was filtered with an antiseptic filter (Millipore) and diluted 10-, 25-, and 100-fold with isotonic saline. A baseline pulmonary function test was performed using a pneumotachometer system with a Lilly head (MasterScreen system, Erich Jaeger Co.), which measures maximum expiratory flow volume. The spirometric flow-volume curve was obtained according to international criteria [19].

ABPT was performed using the method described by Spector et al [20], with some modifications. First, DF solution (1:10 000 w/v) containing 34 ng/mL of *Der f* 1 was administered using a handheld nebulizer (Devilbiss

Table 1. Demographic and Laboratory Data of Patients Included in the Study

No.	Sex	Age, y	Diagnosis	Treatment	Smoking, pack-years	PC <sub>20</sub> , mg/mL	DF sIgE kU/L	DF SPT, mm
1	M	19	BA	LTRA	Never	2.43	18.7	8.0
2	M	21	BA, AR	None	0.2	8.31	31.5	32.0
3	M	22	BA	None	Never	5.0	11.2	11.0
4	M	19	BA, AR, AD	None	Never	0.84	>100.0	8.5
5	M	19	BA	ICS/LABA	Never	10.0	14.3	7.5
6	M	19	BA	None	Never	0.12	25.4	7.0
7	M	20	BA, AR	None	Never	3.15	38.7	30.5
8	M	20	BA	None	1.0	0.28	2.38	5.0
9	M	19	BA, AR, AD	ICS/LABA	Never	1.25	5.53	6.5
10	M	19	BA, AR, AD	ICS/LABA	Never	7.94	26.2	ND
11	M	18	BA	ICS/LABA	Never	1.30	54.3	12.0
12	M	18	BA	None	Never	1.25	53.2	ND
13	M	20	BA	ICS/LABA	Never	7.64	1.72	ND

Abbreviations: AD, atopic dermatitis; AR, allergic rhinitis; BA, bronchial asthma; DF, *Dermatophagoides farinae*; ICS, inhaled corticosteroid; LABA, long-acting β<sub>2</sub>-agonist; LTRA, leukotriene receptor antagonist; ND, not done; PC<sub>20</sub>, methacholine provocation concentration for 20% decrease in forced expiratory volume in the first second; sIgE, specific immunoglobulin E; SPT, skin prick test.

646, Devilbiss Healthcare Inc.) with tidal breathing for 2 minutes. After administration, spirometric data including forced expiratory volume in the first second (FEV<sub>1</sub>) were obtained. A positive test was defined as a 20% decrease in FEV<sub>1</sub> from baseline. If a negative result was observed, the next concentration was administered up to 1:1000 diluents. After the final dose was administered for 2 minutes, serial spirometry was performed at 10, 20, and 30 minutes and at 1, 2, 4, 5, 6, and 7 hours. A significant decrease in FEV<sub>1</sub> 60 minutes after the final DF inhalation was defined as an early response, while a significant decrease after 4 hours was defined as an isolated late response. If both early and late responses were observed, a dual response was recorded. Patients who did not show any significant spirometric changes after 7 hours of spirometric monitoring were considered nonresponders.

### Induced Sputum Analysis

We used a previously reported method for sputum induction [21] with some modifications for processing [22]. Briefly, we checked each patient's peak flow rate (PFR) with a mini-Wright peak flow meter. After administration of bronchodilator (salbutamol 200 µg, 2 puffs), sputum induction was performed with 3% hypertonic saline inhalation for 10 minutes. PFR was checked again if the patient complained of dyspnea. Sputum induction was stopped when a 20% decrease in PFR from baseline was observed. To prevent contamination with squamous epithelial cells, sputum expectoration and collection were performed after cleaning of the oral and nasal cavities.

Induced sputum samples were processed immediately as follows. Mucus components were collected separately from saliva, and the weight and volume of selected samples measured. Then, an equal amount of 0.1% dithiothreitol (Sigma) was added to each mucus sample, vortexed for 15 seconds, and incubated in a warm water bath (37°C) with shaking for 20 minutes. The samples were centrifuged and the supernatant was collected for subsequent measurements and stored at -70°C. Remnant cell components were resuspended in PBS and filtered through a nylon mesh with 60-µm pores to eliminate cell clots. A 10-µL aliquot of resuspension solution was mixed with 10 µL of 0.4% trypan blue solution to count total cell numbers and measure cell viability using a Neubauer counting chamber. Samples were then diluted in PBS to a concentration of 1×10<sup>6</sup> cells/mL, and cytoSpin was performed at 450 rpm for 6 minutes to prepare cytology slides. After staining the slides with Wright's stain, over 400 inflammatory cells were counted differentially under a light microscope.

### Measurement of YKL-40, Eosinophil Cationic Protein, and Cytokines

YKL-40 levels were measured in IS and serum by enzyme-linked immunosorbent assay (ELISA) (Quidel) (detection limit, 20 ng/mL) [15].

Eosinophil cationic protein (ECP) was also measured in IS with the commercially available ImmunoCAP assay (Phadia). The levels of other inflammatory cytokines, including interleukin (IL) 4 (detection limit, 10 pg/mL), IL-5 (detection limit, 3.0 pg/mL), IL-13 (detection limit, 32 pg/mL), IL-33 (detection limit, 28 pg/mL), and tumor necrosis factor (TNF) α (detection limit, 0.5 pg/mL) were also checked in IS and serum by ELISA (R&D Systems).

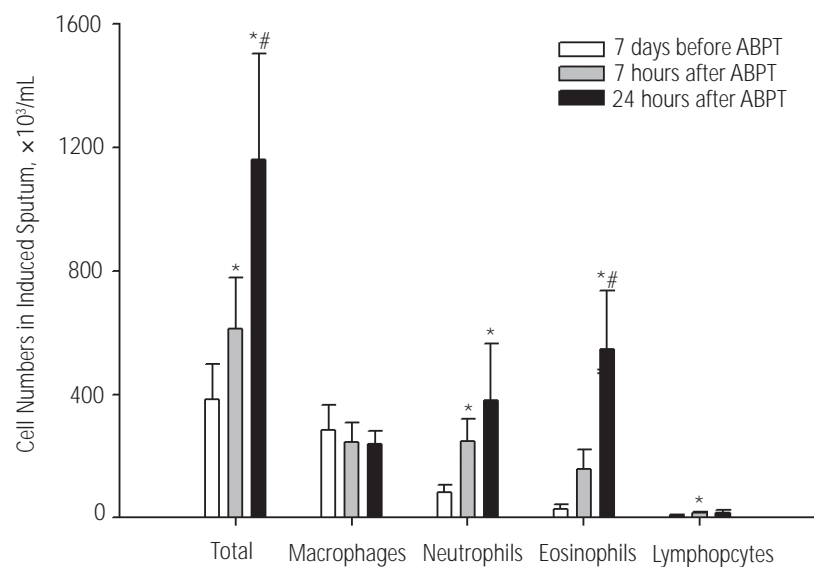
### Statistical Analysis

All data were analyzed using SPSS statistical software (version 12.0). The Wilcoxon test was used for comparing medians, and the Spearman rank test was used for correlation analysis. Data are presented using means (SD).

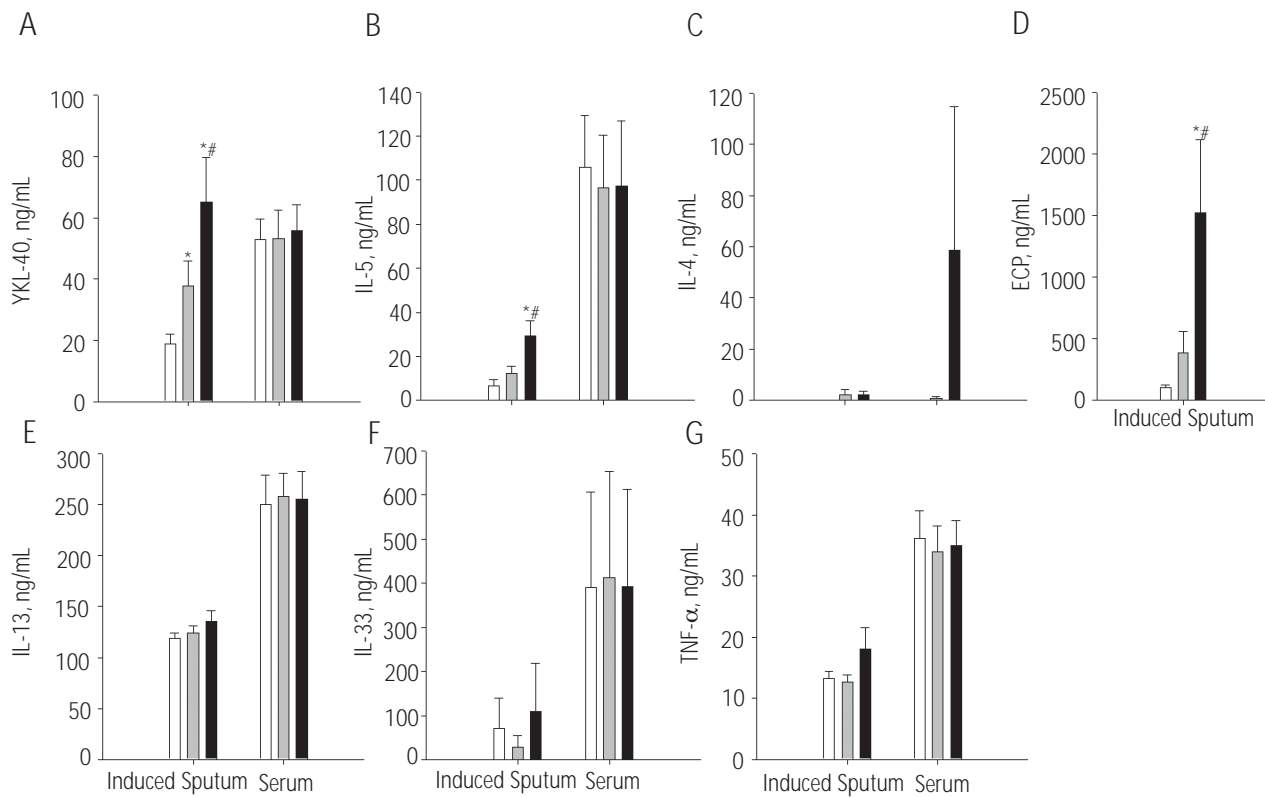
## Results

### Cytological Analysis of Induced Sputum

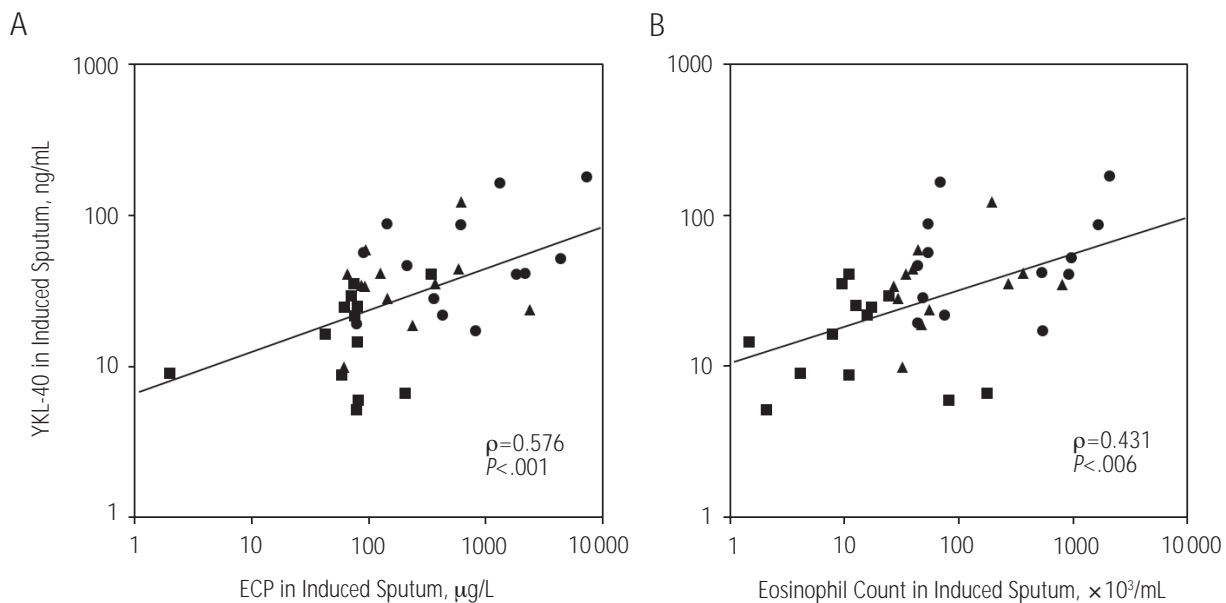
Total cell count increased in a time-dependent manner after ABPT (baseline, 384.7 [407.3] ×10<sup>3</sup> cells/mL; 7 hours, 611.9 [593.1] ×10<sup>3</sup> cells/mL; 24 hours, 1160.5 [1232.8] ×10<sup>3</sup> cells/mL). Significant increases were also detected after this test for eosinophil count (baseline, 29.1 [49.4] ×10<sup>3</sup> cells/mL; 7 hours, 157.9 [223.7] ×10<sup>3</sup> cells/mL; 24 hours, 546.0 [685.7] ×10<sup>3</sup> cells/mL) and neutrophil count (baseline, 84.0 [81.9] ×10<sup>3</sup> cells/mL; 7 hours, 247.2 [269.8] ×10<sup>3</sup> cells/mL; 24 hours, 381.4 [658.5] ×10<sup>3</sup> cells/mL) (Figure 1).



**Figure 1.** Inflammatory cell counts in induced sputum. Total cell, neutrophil, and eosinophil counts increased significantly in a time-dependent manner after the allergen bronchial provocation test (ABPT). All data are represented by mean (SEM). \*:  $P < .05$  compared to baseline, #:  $P < .05$  compared to 7 hours after ABPT.



**Figure 2.** Enzyme-linked immunosorbent assay results of YKL-40, inflammatory cytokines, and eosinophil cationic protein (ECP) by immunoCAP in induced sputum (IS) and serum. YKL-40 (A), IL-5 (B), and ECP (D) levels increased significantly in a time-dependent manner in IS but not in serum after the allergen bronchial provocation test (ABPT). Interleukin (IL) 4 (C), IL-13 (E), IL-33 (F), and tumor necrosis factor (TNF)  $\alpha$  (G) levels were not changed in IS or serum. All data are represented as means (SEM). \* $P < .05$  compared to baseline; # $P < .05$  compared to 7 hours after ABPT.



**Figure 3.** Scatter plots of YKL-40 vs eosinophil cationic protein (ECP) (A) and eosinophil count (B) in induced sputum for all paired samples. Each plot shows a substantial positive correlation. Closed squares represent baseline, closed triangles represent 7 hours after the allergen bronchial provocation test (ABPT), and closed circles represent 24 hours after ABPT.

Table 2. Correlation Analysis of YKL-40 and Other Variables in Induced Sputum

	ECP	IL-5	Macrophages	Neutrophils	Eosinophils
Correlation coefficient (rho)	0.576	0.304	-0.031	0.245	0.431
P value	<.001	.060	.850	.132	.006

Abbreviations: ECP, eosinophil cationic protein; IL, interleukin

### Changes in YKL-40 Levels in IS and Serum After ABPT

YKL-40 levels in IS were increased significantly at 7 and 24 hours after ABPT (baseline, 18.7 [11.8] ng/mL; 7 hours, 37.8 [29.7] ng/mL; 24 hours, 65.0 [52.9] ng/mL). However, there were no significant differences in serum levels (baseline, 53.0 [23.7] ng/mL; 7 hours, 53.1 [34.4] ng/mL; 24 hours, 55.9 [30.6] ng/mL) (Figure 2A).

### Changes in Cytokine and ECP Levels in IS and Serum After ABPT

Only IL-5 levels were significantly increased in IS at 24 hours after ABPT (baseline, 6.6 [9.6] pg/mL; 7 hours, 12.1 [13.1] pg/mL; 24 hours, 29.3 [24.7] pg/mL) (Figure 2B). IL-4 was not detected in the samples and there were no significant differences in IL-13, IL-33, or TNF- $\alpha$  levels in either IS or serum (Figure 2C, E, F, G). ECP levels were significantly increased in IS at 7 hours and 24 hours after ABPT (baseline, 96.4 [85.5]  $\mu$ g/mL; 7 hours, 380.9 [634.5]  $\mu$ g/mL; 24 hours, 1520.8 [2126.5]  $\mu$ g/mL) (Figure 2D).

### Correlation Analysis of YKL-40 Levels and Other Variables in Induced Sputum

Eosinophil count and ECP levels were positively correlated with YKL-40 levels in IS ( $\rho=0.431$ ,  $P=.006$  for eosinophil count;  $\rho=0.576$ ,  $P<.001$  for ECP levels) (Figure 3). IL-5 levels and other inflammatory cell counts showed no significant correlation (Table 2).

## Discussion

Chitin, which consists of *N*-acetylglucosamine polymers, is the second most abundant polysaccharide in nature. It is an important component of the cell wall of fungi, the exoskeleton of shellfish and insects, and the sheath of nematodes [23]. Thus, human beings must have chitinase to protect their bodies from many kinds of pathogens containing chitin. Chitinase is a highly conserved endogenous enzyme found in insects, mammals, and many other organisms. The 2 active chitinases in humans, acidic mammalian chitinase and chitotriosidase, both have enzymatic activity for the degradation of chitin [23].

YKL-40 is known as a chitinase-like protein because it can bind to chitin but is unable to degrade it. Associations between YKL-40 and allergic diseases have been reported. Chupp et al [15] described a positive correlation between elevated serum YKL-40 levels and asthma severity, and the

same group also reported that a genetic variation of the *CHI3L1* gene encoding YKL-40 was significantly associated with serum YKL-40 levels and asthma susceptibility [24]. Furthermore, a single nucleotide polymorphism in the promoter region of the *CHI3L1* gene has been associated with the atopic phenotype in children [25].

In this study, we observed an increment in YKL-40 levels in IS but not in serum at 24 hours after ABPT. The main cell sources of YKL-40 are bronchial epithelial cells, macrophages [26], and neutrophils [27]. ABPT is used to evaluate allergen-induced inflammation in the laboratory. Like other insects and spiders, house dust mites contain chitin in their exoskeleton. However, YKL-40 elevation in IS might be an independent response to chitin, as chitin-free allergens also promote YKL-40 elevation in BAL fluid after administration of the segmental bronchial challenge test [28].

Our results strongly suggest that allergen exposure may rapidly induce YKL-40 secretion from epithelial cells, macrophages, and other cells. The same pattern of increase observed for YKL-40 was also observed for IL-5 and ECP in IS, suggesting that YKL-40 may increase allergic eosinophilic inflammation by inducing the accumulation and activation of local dendritic cells and reducing apoptosis of  $T_H2$  cells [16]. In this study, we used dithiothreitol for mucolysis of IS and therefore cannot exclude the possibility that this may have affected the measurement of cytokines by ELISA. IL-13 plays a critical role in relation to YKL-40 as it can induce YKL-40 expression from epithelial cells and macrophages, and IL-13-induced fibrosis may be mediated by YKL-40 [29]. Further studies are needed to better understand the relationship between YKL-40 and allergic inflammation (exacerbated by allergens, viruses, smoking, or pollution) and tissue remodeling.

Our results are not consistent with those of Kuepper et al [28], who reported that YKL-40 levels in BAL fluid were markedly increased 24 hours but not 10 minutes after segmental allergen challenge. In the present study, we also measured YKL-40 in serum but did not find any changes in YKL-40 levels after ABPT, thus, diminishing the possibility of using serum YKL-40 as a biomarker for uncontrolled asthma. In contrast to our results, Kuepper et al reported that YKL-40 in serum was definitely increased 24 hours after allergen challenge, and other investigators have also reported that serum YKL-40 levels are higher in exacerbated asthma [30] and severe asthma [15]. However, contradictory results have also been reported [31].

The present study has limitations. First, we did not check YKL-40 levels in serum or IS after more than 24 hours following ABPT. Second, long-lasting uncontrolled inflammation can induce the persistent release of YKL-40



from bronchial epithelial cells, which could affect serum levels of YKL-40. ABPT is a good model for studying allergen-induced allergic inflammation. However, viral infection and pollution, in addition to allergens, are also equally important aggravating and/or etiologic factors in real-life asthma. Thus, ABPT does not sufficiently reflect real clinical situations of asthma exacerbation. Some investigators have studied sputum YKL-40 levels in asthma and COPD patients and shown that YKL-40 and chitinase are increased in COPD patients but not in asthmatics compared to healthy controls [11,32]. Therefore, whether or not YKL-40 is clinically related to the pathogenesis of asthma remains to be elucidated.

In conclusion, YKL-40 levels in IS increased during the early phase of allergic inflammation induced by allergen exposure. This finding suggests that YKL-40 may play an important role in the initiation of allergic inflammation and that it may be useful as an acute phase biomarker in allergen-induced asthma exacerbations.

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