

Original article

Evaluation of cytokine mRNA in induced sputum from patients with allergic rhinitis: relationship to airway hyperresponsiveness

Background: Although airway hyperresponsiveness (AHR) is a characteristic feature of asthma, it is also frequently present in allergic rhinitis (AR). However, the pathogenesis of AHR is unclear and the roles of cytokines in the airway have not been well established in AR. We sought to compare cytokine mRNA levels in the sputum of AR patients with or without AHR and those of asthma patients, and to evaluate whether differences in cytokine levels are associated with the development of an abnormal airway response and the absence of respiratory symptoms in AR patients with AHR.

Methods: Airway cells were obtained by sputum induction from 18 AR patients with AHR, 58 AR patients without AHR, and 27 asthma patients. Airway cell cytokine levels, interleukin (IL) -4, IL-5, IL-13, vascular endothelial growth factor (VEGF), and interferon- γ (IFN- γ), were studied at the mRNA level by RT-PCR.

Results: Vascular endothelial growth factor and IL-5 mRNA levels were significantly higher in AR patients with AHR than in AR patients without AHR, but these were lower than those of asthmatic patients. Eosinophils were significantly higher in AR patients with AHR and in asthmatic patients than in AR patients without AHR. Interleukin-4, IL-13, and IFN- γ levels were not elevated in AR patients with or without AHR vs asthma patients.

Conclusions: These findings suggest that VEGF and IL-5 can be important determinants of the development of AHR in AR patients and that lower levels of other cytokines may be associated with the absence of asthmatic symptoms in AR patients with AHR.

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Key words: airway hyperresponsiveness; allergic rhinitis; mRNA; sputum; vascular endothelial growth factor.

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Accepted for publication 9 August 2007

Asthma and allergic rhinitis (AR) are frequently encountered comorbid conditions (1). The prevalence of AR in asthma patients has been reported to be as high as 80% and the prevalence of asthma in AR has been reported to range from 10% to 43% (1, 2). The concepts of 'one airway, one disease' and of 'allergic rhinobronchitis' have been suggested to emphasize the link between asthma and AR (3, 4). Currently, AR is generally considered to be a risk factor of asthma development (2, 5, 6).

Airway hyperresponsiveness (AHR) is a characteristic feature of asthma. It describes the tendency of bronchi to narrow excessively and readily in response to various provocative stimuli (7). However, a significant proportion of AR patients have AHR (8, 9). In one study, 39% of AR patients were found to show AHR to methacholine (9). In nonallergic rhinitis with eosinophilia syndrome (NARES), 46% of patients with NARES but without histories of respiratory symptoms had measurable AHR (10). Moreover, the simultaneous presence of AHR and

AR was also found to be associated with a high probability of developing asthma (11).

Interleukin-13, IL-5, eosinophils, and vascular endothelial growth factor (VEGF) are considered to be causative factors of AHR in asthma (12–16). T helper (Th) 2 cytokines (IL-4, IL-5, IL-13) play important roles in allergic pathophysiology, and interferon- γ (IFN- γ) (a Th1 cytokine) may cause severe airway inflammation (17, 18). However, the determinants of AHR in AR have not been identified, and it is not known whether AHRs of asthma and AR have the same pathophysiologies. To our knowledge, few studies have evaluated cytokine levels in the induced sputum of AR patients with AHR. Analysis of induced sputum is a noninvasive means of obtaining viable cells from the lower airways for evaluating airway inflammation (19). Moreover, sputum can be used to measure cytokine levels at the protein, and mRNA levels. Several studies have examined the relationship between sputum cytokine mRNAs and airway inflammation in

asthma (18, 20, 21), and it is evident that analyses of sputum cytokine mRNA levels provide a useful, noninvasive means of exploring the chronic inflammation associated with asthma and other lung disorders (21).

There are many possible explanations for the apparent absence of symptoms in patients with increased AHR (7), e.g. the cutoff value for AHR is too high, intermittent AHR, poor perception of symptoms, too little inflammation/remodeling, weak diurnal variations in expiratory flows, and others. However, the reasons for the absence of respiratory symptoms are unclear.

The purpose of this study was to compare cytokine mRNA levels in the induced sputum of AR patients with or without AHR and asthmatic patients, and to evaluate whether differences in cytokine expressions are associated with the development of AHR and the absence of respiratory symptoms in AR patients with AHR.

Methods

Subjects

One hundred and three patients attending the outpatient allergy clinic of Seoul National University Hospital were enrolled. All AR patients had perennial rhinorrhea, sneezing, and/or nasal obstruction, but did not have asthma symptoms. All AR patients with AHR ($n = 18$) had a provocative concentration of methacholine causing a 20% fall in forced expiratory volume in 1 s (PC_{20}) of ≤ 16 mg/ml. All denied any past or present symptoms suggestive of asthma including intermittent dyspnea, wheezing, or a recurrent cough, and any respiratory infection during the month preceding this study. Fifteen of the 18 AR subjects with AHR were non-smokers, one was a current smoker, and two were ex-smokers. The AR patients without AHR ($n = 58$) had normal spirometry and airway responsiveness ($PC_{20} > 16$ mg/ml), no history of respiratory diseases, no current or past symptom suggesting asthma, and no respiratory infection during the month before enrollment. Forty-four of the 58 AR subjects without AHR were nonsmokers, six were current smokers, and eight were ex-smokers.

Asthmatic patients ($n = 27$) with mild or moderate persistent symptoms received inhaled corticosteroid and/or intermittent inhaled bronchodilator. They were not taking systemic steroids and had not experienced a respiratory infection or an asthma exacerbation during the month preceding this study. Nineteen of the 27 asthma patients were nonsmokers, four were current smokers, and four were ex-smokers. Asthma was defined based on clinical symptoms according to the criteria suggested by the American Thoracic Society (22). This study was approved by the Institutional Review Board of Seoul National University Hospital and all participants provided informed consent.

Procedures

Skin prick tests were performed using a battery of 16 common inhalant allergens including *Dermatophagoides farinae*, *D. pteronyssinus*, two-spotted mite, Tyrophagus, cat dander, cockroach, ragweed pollens, mugwort pollens, mixed trees pollens, mixed grasses pollens, and mixed fungi spores (Allergopharma, Reinbek, Germany), as previously described (23). Atopy was defined as the presence of at least one positive response to a skin prick test (wheal diameter of allergen/wheal diameter of histamine ≥ 1 and a mean

wheal size ≥ 3 mm). Inhalant allergens were divided into five categories: house dust mite, tree pollen, grass pollen, animal danders, and molds. Atopic indices were defined as the numbers of positive inhalant allergen categories (0–5) by skin prick testing (24). All AR patients were sensitive to at least one indoor allergen (house dust mite, animal danders, or molds). They could be exposed to allergens they were sensitive to during the study procedures. Methacholine bronchial provocation test (MBPT) was performed as previously described (23). All participants discontinued their medications at least 1 week before MBPT. Methacholine AHR is defined as PC_{20} , and was regarded as positive when ≤ 16 mg/ml.

Sputum induction

After pretreatment with 400 μ g inhaled salbutamol (VentolinTM, GlaxoSmithKline, Brentford, UK), 4.5% hypertonic saline was administered using an ultrasonic nebulizer with output set at 4.5 ml/min (Omron Co., Tokyo, Japan), and this saline administration was repeated up to four times at intervals of 5 min unless a fall in forced expiratory volume in 1 s (FEV_1) of $>10\%$ occurred, in which case the procedure was stopped.

Sputum processing

Dithiothreitol (DTE, 0.01 M) was added to sputum samples, vortex mixed, shaken for 20 min at room temperature, filtered through a 52 mm nylon gauze to remove debris and mucus, and then centrifuged at $450 \times g$ for 10 min. Cell pellets obtained were resuspended in phosphate buffer saline to a volume equal to the original sputum plus DTE. Total cell counting was carried out using a hemocytometer and cell concentrations were then adjusted to 1.0×10^6 cells/ml. Cytospins were prepared by adding 60 μ l of this cell suspension to Shandon II cytocentrifuge cups (Shandon Southern Instruments, Sewickley, PA, USA) and spun for 5 min at 42 g. Slides were stained with Diff Quik solution (Sysmex Co., Kobe, Japan). Leukocytes, bronchial epithelial cells and squamous cells were counted. To determine cell differentiation, 300 nucleated cells per slide were counted and these counts were expressed as percentages of intact round nucleated cells, excluding squamous epithelial cells. Sputum samples that contained more than 20% squamous epithelial cells were not analyzed.

RNA extraction

Cells were isolated by centrifugation and then stored in 1 ml Trizol (Gibco, Carlsbad, CA, USA) at -80°C . RNA was extracted using phenol/chloroform extraction and ethanol precipitation method according to the manufacturer's instructions (Gibco). RNA contents of solutions were quantified by optical density at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The ratio (260/280 nm) was always more than 1 : 8.

RT-PCR

Total RNA (2 μ g) was used to synthesize first strand cDNA from oligo-dT-primed RNA by RT using reverse transcriptase (Promega, Madison, WI, USA). Briefly, total RNA mixtures were incubated with 200 U-MLV reverse transcriptase, 20 U RNasin, 0.5 mM dNTPs, 25 μ g/ml primer dT, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂, in a final volume of 50 μ l for 60 min at 42°C . Reaction mixtures for the PCR of β -actin and cytokines

contained 2 µl of sample, 0.5 mM dNTP, 0.5 U *Taq* polymerase, 1 µg of each primer, 50 mM Tris-HCl, 3 mM MgCl₂, 75 mM KCl 0.01% gelatin in a final volume of 100 µl. Polymerase chain reaction was performed on a thermal cycler (Personal Cycler Biometra, Gottingen, Germany). Reaction times for β-actin were 95°C/50 s, 58°C/50 s, and 72°C/50 s for 33 cycles, followed by a 5 min extension at 72°C. Reaction times for IL-4, IL-5, IL-13, VEGF and IFN-γ were 95°C/30 s, 55–58°C/30 s, and 72°C/30 s for 35–40 cycles, followed by a 10 min extension at 72°C. Primer sequences for β-actin were 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' for the downstream primer, and 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3' for the upstream primer. Primer sequences for cytokines were as follows: for IL-4, 5'-CTG CAA ATC GAC ACC TAT TA-3' for the 5' primer, and 5'-GAT CGT CTT TAG CCT TTC-3' for the 3' primer; for IL-5, 5'-CTG AGG ATT CCT GTT CCT GT-3' for the 5' primer, and 5'-CAA CTT TCT ATT ATC CAC TC-3' for the 3' primer; for IL-13, 5'-ACA GCT GGC ATG TAC TGT GC-3' for the 5' primer, and 5'-CAA CTT TCT ATT ATC CAC TC-3' for the 3' primer; for IFN-γ, 5'-GGT CAT TCA GAT GTA GCG GA-3' for the 5' primer, and 5'-GCG TTG GAC ATT CAA GTC AG-3' for the 3' primer, for VEGF, 5'-GAG TGT GTG CCC ACT GAG GAG TCC AAC-3' for the 5' primer, and 5'-CTC CTG CCC GGC TCA CCG CCT CGG CTT-3' for the 3' primer. Amplifications resulted in products of 200–600 bp, as determined by electrophoresis in 2% Tris-Acetate-EDTA (TAE) agarose gel containing bromide ethidium. Cytokine concentrations were measured using BioID image analysis software (Vilber-Lourmat, Marne-La-Vallee, France). All results were normalized vs β-actin to compensate for differences in cDNA amounts.

Statistical analysis

Results are expressed as means ± SEM for age, atopic index, FEV₁ and forced vital capacity (FVC). The PC₂₀ values are expressed as geometric means ± SEM. Kruskal-Wallis and Mann-Whitney *U*-tests were used to compare cytokine mRNA levels and percentages of sputum eosinophils. Correlations were examined using Spearman's nonparametric test or Pearson's test as appropriate. Results were considered significant when *P*-values were <0.05. Data were analyzed using SPSS v. 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Subjects characteristics

Allergic rhinitis patients without AHR were aged from 14 to 66 years (mean 35.2 ± 1.9), AR patients with AHR 14–66 years (mean 31.4 ± 3.7), and asthmatic patients 18–65 years (mean 45.5 ± 2.9). Asthmatic patients are older than the other two patient groups (*P* < 0.05, Table 1). Atopic indices were 1.26 ± 0.07 in AR patients without AHR, 1.78 ± 0.25 in AR patients with AHR, and 1.38 ± 0.25 in asthmatic patients. No statistical differences among the atopic indices of the three groups were found (*P* > 0.05, Table 1). Geometric means of PC₂₀ were 2.99 ± 0.2 in AR patients with AHR and 2.01 ± 0.3 in asthmatic patients. The PC₂₀ of patients with AR with AHR was higher than that of asthmatic patients (*P* < 0.05, Table 1). Basal FEV₁ was lower in

Table 1. The characteristics of study subjects

	AR–	AR+	Asthma	<i>P</i> -value
Number	58	18	27	
Sex				
Male	39	12	15	
Female	19	6	12	
Age (years)	35.2 ± 1.9	31.4 ± 3.7	45.5 ± 2.9*	<0.05*
PC ₂₀ (mg/ml)		2.99 ± 0.2	2.01 ± 0.3	<0.05
Atopic index (N+)	1.26 ± 0.07	1.78 ± 0.25	1.38 ± 0.25	NS
FEV ₁ (%)	96.4 ± 1.7†	87.7 ± 1.9	87.4 ± 2.5	<0.05†
FVC (%)	96.5 ± 1.7	89.9 ± 2.0	92.3 ± 2.7	NS
Smoking (nonsmoker/ smoker/ex-smoker)	44/6/8	15/1/2	19/4/4	

Results are expressed as means ± SEM for age, forced expiratory volume in 1 s (FEV₁), forced vital capacity (FVC), and atopic index, and provocative concentration of methacholine causing a 20% fall in FEV₁ (PC₂₀) values are expressed as geometric means ± SEM. Results were considered significant when *P*-values were <0.05.

AR–: allergic rhinitis (AR) patients without airway hyperresponsiveness (AHR); AR+: AR patients with AHR; NS: nonsignificant.

*Asthma vs AR with AHR or without AHR.

†AR without AHR vs AR with AHR or asthma.

AR patients with AHR and in asthmatic patients than in AR patients without AHR (*P* < 0.05, Table 1). No significant differences were observed among the three groups in terms of basal FVC values (*P* > 0.05, Table 1).

Comparisons of cytokine mRNA levels and cell numbers in induced sputum

Vascular endothelial growth factor mRNA levels were detected in all the three groups, but not in three AR patients without AHR. Vascular endothelial growth factor mRNA levels were significantly higher in AR patients with AHR than in AR patients without AHR (*P* = 0.031), but these were lower than in asthmatic patients (*P* < 0.001, Fig. 1).

Interleukin-5 mRNA levels were detectable in all AR patients with AHR and in almost all asthmatic patients (except two with no or <1.3% sputum eosinophilia). Interleukin-5 mRNA expressions were undetectable in 40% of AR patients without AHR. Interleukin-5 mRNA levels were higher in AR patients with AHR than in AR patients without AHR (*P* = 0.005), but these were both lower than in asthmatic patients (*P* < 0.001, Fig. 1). Interleukin-4, IL-13, and IFN-γ mRNA levels were not different in AR patients with or without AHR (*P* > 0.05), and all were lower than in asthmatic patients (*P* < 0.001, Figs 2 and 3).

Significantly higher percentages of sputum eosinophils were found in AR patients with AHR and in asthmatic patients than AR patients without AHR (*P* < 0.001, Fig. 3). No significant difference was found between AR patients with AHR and asthmatic patients in terms of percentages of sputum eosinophils (*P* > 0.05, Fig. 3). No correlation was found between percentages of sputum

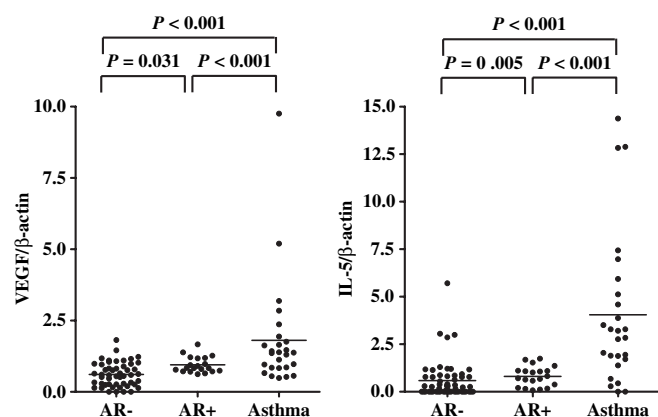


Figure 1. Comparisons between the three study groups in terms of vascular endothelial growth factor (VEGF) and interleukin-5 (IL-5) mRNA levels in induced sputum. Levels of VEGF and IL-5 mRNA were higher in allergic rhinitis (AR) with airway hyperresponsiveness (AHR) than in AR without AHR. They were higher in asthmatic patients than AR with or without AHR. AR-: AR patients without AHR; AR+: AR patients with AHR.

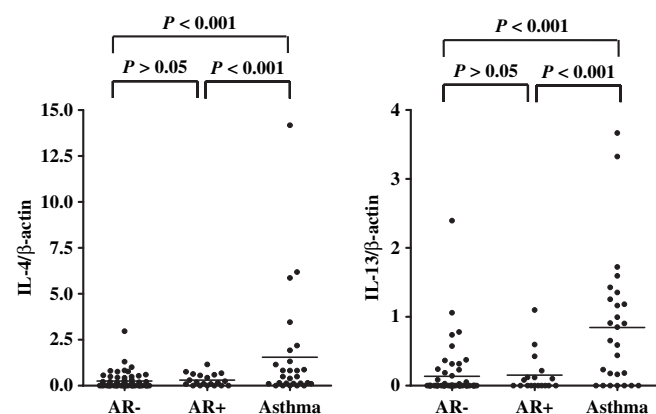


Figure 2. Comparisons between the three study groups in terms of interleukin-4 (IL-4) and IL-13 mRNA levels in induced sputum. Levels of IL-4 and IL-13 mRNA were higher in asthma than in allergic rhinitis (AR) with or without airway hyperresponsiveness (AHR). No statistical difference was found between AR with or without AHR in terms of IL-4 and IL-13 mRNA levels. AR-: AR patients without AHR; AR+: AR patients with AHR.

eosinophils and individual cytokine mRNA levels in asthmatic patients or in AR patients with AHR ($P > 0.05$, data not shown).

Discussion

Interleukin-13, IL-5, eosinophils and VEGF are considered to be important factors of AHR in asthma (12, 15, 16). Interleukin-13 has been found to be necessary and

sufficient for allergic asthma expression in several studies (12, 17, 18, 25). Interleukin-13 can induce AHR independently of eosinophils and IgE (12), and in eotaxin/IL-5 double knock-out mice (26). Interleukin-13 levels were also found to be higher in the bronchial submucosa of asthmatic patients than in eosinophilic bronchitis (EB) patients (25). The role of IL-5 and eosinophils in AHR is somewhat controversial. Sputum IL-5 mRNA levels in asthmatic patients have been found to correlate with AHR (18), and eosinophils were found to contribute to AHR in asthma and in AR (27). However, anti-IL-5 antibody decreased peripheral blood and sputum eosinophil levels but does not affect AHR in asthmatic patients (28). Airway hyperresponsiveness is the cardinal difference between EB and asthma. Eosinophilic bronchitis patients without AHR were found to have similar IL-5 and eosinophil levels in sputum as asthmatic patients, but IL-13 levels were higher in asthmatic patients than in EB patients (17).

Elevated levels of VEGF have been detected in induced sputum and bronchial tissues and can induce AHR in asthma (29, 30). Moreover, VEGF could induce an asthma-like phenotype through IL-13-dependent and -independent pathways. In IL-13 knock-out mice, VEGF was found to induce AHR, tissue and bronchoalveolar lavage inflammation, neovascularization, myocyte hyperplasia, subepithelial fibrosis, and dendritic cell alterations (15). Airway remodeling in asthma can be caused by innate immunity and VEGF may be associated with these alterations (15).

In the present study, VEGF and IL-5 mRNA levels were found to be higher in the sputum of AR patients with AHR than in AR patients without AHR, although these levels were lower than in asthmatic patients. On the other hand, IL-13 mRNA levels were not different in AR patients with or without AHR. Eosinophils were increased in the sputum of AR patients with AHR. Mild increases in IL-5 mRNA levels seemed to induce eosinophil accumulation, which concurs with the findings of a previous study showing that the presence of AHR in AR patients is associated with an increase in eosinophil numbers (27). We suggest that the presence of AHR in AR can be associated with the increase of VEGF and IL-5.

There are many possible explanations for the apparent absence of symptoms in patients with elevated AHR (7). In the present study, low levels of IL-4, IL-5, IL-13, VEGF and IFN- γ appeared to be associated with the absence of respiratory symptoms in AR patients with AHR. Interleukin-4, IL-5, IL-13, VEGF, and IFN- γ are important in the pathophysiology of asthma. Interleukin-4 plays a role in Th2 differentiation, IL-5 recruits eosinophils from bone marrow, IL-13 plays a critical role in the pathophysiology of asthma, VEGF can cause asthma-like symptoms, and IFN- γ may cause severe airway inflammation. Interleukin-4, IL-13, and IFN- γ cytokine levels in AR patients with AHR were as low as in AR patients without AHR, indicating that Th2 and

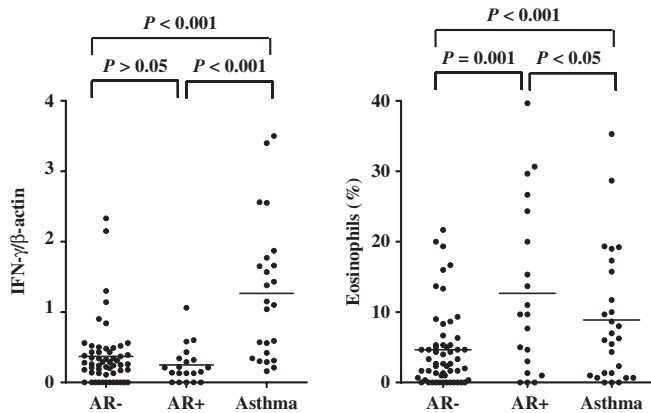


Figure 3. Comparisons between the three study groups in terms of eosinophils and interferon- γ (IFN- γ) mRNA levels in induced sputum. Levels of IFN- γ mRNA were higher in asthma than in allergic rhinitis (AR) with or without airway hyperresponsiveness (AHR). No statistical difference was found between AR with or without AHR in terms of IFN- γ mRNA levels. The percentages of eosinophils were higher in AR with AHR and in asthma than in AR without AHR. No statistical difference was observed between AR with AHR and asthma in terms of eosinophil percentages. AR-: AR patients without AHR; AR+: AR patients with AHR.

Th1 cytokines might simultaneously be reduced in the airways of AR patients with or without AHR. Moreover, VEGF and IL-5 mRNA levels in AR patients with AHR were lower than in asthmatic patients. We speculated that mild increases of VEGF and IL-5 alone could induce mild AHR but not respiratory symptoms enough if the other cytokines – IL-13, IL-4, and IFN- γ were not increased. In a previous study, VEGF induced AHR and airway inflammation independent of IL-13, but the ability of VEGF to induce mucus metaplasia was completely abrogated in the absence of IL-13 (15). Airway mucus hypersecretion can cause more sputum production, cough induction, airway narrowing, and dyspnea, which are all important asthma symptoms, and excessive mucus production can lead to significant airflow limitations in asthma (31, 32). More mucus is present in the central and peripheral airways of both chronic and severe asthmatics

than in normal controls (33). In addition, mild and moderate asthma are associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression (34). In the present study, the lack of an increase in IL-13 levels in AR with AHR may be associated with normal sputum production and normal mucus secretion may contribute to the absence of asthma symptoms in AR with AHR. Lower levels of IL-13 and of other cytokines may explain why AR with AHR differs from asthma with respect to the absence of respiratory symptoms. Further study of this issue is required.

Asymptomatic AHR is considered to be a risk factor for the development of asthma. One study showed that over a 3-year period, subjects with asymptomatic AHR had a greater frequency of asthma symptom development than normoresponsive subjects (24). Moreover, asymptomatic children with AHR are more likely to develop asthma and atopy later in life than asymptomatic children without AHR (35). Allergic rhinitis is also considered an important risk factor for asthma (5). We believe that AR and AHR can act synergistically or additively during the development of asthma (11), and the presence of both AR and AHR can increase the risk of asthma. A prospective study on the prevalence of asthma development is needed in AR with AHR.

The mechanisms underlying AHR and the absence of respiratory symptoms in AR remain poorly understood. To the best of our knowledge, this is the first study to evaluate cytokine expressions in the induced sputum of AR patients with or without AHR. Vascular endothelial growth factor and IL-5 were found to be associated with AHR in AR, and the attenuated expressions of IL-13 and of other cytokines may be associated with the absence of respiratory symptoms in AR with AHR. Further studies will be needed to confirm these observations.

Acknowledgments

This work was supported by a Korean Health 21 R&D Project grant (03-PJ10-PG13-GD01-0002) and by a Clinical Research Center Obstructive Airway Disease grant (0412-CR03-0704-0001) from the Korean Ministry of Health and Welfare.

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