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The association of plasma cytokines including VEGF with recurrent wheezing in allergic patients

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Abstract

Background: Various cytokines have been studied to determine their functions in the pathogenesis of allergic diseases and their potential as therapeutic targets, but the roles and clinical applicability of many of these cytokines still remain unclear.

Objective: We aimed to measure the plasma levels of eight cytokines known to be relevant to allergic diseases, and to determine their association with the diagnostic characteristics of allergic patients.

Methods: The levels of a panel of eight cytokines (IL-5, IL-10, IL12p70, Leptin, CXCL5/ENA-78, CCL2/MCP-1, PDGFBB, and VEGF) were measured in plasma obtained from 83 allergic patients. We investigated whether the cytokine levels differed between children and adults. Statistical analyses were then performed to examine their association with the diagnostic characteristics of allergic patients.

Results: The levels of leptin, CCL2/MCP-1, PDGFBB, and VEGF were significantly higher in adult patients with allergic rhinitis than in children. Among patients with asthma, the levels of leptin and PDGFBB were elevated in adults. PDGFBB and VEGF levels were significantly associated with asthma. Interestingly, there was a significant association between VEGF level and recurrent wheezing regardless of the analyzed conditions. The levels of VEGF and PDGFBB or CCL2/MCP-1 showed a significant increase together in the presence of recurrent wheezing in child patients.

Conclusion: The plasma levels of four cytokines, particularly VEGF, showed significant associations with some diagnostic characteristics in allergic patients. We suggested that plasma VEGF, which performs pleiotropic functions in allergic responses, could serve as a serological marker relevant to recurrent wheezing in allergic patients.

Key words: Recurrent wheezing, VEGF, CCL2/MCP-1, PDGFBB, Allergic disease

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Abbreviations

AHR	airway hyperresponsiveness
ASM	airway smooth muscle
CCL2	chemokine C-C motif ligand 2
CXCL5	chemokine C-X-C motif ligand 5
ENA-78	epithelial cell-derived neutrophil activating peptide-78
IL	Interleukin
MCP-1	monocyte chemoattractant protein 1
PDGFBB	platelet-derived growth factor BB
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor

Introduction

Cytokines are well known to be essential mediators involved in the immunological network for allergic responses, playing pivotal roles in the crosstalk among diverse cells by inducing or modulating cell activation, differentiation, and migration.[1,2] Th2 cytokines such as interleukin (IL)-4, IL-5, and IL-13 are the most fundamental cytokines in the mechanisms underlying the development and perpetuation of allergic responses, and several proinflammatory cytokines, including IL-17 and tumor necrosis factor-alpha (TNF-α), have been implicated in the development and promotion of allergic inflammation.[3,4] Vascular endothelial growth factor (VEGF) and platelet-derived growth factor BB (PDGFBB), as growth factors belonging to a wide range of cytokines, also are considered to contribute to allergic inflammation and airway remodeling.[5-7] Many cytokines are being studied to determine their specific functions in the pathogenesis of allergic diseases

and their potential as therapeutic targets.[8,9] However, studies on cytokines associated with allergic diseases are still needed because the roles and clinical applicability of many cytokines in allergic diseases remain to be determined.

Allergic diseases are complicated and heterogeneous disorders that present with chronic and recurrent symptoms due to allergic inflammation, resulting in the need for individualized treatment approaches instead of standardized management. Previous studies have reported several biomarkers for asthma from various sources such as the airway, exhaled breath, and blood.[10] In particular, it has been proposed that plasma cytokine levels could facilitate the diagnosis of allergic disease and certain phenotypes because measurement of these mediators in blood is relatively non-invasive and much easier to perform.[11] In our previous study, we found that some plasma cytokines showed different patterns between allergic and healthy children.[12]

The aims of this study were to measure the plasma levels of eight cytokines that were known to be clearly or partially relevant to allergic diseases, and to evaluate the association of these cytokines with the diagnostic characteristics of allergic patients. We also performed some statistical analyses to examine whether there were several cytokines that could show diagnostic potential as serological markers for allergic disease or certain phenotypes.

Methods

Study subjects

Eighty-three allergic patients (53 children and 30 adults) were recruited in cooperation with Asan Medical Center and Seoul National University Hospital according to the protocol and the criteria of previous study.[12] Briefly, all exhibited more than one of the four allergic diseases (asthma, allergic rhinitis, atopic dermatitis, and food allergy) defined by physician's diagnosis. Plasma samples were obtained from all participants and examined by the Multiplex assay. This study was approved by the Institutional Review Board (2015-05-CON-15-P-A), and written informed consent was obtained from each patient prior to participation.

Assessment of plasma cytokines

In plasma samples from the 83 allergic patients, the levels of a panel of eight cytokines, including two chemokines and two growth factors, were measured using a customized ProcartaPlex™ cytokine assay kit (eBioscience, Vienna, Austria) according to the manufacturer's instructions. Briefly, the capture antibody beads were first added on a pre-wetted filter plate and then incubated with samples or standard with shaking. After washing the plate, premixed detection antibodies were added and incubated in the dark for 30 minutes. After washing again, the plate was finally incubated with streptavidin-PE, which was followed by analysis with a Luminex instrument (Bio-Plex® 200 systems; Bio-rad, Hercules, CA, USA). The following cytokines were assessed: IL-5, IL-10, IL-12p70, leptin, chemokine C-X-C motif ligand 5 (CXCL5)/epithelial cell-derived neutrophil activating peptide-78 (ENA-78), chemokine C-C motif ligand 2 (CCL2)/monocyte chemoattractant protein (MCP)-1, PDGFBB, and VEGF. The data for four cytokines—IL-5, IL-10, IL-12p70, and CXCL5/ENA-78— were finally excluded from data analysis; values for IL-5, IL-10, and IL-12p70 were out of range for the detection level, while values of CXCL5/ENA-78 were unsuitable for analysis due to extensive variation among individuals.

Statistical analysis

Log-transformation was used for all statistical analyses because the distribution of cytokine levels was skewed. To determine the significance of the plasma levels of cytokines between the two groups under various conditions, the Mann-Whitney test was performed in GraphPad Prism 5 software. To assess associations among each cytokine level and diagnostic characteristics, simple and multiple linear regression analyses were performed. In multiple linear regression, variance inflation factors (VIFs) were used to check for multicollinearity. Hotelling T^2 tests were used to test for combined changes in two cytokine levels under various conditions in the analyzed groups. Linear regression and Hotelling T^2 tests were performed using R software. All statistical differences were considered significant at $p < 0.05$.

Results

Demographics of subjects

A total of 83 allergic patients (53 children and 30 adults) participated in this study. Their demographic and diagnostic characteristics are summarized in **Table 1**. The mean age of the children was 6.8 years, and 19 of them were female, whereas the mean age of the adult participants was 47.7 years, and 18 of them were women. Allergic rhinitis (69.8%)

Table 1. Demographic and diagnostic characteristics of study participants

Characteristics	Children (0-19 yrs)	Adults (20-79 yrs)	Total
Overall (n)	53	30	83
Age (Mean, Range)	6.8 (0-16)	47.7 (21-79)	21.6 (0-79)
Gender (n, %)			
Male	34 (64.2)	12 (40)	46 (55.4)
Female	19 (35.8)	18 (60)	37 (44.6)
Diagnosis (n, %)			
Asthma	29 (54.7)	28 (93.3)	57 (63.9)
Allergic rhinitis	37 (69.8)	25 (83.3)	62 (74.7)
Atopic dermatitis	31 (58.5)	7 (23.3)	38 (45.8)
Food Allergy	15 (28.3)	5 (16.6)	20 (24.0)
Comorbidities (n, %)			
Single disease only	13 (24.5)	4 (13.3)	17 (20.5)
Two allergic diseases	20 (37.7)	16 (53.3)	36 (43.4)
More than three	18 (34.0)	9 (30.0)	27 (32.5)
Recurrent wheezing (n, %)	34 (64.2)	14 (46.7)	48 (57.8)

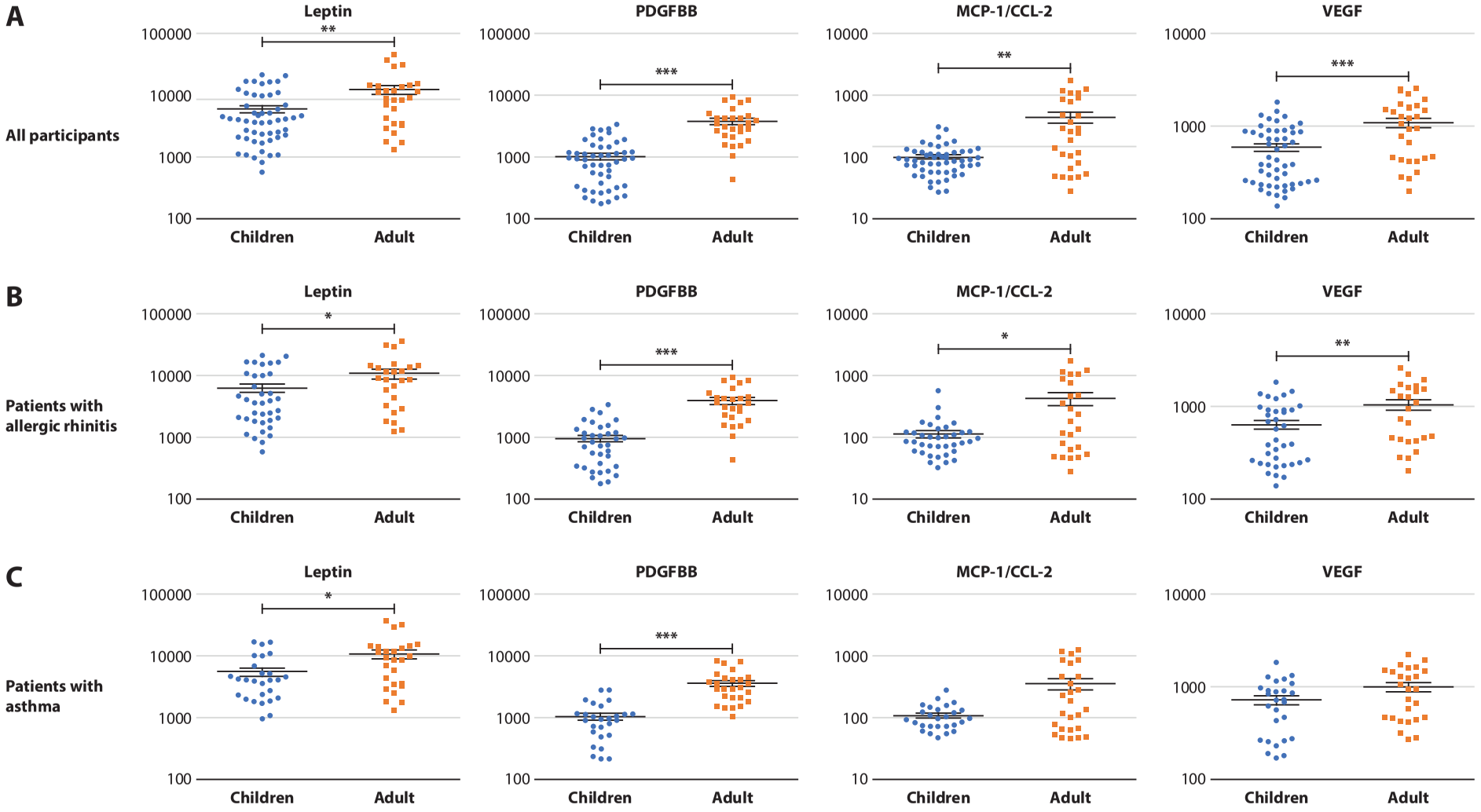

Figure 1. Comparison of plasma cytokine levels between children and adults with allergic conditions

The plasma levels of the four cytokines were analyzed simultaneously using the Multiplex kit. All cytokine concentrations were log-transformed. Between two groups: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

was the most common condition in children, and the percentages of asthma (54.7%) and atopic dermatitis (58.5%) were similar in children, while asthma (93.3%) and allergic rhinitis (83.3%) were the most prevalent conditions in adult participants. Only 20.5% of the patients had a single allergic disease; thus, most participants had two or more allergic diseases. More than half of all participants (64% in children and 46% in adults) exhibited recurrent wheezing.

Plasma cytokine levels in children and adults with allergic diseases

Only four cytokines (leptin, CCL2/MCP-1, PDGFBB, and VEGF), whose levels were within the measurement range, were considered and assessed to investigate whether their plasma levels differed between children and adult patients. All four cytokines showed significantly higher levels in adults than in children (**Figure 1A**). In assessments of patients with allergic rhinitis or asthma, which showed high incidences in both children and adults, the levels of all four cytokines were significantly higher in adult patients with allergic rhinitis like in the overall participant group. Among patients with asthma, however, only leptin and PDGFBB showed significantly higher levels in adults than in children (**Figure 1B and 1C**). There was no significant difference between children and adults with regard to the presence of comorbidities or recurrent wheezing (data not shown).

Table 2. Simple linear regression analysis of plasma cytokines in all participants

Characteristics	Leptin		CCL2/MCP-1		PDGFBB		VEGF	
	Estimate (SE)	*p* value	Estimate (SE)	*p* value	Estimate (SE)	*p* value	Estimate (SE)	*p* value
Gender								
Female	0.609 (0.21)	**0.0049**	0.424 (0.21)	**0.0467**	0.254 (0.242)	0.2970	0.129 (0.168)	0.4440
Diagnosis								
Asthma	0.29 (0.235)	0.2200	0.446 (0.225)	0.0507	0.84 (0.244)	**0.0009**	0.458 (0.174)	**0.0100**
Allergic rhinitis	0.069 (0.253)	0.7840	0.234 (0.245)	0.3410	0.157 (0.278)	0.5750	0.08 (0.193)	0.6810
Atopic dermatitis	-0.087 (0.22)	0.6940	-0.194 (0.214)	0.3650	-0.465 (0.237)	0.0534	-0.16 (0.168)	0.3430
Food allergy	0.203 (0.256)	0.4310	0.377 (0.246)	0.1300	0.269 (0.281)	0.3420	0.184 (0.195)	0.3480
Comorbidities								
Asthma only	0.155 (0.492)	0.7540	0.892 (0.465)	0.0586	0.621 (0.507)	0.2240	0.816 (0.361)	**0.0265**
Asthma + one allergic disease	0.264 (0.282)	0.3530	0.259 (0.266)	0.3340	1.04 (0.291)	**0.0006**	0.407 (0.207)	0.0525
Asthma + two allergic diseases	0.286 (0.3)	0.3430	0.45 (0.283)	0.1160	0.658 (0.309)	**0.0363**	0.372 (0.22)	0.0941
Asthma + three allergic diseases	0.491 (0.429)	0.2560	0.788 (0.405)	0.0555	0.815 (0.442)	0.0691	0.631 (0.315)	**0.0485**

Log-transformation was used for this statistical analysis. Bolded results are statistically significant at $p < 0.05$.

Table 3. Linear regression analysis of plasma cytokine levels in various subgroups of the study population according to the presence of recurrent wheezing

Linear regression	Group	Leptin		CCL2/MCP-1		PDGFBB		VEGF	
		Estimate (SE)	*p* value	Estimate (SE)	*p* value	Estimate (SE)	*p* value	Estimate (SE)	*p* value
Simple	All participants	-0.04 (0.222)	0.8560	0.279 (0.214)	0.1960	0.396 (0.241)	0.1050	0.401 (0.164)	**0.0168**
	Patients with asthma	0.227 (0.271)	0.4060	0.374 (0.268)	0.1670	0.087 (0.269)	0.7490	0.4 (0.2)	**0.0499**
	Children	-0.246 (0.267)	0.3610	0.487 (0.157)	**0.0031**	1 (0.232)	**0.0001**	0.802 (0.17)	**< 0.0001**
	Children with asthma	0.067 (0.377)	0.8600	0.622 (0.177)	**0.0016**	0.78 (0.302)	**0.0154**	1 (0.255)	**0.0005**
Multiple*	All participants	0.223 (0.202)	0.2746	0.460 (0.207)	**0.0293**	0.634 (0.211)	**0.0036**	0.490 (0.163)	**0.0035**
	Patients with asthma	0.475 (0.246)	0.0593	0.553 (0.266)	**0.0427**	0.326 (0.230)	0.1627	0.494 (0.205)	**0.0194**
	Children	0.162 (0.267)	0.5455	0.531 (0.183)	**0.0057**	0.717 (0.255)	**0.0072**	0.783 (0.203)	**0.0004**
	Children with asthma	0.516 (0.364)	0.1695	0.589 (0.207)	**0.0088**	0.527 (0.301)	0.0930	0.885 (0.292)	**0.0057**

Log-transformation was used for this statistical analysis. Bolded results are statistically significant at $p < 0.05$.
*These data were adjusted for age, asthma diagnosis, sex, and body mass index (BMI).

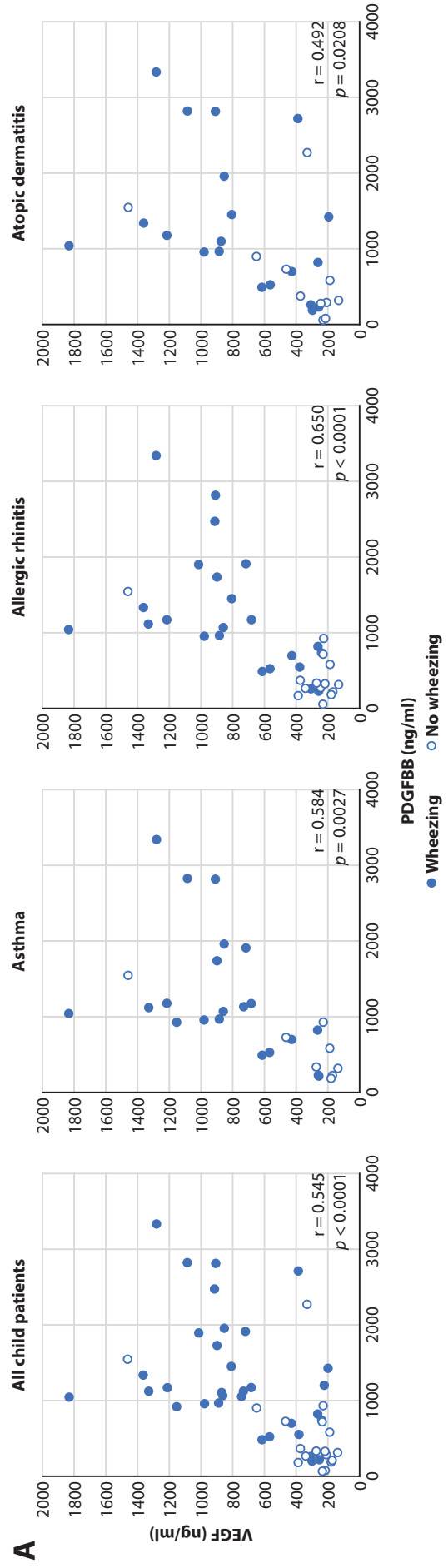

Figure 2. The correlation of each cytokine level with VEGF and recurrent wheezing
Scatterplots for each cytokine level in individuals according to the presence of recurrent wheezing in all child participants, and in the three subgroups (children with asthma, allergic rhinitis, and atopic dermatitis). PDGFBB and VEGF (A), and CCL2/MCP-1 and VEGF (B) were described respectively. The r-value from Pearson's correlation analysis and *p*-value from Hotelling T^2 tests were shown.

Statistical association between plasma cytokines and diagnostic characteristics

A simple linear regression analysis was performed to examine the association between the diagnostic characteristics and the plasma levels of each cytokine. As shown in **Table 2**, leptin and CCL2/MCP-1 levels were significantly associated with female gender, and PDGFBB and VEGF levels were significantly associated with asthma. PDGFBB and VEGF levels were also significantly associated with the occurrence of asthma comorbid with another allergic diseases. Interestingly, our results showed a significant association between VEGF levels and recurrent wheezing even when the analyzed group was changed from all participants to asthma patients, children, or children with asthma (**Table 3**). In children, CCL2/MCP-1 and PDGFBB levels were also significantly associated with recurrent wheezing regardless of the presence of asthma. After adjustment for age, asthma diagnosis, sex, and body mass index by multiple linear regression analysis, VEGF and CCL2/MCP-1 levels were significantly associated with recurrent wheezing in all of the four analyzed groups, including all participants, asthma patients, children, and children with asthma, while PDGFBB levels showed a significant association with recurrent wheezing in all participants and in children (**Table 3**).

Since VEGF levels showed significant associations under various conditions, we performed multivariate analysis to examine the correlation between the levels of each cytokine with VEGF and recurrent wheezing. We found that the levels of PDGFBB and VEGF significantly increased together in the presence of recurrent wheezing in all children, and that this finding was also observed in subgroups categorized according to the allergic diseases the children suffered from, except food allergy (**Figure 2A**). CCL-2/MCP-1, similar to PDGFBB, showed significantly increased levels together with VEGF in the presence of recurrent wheezing in all conditions in children (**Figure 2B**). However, there was no significance in the relationship of leptin with VEGF levels and recurrent wheezing in children. We then investigated whether these significant associations between the increased cytokines in children were also present among the overall study population or in adult patients; however, there was no such significance even though the individual levels of cytokines were higher in the adult group than that in the children group (data not shown).

Discussion

In this study, the plasma levels of all four cytokines—leptin, CCL2/MCP-1, PDGFBB, and VEGF—were shown to be significantly elevated in all adult participants and patients with allergic rhinitis. Leptin and VEGF also showed higher plasma levels in adult asthma patients than in children with asthma. There are several studies showing that all four of these cytokines have correlations with inflammation in common. VEGF has been reported to induce allergic inflammation and upregulation of subsequent Th2 inflammatory responses,[5,6] and CCL2/MCP-1 is known to be a potent chemotactic factor for monocytes and leukocytes, resulting in enhanced inflammation.[13] PDGFBB has been reported to show proinflammatory functions inducing the secretion of inflammatory cytokines in fibroblasts,[14] and leptin is considered to be an important mediator upregulating proinflammatory factors.[15] Immunological changes with aging have been reported to occur with accumulation of the stimulations caused by repeated infections and exposure to antigens,[16] and aging is accompanied by an increase in the levels of proinflammatory cytokines and reductions in the levels of anti-inflammatory cytokines due to immune, hormonal, and adipose changes, leading to chronic inflammation.[17] We, therefore, speculate that the differences in the levels of these cytokines between adults and children in this study could be due to age-related changes in the immune system, rather than because of their functions in allergic inflammation.

We found significant associations between plasma cytokine levels and some diagnostic characteristics by statistical analysis. In particular, our findings showed significant associations between VEGF levels and recurrent wheezing in various conditions. These results seemed to be consistent with previous studies that showed the associations between VEGF levels and wheezing. Several studies have indicated that the plasma level of VEGF is elevated in young children with repeated wheezing episodes,[18] and that its serum level is also increased in children with mycoplasma pneumonia and wheezing.[19] Studies on the genetic polymorphisms in children with recurrent wheezing have shown a significant association between the risk of wheezing and polymorphisms of VEGF-related genes,[20] although levels of VEGF released by airway epithelial cells do not differ between children with a history of wheezing and those without wheezing.[21] Most of the previous studies on recurrent wheezing were conducted on infants and children because this was considered to be a respiratory symptom that mainly manifested in these populations, but our results revealed a significant association between VEGF levels and recurrent wheezing in all participants, including children and adults, as well as after adjustment for age. We thus suggest that VEGF could have clinical applicability as a serological marker relevant to recurrent wheezing in allergic patients.

VEGF was originally known as one of the most important angiogenic factors rather than an inflammatory mediator as described above. Interestingly, the three other cytokines that showed significant associations in this study were also involved in angiogenesis via VEGF. PDGFBB can not only promote angiogenesis in the tumor environment,[22] but is also involved in the signaling pathway with VEGF, contributing to hypoxia-induced angiogenesis of pulmonary artery endothelial cells.[23] CCL2/MCP-1 has been reported to induce the expression of the VEGF-A gene, mediating VEGF-A-triggered angiogenesis,[24] while leptin can display a synergistic effect with VEGF in the stimulation of angiogenesis.[25] Angiogenesis, the growth or formation of new blood vessels, has been considered as a crucial feature of airway inflammation and remodeling in asthma. Studies on the relationship between angiogenesis and inflammation have reported that angiogenesis contributes not only to changes in pathologic conditions resulting in chronic inflammation, fibrosis, and tumor growth, but also to the perpetuation of chronic inflammation by promoting the migration of inflammatory cells.[26] This relationship has been ascertained by another study in which chronic pulmonary inflammation resulted in accelerated angiogenesis only in bronchial asthma,

but not in chronic obstructive pulmonary disease and another pulmonary chronic inflammatory disease, and TNF-α, which is known to be an inflammatory cytokine, and CXCR2 chemokines involved in neutrophilic inflammation promoted *in vitro* angiogenesis of human pulmonary endothelial cells.[27] The combined increase in PDGFBB and VEGF levels, or MCP-1/CCL2 and VEGF levels in this study is thus thought to be accounted by these relationships between angiogenesis and inflammation in allergic environments, suggesting that angiogenesis influences the recurrent wheezing in allergic patients.

Airway remodeling, which was observed in preschool children with severe recurrent wheeze, is characterized by subepithelial fibrosis, increased angiogenesis, and airway smooth muscle (ASM) hypertrophy. ASM cells, as well as airway inflammatory cells, can secrete angiogenic factors resulting in angiogenesis, which is linked to airway hyperresponsiveness (AHR). Therefore, ASM cells can be assumed to be related to wheezing via their activity in promoting angiogenesis and airway remodeling. Interestingly, several studies have indicated that the four cytokines assessed in this study are associated with ASM cell hyperplasia and its function related to vascularization in the airway.[28-31] Evidence has shown that ASM cells are not only considered as the main effector of AHR but are also involved in bronchial inflammation and remodeling,[32] and recurrent wheezing may affect lower lung function as a result of airway narrowing and structural changes in the airway wall.[33] We thus assumed that there is an association between recurrent wheezing and ASM cells via angiogenic and inflammatory cytokines such as VEGF. Some studies, however, have reported that there was no significant relationship between ASM and wheeze in preschool children,[34] so it remains unclear whether ASM cells do perform functions related to wheezing in asthma patients, especially children. Therefore, we believe that further studies are needed to determine the crosstalk between the cytokines and ASM cells with regard to angiogenesis or inflammation, which will clarify their relationship in allergic patients with recurrent wheezing.

This study had some limitations, namely, relatively small number of participants; the study population composed of only allergic patients with physician's diagnosis and did not include normal controls; lack of physiological confirmation; and significant associations obtained only by statistical analysis, which requires validation by functional or pathophysiological studies. We also could not access the medication data of all participants to determine whether the prescription of glucocorticosteroids for treatment affected the difference in the cytokine levels between children and adult patients.[35] However, if the plasma level of VEGF measured from normal subjects in our previous study[12] could be considered as a baseline level of VEGF in Korean population, the VEGF levels measured in this study may be high enough to represent the relevance of plasma VEGF in allergic patients even though it was debatable to directly compare the values in each of the two studies. Therefore, our results imply that VEGF level in plasma is likely to be utilized as a serological marker in allergic patients with recurrent wheezing.

Conclusion

In summary, we found that the plasma levels of four cytokines that play roles in angiogenesis and inflammation were higher in adults than in children with allergic conditions. In particular, our results showed that plasma VEGF levels were associated with recurrent wheezing in various subgroups of the study population, and had increased together with PDGFBB and CCL-2/MCP-1 in the presence of recurrent wheezing in all conditions in children. Taken together, our findings could contribute to expand our knowledge on the roles of cytokines in allergic diseases by elucidating the relationships among cytokines in allergic environments, and we suggest the potential of VEGF as a serological marker that can be used to figure out the aspect of recurrent wheezing in allergic patients. We additionally expect further studies to determine the potential of angiogenic and/or inflammatory cytokines as therapeutic targets in allergic diseases.

Acknowledgements

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Authors' contributions

- Study conception and design: WSC, EJK, JKL
- Acquisition of data: JHD, SHL
- Analysis and interpretation of data: WSC, KPK, YSK
- Drafting of the article: WSC, JHD, KPK
- Critical revision: DY, JKL
- All authors read and approved the final manuscript.

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