VEGF and Activated Eosinophils in Children with Asthma and Eosinophilic Bronchitis

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=Abstract=

Purpose : Vascular endothelial growth factor (VEGF) is an important mediator of airway inflammation and remodeling in asthma. We aimed to explore whether VEGF is expressed at elevated levels in asthmatic airways or eosinophilic bronchitis (EB) and associated with eosinophilic inflammation, pulmonary function, and airway hyperresponsiveness (AHR) in children.

Methods : One hundred seventeen asthmatic children, 77 children with EB, and 84 healthy children were enrolled. Sputum supernatants were collected and VEGF and eosinophil cationic protein (ECP) levels were measured.

Results : Asthmatic children had significantly higher levels of VEGF in induced sputum [89.04 (29.95-178.09) pg/mL] compared to children with EB [25.30 (11.02-80.23) pg/mL] and healthy children [37.37 (16.56-71.30) pg/mL; P=0.0003]. VEGF in sputum positively correlated with sputum ECP (r=0.524; P<0.0001). Negative significant correlations were found between sputum VEGF and FEV_1, (r=-0.252; P=0.001) or post-bronchodilator FEV_1 (r=-0.181; P=0.038) whereas nonsignificant correlations were found between sputum VEGF and sputum eosinophils.

Conclusion : Our findings suggest that VEGF is associated with activated eosinophils in the asthmatic airway, but not EB. Sputum VEGF could be a supportive marker that represents activation of airway eosinophils and persistent airflow limitation in asthmatic children. **[Pediatr Allergy Respir Dis(Korea) 2009;19:173-182]**

Key Words : Childhood asthma, Eosinophil cationic protein, Eosinophilic bronchitis, Vascular endothelial growth factor

Introduction

Airway remodeling and eosinophilic airway inflammation, which are pathologic features that are characteristic of asthma in adults, are

already present in children with asthma as well as preschool wheezers.[1-3] Evidence has shown that eosinophils play a key role in airway remodeling, producing a wide range of proteins in fibrogenesis and angiogenesis, particularly transforming growth factor (TGF)-β, as well as cytokines.[3-5] Angiogenesis and microvascular remodeling has recently attracted considerable attention as a component of airway remodeling such as increased inner airway

thickness or luminal narrowing and AHR.[6-8] VEGF is one of the most potent pro-angiogenic factors; it plays a central role in mediating the process of pathogenesis and increases vascular permeability.[8-10] It has been reported that increased VEGF in the airway has been implicated in the pathogenesis of asthma.[11] Recently, it has been reported that VEGF modulates immune cell function by inhibiting dendritic cell activity and T-cell development and by stimulating eosinophil chemotaxis.[12, 13] These observations suggest that VEGF is associated with eosinophilic inflammation in asthma. However, as far as we know, few studies examined whether VEGF is associated with eosinophilic inflammation in the asthmatic airway.

There is another eosinophilic airway disease, nonasthmatic eosinophilic bronchitis (EB), which has emerged from the study of chronic cough, EB is also characterized by increased exhaled nitric oxide, increased basement membrane thickening, and normal spirometry without any evidence of AHR.[14, 15] The immunopathologic features of EB are distinct from those of asthma, which has no evidence of overexpression of interleukin (IL)-4 or -13 by mast cells or mast cell colonization to airway smooth muscle, implicated in the pathogenesis of asthma.[15-17] However, the pathogenesis of EB is still unclear, especially in children. To our knowledge, no study has quantified sputum VEGF levels in children with EB and compared them to subjects with asthma or controls nor examined whether VEGF is associated with eosinophilic inflammation in EB.

We therefore aimed to determine whether VEGF is expressed at elevated levels in asthmatic airways or EB and associated with eosinophilic inflammation, pulmonary function, and AHR in children.

Methods

1. Subjects

There were 278 children prospectively enrolled from Severance Childrens Hospital in Seoul from May 2007 to Nov 2008; 117 had asthma, 77 had EB and 84 were controls. Asthma was diagnosed according to the American Thoracic Society (ATS) criteria.[18] We classified the severity of asthma following GINA guidelines.[19] There were 42 mild intermittent, 47 mild persistent, 24 moderate persistent and 4 severe persistent asthmatic patients. EB was diagnosed on the basis of the following criteria: 1) FEV_1 and forced vital capacity (FVC)>85% without variable airway obstruction that could be demonstrated by a negative response to a short-acting bronchodilator (increase in FEV_1<12%); 2) absence of airway hyperreactivity (>16 mg/mL of methacholine); 3) sputum eosinophilia> 3%; and 4) no abnormality in the lung parenchyma on simple chest radiograph.[20, 21] Atopy was defined as a positive skin test result upon exposure to more than 1 extract of 12 common aeroallergens and nonatopy was defined as a negative test result with serum IgE concentrations less than 150 IU/mL. Allergens included 2 types of house dust mites, cat and dog epithelial, mold, and pollen antigens (Torii & Co., Tokyo, Japan). Saline was used as a negative control and 0.5% histamine HCl was used as a positive

control. Wheal diameter was measured after 15 minutes, and a positive reaction was defined as a wheal diameter larger than 3 mm.[22, 23] Seventy-five asthmatic children were enrolled after administration of inhaled corticosteroids for more than 2 weeks, and the remaining were without any controller medications including inhaled corticosteoids or leukotriene modifiers. Patients treated with systemic corticosteroids due to asthma exacerbation in the preceding month were excluded. Control subjects were age-matched healthy children who visited the hospital for general health workup or vaccination and had no history of wheezing, recurrent or chronic diseases, infection in the preceding 2 weeks, or hyper-responsiveness to methacholine.

This study was approved by the Institutional Review Board of Severance Hospital. Written consent for participation was obtained from parents with verbal assent from children.

2. Spirometry and methacholine challenge test

Spirometry was performed and flow volume curves were obtained according to ATS guidelines (VIASYS Healthcare Inc., Conshohocken, PA, USA).[24]

A methacholine challenge test was performed according to standardized procedure.[18] Each subject inhaled increasing concentrations of methacholine (0.075, 0.15, 0.31, 0.62, 1.25, 2.5, 5, 10, and 25 mg/mL) nebulized by a dosimeter (Dosimeter MB3 Mefar, Brescia, Italy) until FEV_1 was reduced by 20% from a postnebulized saline value. Airway response to methacholine was expressed as the provocative concentration, causing a 20% decrease in FEV_1 (PC_{20} in mg/mL), and was calculated by linear interpolation of a log-dose response curve.

3. Sputum induction and processing

Sputum induction and processing were performed as previously described by Yoshikawa et al..[25] All subjects were instructed to wash their mouths thoroughly with water. They then inhaled 3% saline nebulized in an ultrasonic nebulizer (NE-U12; Omron Co., Tokyo, Japan) at maximum output at room temperature. They were encouraged to cough deeply at 3-minute intervals thereafter. After sputum induction, spirometry was repeated. If the FEV_1 fell, subjects were required to wait until it returned to baseline value. Sputum samples were kept at 4℃ for no more than 2 hours before further processing. A portion of the sample was diluted with PBS containing 10 mmol/L of dithiothreitol (WAKO Pure Chemical Industries Ltd., Osaka, Japan) and gently vortexed at room temperature for 20 minutes. After centrifugation at 400 g for 10 minutes, the cell pellet was resuspended. We performed sputum viability determination with the trypan blue exclusion method to ensure that viability was adequate. Total cell counts were performed with a hemocytometer and slides were prepared with a cytospin (Cytospin3; Shandon, Tokyo, Japan) and stained with May-Grünwald-Giemsa for differential cell counts. Differential cell counts were performed by counting 400 nonsquamous cells by 2 observers who were blind to clinical details. The supernatant was stored at −70℃ for subsequent assay for VEGF and ECP.

4. Measurement of blood eosinophils, serum total IgE and ECP and sputum ECP and VEGF

The NE-8000 system (Sysmex, Kobe, Japan) was used to count eosinophils automatically in peripheral blood while serum total IgE and ECP and sputum ECP were measured with the CAP system (Phadia, Uppsala, Sweden). Sputum VEGF was measured individually with ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturers instructions. The minimum detection limit of VEGF was 7.8 pg/mL. All assays were performed in duplicate for each sample, with the mean values reported here.

5. Statistical analysis

Numerical variables were expressed as means and standard deviations. Numerical parameters with non-normal distribution (blood eosinophil count; serum total IgE and ECP; and sputum eosinophil count, ECP, and VEGF levels) were log-transformed before analysis. Comparisons between subjects with asthma, EB, and controls were evaluated by 1-way ANOVA. Correlations between sputum VEGF levels and lung function, AHR, or markers of atopy, especially eosinophilic inflammation, were calculated with Pearson's correlation test. Multiple regression analysis was performed to assess the contribution of age and sex.

A P-value<0.05 was considered statistically significant. Statistical Package for the Social Sciences software (version 13.0, SPSS Inc., Chicago, IL, USA) was used for all analyses.

Results

1. Subjects characteristics

Patient clinical characteristics are shown in Table 1. Pulmonary function parameters including FEV_1 (P<.00.0001), post-bronchodilator (postBD) FEV_1 (P=0.008), FEV_1/FVC (P<0.0001), and forced expiratory flow at 25-75% of FVC (FEF_{25-75}) (P<0.0010) showed significantly lower levels in asthmatics compared to EB and control subjects. Blood eosinophil counts increased in asthmatics compared to control subjects (P<0.0001) whereas serum ECP did not show any difference among the groups. Sputum eosinophil and ECP showed greater levels in subjects with asthma and EB than controls (P<0.0001). In addition, sputum ECP was increased in asthmatics compared to EB subjects (P=0.004). There were no differences between asthmatics without inhaled corticosteroid (ICS) therapy and those with ICS therapy in age, sex, pulmonary function parameters, total IgE, blood eosinophils, serum ECP and sputum eosinophils. Sputum ECP was increased in asthmatics without ICS therapy compared to ICS users (P<0.001, data not shown).

2. Sputum VEGF levels in subjects with asthma, EB, and controls

Sputum VEGF (measurements shown in Fig. 1, Table 1) was significantly greater in subjects with asthma [median (interquartile range), 89.04 (29.95-178.09) pg/mL] than in those with EB (25.30 [11.02-80.23] pg/mL; P=0.0017) and controls [37.37 (16.56-71.30) pg/mL; P=0.0003]. Sputum VEGF levels were much higher in asthmatic children before treatment [106.05 (49.07-200.64) pg/mL] than

Table 1. Characteristics of Subjects

	Asthma	EB	Control
Subjects	117	77	84
Age, yr	9.1±2.3	9.0±2.8	9.4±2.5
Sex ratio, M/F	77/40	52/25	46/38
With/without atopy	99/18	58/19	50/34
FEV_1, % predicted	87.7±15.0[*]	96.3±10.7	98.6±11.4
Post BD FEV_1, % predicted	94.5±15.9[†]	100.5±11.9	101.4±10.2
FEV_1/FVC, %	88.4±11.1[*]	93.4±6.4	92.9±6.7
FEF_{25-75}, % predicted	82.6±30.2[*]	103.7±26.5	101.6±24.0
Total IgE, log IU/mL	2.56±0.58[*]	2.28±0.53	2.15±0.67
Blood eosinophil, log μL-1	2.59±0.36[‡]	2.48±0.35	2.36±0.44
Serum ECP, log μg/L	1.33±0.39	1.34±0.39	1.25±0.44
Sputum eosinophil, log %	1.18±0.53[‡]	1.21±0.34[‡]	0.19±0.24
Sputum ECP, log μg/L	2.29±0.69[‡, ∫]	2.00±0.66[‡]	1.57±0.59
Sputum VEGF, pg/mL	89.04 (29.95-178.09)[∥,¶]	25.30 (11.02-80.23)	37.37 (16.56-71.30)

Data are represented as mean±SD, median (interquartile range) or absolute numbers.
[*]$P<0.0001$ compared to subjects with EB and controls
[†]$P=0.008$ compared to subjects with EB and controls
[‡]$P<0.0001$ compared to controls
[∫]$P=0.004$ compared to subjects with EB
[∥]$P=0.0017$ compared to subjects with EB
[¶]$P=0.0003$ compared to controls
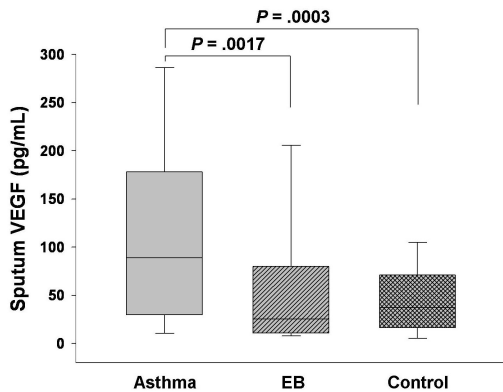Abbreviation : EB, eosinophilic bronchitis

Fig. 1. Comparison of sputum VEGF levels among the asthma, EB, and control groups. Sputum VEGF was significantly greater in subjects with asthma than EB (*P*=0.0017) and controls (*P*=0.0003).

ICS users [43.75 (16.08-147.45) pg/mL; $P<0.0001$] and EB ($P<0.0001$) and control subjects ($P<0.0001$). Sputum VEGF was not significantly different among the groups divided by severity in all asthmatic children. Moreover, there was no difference in sputum VEGF among the groups divided by severity in each group of asthmatic children with and without ICS treatment (data not shown).

3. Correlations between VEGF and eosinophilic inflammation

Sputum VEGF level was positively correlated with sputum ECP (r=0.524; $P<0.0001$), and this association was not significantly affected by adjustment for age and sex (Fig. 2). However, there was no association between sputum VEGF and sputum eosinophil count as well as systemic eosinophilic inflammation represented by blood eosinophil count and serum ECP (data not shown).
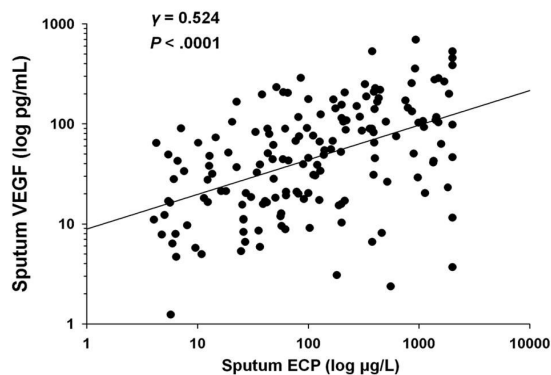
4. Relationship between sputum VEGF

Fig. 2. Correlations between VEGF and ECP in sputum. Sputum VEGF levels were positively correlated with sputum ECP (r=0.524; *P*<0.0001).

with pulmonary function and AHR

A negative significant correlation was found between sputum VEGF and FEV_1 (r=-0.252; P=0.001) and this correlation was not affected by adjustment for age and sex. Another negative significant correlation was found between sputum VEGF and postBD FEV_1 (r=0.181; P=0.038) but this correlation disappeared after adjustment for age and sex. There was no relationship between sputum VEGF and other pulmonary function parameters such as $FEV_1/$FVC or FEF_{25-75} or AHR represented by PC_{20} (data not shown).

4. Relationship between sputum ECP with pulmonary function and AHR

Negative significant correlations were found between sputum ECP and FEV_1 (r=-0.255; $P<$0.0001) or postBD FEV_1 (r=0.237; P=0.002) and these correlations were not affected by adjustment for age and sex. There was no relationship between sputum ECP and other pulmonary function parameters such as FEV_1/FVC or FEF_{25-75} or PC_{20} (data not shown).

Discussion

We have demonstrated that vascular remodeling represented by sputum VEGF notably increased in asthmatic children compared to control subjects. In addition, we observed a significant association between VEGF and activated eosinophils represented by ECP in the asthmatic airway. However, sputum VEGF in EB was similar level to that of controls, different from that of asthma.

Recent studies in the airway of asthmatic patients have revealed that the level of VEGF is increased in the sputum of asthmatic subjects in comparison to that of control subjects, even in children.[26, 27] With regard to EB in adults, a previous study reported that sputum VEGF levels and vascular permeability index are significantly higher in subjects with asthma than patients with EB and normal controls.[28] These observations suggest that VEGF may be a key element in the differences in airway function, such as AHR, between asthma and EB. The results of this study correspond with ours, which reports that sputum VEGF levels are increased in subjects with asthma compared to EB and control subjects. On the other hand, conflicting results have reported that sputum VEGF concentration is increased in asthma and EB compared to controls, and was inversely related to postBD FEV_1 in asthma.[29] These findings suggest that vascular remodeling related to VEGF is a feature of both of asthma and EB. In this regard, vascular remodeling and VEGF may be associated with airflow obstruction but not AHR. However, our results supported the association between

VEGF and both airflow obstruction and AHR. A plausible explanation for these conflicting results is difference in characteristics of asthma patients. Our patients are children, not adults, and milder in symptom. The limitation of this study is that the enrolled subject number is too small, and the number of moderate to severe persistent asthma patients is also minimal. Further studies are needed.

It has been reported that sputum VEGF correlated inversely with AHR and lung function.[28] Our results showed that sputum VEGF had negative correlations with FEV_1 and postBD FEV_1, which represents irreversible airflow limitation.[5] The findings of the present study correspond well with the role of VEGF in airway remolding in previous studies.[6-8] In addition, our report showed that sputum VEGF is involved in the presence of AHR but not in its severity. A recent study showing that AHR is dissociated from airway structural remodeling partly supports our present results.[16]

There is growing evidence that eosinophils play a key role in airway remodeling.[3-5] On the other hand, it has also been reported that eosinophils might not be directly involved in causing AHR.[30] This finding could explain why there are similar airway eosinophil counts in both asthma and EB irrespective of AHR. However, activation of eosinophils represented by ECP in the airway increased in the asthmatic airway compared to EB and controls. These findings suggest that activated eosinophils might be involved in AHR but not the inactivated form. Further investigations on the association between airway remodeling and AHR will be needed.

Recent studies indicate that eosinophil infiltration is reduced by administration of anti-VEGF receptor antibodies in a murine model of toluene diisocyanate-induced asthma and VEGF induces eosinophil migration and ECP release.[13, 31] These findings suggest a positive feedback loop between VEGF release by eosinophils and mast cells and VEGF enhancing activation of mast cells and eosinophils.[12] In our study, sputum was positively associated with sputum ECP but not sputum eosinophil counts and systemic eosinophilic inflammation such as blood eosinophil count or serum ECP. These findings suggest that VEGF would be connected to activated eosinophils, and this association can be confined to the airway inflammation, not systemic inflammation. In addition, no relationship between sputum VEGF and sputum eosinophil counts in our results implicates that activation of eosinophils is important. In this regard, the present study also supports a positive feedback loop between VEGF and activation of eosinophils.

In conclusion, our findings suggest that VEGF is associated with activated eosinophils in the asthmatic airway, but not EB. Sputum VEGF could be a supportive marker that represents activation of airway eosinophils and persistent airflow limitation in asthmatic children.

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한 글 요 약

소아 천식과 호산구성 기관지염에서 혈관 내피세포 성장인자(VEGF)의 의의

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김경원 · 지혜미 · 이경은 · 박여훈
최봉석 · 손명현 · 김규언

목 적 : 혈관 내피세포 성장인자(Vascular en-dothelial growth factor, VEGF)는 기도개형에 중심적인 역할을 하며, 혈관의 투과성을 증가시켜 천식에서 기도 벽의 부종에 기여할 뿐 아니라 기관지과민성에 관여하는 것으로 알려져 기관지 천식의 중요한 매개물질로 주목받고 있다. 본 연구에서는 천식 및 호산구성 기관지염 환아에서 이러한 VEGF의 임상적인 의의와 기관지과민성 및 폐기능에 미치는 영향에 대해 알아보고자 하였다.

방 법 : 만 6세에서 14세 사이의 278명을 대상으로 하였다. 천식 환아는 117명, 호산구성 기관지염 환아는 77명, 대조군은 84명이었다. 전체 대상자에서 폐기능 검사, 메타콜린 흡입 유발시험, 피부 단자 시험을 시행하였고, 혈액 내 호산구수, 혈청 총 IgE, ECP, 유도 객담 내 호산구수 및 유도 객담 상층액의 ECP, VEGF 농도를 측정하였다.

결 과 : 천식군의 유도 객담 VEGF 농도는 89.04 (29.95-178.09) pg/mL, 호산구성 기관지염군은 25.30 (11.02-80.23) pg/mL, 대조군은 37.37 (16.56-71.30) pg/mL로 천식군에서 호산구성 기관지염군과 대조군에 비해 의미있는 증가를 보였다(P=0.0003). 또한 유도 객담 VEGF 농도는 유도 객담 내 ECP 농도와 유의한 양의 상관관계를 나타내었으나(r=0.524; P<0.0001), 유도 객담 내 호산구수와는 관련이 없었다. 또한 유도 객담 내 VEGF는 FEV_1, (r=-0.252; P=0.001) 및 post – bronchodilator FEV_1 (r=-0.181; P=0.038)와 의미있는 음의 상관관계를 나타내었다.

결 론 : VEGF는 소아 천식에서 기도 내 호산구 활성화와 연관이 있는 것으로 생각되며, 특히 유도 객담 VEGF는 호산구성 기도 염증의 정도 및 기류제한을 반영하는 보조적인 지표가 될 수

있을 것으로 생각된다.

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