

Imbalance between vascular endothelial growth factor and endostatin levels in induced sputum from asthmatic subjects

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Asthma, rhinitis,
other respiratory
diseases

Background: Angiogenesis has recently attracted considerable attention as a component of airway remodeling in bronchial asthma. Vascular endothelial growth factor (VEGF) is highly expressed in asthmatic airways, and its contribution to airway remodeling has been reported. Although angiogenesis is regulated by a balance of angiogenic and antiangiogenic factors, the relative levels of antiangiogenic factors in asthmatic airways have not been evaluated.

Objective: We sought to determine whether an imbalance between angiogenic and antiangiogenic factors exists in asthmatic airways.

Methods: We simultaneously measured VEGF and endostatin levels and evaluated their correlation and balance in induced sputum from 18 steroid-naïve asthmatic subjects and 11 healthy control subjects. After initial sputum induction, asthmatic subjects underwent 8 weeks of inhaled beclomethasone dipropionate (BDP; 800 µg/d) therapy, and sputum induction was then repeated.

Results: VEGF and endostatin levels in induced sputum were significantly higher in asthmatic subjects than in control subjects ($P < .001$). There was a significant correlation between VEGF and endostatin levels in both control subjects ($r = 0.995$, $P < .001$) and asthmatic subjects ($r = 0.923$, $P < .001$). Moreover, the VEGF/endostatin level ratio in asthmatic subjects was significantly higher than that in control subjects ($P < .0001$). After 8 weeks of inhaled BDP therapy, the VEGF level in induced sputum in asthmatic subjects was significantly decreased ($P < .001$), whereas the endostatin level was not. A correlation between VEGF and endostatin levels existed even after BDP therapy ($r = 0.861$, $P < .001$). Moreover, the VEGF/endostatin level ratio was significantly decreased to the same level as in the control subjects after BDP therapy ($P < .0001$).

Conclusion: There was an imbalance between VEGF and endostatin levels in induced sputum from asthmatic subjects. This imbalance might play an important role in the pathogenesis of bronchial asthma through its effects on angiogenesis. (*J Allergy Clin Immunol* 2002;110:571-5.)

Key words: Bronchial asthma, vascular endothelial growth factor, endostatin, induced sputum, beclomethasone dipropionate

Asthma is a chronic airway inflammatory disease characterized by airway wall remodeling. Epithelial desquamation, goblet cell hyperplasia, collagen deposition below the basement membrane, smooth muscle hypertrophy-hyperplasia, and growth and proliferation of new blood vessels are recognized as morphologic changes in asthmatic airway walls. It has been reported that both the number and percentage of vessels in bronchial mucosa taken from subjects with even mild asthma were higher than those in control subjects.¹⁻³ These morphometric observations have shown that angiogenesis contributes to the pathogenesis of bronchial asthma.

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors; it stimulates endothelial cell proliferation and induces angiogenesis.⁴ VEGF is widely expressed within many different highly vascularized organs, including the lung.⁵ Because VEGF is a very potent and specific angiogenic factor stimulating endothelial cell proliferation, its relationship to pathogenesis of bronchial asthma has recently been considered. For example, Hoshino et al⁶ reported that VEGF-positive cells are significantly increased in number in the airway mucosa of asthmatic subjects compared with numbers seen in healthy control subjects.

Growth factors for angiogenesis are involved in virtually all aspects of lung development and response to inflammation. Imbalances of these factors are increasingly implicated in a wide spectrum of inflammatory diseases. Imbalances of growth factors for angiogenesis in the lung result in a situation in which the expression or activity of one growth factor predominates over that of another, usually of opposing effect, within the same anatomic compartment, such as airways. It is thus important to simultaneously measure growth factors and opposing factors. However, no reports have been published describing levels of antiangiogenic factors in asthmatic airways. Endostatin is a strong endogenous inhibitor of angiogenesis⁷ and is produced by various types of cells. This study was designed to determine whether an imbalance between angiogenic and antiangiogenic factors exists in asthmatic airways. Therefore we simultaneously measured the levels of VEGF and endostatin and evaluated their correlation and balance in induced sputum from asthmatic and control

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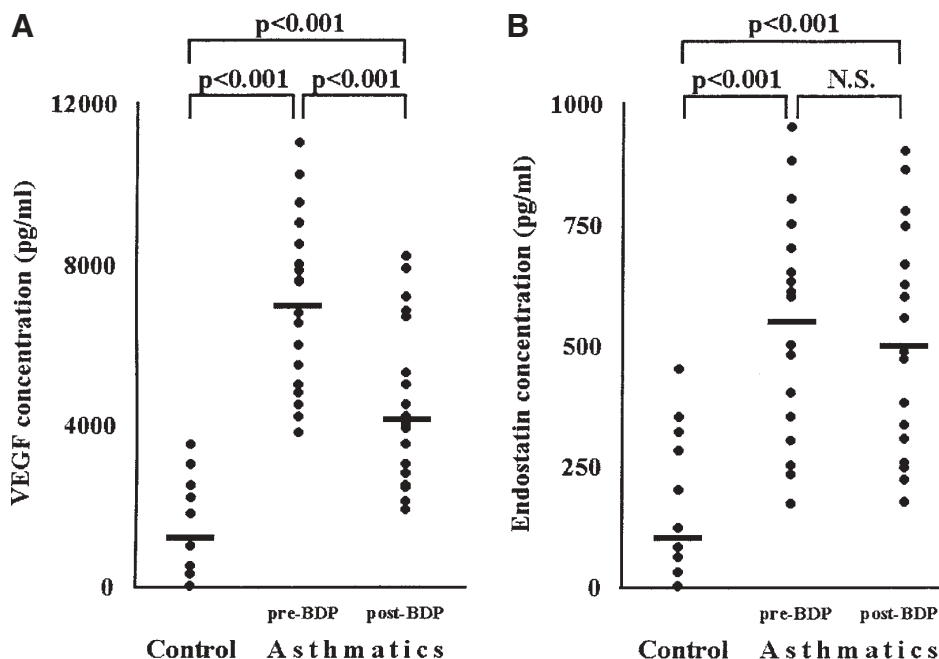


FIG 1. A, Comparison of VEGF concentrations in induced sputum among control subjects, pre-BDP treatment asthmatic subjects, and post-BDP treatment asthmatic subjects. B, Comparison of endostatin concentrations in induced sputum among control subjects, pre-BDP treatment asthmatic subjects, and post-BDP treatment asthmatic subjects.

Abbreviations used

BDP: Beclomethasone dipropionate
IQR: Interquartile range
VEGF: Vascular endothelial growth factor

subjects. In addition, we evaluated the effects of 8 weeks of beclomethasone dipropionate (BDP) therapy on the levels of these factors in asthmatic subjects.

METHODS

Subjects

Eighteen asthmatic subjects and 11 normal control subjects were included in the study. Asthmatic subjects had no history of respiratory infection or asthma attack for at least the 1-month period preceding the study. All asthmatic subjects were lifelong nonsmokers and met the American Thoracic Society standards for asthma.⁸ In short, they all had episodic cough, wheezing and dyspnea, normal chest roentgenography results, and bronchial hyperresponsiveness to cholinergic agents. They also exhibited reduced FEV₁ and percent FEV₁ during attacks and an increase of 20% or greater in FEV₁ in response to a bronchodilator. Normal control subjects were healthy nonsmoking volunteers without a history of lung disease. Methacholine inhalation challenge testing was performed for all subjects. All challenge tests were performed in the afternoon to eliminate effects of diurnal variation. After baseline spirometry and inhalation of diluent to establish the stability of FEV₁, the subjects were instructed to take slow inspirations in each set of inhalations. All asthmatic subjects in this study exhibited bronchial hyperresponsiveness to methacholine. Their regular medication consisted of short-acting β_2 -agonists for rescue use as needed, theophylline, or both, and none were receiving oral or inhaled corticosteroids.

They had episodic symptoms, but their symptoms were well controlled with short-acting β_2 -agonist rescue use not more than once a day. Medications were not changed during the 1-month period preceding the study and were withdrawn for at least 12 hours before the methacholine challenge test and sputum induction. Atopy in asthmatic subjects was defined as one or more positive skin prick test responses to 12 common allergens. All subjects provided written informed consent for participation in the study, which was approved by the Ethics Committee of Osaka City University.

Sputum induction and processing

Sputum induction and processing were performed as previously described by Yoshikawa et al.⁹ After methacholine inhalation challenge testing, spirometry was performed before inhalation of 200 μ g of albuterol administered through a metered-dose inhaler. 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 FEV₁ fell, the subjects were required to wait until it returned to baseline value. The sputum samples were kept at 4°C for no more than 2 hours before further processing.

A portion of the sample was diluted with PBS containing 10 mmol/L dithiothreitol (WAKO Pure Chemical Industries Ltd, Osaka, Japan) and gently vortexed at room temperature for 20 minutes. After centrifugation at 400g 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 stain for differential cell counts. The differential cell counts were performed by counting of 400 nonsquamous cells by 2 observers in a manner blind to clinical details. The supernatant

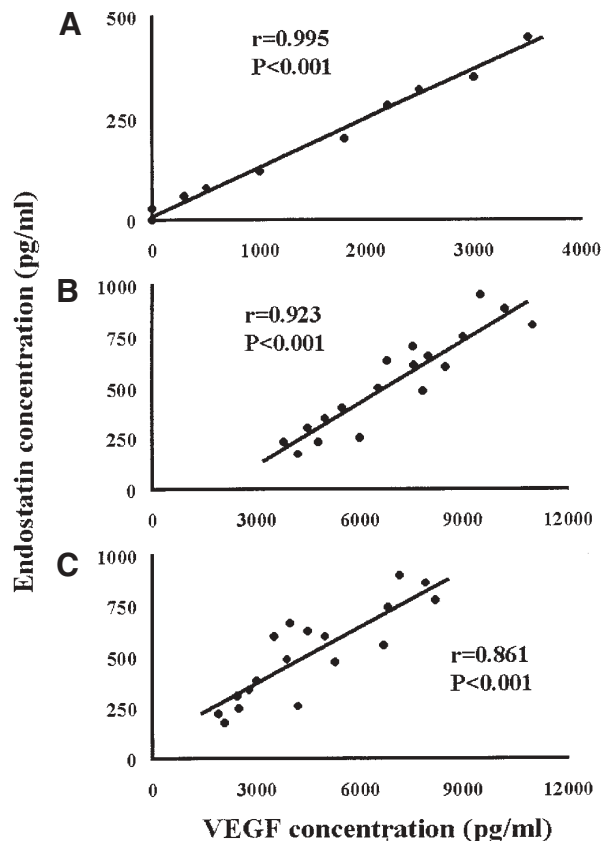


FIG 2. Correlation between VEGF concentration and endostatin concentration in control subjects (**A**), pre-BDP treatment asthmatic subjects (**B**), and post-BDP treatment asthmatic subjects (**C**). There were significant correlations between VEGF and endostatin levels in each condition.

was stored at -70°C for subsequent assay for VEGF, endostatin, and eosinophil cationic protein. VEGF concentration was measured with an ELISA kit (R&D system Inc, Minneapolis, Minn), and endostatin concentration was measured with a separate ELISA kit (Cytimmune Science Inc, College Park, Md). Eosinophil cationic protein concentration was measured with an RIA kit (Pharmacia Diagnostics, Uppsala, Sweden).

After the first sputum induction, asthmatic subjects received 8 weeks of inhaled BDP (800 $\mu\text{g}/\text{d}$; Glaxo Wellcome, Tokyo, Japan) administered through a metered-dose inhaler. Sputum induction and processing were then repeated in the same way.

Statistical analysis

Data are expressed as median and interquartile range (IQR). Differences among groups were examined by means of 1-way ANOVA, followed by the Bonferroni test. The significance of correlations was evaluated by determining Spearman rank correlation coefficients. A P value of less than .05 was considered significant.

RESULTS

Clinical characteristics and results of examination in the 18 asthmatic and 11 control subjects are shown in Table I. The VEGF level in induced sputum was significantly higher in asthmatic subjects (7190 pg/mL [IQR, 4950-8125 pg/mL]) than in control subjects (1000 pg/mL [IQR, 150-2350 pg/mL], $P < .001$; Fig 1, A).

Similarly, the endostatin level in induced sputum was significantly higher in asthmatic subjects (515 pg/mL [IQR, 337.5-662.5 pg/mL]) than in control subjects (120 pg/mL [IQR, 45-300 pg/mL], $P < .001$; Fig 1, B). There was a significant correlation between VEGF and endostatin levels in both control subjects ($r = 0.995$, $P < .001$; Fig 2, A) and asthmatic subjects ($r = 0.923$, $P < .001$; Fig 2, B). Moreover, the VEGF/endostatin level ratio in asthmatic subjects (13.75 [IQR, 12.08-16.02]) was significantly higher than that in control subjects (7.81 [IQR, 6.25-8.33], $P < .0001$; Fig 3).

After 8 weeks of inhaled BDP therapy, we repeated sputum induction in all asthmatic subjects. The VEGF level in induced sputum in asthmatic subjects (4100 pg/mL [IQR, 2850-6350 pg/mL], $P < .001$) was significantly decreased but still higher than that of control subjects ($P < .001$). However, the endostatin level in induced sputum in asthmatic subjects (520 pg/mL [IQR, 312.5-655 pg/mL]) was not significantly decreased. There remained a significant correlation between VEGF and endostatin level, even after BDP therapy ($r = 0.861$, $P < .001$; Fig 2, C). However, the VEGF/endostatin level ratio was significantly decreased to the same level as seen in the control subjects after BDP therapy (8.5 [IQR, 8.0-10.49], $P < .0001$; Fig 3).

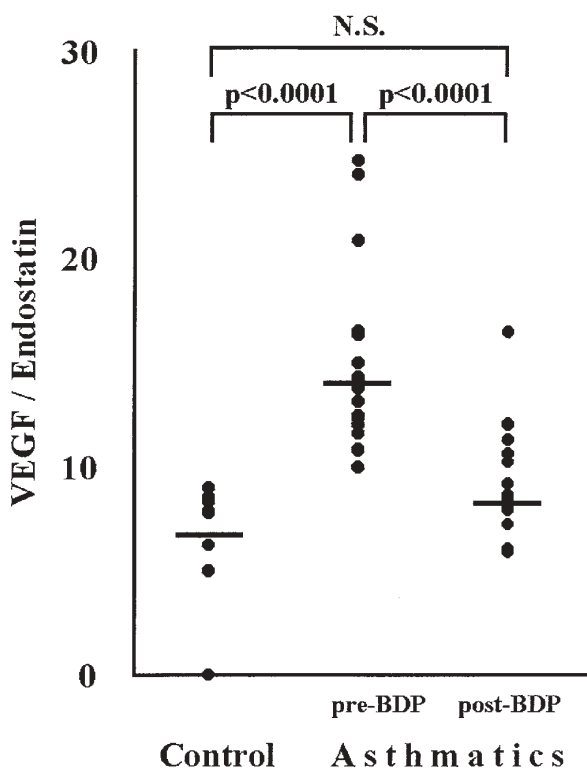


FIG 3. Comparison of the VEGF/endostatin level ratio in induced sputum among control subjects, pre-BDP treatment asthmatic subjects, and post-BDP treatment asthmatic subjects.

TABLE I. Clinical characteristics of asthmatic and control subjects

	Asthmatic subjects (n = 18)	Control subjects (n = 11)
Sex (M/F)	12/6	9/2
Age (y)	33.5 (28.8-40.5)	37 (26.5-40.5)
Atopy (yes/no)	13/5	0/11
FEV ₁ (%)	89.9 (84.8-93.5)*	108.7 (105.3-109.6)
Sputum eosinophils (%)	17.5 (13.5-22.8)*	0.6 (0.4-1.1)
Sputum eosinophil cationic protein (pg/L)	720 (505-893)*	100 (65-150)
PC ₂₀ methacholine (mg/mL)†	2.1 (0.2)*	>10

Values are given as median (IQR) where shown.

* $P < .0001$, asthmatic versus control subjects.

†Value given as geometric mean (log SEM).

DISCUSSION

The most striking findings of this study were that VEGF and endostatin levels in induced sputum were significantly higher in asthmatic than in control subjects and that a significant correlation existed between VEGF and endostatin level in both control and asthmatic subjects. However, the VEGF/endostatin level ratio was significantly higher in asthmatic than in control subjects. This is the first report of an imbalance between VEGF and endostatin level in induced sputum in asthmatic subjects.

Yong and Hern¹⁰ reported that the VEGF level in sputum is significantly increased, even in stable asthmatic subjects, and our results are in good agreement with theirs. Although VEGF has recently attracted attention with regard to its role in the pathogenesis of asthma, the balance of angiogenic to antiangiogenic factors and the participation of antiangiogenic factors in the pathogenesis of bronchial asthma have not been investigated. Endostatin directly inhibits endothelial growth and migration and promotes apoptosis, antagonizes the angiogenesis-promoting effects of VEGF,¹¹ and is thought to play a role in the homeostatic angiogenic regulatory network.¹²⁻¹⁴ Angiogenesis is thought to depend on the local balance of angiogenic and antiangiogenic factors.^{12,15} The correlation between VEGF and endostatin levels was previously evaluated in serum in renal cell carcinoma¹⁶ and in vitreous fluid in diabetic retinopathy.¹⁷ In the present study we found a good correlation in induced sputum from normal control and asthmatic subjects. These findings suggest that homeostatic regulation might control angiogenesis in the airways of both normal control and asthmatic subjects. However, we also found an imbalance between VEGF and endostatin levels in asthmatic airways. This imbalance might be related to angiogenesis as a component of airway remodeling.

Corticosteroids are key agents in the treatment of inflammatory airway disease, such as bronchial asthma. VEGF levels in induced sputum were significantly decreased after 8 weeks of BDP therapy, whereas endostatin levels were not. The significant correlation between VEGF and endostatin levels remained, even after BDP therapy. However, the VEGF/endostatin level ratio was significantly decreased to the same level as seen in control subjects after 8 weeks of BDP therapy. Bandi and Kompella¹⁸ reported that VEGF secretion and VEGF mRNA expression were inhibited through a glucocorticosteroid receptor-mediated mechanism in airway and alveolar epithelial cells. Our finding of decreased VEGF levels after BDP therapy is in good agreement with this. It has been previously reported that the vascularity of asthmatic airway mucosa was decreased by inhaled corticosteroids.^{19,20} In the present study we found that the imbalance between VEGF and endostatin levels in asthmatic subjects was reversed by inhaled corticosteroids. Thus changes in the VEGF/endostatin level ratio might play a role in the changes in vascularity in airway mucosa caused by inhaled corticosteroids.

In conclusion, we found a significant correlation between VEGF and endostatin levels in induced sputum. However, there was an imbalance between VEGF and endostatin levels in steroid-naïve asthmatic subjects. This imbalance was reversed by 8 weeks of BDP therapy. These results suggest that inhaled corticosteroids might be clinically useful in the treatment of angiogenesis in asthmatic airways. However, serial measurement of VEGF and endostatin levels, the imbalance between angiogenic and antiangiogenic factors, and the relationship between these parameters and morphologic changes in bronchial mucosa should be examined in future studies.

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