A Comparative Study of Two Angiogenic Factors*

Vascular Endothelial Growth Factor and Angiogenin in Induced Sputum From Asthmatic Children in Acute Attack

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Background: Angiogenesis is a prerequisite for airway remodeling in bronchial asthma. Several factors may play important roles in inflammation and angiogenesis through effects on inflammatory cell infiltration or neovascularization.

Objectives: (1) To determine the levels of vascular endothelial growth factor (VEGF) and angiogenin in sputum supernatants of asthmatic children during the acute attack and 6 weeks after start of therapy; and (2) to correlate their levels with the degree of asthma severity.

Subjects and methods: Twenty asthmatic children with acute attack (mean age, 9.6 ± 3.5 years [± SD]) and 12 sex- and age-matched healthy control children were enrolled in the study. Sputum supernatants were collected for determination of VEGF and angiogenin levels. Serum samples were withdrawn for IgE measurement. The above tests were performed using an enzyme-linked immunosorbent assay. The FEV₁ was measured using spirometry. VEGF, angiogenin, and FEV₁ estimations were repeated for asthmatic children 6 weeks after start of therapy.

Results: During the acute attack, asthmatic children had significantly higher levels of VEGF and angiogenin than in healthy control children (p < 0.001). VEGF and angiogenin levels showed more elevation with increase in asthma severity (p < 0.001). A significant positive correlation existed between both angiogenic factors (r = 0.98, p < 0.001). A negative significant correlation was found between FEV₁ percentage of predicted and both VEGF (r = −0.99, p < 0.001) and angiogenin (r = −0.97, p < 0.001). A nonsignificant correlation was found between serum IgE and sputum VEGF (r = 0.09, p > 0.05). Although there was a significant decrease in the levels of both VEGF and angiogenin after 6 weeks of treatment with corticosteroid inhalation therapy, the levels did not reach normal control levels (p < 0.001 and p < 0.05, respectively).

Conclusions: Our results show that both VEGF and angiogenin levels were elevated in children with acute asthma. The study also suggests that increased severity of bronchial asthma in children is associated with the expression of both angiogenic factors, which are implicated in asthma pathogenesis. After 6 weeks of therapy, the levels of both angiogenic factors showed significant decrease.

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Key words: angiogenesis; angiogenin; asthma; inhaled corticosteroid; vascular endothelial growth factor

Abbreviation: VEGF = vascular endothelial growth factor

Angiogenesis and microvascular remodeling are known features of chronic inflammatory diseases such as asthma and chronic bronchitis.¹ Angiogenesis is the growth of new blood vessels from existing vessels, whereas microvascular remodeling is characterized by hypertrophy and hyperplasia of airway...
smooth muscle, increase in mucous glands, and thickening of the reticular basement membrane as well as structural alterations—usually enlargement—of arterioles, capillaries, or venules, without the formation of new vessels. This is thought to contribute to irreversible airway obstruction, which is one of the factors that make the treatment for asthma difficult.

The mechanism responsible for the formation of new vessels and the remodeling of the existing vessels is unknown. Mediators and inflammatory cells can be involved in different ways such as histamine and bradykinin, which can induce vasodilation, as well as some cytokines present in the airways that could determine the formation of new blood vessels. The increase in the number and size of vessels can contribute to thickening of airway wall, which leads to critical narrowing of bronchial lumen when bronchial smooth-muscle contraction occurs. The formation of new vessels and the remodeling of the existing vessels are likely to be induced by multiple growth factors, including vascular endothelial growth factor (VEGF) and angiogenin.

VEGF is widely expressed within many different highly vascularized organs such as the lungs. VEGF is one of the most potent proangiogenic cytokines, which plays a central role in mediating the process of angiogenesis. VEGF also increases vascular permeability so that plasma proteins can leak into the extravascular space, leading to edema and profound alterations in the extracellular matrix.

Angiogenin, which was first isolated from the conditioned medium of colonic carcinoma cells grown in culture, is a member of the ribonuclease superfamily, which is normally present in the circulation. It is one of the most potent tumor-derived angiogenic factors. Moreover, angiogenin plays a role in a number of nonmalignant vasculoproliferative pathologic conditions. It has been implicated as a mitogen for vascular endothelial cells, an immune modulator with suppressive effects on polymorphonuclear leukocytes, an activator of certain protease cascades, as well as an adhesion molecule. In the present work, our aims were as follows: (1) to determine the levels of both angiogenic factors, VEGF and angiogenin, in the sputum of asthmatic patients during the acute attack and 6 weeks after start of therapy; and (2) to correlate these levels with the degree of asthma severity.

Materials and Methods

Twenty well-documented asthmatic children were enrolled in the present study. They were recruited from the Pediatric Chest Clinic, Ain Shams University Hospital (14 male and 6 female children; age range, 5 to 16 years; mean, 9.6 ± 3.5 years [± SD]).

Diagnosis of asthma was made according to the criteria suggested by the American Thoracic Society. All patients had episodic cough, wheezing, dyspnea, blood eosinophilia, and normal chest radiograph results. The patients also exhibited reduced FEV1 and bronchial hyperresponsiveness to cholinergic agents. Following Global Initiative for Asthma classification, the children were further classified according to severity of asthma: mild persistent (n = 7), FEV1 ≥ 80% of predicted and at least one of the following: asthma symptoms more than once a week but less than once per day, and nighttime asthma more than twice a month but less than once a week; moderate persistent (n = 9), FEV1 ≥ 60% but < 80% of predicted, daily symptoms, or nighttime asthma more than once a week; and severe persistent (n = 4), FEV1 < 60% of predicted, or physical activities limited by asthma. The patients were maintained on β2-agonists (long- and/or short-acting rescue treatment), and none were receiving oral or inhaled corticosteroids for at least 1 month before presentation. All patients presented to hospital with an acute asthmatic attack as defined by National Asthma Education and Prevention Program guidelines: cough, shortness of breath, wheezing, chest tightness, use of accessory muscles, and suprasternal retractions. Patients with lower respiratory tract infections were excluded from the study. Patients received inhaled short-acting β2-agonists by metered-dose inhaler or nebulizer and oxygen therapy. Patients who did not show improvement with the above regimen received IV methylprednisolone, 1 to 2 mg/kg. After recovery from acute attack, all patients received inhaled corticosteroids (100 to 250 µg of fluticasone propionate) for 6 weeks. β2-Agonists were added to the treatment regimen for moderate and severe cases.

Twelve age- and sex-matched healthy children attending the Pediatric Clinic, National Research Center for routine follow-up were considered as a control group. Informed consent was obtained from all parents and approved by the ethics committee of Ain Shams University Hospital and National Research Center.

Sputum Induction and Processing

Two sputum samples were obtained from each patient: one immediately after admission to the hospital, and the other 6 weeks after treatment. For healthy children, only one sample containing normal bronchial secretions was collected.

Sputum induction and processing were performed as previously described by Asai et al. All subjects were instructed to wash their mouths thoroughly with water. They then inhaled 3% saline solution nebulized in an ultrasonic nebulizer at maximum output, at room temperature under close medical supervision. They were encouraged to cough deeply at 3-min intervals thereafter. The sputum samples were kept at 4°C for no more than 2 h before further processing. A portion of the sample was diluted with phosphate-buffered saline solution containing 10 mmol/L dithioeryitol and gently vortexed at room temperature for 20 min. After centrifugation at 400g for 10 min, the supernatant was stored at −70°C for subsequent assay for VEGF and angiogenin. No material was added to stimulate rupture of cells.

Blood Sample Collection

Three milliliters of whole blood was withdrawn from every subject via venipuncture and was prepared for the different laboratory investigations as appropriate. All of the studied children completed the following: (1) full history taking and thorough clinical examination; (2) chest radiography (posteroanterior and lateral views [for patients only]); (3) pulmonary function testing using spirometry (MedGraphics 1070 series 2E/1085; Medical Graphics; St. Paul, MN) with emphasis on FEV1 percentage of
predicted as an indicator of small airway function; (4) laboratory investigations, including the following: (A) erythrocyte sedimentation rate using the Westergren method; (B) complete blood picture, including total and differential leukocytic counts, especially the band cells and absolute eosinophil and basophil counts (Coulter Jr); cell counter; Coultronics; Hialeah, FL); blood sampling of all subjects was performed at the same time daily (10 AM) to avoid diurnal variations in eosinophil counts; (C) serum total IgE, measured by enzyme-linked immunosorbent assay (Medix Biotech, A Genzyme Company, San Carlos, CA); the value of IgE used for data analysis was the percentage from the highest normal value for age,13 as follows: patient's actual value/highest value of IgE used for data analysis = the percentage from the total IgE, measured by enzyme-linked immunosorbent assay. Standards and samples are pipetted into the wells, and any VEGF detected in each sample is compared to a VEGF standard curve. The kit is highly sensitive—it detects angiogenin levels < 6 pg/mL—and is highly specific, with no cross-reactivity with other cytokines.

### Statistical Analysis

A standard computer program (SPSS for Windows, Release 10.0; SPSS, Chicago, IL) was used for data entry and analysis. All numeric variables were expressed as mean ± SD. Comparison of different variables in various groups was done using Student t test and Mann Whitney test for normal and nonparametric variables, respectively. Comparisons of multiple subgroups were done using analysis of variance and Kruskall-Wallis tests for normal and nonparametric variables, respectively. Pearson and Spearman correlation test were used for correlating normal and nonparametric variables, respectively. For all tests, p < 0.05 was considered significant. Graphic presentation of the results was also done.14

### Results

Results of the present study are illustrated in Tables 1–4 and Figures 1–3. The mean levels of VEGF and angiogenin showed a highly significant increase in acute asthmatics vs healthy children (p < 0.001) [Table 1].

Studying the correlation between VEGF and angiogenin levels during the acute asthmatic attack revealed a highly significant positive correlation (r = 0.98, p < 0.001) [Fig 1]. After 6 weeks of asthma therapy, this correlation still existed (r = 0.64, p < 0.05).

### Table 1—Mean Levels of VEGF and Angiogenin in Children During Asthmatic Attack, After Treatment, and in Healthy Children*

<table>
<thead>
<tr>
<th>Variables</th>
<th>During Asthma Attack (n = 20)</th>
<th>After Treatment</th>
<th>Healthy Children (n = 12)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF, ng/mL</td>
<td>65.4 ± 15.64</td>
<td>37.95 ± 18.44</td>
<td>12.53 ± 9.67</td>
<td>&lt; 0.001†</td>
</tr>
<tr>
<td>Angiogenin, ng/mL</td>
<td>6.37 ± 1.7</td>
<td>4.92 ± 1.7</td>
<td>3.8 ± 1.37</td>
<td>&lt; 0.001‡</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.
†Difference in the mean levels of VEGF and angiogenin between patients during the acute asthmatic attack (n = 20) and healthy children (n = 12).
‡Difference in the mean levels of VEGF and angiogenin between patients during the acute asthmatic attack (n = 20) and after 6 weeks of therapy.
§Difference in the mean levels of VEGF and angiogenin between patients after 6 weeks of therapy and healthy children.

### Table 2—Mean Levels of FEV1, VEGF, and Angiogenin (During Acute Asthmatic Attack) According to Asthma Severity*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mild Asthma (n = 7)</th>
<th>Moderate Asthma (n = 9)</th>
<th>Severe Asthma (n = 4)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1, % predicted</td>
<td>84 ± 2.8</td>
<td>73.8 ± 5.1</td>
<td>59.3 ± 3.5</td>
<td>&lt; 0.001†</td>
</tr>
<tr>
<td>VEGF, ng/mL</td>
<td>48.7 ± 6.8</td>
<td>68.5 ± 6.7</td>
<td>87.00 ± 5.3</td>
<td>&lt; 0.001‡</td>
</tr>
<tr>
<td>Angiogenin, ng/mL</td>
<td>4.6 ± 0.7</td>
<td>6.6 ± 0.8</td>
<td>8.9 ± 0.5</td>
<td>&lt; 0.001§</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.
Severe characteristic changes occur in the bronchial wall in asthma, including specific changes to the vasculature. These result in an increase in vessel numbers per unit area, as well as increased vessel activity suggested by vasodilation, vessel leakage, and cellular margination with transmigration to target tissues. This combined action in asthma leads to airway wall thickening and reduced airway flow. Each component of the vascular response has been shown to be controlled by a range of inflammatory mediators and growth factors.1

In the current work, we studied two angiogenic factors: VEGF and angiogenin, with different modes of action on the bronchial mucosa. The mean levels of both factors in induced sputum from asthmatic children were significantly higher than in healthy children (p < 0.001) [Table 1]. This result was in agreement with many authors,2,4,5,12,15 who also hypothesized that both VEGF and angiogenin are considered among the most important factors that contribute to airway hyperresponsiveness by inducing chronic airway remodeling, thus leading to irreversible airway changes in asthma. Contrary to our results, Demoly et al16 found no significant difference between asthmatic and control subjects in VEGF levels in BAL fluid. This discrepancy might be explained by the difference in samples examined (ie, sputum vs BAL). BAL fluids reflect alveolar rather than bronchial surface composition; in asthma, the bronchi rather than the alveoli are involved in the epithelial injury repair.

Moreover, a positive correlation existed between levels of VEGF and angiogenin in induced sputum (Fig 1). McDonald2 confirmed that both VEGF and angiogenin play complementary and coordinated roles in vascular growth and remodeling, and both have powerful effects on vascular function.

Both VEGF and angiogenin levels were correlated

<table>
<thead>
<tr>
<th>Variables</th>
<th>Asthmatic Children (n = 20)</th>
<th>Healthy Children (n = 12)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocyte count, 10³/µL</td>
<td>11.105 ± 3.117</td>
<td>6.308 ± 1.326</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Eosinophilic count, µL</td>
<td>504.95 ± 178.5</td>
<td>67.67 ± 13.78</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lymphocytic count, µL</td>
<td>3,515.15 ± 1.185</td>
<td>1,633.3 ± 377.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IgE, %</td>
<td>339.6 ± 447.1</td>
<td>76.9 ± 39.7</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.

**Table 3—Mean Serum Total and Differential (Eosinophilic and Lymphocytic) Counts and IgE Percentage in Patients and Healthy Children**

**Table 4—Correlation Between Peripheral Eosinophils and VEGF, and Between Peripheral Lymphocytes and Angiogenin in Asthmatic Children**

<table>
<thead>
<tr>
<th>Variables</th>
<th>IgE</th>
<th>Eosinophilic Count</th>
<th>Lymphocyte Count</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>r = 0.09</td>
<td>r = 0.911</td>
<td></td>
<td>&lt; 0.05†</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>r = 0.566</td>
<td></td>
<td></td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*Correlation between VEGF and IgE percentage.
†Correlation between VEGF and eosinophils.
with the severity of asthma (Table 2). Each angiogenic factor was independently correlated with FEV₁ (as an indicator of small airway obstruction). A negative significant correlation was found between VEGF levels in induced sputum and FEV₁ percentage of predicted (Fig 2). In accordance with the above results, Salvato¹⁷ and Vrugt et al.¹⁸ showed that severe cases had more vessels than patients with mild-to-moderate asthma, which suggests a possible relation between vascular remodeling of the airway wall and asthma severity. Asai et al.⁵ as well demonstrated that asthmatics with elevated VEGF levels might exhibit airflow limitation through thickened edematous airway wall by increased permeability. Similar to VEGF, there was an inverse correlation between angiogenin levels and FEV₁ percentage of predicted (Fig 3). It was hypothesized that angiogenin may contribute to airway hyperresponsiveness by inducing chronic airway remodeling.⁴

A complete blood analysis was performed for all patients in the present study (Table 3). A positive correlation was found between peripheral eosinophils and VEGF levels; similarly, a positive correlation existed between peripheral lymphocytes and angiogenin levels (Table 4). These results confirm the cellular source of both VEGF and angiogenin. In agreement, Horiuchi and Weller¹⁹ reported that VEGF is expressed in peripheral eosinophils, which suggests a strong link between increased levels of VEGF and eosinophilic airway inflammation. However, Ahmed²⁰ found a nonsignificant correlation between sputum VEGF and serum eosinophilic count, which was explained by the small sample size used in his study. Previous work²¹ reported that angiogenin is a mediator produced by a wide variety of cells such as macrophages, endothelial cells, and peripheral blood lymphocytes. Boesiger and coworkers²² noted that mast cells can also play a role in inducing the neovascularization process through the release of proangiogenic factors such as histamine, heparin, and VEGF (following IgE-dependent up-regulation). Ahmed²⁰ confirmed the presence of a significant positive correlation between sputum VEGF and total serum IgE. However, in the present study, a nonsignificant correlation existed between the levels of IgE in serum and sputum VEGF (Table 4). Sly¹³ explained that the total IgE levels are not always informative of disease activity, although closely related to pathogenesis. Furthermore, circulating IgE does not always reflect the actual IgE population attached to mast cells.

![Figure 1](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/22040/)  
**Figure 1.** The correlation between VEGF and angiogenin levels in asthmatic patients.

![Figure 2](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/22040/)  
**Figure 2.** The correlation between VEGF levels and FEV₁ percentage of predicted in asthmatic patients.

![Figure 3](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/22040/)  
**Figure 3.** The correlation between angiogenin levels and FEV₁ percentage of predicted in asthmatic patients.
Levels of both VEGF and angiogenin were re-evaluated 6 weeks after asthma therapy, which consisted mainly of inhaled corticosteroids (fluticasone propionate) for all patients and β2-agonists in selected cases as needed. There was a significant decrease in the mean levels of both angiogenic factors compared to values recorded during the acute asthmatic attack, yet they did not reach normal control levels (Table 1). Previous work by many authors\(^5\) has suggested that inhaled corticosteroids have a role as key agents in the treatment of inflammatory airway disease (such as asthma) and angiogenesis. Bandi and Kompella\(^24\) reported that VEGF secretion and VEGF messenger RNA expression were inhibited through a glucocorticosteroid receptor-mediated mechanism in the airway and the alveolar epithelial cells. Furthermore, glucocorticoids can reverse the remodeling and decrease vascularity of the asthmatic airway mucosa induced by angiogenin production.\(^2\)

In conclusion, VEGF and angiogenin levels in induced sputum supernatants were increased in acute asthmatics, and their levels were associated with the degree of airway narrowing and asthma severity. Therefore, both factors could be used as serologic indicators of the ongoing process of allergic inflammation and asthma severity. Although the levels of both angiogenic factors were decreased after asthma treatment (6 weeks in duration), yet their levels did not reach normal as a result of the sustained disordered airway function in these patients. We recommend further studies to demonstrate the exact effect of asthma treatment (with special emphasis on inhaled corticosteroids) on angiogenesis in asthmatic children. Future studies to develop novel therapeutic agents that prevent angiogenic factor production may be needed to restore normal lung function in asthmatic subjects.

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