

Vascular endothelial growth factor overexpression in induced sputum of children with bronchial asthma

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Vascular endothelial growth factor (VEGF) induces angiogenesis and increases vascular permeability participating in narrowing of the airway lumen that follows lung injury. We sought to investigate the expression of VEGF in induced sputum during and after recovery from acute episodes of bronchial asthma in children. Eighteen asthmatic children with acute attacks of varying severity were subjected to VEGF estimation by an enzymatic immunoassay in induced sputum. They were followed up till complete remission of symptoms and signs and were then retested. VEGF was also estimated in sputum induced from age 34 and sex-matched healthy children enrolled as a control group. The sputum VEGF levels during acute asthma [median = 71 ng/ml; mean (s.d.) = 114.6 (121.8) ng/ml] were significantly higher than the levels estimated during remission [median = 50 ng/ml; mean (s.d.) = 45.7 (24.2) ng/ml] and both were higher than the corresponding levels of the control group [median = 36 ng/ml; mean (s.d.) = 31.3 (17.2) ng/ml]. VEGF levels during asthmatic episodes correlated positively to the recovery levels ($r = 0.6$, $p = 0.009$). The patients' VEGF expression did not vary with asthma severity, serum total IgE concentration, peripheral blood eosinophil count, or erythrocyte sedimentation rate of patients. Children on corticosteroids inhalation therapy at enrollment had sputum VEGF levels that were comparable to those on other therapies. The increased expression of sputum VEGF in asthmatic children reinforces the concept that it might have a pathogenetic role in bronchial asthma and may represent a biomarker of airway inflammation.

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Airway inflammation and the initiation of the injury repair process (remodeling) are fundamental and inter-related to pathogenic processes in asthma (1). The process of remodeling is usually associated with specific changes in the vasculature, resulting in an increase in vessel number per unit area, vasodilatation, vessel leakage, and cellular margination with transmigration to target tissues. This combined action in asthma leads to airway wall-thickening and reduced airflow (2). Each component of the vascular response is controlled by a range of inflammatory mediators

and growth factors (3). Among endothelial cell-specific growth factors is the vascular endothelial growth factor (VEGF) which is one of the most potent proangiogenic cytokines that play a central role in mediating the process of angiogenesis (4, 5). It increases microvascular permeability so that plasma proteins can leak into the extravascular space leading to mucosal edema, narrowing of the airway diameter, and profound alterations in the extracellular matrix (6–8). VEGF was reported to mediate endothelial cell injury during asthma exacerbation induced by exercise

challenge (9). It is also considered to have a critical role in T-helper 2 cell inflammation, cytokine production, and physiologic dysregulation in the lung (10). The number of VEGF-positive cells in bronchial biopsy specimens was found to be positively correlated with the number of vessels, mast cells, and basement membrane thickness (11). Beside its potential role in bronchial asthma, there is increasing evidence that VEGF plays a role in several acute and chronic lung diseases, such as acute lung injury, severe pulmonary hypertension, and emphysema (12).

The critical role of VEGF in the formation of new blood vessels and remodeling of existing ones in the process of allergic inflammation has stimulated us to determine its expression in induced sputum from children with bronchial asthma and investigate its possible association with disease activity, severity and treatment received.

Subjects and methods

Study population

This longitudinal study comprised 18 asthmatic and 34 normal children. The asthmatic children were recruited consecutively from the Pediatric Allergy and Immunology Unit, Children's Hospital, Ain Shams University Cairo, Egypt. The diagnosis of asthma was established according to the American Thoracic Society criteria (13). The patients were 13 males and five females and their ages ranged from 6 to 16 yrs [mean (s.d.) = 9.8 (3.6) yrs]. They were enrolled during exacerbation and were then followed up till complete subsidence of symptoms and signs to be re-tested. The duration of time between the first and second sputum sampling ranged from 14 to 26 d (median = 18 d). The patients were classified according to severity of exacerbation into nine children with mild, seven with moderate and two with acute severe asthma exacerbation and were classified during stability into four patients with mild intermittent, six with mild persistent, and eight with moderate persistent asthma (14). At the time of initial sampling, 12 children inhaled corticosteroids (ICS, fluticasone propionate 100–250 µg/d) while six children were not receiving any ICS. Their chest radiographs at enrollment were normal and cases with concomitant illness were excluded from the study. The control group comprised 34 healthy children (26 males and 8 females). Their ages ranged from 6 to 16 yrs [mean (s.d.) = 10.7 (3.2) yrs]. These children had no present or past history suggestive of asthma or other allergies, no family history of atopy, and were free from pulmonary or any

chronic diseases. An informed consent was obtained from the parents or care givers of each subject prior to enrollment and the study protocol gained approval from the local ethics committee of the Department of Pediatrics, Ain Shams University.

Study measurements

Sputum induction and processing for VEGF estimation. Sputum induction and processing were performed as previously described (15–17). Subjects were instructed to wash their mouth thoroughly with water. After inhalation of 100 µg salbutamol by a metered-dose inhaler and spacer, they inhaled 3% saline at room temperature for 5 min through an ultrasonic nebulizer at maximum output under close medical observation. They were then encouraged to cough deeply at 3-min intervals. A sputum sample was considered adequate if the patient was able to expectorate at least 2 ml of sputum. The sputum samples were kept at 4°C for no more than 2 h before further processing. Each sample was diluted three-fold with phosphate buffer solution containing 10 mmol/l dithiothreitol (final concentration: 1 mmol/l) and was gently vortexed at room temperature for 20 min. After centrifugation at 400 rpm for 10 min, the supernatant was stored at –70°C for subsequent VEGF assay. VEGF concentration in induced sputum was measured using an *in vivo* enzyme immunoassay for the detection of total human VEGF in biological fluids (ACCUCYTE Human VEGF, Cytimmune Sciences Inc, Rockville, MD, USA) according to the manufacturer's instructions.

Blood sampling and testing. Five ml of blood was aseptically collected in aliquots by venepuncture from each subject in sterile vacutainer tubes for blood cell counting, erythrocyte sedimentation rate (ESR) estimation and serum total IgE assay. The serum for IgE estimation was separated by centrifugation at 1500 rpm for 15 min after 2 h of incubation at 25°C and then collected and stored at –20°C until assayed.

Serum total IgE was measured by enzyme-linked immunosorbent assay (Genzyme Diagnostics, Medix Biotech, San Carlos, CA, USA). A serum IgE level was considered elevated if it exceeded the highest reference value for age (18). The value used in correlation analysis was the percentage from the highest normal value for age (patient's actual value/ highest normal value for age multiplied by 100) (19). Subjects in the control group with elevated serum total IgE levels were excluded from the study. A complete

blood count including total and differential leukocyte counting was performed using a cell counter (Beckman Coulter MicroDiff 18, Fullerton, CA, USA). Blood sampling of all subjects was performed at the same time daily (10 AM) to avoid diurnal variation of eosinophil count.

Statistical analysis

The results were statistically analyzed via a standard computer program (SPSS, Version 10 for Windows, Chicago, SPSS Inc., IL, USA). The data are expressed as median, interquartile range, mean, and standard deviation (s.d.). For analysis of the non-parametric data, Mann–Whitney, Wilcoxon Signed Rank, and Kruskal–Wallis tests were used. Correlations were evaluated by the Spearman's rank correlation coefficient. For all tests, p-values less than 0.05 were considered significant.

Results

Analysis of sputum VEGF results according to disease activity

Vascular endothelial growth factor levels in induced sputum of the asthmatic children at enrollment (during exacerbation) ranged between 40 and 440 ng/ml [median = 71; mean (s.d.) = 114.6 (121.8) ng/ml]. These values were significantly higher than the corresponding values of the same patients when studied after complete subsidence of symptoms and signs which ranged from 2.4 to 84 ng/ml [median = 50; mean (s.d.) = 45.7 (24.2) ng/ml]. The healthy children had significantly lower sputum VEGF levels [range = 2.2–52 ng/ml; median = 36; mean (s.d.) = 31.3 (17.2) ng/ml] compared with the patients' values whether during asthma exacerbation or quiescence (Fig. 1).

Analysis of sputum VEGF results according to asthma severity

The sputum VEGF levels were comparable among asthmatic children after being classified according to severity of exacerbation. Sputum VEGF concentrations of patients in mild exacerbation ranged between 42 and 440 ng/ml [median = 56; mean (s.d.) = 103.2 (127.3) ng/ml]. The corresponding levels of children who presented with moderate exacerbation ranged from 40 to 430 ng/ml [median = 86; mean (s.d.) = 138.3 (138.8) ng/ml] and those with acute severe asthma ranged from 80 to 86 ng/ml [median = 83; mean (s.d.) = 83 (4.2) ng/ml] respectively. The differences were statistically insignificant (Fig. 2).

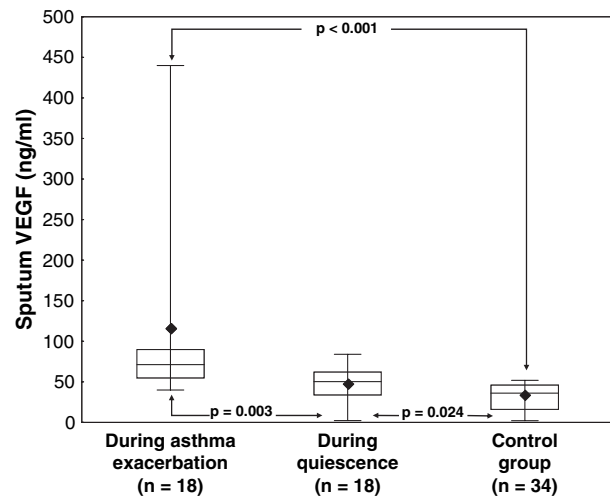


Fig. 1. Sputum vascular endothelial growth factor levels in the studied sample. The horizontal lines indicate the median values and boxes enclose the interquartile range (25th and 75th percentiles). The ranges are marked as maximum and minimum and the dots represent the mean values.

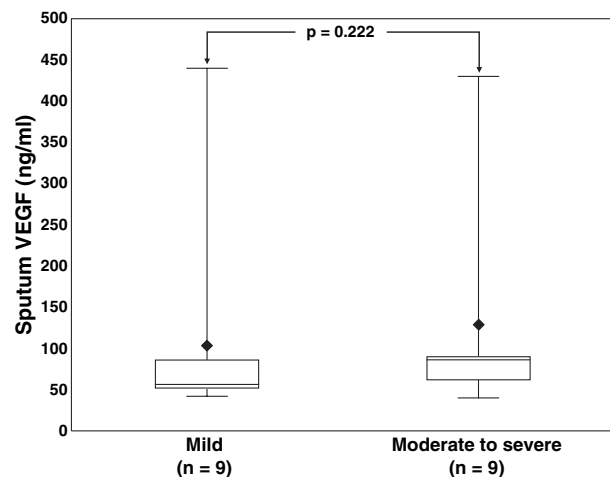


Fig. 2. Variation of sputum vascular endothelial growth factor levels according to exacerbation severity.

Vascular endothelial growth factor expression did not vary according to severity grading during stability as well. Sputum VEGF levels of patients with mild intermittent asthma ranged between 27 and 54 ng/ml [median = 41; mean (s.d.) = 40.8 (12.4) ng/ml]. The corresponding levels of patients with mild persistent and moderate persistent asthma ranged from 2.4 to 72 ng/ml [median = 21; mean (s.d.) = 30.6 (30.8) ng/ml] and from 34 to 84 ng/ml [median = 61.5; mean (s.d.) = 59.6 (15.9) ng/ml], respectively (Fig. 3).

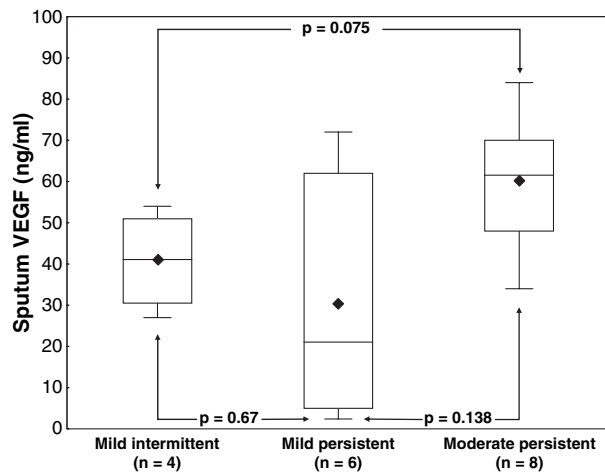


Fig. 3. Variation of sputum vascular endothelial growth factor levels according to asthma grading during stability.

Table 1. Variation of sputum VEGF according to serum total IgE and inhaled corticosteroid therapy

Variable	VEGF (exacerbation) median (mean $\pm$ s.d.)	VEGF (stability) median (mean $\pm$ s.d.)
High IgE	83.0 (139.5 $\pm$ 144.0)	56.5 (52.2 $\pm$ 22.9)
Normal IgE	59.0 (64.7 $\pm$ 19.2)	34.5 (32.8 $\pm$ 23.4)
Z	0.89	1.4
p	>0.05	>0.05
ICS–	56.5 (121.5 $\pm$ 156.8)	41.0 (38.9 $\pm$ 23.3)
ICS+	83.0 (111.2 $\pm$ 108.3)	57.5 (49.2 $\pm$ 25.0)
Z	1.03	1.12
p	>0.05	>0.05

VEGF, vascular endothelial growth factor levels; ICS, inhaled corticosteroids; Z, Mann-Whitney test result.

Analysis of sputum VEGF results in relation to serum total IgE and corticosteroid inhalation therapy

The sputum VEGF levels were comparable between asthmatic children with elevated serum total IgE and normal IgE whether during asthma exacerbation or stability. The median and mean (s.d.) levels during exacerbation were 83.0; 139.5 (144.0) ng/ml in children with high serum total IgE and 59; 64.7 (19.2) ng/ml in patients with normal levels. The corresponding values after subsidence of exacerbation were 56.5; 52.2 (22.9) ng/ml and 34.5; 32.8 (23.4) ng/ml, respectively.

No significant difference was found between patients who were on ICS therapy at enrollment [median = 83; mean (s.d.) = 111.2 (108.3) ng/ml] and those who were not [median = 56.5; mean (s.d.) = 121.5 (156.8) ng/ml] as far as their sputum VEGF expression was concerned. The same finding was observed during stability [57.5; 49.2 (25.0) ng/ml vs. 41; 38.9 (23.3) ng/ml] (Table 1).

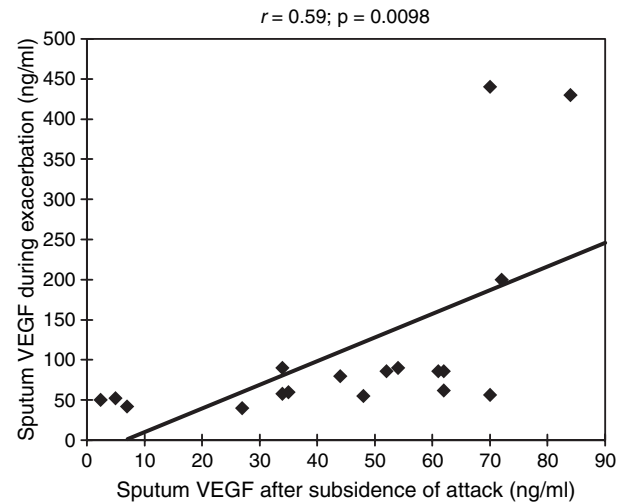


Fig. 4. Positive correlation between sputum vascular endothelial growth factor levels during and after subsidence of asthma exacerbation.

Correlation analysis between sputum VEGF and study parameters

The sputum VEGF levels during asthma exacerbation correlated positively to the levels during remission ($r = 0.59$) meaning that the higher the sputum VEGF during exacerbation, the higher it remained after its subsidence (Fig. 4). Otherwise, no correlations could be elicited between sputum VEGF levels, whether during or after subsidence of exacerbation, and all other numerical data studied such as the ESR, IgE% of normal, hemoglobin concentration, and the red blood cell, platelet, total leukocyte, neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts. Similarly, VEGF levels could not be correlated with the age of patients, duration of illness or time elapsed between the first and second sputum sampling (between enrollment and subsidence of symptoms and physical signs).

Discussion

Sputum VEGF expression during asthma exacerbation was significantly higher than that estimated during quiescence and both were higher than the corresponding level of the control group. The failure of VEGF to decrease to the control level after subsidence of exacerbation may reflect the ongoing process of allergic inflammation in the lungs of asthmatics. VEGF in induced sputum during exacerbation correlated positively to its level during quiescence of symptoms. This finding again suggests that the defect in homeostatic regulation of angiogenesis in asthmatic airways remains the same during remission, although may quantitatively decrease.

The elevation of VEGF in induced sputum of asthmatics is documented in many studies (8, 17, 20–25) even during stability which suggests that overproduction of VEGF is associated with airway inflammation and angiogenesis characterizing the asthmatic disease process. Demoly et al. (26) did not find a significant difference between asthmatic and control subjects in terms of VEGF expression in bronchoalveolar lavage (BAL) fluid. The apparent discrepancy may be because of difference in the material studied. Asthma was found to be associated with enhanced vascularity in the medium but not small airways (27) BAL fluid reflects alveolar more than bronchial surface composition while asthma is associated mainly with bronchial inflammation. Also, the BAL fluid may dilute the substance under study (21).

Clinical severity of asthma did not influence the expression of sputum VEGF in the current study whether during exacerbation or stability. In agreement, some investigators (28, 29) found no relationship between clinical variability and vascularity in patients with asthma. Others (30, 31) reported that severe cases had more vessels than those with mild to moderate asthma suggesting a possible relation between vascular remodeling of the airway and asthma severity. Increased VEGF in induced sputum of adult asthmatics was previously linked to the degree of airway narrowing and airway vascular permeability (21). VEGF-induced hyperpermeability was found to contribute to abnormal airway function in cough variant as well as classic asthma and it was hypothesized that the difference in clinical features of these two disorders depends on airway VEGF levels (32). The discrepancy between the aforementioned data and ours could be age-related. Moreover, our consecutive series did not contain cases with severe persistent asthma and most exacerbations studied were categorized as mild to moderate which may explain the difference in results. It is also possible that sputum VEGF expression as an inflammatory marker is not related to current severity. The same was observed with some other markers such as exhaled nitric oxide, serum eosinophil cationic protein (ECP) and sputum eosinophil count, and ECP level (33).

Vascular endothelial growth factor is expressed in peripheral blood eosinophils as well as local eosinophils of the bronchial mucosa suggesting that eosinophils might be one source of the airway VEGF (16, 34). Eosinophils were reported to express a VEGF receptor whose activation stimulates directed migration and release of ECP (35). Puxeddu et al. (36) stated

that human peripheral blood eosinophils may induce angiogenesis that is partially mediated by their pre-formed VEGF. In the current study, we could not establish a link between sputum VEGF levels and peripheral blood eosinophil counts. Contribution of the local airway eosinophils as well as other types of cells in the airway to the production of VEGF [e.g. epithelial cells (37, 38), mast cells (39), myofibroblast, and smooth muscle cells (40, 41)] may explain its overexpression in our series. Asai et al. (21) demonstrated a significant correlation between VEGF expression and the percentage of eosinophils and ECP level in induced sputum from adult asthmatics and they concluded that the VEGF elevation may be linked to eosinophilic airway inflammation. We did not study the sputum eosinophil counts and this may count partly for the difference as peripheral blood eosinophil counts are not necessarily elevated in every asthmatic subjects and they do not necessarily reflect the airway eosinophil numbers. It was previously postulated that a significant decrease in the number of circulating eosinophils together with a trend towards an increase of sputum eosinophil peroxidase occurs after allergen exposure which could correlate with the early phase of eosinophil recruitment to the lungs (42).

Mast cells can play a role in inducing the neo-vascularization process through the release of proangiogenic factors (43). Mast cells produce and secrete VEGF after IgE-dependent upregulation (39) and VEGF has been shown to stimulate mast cell migration to sites of angiogenesis (44). With this in mind, we sought to determine whether a relationship between the sputum VEGF and serum total IgE levels exists in asthmatic children, but we could not prove such a link. Approximately one half of patients with allergic disease have a total serum IgE level in the normal range (45) and therefore, this parameter is not always informative of asthma activity although closely related to the pathogenesis. As a matter of fact, the circulating total IgE levels do not always reflect the IgE population attached to mast cells (46) and is therefore an unreliable marker of the degree of sensitization.

Inhaled corticosteroids may have *in vitro* anti-angiogenic properties because they directly inhibit the expression of the VEGF gene (47). Bandi and Kompella (38) reported that VEGF secretion and mRNA expression were inhibited through a glucocorticoid receptor-mediated mechanism in airway and alveolar epithelial cells. We did not, however, find an influence for the low dose ICS (100–250 µg fluticasone

propionate) used by our patients before enrollment on the sputum VEGF expression. Some investigators (48, 49) found no difference between steroid naïve newly diagnosed asthmatics and asthmatics on steroids in terms of number of vessels in the airway wall. Others indicated that VEGF levels and VEGF/angiopoietin ratio in induced sputum were significantly decreased after 8 wks of inhaled beclomethasone dipropionate (800 µg/d) (21, 50). Our data conformed with those of Chetta et al. (29) who demonstrated that low doses (100 µg twice a day) of inhaled fluticasone propionate did not demonstrate any effect on thickening of the basement membrane despite significant changes in airway mucosal inflammation and asthma symptom score while 6 wks of higher doses (500 µg twice daily) did reduce the vascular component of airway remodeling in mild to moderate adult asthmatics. Beclomethasone dipropionate was reported to decrease VEGF levels significantly after 1 yr of regular therapy (24). Thus, the difference may be attributed to the lower doses of ICS and/or shorter duration of follow up in our series.

There are various VEGF isoforms and a recent study demonstrated that soluble VEGF isoforms act as a growth and anti-apoptotic factor not only endothelial cells but also on distal lung epithelial cells (51). In the current investigation, we used an assay that measures the total VEGF expression. It could be worthwhile to study the VEGF isoform variability in asthma in relation to airway inflammation and remodeling.

The study has certain limitations for credibility. First, the sample size is too small to come out with solid conclusions. Secondly, the atopic background of asthma in our series was not verified by the results of skin testing or specific IgE to common local environmental allergens and the interpretation of our data is based on the presence of bronchial asthma regardless of its atopic nature. Worth mentioning is that a previous investigation found no difference in sputum VEGF levels between atopic and non-atopic adult asthmatics (21). Finally, to objectively study the effect of ICS on VEGF expression, a prospective study design is needed in which a group of steroid naïve subjects are tested before and after a reasonable period of regular ICS therapy.

In conclusion, our results demonstrate an overexpression of VEGF in induced sputum from a group of mild to moderate asthmatic children. Our data suggest that VEGF plays a role in angiogenesis and remodeling associated with asthma. Therefore, agents that would prevent

VEGF production or block its receptors may provide a conceptual basis for the development of novel therapeutic strategies in childhood asthma. The persistence of VEGF overexpression during asthma quiescence augments the concept of ongoing inflammation and hence, the importance of continuous anti-inflammatory therapy.

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