

Evaluation of angiopoietin 1 and 2, vascular endothelial growth factor, and tumor necrosis factor alpha levels in asthmatic children

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ABSTRACT

Asthma is characterized by chronic airway inflammation that is associated with structural changes termed airway remodeling. Recently, cytokines/mediators that augment inflammation have been attracting attention in this field. The aim of this study was to evaluate serum angiopoietin (Ang)-1, Ang-2, vascular endothelial growth factor (VEGF), and tumor necrosis factor (TNF) alpha values, which have important roles in inflammation, angiogenesis, and remodeling in asthmatic children. We also documented correlations between demographic features, duration of asthma, and pulmonary function test (PFT) parameters. Randomly selected 40 children (20 male and 20 female children, aged 6–16 years) with mild or moderate persistent asthma and 32 healthy children (15 male and 17 female children, aged 6–16 years) enrolled in the study. All asthmatic children had been using inhaled corticosteroids at least for the last 3 months. Serum Ang-1 levels were significantly lower in asthmatic children than those in normal controls. The Ang-1/Ang-2 ratio was also significantly lower in asthmatic children compared with those in normal controls ($p < 0.01$). However, serum Ang-2, VEGF, and TNF-alpha levels were similar in the two groups. A significant positive correlation was found between VEGF and duration of asthma. No correlation between serum Ang-1, Ang-2, VEGF values, and PFT parameters was obtained. On the other hand, significant negative correlation was detected between serum TNF-alpha and forced expiratory volume in 1 second. We have shown that serum Ang-1 levels and Ang-1/Ang-2 ratio were significantly reduced and balance was toward Ang-2 in asthmatic children. This process may lead to inflammation, destabilization of blood vessels, and trigger remodeling.

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Asthma is characterized by chronic airway inflammation that is interrelated to angiogenesis. The angiopoietin (Ang)–Tie system has a key role in maintaining homeostasis of endothelial cells and regulates angiogenesis and inflammation.¹ Resources of major ligands of Tie receptors, Ang-1 and Ang-2, is pericytes and endothelial cells, respectively. Ang-1 phosphorylates and activates Tie-2 receptor resulting in stabilization of vessels, whereas Ang-2 is an antagonist of this receptor and destabilizes vessels.^{2,3} Ang-1 has also anti-inflammatory and antiapoptotic properties. Conversely, Ang-2 enhances inflammatory response and exhibits a substantial role in controlling the responsiveness of endothelium to cytokines such as vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF) α .

In successful angiogenesis and microvascular remodeling, Ang-1 and Ang-2 were shown to regulate function of VEGF.^{4,5} TNF- α , another important proinflammatory cy-

tokine in asthma, enhances production and secretion of Ang-2 from endothelial cells and inhibits Ang-1 expression.^{6,7} Nevertheless, Ang1–Tie signal prevents uncontrolled onset of inflammatory reactions in minor amounts of TNF- α .⁸

Angiogenesis and microvascular remodeling are known features of chronic inflammatory diseases and are responsible for a significant part of remodeling.⁹ As the metabolic need increases, blood vessels enlarge and proliferate to supply nutrients to inflammatory cells and structural cells. Airways containing dilated and congested blood vessels have been shown even in mild forms of asthma.¹⁰ Inflammation as well as remodeling constitute an important part in pathophysiology and pathogenesis of asthma and cause airway obstruction, airflow limitation, and progressive loss of lung function.^{1,11–16}

The aim of our study was to evaluate values of serum Ang-1, Ang-2, VEGF, and TNF- α which have important roles in angiogenesis, remodeling, and inflammation in asthmatic children and to document correlations between clinical features and pulmonary functions.

METHODS

Forty consecutive children with mild ($n = 10$) or moderate ($n = 30$) persistent asthma and 32 healthy children admitted to Baskent University Pediatric Al-

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lergy Department enrolled in the study. All asthmatic children had been using inhaled corticosteroids (ICSs; either fluticasone at 125–250 μg or budesonide at 200–400 μg) for at least the last 3 months. Children with severe asthma or having history of systemic steroid use or acute exacerbation of asthma within 3 months or upper respiratory tract infection at the time of the last polyclinic visit were excluded. All control subjects were healthy with no history of lung disease or any other chronic disease and were not using any medication.

Asthma was diagnosed by a history of intermittent wheezing and the presence of reversible airway obstruction as defined by at least a 12% improvement in forced expiratory volume in 1 second (FEV_1) after bronchodilator administration. Asthma diagnosis was confirmed and patients were graded for asthma severity by examination according to the Global Initiative for Asthma guidelines. Demographic features including body mass index and onset of asthmatic symptoms, animal at home, passive smoking, atopy, and history of allergic rhinitis were recorded in patients.

At admission, blood samples were collected for complete blood cell count, Ang-1, Ang-2, VEGF, and $\text{TNF-}\alpha$ measurements. Complete blood counts were performed using a daily calibrated hemocytometer (Cell-dyn 3700; Abbott Diagnostics, Santa Clara, CA) and total eosinophil counts and eosinophil percentages were recorded. Serum samples, immediately frozen from each case in a -80°C freezer, were thawed at once and analyzed for Ang-1, Ang-2, VEGF, and $\text{TNF-}\alpha$ levels. Ang-1, Ang-2, and VEGF values were measured by ELISA (RayBiotech, Inc., Norcross, GA) and $\text{TNF-}\alpha$ values were measured by Enzyme Amplified Sensitivity Immunoassay (DIAsource ImmunoAssays S.A., Nivelles, Belgium) according to the manufacturer's protocol. Total IgE values of patients were measured by ELISA (DiaMetra, Paciana, Italy).

Standard skin-prick method on the forearm with standardized allergen extracts including house-dust mite, mold mix, tree mix, grass mix, weed mix, cat, and dog had been performed in routine visits. Reactions with an induration at least 3 mm greater than the negative control were considered positive and children with at least one positive test result were considered atopic.

Just before blood sampling, pulmonary function tests (PFTs) were performed in all patients by a spirometer (Flowhandy ZAN 100 USB; Nspire Health, GmbH, Oberthulba, Germany). FEV_1 , forced vital capacity (FVC), FEV_1/FVC , and forced expiratory flow at 25–75% (FEF_{25-75}) were measured.

The study was approved by Başkent University Ethical Committee and written informed consent was obtained from all subjects and their family before the study.

Statistics

Data analyses were performed using the Statistical Package for the Social Sciences, Version 17.0 (SPSS, Inc., Chicago, IL). A value of $p < 0.05$ was considered statistically significant. The results of statistical analysis were expressed as number of observations (n), mean $\pm$ SD (mean $\pm$ SD), median, and minimum–maximum values.

Compliance with the normal distribution of continuous variables was checked with Shapiro–Wilk test. Homogeneity of groups' variances was checked by Levene's test. If parametric test assumptions are available, two independent group means were compared by Student's t -test. Correlations between the variables were analyzed by Pearson correlation coefficient. If assumptions are not available, Mann–Whitney U test was used for comparisons of group medians. Spearman ρ -correlation coefficient was used to evaluate the correlations.

RESULTS

Forty asthmatic children (20 males and 20 female children; mean age, 9.7 ± 2.9 years; range, 6–16 years) and 32 healthy children (15 males and 27 female children; mean age, 11.1 ± 3.1 years; range, 6–16 years) enrolled in the study. Ten of the patients (4 male and 6 female patients) had mild asthma whereas 30 of them (16 male and 14 female patients) had moderate persistent asthma. Mean duration of asthma was 27.2 ± 23.3 (median, 24 months; range, 3–84 months) months and mean age at diagnosis was 7.45 ± 3.1 (median, 6.8 years; range, 1–16 years) years. The mean values for age and body mass index were not different in two groups. Mean values of absolute eosinophil count were 287 ± 251 (median, 186.5; range, 17–1060) in patients and 124.9 ± 94.3 (median, 119; range, 13–453) in controls ($p < 0.01$; Table 1).

Serum Ang-1 levels were significantly lower in asthmatic children than those in control subjects ($p < 0.01$). Similarly, Ang-1/Ang-2 ratio was significantly lower in asthmatic patients ($p < 0.01$), whereas serum Ang-2, VEGF, and $\text{TNF-}\alpha$ levels showed no difference between two groups (Fig. 1; Table 1). Analysis according to the patients' sex revealed Ang-1 levels were significantly lower in asthmatic girls compared with those in healthy girls. However, no significant difference was observed between asthmatic and healthy boys and between asthmatic boys and girls (Table 2). In contrast, healthy boys had significantly lower serum Ang-1 levels compared with those in healthy girls. We also found that serum Ang-1 level increased with age in healthy boys.

We found a significant correlation between serum Ang-2 and VEGF levels in asthmatic patients (Spearman $\rho = 0.335$; $p < 0.05$) without no significant corre-

Table 1 Comparison of serum Ang-1, Ang-2, VEGF, TNF- α levels, Ang-1/Ang-2, and VEGF/Ang-2 ratios; total eosinophil count; and eosinophil (%) of asthmatic and control groups

	Asthma <i>n</i> = 40	Control <i>n</i> = 32	<i>p</i> Value
Ang-1 (pg/mL)			
Mean $\pm$ SD	12,498.5 $\pm$ 7324.5	19,686.7 $\pm$ 11368.9	0.002
Median	10,783.2	17,203	
Ang-2 (pg/mL)			
Mean $\pm$ SD	2490.4 $\pm$ 1353.2	2223.9 $\pm$ 1270	0.397
Median	2262.7	2025	
Ang-1/Ang-2			
Mean $\pm$ SD	6.86 $\pm$ 5.76	11.9 $\pm$ 9.2	0.006
Median	4.99	8.4	
VEGF (pg/mL)			
Mean $\pm$ SD	243.5 $\pm$ 382.3	188.3 $\pm$ 168.8	0.45
Median	130.6	133	
TNF- α (pg/mL)			
Mean $\pm$ SD	8.3 $\pm$ 11.8	7.7 $\pm$ 19.6	0.88
Median	4.8	3.7	
VEGF/Ang-2			
Mean $\pm$ SD	0.13 $\pm$ 0.26	0.12 $\pm$ 0.16	0.92
Median	0.07	0.1	
Total eosinophil count			
Mean $\pm$ SD	287 $\pm$ 251	124.9 $\pm$ 94.3 (<i>n</i> = 26)	0.003
Median	186	119	
Eosinophil (%)			
Mean $\pm$ SD	3.8 $\pm$ 3.5	1.7 $\pm$ 1.2 (<i>n</i> = 26)	0.006

Ang = angiopoietin; VEGF = vascular endothelial growth factor; TNF = tumor necrosis factor.

lation among other parameters. Age at the diagnosis of asthma and serum Ang-2 levels also showed a positive correlation. Moreover, we found positive correlation between duration of asthma and serum VEGF levels ($p < 0.05$).

No significant difference was observed in Ang-1, Ang-2, VEGF, and TNF- α levels among asthmatic children when compared according to the presence of atopy, allergic rhinitis, passive smoking, and family history of asthma as well as age, total eosinophil count, eosinophil percentage, Ang-1, Ang-2, VEGF, TNF- α levels, Ang-1/Ang-2, and VEGF/Ang-2 ratios.

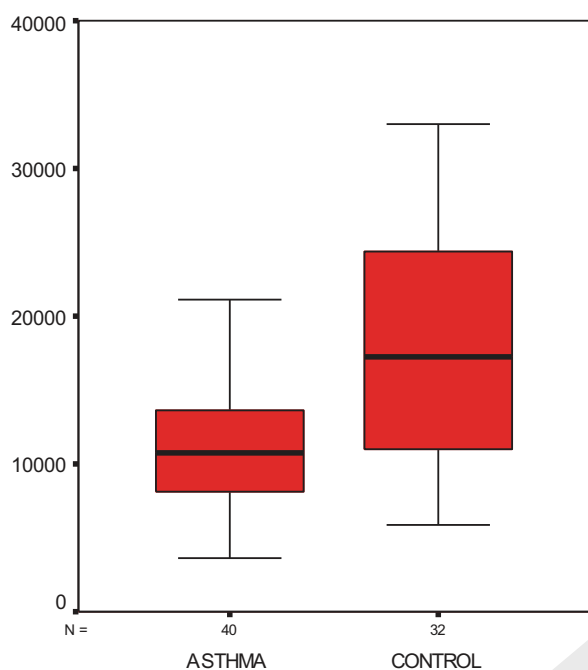
In asthmatic children, PFT revealed mean values of FEV₁, 104.3 $\pm$ 13.9; FEV₁/FVC, 95.4 $\pm$ 5.6; and forced expiratory flow at 25–75%, 110 $\pm$ 21.7. PFTs of mild and moderate persistent asthmatic patients is shown in Table 3. There was no correlation between serum Ang-1, Ang-2, VEGF levels, and PFT parameters. We identified a negative correlation between TNF- α and FEV₁ (Spearman $\rho = -0.387$; $p < 0.05$). Ang's, VEGF, TNF- α levels, and PFT parameters showed no statistically significant difference between mild and moderate asthmatic patients.

DISCUSSION

Cytokines/mediators are of particular importance causing chronic inflammation and the means by which cytokines amplify and perpetuate the inflammatory process in asthma is now emerging. Ang's have been attracting attention now because of their roles in regulating inflammatory response and remodeling. However, there is no study in literature concerning Ang levels in asthmatic children. In our study, we found significantly lower Ang-1 levels and Ang-1/Ang-2 ratio in asthmatic children. Because Ang-1 has anti-inflammatory properties and is responsible for vascular integrity, lower levels of this cytokine may contribute to the pathogenesis of asthma as well as remodeling. A recent study revealed reduced Ang-1 mRNA and protein levels in the lungs of mice sensitized and challenged with ovalbumin.¹⁷ This is the only study that has consistent results with ours regarding low Ang-1 levels so far. Ang-1 expression may be attenuated during the inflammatory process in asthma because of factors such as TNF- α , transforming growth factor β , IL-1 β , and nitric oxide.^{18,19}

Conversely, some other studies revealed increased Ang-1 in airway or sputum of asthmatic subjects that

(A)



(B)

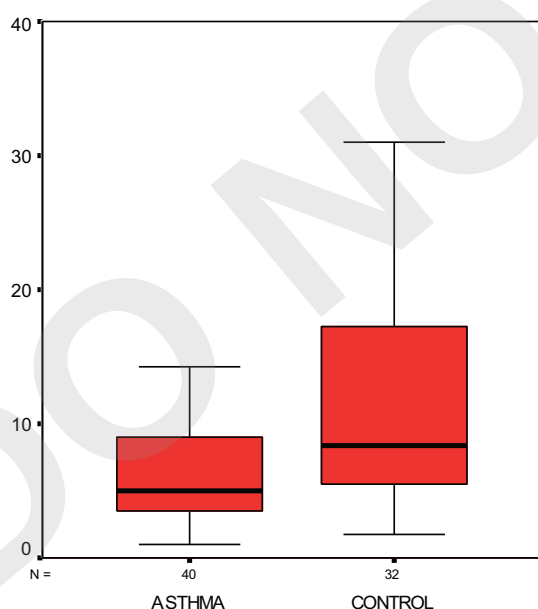


Figure 1. Comparison of serum (A) angiopoietin-1 (Ang-1) levels and (B) Ang-1/Ang-2 ratio between asthmatic subjects and control subjects.

decreased to similar levels but not lower than those in controls after using ICS.^{20–22} Simoes and colleagues¹⁷ speculated that in the course of asthma, after an initial decrease, Ang-1 levels rise to provide vascular permeability and integrity. This opinion may explain why Ang-1 levels were found to be increased in adult studies. As a process of adaptation, Ang-1 levels may fluctuate according to the duration of disease or age at diagnosis that may explain the discrepancy among studies including ours. On the other hand, examination of Ang-1 levels in serum, airway, or sputum might be another reason why we have found lower Ang-1 levels in serum unlike the other studies.

tuating according to the duration of disease or age at diagnosis that may explain the discrepancy among studies including ours. On the other hand, examination of Ang-1 levels in serum, airway, or sputum might be another reason why we have found lower Ang-1 levels in serum unlike the other studies.

Serum Ang-1 levels of asthmatic girls were significantly lower than those in normal controls in our study. However, no significant difference was observed between asthmatic and healthy boys as well as between asthmatic boys and girls. Disappearance of sex difference in asthmatic children may indicate importance of Ang-1 in pathogenesis of asthma. It is well known that prepubertal male children are more prone to develop asthma than prepubertal girls. Supporting this opinion, healthy boys showed lower serum Ang-1 levels compared with healthy girls and serum Ang-1 levels increased with age in only healthy boys.

All asthmatic children in our study had been receiving ICS treatment for at least 3 months. Ang-1 levels have been shown to decrease with ICS treatment; however, Ang-2 levels were not affected.²⁰ Sputum Ang-2 levels were increased in asthmatic subjects in several studies.^{20,22–26} Tseliou and coworkers²⁴ showed that sputum Ang-1 and Ang-2 levels had been significantly elevated in severe refractory asthma compared with those with moderate asthma or those who were healthy. However, in our study serum Ang-2 levels were similar in two groups. We thought evaluating Ang-2 levels in serum, examining mild-moderate asthmatic patients, and, more importantly, involving child age group were the reasons for similar Ang-2 levels.

Ang-1/Ang-2 ratio, determining functional status of vasculature, was found to be more important than Ang-2 level alone for determining effect of Tie-2 signaling.²⁷ Because the balance between Ang-1 and Ang-2 is toward Ang-2, phosphorylation of Tie-2 receptor decreases, leading loss of its protective role.^{17,23,24} Our study showing lower Ang-1/Ang-2 ratio in asthmatic patients supports that Ang-2 may bind to Tie-2 receptor more easily in the absence or low levels of Ang-1 and the pathway may go along with Ang-2 signal. Consequently, antagonist action of Ang-2 may trigger inflammation and angiogenesis by destabilization of vasculature and sensitize vessels to angiogenic and inflammatory factors.^{1,7} Although asthma symptoms were controlled, having lower Ang-1/Ang-2 ratio suggested that balance toward Ang-2 in our patients might have induced inflammation, endothelial permeability, progression of angiogenesis, and the remodeling process. Despite receiving ICS treatment, Ang-1 levels and Ang-1/Ang-2 ratio were lower in asthmatic children in our study. This finding may suggest that this process contributes to the progression of inflammation and augmentation of vascular permeability by activation of the vascular endothelial barrier to increase leakage and

Table 2 Comparison of serum Ang-1, Ang-2, and Ang-1/Ang-2 ratios according to gender

	Male Subjects		<i>p</i> Value	Female Subjects		<i>p</i> Value
	Asthma <i>n</i> = 20	Control <i>n</i> = 15		Asthma <i>n</i> = 20	Control <i>n</i> = 17	
Ang-1 (pg/mL)						
Mean ± SD	13,522 ± 8393.5	15,235 ± 5344	0.50	114,74.3 ± 6120.6	23,614 ± 13,801	0.001
median	11,160.8	14,266		10,279.7	22,637	
Ang-2 (pg/mL)						
Mean ± SD	2642.7 ± 1479.9	2345 ± 1092	0.52	2338 ± 1232.7	2117 ± 1434	0.62
median	2992.1	2389		2046.6	1627	
Ang-1/Ang-2						
Mean ± SD	7.5 ± 6.8	9.2 ± 7.6	0.50	6.2 ± 4.6	14.3 ± 10.1	0.003
median	5.5	6.9		4.1	9.9	

Ang = angiopoietin.

Table 3 PFT of mild and moderate asthmatic children

	Mild Persistent Asthma	Moderate Persistent Asthma*	<i>p</i> Value
Subject no. (M/F)	10 (4/6)	30 (16/14)	
Age (yr)	9 ± 2.8	10 ± 2.9	>0.05
FVC	97.8 ± 12.5	91.9 ± 14	>0.05
FEV ₁	110.8 ± 11.2	102.1 ± 14.1	>0.05
FEV ₁ /FVC	97.8 ± 3.4	94.6 ± 6	>0.05
FEF ₂₅₋₇₅	112.3 ± 14.8	109.2 ± 23.8	>0.05
ICS dose	125 µg of fluticasone or 200 µg of budesonide	250 µg of fluticasone or 400 µg of budesonide	

*Four of the patients had montelukast and the other two had long-acting β -agonist, additionally.

PFT = pulmonary function test; FVC = forced vital capacity; FEV₁ = forced expiratory volume in 1 s; FEF₂₅₋₇₅ = forced expiratory flow at 25–75%; ICS = inhaled corticosteroid.

leukocyte migration into tissues. Once the Ang-1 level is low, negative action of Ang-2 on Ang-Tie system would have been illustrated easily. Our results, once again, emphasized the notion that only asthma symptoms could be controlled but inflammation, progression of asthma, and, probably, remodeling could not be prevented by ICSs. Although all the patients were receiving ICS, it is not clear whether low Ang-1 levels were related to ICS treatment or asthma itself.

Nomura and colleagues²⁶ determined that both VEGF and Ang-2 were important in the remodeling process. We showed correlation between serum VEGF levels and duration of asthma in our study that suggests as asthma progresses and its duration increases, VEGF augments angiogenesis and remodeling gradually.^{28,29} Increased VEGF production in bronchial epithelial cells³⁰ and increased VEGF levels in sputum^{23,31–33} in recent studies indicated increased bronchial vascular mass in children with acute asthma.

In our study, we showed a negative correlation between serum FEV₁ and TNF- α , which has a key role in

the pathogenesis of asthma that might affect respiratory functions by causing inflammation in airways. Increased TNF- α in airways, and TNF- α -mRNA in bronchoalveolar lavage (BAL) of asthmatic patients have been reported similar to our study.^{34,35} Although increased levels are expected in asthma, no difference was present between two groups in our study concerning Ang-2, VEGF, and TNF- α level. Having involved mild-moderate asthmatic children with controlled symptoms, receiving ICSs and no recent asthmatic attack may be the contributing factors that might help to explain obtaining similar results with healthy subjects in our study. Consistent with our observation, after treatment with ICSs, the increased levels of angiogenic factors as well as TNF- α levels have been shown to decrease significantly.^{23,26,36,37} One can conclude that asthmatic children receiving ICS somehow have been controlled, because VEGF and Ang-2 act as proangiogenic factor, whereas TNF- α acts as a proinflammatory factor. However, Ang-1, with anti-inflammatory and antiapoptotic effects, was significantly lower in asthmatic

children in our study. This result may support that perfect control could not be achieved in asthmatic patients.

Ang-1 also has been shown to inhibit inflammation-induced leakage and VEGF-induced permeability in mature blood vessels. Recently, local administration of Ang-1 has been considered as a new therapeutic approach in inflammatory states as well as in asthma.^{17,18,38–43} Because we observed low levels of Ang-1 in asthmatic children, we suggest that administration of an Ang-1 agonist or Ang-2 antagonist would be beneficial. Achieving balance of Ang-1/Ang-2 toward Ang-1 would result in maintenance of vessel integrity, decrease in inflammation, and inhibition of angiogenesis as well as remodeling. We believe finding low Ang-1 levels in serum is very valuable and convenient compared with investigations in BAL and sputum; however, limitation of our study is that levels were obtained only from serum at once. Getting sputum/BAL/lung tissue and serum samples at the same time and comparing these results would give better clues to understand how their levels are related and whether any significant difference presents among them.

In conclusion, lower Ang-1 levels and Ang-1/Ang-2 ratio in asthmatic children reinforce the notion that Ang-1 has an essential role in pathogenesis of asthma concerning inflammation as well as remodeling. These would be important measures in follow-up in asthma as well as other chronic inflammatory diseases. We believe it is worthy to investigate Ang-1, Ang-2, VEGF, and TNF- α and their interrelation, particularly in asthmatic children. By the help of larger, prospective studies and further investigations concerning Ang-1, Ang-2 levels, and Ang-1/Ang-2 ratio, these parameters might be considered in the near future for the follow-up of patients with asthma.

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