

Correlation of plasma transforming growth factor beta 1 with asthma control test

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ABSTRACT

Assessment of asthma with a control test is suggested as a relevant approach for recent years. Ideally, an asthma control test should apply not only to clinical manifestations but also to laboratory markers of inflammation as well. Until now, this could not be performed because of the lack of a confirmed marker which indicates inflammation. A fibrotic mediator TGF-beta 1 has been reported as a key mediator of remodeling in asthma. The aim of this study is to evaluate plasma TGF-beta 1 level in stable asthmatic sufferers and to investigate its correlation with the asthma control test. Stable asthmatic sufferers and healthy controls were recruited for this study. After obtaining demographic information, skin prick and asthma control tests were performed. Blood samples were collected for plasma TGF-beta 1 level. Any contributing factors that may affect plasma TGF-beta 1 level were excluded from both groups. Thirty-five atopic, 35 nonatopic asthmatic sufferers and 15 healthy control subjects were included for this study. The mean age was 38 ± 10 (years) and 61% were female. When the asthmatic group compared with the control group, plasma TGF-beta 1 level was significantly higher in the asthmatic group (41.7 ± 12.6 ng/mL versus 27.6 ± 13 ng/mL) ($p < 0.05$) whereas it was similar among the atopic and nonatopic groups (41.8 ± 14.2 ng/mL versus 41.6 ± 11 ng/mL) ($p > 0.05$). Spearman Correlation Analysis results pointed positive correlation between uncontrolled asthma and plasma TGF-beta 1 level. This study shows that plasma TGF-beta 1 level may be a systemic marker of asthma control.

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Key words: Asthma, asthma control test, atopy, inflammation, remodeling, systemic marker, TGF-beta 1

Asthma is a well-characterized bronchial inflammatory process associated with mast cells, eosinophils, and T lymphocytes within the bronchial wall.¹ Consequently, treatments have been developed to suppress this inflammatory response and some success has been achieved by using corticosteroids, leukotriene receptor antagonists, and mast cell stabilizers.^{2,3} However, some patients experience irreversible or refractory asthma with persistent symptoms and a progressive decline in their lung functions develop despite maximal level of therapy. Histological examination in such patients reveals subepithelial deposition of collagen in the airways, hypertrophy, and hyperplasia in smooth muscle cells, mucus gland hyperplasia, and angiogenesis, which has been described as remodeling according to the National Institute of Health Workshop.^{3,4} The mechanisms responsible for both the remodeling and the inflammation are incompletely understood, but some clinical studies indicate that inflammation is related to the severity of the disease.^{5–7} Despite these results, the classification of asthma severity is no

longer recommended except newly diagnosed patients and the assessment of asthma control with tests is reported as a relevant approach according to the recent guidelines.³ Ideally, the asthma control test should apply not only to clinical manifestations, but also to laboratory markers of inflammation and pathophysiological features of disease as well. Unfortunately, this could not be performed due to the cost and the unavailability of a confirmed marker, which indicates inflammation until now.⁸

Transforming growth factor (TGF) β is a potent immunoregulatory protein family. In mammalian cells, there are three subtypes of TGF- β ligands, $\beta 1$, $\beta 2$, and $\beta 3$. They are encoded by separate genes but signal through the same signaling cascade.⁹ TGF- $\beta 1$ is commonly responsible for much of the biological effects.¹⁰ TGF- $\beta 1$ is also believed to be a potent immunomodulatory agent that has both inductive and suppressive effects.¹¹ The effects of TGF- $\beta 1$ protein vary with the state of activation, differentiation of the target cell, and the presence of other stimuli in the local microenvironment. In addition, it has been shown that TGF- $\beta 1$ can exert its seemingly antagonistic effects *via* different effector pathways.¹² Major cellular sources of TGF- $\beta 1$ in asthmatic airways include activated eosinophils, bronchial epithelial cells, and fibroblasts. Mast cells, smooth muscle cells, and T lymphocytes are also known to produce TGF- $\beta 1$ on activation.^{1,13} TGF- $\beta 1$ is

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a potent chemoattractant for several cell types including T lymphocytes, fibroblasts, and eosinophils.¹³ It has been known as the most potent chemoattractant for direct collagen synthesis. Furthermore, TGF- β 1 stimulates the fibroblasts and the myofibroblasts, which increases the synthesis of many extracellular matrix components such as collagen, fibronectin, and proteoglycans.¹⁴ TGF- β 1 also participates in the pathogenesis of many diseases related to fibrosis. Increased tissue and plasma levels have been reported in hepatocellular carcinoma, pseudoexfoliative syndrome, inflammatory bowel disease, and systemic sclerosis.^{15–18} It has also been reported to play a key role in the remodeling phase of asthma according to the immunocytochemical studies.^{19–22} The aim of this study was to evaluate the plasma TGF- β 1 level in stable nonatopic and atopic asthmatic patients and to investigate its correlation with the atopy, level of disease control, duration of the disease, and the frequency of attacks.

METHODS

Stable asthmatic patients attending our department were recruited for this prospective case-control study. Asthma was defined and classified according to the recent guidelines.³ Exacerbations of asthma were defined as episodes of progressive increase in shortness of breath, cough, wheezing, chest tightness, or some combination of these symptoms. After selecting the group age, gender, duration of asthma, respiratory function test parameters, the frequency of attacks per year, daytime symptoms, limitation of activities, nocturnal symptoms, the need for rescue treatment, and the medications used were all recorded. Asthma control test was then performed using prospectively collected data according to Global Initiative for Asthma guidelines. If the patients exhibit two or less per week daytime symptoms, no limitation of activities, no nocturnal symptoms, two or less per week need for rescue treatment, normal pulmonary function tests, and no exacerbation, the case was defined as controlled. If the patients exhibit symptoms more than twice per week during the daytime, limitation of activities, or nocturnal symptoms in any week, a need for rescue treatment more than twice per week, predicted or personal best (if known) peak expiratory flow or forced expiratory volume at 1 second of $< 80\%$, or one or more exacerbation per year, the case was accepted as partly controlled. Finally, if three or more factors of partly controlled asthma present in any given week, it was confirmed as uncontrolled.⁸ The patients also undergo skin-prick tests. If a wheal response of ≥ 3 mm in diameter develops after 15 minutes to one or more of the allergens in the presence of both negative (0, 9% saline) and positive (histamine acid phosphate) con-

trols, the test was considered as a positive response.²³ Patients with positive skin-prick test results and accompanying clinical information were accepted as atopic.

Exclusion criteria for the patient group were as follows: age being < 18 years; smoking or past smoking history; pregnancy; negative reversibility or bronchoprovocation test; febrile illness; any infection or asthma exacerbation within the previous 4 weeks; the use of antihistaminic, oral, or parenteral steroid within the previous 4 weeks; any pulmonary comorbidity such as chronic obstructive pulmonary disease, bronchiolitis obliterans, sarcoidosis, silicosis, etc.; any systemic disease such as acute, chronic liver disease; any malignancy; inflammatory bowel disease; or amyotrophic lateral sclerosis. Healthy individuals were selected from similar age and gender groups.

Exclusion criteria of the control group were age being < 18 years, asthma or atopy in medical history, asthma or atopy in family history, any infection within the past 4 weeks, and smoking or past smoking history. Participants who refuse to take part in the study were also excluded from both the patient and the control groups. The research ethics committee of the hospital approved the study protocol and written informed consent documents were obtained from all of the participants.

Blood samples were collected from the patient group for whole blood count, serum IgE level, and plasma TGF- β 1 level and only for plasma TGF- β 1 level measurement from the control group with utmost care preventing hemolysis. Whole blood count and serum IgE level measurements were performed with a Coulter GEN-ES and Tosoh AIA 21 devices (Tosoh Corp., Tokyo, Japan), respectively.

Plasma TGF- β 1 Measurement

The blood samples collected in EDTA tubes for plasma TGF- β 1 measurement were first put into centrifuge at $1000 \times g$ for 10 minutes at 4°C and sera were extracted. Then, the sera were separated from the cells within an hour and put in the centrifuge at $3000 \times g$ for 10 minutes to eliminate platelets. After collecting platelet-free plasma, samples were frozen at -70°C until testing began.

Plasma TGF- β 1 measurement was performed with a solid-phase sandwich ELISA kit prepared by BioSource International Immunoassay (Camarillo, CA). Before the measurement, a sample extraction was performed to release active TGF- β 1 from latent complexes, thus making it accessible for measurement in the immunoassay according to the manufacturer's instructions. For this step, 0.1 mL of each sample was added with 0.3 mL of extraction solution containing ethanol 70% and 1 N HCl 30% in a polypropylene tube. Then,

the mixture was vortexed and incubated 10 minutes at room temperature with continuous shaking. The sample was then put in the centrifuge at $1000 \times g$ for 10 minutes, and the collected supernatant was diluted 10-fold with standard diluent buffer in polypropylene. Control was added to the microtiter wells covered with anti-TGF- β 1 anticore. Later, biotinylated anti TGF- β 1 solution was pipetted to each of the wells. TGF- β 1 antigen binding to immobile anticore in each of the wells was followed by the biotinylated anticore binding to the antigen. Each well was then incubated for 3 hours at room temperature, and then the wells were washed four times. After adding 100 μ L of streptavidin-peroxidase, the sandwich was completed. The optical density of the sample-enzyme complex, which was measured at 450 nm, was correlated with the TGF- β 1 concentration in the sample. The minimum level of detection of TGF- β 1 with this ELISA technique was 10 pg/mL.

Statistical Analysis

SPSS for Windows 10.0.1 software (SPSS Inc., Chicago, IL) was used for the statistical analysis. Results were presented as mean $\pm$ SD and percentiles. Differences in parametric and nonparametric values were tested with Student's *t*-test and Mann Whitney *U* test, respectively. Categorical variables were compared with χ^2 . Kruskal-Wallis tests were used for nonparametric analysis. For the comparison of three groups, one-way ANOVA test was used. If the value of *p* was <0.05 in those tests, *post hoc* comparison tests were used for the analysis. A receiver operating characteristic curve for the assessment of the predictive value of TGF- β 1 level on uncontrolled disease was performed and the area under the curve was calculated. Correlation was investigated with Pearson tests for parametric values and Spearman correlation tests for nonparametric values. A value of *p* < 0.05 was considered a statistically significant difference.

RESULTS

Thirty-five atopic and 35 nonatopic asthmatic patients and 15 healthy control subjects were enrolled for the study. Mean age was 38 ± 10 years (20–60 years, minimum-maximum) and male/female ratio was 36:64%. The most common allergen was house-dust mites (57%) followed by mold (48%) in the atopic group according to skin-prick test results presented in Table 1.

When the asthmatic subjects were compared with the control group, mean age and gender were found to be similar (*p* > 0.05) but plasma TGF- β 1 levels were significantly higher in the asthmatic group (41.7 ± 12.6 versus 27.6 ± 13.0 ; *p* < 0.05). Comparison of the atopic and nonatopic asthmatic patients is presented in Table

Table 1 Skin-prick test results of the atopic asthmatic patients

Allergens	Number <i>n</i> = 35 (%)
House-dust mites	7 (20)
Pollens	1 (3)
Mold	5 (14)
Other (cats, dogs, cockroaches, or grass)	2 (6)
House-dust mites + pollen	2 (6)
House-dust mites + mold	6 (17)
House-dust mites + other	3 (8)
Pollen + mold	1 (3)
Pollen + other	2 (6)
House-dust mites + pollen + other	1 (3)
House-dust mites + mold + other	1 (3)
Pollen + mold + other	4 (11)

House-dust mites: Dermatophagoides pteronyssinus and Dermatophagoides farinae.

Pollen: Tree pollens (Betulaceae: alder, birch, hazel, and hornbeam).

Other: Cats, dogs, cockroaches, or grass.

2. Age, gender, the control level of disease, the medications used, the duration of asthma, attack frequency per year, blood eosinophils, and plasma TGF- β 1 levels were similar (*p* > 0.05), whereas serum IgE level was statistically higher among the atopic patients compared with the nonatopic patients (*p* < 0.05 ; Table 2).

When the asthmatic patients were classified according to the level of asthma control age, gender, duration of disease, and serum IgE levels were similar while the plasma TGF- β 1 level and the frequency of attacks were significantly higher among the uncontrolled group compared with the controlled and partly controlled groups (*p* < 0.05 ; Table 3). When the relationship between uncontrolled disease and plasma TGF- β 1 levels were compared with the receiver operating characteristic curve, ≥ 40 ng/mL, the cutoff level was found to be the value with highest sensitivity and specificity. Among 26 uncontrolled patients, 81% had a plasma TGF- β 1 level of ≥ 40 ng/mL and among the remaining 44 controlled and partly controlled patients, only 47% had a plasma TGF- β 1 level of ≥ 40 ng/mL (*p* < 0.05 ; Fig. 1). Spearman correlation analysis results pointed out that the two independent variables that cause increased plasma TGF- β 1 levels were the attack frequency per year (*r* = 0.350; *p* = 0.001) and the uncontrolled disease (*r* = 0.449; *p* = 0.001).

CONCLUSION

The main finding of the present study is the increased level of plasma TGF- β 1 in asthmatic patients and its positive correlation with the attack frequency

Table 2 Comparison of atopic and nonatopic patients

	Atopic Patients (n = 35)	Nonatopic Patients (n = 35)	p Value
Age (mean/yr)	39 ± 10	36 ± 9	0.431
Gender (female/male)(n)	23/12	22/13	0.500
Controlled asthma, n (%)	12 (34%)	9 (25%)	0.603
Partly controlled asthma, n (%)	11 (32%)	12 (35%)	0.500
Uncontrolled asthma, n (%)	12 (34%)	14 (40%)	0.805
Short-acting β_2 -agonist as needed	5 (14)	4 (11%)	0.726
Inhaled corticosteroid monotherapy	7 (20%)	9 (26%)	0.576
Inhaled corticosteroid + LABA	11 (32%)	8 (23%)	0.427
Fixed combination	12 (34%)	14 (40%)	0.803
Leukotriene receptor antagonist	1 (3%)	2 (6%)	0.562
Duration of asthma (yr)	9.7 ± 8.6	8.5 ± 7.6	0.370
Frequency of attacks per year	6.7 ± 7	6.9 ± 7	0.794
Blood eosinophils (mm ³)	503 ± 604	383 ± 364	0.319
Serum IgE levels (IU/mL)	361 ± 412	192 ± 331	0.021
Plasma TGF- β 1 level (ng/mL)	41.8 ± 14.2	41.6 ± 11.0	0.938

LABA = long-acting β_2 agonist; TGF = transforming growth factor.

Table 3 Comparison of patients according to the level of asthma control

	Controlled n = 21	Partly Controlled n = 23	Uncontrolled n = 26	p Value
Age (yr)	38 ± 9	37 ± 9	41 ± 10	0.415
Female/male (%)	72/28	70/30	54/46	>0.05
Duration of asthma (yr)	7.8 ± 7.9	10.3 ± 8.0	9.1 ± 8.4	0.592
Frequency of attacks per year	3.8 ± 5	6.4 ± 7	9.5 ± 7.5	0.020
Serum IgE levels (IU/mL)	224 ± 318	274 ± 441	322 ± 378	0.686
Plasma TGF- β 1 level (ng/mL)	35.3 ± 12	39.2 ± 9	49.0 ± 12	0.001

TGF = transforming growth factor.

and uncontrolled disease. TGF- β 1 and its histological correlation with tissue fibrosis have been proven with many animal and human studies.^{24–27} Many cells in the human lung are known as potent sources of TGF- β 1.¹³ The first study, which indicates the relationship of TGF- β 1 and asthma, was performed by Vignola and found that TGF- β immunoreactivity was highly increased in epithelium and submucosa in chronic bronchitis and asthma. Moreover, a significant correlation was found between the number of epithelial or submucosal cells expressing TGF- β , the thickness of basement membrane, and fibroblast number in both of the patient groups.²⁰ Then, Magnan *et al.* reported TGF- β staining in the inflammatory cells beneath the basement membrane in the bronchial biopsy of asthmatic patients and concluded these results to be related with subepithelial fibrosis.²¹ After that, Minshall *et al.* reported that the increase of submucosal TGF- β 1 mRNA was directly related with the disease severity in asthma.²² Kokturk *et al.* confirmed these results.²⁷

The first study, which evaluates plasma TGF- β 1 level, showed a significant increase due to thoracic radiotherapy-related fibrosis.²⁸ Elevated levels were also reported in patients with bronchiectasis and idiopathic pulmonary fibrosis.^{29,30} Joseph *et al.* reported an increase of plasma TGF- β 1 level in nonatopic asthmatic patients compared with the atopic and normal healthy subjects.³¹

The increase in plasma TGF- β 1 level in asthmatic subjects in our study supports the findings of Joseph *et al.* but some of the differences should be mentioned. First, although Joseph indicated an increase of plasma TGF- β 1 level in nonatopic asthmatic patients compared with the atopic and normal healthy subjects, we could not find any difference between atopic and nonatopic patients. This variation may be because of the different methods of two studies while describing atopy. The patients were classified as atopic or nonatopic based on the presence or absence of serum-specific IgE directed to common allergens in that study but

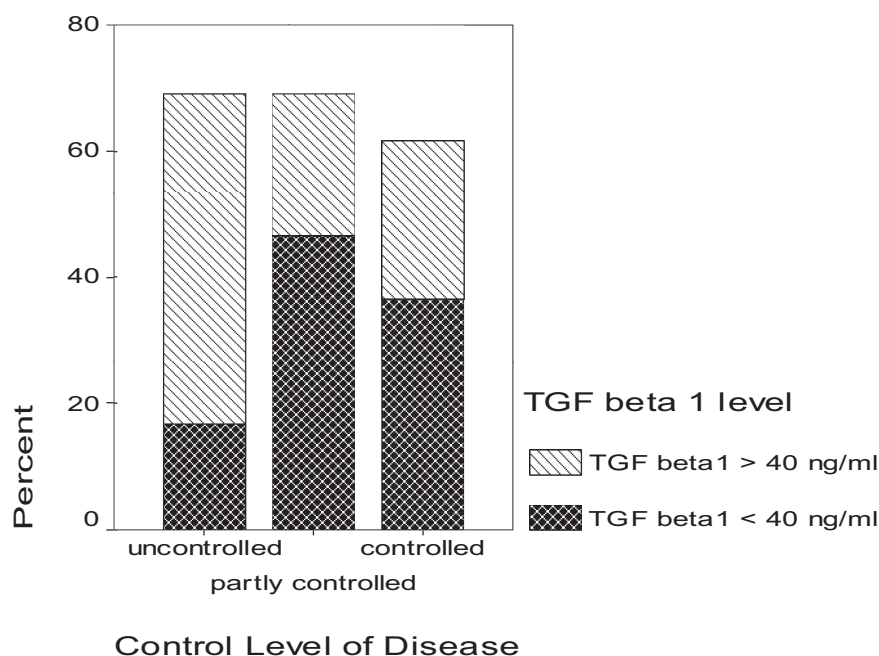


Figure 1. Comparison of transforming growth factor $\beta 1$ and levels of the asthma control test.

we performed skin-prick tests. Second, plasma TGF- $\beta 1$ level was independent from the disease severity and duration in that study but we found positive correlation between plasma TGF- $\beta 1$ level and asthma control test, which may reflect airway inflammation. Interestingly, the authors reported leukocytosis among atopic patients in accordance with increased TGF- $\beta 1$ level. Therefore, it was suggested that nonatopic asthmatic patients exhibit an altered inflammatory response, perhaps to a respiratory infection rather than fibrosis and continuing inflammation. We did not investigate any relationship between plasma TGF- $\beta 1$ level and leukocyte number because it was not related to our hypothesis.³¹ A very recent study also confirmed the increased levels of TGF- $\beta 1$ in induced sputum in asthmatic patients despite treatment with inhaled corticosteroids and it was associated with airflow obstruction and airway wall thickening.³² Our study also supports these results.

The higher levels of circulating active TGF- $\beta 1$ in asthmatic patients might be attributable to several factors. Because both the bronchial epithelial cells and many of the inflammatory cells located in submucosa release TGF- $\beta 1$, high plasma TGF- $\beta 1$ levels may be caused by bronchial epithelial cell injury or ongoing submucosal inflammation. Increased TGF- $\beta 1$ levels correlated with the level of asthma control and the frequency of attacks in our study supports this hypothesis. TGF- $\beta 1$ release due to uncontrolled disease and recurrent attacks may result in prolonged airway inflammation and remodeling in asthma. In a recent study, Lim *et al.* reported that the induction of airway remodeling of nasal mucosa by repetitive allergen chal-

lenge in a murine model of allergic rhinitis resulted with airway remodeling. Moreover, the administration of dexamethasone significantly inhibited these histological changes.³³

Our study has some limitations. Although we have excluded patients who have any systemic disorder related with TGF- $\beta 1$ release, local TGF- $\beta 1$ levels such as bronchial lavage or biopsy specimens might give more specific results. We could not perform bronchoscopy on the study group because of ethical reasons. Other noninvasive markers of airway inflammation such as exhaled nitric oxide may have been useful to specify the source of TGF- $\beta 1$. Furthermore, all of our patients were in a stable phase. Plasma TGF- $\beta 1$ levels during the attack period might also be interesting. Finally, these results reflect only a small sample size of the population.

In conclusion, this is the first study that shows positive correlation between the asthma control test and plasma TGF- $\beta 1$ level. Plasma TGF- $\beta 1$ level may be a useful surrogate marker of airway inflammation in stable asthma and a ≥ 40 -ng/mL level may reflect an uncontrolled disease. Monitoring plasma levels may be used not only to achieve and maintain asthma control but also to prevent exacerbations. Additionally, TGF- $\beta 1$ may be a target of therapy. Additional studies, evaluating the treatment response according to TGF- $\beta 1$ and asthma control test, could shed more light on this topic.

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