Transforming Growth Factor-β1 Levels in Induced Sputum Samples of Asthmatic Subjects

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Abstract

The histopathology of bronchial asthma is associated with structural changes within the airways. Transforming growth factor beta TGF-β_1 is a potent profibrotic cytokine which may contribute to the airway remodelling. To assess the clinical importance of TGF-β_1 in asthma we have measured basal TGF- β_1 concentrations in induced sputum and serum samples of asthmatic and healthy subjects using ELISA (PROMEGA, USA). 15 asthmatic patients (3 males, 12 females, mean age 37±13 yrs) and 5 controls were included. The mean TGF-β_1 was 3.09±0.21 ng/ml in sputum samples of asthmatics and 2.89±0.09 ng/ml in those of controls ($p>0.05$); 3.07±0.15 ng/ml in serum of asthmatics and 2.89±0.20 ng/ml in those of controls ($p>0.05$). There were no significant correlations between sputum TGF-β_1 concentration and number of total nonsquamous cells, number of eosinophils, number of lymphocytes, percent of lymphocytes in sputum samples, however percent of eosinophils slightly but insignificantly correlated with sputum TGF-β_1 levels ($p<0.09$, $r=0.44$). There was no difference between serum TGF-β_1 and sputum TGF-β_1 levels either in asthmatics or in controls. Furthermore, serum and sputum TGF-β_1 levels did not correlate with asthma severity. We conclude that TGF-β_1 levels in serum and sputum samples have no predictive value in diagnostic use and reflection of the asthma severity.

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Introduction

Transforming growth factor TGF-β_1 is a 25 kD homodimeric protein that plays a complex role in regulating growth and differentiation in many tissues, including the lungs. The mammalian TGF-β_1 family comprises three isoforms such as TGF-β_1 ,TGF-β_2, TGF-β_3. TGF-β_1 is present in normal human lung tissue and BAL fluid, and increased levels have been detected in acute lung injury, lung fibrosis, pulmonary vascular diseases, COPD, and sarcoidosis (1-5). Bronchial asthma is characterized by airway structural changes, including mucosal inflammatory infiltration and subepithelial collagen deposition, that may represent the morphological basis for the chronicity of the disease (6,7). There is now accumulating evidence that TGF-β_1 is responsible for this airway wall remodelling. TGF-β_1 has a role of regulating inflammatory and immunologic responses by induction of cellular and molecular events in the subepithelial fibrosis of bronchial asthma including the

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collagen deposition and the increase of fibroblasts beneath the basement membrane (8). The number of epithelial or submucosal cells expressing TGF correlated with basement thickness and fibroblast number in asthma (5,9).

Recently, several reports have demonstrated that eosinophils in the airway mucosa represent a major source of TGF-β_1 (5,10-12). Furthermore, expression of TGF-β_1 is evident in circulating eosinophils from hypereosinophilic individuals (13). Since bronchial asthma is associated with eosinophilic infiltration and activation in the airways, the expression of TGF-β_1 by these cells may result in fibroblast activation and collagen deposition seen in this disease. It has been shown that bronchial biopsy tissues from severe asthmatics overexpressed TGF-β_1 mRNA more than normal subjects and the main source of the mRNA was eosinophils (11). In addition, TGF-β_1 levels in the BAL fluid are elevated in atopic asthmatics and these levels increased in response to allergen exposure (14). However, the presence of TGF-β_1 on the tissue of airway wall from asthmatics did not correlate with the clinic severity of asthma (15).

Induced sputum is a useful non-invasive technique to study airway inflammation in asthma. The clinical importance of TGF-β_1 levels in induced sputum samples of asthmatics has been poorly studied. It is reasonable to expect that asthmatic airways have higher TGF-β_1 expression in the airway wall and thereby have higher TGF-β_1 in the induced sputum samples. To assess a potential role of TGF-β_1 in asthma, we have measured TGF-β_1 concentrations in induced sputum and serum samples of asthmatic and healthy subjects. Furthermore, we investigated the association between TGF-β_1 levels and the baseline lung function, the number and the differentiation of cells in induced sputum samples, and other clinical indices.

Material and Methods

Subjects

A total of 15 nonsmoker subjects (12 females and 3 males) with a history of asthma and 5 healthy normal subjects were included in the study. Before entry, subjects were screened with histories, physical examinations, pulmonary function tests, including prebronchodilator and postbronchodilator lung function tests, methacholine challenge test (in the mild and moderate group), symptom questionnaire, daily PEFR (Peak expiratory flow rate) measurements, complete blood counts, serum IgE levels (by using ELISA), skin prick tests, and chest radiographs. The subjects were defined as atopic by a positive skin prick test (>2mm weal response) to common allergens and/ or high IgE levels.

Subjects were not studied within 3 months of having had an upper respiratory tract infection or known allergen exposure. Subjects were not excluded from the study if they were receiving anti-asthmatic drugs, but they were required to have been on a stable regime for 3 months and to continue the same dosage of all medications over the course of the study. Informed consent was obtained from each subject before the study.

Pulmonary function was assessed with a spirometer (Sensormedics, S3513,Ca,USA). Each data point was the average of at least 3 reproducible measurements (variability<5%). To induce bronchial provocation, the nebulizer was attached to a dosimeter (Mediprom FDC 88, FRANCE), which consisted of a breath-activated solenoid valve and a source of compressed air (pressure 20 psi). Methacholine (Sigma Chemical Co,USA) was freshly prepared in 0.9% NaCl to produce a range of doubling doses of 0.125-16 mg/ml concentrations. The test was terminated when the FEV_1 fell at least 20% below the baseline value recorded upon inhalation of normal saline. The PD20 (provocative dose of drug producing 20% fall in FEV_1 from the post-saline baseline value) was calculated.

Asthmatics were divided into 3 subgroups according to symptom level and PEFR variability. Mild asthmatics: Mild symptoms, FEV_1 80% of predicted, and daily PEFR variability was <20%. Moderate asthmatics: Moderate symptoms, FEV_1 between 60-80% of predicted, and daily PEFR variability was >30%. Severe asthmatics: Severe symptoms, FEV_1 <60% and their PEF variability was >30%.

Sputum induction, sputum processing and biochemical assays

All subjects were premedicated with inhaled 200 g salbutamol, and then PEFR measurements of subjects were performed and repeated every 5 minutes throughout the sputum induction procedure. Sputum induction was performed with an aerosol of hypertonic saline (5%) generated by an ultrasonic nebulizer for approximately 30 minutes. The volume of the induced sputum sample was determined and an equal volume of dithiotrietol 10% (DTT) was added. The samples were then mixed gently by a vortex mixer and placed in a shaking water bath at 37°C for 30 minutes to ensure complete homogenization. We collected the entire induced sputum (plugs plus fluid component). The microliters of the homogenized sputum was used to determine the total cell counts of the samples using a standard hemocytometer. The remainder of the homogenized sputum was centrifuged at 1800 rpm for 5 minutes. The supernatants were frozen at -70°C for further TGF-β_1 analysis. The cell pellets were resuspended in PBS (phosphate-buffer-saline) solution and centrifuged. The remaining cell pellets were cytocentrifuged and stained with Wright stain. On each sputum slide, at least 500 nonsquamous cells were counted. The concentrations of TGF-β_1 were measured by ELISA method (PROMEGA,USA).

Statistical analysis

Data are expressed as the mean and the standard deviation. The Mann-Whitney U test was used to assess differences between healthy and asthmatic subjects. The Kruskal-Wallis test was used to assess differences within asthmatic subgroups. The Spearman Rank correlation was calculated to assess the correlation between TGF-β_1 levels and symptom scores, PEFR, PD20 values, eosinophilia, IgE level, sputum cell profiles.

Table : 1 Clinical characteristics of patients

	Asthmatics	Controls
Male	3	2
Female	12	3
Age	37.4±13.2	30.2±9.1
FEV_1 (L) (%pred.)	2.19±0.84 3.26±0.77	(77±26) (93±14)
FEV_1/FVC %	74±19	84±2
Bronchodilator response (%)	16±2.3	-
Methacholine PD20 (mg/ml)	2.99±4.9	-

X ± SD
pred : predicted
NS:Nonsignificant

Results

The clinical characteristics of the patients are summarized in Table I. In the asthmatic group there were 15 patients (3 males, 12 females), and in the control group the number of subjects was 5 (2 males, 3 females). There were 5 patients in the mild group, 5 in the moderate group, and 5 in the severe asthmatic group. All patients were atopic. The mean total IgE level was IU/mL (normal <94 IU/mL). The methacholine challenge test was performed in the mild and moderate group (n=10). The mean PD20 level was 2.99 ± 4.89 mg/ml. Nine patients were on inhaled steroid, 1 on oral steroid, 1 on inhaled nedocromil sodium, and 4 on prn inhaled salbutamol treatment. The mean volume of sputum produced by the asthmatic subjects was not significantly different from the mean volume of sputum produced by the healthy subjects (14.9 ± 3.1 ml versus 16.1 ± 4.6 ml respectively, $p>0.05$).

The sputum cell profiles are shown in Table II. The mean percentage of eosinophils was higher in the sputum samples of asthmatics ($p<0.001$), the mean percentage of lymphocytes and macrophages were relatively raised in controls over asthmatics ($p<0.05$, and $p<0.01$ respectively).

There was no significant difference in sputum TGF-β_1 levels between asthmatics and controls (3.09 ± 0.21 ng/ml versus 2.89 ± 0.09 ng/ml respectively ($p>0.05$). The mean TGF-β_1 concentration was 3.07±0.21 ng/ml in serum of asthmatics and 2.89 ± 0.20 mg/ml in serum of controls ($p>0.05$).

Table : 2 Cell Profiles in Sputum Samples of Asthmatics and Controls

	Patient Group	Control Group	P value
Total cell count ($x10^6$/ml)	24.8 ± 18.5	4.9 ± 4.8	P <0.005
Neutrophil ($x10^6$/ml) (%)	3.2 ± 4.4 (14.3 ± 15.9)	0.28 ± 0.23 (10.8 ± 7.1)	p <0.01 (NS)
Eosinophil ($x10^6$/ml) (%)	12.3 ± 14.7 (41.4 ± 26.3)	0.08 ± 0.16 (1.3 ± 1.9)	p <0.001 (p <0.001)
Lymphocyte ($x10^6$/ml) (%)	1.8 ± 2.4 (7.5 ± 5.0)	0.50 ± 0.53 (14.5 ± 7.5)	NS (p <0.05)
Macrophage ($x10^6$/ml) (%)	6.5 ± 5.0 (35.4 ± 23.2)	2.3 ± 2.5 (73.5 ± 12.0)	p <0.05 (p <0.01)

X±SD
NS : Nonsignificant

There was no significant correlation between TGF-β_1 concentrations and percentages of lymphocytes, numbers of eosinophils, numbers of lymphocytes in sputum samples. However, there was statistically insignificant but slightly positive correlation between sputum TGF-β_1 levels and percent of eosinophils ($p < 0.09$, $r = 0.44$).

There was no significant difference between serum TGF-β_1 and sputum TGF-β_1 values in asthmatics and in controls.

No significant correlations were found between TGF-β_1 levels and FEV_1 and FEV_1 %pred, FEV_1/FVC, PEFR, symptom scores, peripheral blood eosinophilia. The use of anti-asthma drugs have no influence on sputum and serum TGF-β_1 data in asthmatics.

Discussion

In this study, we demonstrated that TGF-β_1 is present in sputum samples of asthmatics and normal subjects. However, TGF-β_1 levels in sputum and serum samples are not increased in asthmatic patients over controls and do not correlate with clinical asthma status.

Airway wall remodeling is a crucial feature in asthma pathophysiology. TGF-β_1 is potentially important in this regard since TGF-β_1 has been demonstrated by immunostaining in epithelium and submucosa of human airways, it correlated with basement thickness and fibroblast number (5,9) It is known that TGF-β_1 can be generated by many cells such as platelets, smooth muscle cells, macrophages, epithelial cells, fibroblasts, and eosinophils 5,10-12,16,17). Airway epithelial cells are potentially important in repair process because of their location and their known functions. These cells are the first cells of the airway exposed to a variety of inhaled substances such as cigarette smoke, inhaled antigens, and infectious agents associated with the development of such diseases as asthma. TGF-β_1 has a role in subepithelial fibrosis in asthma due to its well known function as a stimulator of extracellular matrix protein synthesis (18). Aubert JD et al showed that TGF-β_1 precursor was immunolocalised throughout the airway wall including the epithelium and in alveolar macrophages. The mature TGF-β_1 was localised primarily within the connective tissue of the airway wall. However, they couldn't find any clear difference in expression between airways of asthmatics and those of smokers with and without COPD (19). EG2 positive eosinophils represented the major source of TGF-β_1 mRNA and immunoreactivity (10). Furthermore, eosinophils in nasal polyp tissue overexpressed TGF-β_1 (12). Similarly, we could find slightly but not statistically significant correlation between percent of eosinophils and TGF-β_1 in sputum samples.

Minshall et al found that asthmatic individuals exhibited a greater expression of TGF-β_1 mRNA and immunoreactivity in the airways mucosa than normal control subjects and this incline was directly related to the severity of the disorder. However, we could not find any difference in TGF-β_1 levels between asthmatics and controls. This result is supported by showing similar expression of TGF-β_1 in bronchial mucosal samples of asthmatic subjects and healthy controls (9). Additionally, Chu HW could not show any difference of TGF-β_1 immunoreactivity in lung biopsies of asthmatics and controls (15).

It may be argued that there is a discrepancy between TGF-β_1 immunoreactivity on biopsy specimens and TGF-β_1 levels in epithelial lining fluid, sputum, and BAL. Basal concentrations of TGF-β_1 in BAL fluid are increased in atopic asthma in comparison with nonasthmatic controls (8.0 versus 5.5 pg/ml). Furthermore, 24 hours after segmental bronchoprovocation, TGF-β_1 concentrations were higher at the allergen-challenged sites (14).

In addition, it has been shown that TGF-β_1 in BAL fluid was significantly higher in status asthmaticus when compared with controls and stable asthmatics (20).Interstingly, in the same study, stable asthmatics have lower levels of TGF-β_1 than patients with status asthmaticus. In the present study, we evaluated only stable asthmatic subjects, thus we may not find higher sputum TGF-$\beta_1$levels in our patients over controls. Redington et al reported low levels of TGF-β_1 in BAL samples when compared to those in the sputum samples of our study. This is likely due to the dilution of cytokine in saline during the BAL procedure (14). Adachi et al have recently shown that TGF-β_1 is present in sputum of patients with bronchial asthma by using ELISA (21). We found 3.0 ± 0.21 ng/ml TGF-β_1 in sputum samples of asthmatics. This low level may be due to processing protocol of sputum samples in different studies and different study populations.

One of the reasons for the current interest in measuring TGF-β_1 in lungs is the hope that it might be used to predict the severity of asthma. Chu HW evaluated TGF-β_1 immunoreactivity in lung tissue of mild, moderate and severe asthmatics and no significant correlations were found between collagen deposition and eosinophil count, TGF- β_1 expression level, FEV_1, and duration of asthma (15). This view was encouraged by our results. No significant correlations were found between TGF-β_1 levels and FEV_1, FEV_1 %pred, FEV_1/FVC, PEFR, symptom scores, peripheral blood eosinophilia. In contrast, Minshall et al showed positive correlation between expression of TGF-β_1 and severity of asthma (10).

Many of the adhesive interactions between T lymphocytes and endothelial or epithelial cells are mediated by integrins. TGF-β_1 upregulated integrin expression on T cells in asthmatics and thereby contributed to the selective accumulation of these cells in inflammatory lung diseases (22,23). In contrast, TGF-β_1 did not correlate with the number and percentage of lymphocytes in our study.

In conclusion, we have demonstrated that sputum and serum levels of TGF-β_1 are not increased in asthmatic patients over controls and not correlated with the severity of the asthma. Further studies are needed to investigate the local and systemic levels of TGF-β_1 in stable asthmatics and in acute asthmatic attacks to understand the exact role of TGF-β_1 in the airways process in asthma.

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