

Effect of TGF β 1 and TIMP2 on Disease Activity in Asthma and COPD

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ABSTRACT

The process of bronchial tissue repair/remodeling depends on balance between production and degradation achieves the regulation of extracellular matrix turnover. We designed this study to evaluate relation between Transforming Growth Factor β 1 (TGF β 1) and Tissue Inhibitory of Metaloproteinase 2 (TIMP2) as two main tissue mediators on activity and reversibility of asthma and chronic obstructive pulmonary disease (COPD).

In a cross sectional study we evaluated TIMP2 and TGF β 1 expression in two groups of 29 asthmatic (14 males and 15 females) and 13 male COPD patients using semi-quantitative PCR on induced sputum samples. The relation between TIMP2 and TGF β 1 and PFT indices and disease free period were assessed. The COPD patients with raised expression of both TGF β 1 and TIMP2 had better pulmonary function test (PFT) indices and also longer disease free period. In contrast patients with chronic asthma could remain in well pulmonary function status with raised TIMP2 and decreased TGF β 1 expression.

We supposed that underlying inflammatory process is the main reason for the different effect of cytokines in asthma and COPD. It raises concern about critical role of corticosteroids consumption on various cytokines expression. Furthermore TGF β 1 may be served as a biomarker in sputum for assessing disease activity and evidence based prescribing corticosteroids in patients with COPD and asthma.

Key words: Asthma; COPD; TGF β 1; TIMP2

INTRODUCTION

Airway remodeling is an irreversible and dynamic process which can lead to more rapid decline in lung function over time.

The process of bronchial tissue repair/remodeling depends on balance between production and degradation achieves the regulation of extracellular matrix (ECM) turnover. The ECM degradation is dependent on the family of proteinases termed matrix metalloproteinases (MMPs)¹⁻³ Airway epithelium and inflammatory cells like macrophages are important sources of transforming growth factors beta-1 (TGF β 1) in addition to other growth factors that have potent effects on airway reconstruction. The role of TGF β 1 in pathologies of

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several lung disorders including pleural disease, adult respiratory distress syndrome and fibrotic tissue remodeling is well known.⁴ Also, increased TGF β 1 concentration in BAL fluid of patients with chronic obstructive pulmonary disease (COPD) and asthma has been reported. TGF- β -eosinophil-epithelial cell interactions may be implicated in bronchial tissue repair and remodeling.

TIMPs inhibit MMP to regulate its activities. TIMP-1 and -2 are two main forms that contribute in pulmonary diseases with alterations of alveolar structure in asthma or COPD. This balance is necessary for optimal function whereas any over/under production of related proteinases can yield tissue damage and undesirable changes in ECM. However, some pathology in respiratory airway could disturb the mentioned balance. This would promote the pathological process and inhibit the disease recovery.

Understanding the contribution of cytokines in activity of obstructive lung disease i.e., asthma and COPD may guide us to designing new drugs. Also it can provide new insight to reevaluate efficacy of the conventional drugs like corticosteroids in this field. Hence we designed this study to assess relation between TGF β 1 and TIMP2 as two main tissue mediators on activity and responsibility of asthma and COPD in medication. We considered a unique group of chemical exposed COPD patients with same underlying etiology to made more consistent group.

PATIENTS AND METHODS

In a cross sectional study we evaluated TIMP2 and TGF β 1 in two groups of 29 asthmatic (14 males and 15 females) and 16 male COPD patients. Three COPD patients were excluded because of incomplete data and 13 remaining completed the study. The ethics committee of the Baqiyatallah University of Medical Sciences approved this study.

Asthma Group

Case selection for this group was based on the guidelines of Global Initiative for Asthma 2006 (GINA).⁵ Inclusion criteria were intense subjective breathlessness, audible wheezing on auscultation and a morning peak expiratory flow (PEF) <70% of the personal best value in the previous 3 months, nonsmokers or with a smoking history of <10 pack per year, episodic cough, wheezing, dyspnea, normal chest

radiograph results and an increase of 20% or greater in FEV1 in response to a bronchodilator. Oral corticosteroids had been withdrawn for at least 2 months prior entrance to the study in stable patients. Exclusion criteria were presence of concomitant acute illnesses such as pneumonia and/or requiring regular systemic corticosteroid therapy and regular medication consisting of short-acting β 2-agonists.

COPD Group

Case selection for this group was based on the diagnosis of COPD due to exposure to sulfur mustard as a chemical weapon during Iraq-Iran war. This group of COPD patients was considered for current study because of following reasons: 1) they had history of single exposure to a chemical weapon, 2) they did not have other risk factors, 3) the pathology was restricted to airways and they did not have emphysema, 4) they were stable COPD patients. Inclusion criteria were exposure to mustard gas 15–16 years ago during the Iran-Iraq war led to bronchiolitis confirmed from high-resolution computed tomography (HRCT) scan, severe respiratory symptoms immediately after their initial mustard gas exposure, rhinorrhea, sore throat, hoarseness, cough, chest tightness, and dyspnea. Bronchiectasis, airway stricture, or fibrosis were excluded by high-resolution computerized tomography scan of the chest.⁶ Also history of exposure to asbestos, coal dust, silicone, or cotton dust considered as exclusion criteria.

Disease Free Period

All patients involved in the study were under observation by an expert pulmonologist and the disease free period was defined as remission of asthma in a symptom free state (loud audible wheezing) and absence of treatment on a routine maintenance therapy in both diseases.⁷

Pulmonary Function Test

Pulmonary Function Test (PFT) was performed before the sputum induction on the same day according to American Thoracic Society (ATS) guidelines.⁸

Sputum Induction

Induced sputum was carried out according to the method of Pin et al., 1992, with slight modification.⁹ FEV1 and vital capacity were measured before and 10 minutes after salbutamol inhalation (two puffs, 200 ug)

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and then every five minutes during inhalation of hypertonic saline solution. The hypertonic saline was nebulised with an ultrasonic nebulizer (Fisoneb, Fisons, Pickering, Ontario) on the maximum setting and was inhaled for five minute periods for up to 30 minutes. The concentration of saline was increased at intervals of 10 minutes from 3% to 4% to 5%. Nebulisation was discontinued with declining of FEV1 more than 20% or occurrence of troublesome symptoms. Subjects were rinsed their mouths and throats carefully and then collected their sputa. After collection of sufficient amount of sample the nebulization was stopped.

Sputum Processing

The samples were kept in 4°C overnight until processing within 2h. After volume measurement of collected sputum, it was treated with freshly prepared 0.1% dithiothreitol (DTT) (Sputolysin; Calbiochem Corp., San Diego, CA) solution by 1:4 ratios in volume.¹⁰ The samples were mixed gently by vortex mixer and placed in a shaking water bath at 37°C for 15 minutes. Then it was mixed again for 15 seconds.¹¹ 100 microlitres was considered for cellular count. A blind observer counted 500 non-squamous epithelial cells and cell differentials per sputum preparation by Wright staining. Then it was centrifuged for 5min (2000rpm) (SIGMA centrifuge, Germany). Supernatant was frozen at -80°C for later analysis.

RNA Extraction

RNA could be isolated from 23 of the 29 asthma and 13 of the 16 COPD processed sputum Samples. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After homogenization of processed sputum sample, the chlorophorm was added and centrifugation done. Next the aqueous phase (containing RNA) was separated and diluted by isopropanol and centrifuged again. Then precipitated RNA was dissolved in ethanol 75% and centrifuged. 20 microlitres RNAase- free water was added to isolated RNA and the quality and quantity of RNA were established by electrophoresis and spectrophotometry (ND-1000; NanoDrop, Wilmington, DE), respectively.

cDNA Synthesis

Reverse transcription was performed by CycleScript RT PreMix (dN6) kit (BIONEER, Daejeon, South

Korea) with 500 ng of total extracted RNA according to manufacturer's instructions.

Primer Design

Primer sets for TGFβ1, TIMP2, and β-actin (control gene) are shown in table 1.

Semi Quantitative PCR

PCR process by Taq DNA polymerase (Cinagene, Tehran, Iran) was performed with MasterCycler PCR system (Eppendorf, Hamburg, Germany). By initial denaturation (30 sec at 94°C), annealing (30 sec at 62°C for TGFβ1, 60°C for TIMP2 and 59°C for β-actin), extension (1 min at 72°C) and terminal extension (5 min at 72°C) cDNA was subjected to 33 cycles of PCR and the products were separated in a 2% agarose gel and dyed with ethidiumbromide and then detected under UV light.

All results were normalized with β-actin expression to compensate for differences in the amount of cDNA and then made into quantitative measures using special Scion-Image Analysis software. The expression of TGF Beta 1, TIMP2 and Beta-actin of 4 COPD and 4 asthmatic patients in agarose gel electrophoresis images are shown in figure 1.

Statistical Analysis

Data were presented as mean and standard deviation and were analyzed with Chi-square tests. P values <0.05 were considered statistically significant. Statistical analysis was performed with SPSS version 11.0 software (SPSS Inc., Chicago, IL).

RESULTS

Totally 45 patients were participated in this study. There were 29 asthmatic patients (14 males and 15 females) and 16 male COPD patients.

The mean and standard deviation (mean±SD) of age were 45.7±6.7 and 43.15±5.6 in the asthma and COPD patients, respectively. There was no statistically difference between the age of two groups (p>0.05). There was no smoker in asthma group.

The relation between TIMP2 and TGFβ1 and PFT indices and disease free period were assessed. There were statistical relation between TIMP2 and/or TGFβ1 and PFT indices in the asthmatic group (p<0.05; Table 2). There was no relation in COPD patients for mentioned parameters (p > 0.05; Table 3).

Table 1. Primer design and sequence for TGFβ1, TIMP2 and β-actin

Product size	Annealing Tm	Sequence	Name	NCBI code
242bp	62	5'TCAAGCAGAGTACACACAGC3'	TGFβ1 Forward primer	NM_000660
	62	5'GCACAACCTCCGGTGACATC3'	TGFβ1 Reverse Primer	NM_000660
182bp	60	5'TGCACATCACCTCTGTGAC3'	TIMP2 Forward Primer	NM_003255
	60	5'TGTTCTTCTCTGTGACCCAG3'	TIMP2 Reverse Primer	NM_003255
119bp	59	5'TTCTACAATGAGCTGCGTGTGG3'	β-ACTIN Forward primer	NM_001101
	59	5'GTGTTGAAGGTCTCAAACATGA3'	β-ACTIN Reverse primer	NM_001101

According to the Global initiative for chronic Obstructive Lung Disease criteria,¹² 8 patients had mild obstructive pattern, 2 moderate obstructive and 3 severe obstructive in COPD group.

Also there were no statistical relation between disease free period (months) and TIMP2 and/or TGFβ1 in the asthma (p=0.54) and COPD (p=0.35) groups (Table 4).

Table 2. Relation between PFT status and expression of TGFβ1 and/or TIMP2 in Asthma

	TGFβ1 and/or TIMP2*	N	Mean	SD	P value
FVC %	.00	4	80.800	8.0850	0.041
	TGFβ1	8	61.313	22.8510	
	TIMP2	5	86.440	10.3751	
	2.00	6	87.733	18.5151	
FEV1 %	.00	4	81.650	9.6404	0.013
	TGFβ1	8	51.713	25.2985	
	TIMP2	5	89.060	18.8142	
	2.00	6	84.500	19.8666	
MMEF %	.00	4	65.000	15.5411	0.038
	TGFβ1	8	30.763	21.8302	
	TIMP2	5	62.840	24.4764	
	2.00	6	60.567	25.9307	

*.00: Decreased expression of TGFβ1 and TIMP2

2.00: Increased expression of TGFβ1 and TIMP2

Table 3. Relation between PFT status and expression of TGFβ1 and/or TIMP2 in COPD

	TGFβ1 and/or TIMP2*	N	Mean	SD	P value
FVC %	.00	5	73.980	14.6293	0.202
	TGFβ1	2	90.350	1.4849	
	TIMP2	2	70.150	5.0205	
	2.00	4	83.125	5.1970	
FEV1 %	.00	5	60.920	26.7611	0.132
	TGFβ1	2	89.400	1.8385	
	TIMP2	2	57.200	23.0517	
	2.00	4	90.837	10.4527	
MMEF %	.00	5	41.080	32.4863	0.133
	TGFβ1	2	72.250	7.1418	
	TIMP2	2	41.150	28.3550	
	2.00	4	97.825	42.0769	

*.00: Decreased expression of TGFβ1 and TIMP2

2.00: Increased expression of TGFβ1 and TIMP2

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Table 4. Relation between disease free period (months) and TGFβ1 and/or TIMP2 in asthma and COPD groups

	TGFβ1 and/or TIMP2*	N	Mean	SD	P value
COPD	0	5	3.60	4.980	
	1	4	8.75	10.500	0.351
	2	4	11.00	6.683	
Asthma	0	4	11.25	5.058	0.545
	1	12	6.83	7.530	
	2	6	8.67	6.653	

*0: Decreased expression of TGFβ1 and TIMP2

1: Decreased expression of TGFβ1 or TIMP2

2: Decreased expression of TGFβ1 and TIMP2

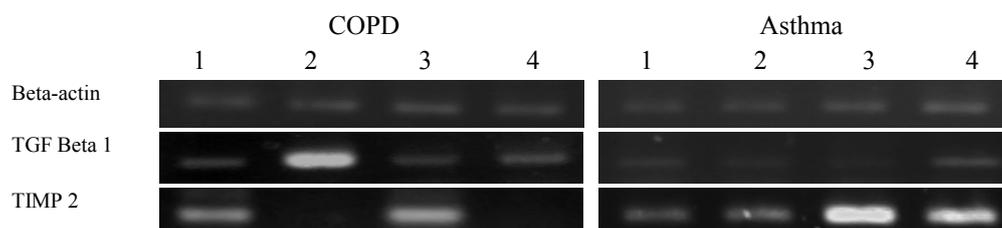


Figure 1. The expression of TGF Beta 1, TIMP2 and Beta-actin of 4 COPD and 4 asthmatic patients in agarose gel electrophoresis images.

DISCUSSION

This study offered excellent opportunity to declare effect of modifying factors on disease activity in two groups of COPD and chronic asthma. As our main finding the COPD patients with maintenance of TGFβ1 and TIMP2 expression had better PFT's indices and also better sign and symptom. They also had longer disease free period. In contrast patients with chronic asthma could remain in well pulmonary function status in which TIMP2 had been raised but TGFβ1 had been decreased. On the other hand asthmatic patients with decreased expression of TGFβ1 had better condition as well as longer disease free period than other ones with increased expression of TGFβ1 as a fibrogenic cytokine. Although there was no statistically relation between some parameters, may be because of small sample size, according to clinical opinion these findings are very interesting and have clinical significance. These results mirror the heterogeneity of the disease in terms of triggering agents, individual genetic variability, history and activity of the disease. We suppose that in such a complex and multifactorial disorder understanding of the exact mechanism and all cofactors are very difficult and can differ from one patient to another.¹³

There is agreement that small airway remodeling contributes to airflow limitation in COPD.^{14,15} Others found significant increase in TGFβ1 concentration in BAL fluid of patients with COPD like symptoms compared to controls.^{6,16} It has been suggested that TGFβ-eosinophil-epithelial cell interactions contribute to bronchial tissue repair/remodeling in asthma via protease network.^{17,18} TGFβ promotes the transformation from fibroblast to myofibroblast as well as enhancing the survival of myofibroblasts by inhibiting interleukin (IL)-1b-induced apoptosis.^{19,20} Activated smooth muscle cells and fibroblasts can release ECM proteins in response to TGFβ.^{19,21-23} TGFβ also has indirect effects through the up regulation of inhibitors of ECM proteases, such as TIMP1 and TIMP3, and the down regulation of degrading ECM proteases (e.g., interstitial collagenase).²⁴⁻²⁶ TIMPs, specific MMP inhibitors, are secreted from many cells in four types to interact with active forms of MMPs leading to regulation of their activities. Each TIMP inhibits MMPs via tight, non-covalent binding in a 1:1 manner.^{1-3,27} K. Hirano and colleagues reported TIMP-1 and 2 as main participants of family in pulmonary diseases with alterations of alveolar structure or abnormal remodelling responses as seen in asthma or

COPD.²⁸ TIMP2 is secreted and complexed to MMP2 in fibroblasts. TIMP inhibits MMP2 and is involved to docking pro-MMP2 to the cell surface that is activated by membrane MMP1.²⁹ Recent data show that TIMP proteins are inversely correlated with reduced FEV1 in COPD. Mercer and colleagues investigation on MMP9/TIMP1 balance in the sputum of COPD subjects demonstrated the MMP9/TIMP1 balance in favour of TIMP1 in stable condition, whereas, elevated levels of MMP9 during exacerbations.³⁰ TGF β has been implicated as a key mediator in asthma, responsible for a number of remodeling events. TGF β 1 over activation in asthma may not rely exclusively on its increased expression, but may be related to different alterations in other points of regulation that modulate TGF β 1 activity.¹³ TGF β also has indirect effects through the up regulation of inhibitors of ECM proteases, such as TIMPs, and the down regulation of degrading ECM proteases, such as interstitial collagenase.²⁴⁻²⁶ Alveolar macrophages would continue higher secretion of TGF β 1 after resolving inflammation and the airway epithelium would begin to reexpress TGF β 1. Increased TGF β 1 may subside remnant inflammation and consequently help the healing response. In summary regulation of TGF β 1 action would yield restoration of airway wall integrity and function.^{13,31}

It should be noted that underlying pathology in asthmatic and COPD are different. It seems the main reason for this difference is presence or absence of inflammation. While inflammation plays major role in asthma, there is no main inflammatory component in COPD patients following exposure to mustard gas.³² Furthermore, glucocorticosteroids have cardinal effect on inflammatory mediator cells and cytokines. They are the mainstay in therapy for the majority of mild to severe, persistent asthmatic subjects.

Dexamethasone, for example, has been shown to block TGF β -induced collagen synthesis in rats.³³ Consequently, asthmatic patients get benefit from corticosteroid therapy by anti inflammatory effect and suppressing TGF β 1 expression. But interestingly in COPD patients because of related non-inflammatory process and optimal balance in both increased TGF β 1 and TIMP2 not only corticosteroids consumption has therapeutic effect but also by subsiding TGF β 1 can lead to imbalance of modulator that can disturb well regulated interaction of different involved factors and as a result worsen the pathology and disease in COPD patients.

This study with such a small sample size cannot answer many unresolved questions. However it can be served as a trigger for more studies on human that can lead to further explorations for using practical and therapeutic methods. Further studies using tissue sampling to evaluate more evidence such as fibrosis and epithelial dystrophies would be helpful. Also quantitative measurements of TGF β 1 and TIMP2 are necessary to better estimation of correlation between disease activity parameters and expression of mediators.

CONCLUSION

Lower expression of TGF β 1 and higher expression of TIMP2 in asthma but higher expression of both TIMP2 and TGF β 1 in COPD have showed better PFT's indices and longer disease free period as well. It seems TGF β 1 has bidirectional effect on activity of disease in asthma and COPD.

TGF β 1 may be considered as a biomarker in sputum for assessing disease activity and evidence based prescribing of corticosteroids in patients with COPD and asthma.

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