

## Increased Expression of Transforming Growth Factor- $\beta$ and Receptors in Primary Human Airway Fibroblasts from Chemical Inhalation Patients

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### ABSTRACT

The widespread use of sulfur mustard (SM) as a chemical warfare agent in the past century has proved its long-lasting toxic effects. Despite a lot of research over the past decades on Iranian veterans, there are still major gaps in the SM literature. Transforming growth factor (TGF- $\beta$ ), a cytokine that affects many different cell processes, has an important role in the lungs of patients with some of chronic airway diseases, especially with respect to airway remodeling in mustard lung.

Primary airway fibroblasts from epibronchial biopsies were cultured, and gene expression of TGF- $\beta$ 1, TGF- $\beta$ 2, T $\beta$ R-I and T $\beta$ R-II in fibroblasts of SM injured patients and controls were investigated. Expression of TGF- $\beta$ s and receptors was measured by RT-PCR. Protein level of TGF- $\beta$ 1 was surveyed by western blot.

Our findings revealed that expression levels of TGF- $\beta$ 1, TGF- $\beta$ 2, T $\beta$ R-I and T $\beta$ R-II were upregulated in the airway fibroblasts of SM exposed patients in comparison with control samples. TGF- $\beta$ 1 expression was shown to be markedly increased in primary lung fibroblasts of chemically injured patients.

Our novel data, suggested that over-expression of TGF- $\beta$  molecule and receptors in primary airway fibroblasts of mustard gas injured patients may be involved in progression of airway remodeling of these patients.

**Keywords:** Airway Remodeling; Fibroblasts; Sulfur Mustard; TGF- $\beta$

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## INTRODUCTION

Sulfur mustard (SM) is a strong vesicant agent which has been used in several military conflicts. The widespread use of sulfur mustard as a chemical warfare agent in the past century has proved its long-lasting toxic effects. It may also be used as a chemical terrorist agent. Therefore, all health professionals should have sufficient knowledge and be prepared for any such chemical attack.<sup>1</sup> In the Iran-Iraq war there was extensive usage of SM by Iraqi ex-regime against Iranian civilians and military forces.<sup>2-6</sup> SM is an alkylating agent that affects DNA synthesis, and, thus, delayed complications have been observed.<sup>7</sup> However, despite lots of research over the past decades on Iranian veterans, there are still major gaps in the SM literature.

Transforming growth factor (TGF- $\beta$ ), a cytokine that affects many different cell processes, has an important role in the lungs of patients with some of chronic airway diseases, especially with respect to airway remodeling.<sup>8</sup> Significant structural alterations of the bronchial wall (collectively termed airway remodeling) have been repeatedly demonstrated in many patients with asthma and may lead to a state of irreversible airway obstruction.<sup>9</sup> This morphologic change is believed to be caused by increased collagen (types I, III, and V) deposition by the airway fibroblasts.<sup>10</sup> Among the numerous growth factors that are active in airway inflammation and tissue repair, TGF- $\beta$  may have a prominent role in altering fibroblast activities linked to airway remodeling.<sup>11</sup>

Airway remodeling is characterized by defective extracellular matrix (ECM) turnover. With respect to increased ECM accumulation, TGF- $\beta$  has evolved as a key molecular mediator stimulating ECM accumulation.<sup>12</sup> Recent studies have clarified some of the mechanisms involved in TGF- $\beta$ 1 stimulation of airway remodeling. TGF- $\beta$ 1 activates gene transcription via binding to two specific subfamilies of cell trans membrane receptors; Type I (TbR-I) and type II receptors (TbR-II), part of the activin receptor-like kinase (ALK) family. TGF- $\beta$  receptor I phosphorylates members of the Smad protein family to initiate nuclear

translocation and transcription.<sup>13</sup> There is a sample evidence suggesting that fibroblasts are the main cellular source of extracellular matrix deposition.<sup>14</sup> Aberrant or abnormal phenotypes of fibroblasts have been described in several fibrotic disorders.<sup>15</sup> Altered fibroblast phenotypes have been described in fibrotic diseases of the lung.<sup>16</sup> This is likely due to an abnormal response to TGF- $\beta$ , an important profibrotic signaling molecule.<sup>17</sup> Raghu and coworkers have reported that fibroblasts isolated from regions of lungs with early fibrosis in patients with idiopathic pulmonary fibrosis have greater proliferative potential than those obtained from regions of dense fibrosis or from normal lungs.<sup>18</sup>

The goal of the present study was to denote abnormal expression of TGF- $\beta$ 1, TGF- $\beta$ 2, TbR-I and TbR-II in primary airway fibroblasts of SM injured people in comparison with normal individuals.

## MATERIALS AND METHODS

### Subjects and Selection and Biopsy Handling

Bronchial biopsy specimens were obtained from male SM exposed patients and control subjects. The exposed patients were individuals who had a documented encounter with SM during the Iran-Iraq war. Tissue was donated by volunteers who had given their informed consent to participate in the study, and this survey was conducted in accordance with a protocol approved by Baqiyatallah Medical Sciences University (BMSU) ethics committee. Bronchial tissues from five SM exposed patients and five normal controls were obtained for fibroblast isolation. Data such as age were obtained, as is shown in Table 1. Cases with positive history of cigarette smoking, history of pulmonary disease such as asthma, lung cancer or respiratory infections and history of exposure to toxic agents elderly patients and addicts, were excluded from our study.

In respect to obtaining biopsies from the bronchi wall, the flexible fiberoptic bronchoscopy (FFB) (Olympus BF1T, Tokyo, Japan) was performed. Patients were topically anaesthetized by applying 2%

**Table 1. Characteristics of SM-injured patient and control groups samples.**

Age(years)	Control group subjects n=5	SM-injured patients n=5	P-value
Age range	43-55	42-54	0.67
Age (mean $\pm$ SD)	49.4 $\pm$ 5.6	47.8 $\pm$ 5	

Lidocaine throughout the bronchoscopy. Supplemental oxygen was administered throughout the procedure; in addition, the heart rate and oxygen saturation were also monitored.

To obtain endobronchial biopsy samples, bronchoscope forceps (Olympus, Tokyo, Japan) were passed through the route of fiberoptic bronchoscopy to reach the segmental and subsegmental carinae. Each biopsy specimen was placed in DMEM culture medium for transport to laboratory. All containers and medium were sterile.

### Primary Cell Culture

Primary human airway fibroblasts (HAF) were obtained by outgrowth from bronchial biopsies (explant technique). As mentioned in the protocol by Takashima,<sup>20</sup> with little change, briefly after washing biopsies with PBS, biopsies were cut into several small pieces and incubated in a 6 well tissue culture dish, containing complete culture medium (DMEM, supplemented with 10%FBS, 1% HEPES buffer solution 1 M, 1% nonessential amino acid mixture 100X and 1% penicillin/streptomycin). The tissue pieces were incubated in a humidified incubator at 37 C° with 5% carbon dioxide and cultured until fibroblasts migrated from the tissue and proliferated on the base of the culture dish cell (8–14 days), the medium was changed every 2–3 days. Upon reaching 50% confluency, the tissue pieces were removed and the cells were trypsinised and seeded in T25 flasks (Containing DMEM with 10% FBS) for subsequent cell passages. Isolated cells were characterized as fibroblasts by morphological appearance.<sup>19,20</sup> Experiments were performed on fibroblasts of passage 5/6 with confluent growth. Given the potential concern for changes in cell function, including enhanced senescence, which may occur after several passages.

### RNA Extraction

Total RNA from cultured fibroblasts was prepared with TriPure isolation reagent (Roche Diagnostics, Germany), according to the manufacturer's recommendations. For the first step, after decanting culture medium from cells, Tripure Isolation Reagent was added directly to T25 flasks with confluent growth of fibroblasts, the harvest cell lysate was used for subsequent stage, at last isolated RNA was eluted in RNase-free water, with the concentration and purity being determined by measuring OD at 260 nm by

NanoDrop spectroscopy (ND-1000, Wilmington, DE), and total RNA quality was checked on agarose gel stained with ethidium bromide.

### cDNA Synthesis

500 ng of total extracted RNA was reverse-transcribed to create single-strand complementary DNA using reverse transcriptase kit, CycleScript RT PreMix (dN6) (Bioneer, Korea) according to manufacturer's instruction.

### Semi Quantitative PCR

The following specific primer pairs, purchased from Bioneer (South Korea), were designed and used for PCR amplification:

TGFβ1 Primer

forward, 5'TGTGACAGCAGGGATAACAC3'

reverse, 5'TGAAGCAATAGTTGGTGTCC3'

TGFβ2 Primer

forward, 5'TTGACGTCTCAGCAATGGAG3'

reverse, 5'TCAGTTACATCGAAGGAGAGC3'

TbR-I Primer

forward, 5'TGCTGCAATCAGGACCATTG3'

reverse, 5'TCCTCTTCATTTGGCACTCG3'

TbR-II Primer

forward, 5'TGCTCACCTCCACAGTGATC3'

reverse, 5'TCTGGAGCCATGTATCTTG3'

and β-actin as a house keeping gene,

forward, 5'TCATGAAGATCCTCACCGAG3'

reverse, 5'TTGCCAATGGTGTGACCTG3'.

For PCR, 1 µl of the resulting cDNA were validated with PCR in a volume of 25 µl, containing 2.5 µl 10X buffer (Takara, Japan), 5 PM deoxynucleoside triphosphate (Takara, Japan), 0.3 µl Taq polymerase Enzyme (Takara, Japan) and 10 PM Primer Mix. PCR process was performed with asterCycler PCR system (Eppendorf, Hamburg, Germany). By initial denaturation (30 sec at 95°C), annealing (30 sec at 59°C for TGFβ1, 57°C for TGFβ2 and TbR-II, 55°C for TbR-I 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. PCR products were separated in a 2% agarose gel and dyed with ethidium bromide and then detected under UV light. All results were normalized with β-actin expression to compensate for differences in the amount of cDNA. For quantitative measures and evaluation, PCR results on gel were checked by densitometry by using special

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Image Analysis software (Scion Corporation, Frederick, MD)

### Western Blotting

Total protein was extracted from monolayer fibroblast cells with TriPure isolation reagent (Roche Diagnostics, Germany), according to the manufacturer's recommendations. Briefly, after decanting culture medium from cells, cells were washed and lysed with Tripure by adding Tripure Isolation Reagent directly to T25 flasks with confluent growth of fibroblasts. The cell lysate was used for subsequent stage. Eventually total extracted proteins were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Roche Diagnostics, Germany). The membrane was blocked with 5% skimmed milk and incubated overnight at 4°C. The membrane was then washed before being incubated at room temperature with the primary mouse monoclonal antibody to TGF- $\beta$ 1 (1:200, Santa Cruz Biotechnology, CA, USA). After 2 h, membrane was washed and incubated for 1 h at room temperature

with a horseradish anti-mouse antibody (1:1000, Santa Cruz Biotechnology, CA, USA).

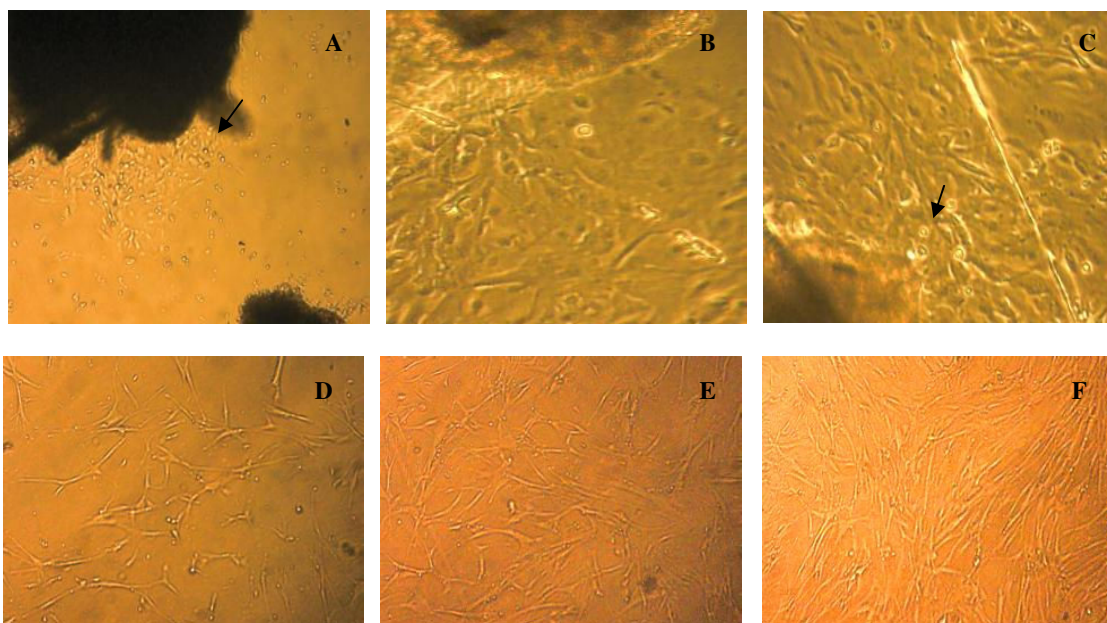
### Statistical Analysis

To compare differences between SM injured group and unexposed group, comparison of two populations was made with Student's T test via SPSS software version 16.0 (SPSS, IL). Data were considered significant at  $p < 0.05$ .

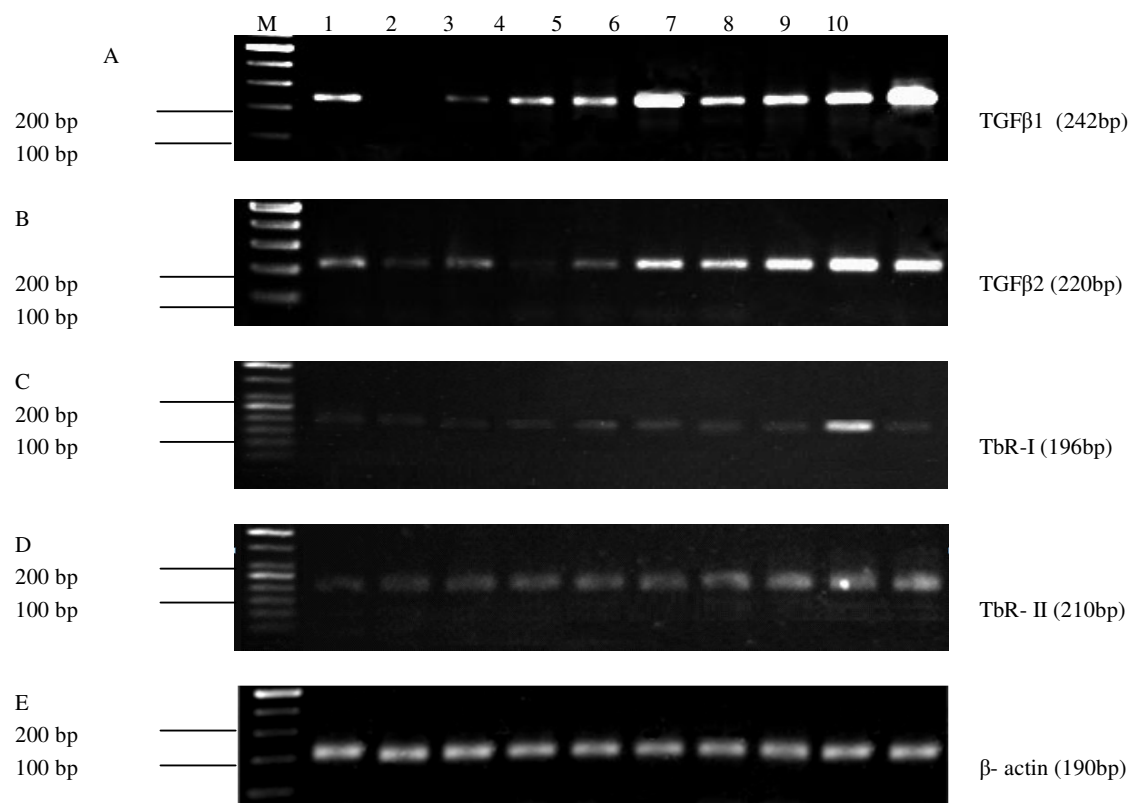
## RESULTS

### Subject Characteristics

Totally 10 individuals took part in the study. There were five SM exposed patients and five normal controls. The mean and standard deviation (mean $\pm$ SD) of age were 47.8 $\pm$ 5 and 49.4 $\pm$ 5.6 in the exposed patients and normal controls, respectively. There was no statistically difference between the age of two groups ( $p > 0.05$ ).



**Figure 1.** Microscopic appearance of fibroblast cultures established from biopsy using the explant culture system. Cells can be observed growing out from the tissue within a couple of days and by the end of the 2<sup>nd</sup> wk, few cells other than fibroblasts remained, as seen here (A,B,C) biopsy explant, day 5; (D) Dissociation culture, day 1; (E) dissociation culture, day 5; (F) dissociation culture, day 14. Note that fibroblasts migrated out from the edge of specimen (panel B,C) and became confluent. In dissociated-cell cultures, fibroblasts attached (panel D), spread on culture plates (panel E), and became confluent (panel F). Arrows show elongated cells with fibroblastic morphology.



**Figure 2.** Expression levels of TGF- $\beta$ 1, TGF- $\beta$ 2, TbR-I and TbR-II in primary airway fibroblast of patients with long term exposure to sulfur mustard. Total RNAs were purified and then analyzed by Semiquantitative RT-PCR. This panel shows gel bands in order to PCR amplification products of TGF- $\beta$ 1 (242bp), TGF- $\beta$ 2 (220bp), TbR-I (196bp), TbR- II (210bp) and  $\beta$ -actin (190 bp) transcripts. (A) marked increase in TGF- $\beta$ 1 expression levels of SM exposed patients (Lanes 5-10) was recognized in comparison to expression level of unexposed ones (Lanes 1-5). (B) The density band of TGF- $\beta$ 2 in SM exposed patients also shows highlight deference with mRNA of unexposed group. TbR-I(C) and TbR-II(D) also shows the deference in mRNA expression level. (E) B-actin was used as internal control. Lane M shows DNA ladder (100bp in A, B, E and 50bp in C,D).

#### Expression of TGF- $\beta$ mRNA and Protein in Primary Fibroblasts

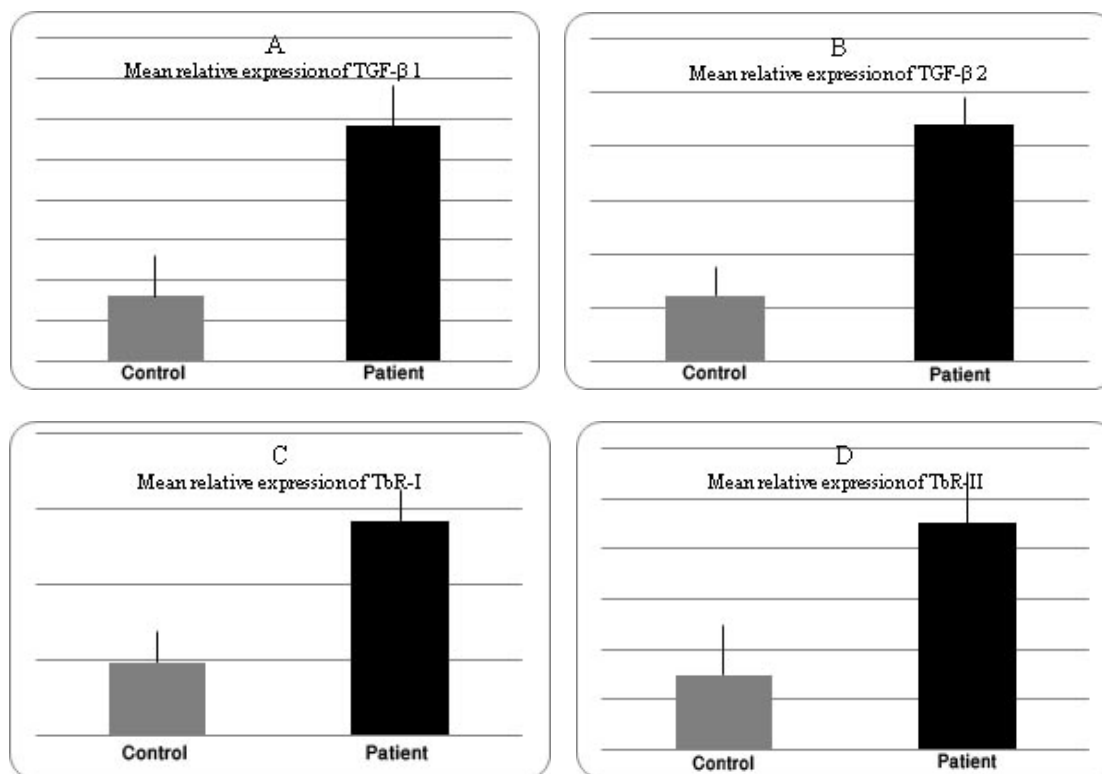
The expression of TGF- $\beta$ 1, TGF- $\beta$ 2, TbR-I, TbR-II mRNA and TGF- $\beta$ 1 protein were assessed by RT-PCR and Western blotting.

Semiquantitative RT-PCR were performed on RNA isolated from control fibroblasts and chemical fibroblasts. Figure 2 shows the mRNA expression levels of TGF- $\beta$ 1, TGF- $\beta$ 2, TbR-I, TbR-II and Beta-actin of SM exposed and unexposed ones obtained from semiquantitative RT-PCR. The size of the PCR products for TGF- $\beta$ 1, TGF- $\beta$ 2, TbR-I, TbR-II and  $\beta$ -actin were 242, 220, 196, 210 and 190 bp, respectively.

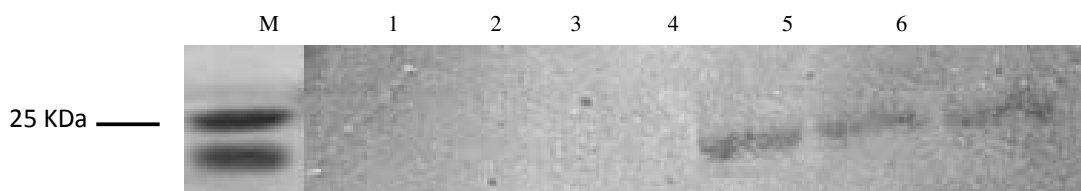
The significant TGF- $\beta$ 1, TGF- $\beta$ 2, TbR-I and TbR-II expression of SM exposed patients compared to unexposed ones was observed ( $p < 0.05$ ) Figure 3.

Western blotting analyses revealed similar trends in mRNA expression. In Western blot experiments, no detectable level of TGF- $\beta$ 1 was found in control primary lung fibroblasts (figure 4 line 1-3). In contrast, western blotting analysis showed that TGF- $\beta$ 1 expression was markedly increased in primary lung fibroblasts of chemical patients (Figure 4 line 4-6).

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**Figure 3.** The mean expression of target genes in SM exposed patients and unexposed ones. (A,B) The mean expression of TGF- $\beta$ 1 and TGF- $\beta$ 2 in SM injured patients, which is increased significantly in comparison to unexposed samples. (C, D) The mean expression of TbR-I and TbR-II in SM injured samples that were also increased significantly in comparison with unexposed group. ( $P < 0.05$ )



**Figure 4.** Western blot analysis revealed the presence of TGF- $\beta$ 1 (25 kDa) in the primary fibroblasts of SM exposed patients (line 4-6). Line 1-3 are related to the control samples. The cells were rinsed with phosphate-buffered saline; total extracted proteins were harvested and analyzed by Western blotting to indicate TGF- $\beta$ 1 protein

## DISCUSSION

Exposure of sensitive respiratory structures to sulfur mustard can lead to persistent lung disease. The greatest discomforts and symptoms are of chronic cough, dyspnoea and sputum production.<sup>21,22</sup> Airway remodeling followed by inhalation of mustard gas in veterans with chronic obstructive pulmonary disease

(COPD) has been reported.<sup>23</sup> Airway remodeling was considered, for the first time 75 years ago by Huber in the study and description of fatal asthma.<sup>24</sup> In airway remodeling besides increases in the collagen layer, vessels and elastic tissue in the airway wall, considerable amount of extracellular matrix is observed, probably due to increased synthesis and secretion by airways fibroblasts and other structural cells.<sup>25</sup> Various

researches have shown that TGF- $\beta$  can be effective as a therapeutic target. TGF- $\beta$  is well known as an important cytokine in the process of airway remodeling in lung diseases.<sup>26</sup>

In the present study, we found that the mRNA levels of TGF- $\beta$ 1, TGF- $\beta$ 2, T $\beta$ R-I and T $\beta$ R-II mRNA in primary human airway fibroblasts were significantly higher in exposed patients compared with controls. Western blot also showed that the TGF- $\beta$ 1 protein expression was more intensely detected in fibroblast cells from exposed patients.

The recent description of increased TGF- $\beta$  expression in asthma suggests that this cytokine plays a role in asthmatic airways.<sup>27</sup> Later investigations describe the over expression of TGF- $\beta$ 1 mRNA and proteins in bronchial biopsies from subjects with severe and moderate asthma, compared with controls without asthma.<sup>28-32</sup>

TGF $\beta$ 1 maybe served as a biomarker in sputum for assessing disease activity in patients with COPD and asthma.<sup>33</sup>

Using semi-quantitative RT-PCR techniques and Immuno-histochemistry, increased TGF- $\beta$ 1 gene expression in epithelial biopsies of smokers and people with chronic obstructive pulmonary disease (COPD) has been showed.<sup>34</sup> It is likely that lung fibroblasts participate in the increased expression of TGF- $\beta$ . Higher TGF- $\beta$ 1 levels are also reported in the bronchoalveolar lavage (BAL) fluid of stable atopic asthmatics,<sup>35</sup> SM exposed patients,<sup>36</sup> than in that of control subjects. Bergmann et al (1998), also showed that TGF- $\beta$ 1 mRNA in BAL cells from patients with obstructive lung transplantation Bronchiolitis, increased compared with control subjects.<sup>37</sup>

Utilizing semi-quantitative RT-PCR and western blot techniques, we denoted elevated levels of TGF- $\beta$  variants and receptors in primary human airway fibroblasts in SM exposed patients. These findings are consistent with the report that increased expression of growth factors, such as TGF- $\beta$ ,<sup>38,39</sup> have been observed in lung fibrosis and may drive the excessive matrix deposition and cell proliferation. TGF- $\beta$  is thought to be particularly involved in the development of subepithelial fibrosis, since TGF- $\beta$  *in vitro* stimulates fibroblast proliferation,<sup>40</sup> promotes matrix protein synthesis and increases the expression of receptors for extracellular matrix components.<sup>41</sup> Irrespective of the mechanism involved, a consistent biological observation is that in models of wounding and injury,

T $\beta$ Rs are increased in expression by fibroblasts, airway smooth muscle cells (ASMCs) and pulmonary vascular smooth muscle (VSM).<sup>42-44</sup> Since both T $\beta$ R-I and T $\beta$ R-II are required for signal transduction of TGF- $\beta$ , the increased expression of T $\beta$ R-I and T $\beta$ R-II is likely to have resulted in increase of connective tissue synthesis and deposition by fibroblasts.<sup>45</sup>

Several authors have related TGF- $\beta$  expression to signs of airway remodeling. One study comparing the relative expression of TGF- $\beta$  in the remodeling of the airway wall found a weak but statistically significant correlation between the number of fibroblasts and the expression of TGF- $\beta$ 1 in asthmatic patients, but not in controls, suggesting that the role of TGF- $\beta$ 1 is preponderant in airway wall remodeling.<sup>46-48</sup> We demonstrated an increased expression of TGF- $\beta$ 1, TGF- $\beta$ 2, T $\beta$ R-I and T $\beta$ R-II mRNA in primary human airway fibroblasts SM exposed patients. Taken together, these results indicate that TGF- $\beta$ s and their receptors have a major role in the remodeling of the airway wall. These findings confirm what we have reported through this article: TGF- $\beta$  might play an important role in the chronic effects of SM exposure and it was suggested that airway fibroblasts might be involved in the processes of airway remodeling and the resultant obstructive changes in the airways by affecting TGF- $\beta$  pathway.

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