ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
September 2011; 10(3): 147-154.

Smad Molecules Expression Pattern in Human Bronchial Airway Induced by Sulfur Mustard

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Received: 1 February 2011 ; Received in revised form: 1 June 2011 ; Accepted: 10 June 2011

ABSTRACT

Airway remodelling is characterized by the thickening and reorganization of the airways seen in mustard lung patients. Mustard lung is the general description for the chronic obstructive pulmonary disease induced by sulfur mustard(SM). Pulmonary disease was diagnosed as the most important disorder in individuals that had been exposed to sulfur mustard. Sulfur mustard is a chemical warfare agent developed during Wars. Iraqi forces frequently used it against Iranian during Iran –Iraq in the 1980–1988. Peribronchial fibrosis result from airway remodeling that include excess of collagen of extracellular matrix deposition in the airway wall. Some of Smads families in association with TGF-β are involved in airway remodeling due to lung fibrosis. In the present study we compared the mRNA expression of Smad2, Smad3, and Smad4 and Smad7 genes in airway wall biopsies of chemical-injured patients with non-injured patients as control.

We used airway wall biopsies of ten unexposed patients and fifteen SM-induced patients. Smads expression was evaluated by RT-PCR followed by bands densitometry.

Expression levels of Smad3 and Smad4 in SM exposed patients were upregulated but Smad2 and Smad7 was not significantly altered.

Our results revealed that Smad3, and 4 may be involved in airway remodeling process in SM induced patients by activation of TGF-β. Smad pathway is the most represented signaling mechanism for airway remodeling and peribronchial fibrosis. The complex of Smads in the nucleus affects a series of genes that results in peribronchial fibrosis in SM-induced patients.

Keywords: Bronchial Airway; Smad; Sulfur Mustard; TGFβ

INTRODUCTION

Airway remodelling is characterized by the thickening and reorganization of the airways seen in mustard lung patients.[1,2] The characteristics of airway remodelling include subepithelial fibrosis, basement

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membrane thickening, mononuclear cell infiltration of the lamina propria, myofibroblast hyperplasia, myocyte hyperplasia and hypertrophy, together with epithelial damage, goblet cell metaplasia and oedema.[1,3] Mustard lung is the general description for the chronic obstructive pulmonary disease induced by sulfur mustard.[4]

Sulfur mustard (SM) is a chemical warfare agent developed during world Wars. Its production is easy and its vesicant property bestows the highest military significance. More recently, SM was used by Iraqi forces against Iranian troops and civilians as well as Iraqi Kurdish civilians during the Iraq-Iran war 1984–1988.[5-7]

SM is capable of producing severe chemical injuries primarily in three major organs: skin, eyes, and lungs. More than 45,000 of 100,000 exposed patients are suffering from late effects of SM after almost 25years post-exposure.

Previous studies have clarified that bronchiolitis obliterans (BO) remains as a main respiratory clinical complication in patients exposed to SM. Moreover, all the patients exhibited symptoms of BO were confirmed by high-resolution computerized tomography (HRCT), scan data characterized by expiratory air trapping of more than 25% and mosaic parenchymal attenuation, and biopsy samples taken in previous studies.[8,9] peribronchial fibrosis that result from airway remodeling includes excess of collagen of extracellular matrix deposition in the airway wall. [10]

Some of Smad family members in association with TGF-β are involved in airway remodeling due to lung fibrosis.[11, 12]

TGF-β has a role in most pulmonary disorders.[3,13] TGF- β administration induces peribronchial collagen deposition.[14] This cytokine has increased in bronchoalveolar lavage as well as airway biopsy samples of individuals that were exposed to sulfur mustard. [13] TGF-β signaling pathway, centrally involved in smad proteins. Smads protein are major signaling molecules acting downstream of TGF-β receptors and are essential in the regulation of the transcription of genes involved in ECM remodelling and repair.[15]

Smads are categorized into 3 classes: R-Smads (Regulatory-smads include Smads 1, 2, 3, 5 and 8), Co-Smads (Common-partner Smads include Smad4) and I-Smads (Inhibitory Smads include Smads 6 and 7).

Smads 2, 3 are direct substrates for type 1 TGF-β receptors (TGF-βRI).[16] Smads 2, 3 bind to SARA (Smad anchor for receptor activation) and hold them close to plasma membrane. [17] When a dimeric TGF-β molecule binds to type II receptors transphosphorylate Type I receptors.[17-20] Type I receptor kinases phosphorylate Smads 2, 3 and then the phosphorylated Smad3 and Smad2 are released by SARA. Smad2, 3 combine with Smad 4 and form a heterotrimeric complex containing two Smad 2 or 3 molecules and one Smad 4 molecule. This complex translocate into the nucleus[16, 17] and activate a series of genes involved in matrix expression and cell differentiation and proliferation.[21, 22] And excessive deposition of ECM induce fibrosis (Figure 1). In the present study, in order to clarify the significance expression of intracellular mediators of Smads in airway wall of people exposed to SM, we examined the mRNA expression for smad2, smad3 and smad4 genes in airway wall biopsies of chemical-injured patients by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR), and compared it with control group.

MATERIALS AND METHODS

Sampling

In this study, 15 SM exposed patients and 10 healthy participants enrolled as a control group. The documented evidence of chemical exposures by the military health services confirmed exposure to SM. These people showed respiratory symptoms immediately after the exposure without symptom-free periods. High-resolution computerized tomography (HRCT) scan has indicated bronchiolitis in these individuals. This survey was conducted in accordance with a protocol approved by BaqiyatallahUniversity of Medical Sciences ethics committee. Both groups signed a consent form.

In this study, exclusion criteria were people who had cancer, diabetes, other chronic pulmonary diseases (such as asthma), autoimmune diseases (such as rheumatoid arthritis), pneumonia, elderly, organ transplant recipients, or patients with occupational history of toxic fume exposure, smokers and addiction. The characteristics of subjects are presented in table 1. A

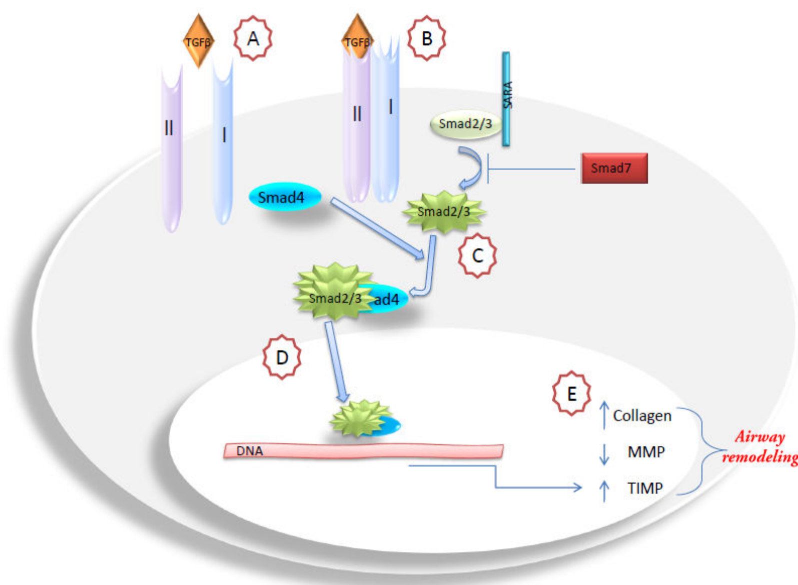


Figure1. The transforming growth factor (TGF)-β-Smad pathway. TGF-β bind to type II and type I receptors (A).Ligand binding induces the formation of heterotetrameric Complexes (B). Type I receptors then activate R-Smads (Smad2/3), which associate with Co-Smad (Smad4) (C). The R-Smad and Co-Smad complexes translocate to the nucleus, and regulate the expression of target genes in cooperation with transcription factors, co-activators and co-repressors (D). Smads result in ECM deposition and thickening of bronchial through MMPs repression, TIMP up regulation and contraction of three-dimensional collagen gels (E). Smads molecule with effect on the series of genes results in airway remodeling and peribronchial fibrosis (D).

A Physician performed the bronchoscopic examination using fiber flexible fiberoptic bronchoscope (Olympus, Japan) and obtained a biopsy of the segmental and sub-segmental carinae.

The subjects were anesthetized by 2% lidocaine and intravascular midazolam and slept lightly during the process. During the process, supplemental oxygen was given and the oxygen saturation was checked by continuous pulse oxymeter. The taken biopsies were immersed in Tripure Isolation Reagent (Roche applied science, Germany).The biopsies were stored in -80 until mRNA extraction.

mRNA Extraction and RT PCR

At first collected specimens were homogenized in Tripure Isolation Reagent (Roche applied science, Germany). Total mRNA was isolated from collected specimens using manufacturer's recommendation. The quantities of purified mRNAs were verified by Nanodrop spectrophotometer (ND-1000, Wilmington, DE) and the qualities of them were verified by electrophoresis in 1% agarose gel (Sinnagene, Tehran, Iran).

500 ng of these total mRNA were used for cDNA synthesis using Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA).This reaction was done in a master cycler thermal cycler (Eppendorf, Germany).

Primers designed for Smad2, 3, 4 and 7 genes are listed in table 2.Then RT- PCR reaction was performed in 25-µL reaction mixture containing 10X buffer (Takara, Japan): 1.5 mM $MgCl_2$, 0.5 µL of first-strand cDNA, 0.5 U of recombinant Taq DNA polymerase (Cinagene), 200 µM of each deoxynucleoside triphosphate, and 4 µM of each primer.

Initial denaturation of cDNA was done at 94◦C for 5 minutes. following cycle was repeated for 35 times consisting of denaturation at 94◦C for 30 seconds, annealing as described in table 2 for 30 seconds, and polymerization at 72◦C for 30 seconds followed by a 5-minute terminal extension at 72◦C and interested genes were amplified with this method

Table 1. Subject characteristics

Group	N	Sex (M/F)	Age range	Age mean±SD	*P*
Control group	10	10/0	31-58	48.0±9.4	0.82
SM-injured group	15	15/0	38-59	47.3±5.8	

Table 2. Sequence and characteristics of PCR Primers

Gene and Gene Bank ID	Primer	Primer sequence (5' to 3')	Annealing Tm	PCR Product Length
Smad2 (NM_001135937.1)	Forward	TGGATGACTATACTCACTCCA	60	180 bp
	Reverse	TAGTAGGAGATAGTTCTGCTG	60	
Smad3 (NM_001145104.1)	Forward	TGGTGCTCCATCTCCTACTA	60	267 bp
	Reverse	TACAGTTGGGAGACTGGACA	60	
Smad4 (NM_005359.5)	Forward	TGGCCTGTTCACAATGAGCT	60	219 bp
	Reverse	TTCTGTCCTGTGGACATTGG	60	
Smad7 (NM_005904.2)	Forward	TTCTTCTGGAGCCTGGGGA	60	250 bp
	Reverse	TTGTACACCCACACACCATC	60	
Beta actin (NM_001101)	Forward	TCATGAAGATCCTCACCGAG	59	190 bp
	Reverse	TTGCCAATGGTGATGACCTG	59	

Table 3. Increases in Smad 3, 4 expression in SM-exposed patients in comparison with control group

Gene name	SM-exposed cases vs. unexposed cases (fold changes)	*P*
Smad 3	2.2	0.003*
Smad 4	1.7	0.023*

*Statistical significance: $P < 0.05$.

The master cycler thermal cycler (Eppendorf, Germany) was used for this process. PCR products were electrophoresed in 2% agarose gel (Cinnagene, Tehran, Iran) then were stained with ethidium bromide and were expressed with UV ray in gel documentation (Bio-Rad Laboratories, CA).The density of bands obtained from capturing picture were determined by scion image software(Scion Corporation, Frederick, MD) and the data were obtained.

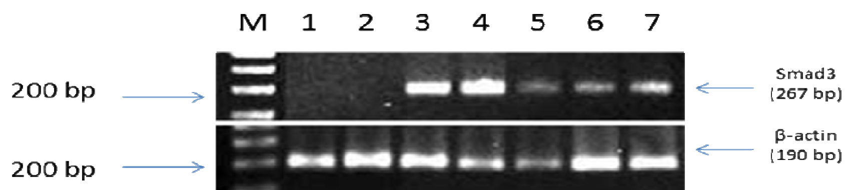
Statistical Analysis

We used SPSS 16 for statistical analysis and the results were analyzed by performing T-tests and Mann-Whitney test. $P < 0.05$ was considered as statistically significant.

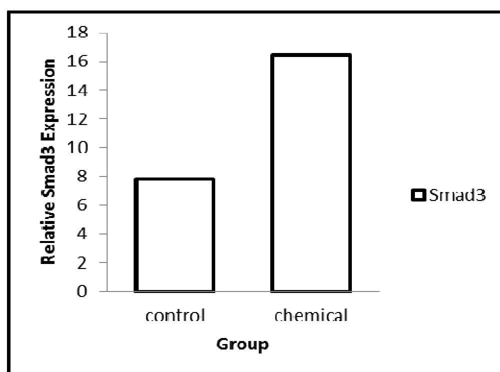
RESULTS

In the present study, 15 airway biopsies of SM exposed and 10 samples of control unexposed people were collected. Average age of SM-inhalated patients and control people were 48 and 47 years. There was not statistically difference between the age of two groups ($p>0.05$).

Semiquantitive RT-PCR was used to determine Smad2, Smad3, Smad4 and Smad7 gene expression variation between control samples and case samples. The size of desirable fragments for β-actin, Smad2, Smad3, Smad4 and Smad7 were 190, 180, 267, 219 and 250 bp respectively. We have normalized our results with β-actin that was a housekeeping gene. After statistical examination, we found that Smad3 gene expression in SM-inhalated was higher (2.2 folds) than unexposed group and there was significant difference in Smad3 gene expression between two groups ($p=0.003$) (Figure 2 and Table 3).

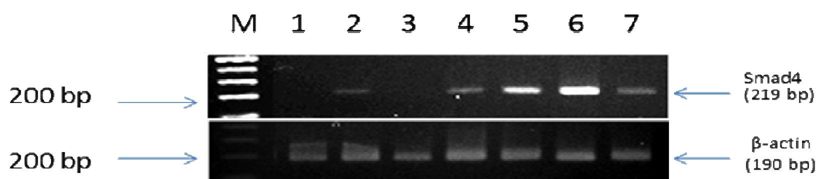


(a)

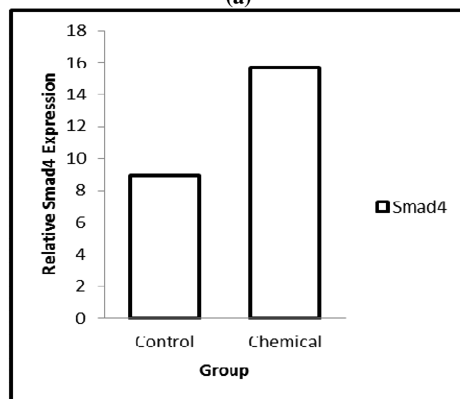


(b)

Figure 2. Upregulation of Smad3 in SM-injured patients. Gene expressions (a) were measured by semi quantitative RT-PCR. Smad3 was upregulated in SM-injured patients (Lanes 3–7). Only 7 samples have been shown, compared to two normal samples (Lanes 1 and 2). M: 100-bp marker. (b) Ratio of Smad3/beta-actin has also been shown by a histogram.



(a)



(b)

Figure 3. Upregulation of Smad4 in SM-injured patients. Gene expressions (a) were measured by semiquantitative RT-PCR. Smad4 was upregulated in SM-injured patients (Lanes 3–7). Only 7 samples have been shown, compared to two normal samples (Lanes 1 and 2). M: 100-bp marker. (b) Ratio of Smad4/beta-actin has also been shown by a histogram.

Smad4 gene expression in case group was also higher (1.7 folds) than control group. There was significant difference in Smad4 gene expression between two groups (p=0.023) (Figure 3 and Table 3); however there was not significant difference in Smad2 and Smad7 genes expression between two groups (p=0.243, p=0.978).

DISCUSSION

With regard to importance of lung disease in individuals that had been exposed to sulfur mustard, we studied the molecular mechanisms leading to structural alterations and pathological symptoms observed in the lungs of these people. TGF- β regulates different processes, including tissue remodeling and repair, cell apoptosis and survival, extracellular matrix production, and inflammation. TGF-β has a role in most respiratory disorders and it is an early marker of BO.[13, 23]

We decided to investigate Smad molecules as downstream of TGF-β signaling pathway. Hence, we examined Smad2, 3 mRNA levels as the R-smads and Smad4 mRNA levels as co-Smad and Smad7 as I-Smad in the TGF-β/Smad signaling pathway in the airway wall biopsies.

The genes expression of Smad3 and Smad4 were 2.2 folds and 1.7 folds higher in chemically injured patients comparing to control control patients respectively, whereas Smad2 gene expression was not significantly different from control patients.

The recent reports have suggested that TGF-β/Smad signaling pathway are involved in pulmonary diseases such as COPD and result in peribronchial fibrosis.[15]

Zandvoort et al have shown Smad 7 expression (inhibitory Smad of the TGF- β -induced Smad pathway) was decreased in bronchial epithelial cells in patients with stage II, as well as stage IV, COPD and have indicated that there is possibly less inhibition of the TGF-β/Smad pathway.[15] Anne V. Gonzales and et al have demonstrated that lung fibroblasts, isolated from rats after exposure to bleomycin (a potential agent used for lung fibrosis), showed increased level of activation of the receptor-regulated Smad3 and showed decreased level of the inhibitory Smad7. [24] The absence of Smad3 signaling molecule may mediates the reduction of the ECM (that is mostly composed of collagen) via direct blockade of the TGF- β /Smad pathway. Also, Smad3 signaling is involved in the maintaining the balance between protease such as MMPs and anti-protease such as TIMPs activity because Smad3 deficiency leads to an TGF-β inability to negatively regulate the expression of certain proteases and a decrease in ECM deposition.[2, 24]

In another study, Annie V Le et al have used Smad3 deficient mice for allergen-induced airway remodeling and have shown decreasing of airway remodeling (collagen deposition, smooth muscle layer, and mucus production) in Smad3-deficient mice compared to WT mice.[25]

Some researchers have shown that TGF-β is necessary for the transformation of fibroblasts to myofibroblasts. The collagen is mostly produced by myofibroblasts. TGF-βs are mainly activated by the phosphorilation of TGF- β type 1 and type2 receptors and the subsequent phosphorilation and translocation of R-Smad (Smad2 and Smad3) to the nucleus in which they regulate gene transcription. Sarah J. McMillan et al have suggested that administration of anti TGF-β Antibody modulated active TGF-β signaling, resulting in reduced collagene deposition.

Also, TGF-β induced in vitro differentiation of myofibroblasts and collagen production by a Smad2 dependent mechanism. Inflammation and remodeling can be uncoupled during the development airway inflammation because treatment with anti-TGF-β antibody reduced the airway remodeling development but not inflammation.[26] All these studies have shown that TGF-β/Smad signaling pathway is active in obstructive pulmonary disease by high expression of R-Smad (Smad2 or Smad3) or suppression of I-Smad (Smad7). We have indicated increasing of Smad3 and 4 expression; whereas Smad7 gene expression has not altered. Then, we have resulted TGF-β/Smad signaling pathway is active in treatment group. So our study has similar result with previous study.[15]

In conclusion our study demonstrated that expression of Smad 3,4 at mRNA levels in sulfur mustard injured veterans significantly increased compared to control group indicating that maybe Smad 3,4 have been activated by TGF-β in SM-inhalated patients. Smad pathway is the most represented signaling mechanism for airway remodeling and peribronchial fibrosis. Smads may result in ECM deposition and thickening of bronchial through MMPs repression, TIMP up regulation and contraction of three-dimensional collagen gels. However, further and complementary studies are required to clarify underlying mechanisms of how SM exerts cytotoxicity.

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