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RESEARCH ARTICLE

The effects of sulfur mustard on expression of TGF-βs variants in lung epithelial cell line

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Abstract

Sulfur mustard (SM) is a blister-forming agent and can cause damages in various momentous human organs. Previous studies have demonstrated that chemical and mechanical injuries of epithelial cells cause to give rise the secretion of TGF-β1 and TGF-β2. These cytokines play a key role in respiratory remodeling due to SM. In this study, we investigated the impact of SM on the expression level of TGF-β isoforms and their receptors *in vitro* using reverse transcriptase polymerase chain reaction and western blotting. Our finding revealed the significant increase at concentrations of 25 μl/ml SM for 30 min and 60 min and also 100 μl/ml for 60 min for TGF-β1, 25, 50 and 100 μl/ml SM for 30 min for TGF-βr1 and after exposing with 100 μl/ml SM for both 30 and 60 min for TGF-β2 ($p < 0.05$). Data from western blotting showed the increase of TGF-β1 expression at the level of protein as the same pattern as the mRNA level. *In vitro* short-time exposure of fibroblast to SM can induce the expression of TGF-β1, TGF-β2 and TGF-βR1 denoting that over-expression of TGF-β isoforms and their receptors leads to differentiation and collagen production, causing in airway remodeling and fibrosis.

Keywords

Receptors, sulfur mustard, TGF-β

History

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Introduction

Sulfur mustard (SM) [bis-2-(chloroethyl) sulfide] is a hazardous vesicant agent, which is known as a potent chemical weapon (1). For the first time, this destructive agent has been used by military forces during World War I. Recent extensive use of SM exerted in the Iraq–Iran war in the 1980's by Iraqi military forces that victimized over 100 000 civilian and military personnel in Iran (2). This component is a bifunctional alkylating agent, being covalently able to react with the essential cellular macromolecules such as DNA, RNA and protein, and finally modifies them (3).

Various organs such as the skin, respiratory tract and eyes are suffered from SM. The late implication of SM exposure is more serious (4). Airway remodeling is an irreversible and dynamic process causing to modify the structure of the airway. These alterations are subepithelial fibrosis, enlargement of submucosal gland, epithelial damage, myofibroblast and myocyte hyperplasia, infiltration of mononuclear blood cells to a lamina propria and edema, which leads to the reorganization and thickening of the airway (5–7). Remodeling was frequently seen in victims after the inhalation of SM. Furthermore, it is accompanied with the chronic obstructive pulmonary diseases (COPDs) (3,7,8). It has been reported that TGF-β has an important role in the pathogenesis of numerous pulmonary disease such as fibrosis (9). The bronchoalveolar lavage and biopsy from the airway of victims exposed to SM contain high amounts of TGF-β (8–10). TGF-β belongs to a dimeric polypeptide growth factor family, and various human cells such as epithelial, endothelial hematopoietic and connective-tissue cells are able to produce this cytokine. In addition, all cells have its receptors. TGF-β involves in justifying several biological functions including the cell proliferation and differentiation, angiogenesis, wound healing, etc. Three isoforms have been known for TGF-β termed as TGF-β1, TGF-β2 and TGF-β3 (11). Although these isoforms have similar structure and can influence on cells *via* the same receptors, the expression level and their function are discrepant (12).

Data from previous *in vitro* studies have demonstrated that chemical and mechanical injuries of epithelial cells cause to give rise the secretion of TGF-β1 (13) and TGF-β2 (14). At the pathological condition during pulmonary disorder, the production of this cytokine gives rise to fibrosis due to increasing the proliferation of epithelial cells and myofibroblast producing collagen (15). Excess deposition of collagen

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in the extracellular matrix leads to thickening of the epithelium and its remodeling (16).

In order to clarify the significance expression of TGF-β in the airway wall of individual exposed to SM, in this study, we aim to assess the amount of mRNA expression of *TGF-β1*, *TGF-β2* and *TGF-βr1* genes in the airway epithelial cell line after exposure to SM using reverse transcriptase polymerase chain reaction (RT-PCR). In addition, TGF-β 1 protein concentration was determined by western blotting analysis.

Materials and methods

Cell culture

An epithelial lung cell line, L105, was obtained from the Pasteur Institute (Tehran, Iran). This cell line was cultured in RPMI 1640 medium containing 10% FBS 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C and 5% Co_2. All the cell culture reagents were purchased from the Gibco Company (Langley, UK).

Cell toxicity assay

To evaluate the cytotoxicity effect of SM on L150 cell, the MTT test was performed. Briefly, after the cells reached 80% confluency, the cells were detached by trypsin/EDTA and counted using trypan blue staining, then 10^4 cells were transferred to each well of 96-well plate. After 24 h, the cells were exposed to with 25, 50 and 100 µl/ml of SM for three different times, 30 min, 60 min and 16 h. After passing the desired time, the medium of each well was replaced by 200 µl of new media and then 20 µl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (Sigma-Aldrich, Schnelldorf, Germany) was added to each well and incubated for 4 h at 37 °C. In the next step, supernatant of each well was replaced by 100 µl of dimethyl sulfoxide and immediately the optical density of each one was read by ELISA reader at 570 ηm. All tests were performed three times. Cells treated with absolute ethanol as SM solvent, and PBS were assessed as controls.

Exposure to SM and RNA extraction

To analyse the effect of SM exposure on the gene expression, cells were treated with 25, 50, 100 µl/ml. After 30 min, 60 min and 16 h, the cells were collected in the clean micro-tube. RNA was extracted using a solution TriPure Isolation reagent (Roche, Mannheim, Germany) according the manufactures' instruction. The quality and the quantity of the extracted RNA were investigated by electrophoresis on the 1% agarose gel and NanoDrop spectrophotometer (NanoDrop, Wilmington, DE) at 260 ηm, respectively.

Gene expression analysis

To synthesize the cDNA, 500 ηg RNA from each sample were amplified using Bioneer kit (Takara, Tokyo, Japan). By 12 cycles, each cycle consisting of 30 s at 25 °C, 4 min at 25 °C and 30 s at 72 °C, followed by a single 5-min heat inactivation at 95 °C.

One microliter of each cDNA was amplified in 20 µl of mixture reaction containing 10 × reaction buffer, 0.2 mM of the deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 10 ρmol of each of the primers (Table 1) and 1.5 U of Taq DNA polymerase (Cinnagen, Tehran, Iran). PCR procedure was performed by Eppendorf asterCycker (Hamburg, Germany) with an initial denaturation step of 6 min at 94 °C; 35 cycles of 30 s at 94 °C, 45 s at specified annealing temperature for each primer and 45 s at 72 °C followed by 5 min final extension at 72 °C.

To visualize the PCR product, the electrophoresis was carried out on 1.5% gel agarose staining by ethidium bromide. All results were normalized with β-actin expression to compensate for differences in cDNA amount. Image analysis (using Scion Image software) was done to obtain quantitative data (Scion Corporation, Frederick, MD).

Western blotting

To assess the expression amount of TGFβ1 protein, the western blotting method was performed. To separate proteins, an equal amount of L105 cells lysate (lysed by lysis buffer containing 10 mM triton X-100 pH 7.5 and 150 mM NaCl) and purified exosomes were loaded onto SDS-PAGE containing 12.5% polyacrylamide with 0.1% SDS. After resolving, proteins were electrophoretically transferred to a PVDF (Roche) and then blocked with 5% skim milk solution in Tris buffered saline with 0.1% tween-20 (TTBS) for overnight at 4 °C. To specifically detect TGF-B1, the membrane was incubated with mouse anti-TGF-B1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature and then washed by TTBS three times. In the next step, the anti-mouse IgG conjugated with horseradish

Table 1. Gene expression analysis.

The length of fragment	Annealing temperature	Primer sequences	Primer type	Gene name
242 bp	59 °C	5′TCAAGCAGAGTACACACAGC3′	F	TGFβ1
		5′GCACAACTCCGGTGACATC3′	R	
220 bp	57 °C	5′TTGACGTCTCAGCAATGGAG3′	F	TGFβ2
		5′TCAGTTACATCGAAGGAGAGC3′	R	
167 bp	58 °C	5′TCAGCATTCACTGTCCATGTC3′	F	TGFβ3
		5′TAGATGAGGGTTGTGGTGATC3′	R	
190 bp	56 °C	5′TGCTGCAATCAGGACCATTG3′	F	TGFβR1
		5′TCCTCTTCATTTGGCACTCG3′	R	
210 bp	57 °C	5′TGCTCACCTCCACAGTGATC3′	F	TGFβR2
		5′TCTGGAGCCATGTATCTTGC3′	R	
190 bp	59 °C	5′TCATGAAGATCCTCACCGAG3′	F	actin-β
		5′TTGCCAATGGTGATGACCTG3′	R	

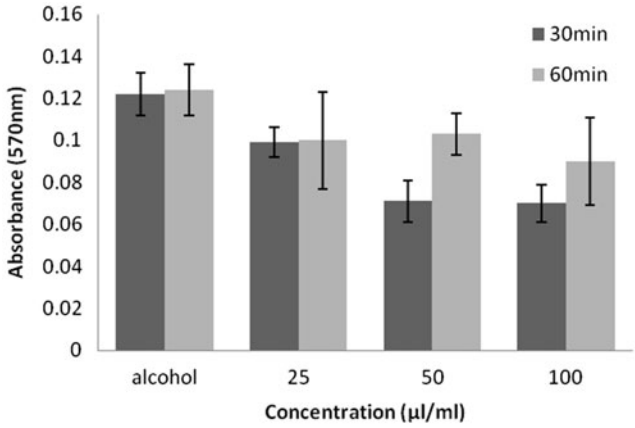

Figure 1. The cell cytotoxicity rate of treated L150 cells with the sulfur mustard. The cells were treated with 25, 50 and 100 µl/ml of sulfur mustard for 30 and 60 min. Furthermore, equal amount of alcohol were examined as solvent controls. After 30 min, significant decrease proliferation was determined in all concentrations of the sulfur mustard after 30 min and at concentration of 50 and 100 µl/ml after 60 min ($p < 0.05$).

peroxidase (Santa Cruz Biotechnology) was added to the membrane and incubated for 1 h at room temperature. To visualize the interested protein, DAB/$NiCl_2$, a chromogenic stain, was utilized.

Results

MTT results

Figure 1 shows the outcomes from the cytotoxicity evaluation of three different doses of SM. All concentrations significantly showed the harmful effect on the proliferation of exposed cells compared to alcohol control as a solvent after both 30 and 60 min ($p < 0.05$) except 25 µl/ml of SM after 60 min that had no impact on the cells. We found that the cytotoxic effect of SM was a dose-dependent at the time of 30 min.

Gene expression

In this study, we used RT-PCR for evaluating the expression of TGF-β1, TGF-β2 and TGF-βR1 in the epithelial cells exposed to SM. Figure 1 illustrates examples of gel electrophoresis of PCR products. As presented in Figure 2, upregulation of TGF-β1 was significantly demonstrated after exposure to 25 µl/ml SM for 30 min and 60 min and

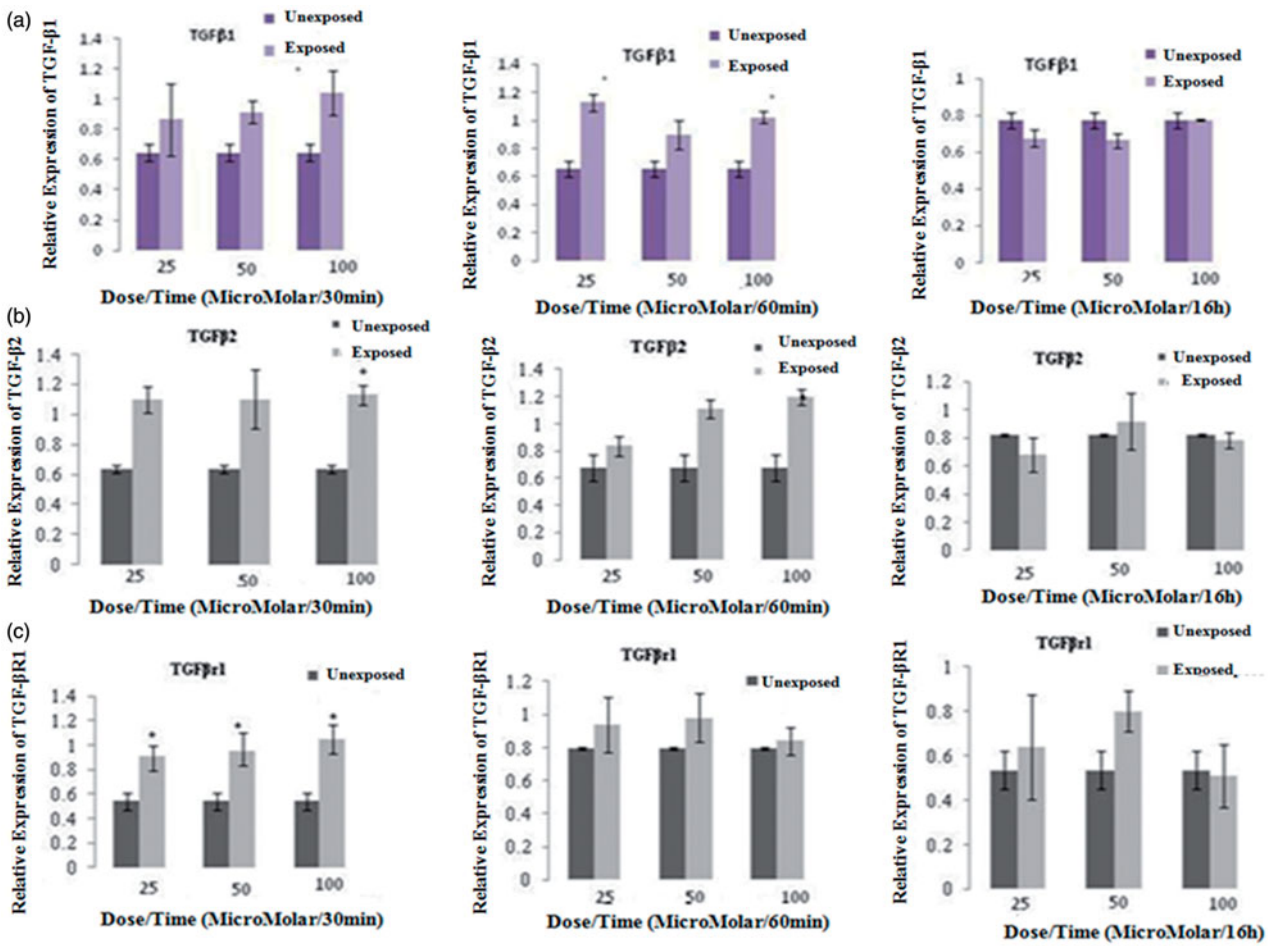

Figure 2. Effect of sulfur mustard on the expression of TGF-β1, TGF-β2 and TGF-βR1. The mRNA levels of described gene were relatively measured by RT-PCR in the L150 cell exposed to the three different concentrations of SM (25, 50 and 100 µl/ml) after 30 and 60 min. β-actin was tested as a housekeeping gene, and the results showed as a ratio of desired gene expression/β-actin expression. SM gave rise the expression of TGF-β1 (a) and TGF-β2 (b) at the concentration of 100 µl/ml after 30 and 60 min. All three concentrations of SM after 30 min caused to up-regulate the TGF-βR1 (c).

Figure 3. The finding from Western blotting.

also 100 µl/ml for 60 min ($p < 0.05$). Furthermore, we observed a significant increase of TGF-β2 expression after exposing with 100 µl/ml SM for both 30 and 60 min (Figure 3) ($p < 0.05$). According our results showed in Figure 2, exposing the cells to all mentioned concentrations for 30 min can give rise the expression of TGF-βR1, significantly ($p < 0.05$). We showed no TGF-βR2 PCR products in both test and control groups.

Since 60-min exposure to SM lead to increase the expression of TGF-β1 more than other times, we assessed whether this event can cause to alter the expression of TGF-β1 in protein level. Data from western blotting showed the increase of protein expression as the same pattern as the mRNA level. Figure 3 shows the finding from western blotting.

Discussion

COPD is the common implication after the inhalation of SM that manifests with the chronic cough, dyspnoea and over-production of sputum (17,18). Furthermore, airway remodeling is a pathological feature that has been demonstrated in veterans suffered from COPD (19). Several previous studies have been reported that TGF-β isoforms involved in the modulation of airway remodeling process (5). Despite the contribution of this cytokine in the decrease of the acute inflammatory responses and triggering the tissue repairmen, its pathologic effect is to emerge fibrotic diseases in high levels. To transduct the TGF-β signaling, the presence of TGF-βR1 and TGF-βR2 is necessary (20). Raised level of TGF-β was found in the bronchoalveolar lavage fluids collected from obliterative bronchiolitis patients, which is the main complication in the victim exposed to SM. This event can lead to pulmonary fibrosis (21).

Previous finding from SM-exposed patients and reports from other studies performed on asthma and COPD patient identified that TGF-β and its receptors could play a predominant role in the long-term impact of airway remodeling. We showed that the expression levels of TGF-β1, TGF-β2, TGF-βR1 and TGF-βR2 was to rise in human airway fibroblast of patients exposed to SM compared to a control group.

Several studies have demonstrated that different molecules were affected by Ms (4,22,23). Similar to our findings, Takizawa et al. and Torrego et al. viewed a higher expression level of TGF-β1 in the epithelial cells located in airways in the COPD patients and after challenging with allergen, respectively (21,24). In this study, we found that *in vitro* short-time exposure of epithelial to SM can induce the expression of TGF-β1, TGF-β2 and TGF-βR1, both at mRNA and protein levels. Phipps et al. reported that airway remodeling occurs during the first 24 h after allergen challenging, suggesting the responsibility of epithelium for this alteration (25). We have also revealed the elevated expression levels of TGF-β1 and two following exposure to SM after 30 and 60 min. Furthermore, data obtained from Torrgo demonstrated increased the TGF-β2 but not TGF-β1 levels following allergen exposing within 24 h (21). It is described that SM similar to allergen has a capability to induce remodeling signaling in a short-term even lower than 24 h. In fact, it is reported that a rise of tenascin and fibronectin level expression of in the extracellular matrix occurs owing to the release of both TGF-β1 and TGF-β2 from bronchial epithelial cells (26).

Moreover, alteration in the gene expression was a dose-dependent. Furthermore, these results denoted that increase of TGF-β may be a protective behavior of airway epithelial for repairing tissue *via* interaction between epithelial–eosinophil–TGF-β as well as increase survival time of myofibroblast through suppressing IL-1b mediated apoptosis. But continuous over-expression of TGF-β isoforms and their receptors leads to differentiation and collagen production, resulting in airway remodeling and fibrosis. An animal model air way remodeling that induced by TGFβ1, demonstrated that it causes airway fibrosis and increased collagen I and III mRNA (27). In recent years, many potential antifibrotic treatment strategies have emerged from molecular studies of profibrotic disease that affects the skin and multiple internal organs, including the lungs, kidneys, gut and heart. Blocking TGF-β1 secretion suggesting that anti-TGF-β therapeutics may be beneficial to pulmonary fibrosis patients.

Conclusion

Overall, we demonstrated that increase TGF-β level is one of the early momentous events taking place in pulmonary epithelial cells following SM exposure and is a dose-dependent. What is more, it can actuate airway remodeling and in the long run, fibrosis. Drugs that inhibit TGF-β may be especially helpful to a molecularly defined patients.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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