Trefoil Factor Family 1 Is Involved in Airway Remodeling of Mustard Lung

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ABSTRACT

Human airway is generally destructed by inhaled factors and is the barrier to the external environment and has a crucial role in protection of the internal homeostasis of the lung. Sulfur mustard (SM) is a chemical warfare that is capable of producing severe chemical injuries primarily in the lungs. Trefoil factor family (TFFs) plays major roles in epithelial repair and homeostasis, particularly in the lung. This study attempted to verify the role of TFFs in airway induced by sulfur mustard.

Fifteen patients exposed to SM and 10 non-exposed subjects were enrolled in this study. Bronchoscopy was carried out and endobronchial biopsy specimens were taken. The TFFs gene expressions were evaluated by RT-PCR. The results revealed that the TFF1 was overexpressed in exposed subjects in comparison to non-exposed subjects.

In conclusion, TFF1 displays a distinct protein expression pattern in the developing of airway remodeling due to SM inhalation and plays an important role in maintaining the airway epithelium function.

Keywords: Airway remodeling; Sulfur mustard; Trefoil factor family

INTRODUCTION

Human airway surface epithelium is a barrier to the external environment and has a crucial role in protection of the internal homeostasis of the lung. However it could be destructed by inhaled factors (viruses, bacteria, hazardous gasses).1

Considerable lines of defense protect the surface of airway epithelium against severe trauma. These defense mechanisms such as normal epithelial repair require restitution (cell migration) and regeneration via proliferation and differentiation.2

Airway remodeling is a complex process of structural changes such as subepithelial fibrosis, increased smooth muscle mass, enlargement of glands, neovascularization, and epithelial alterations, which involves both large and small airways in various diseases.3 These structural changes are characterized by the wall thickening due to increasing extracellular matrix deposition, particularly in the reticular basement membrane region, lamina propria and submucosa, subepithelial fibrosis, epithelial goblet cell hyperplasia
and metaplasia, mucus metaplasia, myofibroblast hyperplasia and increase in bronchial smooth muscles, new blood vessels and epithelial hypertrophy. One reason for this pathophysiological event is harmful agents inhalation.

Sulfur mustard (SM) is a chemical warfare agent that is capable of producing severe chemical injuries primarily in the lungs. Previous studies have clarified that bronchiolitis obliterans (BO) remains as the 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) and biopsy samples taken in previous studies. Suggestive scan findings included expiratory air trapping of more than 25% and mosaic parenchymal attenuation. Histo pathological findings comprised peribronchial fibrosis, increasing mucine secretion from goblet cell, excessive deposition of collagen extracellular matrix in the airway wall due to airway remodeling. These pathological characteristics in lung due to SM inhalation is called “mustard lung”.

Trehf oil factor family (TFFs) plays major roles in epithelial repair and homeostasis, particularly in the lung and mucous-cell (for example, goblet cell). TFFs are typical secretory products of mucous epithelia. Three TFF peptides are known in humans: TFF1, TFF2, and TFF3. Their expression patterns vary characteristically and were studied particularly in the lung and gastrointestinal tract. TFF peptides are expressed in a wide variety of chronic inflammatory diseases. Also our previous study and other related studies reported that higher rates of production of these peptides are observed in airway cells from patients with mustard lung.

Mucins are secreted all over the respiratory tract and have an important role in the mucociliary clearance and defense system. TFFs are also present in secretory cells of the human respiratory tract. TFF1 and TFF3 are expressed in a cell-specific fashion and are secreted in normal and inflamed airways. Allergen exposure like asthma leads to the trans-differentiation of Clara cells regarding upregulation in TFF1 expression and secretion by airway secretory gland. In another research work, it was demonstrated that TFF2 protein expression was increased and widely distributed in lung tissue from mice model with chronic allergie airway disease (AAD), compared with control mice.

In an high throughput assay on airway wall of patients that induced by sulfur mustard reveal that mucin hyper secretion pathway is active via TFFs. TFFs acts as a ligand and binds to ErbB1/ErbB2 complex. Heterodimerization of ErbB1/ErbB2 stimulate ERK kinase cascade. This ligand-receptor complex regulates down-stream signaling cascades. Also, EGF or other EGFR ligands and trefoil factor family (TFF) peptides support airway remodeling processes synergistically (Figure 1).

Figure 1. A simplified schematic description of the ErbB1/ErbB2 pathway via binding TFFs in airway remodeling of the mustard lung. To initiate signaling, secreted TFFs binds to ErbB1/ErbB2 receptor and acts on adjacent mucosal cell populations via ERK/MAPk cascade activation. (depicted using “Pathway Builder Tool 2.0”)
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Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Sex (M/F)</th>
<th>Age range</th>
<th>Age mean±SD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>10</td>
<td>8/2</td>
<td>39.0–44.0</td>
<td>41.3 ±2.5</td>
<td>0.64</td>
</tr>
<tr>
<td>SM-injured group</td>
<td>15</td>
<td>15/0</td>
<td>36.0–58.0</td>
<td>43.2 ±6.4</td>
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</table>

According to the pathologic expression of TFFs during chronic inflammatory diseases and upon the mucus hyper secretion in patients with asthma and mustard lung disease, we present evidence that secreted TFF1 and mucins contribute to the mucociliary defense system of the airways.

MATERIALS AND METHODS

Samples

Fifteen patients (Table 1) who had suffered from delayed respiratory complications due to the exposure to SM through the 1988–1995 Iran-Iraq war were grouped as the SM-injured. The SM-exposure was confirmed through documented evidence of chemical exposures by the military health services at the time of contact and beginning of respiratory symptoms instantaneously after the exposure without symptom-free periods. On the other hand, ten SM unexposed individuals were enrolled as the control group, who were confirmed as normal by chest X-ray films and HRCT scan as well as regular pulmonary function tests. All participants signed an informed written consent. This study was conducted in accordance with a protocol approved by Baqiyatallah University of Medical Sciences ethics committee (No. 000489). A summary of the two subject characteristics is shown in Table 1.

In this study, patients with other chronic pulmonary diseases (such as asthma), lung cancer, autoimmune diseases (such as rheumatoid arthritis), diabetes mellitus, pneumonia, or acute infective bronchitis were excluded from the examination target. In addition smokers, addicts, elders, organ transplant recipients, or patients with occupational history of toxic fume exposure were also excluded.

Endobronchial Biopsy Sampling

All participants underwent the bronchoscopic examination via a flexible fiberoptic bronchoscope (Olympus BF1T, Tokyo, Japan). Endobronchial biopsy samples were taken by a bronchoscopic forceps (Olympus, Tokyo, Japan) through the bronchoscope. 2% lidocaine was used to anesthetize the upper respiratory tract. Before beginning the procedure, 0.75 mg atropine was given to each case intramuscularly. Supplemental oxygen was given during the procedure, and the oxygen saturation was monitored continuously by the pulse oxymeter. Biopsy samples were obtained from segmental and subsegmental carinae of the right or left lower lobe and they were immediately immersed in Tripure Isolation Reagent (Roche applied science, Germany) at 4˚C and maintained at −80˚C till RNA extraction.

Total RNA Extraction and cDNA Synthesis.

Total RNAs from biopsy samples were isolated by Tripure Isolation Reagent (Roche Applied Science, Germany) according to the manufacturer’s recommendation. The extracted total RNAs were suspended in 20 μl RNase-free water and stored at −80˚C for subsequent procedures. The quantity and quality of purified RNAs were verified by Nanodrop spectrophotometer (ND-1000, Wilmington, DE) and electrophoresis in 1% agarose gel (Cinnagene, Tehran, Iran), respectively. The purified totalRNAs were used as templates for cDNA synthesis. Reverse transcription was carried out by SuperScript III reverse assessment of TFFs Gene Expression.

Semiquantitative PCR was performed using Taq DNA polymerase (Takara, Japan) in a PCR system (mastercycler ep, Ependorf, Germany), by initial denaturation (30 s at 95 °C), annealing (30 s at 55 °C for human GSTs and 59 °C for b-actin), extension (1 min at 72°C) and terminal extension (5 min at 72 °C) in 30 cycles. Primer set for the human TFFs were shown in table 2.

PCR products were separated in 2% agarose gel and dyed with ethidium bromide and then detected under UV light. All results were normalized with b-actin expression to compensate for differences in the amount of cDNA. For quantitative measures and evaluation, Density of PCR results on gel were determined by.
Table 2. Sequence and characteristics of PCR Primers.

<table>
<thead>
<tr>
<th>Product length (bp)</th>
<th>Annealing Tm</th>
<th>Primer sequence (5′ to 3′)</th>
<th>Genes</th>
</tr>
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<tbody>
<tr>
<td>163 bp</td>
<td>60°C</td>
<td>5′CACCATGGAGAACAAGGTGA3′</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′CAGCCCTTTATTGACACT3′</td>
<td></td>
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<td>209 bp</td>
<td>60°C</td>
<td>5′TGGATGCTGTTCGACTCCA3′</td>
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<td></td>
<td></td>
<td>5′TCCACAGACTTCGGAGAAGAA3′</td>
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<td>232 bp</td>
<td>62 C°</td>
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<td>190 bp</td>
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<td>5′TCATGAAGATCCTCACCGAG3′</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′TTGCCAAATGATGAGCCTG3′</td>
<td>R</td>
</tr>
</tbody>
</table>

using special Image Analysis software (Scion Corporation, Frederick, MD).

Statistical Analysis

Results are shown as mean±SD of fold changes of each gene which were analyzed using the SPSS statistical package (version 13.0; SPSS Inc., Chicago, IL, USA) and Mann-Whitney U test was used to compare the differences between groups (p<0.05).

RESULTS

Evaluation of Total RNA Extraction

The quantity and quality of purified RNAs from air way wall biopsy of subjects were verified and electrophoreses in 1% agarose gel respectively that are shown in Table 3 and Figure2.

Table 3. Total mRNA extracted concentration ng/µl

<table>
<thead>
<tr>
<th></th>
<th>SM-injured group</th>
<th>Control group</th>
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<tbody>
<tr>
<td>1</td>
<td>418</td>
<td>359</td>
</tr>
<tr>
<td>2</td>
<td>598</td>
<td>764</td>
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<tr>
<td>3</td>
<td>379</td>
<td>575</td>
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<td>4</td>
<td>356</td>
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<tr>
<td>5</td>
<td>450</td>
<td>458</td>
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<tr>
<td>6</td>
<td>865</td>
<td>685</td>
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<td>14</td>
<td>564</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>435</td>
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</tr>
</tbody>
</table>

Figure 2. Extracted RNA electrophoresis in 1% agarose gel. 1-5 belonged to SM-injured patients and 6-10 belonged to healthy controls.
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<table>
<thead>
<tr>
<th>Subject</th>
<th>M 1 2 3 4 5 6 7 8 9 10</th>
<th>Gene</th>
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<tbody>
<tr>
<td>SM-injured group</td>
<td></td>
<td>TFF1 163bp</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
<td>TFF1 163bp</td>
</tr>
<tr>
<td>SM-injured group</td>
<td></td>
<td>B-actin 190bp</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
<td>B-actin 190bp</td>
</tr>
</tbody>
</table>

Figure 3. The expressions of TFF1 were assessed by semi-quantitative RT-PCR on total RNA extracted from fresh airway biopsy of healthy controls and SM-exposed individuals. Each group had 10 samples.

Overexpression of TFF mRNA

The expressions of TFFs were assessed by semi-quantitative RT-PCR on total RNA extracted from fresh airway biopsy of healthy controls and SM-injured individuals. TFF1 mRNAs was present at expected sizes of 163, and specific primers were used to achieve full length human TFF1 (Figure 3). TFF1 expression changes in SM-exposed patients in comparison with control group reveal in Figure 4. SM-induced individuals and control group showed expression of TFF1 2.97±1.32 and 12.23±2.97 respectively (Figure 5).

According to these findings, we speculated that TFF1 mRNA 4.11 folds overexpression in airway wall biopsy was due to SM induction. The TFF2 and TFF3 gene expression showed no significant changing between 2 groups (Data not shown).

Figure 4. TFF1 expression changes in SM-exposed patients in comparison with control group. Subjects 1-10 are control and 11-25 are SM-injured group
DISCUSSION

The pathophysiological function of TFFs and their expression patterns in normal and experimental tissues are still poorly known and are under elucidation. TFFs have been shown to be involved in the protection and healing of the gastrointestinal and respiratory tract mucosa.\(^1\)

In the present study, for the first time, RT-PCR analysis revealed a markedly different expression pattern for TFF1 in airway wall of chronic SM-injured patients in comparison with non-SM-injured subjects.

This result is completely in line with recent report on very strong induction of TFF1, whereas the TFF2 and TFF3 expression did not change. This result is abundantly in line with recent report that allergen exposure induce the trans-differentiation of Clara cells in the direction of a TFF1-expressing mucous via goblet cells.\(^1\) Identification of the TFF1 in airway wall is in agreement with previous findings of TFF1 peptide in lung tissue by immunohistochemistry and by mRNA determination.\(^1\)\(^,\)\(^1\)

A recent report revealed that the tree type of TFF were markedly detected by ELISA in broncho alveolar lavage fluid of chronic obstructive lung disease (COPD) patients.\(^8\)

Excitingly Viby and colleagues found the TFFs in patients with COPD, and increased levels of TFF1 and TFF2 in patients with pulmonary malignancies. Goblet cell metaplasia and hyperplasia, together with peribronchial inflammation and fibrosis is characteristic in the pathobiology of COPD.\(^1\) TFF1 upregulation was reported in small cell lung carcinomas, bronchial carcinoids, and adenocarcinomas.\(^1\)\(^,\)\(^2\) Higher TFF1 serum levels were found in patients with goblet cell subtype lung adenocarcinoma.\(^2\)

The observation that TFF1 expression is induced in trans-differentiating Clara cells could be due to a binding site for the transcription factor FoxA2/HNF-3β,\(^2\) which plays a major role in development and inflammation.\(^2\) This finding also confirms our previous finding that the SM lead the airway epithelial cell toward inflammation via Smads\(^1\)\(^,\)\(^8\) andNF-κB pathway.\(^1\)\(^,\)\(^2\)

A recent study shows that SM-induced apoptosis in BAL fluid cells via inhibition of Fas-FasL ligand\(^2\) TFF1 was found to protect cells from apoptosis by partially or completely blocking caspase-3, -6, -8 and -9 activities\(^1\) therefore we speculated that the TFF1 has critical role in antiapototic process in remodeling of airway induced by SM.

Our previous study reported that mucins secretion in airway of SM-injured patients was increased\(^1\) and because TFFs are involved in mucus synthesis and secretion,\(^2\) therefore our finding reveals the associations between the TFF1 and mucus hypersecreation in SM- inhaled patients. A research group reported that the TFFs is involved in gastroesophageal reflux disease\(^2\) and also our previous
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finding reveals that SM frequency of GER and esophagitis was higher in the patients with BO due to SM exposure than the control group. Therefore it is concluded that TTFs may play an important role in GERD induced by SM.

The exact stimulus for the goblet cell increase in the expression of the peptide is not yet elucidated, but the epithelial damage could be a mediator, since TFFs are shown to be involved in protection and regeneration in other tissues. In conclusion, TFF1 displays a distinct protein expression pattern in the developing airway remodeling due to SM inhalation and plays an important role in maintaining the airway epithelium function.

REFERENCES

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