Substance P Potentiates TGF-β1 Production in Lung Epithelial Cell Lines

Roya Yaraee and Tooba Ghazanfari

Department of Immunology, Immunoregulation Research Group, Medical Research Center, Medical School, Shahed University of Medical Sciences, Tehran, Iran

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ABSTRACT

Transforming growth factor-beta (TGF-β) is one of the most important cytokines implicated in growth, differentiation, repair and also the pathogenesis of the lung fibrosis by its stimulatory effect on extracellular matrix deposition. Pulmonary epithelial cells are considered as a source of TGF-β in lung. Substance P (SP), as a neuroimmunomodulator has elevated levels in inflamed airways and although it has significant role in the pathogenesis of the lung fibrosis, but its effect on transforming growth factor-beta (TGF-β) production of the lung epithelial cells (and so its regulatory potential) remains unclear.

In this study TGF-β1 levels in supernatants of the normal (BEAS–2B) and cancerous (A549) lung epithelial cell line cultures at the presence of various concentrations of SP were examined and MTT assay was performed to evaluate cells viability.

We have observed that SP (without any other stimulator) significantly augments TGF-β production of both BEAS and A54 cells and this effect is inhibited by NK1-receptor antagonist (CP-96345). We have also observed that the viability of cells did not significantly affect at the presence of SP.

It can be concluded that SP can directly modulate the release of TGF-β from human bronchial epithelial cell line and thereby participates in various lung functions or pathologic conditions.

Key words: Bronchoepithelial cell line; CP-96345; Substance P; Transforming growth factor-beta (TGF-β)

INTRODUCTION

Transforming growth factor-beta (TGF-β) is one of the important cytokines implicated in the growth, differentiation, repair and also pathogenesis of the lung fibrosis by its stimulatory effect on extracellular matrix deposition.1

Corresponding Author: Roya Yaraee, PhD;
Department of Immunology, Medical School, Shahed University, Tehran, Iran. Tel: (+98 21) 8896 4792, Fax: (+98 21) 8896 6310, E-mail: ryaraee@yahoo.com

In vitro studies show that TGF-β activates fibroblast procollagen gene expression and protein synthesis and down-regulates the production of matrixmetalloproteinases that digest matrix in the interstitium and alveolar spaces.1,2 Lung epithelial cells are able to produce TGF-β.3 Sensory airway nerves contain neuropeptides including substance P—an 11 amino-acid peptide belonging to tachykinin family of the neurotransmitters.4 In inflamed airways, immune cells may form an additional source of tachykinins.5,6 It is documented that elevated levels of
tachykinins and NK receptors have been recovered from the airways of patients with asthma and chronic obstructive pulmonary disease, airway inflammation or exposure to inhaled irritants and there is a role for the tachykinin NK receptors in bronchoconstriction, airway hyperresponsiveness and airway inflammation⁴,⁷ and in proliferative events associated with vascular remodeling.⁸,⁹ Although there are few studies indicating that substance P (SP) modulates the production of various inflammatory cytokines by lung epithelial cell line,¹⁰ but whether SP exerts its role in lung events through its effect on TGF-β₁ production of lung epithelial cell lines and whether NK-1R antagonist could neutralize or reduce the effect remains unclear. In this study we have evaluated the effect of SP on TGF-β₁ production of two different lung epithelial cell lines (BEAS as a normal and A549 as a cancerous cell line) at the presence of the various concentrations of substance P.

**MATERIALS AND METHODS**

**Bronchoepithelial Cells**

- **Normal epithelial cell line**- Human bronchial epithelial cells, BEAS-2B purchased from Pasteur Institute (Tehran, Iran), were maintained in bronchial-epithelial growth medium (BEGM) (Cambrex, USA) which was replaced three times weekly. BEGM consisted of a basal medium supplemented with 2 ml of 13 mg/ml bovine pituitary extract and 0.5 mg/ml hydrocortisone, 0.5 µg/ml human recombinant epidermal growth factor, 0.5 µg/ml epinephrine, 10 mg/ml transferrin, 5 mg/ml insulin, 0.1 µg/ml retinoic acid, 6.5 µg/ml triiodothyronine, 50 mg/ml gentamicin, and 50 µg/ml amphotericin-B (per 500 ml). Cells were grown to 80-90% confluence (37 °C, 5% CO₂/95% air). After brief trypsinization of the confluent cell cultures, the cells were harvested, counted and then 10⁵ cells were plated on 24 well microplates, with either SP (Sigma, USA) at various concentrations diluted in media or media alone as control. After 24 hours, the supernatant was removed for cytokine assay and MTT test was performed for remaining cells.

- **Cancerous epithelial cell line**- A549 was purchased from Pasteur Institute (Tehran, Iran) and was maintained in DMEM media (sigma) supplemented with 10% FCS (Sigma).

**Neuropeptide and Antagonist**

The lyophilized neuropeptide SP and NK-1R antagonist CP-96345 (both from Sigma, USA) were dissolved in injectable-grade deionized water (at stock concentrations of 2x10⁻⁴M and 2x10⁻³M respectively) and after aliquotation were stored at -70°C. Required concentrations of SP or CP-96345 were prepared in the media as working solution and were added to cultures. All media and solutions used were endotoxin free.

**TGF-β Determination**

After 24 hours, supernatants of cultured cells were collected and used to measure latent and activated TGF-β concentrations. Total TGF-β₁ protein was measured using a standard commercial ELISA kit (R&D systems, USA) according to manufacturer’s instructions. Briefly, to activate latent TGF-β₁ to the immunoreactive form, 0.1 ml 1 N HCl was added to 0.5 mL cell culture sample, mixed well and incubated 10 min at room temperature. Then the samples were neutralized by adding 0.1 ml 1.2 N NaOH/0.5 M HEPES and mixed. ELISA test was performed according to manufacturer procedure and the results were read at 450 nm with TiterTek plate-reader. Both activated and natural samples were used for TGF-β assay.

**MTT Reduction Assay**

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay has been used for evaluating cells viability. The method is based on the ability of the living cells to reduce MTT tetrazolium salt into MTT formazan by the mitochondrial enzyme succinate-deshydrogenase. MTT (Merck, Germany) was dissolved in PBS (5mg/ml), filtered and stored at -20°C until use. Following the 24h exposure to SP and replacement of the medium, the cells were incubated in 37°C for 4 h with MTT solution (MTT solution was added to each well at one tenth of its volume (0.5 mg/ml final concentration) and after four hours, the supernatants were gently removed and, MTT formazan was extracted in acidic isopropanol (0.04 N HCl). Measurement of the optical density was performed at 492nm with a TitrTek microplate reader and isopropanol (0.04 N HCl) was used as blank reference.

**Statistical Analysis**

All results are representative of at least 2 separate experiments. The Student’s t-test was used to determine if any increase in TGF-β₁ production obtained by treating cultures with SP was significant compared to control cultures. The minimal level of significance chosen was P<0.05.
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**RESULTS**

The Effect of SP on TGF-β Production (secretion) of the Bronchoepithelial Cell lines

TGF-β production by cancerous lung epithelial cell line (A549) has been up-regulated more than 20 times of control group (Figure 1) at the presence of SP \((P<0.05)\). Maximal response was obtained at concentration of \(10^{-7}\)M of SP. There was significant increase at concentrations of \(10^{-6}\)M and \(10^{-8}\)M as well (TGF-β was activated in supernatants before measuring).

As can be observed in Figure 2, TGF-β production by lung epithelial cells (BEAS) is significantly up-regulated (about 70% more than medium alone) at the presence of SP at concentration of \(10^{-7}\)M \((P<0.05)\) and down-regulated (80%) at concentration of \(-10^{-5}\)M. Maximal response was obtained at a concentration of \(10^{-7}\)M of SP. There was no significant difference at other concentrations.

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**TGF-β Production of Normal (BEAS) and Lung Epithelial cell line at the Presence of Substance P**

<table>
<thead>
<tr>
<th>Substance P Concentrations</th>
<th>(10^{-10}) M</th>
<th>(10^{-9}) M</th>
<th>(10^{-8}) M</th>
<th>(10^{-7}) M</th>
<th>(10^{-6}) M</th>
<th>(10^{-5}) M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF (pg/ml)</td>
<td>8.48±0.98</td>
<td>9.98±2.56</td>
<td>14.4±1.11</td>
<td>10.34±2.07</td>
<td>6.66±1.5</td>
<td>1.64±0.54</td>
<td>8.51±0.22</td>
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<tr>
<td>In supernatant of BEAS cells</td>
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**TGF-β Production of Lung Epithelial cell line (A549) at the Presence of Substance P**

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<th>(10^{-7}) M</th>
<th>(10^{-6}) M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF (pg/ml)</td>
<td>22.70±0.98</td>
<td>25.38±2.56</td>
<td>118.5±1.11</td>
<td>184.86±10.86</td>
<td>180.04±14.3</td>
<td>8.43±0.22</td>
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<tr>
<td>In supernatant of A549 cells</td>
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Figure 1. Normal bronchoepithelial cell line (BEAS) were seeded in 24 well microplates at a density of \(10^5\)/well and were exposed to SP i.e. BEGM medium containing different concentrations of neuropeptide substance P \((10^{-10}\text{-}10^{-5}\)M) or medium alone (control) was added to cultures. TGF-β production was assayed in supernatants of cells 24 hours after incubation with SP using ELISA method. The results are shown as mean +/- SEM.

Figure 2. Cancerous bronchoepithelial cell line (A549) were seeded in 24 well microplates at a density of \(10^5\)/well and were exposed to SP i.e. FCS supplemented medium containing different concentrations of neuropeptide substance P \((10^{-10}\text{-}10^{-6}\)M) or medium alone (control) was added to cultures. TGF-β production was assayed in supernatants of cells 24 hours after incubation with SP using ELISA method. The results are shown as mean +/- SEM.
Substance P Potentiates TGF-beta Production

Inhibition of Substance P induced TGF-β Production of Lung Epithelial cell line (A549) at the Presence of NKR-1 antagonist

<table>
<thead>
<tr>
<th>TGF (pg/ml) in supernatant of A549 cells</th>
<th>Control</th>
<th>SP(10⁻⁷ M)</th>
<th>SP(10⁻⁷ M)+CP96,345 (10⁻⁷ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.43±0.22</td>
<td>184.86±10.86</td>
<td>8.44±0.54</td>
</tr>
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</table>

Figure 3. Cancerous bronchoepithelial cell line (A549) were seeded in 24 well microplates at a density of 10⁵/well and were exposed to SP (at final concentration of 10⁻⁷ M) or SP + CP-96,345 (both at final concentration of 10⁻⁷ M) or medium alone (control). TGF-β production was assayed in supernatants of cells 24 hours after incubation with SP using ELISA method. The results are shown as mean +/- SEM.

CP-96,345 Inhibits over Production of TGF by lung Epithelial Cell line

SP has up-regulated TGF production of normal and cancerous lung epithelial cell lines in a concentration dependent fashion (Figures 1 and 2). As shown in Figure 3, CP-96,345, a concentration of 10⁻⁷M inhibited the additional TGF production of A549 lung epithelial cell line (at the presence of 10⁻⁷M SP). The observed inhibition was about 100%.

The Effect of SP on Vital Activity of the lung Epithelial Cell lines

In order to determine whether the presence of SP could affect bronchoepithelial cells and to ensure that the cells are viable, we have also examined viability of the cells using MTT assay. As determined by MTT assay (Figure 4), BEAS and A549 cell lines do not significantly affect by the presence of SP.

<table>
<thead>
<tr>
<th>Viability of the normal (BEAS) and cancerous (A549) Lung Epithelial cell lines (Absorbances at MTT test)</th>
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<tr>
<td>10⁻(10)</td>
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<tr>
<td>---------</td>
</tr>
<tr>
<td>A549</td>
</tr>
<tr>
<td>BEAS</td>
</tr>
</tbody>
</table>

Figure 4. The viability of the normal (BEAS) and cancerous (A549) bronchoepithelial cells was evaluated 24 hours after addition of SP (various final concentrations from 10⁻³M - 10⁻⁶M) using MTT method. The results are demonstrated as percent of activity comparing to control group (each point is the mean of four separate wells).
DISCUSSION

Transforming growth factor-beta (TGF-β) is known to reduce acute inflammatory immune responses and recent information highlights the role of TGF-β in repair responses that lead to matrix deposition and tissue remodeling and fibrotic response. Overproduction of TGF-β can result in excessive deposition of the scar tissue and fibrosis of the lung and in many chronic inflammatory diseases; TGF-β-mediated tissue remodeling can be a serious complication. High serum TGF-β1 levels are correlated with the development of many fibrotic diseases and the success of various treatments in them is in part due to the ability of these drugs and cytokines to reduce serum levels of TGF-β. In bronchoalveolar lavage fluid samples obtained from patients suffering from obliterative bronchiolitis (OB), elevated expression of TGF-β had been found which is capable of interfering with the expression of the secretory leukoprotease inhibitor (SLPI). OB is also a major problem in victims of the chemical war with sulfur mustard. Release of TGF-β by alveolar epithelial cells may result in pulmonary fibrosis and modulation of the production of transforming growth factor-beta may represent a potentially useful therapeutic strategy for idiopathic pulmonary fibrosis.

Substance P participates in the inflammatory and fibrotic processes of interstitial lung diseases; it has higher concentrations in bronchoalveolar lavage (BAL) fluids from patients with idiopathic pulmonary fibrosis (IPF) than that of healthy. Substance P stimulates proinflammatory gene expression in lung epithelial cells through activation of NF-KB. It augments cytokine-induced fibroblast proliferation and has a role in pathophysiology of bone marrow (BM) fibrosis. It is noteworthy that the spectrum of diseases collectively known as pulmonary fibrosis does not have reliable therapeutic options yet.

Regarding the important role of TGF in various lung phenomena, regulators of its production also possess important placement. The results of this study demonstrated that SP induces bronchial epithelial cells to increase TGF-β production. Although SP is able to modulate cytokine production in various conditions, but there are few reports indicating the effect of SP on TGF-β expression. In the study of Hu and colleagues dermal fibroblasts derived from human normal skin were cultured with 25 ng/ml of substance P and TGF-beta 1 mRNA expression in the fibroblasts was expressed more significantly. According to Marriott et al (1998) SP alone (1 nM) induces TGF-β1 mRNA expression in cultured murine macrophages although diminishes TGF-β1 induction at the presence of lipopolysaccharide or interferon γ but it should be noted that additional stimulators should be considered as completely different condition. The present study extends these data and demonstrates that SP can directly stimulate the release of the inflammatory cytokines in human bronchial epithelial cell lines (i.e. BEAS–2B and A549). It should be noted that high TGF-β production of A549 cells comparing to BEAS cells, may be due to high proliferation rate of this cancerous cell line.

We have also demonstrated in this study that the SP antagonist (CP-96,345) completely inhibited increased TGF-β production of epithelial cell lines, indicating that NK-1R could be involved. It has been suggested from experimental animal studies that NK-1R antagonists are powerful drugs for treatment of inflammatory disease in skin, respiratory, and gastrointestinal tract. Here also inhibition of TGF-β production in bronchial epithelial cells may offer approaches to the design of new antifibrotic therapeutics. Further investigation is needed to determine the mechanism(s) whereby CP-96,345–NK-1R interaction regulates TGF-β in bronchial epithelial cells and would provide insights into the regulatory mechanisms of SP and its role in inflammation and lung fibrosis. Studies of the role of SP in animal model of lung fibrosis and/or inflammation and also in vivo effect of its antagonist in these models will be beneficial.

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REFERENCES


