TGF-beta1-induced Epithelial-Mesenchymal Transition in Airway Epithelial Cells is influenced by Th1, Th2 Inflammation and Asthma Medications

Tracee Wee[1,2], Furquan Shaheen[1], Thomas Abraham[1], Tillie-Louise Hackett[1,3]

[1]UBC James Hogg Research Centre, Institute for Heart + Lung Health, St. Paul's Hospital, Vancouver, BC, CANADA

[2]Experimental Medicine Program and [3]Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver, BC, CANADA

JAMES HOGG RESEARCH CENTRE
University of British Columbia
Institute for HEART + LUNG Health

AllerGen
Allergy, Genes and Environment Network

UBC a place of mind

Abstract

Rationale: Airway remodeling is believed to be an important early morphologic change in the pathological development of asthma. This results in the accumulation of myofibroblasts immediately beneath a thickened epithelial basement membrane. Previous work suggests a potential role for airway epithelial cells in airway remodeling via epithelial-mesenchymal transition (EMT). The functional effects of Th1, Th2 inflammatory cytokines, and asthma medications on this phenomenon have not been studied.

Methods: To observe mesenchymal function in airway and alveolar epithelial cells, the 16HBEo- and A549 cell lines were grown in monolayer culture or seeded in polymerized type I collagen gels and treated with TGFβ1 (10 ng/ml) with or without Fluticasone (10 μM) or Salbutamol (10 μM), in the presence or absence of TNF-α (10 ng/ml) and IL-1β (10 ng/ml), or IL-13 (20 ng/ml) for 48 h. Cells were then lysed for immunoblot analysis. For collagen gel contraction assays, cells were immunostained and imaged by second harmonic generation microscopy to observe fibrillar collagen formation. To study the effect of matrix environment on the EMT process, 16HBEo- cells were treated with TGFβ1 (10 ng/ml) for 24 h on plates coated with BSA (0.4 mg/ml), collagen 1 (0.4 mg/ml) or fibronectin (0.4 mg/ml). RNA was collected and processed for real-time PCR analysis of fibronectin and collagen 1α1 gene expression.

Results: TGFβ1 treatment induced an EMT phenotype in 16HBEo- and A549 cells which were able to contract collagen 1 gels and remodel fibrillar collagen by multiphoton microscopy. The addition of IL-13 exacerbated the observed collagen 1 gel contraction in 16HBEo- cells but not in A549 cells. TGFβ1 reduced E-cadherin and induced fibronectin-EDA and plasma fibronectin protein production in 16HBEo- and A549 cells in the presence or absence of Th1 or Th2 inflammation. Fluticasone inhibited TGFβ1 induced down-regulation of E-cadherin expression but enhanced TGFβ1 stimulated plasma fibronectin expression regardless of Th1 or Th2 inflammation in 16HBEo- cells. However, Fluticasone in A549 cells enhanced TGFβ1 mediated plasma fibronectin and collagen 1α1 production in the absence of inflammation. In contrast, Salbutamol enhanced TGFβ1 induced plasma fibronectin production in both airway and alveolar epithelial cells. A matrix environment of Collagen 1, but not fibronectin and BSA, augmented the mRNA expression of fibronectin and collagen 1α1 induced by TGFβ1.

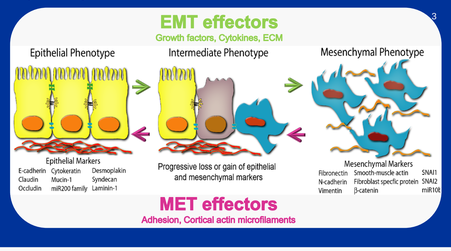
Conclusion: Our data show that TGFβ1 induces functional changes in airway and alveolar epithelial cells that confers mesenchymal cell characteristics, including the production of collagen 1 and the ability to contract collagen gels. Moreover, the surrounding tissue environment, conferred by the lung matrix, inflammation and inhaled medications can also influence the outcome of TGFβ1- induced epithelial plasticity.

Introduction

Asthma is the most common chronic condition in children worldwide[1]. However, despite advances in our understanding of the disease pathogenesis and progression, there is still no cure for the disease.

In addition to airway inflammation, airway remodeling has also been shown to play a critical role in the chronic reversible airflow obstruction observed in asthmatic individuals, and is characterized by impaired epithelial repair, sub-epithelial fibrosis, matrix deposition and mucus cell metaplasia.

Previous work in our group has shown that TGFβ1 can promote EMT in basal airway epithelial cells from both normal and asthmatic individuals[2]. However, the ability of lung epithelial cells to function as fibroblasts (i.e. collagen contraction and production) has not been fully investigated.



Moreover, the effect of airway inflammation derived from either Th1 or Th2 acute exacerbations and standard asthma medications (i.e. steroids and short-acting beta-agonists) on epithelial plasticity are poorly understood.

Hypothesis

We hypothesize that inflammatory cytokines and asthma medications will alter the EMT phenotype induced in airway epithelial cells by TGFβ1.

Specific Aim

The aim of this study was to investigate the extent of TGFβ1-induced EMT on epithelial cells using TGFβ1-treated alveolar and bronchial epithelial cell lines and measuring aspects of fibroblast function. Additionally, as acute exacerbations of asthma are associated with inflammation requiring the use of reliever medications, we will evaluate the effect of Th1 and Th2 inflammatory cytokines, Fluticasone and Salbutamol on TGFβ1-induced EMT.

Methods

Cell Culture

The A549 human adenocarcinoma alveolar epithelial cell line and the 16HBEo-cell line from normal human airway epithelial cells transformed with Simian virus 40 (SV_{40}) provided by Dr Gurtner were used for all cell culture experiments. Both cell lines were grown in monolayer in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in humidified air with 5% CO_2.

Cytokine, Fluticasone and Salbutamol Treatments

A549 and 16HBEo- cells grown to 70% confluence in 6-well tissue culture plates in 1 ml growth media were treated with or without recombinant human (rh) TGFβ1 (10 ng/ml). Additionally, cells were treated with or without the cytokines TNFα (10 ng/ml) and IL-1β (10 ng/ml) or IL-13 (20 ng/ml), and the drugs Fluticasone (10 μM) or Salbutamol (10 μM). Cells were incubated with the corresponding treatments for 48 h at 37°C with humidified air in 5% CO_2.

Collagen Gel Contraction Assay

12-well tissue culture plates were incubated with 1% bovine serum albumin (BSA) in DMEM for 2 h. The media was then replaced with 500 μl of 0.4 mg/ml type I collagen and allowed to polymerize at 37°C for 8 h. The A549 and 16HBEo- cells were trypsinized and seeded at 100,000 cells/ml on the collagen gels with the appropriate cytokines in 1% FBS DMEM. The cells were then cultured for 48 h at 37°C in 5% CO_2. The collagen gels were imaged before and after incubation with the cells to determine the extent of collagen gel contraction quantified using Image-Pro Software (Media Cybernetics, Warrendale, PA).

Multi-Photon Excitation Fluorescence and Second Harmonic Generation Imaging of Fibrillar Collagen

The collagen gels were fixed in 4% paraformaldehyde for 20 minutes, washed in phosphate buffered saline (PBS) with 0.1% saponin and 0.1% Tween 20, then incubated at room temperature with phalloidin conjugated Alexa Fluor 594 for 1 h. After staining, the gels were mounted onto glass slides with spacer plates and sealed with Cytoseal. The images obtained from multi-photon microscopy of the gels were processed on Volocity software (PerkinElmer Inc.).

Matrix Protein Treatments

24-well plates were coated with BSA (0.4 mg/ml), collagen 1α1 (0.4 mg/ml or fibronectin (0.4 mg/ml). 16HBEo- cells were seeded in the plates at 20,000 cells per well, and allowed to adhere for 8 h at 37°C in 5% CO_2 humidified air. The cells were then treated with 10 ng/ml rh TGFβ1 for 24 h at 37°C in 5% CO_2 before RNA was collected.

RNA Isolation and Quantitative Polymerase Chain Reaction

RNA was isolated from treated monolayer cell cultures using RNeasy Plus Mini-Kits. Purified RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription reagents, and gene expression was assessed by real-time polymerase chain reaction (PCR) using TaqMan Universal PCR Master Mix and pre-designed TaqMan Gene Expression Assays performed according to the manufacturer's protocol. Gene expression was calculated using Ct values for the genes of interest and the housekeeping gene GAPDH.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblot

A549 and 16HBEo- cells were lysed in protein extraction buffer with protease and phosphatase inhibitor cocktails. Protein samples were loaded at equal concentrations in a sodium dodecyl sulfate-polyacrylamide gel for electrophoretic resolution of proteins of interest. Proteins in the gels were transferred to 0.45 μm nitrocellulose membranes, and probed with a cocktail of primary antibodies. E-cadherin and fibronectin-EDA protein expression in cell lysates was assessed using the rabbit polyclonal antibody against E-cadherin and the mouse monoclonal antibody against fibronectin-EDA, respectively, under denaturing conditions. Collagen 1α1 and plasma fibronectin expression in cell supernatants was determined using the rabbit polyclonal antibody against collagen 1 and the mouse monoclonal antibody against plasma fibronectin, respectively, in non-denaturing conditions. Alexa Fluor 680 goat anti-rabbit polyclonal antibody and the IRDye 800 conjugated goat anti-mouse polyclonal antibody were used to detect the corresponding primary antibodies. The protein bands were imaged with the Odyssey Infrared Imaging System according to the manufacturer's instructions, and protein band intensities were analyzed with the Odyssey software 1.1 (LI-COR Biotechnology). The results are expressed as protein of interest/β-tubulin ratios or the protein ratios normalized to the corresponding controls.

Statistics

Data are presented as mean ± SEM of at least 3 independent experiments. Paired comparisons were analyzed with the Student paired *t* test and one-way analysis of variance with Tukey post test was used for comparison of group data. All statistical analysis was done using Prism Version 5.04 (GraphPad Software Inc., San Diego, CA), with *P* values less than 0.05 considered statistically significant.

Results

Figure 1. Th1 and Th2 inflammatory cytokines modulate TGFβ1-induced EMT

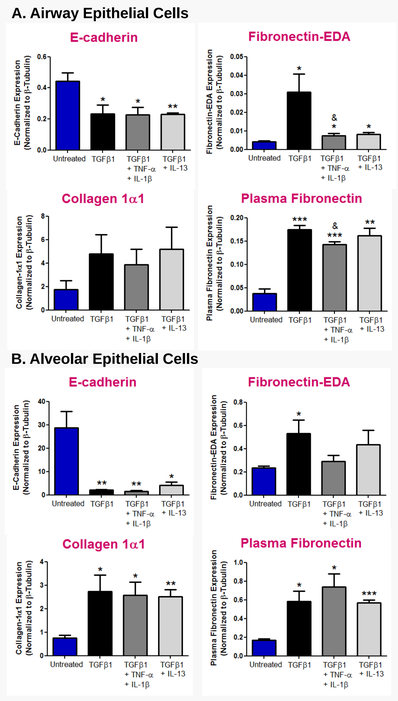


Figure 1. Th1 and Th2 inflammatory cytokines modulate TGFβ1-induced EMT. As models of airway and alveolar epithelial cells, 16HBEo- (A) and A549 (B) cells were used to examine the influence of inflammatory cytokines on the EMT phenotype induced by TGFβ1, respectively. Cells were treated with (black bars) or without (blue bars) TGFβ1, in the presence of TNF-α + IL-1β (dark gray bars) or IL-13 (light gray bars) for 48 h. Cell lysates were collected and used to assess protein expression of E-cadherin and fibronectin-EDA, and supernatants were collected to determine the secreted levels of collagen 1α1 and plasma fibronectin by immunoblot. All densitometry values were normalized to β-tubulin and presented as mean ± SEM for n = 4. * signifies p<0.05, ** signifies p<0.01 and *** signifies p<0.001 compared to the untreated control. & signifies p<0.05 compared to the TGFβ1-treated cells.

Figure 2. IL-13 enhances TGFβ1- induced collagen gel contraction by airway epithelial cells

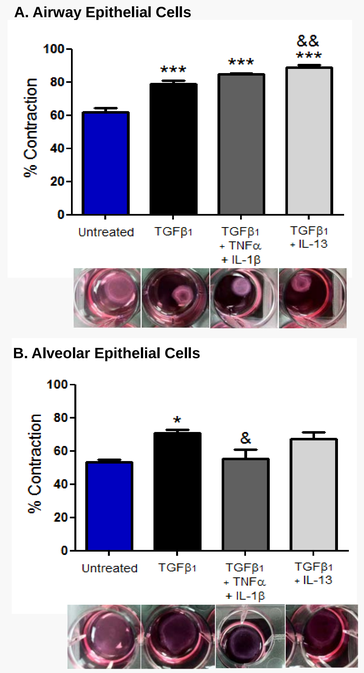


Figure 2. IL-13 enhances TGFβ1-induced collagen gel contraction by airway epithelial cells. To evaluate the ability of airway and alveolar epithelial cells to contract collagen during EMT in the presence of various inflammatory cytokines, 16HBEo- and A549 cells were seeded and incubated in polymerized type I collagen with the appropriate cytokines for 48 h. The collagen gels were imaged before and after incubation with the cells to determine the extent of collagen gel contraction quantified using Image-Pro Software. The amount of collagen contraction was measured as the percent surface area the gel had contracted within the incubation period, and presented as mean ± SEM for all treatments: untreated (blue bars), TGFβ1 (black bars), TGFβ1 + TNF-α + IL-1β (dark gray bars) and TGFβ1 + IL-13 (light gray bars) . 16HBEo- (A) and A549 (B) gel contraction data are supplemented with representative images of gels from n = 4 experiments after the 48 h incubation period. * indicates p<0.05, while *** indicates p<0.001 compared to the untreated control gels. & indicates p<0.05 and && indicates p<0.01 compared to the TGFβ1-treated gels.

Figure 3. TGFβ1-induced airway epithelial cell collagen remodeling is not enhanced by the presence of Th1 or Th2 inflammation

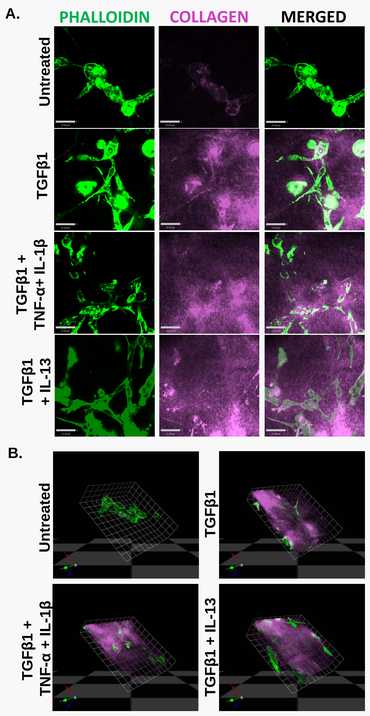


Figure 3. TGFβ1-induced epithelial cell collagen remodeling is not enhanced by the presence of Th1 or Th2 inflammation. To determine the ability of airway epithelial cells during EMT to remodel collagen in the presence of cytokines, 16HBEo- cells cultured for 48 h in polymerized type I collagen with TGFβ1 and various cytokines were fixed and stained with phalloidin conjugated Alexa Fluor 594. The gels were imaged using multi-photon microscopy with second harmonic generation to visualize the amount of fibrillar collagen remodeled by the treated cells. Representative *en face* images (A) of untreated, TGFβ1-treated, TGFβ1 + TNF-α + IL-1β-treated and TGFβ1 + IL-13-treated collagen gels with their corresponding 3D-images (B) were taken. Cells are stained green for phalloidin and fibrillar collagen is coloured magenta.

Figure 4. Fluticasone and Salbutamol can enhance TGFβ1-induced EMT

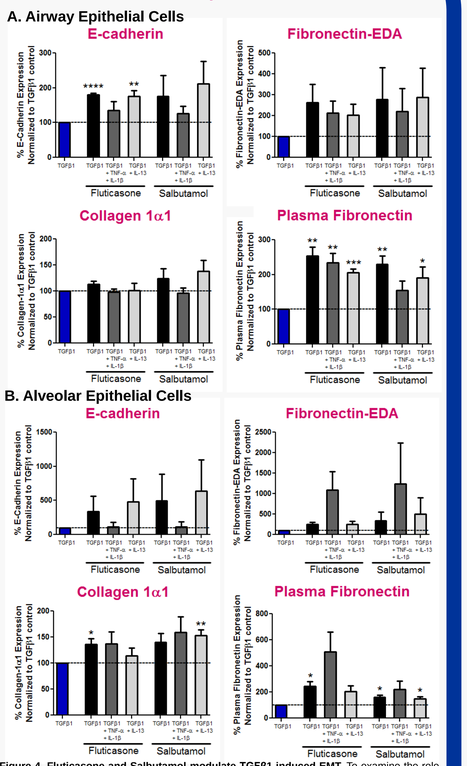


Figure 4. Fluticasone and Salbutamol modulate TGFβ1-induced EMT. To examine the role of steroids and beta-agonists on the TGFβ1-induced EMT phenotype, 16HBEo- and A549 cells were treated with (black bars) or without (blue bars) TGFβ1 10 ng/ml and the drugs Fluticasone or Salbutamol, in the presence of TNF-α + IL-1β (dark gray bars) or IL-13 (light gray bars). After the 48 h treatment incubation, cell lysates were collected for examining protein expression of E-cadherin and fibronectin-EDA, and supernatants were collected for determining the secreted levels of collagen 1α1 and plasma fibronectin by immunoblotting. All densitometry values were normalized to β-tubulin and further normalized to the TGFβ1-treated control, presented as mean ± SEM for n = 3 experiments. * signifies p<0.05, ** signifies p<0.01 and **** signifies p<0.0001 compared to the TGFβ1-treated control.

Figure 5. The presence of collagen 1 enhances TGFβ1-induced expression of ECM proteins in airway epithelial cells

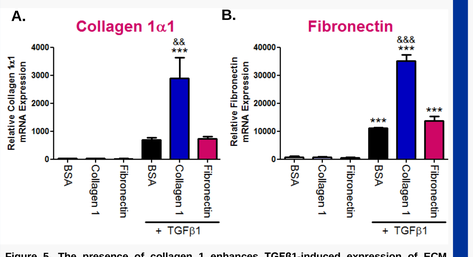


Figure 5. The presence of collagen 1 enhances TGFβ1-induced expression of ECM proteins in airway epithelial cells. 16HBEo- monolayer cultures seeded in various matrix environments were treated with or without TGFβ1 for 24 h and mRNA was harvested and purified for cDNA synthesis. RT-PCR analysis of collagen 1α1 (A) and fibronectin (B) mRNA expression are shown normalized to GAPDH. Gene expression data is presented as mean ± SEM for n = 3 experiments. *** indicates p<0.001 compared to all treatments without TGFβ1. && indicates p<0.01 and &&& indicates p<0.001 compared to TGFβ1-treated cells.

Summary

- Our data indicate that Th1 and Th2 inflammation can influence the contractile function of both airway and alveolar epithelial cells that have undergone EMT *in vitro*, but do not significantly impact the expression of cell markers for EMT (E-cadherin, fibronectin-EDA) or mesenchymal matrix proteins (collagen 1, fibronectin).
- In airway epithelial cells, we found that Fluticasone seems to reverse the TGFβ1-induced downregulation of E-cadherin except in the presence of Th1 cytokines, but also increases plasma fibronectin production. In addition, Salbutamol enhanced TGFβ1-mediated plasma fibronectin production but only in the absence of Th1 inflammation.
- We show that in alveolar epithelial cells, Fluticasone is able to enhance collagen 1α1 and plasma fibronectin production in the absence of inflammation. Salbutamol was able to enhance TGFβ1-induced plasma fibronectin and collagen 1α1 expression in the presence of Th2 inflammation.
- The matrix environment, particularly collagen 1, is also able to enhance mRNA expression of collagen 1α1 and fibronectin in the presence of TGFβ1, suggesting its ability to promote the EMT process.

References

1. **World Health Organization. Asthma Fact sheet No. 307, 2011. Retrieved from http://www.who.int/mediacentre/factsheets/fs307/en/index.html**
2. **Hackett TL, Warner SM, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, et al. Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-β1. Am J Respir Crit Care Med. 2009 Apr;180(2):122-133.**
3. **Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. Curr Opin Allergy Clin Immunol. 2012 Feb;12(1):53-59.**