UNIVERSITI PUTRA MALAYSIA

THE REGULATION OF TUMOUR NECROSIS FACTOR-RELATED APOPTOSIS INDUCING LIGAND (TRAIL) EXPRESSION IN TGFB-INDUCED BRONCHIAL EPITHELIAL MESENCHYMAL TRANSITION (EMT)

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A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE (BIOMEDICAL SCIENCES) WITH HONOURS

DEPARTMENT OF BIOMEDICAL SCIENCES
FACULTY OF MEDICINE AND HEALTH SCIENCES
UNIVERSITI PUTRA MALAYSIA

2021

ABSTRACT

The Regulation of TNF-Related Apoptosis Inducing Ligand (TRAIL) Expression in TGFB-Induced Bronchial Epithelial Mesenchymal Transition (EMT)

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Introduction: Asthma is an airway disease characterized by reversible airflow limitation and airway hyperresponsiveness, meanwhile chronic obstructive pulmonary disease (COPD) is characterized by its persistent airflow obstruction. Besides airway inflammation, structural changes in the airways, known as airway remodelling, also contributes to these asthmatic and COPD features. One of the prominent structural changes in airway remodelling is the alteration that occurs in the airway epithelium. It has been proposed that a biological process known as epithelial-mesenchymal transition (EMT) can contribute to airway remodelling. Increasing evidence suggests an association between airway remodelling and a molecule called tumor necrosis factor–related apoptosis inducing ligand (TRAIL). Although high levels of TRAIL have been reported in bronchial biopsy and bronchoalveolarlavage fluid from asthmatic patients, the biological role of TRAIL in the airway epithelium and its association with EMT still needs further understanding. **Objective:** This study aims to determine the association between EMT induction and TRAIL protein expression in human bronchial epithelial cells following acute and chronic treatment of TGFβ1. **Methodology:** Normal human bronchial epithelial cells (BEAS2B) cells were treated with TGFβ1 for 48 hours to induce acute induction of EMT, meanwhile for chronic induction of EMT, the BEAS2B cells were treated for 2 days, 4 days and 6 days. Western Blot was run to determine the expression of TRAIL and EMT markers (E-cadherin, N-cadherin and Vimentin) following acute and chronic induction of EMT by TGFβ1. **Results:** E-cadherin expression reduced while N-cadherin and Vimentin expression as well as TRAIL expression increased in human bronchial epithelial cells following acute and chronic induction of EMT by TGFβ1. **Discussion:** EMT induction by TGFβ1 in bronchial epithelial cells is successful and consistent with previous studies in which TRAIL is upregulated in human bronchial epithelial cells, that suggests epithelial cells is a source of TRAIL that contributes to the elevated TRAIL levels that has been reported in asthmatic and COPD patients. **Conclusion:** TRAIL expression has a positive correlation with TGFβ1-induced bronchial epithelial EMT.

Keywords: Airway diseases, airway remodelling, Epithelial-Mesenchymal Transition (EMT), TRAIL

ABSTRAK

Regulasi ekpresi TRAIL pada peralihan epitelium-mesenkima yang di induksi oleh TGFβ1 di dalam Sel-Sel Epitelium Bronkus Manusia

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Pengenalan: Asma ialah penyakit saluran pernafasan yang dicirikan oleh had aliran udara boleh balik dan hiperresponsif saluran pernafasan, manakala penyakit paru-paru obstruktif kronik (COPD) dicirikan oleh halangan aliran udara yang berterusan. Selain keradangan saluran pernafasan, perubahan struktur dalam saluran pernafasan, yang dikenali sebagai pembentukan semula saluran pernafasan, juga menyumbang kepada ciri asma dan COPD ini. Salah satu perubahan struktur yang menonjol dalam pembentukan semula saluran pernafasan ialah perubahan yang berlaku pada epitelium saluran udara. Telah dicadangkan bahawa proses biologi yang dikenali sebagai peralihan epithelial-mesenchymal (EMT) boleh menyumbang kepada pembentukan semula saluran pernafasan. Bukti yang semakin meningkat menunjukkan perkaitan antara pembentukan semula saluran pernafasan dan molekul yang dipanggil ligan apoptosis berkaitan faktor nekrosis tumor (TRAIL). Walaupun tahap TRAIL yang tinggi telah dilaporkan dalam biopsi bronkial dan cecair bronchoalveolarlavage daripada pesakit asma, peranan biologi TRAIL dalam epitelium saluran pernafasan dan kaitannya dengan EMT masih memerlukan pemahaman lanjut. Objektif: Kajian ini bertujuan untuk menentukan perkaitan antara induksi EMT dan ekspresi protein TRAIL dalam sel epitelium bronkial manusia berikutan rawatan akut dan kronik TGFβ1. **Metodologi**: Sel-sel epitelium bronkial manusia normal (BEAS2B) dirawat dengan TGFβ1 selama 48 jam untuk mendorong induksi akut EMT, manakala untuk induksi kronik EMT, sel BEAS2B dirawat selama 2 hari, 4 hari dan 6 hari. Western Blot dijalankan untuk menentukan ekspresi penanda TRAIL dan EMT (E-cadherin, N-cadherin dan Vimentin) berikutan induksi akut dan kronik EMT oleh TGFβ1. **Keputusan**: Ekspresi E-cadherin berkurangan manakala ekspresi N-cadherin dan Vimentin serta ekspresi TRAIL meningkat dalam sel epitelium bronkial manusia berikutan induksi akut dan kronik EMT oleh TGFβ1. **Perbincangan**: Induksi EMT oleh TGFβ1 dalam sel epitelium bronkial berjaya dan konsisten dengan kajian terdahulu di mana TRAIL dikawal selia dalam sel epitelium bronkial manusia, yang menunjukkan sel epitelium ialah sumber TRAIL yang menyumbang kepada paras TRAIL yang tinggi yang telah dilaporkan dalam pesakit asma. dan pesakit COPD. **Kesimpulan**: Ekspresi TRAIL mempunyai korelasi positif dengan EMT epitelium bronkial yang disebabkan oleh TGFβ-1.

Kata kunci: Penyakit saluran pernafasan, pembentukan semula saluran pernafasan, Peralihan epitelium-mesekima, TRAIL

Acknowledgement

I would like to thank my esteemed supervisor, Dr. Hanis Hazeera Binti Harith for her invaluable supervision, support, patience and tutelage during the course of my final year project. My gratitude extends to my supervisor's post-graduate student, Raja Zarith Fatiah Binti Raja Malik Rizuan for her forbearing guidance and mentorship throughout the journey of my final year project. Her passion will always be one of my motivation to continue believing in myself. Additionally, I would like to thank my FYP teammate, Mizan Adilah Binti Azhar for a cherished time spent together in the lab and for her utmost patience in helping me with this project. My appreciation also goes out to my family and close friends for their encouragement and support all through my studies, for their endless motivation for me to finish this degree. Without them, I would not think I could finally be able to say that I finally made it until the end.

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LIST OF ABBREVATION

ANOVA	One-way Analysis of Variance
ASM	Airway smooth muscle
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
COPD	Chronic obstructive pulmonary isorder
CS	Cigarette smoke
CTGF	Connective tissue growth factor
DMSO	Dimethyl sulfoxide
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
HGF	Hepatocyte growth factor
IL-1	Interleukin-1
IGF	Insulin-like growth factor
PBS	Phosphate buffered saline
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay
SAD	Small airway diseases

TBST	Tris Buffered Saline with Tween 20
TGFβ-1	Transforming growth factor beta 1
Th2	T-helper 2
TNF-α	Tumour necrosis factor alpha
TRAIL	Tumour necrosis factor related apoptosis inducing ligand

CHAPTER 1

INTRODUCTION

1.1 Background

Asthma and chronic obstructive pulmonary disease (COPD) are among chronic airway diseases that affect millions of children and adults. Asthma is mainly characterized by airway hyperresponsiveness and reversible airflow limitation. Meanwhile, COPD is characterized by persistent airflow obstruction, with an increase of inflammatory response in both lungs and airways. Both of these conditions are contributed mostly by airway inflammation. Other than airway inflammation, many studies found that a pathological feature called airway remodelling to worsen asthmatic and COPD symptoms. Therefore, airway remodelling may be considered a potential target to reverse the symptoms of asthma and COPD.

Epithelial mesenchymal transition (EMT) is one of the underlying mechanisms that contribute to airway remodelling in both asthma and COPD. EMT is a biological process where polarized epithelial cells undergo biochemical changes and gain mesenchymal phenotype (Kalluri & Weinberg, 2009). The hallmark trait of EMT is the decomposition of cell-cell junction (Hackett, 2012). This indicates a progressive loss of epithelial marker such as E-cadherin and gain of mesenchymal markers that include proteins N-cadherin and Vimentin.

TRAIL is a cytokine that is expressed in most cell types. Several studies showed that TRAIL expression is increased, as demonstrated by immunostaining of the epithelium of endobronchial biopsies collected from asthmatic airways (Robertson, et al., 2002). TRAIL is suggested to play a role in the structural changes of the airway. However, the role of TRAIL in airway remodelling is still unclear and it is presently unknown whether TRAIL could directly contribute to airway remodelling through EMT.

Therefore, this study aims to give an insight into the regulation of TRAIL in bronchial EMT using an established cellular model by assessing the correlation between TRAIL expression and EMT induction following acute and chornic treatment with TGFβ-1. This study also aims to discover TRAIL's potential as a molecular target to inhibit airway remodelling.

1.2 Objectives

1.2.1 General Objective

- To determine the association between TGFβ1-induced epithelial mesenchymal transition (EMT) with TRAIL expression in human bronchial epithelial cells.

1.2.2 Specific Objectives

- To determine the association between EMT induction and TRAIL protein expression in human bronchial epithelial cells following acute treatment with TGFβ-1
- To determine the association between EMT induction and TRAIL protein expression in human bronchial epithelial cells following chronic treatment with TGFβ-1

1.3 Hypothesis

TGFβ-1 can upregulate TRAIL expression in the human bronchial epithelial cell and this regulation is correlated with the expression of EMT markers such as upregulation of epithelial markers and downregulation of mesenchymal markers.

CHAPTER 2

LITERATURE REVIEW

2.1 Airway diseases : An Overview

Airway diseases include asthma and chronic obstructive pulmonary disorder (COPD). The characteristics or features of airway disease include variable airflow limitation, airway hyperresponsiveness, chronic airflow limitation as well as airway inflammation. Airway inflammation is the main component that contributes to airway diseases (Hargreave & Parameswaran, 2006). Airway diseases prevalence is reported to has increase recent decades in spite of many therapeutic approach has been discovered.

Other than symptoms such as dyspnea, coughing, wheezing and expectoration, airway diseases also can be presented by different underlying pathophysiological processes. It is important to understand and distinguish the difference to ensure appropriate treatment is administered (Athanazio, 2012). Athanazio, 2012 also stated that clinical history and diagnostic testing can ensure that the diseases are distinguished correctly and effieciently. However, there are many misdiagnosed cases in patients with respistaory symptoms due to atypical case presentation and inadequate etiological investigation. Asthma severity is the magnitude of variable airflow limitation, meanwhile COPD severity is the degree of chronic airflow limitation. (Hargreave & Parameswaran, 2006).

2.1.1 Asthma

Asthma is one of serious non-communicable diseases that affects children and adults. In 2019, asthma has been reported to affect an estimated of 262 million people and caused deaths with a total of 461000 (World Health Organization, 2021). In future, asthma is also predicted to affect around 300 million of people globally and its prevalence is expected to rise (Krings, *et al*., 2021).

Asthma is considered a major world health problem. Asthma has affected people from various ages, from adults to children and has become fatal and will have severe effects if it is uncontrolled. Asthma prevalence is increasing in most countries most importantly in children (Bateman, *et al*., 2007).

Asthma is an airway disease characterized by persistent airway obstruction and airway inflammation. Common symptoms of asthma include tightness of chest, wheezing, and cough. The airway wall of asthma patients constantly undergo structural and inflammatory changes that causes thickening of the bronchial, increase in mucus production and also bronchoconstriction that build up an episodic airflow obstruction (Doeing & Solway, 2013). To further help diagnose asthma, the episodic symptoms after being exposed to incidental allergen, or a confirmed family history of asthma are crucial elements to be examined (Bateman, *et al*., 2007).

Asthma is a chronic inflammatory condition, mainly identified by airway infiltration by activated mast cells and eosinophils, managed by Th2-type T lymphocytes (Lordan & Holgate, 2002).

2.1.2 Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is characterized by its persistent airflow obstruction, with an increase in inflammatory response in both lungs and airways. COPD is one of the leading cause of morbidity and mortality of patients with lung diseases (Yan, *et al*., 2018).

The utmost cause of COPD is cigarette smoking, other crucial environmental factors that cause COPD is exposure to pollution such as biomass cooking, heating and exhaust gas (Gan, *et al*., 2013). Airflow limitation in COPD is progressive and it is not fully reversible (Woodruff, *et al*., 2016).

These features in COPD are mainly caused by airway remodelling and loss of small airways (Chen, *et al*., 2014). Pathological features that include lung dysfunction mainly caused by small airway diseases (SAD), in which airway or bronchial remodelling is a part of it (Lange, *et al*., 2021).

Musocal hyperproduction, epithelial hyperplasia and metaplasis, increase of basement membrane and increased mass of airway smooth muscle (ASM) are among features of airway remodelling in COPD (Yan, *et al.*, 2018).

Another indication for COPD is emphysema, a condition where alveolar compartmentalization is destructed. It is understood to have relation with cigarette smoke (CS) toxicity, extracellular matrix remodelling and protease–antiprotease disequilibrium (Lange, *et al*., 2021).

Individuals with different COPD phenotypes are most likely to be different due to the natural history of their COPD, the initiation, early stages and the disease development (Han, *et al*., 2018).

2.2 Airway remodelling

Airway remodelling can be defined as changes in the structure and organization of airway walls. Airway remodelling process can regulate the lung function and become harmful as it can lead to atypical structure and function of the airway (Dang, *et al*., 2020). It has been recognized that in asthmatic airways, there have been several pathological changes, for example, airway smooth muscle hyperplasia, sub-epithelial fibrosis and thickening of airway epithelium (Munakata, 2006).

According to Munakata 2006, airway remodelling is one of the crucial factors that contributes to the severity of asthma. The pathological changes are also seen in mild and moderate asthmatics, not only in severe asthmatic. Autopsied lungs of asthmatic patients found that thickness of the airway smooth muscle was tripled than in normal controls (Amishima, *et al*., 1998). A SARP3 study by Krings et al showed that there is an association between rate of subsequent exacerbations and decline in lung function with having lesser pointwise regional change of the volumes in lung in severe asthmatic patients (Winkler & Frey, 2021).

2.2.1 Common features of airway remodeling

2.2.1.1 Epithelial alterations

Airway epithelium cells play an important role in making sure that airway function normally. The airway epithelium is the prime site for molecular and histologic changes in airway disease such as COPD. Studies also showed that airway epithelium contributes to initiation and development of COPD (De Rose, *et al*., 2018).

The airway epithelium functions as a physical barrier to protect airway mucosa and bloodstream from being invaded by potential pathogens or noxious agents (Ganesan, *et al*., 2013). The airway epithelium also functions in regulating ion transportation to help maintain airway hydration and for innate immune responses. The competency of pithelial regeneration after an injury is important in tissue homeostasis and to prevent diseases. Airway epithelial dysfunctional caused by recurrent inflammatory stimuli will affect the epithelial regeneration and the ability to restore its barrier functions which leads to abnormal remodelling and structural impairment of the airway (De Rose, *et al*., 2018). Studies showed that in asthmatic airways, an antiapoptotic or apoptotic effect can be produced by TGF-β in airway epithelial cells (Halwani, *et al*., 2011)

2.2.1.2 Increased airway smooth muscle (ASM) mass

Airway smooth muscle (ASM) is a crucial element in airways. ASM has been claimed to have major role in preserving bronchial tone and lung ventilation (Mitzner, 2004) Increase in ASM mass and volume are majorly caused by ASM hyperplasia and hypertrophy (Benayoun, *et al*., 2003). Growth factor such as transforming growth factor (TGF-1), platelet-derived growth factor are among stimuli that generate hyperplasia in cultured ASM cell (Doeing & Solway, 2013).

The addition of new ASM cells might result from increased proliferation, pre-existing ASM or transformation and differentiation of non-ASM cells into ASM cells. Potential ASM progenitor might include multipotent stem cells, the ones located in airway tissues or obtained from peripheral blood. Airway epithelial cells can also transform into a mesenchymal cell phenotype through epithelial mesenchymal transition (EMT) (Berair *et.al.*, 2013).

2.2.1.3 Sub-epithelial fibrosis

Extracellular matrix (ECM) has proteins that help to maintain the airways structure and functions. ECM components that produce ASM and matrix-modifying enzymes can render airway remodelling. Studies showed that deposition of ECM molecules including structural proteins such as collagens I, III, and V along with adhesion proteins such as fibronectin and tenascin are increased in asthmatic airways. Fibroblasts and myofibroblasts are the major source of ECM (Hough, *et al.*, 2020).

Subepithelial fibrosis is a characteristic resulted from remodeling response. The thickened lamina reticularis contains an elevated amount of collagens I, III, and fibronectin. These proteins are speculated to be produced by myofibroblasts (Schmidt, *et al.*, 2003). ECM adhesion protein like fibronectin provides binding sites for cell adhesion receptors. This indicates that ECM proteins gives structural and mechanical support for lung tissue for cell adhesion, migration, activation, and proliferation. However, abnormal accumulation of ECM will alter tissue structure and function and thus contributes to airway remodeling (Hough, *et al.*, 2020).

The role of TGFβ in subepithelial fibrosis is to enhance the differentiation from fibroblasts to myofibroblast cells, and also activate their proliferation activities. Additionally, TGF-b also induces the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases in which both of them counterbalance for ECM replacement. Other than that, TGF-β regulate subepithelial fibrosis through Smad7 pathway signalling.

2.2.2 Epithelial mesenchymal transition (EMT) in airway diseases

Epithelial mesenchymal transition or EMT is a biological process where transition of epithelial cell that are polarized and enclosed within stratified epithelium with cell to cell contact with the basal membrane into a fibroblastoid cell occurs. This transition is aid by molecular reprogramming (Hackett, 2012).

EMT is a source for accumulation of migrating mesenchymal cells, for example fibroblasts and myofibroblasts in which they both stimulate subepithelial fibrosis and deposition of extracellular matrix (ECM) (Lee Yu Zhao, *et al*., 2017).

EMT can be categorized into three types. All types have different function. Type I EMT involved in tissue and organ formation during embryogenesis process (Kalluri R., 2009). EMT type II is linked to mesenchymal cells responsible for wound recovery and regeneration of tissue when there is epithelial stress or injury in tissue (Hackett, 2012). Type III EMT is associated with tumor invasiveness that occurs when migratory phenotype is gained by malignant epithelial (Pain, *et al*., 2014).

EMT can be identified with specific markers such as E-cadherin and zonula occludens-1. Decrease in the expression of these two proteins, which are juction proteins characterize the loss of epithelial phenotype (Lee JM, *et al*., 2006). Meanwhile, markers that can be used to determine the mesenchymal phenotype include N-cadherin, vimentin, a-SMA, desmin, and fibronectin (Okada H, *et al.*, 2000).

There are various growth factors that can catalyze EMT, they include fibroblast growth factor (FGF)2, epidermal growth factor, CTGF, insulin-like growth factor (IGF)2, interleukin (IL)-1 and hepatocyte growth factor (HGF) (Pain, *et al*., 2014).

2.3 TRAIL

2.3.1 Background of TRAIL

TRAIL or tumour necrosis factor-related apoptosis inducing ligand is a cytokine that induce apoptosis and can be found in almost all cell types (Braithwaite et al., 2018) especially in human lung, prostate and spleen (Wiley et al., 1995). TRAIL can also be known as Apo2 ligand (Braithwaite et al., 2018). TRAIL belongs the death receptor ligand family which is a subclass of the tumour necrosis factor family (Wiley et al., 1995).

TRAIL is suggested to stimulate inflammation response and thus play a role in acute and chronic airway inflammation (Braithwaite et al., 2018). It has been found that TRAIL level is increased in bronchoalveolar lavage (BAL) fluid of asthmatic patients after an antigen challenge (Robertson et al., 2002).

2.3.2 Physiological effects of TRAIL in airways

2.3.2.1 TRAIL in epithelium

The effects that TRAIL has on airway epithelium still remains unclear. TRAIL expression was shown in airway cells that include fibroblasts and epithelial (Chaudari, et al., 2006). TRAIL was found to stimulate TGF-β production in fibroblasts which demonstrates that TRAIL may have the ability to increase synthesis of extracelullar

matrix in fibroblasts. TGF-β also plays a role to control epithelial cells growth and differentiation (Bogdanowicz & Pujol, 2000). Fibroblasts are involved in epithelium thickening during airway remodelling (Chaudari, et al., 2006). Other than that, TGF-β was found to be an inducer for squamous differentiation in normal human bronchial epithelial cells (Masui et al., 1986).

2.3.2.2 TRAIL in fibroblasts

TRAIL induces apoptosis at high concentrations, but stimulates proliferation of lung fibroblast at lower concentrations (Yurovsky, 2003). Additionally, production of TGF-β can be stimulated by TRAIL and this leads to production of collagen which is a matrix component (Coker et al., 1997; Fine and Goldstein, 1987; Krupsky et al., 1996; Yurovsky, 2003). TGF-β can also increase matrix growth factors that includes connective tissue growth factor (Kucich et al., 2001; Ricupero et al., 1999). These finding suggest that TRAIL may play a role in regulating extracelullar matrix through stimulation of fibroblasts.

2.3.2.3 TRAIL in airway smooth muscle

Higher TRAIL levels have been observed in both bronchial biopsy and bronchoalveolar lavaage fluid (BAL) from asthmatic patients (Robertson et al., 2002). The human airway smooth muscle cells (HASMC) have been demonstrated as a source of TRAIL production (Robertson et al., 2004). According to Robertson et al., (2004), it was reported by RT-PCR data that TRAIL mRNA was abundantly present in HASMC after being treated with cytokines and glucorcoticoid.

2.3.3 Role of TRAIL in airway disease

2.3.3.1 TRAIL in airway inflammation

Several studies have shown the negative effects of TRAIL in airway inflammation. In a study done by Robertson, et al., (2002), it was revealed that TRAIL levels increased in the BAL fluid in allergic asthmatic subjects after SAC. It was also found that in airway smooth muscle and epithelium, after SAC, TRAIL levels were increased in asthmatic subjects compared to normal control subjects. It was also found that airway inflammation had been reduced after a deletion of TRAIL gene in the ovalbumin-induced model of chronic airway diseases (Collison, et al., 2014). A study by Haw, et al., (2015) also revealed that pro-inflammatory cytokine, chemokine, and COPD-related factor mRNA expression are reduced in TRAIL-deficient mice after an exposure to cigarette smoke. These findings suggest that TRAIL plays a role in regulating airway.

2.3.3.2 TRAIL in airway remodelling

There are limited evidence on the effects of TRAIL in airway remodelling specifically in airway epithelium. However, some studies had shown dentrimental effects of TRAIL in airway remodelling. It was demonstrated that airway remodelling was reduced when TRAIL is absent and when the Th2-driven inflammatory signaling, as well as eosinophils recruitment and mast cells to the airways are reduced (Collison, et al., 2014).

Airway remodeling is characterized by subepithelial fibrosis, myofibroblast hyperplasia, as well as collagen deposition in the basement membrane. It was reported that TRAIL induced TGF-β production in fibroblasts, and thus enhanced extracellular matrix synthesis that contributes to airway remodelling (Robertson et al., 2004). Chaudari et al., 2006 stated that in vitro study showed that TRAIL was expressed in airway cells including fibroblasts and epithelial, endothelial, and smooth muscle cells. Mainly, effects of TRAIL can be seen on apoptosis of cells. It was shown that expression of TRAIL was increased in hepatoma cells in the present of TGFβ-1 (Chaudari, et al., 2006). This demonstrates that TRAIL and TGFβ-1 may cooperatively share certain mechanism in apoptosis which further suggests that they both may also share same mechanism in airway remodelling.

CHAPTER 3

METHODOLOGY

3.1 Materials

Trypan blue and Tween 20 were purchased from Sigma Aldrich, USA. TGFβ-1 were purchased from R&D System, USA. Fetal bovine serum (FBS) was purchased from Gibco, USA. Enhanced Chemiluminescence (ECL) detecting reagent was purchased from Advansta, USA. 40% acrylamide/bis mixed solution 29:1 were purchased from Nacalai Tesque, Japan. Phosphate buffered saline (PBS), tetramethylethylenediamine (TEMED), sodium dodecyl-sulfate (SDS), tris, glycine and dimethyl sulfoxide (DMSO) were purchased from Amresco, USA. Polyvinylidene fluoride (PVDF) membrane was purchased from Milipore, USA. Bicinchoninic acid (BCA) was purchased from Pierce, USA. Protein ladder was purchased from SMOBIO, Taiwan. 4X Laemmli Sample Buffer was purchased from Bio-Rad, USA. Methanol was purchased from Fisher Scientific, USA. Ethanol was purchased from HmBG Chemicals, Germany. Bovine serum albumin (BSA) was purchased from Tocris Bioscience, UK. Rabbit anti E-cadherin, Rabbit anti-α tubulin were purchased from Cell signalling technology, USA. Mouse anti-rabbit IgG HRP-linked were purchased from Abcam, UK. BCA Protein Assay Kit was purchased from Merck, USA.

3.2 Cell culture and sub-culturing

The BEAS2B cells and culture media used in this study was purchased from American Type Culture Collection (ATCC CRL-9609™). BEAS2B cells were maintained in Bronchial Epithelial Basal Medium (BEBM) supplemented with BPE, insulin, Hydrocortisone, GA-1000, Retinoic acid, Transferrin, Triiodothyronine, Epinephrine and hEGF. The cells were cultured at 37℃, 5% CO_2 in humidified condition. Sub-culturing of BEAS2B cells was done when culture reached approximately 70% - 80% confluency. The spent medium was

removed carefully and the cells were washed with PBS for two times. Then, trypsin-EDTA solution was added into the cells and the flask was swirled to help the cells to detach. Then, the flask was incubated for 5 to 10 minutes. After that, media containing FBS was added to stop trypsinization. The cell suspension was transferred into a centrifuge tube and centrifuged at RCF 130g for 5 minutes at 25℃. After centrifugation, supernatant was removed and 1 ml media was further added into the cell pellet and resuspended. The cells were then seeded into new culture flasks.

3.3 Cell counting and cell viability

Three droplets of 10µl Tryphan blue were placed on parafilm. 10µl of cell suspension was mixed with the first Tryphan blue droplet. The mixture was then transferred to the next droplet until the last droplet. After that, 10µl of the final mixture was placed into a hemocytometer and viable cells were counted under a microscope. Cell viability was counted by using following formula. Number of viable cells was divided by four and then multiplied with ten to the power of four and dilution factor.

$$\frac{(Number\ of\ viable\ cells)}{4} \times 10^4 \times 2^{Dilution\ factor}$$

Where,

Dilution factor = 3

3.4 Cell seeding and cell treatment

The BEAS2B cells were seeded with a seeding density of 7 X 10^5 in a 6-well plate one day prior to treatment. The normal control groups for both acute and chronic treatment did not receive any treatment. The TGFβ-1 treatment groups for acute induction were treated with 1, 5, 10 and 20ng/mL of TGFβ-1 respectively for 48 hours. Meanwhile in TGFβ-1 treatment groups for chronic treatment, all groups received 10ng/mL of TGFβ-1. The cells were treated for 2, 4 and 6 days. Each treatment group was paired with its normal control group respectively. To ensure enough nutrients were given to the cells, old media were replaced with fresh media with or without TGFβ-1 every two days.

3.5 Analysis of protein expression

The expression of N-cadherin, E-cadherin, TRAIL and Vimentin in TGFβ-1-induced-BEAS2B cells were detected by analysis of western blot.

3.5.1 Protein extraction and quantification

At the end of the treatment, the cells were treated with RIPA lysis in cold conditions to inhibit protease activity. The bottom of the plate was scratched to detach the cells. The suspension was collected in a conical tube and then centrifuged for 15 to 20 minutes with 130 RCF at 4°C. After centrifugation, the cell lysate was transferred into micropipette tubes.

Protein quantification was done by using Bicinchoninic acid (BCA) protein assay kits to determine the protein concentration of the cell lysate of treatment and control groups. The standard curve used in the protein assay was generated by using 2 mg/mL of Bovine Serum Albumin (BSA) standard. BSA standard solution and working reagent were prepared beforehand.

For BCA assay, 25µL of each standard and protein of treatment and control groups were pipetted in duplicates into a 96-well plate. For each well, 200µL of working reagent were added and mixed by shaking the well gently for 30 seconds. The 96-well plate was placed in a 5% CO_2 incubator at 37℃ for 30 minutes. After incubation, the absorbance of each well was read at wavelength of 560nm using ELISA microplate reader (Tecan, Switzerland).

3.5.2 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

10% polyacrylamide gel and the running buffer were prepared beforehand. The 10% polyacrylamide gel was inserted into the electrophoresis tank. 4µl of protein ladder was added into the first well of the gel for molecular weight reference. The specific amount of each protein sample was mixed with 4X Laemmli buffer and RIPA buffer to obtain a mixture with a protein concentration of 10µg with a final volume of 20µl. 1X running buffer was added into the tank. A voltage of 100V was supplied to the electrode chamber and electrophoresis was carried out until the dye reached the bottom of the gel.

3.5.3 Wet protein transfer

PVDF membrane was cut and soaked in methanol to activate it. The gel from electrophoresis chamber was cut. The PVDF membrane were placed onto the each gel. The gels then were placed onto blotting paper, layered with foam pad. Another set of blotting paper with a foam pad was placed on top of the PVDF membrane. The membrane sandwich was rolled using a roller to remove any bubbles.

The sandwich was then inserted into a cassette and the cassette was inserted into the wet transfer tank filled with 1X transfer buffer. Magnetic stirrer was placed inside the tank to ensure a constant temperature. Ice packs were added into the tank for cooling purposes. The transfer process was carried out at 100V for 1 hour and 30 minutes.

3.5.4 Immunoblotting and visualization

After performing protein transfer, the PVDF membrane was removed carefully and placed into a small container. The membrane then was washed with TBST twice. After washing, the membrane was blocked with blocking buffer containing 5% BSA in 1X Tris Buffered Saline with Tween 20 (TBST) for 1 hour.

After 1 hour, the membrane was rinsed 3 times with 1X TBST for 10 minutes. Then, the membrane was incubated with a primary antibody (1:1000) for α-tubulin, E-cadherin, N-cadherin, TRAIL and Vimentin at 4°C overnight. On the next day, the membrane was rinsed with 1X TBST for 10 minutes thrice and then incubated with secondary antibody which is goat HRP-conjugated anti-rabbit IgG (1:2000) at room temperature for 1 hour. After 1 hour, the membrane was rinsed again with 1X TBST for 10 minutes thrice. Then the membrane was visualized by using a gel documentation system. After that, the membrane was stripped with a stripping buffer for 10 minutes and the stripping process was repeated two times. The membrane was incubated with a chemiluminescent substrate solution and visualized using the Fusion FX gel documentation system.

3.6 Statistical analysis

Data for all sample groups were presented as mean ± SEM with GraphPad Prism version 7. One-way Analysis of Variance (ANOVA) followed by Dunnet's and Student's T-test were carried out to determine statistical significance with a p-value of < 0.05.

CHAPTER 4

RESULT

4.1 Effect of acute treatment of TGFβ-1 on E-cadherin, N-cadherin, Vimentin and TRAIL

In this study, E-cadherin, N-cadherin and Vimentin protein expression were assessed by immunoblotting assay to confirm EMT induction by TGFβ-1 in BEAS2B cells for 48 hours. The control group was not treated with TGFβ-1, meanwhile treatment groups were treated with different concentrations of TGFβ-1. The lowest concentration given was 1ng/ml, followed by 5ng/ml, 10ng/ml and the highest concentration was 20ng/ml.

For epithelial marker E-cadherin expression, the TGFβ-1 treatment groups resulted in a significant downregulation. The lowest expression of E-cadherin can be observed at the highest treatment concentration of TGFβ-1, which was 20ng/ml, with p-value <0.0001. The results of E-cadherin expression produced a p-value less than 0.05 which indicates there is a significant difference between the mean groups except for treatment group with 1ng/ml TGFβ-1 which was not significantly different.

Mesenchymal marker, N-cadherin expression was upregulated when treated with 1ng/ml, 5 ng/ml and 10ng/ml of TGFβ-1. However, there is a slight decrease of N-cadherin expression when treated with 20ng/ml of of TGFβ-1. The highest increase of N-cadherin expression was at 10ng/ml of TGFβ-1 with a p-value less than 0.0001. Another mesenchymal marker, vimentin, was expressed greatly when treated with 20ng/ml of of TGFβ-1. There is a significant different between all mean groups with p-value less than 0.05. These three markers expression strongly suggests that EMT induction in BEAS2B cells by TGFβ-1 was succesful.

Meanwhile, TRAIL expression level in TGFβ-1 treatment group was also significantly upregulated, with p-value 0.004 at concentration of 10ng/ml. Treatment group at 10ng/ml was upregulated by 31% compared to treatment group at 5ng/ml. A slight decrease at concentration of 5ng/ml was observed with p-value of 0.79 which indicates that it is not significantly different compared to normal group. TRAIL expression was seen to increase in BEASB2B cells following the increasing concentration of TGFβ-1 which suggests that this increased TRAIL expression may be correlated with TGFβ-1.

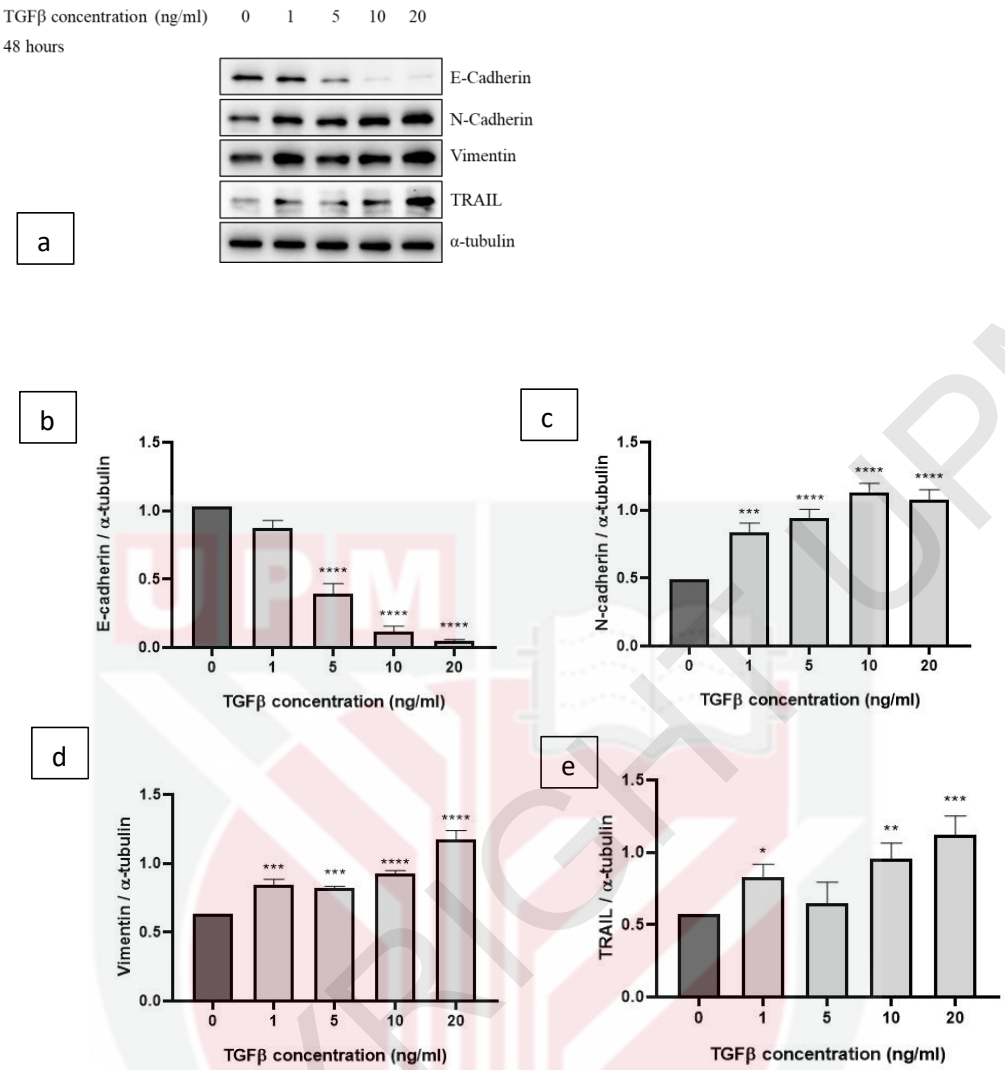

Fig 1. The expression of EMT markers and TRAIL expression in BEAS2B cells upon acute treatment of TGFβ-1

a A representative image of E-cadherin, N-cadherin, Vimentin and TRAIL expression following acute treatment of TGFβ-1 in BEAS2B cell **b** Densitometric analysis of E-cadherin **c** N-cadherin **d** Vimentin expression **e** TRAIL expression following acute treatment of TGFβ-1 in BEAS2B cells. Cells were treated with TGFβ-1 at indicated concentrations for 48 hours. Protein extracts were collected and subjected to Western Blotting. Results were normalized to α-tubulin and presented as the mean ± SEM of four experiments. * represents $p \leq 0.05$ significantly different from the normal group; one-way ANOVA followed by Dunnet's test.

4.2 Effect of chronic treatment of TGFβ-1 on E-cadherin, N-cadherin, Vimentin and TRAIL

For chronic treatment with TGFβ-1, normal control groups did not receive any treatment of TGFβ-1. Meanwhile, treatment groups were treated with 10ng/ml concentration of TGFβ -1 for 2, 4 and 6 days. Each treatment group were paired with its respective normal control groups which did not receive any treatment with TGFβ-1, and old media were replaced with fresh media with or without TGFβ-1 every two days.

For the E-cadherin expression, the TGFβ-1 treatment groups expressed decreasing level of E-cadherin. The expression decreased gradually from day 2 to day 6. By day 6, the treatment group lessen by 98% than normal control. The N-cadherin expression was significantly upregulated from day 2 to day 6 in treatment groups compared to normal groups.. The greatest increase of N-cadherin expression can be observed on day 6, in which treatment group was upregulated by 84% than normal control. TGFβ-1 treatment groups for Vimentin expression also showed similar pattern of upregulation. Treatment group on day 6 was upregulated by 114% higher than the normal control. This result indicates successful chronic treatment of TGFβ-1 in the BEAS2B cell and that the effect of TGFβ-1 was not transient after 48 hours duration of treatment.

Simultaneously, TRAIL expression for the TGFβ-1 treatment groups showed a significant increase in the expression compared to normal groups with a p-value less than 0.0001 on day 6 of treatment. There was only an 8% of upregulation from day 4 of treatment compared to day 2 of treatment with TGFβ-1. The treament group on day 6 was upregulated by 221% than normal control. Upregulation of TRAIL expression demonstrated that TRAIL effects in BEAS2B cells is not temporary even after a longer treatment period.

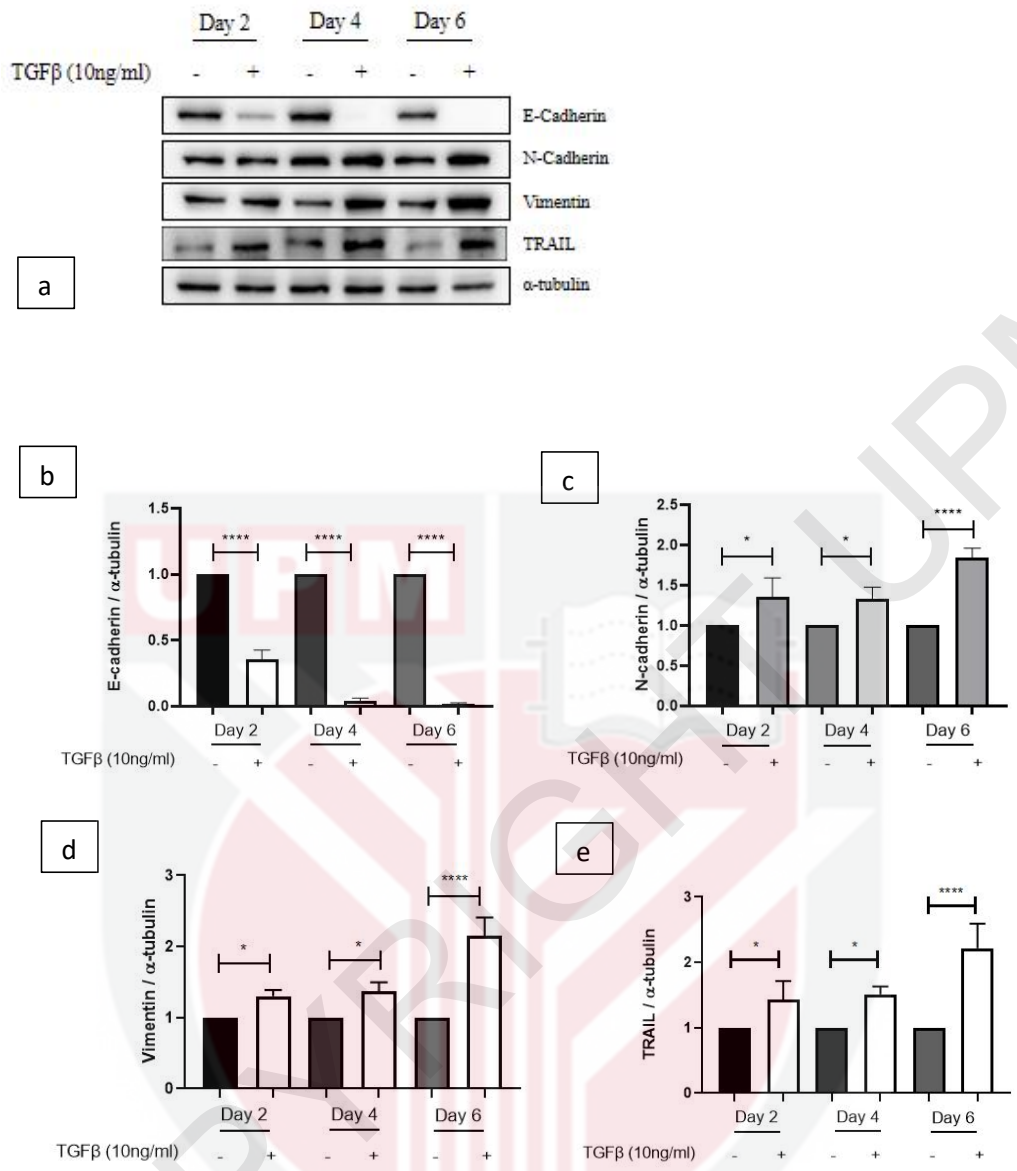

Fig 2. The expression of EMT markers and TRAIL expression in BEAS2B cells upon chronic treatment of TGFβ-1

a A representative image of E-cadherin, N-cadherin, Vimentin and TRAIL expression following TGFβ-1 induced EMT in BEAS2B cell **b** Densitometric analysis of E-cadherin **c** N-cadherin **d** Vimentin expression **e** TRAIL expression following chronic treatment of TGFβ-1 in BEAS2B cells. Cells were treated with 10ng/ml of TGFβ-1 for 3 different timepoints, 2 days, 4 days and 6 days. Protein extracts were collected and subjected to Western Blotting. Results are normalized to α-tubulin and presented as the mean ± SEM of

four experiments. * represents $p \leq 0.05$ significantly different from the normal group; one-way ANOVA followed by Student's T-test.

CHAPTER 5

DISCUSSION

Airway diseases such as asthma and COPD are mainly associated with airway inflammation. However, another pathological feature that contributes to the severity of airway diseases that needs attention is airway remodelling. It was shown that allergic inflammation or atopy alone is not enough for asthma to develop and that airway remodelling is another mechanism that play a significant role in it (Hackett, 2012). One of the mechanisms thought to contribute to airway remodeling is a biological process known as epithelial-mesenchymal transition (EMT).

EMT is a biological process in which a polarised epithelium with cell–cell contacts that is tethered to the basal membrane undergoes differentiation to become fibroblast-type mesenchymal cells. EMT hallmark is a progressive loss of epithelial markers and gain of mesenchymal markers. Bronchial epithelium is a potential source of fibroblasts and myofibroblasts accumulated in chronic remodelling in airways. Bronchial remodelling is a feature of airway diseases such as asthma, COPD and bronchiolitis obliterans (BO) (Pain, *et al*., 2013). These findings suggest that accumulation of fibroblasts that may have been caused by EMT contributes to airway remodelling. Therefore, this study aimed to recognize and understand this feature of airway remodelling which is EMT and how it contributes to airway remodelling.

Exposure to environmental challenges like growth factors and inflammatory mediators promotes the downregulation of epithelial cell-cell adhesions and enhance expression of mesenchymal gene in both in-vitro and in-vivo experiments. It was showed that airway remodelling may be contributed by plastic and dynamic airway epithelium through EMT process in asthma (Hackett, 2012). Previous study demonstrated that adhesion proteins that are necessary for cell–cell contacts are downregulated in asthmatic epithelium

(de Boer, *et al.*, 2008). These data support the hypothesis that abnormalities in the structure of asthmatic airway epithelium could be the factor that increase signalling of mesenchymal cells and thus drives the abnormal responses to tissue remodelling in the asthmatic airway (Holgate, *et al*., 2000). The role of TGF-β was shown to have effects in the remodeling process in asthma and also other inflammatory and immune-mediated lung diseases (Postma & Timens, 2006). Additionally, it was shown that TGF-β induces epithelial–mesenchymal transition in primary airway epithelial cells from patients with asthma (Hackett, *et al*., 2009).

The main interest in this study is to discover the role of TRAIL in EMT, specifically in the context of airway remodelling. TRAIL is a cytokine and a member of the TNF superfamily. TRAIL expression was demonstrated to increase in asthma which proposes that this cytokine has a role in asthmatic airways (Robertson, *et al.*, 2002). It was also found that TRAIL has negative associations with the lung function of asthmatics (Marks et al., 2020). From the information gained on TRAIL, TRAIL has been shown to have a close association with airway inflammation and airway remodelling in lung diseases and thus suggests that investigating TRAIL as a therapeutic target for airway diseases would be worthwhile. Hence, a clear understanding on the role of TRAIL in airway remodelling is needed. This study provides in-vitro evidence on the association between TRAIL and TGFβ1-induced EMT in BEAS2B cells.

The present study resulted in successful induction of EMT by TGFβ-1. From the result, the TGFβ-1 treatment for 48 hours showed a significantly decreased of epithelial marker, E-cadherin and increased expression of both mesenchymal markers, N-Cadherin and Vimentin compared to the normal control group. A decrease in the expression of epithelial proteins such as E-cadherin characterized the loss of the epithelial phenotype (Pain, et al., 2013). Valcourt, et al. (2016) stated that 2 to 7 days is a common period for a full EMT

process to be achieved and that EMT infrequently occurred in response to TGF-β, during a 36 hours time period as concluded by previous in vitro studies on human epithelial cell lines. Various studies had shown that 48 hours is the optimal period for TGFβ-1 to show an effect in EMT induction (Hackett, et al., 2009; Kamitani et al., 2010; Zhu, et al., 2021). There is limited evidence on the chronic treatment of TGF-β on BEAS2B cells line. However, some other studies that used different cell lines related to lung demonstrated that EMT was induced in the present of TGFβ-1. TGFβ-1 appeared to induce EMT in A549 alveolar epithelial cells via activation of Smad2 pathway (Kasai, et al., 2005). Significantly high levels of TGF-β1 were observed in the asthmatic mice compared to control group through ELISA analysis in the BAL fluid (Yang, et al., 2013).

There is no evidence of TRAIL association with TGFβ1-induced EMT. However, there has been an association between TNF-α, a member of the TNF superfamily, with TGFβ1-induced EMT in BEAS-2B cells. It has been found that costimulation with TNF-α enhanced the EMT (Kamitani, et al., 2010). This suggests TRAIL, whose belongs to the family of TNF might as well play a similar role as TNF-α. In present finding, TRAIL expression was upregulated upon both acute and chronic treatment of TGFβ-1 in BEAS2B cell. This demonstrates that TRAIL may be involved in promoting EMT-mediated airway remodelling. Nonetheless, this finding is still not enough to justify that upregulation of TRAIL was due to the exposure of TGFβ-1.

It was also found that TRAIL expression increases in mouse model of chronic cigarette smoke-induced experimental COPD (Haw, et al., 2015). Furthermore, increase of TGF-β and its mRNA were observed in bronchiolar epithelium from COPD patients (Kamitani et al, 2010). Based on these findings, it is possible that structural changes occurred in COPD patients that increases the COPD severity might be due to the increase of TGF-β,

constimulated by increase of TRAIL level. Additionally, TGFβ-1 was demonstrated to participate in the pathogenesis of asthma due to the increased of TGF-β1 serum levels in atopic asthmatic children (Manuyakorn, et al., 2008). However, further investigation is still needed to confirm the role of TRAIL in TGFβ1-induced EMT. Further recommendation for this study include using other TGFβ-1 inhibitor to confirm whether upregulation of TRAIL expression in TGFβ1-induced EMT is correlated with TRAIL itself or to proceed with TRAIL knockdown in cell to further confirm the association between TRAIL and TGFβ-1.

CHAPTER 6

CONCLUSION

TGFβ-1 can upregulate TRAIL expression in the human bronchial epithelial cell and this regulation is correlated with the expression of EMT markers such as upregulation of epithelial markers and downregulation of mesenchymal markers.

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APPENDIX

Statistical analysis

Table 1. Statistical analysis of E-cadherin expression in acute treatment of TGFβ-1 by one-way ANOVA

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	2.350	4	0.5876	F (4, 10) = 79.90	P<0.0001
Residual (within columns)	0.07354	10	0.007354		
Total	2.424	14			

Table 2. Statistical analysis of E-cadherin expression in acute treatment of TGFβ-1 by Dunnet's test

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 1	0.1629	-0.03945 to 0.3653	No	ns	0.1241
0 vs. 5	0.6384	0.4361 to 0.8408	Yes	****	<0.0001
0 vs. 10	0.9175	0.7151 to 1.120	Yes	****	<0.0001
0 vs. 20	0.9843	0.7820 to 1.187	Yes	****	<0.0001

Table 3. Statistical analysis of N-cadherin expression in acute treatment of TGFβ-1 by one-way ANOVA

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

Treatment (between columns)	0.7767	4	0.1942	F (4, 10) = 41.10	P<0.0001
Residual (within columns)	0.04725	10	0.004725		
Total	0.8240	14			

Table 4. Statistical analysis of N-cadherin expression in acute treatment of TGFβ-1 by Dunnet's test

Dunnett's multiple comparisons test	**Mean Diff.**	**95.00% CI of diff.**	**Significant ?**	**Summary**	**Adjusted P Value**
0 vs. 1	-0.3429	-0.5052 to -0.1807	Yes	***	0.0004
0 vs. 5	-0.4480	-0.6102 to -0.2858	Yes	****	<0.0001
0 vs. 10	-0.6408	-0.8030 to -0.4786	Yes	****	<0.0001
0 vs. 20	-0.5883	-0.7505 to -0.4260	Yes	****	<0.0001

Table 5. Statistical analysis of Vimentin expression in acute treatment of TGFβ-1 by one-way ANOVA

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	0.4531	4	0.1133	F (4, 10) = 79.70	P<0.0001
Residual (within columns)	0.01421	10	0.001421		
Total	0.4673	14			

Table 6. Statistical analysis of Vimentin expression in acute treatment of TGFβ-1 by Dunnet's test

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
0 vs. 1	-0.2078	-0.2967 to -0.1188	Yes	***	0.0002
0 vs. 5	-0.1838	-0.2728 to -0.09482	Yes	***	0.0005
0 vs. 10	-0.2897	-0.3787 to -0.2008	Yes	****	<0.0001
0 vs. 20	-0.5347	-0.6236 to -0.4457	Yes	****	<0.0001

Table 7. Statistical analysis of TRAIL expression in acute treatment of TGFβ-1 by one-way ANOVA

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.6058	4	0.1515	F (4, 10) = 13.37	P=0.0005
Residual (within columns)	0.1133	10	0.01133		
Total	0.7191	14			

Table 8. Statistical analysis of TRAIL expression in acute treatment of TGFβ-1 by Dunnet's test

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value

0 vs. 1	-0.2596	-0.5108 to -0.008369	Yes	*	0.0428
0 vs. 5	-0.07574	-0.3270 to 0.1755	No	ns	0.7974
0 vs. 10	-0.3852	-0.6364 to -0.1340	Yes	**	0.0043
0 vs. 20	-0.5513	-0.8026 to -0.3001	Yes	***	0.0003

Table 9. Statistical analysis of E-cadherin expression in chronic treatment of TGFβ-1 by Student's T-test

Unpaired t test	**Day 2**	**Day 4**	**Day 6**
P value	<0.0001	<0.0001	<0.0001
P value summary	****	****	****
Significantly different (P < 0.05)?	Yes	Yes	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed
t, df	t=18.64, df=4	t=63.34, df=4	t=313.4, df=4

Table 10. Statistical analysis of N-cadherin expression in chronic treatment of TGFβ-1 by Student's T-test

Unpaired t test	**Day 2**	**Day 4**	**Day 6**
P value	0.0898	0.0189	0.0002
P value summary	ns	*	***

Significantly different (P < 0.05)?	No	Yes	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed
t, df	t=2.229, df=4	t=3.814, df=4	t=13.70, df=4

Table 11. Statistical analysis of Vimentin expression in chronic treatment of TGFβ-1 by Student's T-test

Unpaired t test	**Day 2**	**Day 4**	**Day 6**
P value	0.0033	0.0078	0.0016
P value summary	**	**	**
Significantly different (P < 0.05)?	Yes	Yes	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed
t, df	t=6.246, df=4	t=4.948, df=4	t=7.594, df=4

Table 12. Statistical analysis of TRAIL expression in chronic treatment of TGFβ-1 by Student's T-test

Unpaired t test	**Day 2**	**Day 4**	**Day 6**
P value	0.0546	0.0020	0.0084
P value summary	ns	**	**
Significantly different (P < 0.05)?	No	Yes	Yes

One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed
t, df	t=2.692, df=4	t=7.201, df=4	t=6.203, df=3