Epithelial to Mesenchymal Transition (EMT) and Airway Remodelling after Human Lung Transplantation.


Applied Immunobiology and Transplantation Research Group, Institute of Cellular Medicine, Newcastle University, United Kingdom.

*Corresponding Author:

Dr Andrew J. Fisher
Applied Immunobiology and Transplantation Research Group
Institute of Cellular Medicine
Medical School
Newcastle University
United Kingdom
NE2 4HH.

Tel. - 0191 222 8266
Fax. - 0191 222 8988

E-mail - A.J.Fisher@newcastle.ac.uk
Key Words

Epithelial to Mesenchymal Transition, Obliterative Bronchiolitis, Tumour Necrosis Factor-α, Transforming Growth Factor-β1, Lung Transplantation.

Word Count

2993
Abstract

Aberrant epithelial repair is a key event in the airway remodelling which characterises obliterative bronchiolitis (OB) in the transplanted lung. The potential for airway epithelium from lung transplant recipients to undergo epithelial to mesenchymal cell transition (EMT) was assessed in culture and in vivo in lung allograft tissue. Changes in epithelial and mesenchymal marker expression was assessed after stimulation with TGF-β1 alone or in combination with TNFα and compared to untreated controls. Cells ability to deposit extra-cellular matrix, secrete matrix metalloproteinases and invade collagen were investigated. Immunolocalisation of epithelial and mesenchymal markers were compared in airway tissue from stable recipients and those with OB. Untreated cells maintain epithelial morphology and phenotype. TGF-β1 reduced expression of epithelial markers, increased expression of vimentin and fibronectin, promoted collagen I and fibronectin deposition and increased MMP-9 production. Co-treatment with TNFα dramatically accentuated phenotypic and some functional features of EMT. Airway epithelial biopsies from recipients with OB demonstrated significantly increased staining for mesenchymal markers and significantly reduced E-cadherin staining compared to stable recipients. These observations support a potential role for EMT in development of OB after lung transplantation and suggest a potential link between inflammatory injury and TGF-β1 driven airway remodelling.
Introduction

Lung transplantation provides a valuable therapeutic option for selected patients with end-stage lung disease [1], however long term survival remains limited to a median of 5 years by the development of bronchiolitis obliterans syndrome (BOS) [2]. The pathological lesion of BOS is obliterative bronchiolitis (OB) which is characterised by aberrant epithelial repair and airway remodelling leading to obstruction due to deposition of extracellular matrix (ECM) as a result of an excessive fibroblastic response.

The origin of the fibroblasts responsible for the deposition of ECM remains unclear with in situ proliferation of resident fibroblasts and recruitment of circulating progenitor cells proposed as potential sources [3]. Evidence from animal models of airway obliteration suggests epithelial injury and importantly a failure to re-establish an intact epithelium may be critical to the pathogenesis [4].

Epithelial cells can respond to injury in a number of ways including repair, necrosis and apoptosis. However, epithelial to mesenchymal transition (EMT) is increasingly recognised as an alternative response to injury. During EMT, cells lose epithelial properties and gain phenotypic properties of a mesenchymal cell including production of matrix metalloproteinases (MMPs) and deposition of ECM [5]. Recent data from animal models and epithelial cell lines suggests EMT may be an important response in lung epithelium at the alveolar level [6-8], however data using primary human lung epithelium has been limited to one previous pilot study [9].
Transforming growth factor-beta 1 (TGF-β1) is implicated in driving fibrosis in lung, liver and kidney but a mechanism by which TGF-β1 may contribute to OB pathogenesis remains to be elucidated. Previous studies show that TGF-β1 expression is increased in recipients with OB [10-13] and in an animal model of airway obliteration, interrupting TGF-β1 binding to its receptor reduced intraluminal airway matrix deposition [14].

TGF-β1 signals predominantly via the SMAD signalling pathway [15-17], but can also activate the Mitogen activated protein kinase (MAPK) pathway [18-20]. However, activation of the MAPK signalling pathway by pro-inflammatory stimuli such as TNFα [21] raises the possibility of cross-talk between TGF-β1 signalling and inflammatory signalling in epithelium. EMT may therefore provide a plausible link between excessive inflammation, TGF-β1 activity and the development of OB. We hypothesise that airway epithelial cells in the transplanted lung may undergo TGF-β1 driven EMT and that this may be exaggerated by pro-inflammatory stimuli such as TNFα.

We have previously demonstrated preliminary evidence of EMT in the airways of lung transplant recipients by showing increased expression of the single mesenchymal marker S100A4 in airway epithelium from stable lung allografts [9]. This study aims to extend these initial observations by comprehensively assessing the ability of airway epithelial cells from lung transplant recipients to undergo EMT and evaluating the evidence for EMT in tissue sections from stable lung transplant recipients and those with OB.
Materials and Methods

Cell Culture

Primary bronchial epithelial cells (PBEC) from stable lung transplant recipients were isolated as previously described [22].

Cell Treatments

Cells were cultured in media alone or treated with TGF-β1 (0.1-20ng/ml) and/or TNFα (1-20ng/ml) for 72 hours. Protein concentrations were determined using the BCA protein assay kit (Perbio, UK).

Western Blotting

Total cell lysates (5-60μg) were separated on 4-12% bis-Tris gels (Invitrogen, UK). Membranes were incubated with primary antibodies and detected with horseradish peroxidase (HRP)-labelled IgG conjugates (Abcam, UK). Antibody complexes were visualised using the SuperSignal West Pico chemiluminescent kit (Perbio, UK). Results are normalised to β-tubulin as appropriate.

Immunofluorescence

Treated cells fixed in 4% (w/v) paraformaldehyde or explanted lung tissue were incubated with primary antibodies and detected using an appropriate fluorochrome-linked secondary antibody. DAPI was used as a nuclear counterstain. Images acquired using a LSM 510 laser scanning confocal microscope.

Gelatin Zymography
MMP-9 activity in conditioned media from cells treated as indicated were analysed as previously described [9].

**Invasion Assay**

The invasive potential of cells was evaluated by assessing penetration of Matrigel coated filters. Briefly, cells were treated with TGF-β1 (10ng/ml) and/or TNFα (20ng/ml) for 72 hours, 10000 cells loaded in the invasion chamber and incubated for 24 hours at 37°C. The number of invasive cells was determined following Diff-Quik (Dade Behring, UK) staining and scored at bright field microscopy.

**TCA Protein Precipitation**

Trichloroacetic (TCA, 100% w/v) was added to conditioned media from treated cells and incubated at 4°C for 10 minutes. The protein precipitate was pelleted, washed twice in acetone and dried by heating to 95°C for 10 minutes. The pellet was suspended in sample buffer and separated by SDS-PAGE.

**Human Lung Tissue Sampling**

This use of human tissue was approved by Local Research Ethics Committee. Normal control tissue was obtained from donor lungs assessed for, but not accepted for, use in clinical lung transplant. Stable lung transplant recipients underwent transbronchial biopsy by an established technique. Tissue from recipients with OB was obtained from explanted lung at the time of retransplantation. All lung samples were fixed in 10% formalin, embedded, sectioned and stained with H&E. Acute rejection and other pathological changes in stable patients were excluded and OB confirmed in explanted lungs by an experienced pulmonary histopathologist.
Sequential sections from stable recipients, OB recipients and normal control lungs were stained with antibodies against E-cadherin, vimentin and α-smooth muscle actin (Dako) using a modified immunoperoxidase method (Envision; Dako). The extent of positive bronchial epithelial staining was assessed in five non-overlapping high power fields as previously reported [23]. Image analysis software was used to capture the positive staining (Adobe Photoshop CS3 Extended).

**Statistical Analysis**

Results are presented as mean ± standard error of the mean (SEM). The differences between groups was analysed using an ANOVA. A p-value of <0.05 was considered significant.
Results

Effect of TGF-β1 and TNFα on epithelial cell morphology and protein expression

Untreated or TNFα treated cells maintained a uniform ‘cobblestone’ appearance characteristic of epithelial cells (Figure 1 i, and iii respectively). The cells demonstrated high expression of the epithelial marker cytokeratin 19 (Figure 1 v, and vii) and little or no expression of the mesenchymal markers vimentin (Figure 1 ix and xi) or fibronectin (Figure 1 xiii and xv). Treatment of the cells with TGF-β1 markedly changed cell phenotype to a more elongated bi-polar phenotype characteristic of a mesenchymal cell (Figure 1 iii). This morphological change was associated with a downregulation in cytokeratin-19 expression (Figure 1 vi) together with the upregulation of vimentin (Figure 1 x) and fibronectin (Figure 1 xiv) expression. Co-treatment of the cells with TGF-β1 and TNFα dramatically accentuated the phenotype change (Figure 1 iv) and further increased the expression of vimentin (Figure 1 xii) and fibronectin (Figure 1 xvi) compared to TGF-β1 alone.

Western blotting was used to quantify the changes in protein expression (Figure 2). Untreated cells expressed high levels of E-cadherin, but expressed little vimentin or fibronectin. Treatment with TGF-β1 induced a significant downregulation in E-cadherin expression (Figure 2B, i) and a marked increase in fibronectin (Figure 2B, ii) and vimentin (Figure 2B, iii) expression. The protein changes seen with TGF-β1 were significantly accentuated by co-culture with TNFα (*p<0.05, n=4). Treatment of the epithelial cells with TNFα alone (p>0.05, n=4) had no effect on the expression of any of the proteins examined.
Effect of TGF-β1 and TNFα concentrations on EMT marker expression

The effect of increasing concentrations of TGF-β1 (0.1 ng/ml - 20 ng/ml) on the production of pro-MMP-9 was assessed using gelatin zymography. Control cells and cells treated with 0.1ng/ml of TGF-β1 do not secrete pro-MMP-9. However, cells treated with >1ng/ml of TGF-β1 secrete pro-MMP-9 in a dose dependant manner (Figure 3A). The accentuating effects of TNFα on EMT were further evaluated using lower doses of TGF-β1 (1ng/ml and 3ng/ml) by western blotting and zymography. A higher dose of TNFα (20ng/ml) is required to accentuated EMT in the presence of a low dose of TGF-β1 (1ng/ml) but if the dose of TGF-β1 is increased to 3ng/ml (Figure 3B) or 10ng/ml (Figure 3C) a dramatic accentuating effect is achieved with as little as 1ng/ml of TNFα.

Effect of TGF-β1 and TNFα on the invasive potential of epithelial cells

To assess if airway epithelial cells are undergoing true functional EMT, their ability to penetrate a Matrigel coated filter was investigated (Figure 4A). Very few untreated cells had the ability to penetrate the filter, however the number of invasive cells increased 3 fold following treatment with TGF-β1 (p<0.05, n=4). TNFα alone had no effect on the number of invasive cells compared to untreated cells, yet co-treatment of the cells with TGF-β1 and TNFα further increased the number of invasive cells 2 fold compared to TGF-β1 alone (*p<0.05, n=4).

The effect of TGF-β1, in the presence or absence of TNFα, on the production of pro-MMP-9 was assessed using gelatin zymography (Figure 4B, C). Control cells secrete a low level of pro-MMP-9 that is increased 2 fold by treatment with TGF-β1. Co-treatment with TNFα resulted in a further 2 fold increase in the secretion of pro-MMP-9 compared to cells treated with TGF-β1 alone (*p<0.05, n=4).
Effect of TGF-β1 and TNFα on epithelial cells deposition of Extracellular Matrix

Untreated cells or cells treated with TNFα did not secrete collagen type I or fibronectin as assessed by TCA precipitation (Figure 5A, B). However cells treated with TGF-β1 were able to secrete both collagen type I and fibronectin. Interestingly, co-treatment of the cells with TGF-β1 and TNFα had no significant effect on the secretion of fibronectin (p>0.05, n=4) but significantly decreased the secretion of collagen type I (*p<0.05, n=4) when compared to TGF-β1 alone.

Expression of markers of EMT in vivo in airway epithelium

Transbronchial biopsies were obtained from 6 (5M:1F) lung transplant recipients with stable allograft function between 6 and 12 months post transplant. All 6 subjects were free of acute rejection, infection and were BOS stage 0 (>90% baseline FEV1). Biopsies of OB affected lung were obtained from 8 (3M:5F) lung transplant recipients undergoing re-transplantation for advanced BOS stage 3 (<40% baseline FEV1), between 32 and 116 months after their first transplant. Normal control lung tissue was obtained from 6 unused donor lungs. Biopsies were subjected to immunolocalisation studies for E-cadherin (Figure 6A, i-iii), α-smooth muscle actin (α-SMA) (Figure 6A, iv-vi) and vimentin (Figure 6A, vii-ix). The percentage of small or medium sized airway epithelium staining positive for each marker was quantified and compared to non-transplanted control lungs (Figure 6B). Epithelium from normal control tissue was highly positive for E-cadherin (68.53% ± 2.54, n=6) but expressed little α-SMA (6.69% ± 2.28, n=6) or vimentin (2.05% ± 0.54, n=6). Epithelium from stable transplant recipients show a significant decrease in E-cadherin expression (51.81% ± 2.28, *p<0.05 n=6) and increase in vimentin expression (6.42% ± 0.77,
*p<0.05 n=6) compared to control tissue. However, α-SMA expression was not significantly different in the epithelium of stable transplant recipients compared to normal epithelium (4.89% ± 2.43, p>0.05 n=6). Epithelium in explanted lung tissue from patients with BOS showed a marked decrease in E-cadherin expression (40.06% ± 3.98, *p<0.05 n=8) and a significant increased expression of α-SMA (21.21% ± 4.34, *p<0.05 n=8) and vimentin (12.61% ± 2.32, *p<0.05 n=8) compared to epithelium in both normal tissue and stable recipients.

To further evaluate EMT in vivo, lung tissue from patients with BOS was analysed for co-expression of E-cadherin and α-SMA or S100A4 using confocal microscopy. Figure 7A shows an area of intact airway epithelium with clear E-cadherin expression at the junction between cells. In a number of the cells expressing E-cadherin we also see the expression of the early marker of EMT, S100A4 (see arrows) showing that these cells co-express both epithelial and mesenchymal markers. By comparison, in a more damaged area of epithelium (Figure 7B) we see that E-cadherin, although still present, is significantly reduced in intensity and very differently distributed throughout the cell. Furthermore, certain cells in the damaged epithelium also express αSMA, a late marker of EMT, co-localising in E-cadherin positive cells (see arrows).
Discussion

In our study, we have adopted a multifunctional approach to identification of EMT ex-vivo using cell morphology, upregulation of mesenchymal proteins, down regulation of epithelial proteins, secretion of ECM and a functional assessment of invasive and migratory capacity. This multifunctional approach is likely to be much more robust than reliance on any single marker of EMT. In addition our quantitative immunohistochemistry has demonstrated reduced epithelial E-cadherin expression and increases in vimentin and α-SMA expression in the airway epithelium of patients suffering from BOS compared to stable recipients and control lung samples.

The results of this study add significantly to the understanding gained from our previously reported pilot observations in the lung transplant airway [9]. The present study provides a comprehensive assessment of the ability of airway epithelial cells in the lung transplant to undergo EMT. Our observations offer significant insight into what might be an important mechanism in the development of OB. The hypothesis that EMT represents a common final pathway in the development of airway remodelling after lung transplant is attractive. It could explain why a broad range of possible insults to the transplanted lung, including both alloimmune driven injury and non-alloimmune injury, could produce a similar pathological endpoint.

TGF-β1 has been widely implicated in driving fibrosis in a variety of organs such as the liver and kidney but a mechanism by which this may occur in OB has not been fully elucidated. It has been previously observed that TGF-β1 expression is increased in patients with OB [10-13]. In addition, studies in the rodent heterotrophic tracheal transplant models of OB have shown that interrupting TGF-β1 binding to its receptor
or inhibiting its downstream signalling via the SMAD pathway reduces intraluminal airway matrix deposition [14].

It is well recognised in clinical practice that BOS is often first diagnosed or progresses more rapidly following a significant episode of airway injury such as a viral or bacterial infection and these sources of acute inflammation in the transplant airway may be very important in accentuating the process already driving OB. It has been previously suggested that repeated injury and persistent inflammation in the epithelium coinciding with defective regeneration is likely to lead to excessive fibroproliferation and obliteration of small airways in OB [24]. The role played by pro-inflammatory cytokines in driving excessive fibroproliferation is unclear and our data may highlight a possible novel mechanism in the lung.

Alveolar macrophages play a crucial role in initiating an acute inflammatory response in the lung and on activation produce a multitude of pro-inflammatory cytokines and growth factors [25]. A prominent product produced by macrophages is the pro-inflammatory cytokine TNFα which has been previously been reported to play a role in fibrotic disorders [26]. Also, an elevation in the level of intracellular TNFα found in CD4+ and CD8+ T-cells during acute lung transplant rejection has been reported [27] and TNFα produced by activated macrophages can increase metastatic potential in human colonic epithelial organoid models of colon cancer by accentuating EMT [28]. The addition of TNFα to cultures containing TGF-β1 significantly increased the expression of vimentin and fibronectin while further decreasing the expression of E-cadherin and cytokeratin-19 when compared to TGF-β1 alone. Our observations together with the observations of previous studies described above, suggests a
possible link between acute inflammation and accentuated TGF-β1 driven epithelial remodelling.

The demonstration of EMT in cultured cells always causes debate as to whether the observations represent true EMT or simply a ‘stress-response’ of cells in culture. It is our opinion that the demonstration of a true transition from an epithelial phenotype to a mesenchymal phenotype requires analysis of functional properties as well as protein expression and morphology. The production and secretion of MMP is one functional characteristic of mesenchymal cells. The ability of our ex-vivo cultures to acquire an invasive phenotype was analysed and TGF-β1 induces a threefold increase in the number of invasive cells and this is associated with an increased production and secretion of pro-MMP-9. As with protein expression, co-treatment of the cells with TGF-β1 and TNFα further accentuated the effects of TGF-β1 alone with an increase in both pro-MMP-9 production and the number of invasive cells observed. These observations support our hypothesis that epithelial cells in the airway may undergo EMT, penetrate the basement membrane and migrate into the sub-epithelial layer to lay down excessive connective tissue.

The cells that drive the obstruction of the bronchioles in OB are capable of depositing ECM proteins such as fibronectin and collagen I. We confirmed in our study that TGF-β1 induced a marked increase in collagen I secretion in our cells, however, in the presence of TNFα this was inhibited. This result is in agreement with previous studies showing that TNFα can inhibit collagen production both in vivo and in vitro [29-31]. Interestingly, although TGF-β1 induced an increase in fibronectin deposition the addition of TNFα had no significant effect on this phenomenon suggesting
different pathways may control collagen I and fibronectin deposition. This raises the intriguing possibility that episodic release of TNF-α in the presence of TGF-β1, such as could occur in response to repeated inflammatory insults to the lung, could drive migration and invasion of transformed epithelial cells but not deposition of collagen until the levels of TNF-α fall again in which case the transformed cells could then drive fibrosis.

Our demonstration of changes in expression levels of E-cadherin, vimentin and α-SMA in the epithelium of OB lung when compared to stable transplant recipients as well as our demonstration of co-localisation of epithelial and mesenchymal markers in individual cells in the epithelium of OB lung supports our hypothesis. In this study areas of intact epithelium were analysed in OB, stable and control lung. We did not analyse severely remodelled airways in the OB lung as these areas represent advanced lesions where the identification of the epithelium is impossible. Instead our approach allowed a direct comparison between epithelial and mesenchymal marker expression in our study groups.

We believe these observations represent a significant increase in the understanding of the potential for airway epithelium to undergo EMT and additionally highlight a possible synergistic action between acute inflammation and the ability of TGF-β1 to drive EMT. The mechanism driving this synergy needs more investigation to identify possible therapeutic targets that may interfere with these mechanisms.
References


fibrosis model derived from an alpha-smooth muscle actin-Cre transgenic mouse. 

Respir Res. 2007;8:1.


Figure 1

Phenotype and protein changes in primary bronchial epithelial cells (PBEC) induced by TGF-β1 and TNFα

PBEC were either left untreated (panel 1), treated with TGF-β1 (10ng/ml, panel 2), treated with TNFα (20ng/ml, panel 3) or treated with TGF-β1 and TNFα (panel 4) for 72 hours and the phenotype of the cells (i-iv), the expression of cytokeratin-19 (v-viii), fibronectin (ix-xii) and vimentin (xiii-xvi) monitored using immunocytochemistry. Cells left untreated (i) or treated with TNFα (iii) maintained the ‘cobblestone’ appearance characteristic of epithelial cells, express cytokeratin-19 (v and vii respectively) but do not express fibronectin (ix and xi respectively) or vimentin (xiii and xv respectively). Cells treated with TGF-β1 begin to lose cell-cell contact and adopt an elongated phenotype (ii), down regulate cytokeratin-19 expression (vi) and increase fibronectin (x) and vimentin (xiv) expression. Co-treatment of the cells with TGF-β1 and TNFα induces a more dramatic change in phenotype (iv) and further downregulated the expression of cytokeratin 19 (viii) and upregulated the expression of fibronectin (xii) and vimentin (xvi) compared to TGF-β1 alone. All images acquired on a Leica confocal microscope (x 63 magnification).

Figure 2

Change in protein expression induced by TGF-β1 and TNFα in human primary bronchial epithelial cells (PBEC) from the transplanted lung
PBEC were treated with TGF-β1 (10ng/ml) and / or TNFα (20ng/ml) for 72 hours and the expression of E-cadherin, fibronectin and vimentin assessed using immunoblotting. (A) Untreated cells and cells treated with TNFα express high levels of E-cadherin but little to no fibronectin or vimentin (Lanes 1 and 3 respectively). TGF-β1 treated cells down regulate E-cadherin expression and upregulate fibronectin and vimentin expression (Lane 2). Cells treated with TGF-β1 and TNFα further down regulate E-cadherin expression and upregulate fibronectin and vimentin expression compared to TGF-β1 alone (Lane 4). β-Tubulin is shown as a loading control.

(B) Quantitation of the relative band density per treatment ± s.e.m. (n=4, *p<0.05). (i) E-cadherin, (ii) fibronectin, (iii) vimentin. All results normalised to β-tubulin.

Figure 3

Accentuation of TGF-β1 driven EMT in human primary bronchial epithelial cells (PBEC) from the transplanted lung with low doses of TNFα.

(A) Cell culture media from cells treated with TGF-β1 (0 - 20ng/ml) for 72 hours was examined for pro-MMP-9 expression using gelatin zymography. PBEC treated with increasing concentrations of TGF-β1 secrete elevated levels of pro-MMP-9 into the cell culture media compared to untreated cells.

(B) PBEC were treated with TGF-β1 (1ng/ml or 3ng/ml) ± TNFα (1ng/ml or 20ng/ml) for 72 hours. The production of pro-MMP-9 was assessed using gelatin zymography and the expression of E-cadherin and fibronectin assessed using immunoblotting. Untreated cells express high levels of E-cadherin but little to no fibronectin and produce little pro-MMP-9 (Lanes 1). Cells treated with 1ng/ml of TGF-β1 down regulate E-cadherin expression and upregulate fibronectin and pro-MMP-9 expression
Addition of 1ng/ml of TNFα upregulates pro-MMP-9 expression compared to TGF-β1 alone but has no effect on E-cadherin or Fibronectin expression (Lane 3). Addition of 20ng/ml of TNFα further upregulates pro-MMP-9 expression and decreases E-cadherin expression but has no effect on Fibronectin expression (Lane 4). Cells treated with 3ng/ml of TGF-β1 down regulate E-cadherin expression and upregulate fibronectin and pro-MMP-9 expression (Lane 5). Addition of TNFα at 1ng/ml down regulates E-cadherin expression and upregulates fibronectin and pro-MMP-9 expression (Lane 6). Addition of 20ng/ml of TNFα further upregulates pro-MMP-9 expression but has no effect on E-cadherin and Fibronectin expression (Lane 7).

(C) PBEC were treated with TGF-β1 (10ng/ml) ± TNFα (1-20ng/ml) for 72 hours. The production of pro-MMP-9 was assessed using gelatin zymography and the expression of E-cadherin, vimentin and fibronectin assessed using immunoblotting. Untreated cells express high levels of E-cadherin but little to no fibronectin, vimentin or pro-MMP-9 (Lanes 1). Cells treated with TGF-β1 down regulate E-cadherin expression and upregulate fibronectin, vimentin and pro-MMP-9 expression (Lane 2). Addition of TNFα induces a dose dependant decrease in E-cadherin expression and a dose dependant increase in fibronectin, vimentin and pro-MMP-9 expression (Lane 3-5).

**Figure 4**

TGF-β1 and TNFα induce increased invasive potential in human primary bronchial epithelial cells (PBEC) from the transplanted lung.
(A) PBEC were treated with TGF-β1 (10ng/ml), TNFα (20ng/ml), or TGF-β1 and TNFα for 72 hours and the number of invasive cells assessed using a Matrigel invasion assays. Quantitation of the mean number of invasive cells per treatment ± s.e.m. (n=4, *p<0.05). Untreated or TNFα treated cells show little to no invasion. TGF-β1 treated cells show an increase in invasion that is further accentuated by co-treatment of the cells with TGF-β1 and TNFα.

(B) Cell culture media from cells treated with TGF-β1 (10ng/ml) ± TNFα (20ng/ml) for 72 hours was examined for pro-MMP-9 expression using gelatin zymography. PBEC treated with TGF-β1 secrete an elevated level of pro-MMP-9 into the cell culture media compared to untreated cells (Lane 2 and 1 respectively). Treatment of the cells with TGF-β1 and TNFα accentuated the release of pro-MMP-9 seen with TGF-β1 alone (Lane 3).

(C) Quantitation of the relative band density of pro-MMP-9 per treatment ± s.e.m. (n=4, * p<0.05).

Figure 5
Deposition of ECM by human primary bronchial epithelial cells (PBEC) from the transplanted lung

PBEC were treated with TGF-β1 (10ng/ml), TNFα (20ng/ml), or TGF-β1 and TNFα for 72 hours and the deposition of collagen I and fibronectin assayed using TCA precipitation. (A) PBEC treated with TNFα or left untreated do not deposit collagen I or fibronectin (Lanes 3 and 1 respectively). TGF-β1 treated PBEC deposit collagen I and fibronectin (Lane 2). Co-treatment of the cells with TGF-β1 + TNFα inhibits the
TGF-β dependant deposition of collagen I while having no effect on fibronectin deposition (Lane 4).

(B) Quantitation of the relative band density per treatment ± s.e.m. (n=4, *p<0.05).
(i) Collagen I, (ii) Fibronectin.

Figure 6
Expression of markers of EMT in vivo in human lung

A) Lung sections from normal control lung (i, iv and vii), from stable transplants (ii, v and viii) and OB affected tissue (iii, vi, ix) were stained for E-cadherin (i-iii), α-SMA (iv-vi) and vimentin (vii-ix). An IgG control is shown (x). All sections shown at magnification x100.

B) The percentage of the epithelium stained positive for E-cadherin (i), α-SMA (ii) and vimentin (iii) was quantified. Epithelium from normal lungs are strongly positive for E-cadherin (68.53% ± 2.54, n=6) and express little α-SMA (6.69% ± 2.28, n=6) and vimentin (2.05% ± 0.54, n=6). Epithelium from stable transplant recipients show a decrease in E-cadherin (51.81% ± 2.28, *p<0.05 n=6) expression and increase in vimentin (6.42% ± 0.77, *p<0.05 n=6) expression. α-SMA expression was not significantly different in stable transplant recipients compared to normal (4.89% ± 2.43, p>0.05 n=6). Epithelium from OB lungs show a decrease in E-cadherin expression (40.06% ± 3.98, *p<0.05 n=8) and increased expression of α-SMA (21.21% ± 4.34, *p<0.05 n=8) and vimentin (12.61% ± 2.32, *p<0.05 n=8) compared to normal control lung and stable transplant recipients.

Figure 7
Co-expression of E-cadherin and S100A4 / α-Smooth Muscle Actin (α-SMA) in epithelial cells in airway from recipients with obliterative bronchiolitis.

Sections from OB lung were stained for E-cadherin and S100A4 (Figure 8A) or E-cadherin and α-SMA (Figure 8B). A) In an area of intact airway epithelium, E-cadherin (green) expression at the interface between cells is clearly visible. In some cells the expression of S100A4 (red) (see arrows) is co-localised with E-cadherin. B) In a more damaged area of epithelium we see reduced E-cadherin (green) expression associated with an increase in the expression of αSMA (red) (see arrows). Image acquired on a Leica confocal microscope (x 63 magnification).
**Supplementary figure 1**

Quantitation of the relative band density per treatment ± s.e.m. (*p<0.05, n=5). (i) E-cadherin, (ii) fibronectin, (iii) vimentin. All results normalised to β-tubulin.

**Supplementary figure 2**

Quantitation of the relative band density per treatment ± s.e.m. (*p=0.0036, n=5). (i) Collagen I (ii) Fibronectin.

**Supplementary figure 3**

Quantitation of the relative band density of pro-MMP-9 per treatment ± s.e.m. (*p=0.0001, n=5).
Antibodies and Reagents

Recombinant human TGF-β1 (100-21) was purchased from Peprotech (London, UK).
Recombinant human TNFα (T-0157) was purchased from Sigma (St. Louis, MO).
Antibodies to vimentin (M7020), E-cadherin (M3612) S100A4 (A5114) and cytokeratin-19
(M0888) were purchased from Dako Cytochemistry (Glostrup, Denmark). Antibodies to α-
smooth muscle actin (A2547), and fibronectin (F3648) were purchased from Sigma.
Collagen I (sc-8783) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz,
CA). All other reagents unless otherwise stated were of the highest available purity from
commercial sources.

Cell Culture

Briefly, bronchial brushings from subsegmental bronchi were suspended into sterile
phosphate buffered saline. Subsequently, an equal volume of RPMI supplemented with 10%
foetal calf serum (FCS) was added and the cells pelleted at 1,000 x g for 5 minutes. The
pellet was re-suspended in small airway growth media (SAGM) supplemented with SAGM
SingleQuots (both purchased from Lonza) and 100U/ml penicillin - 100μg/ml streptomycin
and transferred to a T-25 flask pre-coated with 0.5% collagen (Nutacon). Cells were
maintained at 37°C in a humidified 95% air / 5% CO2 incubator until confluence. Cells were
not advanced beyond P4 to ensure a uniform epithelial phenotype.

Gelatin Zymography

To assay for pro-MMP-9 secretion conditioned media from cells treated as indicated were
separated on an 8% SDS-PAGE gel containing 0.1% gelatin. Following electrophoresis, gels
were incubated in 2.5% (v/v) Triton X-100 for 30 minutes and then overnight in developing
buffer (50mM Tris-HCl, 0.2M NaCl, 5mM CaCl2) at 37°C. Gels were stained with
Coomassie blue stain (40% methanol, 10% acetic acid, 0.05% Coomassie blue) and destained (40% methanol, 10% acetic acid) until the desired contrast was achieved.

**Human Lung Tissue Sampling**

This use of human tissue was approved by Local Research Ethics Committee.

Normal control tissue was obtained from donor lungs assessed for, but not accepted for, use in clinical lung transplant. Stable lung transplant recipients underwent transbronchial biopsy by an established technique. Tissue from recipients with OB was obtained from explanted lung at the time of retransplantation.

All lung samples were fixed in 10% formalin, embedded, sectioned and stained with H&E. Acute rejection and other pathological changes in stable patients were excluded and OB confirmed in explanted lungs by an experienced pulmonary histopathologist. Sequential sections from stable recipients, OB recipients and normal control lungs were stained with antibodies against E-cadherin, vimentin and α-smooth muscle actin using a modified immunoperoxidase method (Envision; Dako).

Areas of intact airway epithelium were identified under high power (magnification x100) as close as possible to the same area of epithelium in each section and for each marker. Five high power fields per case were evaluated. The airway epithelium was identified visually and isolated from the rest of the image using image analysis software (Adobe Photoshop CS3). The area of positive staining within the isolated epithelium was then identified using a densitometry based analysis for each field. The five high power fields were averaged for each marker in each case and used in the analysis between different patient groups.
Supplementary File 1

(i)

(ii)

(iii)
Supplementary File 2

(i)

Relative band density

No  TGF-β1  TNFα  TGF-β1 + TNFα

Collagen I

(ii)

Relative band density

No  TGF-β1  TNFα  TGF-β1 + TNFα

Fibronectin
Supplementary File 3

![Graph showing relative band density for MMP-9 with No, TGF-β1, and TGF-β1 + TNFα treatments.](http://thorax.bmj.com/)

- No
- TGF-β1
- TGF-β1 + TNFα
Epithelial to Mesenchymal Transition (EMT) and Airway Remodelling after Human Lung Transplantation.

Lee A Borthwick, Sean M Parker, Kathrine A Brougham, Gail E Johnson, Marta R Gorowiec, Chris Ward, James Lordan, Paul Corris, John A Kirby and Andrew J Fisher

Thorax published online February 12, 2009

Updated information and services can be found at:
http://thorax.bmj.com/content/early/2009/02/12/thx.2008.104133

These include:

Supplementary Material
Supplementary material can be found at:
http://thorax.bmj.com/content/suppl/2009/09/09/thx.2008.104133.DC1

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
- Editor's choice (127)
- Screening (oncology) (407)
- Transplantation (184)
- Bronchiolitis (112)
- Bronchitis (235)
- Cardiotoracic surgery (676)
- Interstitial lung disease (559)
- TB and other respiratory infections (1273)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/