Epithelial-mesenchymal transition in primary human bronchial epithelial cells is Smad-dependent and enhanced by fibronectin and TNF-α

Joana Câmara, Gabor Jarai*

Abstract

Background: Defective epithelial repair, excess fibroblasts and myofibroblasts, collagen overproduction and fibrosis occur in a number of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis. Pathological conversion of epithelial cells into fibroblasts (epithelial-mesenchymal transition, EMT) has been proposed as a mechanism for the increased fibroblast numbers and has been demonstrated to occur in lung alveolar epithelial cells. Whether other airway cell types also have the capability to undergo EMT has been less explored so far. A better understanding of the full extent of EMT in airways, and the underlying mechanisms, can provide important insights into airway disease pathology and enable the development of new therapies. The main aim of this study was to test whether primary human bronchial epithelial cells are able to undergo EMT in vitro and to investigate the effect of various profibrotic factors in the process.

Results: Our data demonstrate that primary human bronchial epithelial cells (HBECs) are able to undergo EMT in response to transforming growth factor-beta 1 (TGF-β1), as revealed by typical morphological alterations and EMT marker progression at the RNA level by real-time quantitative polymerase chain reaction and, at the protein level, by western blot. By using pharmacological inhibitors we show that this is a Smad-dependent mechanism and is independent of extracellular signal-related kinase pathway activation. Additional cytokines and growth factors such as tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL1β) and connective tissue growth factor (CTGF) were also tested, alone or in combination with TGF-β1. TNF-α markedly enhances the effect of TGF-β1 on EMT, whereas IL1β shows only a very weak effect and CTGF has no significant effect. We have also found that cell-matrix contact, in particular to fibronectin, an ECM component upregulated in fibrotic lesions, potentiates EMT in both human alveolar epithelial cells and HBECs. Furthermore, we also show that the collagen discoidin domain receptor 1 (DDR1), generally expressed in epithelial cells and HBECs. Furthermore, we also show that the collagen discoidin domain receptor 1 (DDR1), generally expressed in epithelial cells, is downregulated during the EMT of bronchial epithelium whereas DDR2 is unaffected. Our results also suggest that bone morphogenetic protein-4 is likely to have a context dependent effect during the EMT of HBECs, being able to induce the expression of EMT markers and, at the same time, to inhibit TGF-β induced epithelial transdifferentiation.

Conclusions: The results presented in this study provide additional insights into EMT, a potentially very important mechanism in fibrogenesis. We show that, in addition to alveolar epithelial type II cells, primary HBECs are also able to undergo EMT in vitro upon TGF-β1 stimulation via a primarily Smad 2/3 dependent mechanism. The effect of TGF-β1 is potentiated on fibronectin matrix and in the presence of TNF-α, representing a milieu reminiscent of fibrotic lesions. Our results can contribute to a better understanding of lung fibrosis and to the development of new therapeutic approaches.

* Correspondence: gabor.jarai@novartis.com
Novartis Institutes for BioMedical Research, Respiratory Disease Area, Wimblehurst Road, Horsham, RH12 5AB West Sussex, UK

© 2010 Câmara and Jarai; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Background

Epithelial-mesenchymal transition (EMT) is the trans-differentiation of epithelial cells into mesenchymal cells. During this process, epithelial cells are removed of cellular polarity and epithelial cell-cell, as well as cell-matrix adhesion contacts are remodelled. Markers of polarized epithelial cells, such as E-cadherin and some cytokeratins, are lost whereas markers of mesenchymal cells such as vimentin, N-cadherin or of myofibroblasts, as α-smooth muscle actin (α-SMA) are acquired [1]. As a result of these changes, epithelial cells are converted into motile fibroblasts to myofibroblasts (FMT) that are key cells in both the degradation and de novo synthesis of the extracellular matrix (ECM). The trans-differentiated cells are able to migrate through basement membranes into other areas in the tissue. EMT has been shown to play a role in organogenesis during embryonic development and is an important biological process in normal wound healing [2]. However, disregulated EMT also appears to occur in disease, contributing to cancer progression and metastasis, as well as to the pathogenesis of chronic degenerative fibrotic disorders in several organs including the lung [3,4].

Dysfunction of the lungs and conducting airways at different levels may lead to a number of respiratory diseases such as asthma, cystic fibrosis, chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis and an important role for EMT has been proposed in the pathogenesis of many of these [5-8]. Particularly, recent studies have identified important mechanisms that control the mesenchymal trans-differentiation of lung alveolar epithelial cells (AECs) in pulmonary fibrosis. It has been shown that both rat and human lung AECs can undergo EMT in response to exposure to transforming growth factor (TGF)-β1 in culture [9-11]. The EMT of alveolar cells was later demonstrated in vivo by generating transgenic mice expressing β-galactosidase exclusively in lung epithelial cells and following their fate after inducing lung fibrosis by intranasal instillation of adenoviral TGF-β1 [12]. Further evidence for the re-occurrence of EMT in adult tissue, as well as for its possible role in pathogenesis, was obtained from lung tissue from idiopathic pulmonary fibrosis (IPF) patients by co-localization of myofibroblast and epithelial markers in AECs overlying fibroblastic foci, which are key features of the disease and are important for both diagnosis and disease progression [11].

A number of growth factors and cytokines are able to induce EMT in vitro. TGF-β is a central regulator of pulmonary inflammation and fibrosis [13]. TGF-β1 has been reported to induce EMT in AECs as well as the differentiation of fibroblasts to myofibroblasts and was also shown to be upregulated in IPF patients [11,14-16]. Several other factors have also been identified that appear to act in concert with or to modulate TGF-β activity. Whereas on its own IL (interleukin)1β does not induce EMT in A549 cells and similarly, tumour necrosis factor (TNF)-α has only a mild effect, when combined with TGF-β the two factors enhance EMT in type II AECs in both rat-derived cells and in the human A549 cell line. Similarly, connective tissue growth factor (CTGF) is produced by fibroblasts and bronchial epithelial cells and is also known to regulate fibroblast proliferation and collagen deposition in coordination with TGF-β [17,18].

The molecular mechanisms underlying EMT in the lung have not been explored in great detail. TGF-β1 is known to signal through two main pathways: the canonical Smad-dependent pathway and the alternative mitogen-activated protein kinase (MAPK) pathway [19]. Both of these have been implicated in EMT and FMT [7,20-22]. In addition, bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, are involved in a variety of biological functions including lung morphogenesis [23]. The balance and interplay between BMP and TGF-β signalling has been demonstrated to be important to development [24]. Smad2 and Smad3 are activated by activin receptor-like kinase (ALK)-4, -5 and -7 receptors in response to TGF-β ligands, whereas Smad1, Smad5 and Smad8 are activated by ALK-1, -2, -3 and -6 in response to BMPs [25]. Several BMPs, particularly BMP7 and BMP4, have been implicated in EMT in various organs such as the kidney and lung [26,27].

The ECM also appears to be playing key roles in fibrosis not only as a major cause of the disruption of tissue architecture but also due to its ability to activate cellular pathways. The main ECM components of lung basement membranes are collagen IV and laminin [28]. For example, in IPF lung basement membranes are disrupted and the expression of collagen I, collagen III and fibronectin is upregulated [29-31]. Furthermore, fibronectin has been recently shown to drive the EMT of mouse AECII [12]. Two main families of receptors for ECM proteins have been implicated in lung cancer and fibrosis [32]: integrins and the discoidin domain receptors (DDRs). While integrins can sense various ECM proteins, DDRs appear to play a more specialized role. They are receptor tyrosine kinases that function as collagen receptors and have intrinsic signalling properties [33]. DDR1 is expressed primarily in epithelial tissues, whereas the other member of the subfamily, DDR2, is mostly present in mesenchymal cells [34]. The differential expression of DDR receptors appears to be context and disease dependent. Human nasopharyngeal carcinoma and HAK-1B hepatoma cells showed overexpression of DDR2, whereas DDR1 expression was unaltered.
In contrast, in human lung cancer tissue DDR1 was upregulated, whereas DDR2 expression was unaffected [37]. As collagen receptors, DDr may play a role in mechanisms related to excessive collagen deposition, such as EMT or FMT in fibrosis. However, they have not yet been explored in this context.

Another mechanism that is proposed to contribute to the remodelling of diseases is the disturbed physiological balance between matrix formation and degradation. This equilibrium appears to be lost in fibrotic diseases and several matrix metalloproteases (MMPs), involved in both matrix turnover and growth factor activation, such as MMP-2 and MMP-9, have been shown to be upregulated [38]. These two MMPs also play a role in the cleavage and activation of the main profibrotic growth factor, TGF-β, and, hence, they may be of particular importance [39].

The identification of the specific epithelial cell types that are able to undergo EMT, and the underlying mechanisms, has recently been a matter of intense investigation as this may be an important process by which fibroblast numbers increase, constituting the fibroblastic foci correlated with severity of pulmonary fibrosis [40]. As described above, most of the work on lung EMT has focused on the alveolar epithelium. The increased expression of the mesenchymal marker S100A4 in airway epithelium biopsy samples from lung transplant patients suggests that the epithelium lining the conducting airways might also be able to differentiate into mesenchymal cells [41]. In the current study, we asked whether primary human bronchial epithelial cells, similarly to AECs, can also undergo EMT. We demonstrate that these cells can phenotypically convert to mesenchymal cells, an effect potentiated in a pathological environment reminiscent of fibrotic disease composed of TGF-β1 and fibronectin. Furthermore, we show that this is a Smad-dependent mechanism and implicate BMP4 and DDR1 in bronchial epithelial cell transdifferentiation.

Materials and methods

Cell culture

A549 cells (ATCC) were maintained in DMEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and human bronchial epithelial cells (HBECs; Lonza, Basel, Switzerland) were maintained in BEGM medium (Lonza), both at 37°C in the presence of 5% CO2 in a humidified incubator. Except for the experiments shown in Figure 1, all further experiments with HBECs were performed in BEGM medium only, unless indicated otherwise.

EMT assay

A549 cells were seeded at a density of 6 x 10⁴ cells/well (12-well plates) in DMEM 1% FBS and HBECs at a density of 10⁶ cells/well in 1:100 BEGM:BEBM (six-well plates). Cells were allowed to adhere for 1 day and then changed into media containing 5 ng/ml of TGF-β1 (R&D Systems, MN, USA). A549 cells were differentiated for 48 h and HBECs for 3-5 days. Human BMP4 recombinant protein (5 or 50 ng/ml, R&D Systems) was used in some EMT experiments.

EMT assay in the presence of pharmacological inhibitors

A549 and HBECs were incubated with pharmacological inhibitors for 1 h prior to the addition of TGF-β1 (5 ng/ml) for EMT induction for 48 h or 3-5 days, respectively (see legends for Figures 1, 2, 3, 4, 5 for further description). The Smad pathway inhibitor SB431542 (10 μM, Sigma-Aldrich) and the ERK pathway inhibitor PD98059 (10 μM, Calbiochem) were used.

Western blotting

Cells were lysed in a buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris pH 8.0, 1 mM sodium orthovanadate, 5 mM NaF and protease inhibitor cocktail (Sigma, NY, USA). Lysates were subjected to SDS-PAGE on a 4-10% Bis-Tris gels (NuPAGE, Invitrogen). They were then transferred onto nitrocellulose membranes (Invitrogen) and blocked in Tris-buffered saline (TBS), 0.1% Tween-20, 5% non-fat dried milk for 1 h at room temperature (RT). The blots were then washed three times in TBS 0.1% Tween (TBS-T) and incubated for 1 h at RT with a horseradish peroxidase-labelled secondary antibody (Invitrogen). After repeated washing in TBS-T the immunoreactive proteins were detected by chemiluminescence (ECL; Pierce, L, USA) according to the manufacturer’s instructions. The following antibodies were used: E-cadherin (Abcam, MA, USA), N-cadherin (Zymed, CA, USA), vimentin (Abcam), α-SMA (Sigma), MMP-2 (R&D Systems), pSmad2 (Cell Signaling, MA, USA), pSmad1/5/8 (Cell Signaling), DDR1 (R&D Systems), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, CA, USA).

Real-time quantitative polymerase chain reaction (PCR)

Total RNA was prepared from cells using the RNeasy plus mini kit (Qiagen), according to manufacturer’s instructions and RNA was reverse transcribed into cDNA (Applied Biosystems, CA, USA). The cDNA was then amplified by real-time quantitative TaqMan PCR in a reaction containing 1× Taqman Universal PCR master mix, 1 μM primers, 0.3 μM probe and analysed using an ABI Prism 7900 sequence detector (Applied Biosystems). The house-keeping gene GAPDH was used as internal control. Data were normalized to GAPDH and expressed as fold change over control (Table 1).

Statistical analysis

The data show the mean ± standard deviation of at least three independent experiments (n = 3), unless otherwise indicated. Statistical significance was determined using
one-way ANOVA or Student's t test. Statistical analysis was performed using GraphPad Prism software.

Results

TGF-β1 induces EMT in HBECs

In order to address the question of whether EMT can occur in AECs we first stimulated HBECs with TGF-β1 for 3-5 days. We observed a dramatic upregulation of collagen I mRNA expression and downregulation of the epithelial marker E-cad, as well as an enhanced expression of the metalloprotease MMP-2 after EMT induction (Figure 1a). Similarly, an increased expression of several mesenchymal markers including N-cad, vimentin, MMP-2 and of the myofibroblast marker α-SMA was detected at the protein level (Figure 1b). In contrast, a reduced expression of E-cad was observed in the presence of TGF-β1 (Figure 1b). We concluded from these data that TGF-β1 induces EMT in HBECs as revealed by the downregulation of epithelial markers and an upregulation of mesenchymal markers at both the RNA and the protein level. A concomitant upregulation of phospho-Smad2 with EMT induction in HBECs prompted us to investigate which signaling pathways were involved in this phenomenon.

Smad inhibition prevents TGF-β1 induction of EMT in HBECs

In order to determine which of two main pathways, the Smad-dependent pathway or MAPK/ERK1/2 pathway - both of which can be activated by TGF-β1, were involved in EMT in HBECs, we treated cells with pharmacological inhibitors for each pathway prior to the induction of EMT by TGF-β1. For the inhibition of the Smad pathway, we used SB431542, a potent and specific inhibitor of TGF-β type I receptor kinases (ALK-4,-5,-7), therefore an inhibitor of TGF-β signalling with no effect on ALK-1,-2,-3 and -6 and, hence, BMP signalling [42]. We found that treatment of HBECs with SB431542 before the addition of TGF-β1 prevented EMT, as demonstrated by the unchanged expression of marker proteins and their mRNA (Figure 2b and 2d) as well as by the preserved cellular morphology (Figure 2a). In contrast, the MEK/ERK inhibitor PD98059 failed to fully inhibit EMT (Figure 2a and 2b). Taken together, these results show that HBECs can undergo EMT in vitro in response to TGF-β1 via a primarily Smad-dependent mechanism.

BMP4 is involved in EMT in HBECs

Two BMPs, BMP7 and BMP4, have so far been implicated in EMT in various organs. BMP7, and not BMP4,
Transforming growth factor (TGF)-β1-induced epithelial mesenchymal transition (EMT) in human bronchial epithelial cells (HBECs) is Smad-dependent. HBECs that have been pre-incubated with the activin receptor-like kinase (ALK)-5 inhibitor SB431542 (SB, 10 μM) 1 h before the addition of TGF-β1 (5 ng/ml) are unable to undergo EMT after 5 days of differentiation. By contrast, mitogen-activated protein kinase inhibition by PD98059, a MEK inhibitor (PD, 10 μM), only partially inhibits EMT. (a) Representative phase contrast images (10× magnification, scale bar = 10 μM) show that HBECs in the presence of TGF-β1 lose cell-cell contact, become more sparse and change into an elongated fibroblastoid morphology. This effect is inhibited after pre-incubation of the cells with the ALK-4,-5,-7 inhibitor SB but not by the MEK inhibitor PD. (b) Western-blot analysis shows that SB completely abolishes TGF-β1 gene regulation of E-cad and the phosho-Smad2 signal is fully inhibited in the presence of SB. An antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (c) Western-blot analysis shows that the upregulation of matrix metalloprotease (MMP)-2 by TGF-β1 is partially inhibited by the presence of bone morphogenetic proteins (BMP)4 confirming the quantitative polymerase chain reaction (qPCR) results shown in (d). Phosho-Smad1/5/8 shows active BMP signalling in the presence of BMP4 which is inhibited in the presence of TGF-β1. An antibody against GAPDH was used as a loading control. (d) q(PCR) analysis shows that SB inhibits gene expression changes of collagen I α1, MMP2, MMP-9 and E-cad induced by TGF-β1 (top 2 panels). BMP4 (50 ng/ml) induces a significant upregulation of collagen I in the presence of TGF-β1, as compared to TGF-β1 alone (top left panel) and has no significant effect on E-cad expression either alone or in combination with TGF-β1 (top right panel). In contrast, the upregulation of MMP-2 and MMP-9 expression in the presence of TGF-β1 is significantly inhibited by concomitant BMP4 treatment (bottom 2 panels). The relative expression level of each gene was normalized to GAPDH mRNA in the same sample. Statistical significance was determined by one-way ANOVA followed by Tukey test; ***P < 0.001.
has been reported to reverse TGF-β1-induced EMT in the kidney and to induce mesenchymal to epithelial transition [27,43]. However, the evidence for the importance of BMP7 in the lung is less convincing, as BMP7 does not appear to inhibit EMT in vitro in A549s or in vivo in the bleomycin model [44]. Similarly, previous data on the role of BMP4 in EMT in the lung is rather conflicting. Whereas BMP4 was shown to induce EMT in the BEAS2B epithelial cell line, overexpressing Gremlin, an inhibitor of BMP4 signalling, also appeared to enhance EMT in A549 cells [26,45].

In order to determine whether BMP4 may also play a role in fibrosis pathogenesis of the airways via modulating EMT in HBECs, we incubated human BMP4 recombinant protein alone or before inducing EMT with TGF-β1 in HBECs. We found that BMP4 enhanced collagen I mRNA expression in the presence of TGF-β1 (Figure 2d). In contrast, the mRNA expression of the metalloproteases MMP-2 and MMP-9 induced by TGF-β1 was inhibited by BMP4 (Figure 2d). An antagonistic effect of the BMP4 protein upon the TGF-β1-mediated upregulation of MMP2 protein was also confirmed by western blot (Figure 2c) and by assessing cellular morphology using phase contrast microscopy (data not shown). In order to assure that BMP signalling was active in the presence of BMP4 recombinant protein, we used an antibody that cross-reacts with the phosphorylated forms of Smads 1, 5 and 8, downstream effectors of BMP signalling. We found increased phosphorylation of Smad2/3 in the presence of both low (5 ng/ml) and high (50 ng/ml) concentrations of human BMP4 recombinant protein, an effect that could be abrogated if TGF-β1 was concomitantly added (Figure 2c). These results suggest a possible interplay between the BMP and TGFβ pathways during EMT in HBECs.

**TNF-α enhances the effect of TGF-β1 on EMT induction in HBECs**

Next, we asked whether other cytokines and growth factors previously implicated in interstitial lung disease, such as TNF-α, IL1β and CTGF [46,47], alone or in combination with TGF-β1, would induce or alter the progression of EMT in HBECs. While TGF-β1 is involved in several biological processes and in EMT in many developmental and pathological scenarios, other
mediators are likely to modulate TGF-β signalling conferring context specificity. Indeed, we found that TNF-α markedly enhanced the effect of TGF-β1 on EMT, whereas IL1β appeared to have a weak effect on MMP-9 expression but not on the expression of MMP-2 or E-cad (Figure 3a and 3b). Interestingly, CTGF, a profibrotic growth factor and a downstream effector of TGF-β signalling, had no significant effect when added alone or in combination with TGF-β1.

Disease associated matrix enhances EMT in HBECs

In order to investigate if, in addition to soluble factors, cell-matrix contact would also modulate EMT in human airway epithelial cells we tested the effect of ECM components that have been associated with fibrotic lesions in IPF. Initially we tested whether type II A549s were affected by fibronectin and we found that alone it significantly upregulated mRNA expression of two important EMT markers, collagen I and MMP-2 (Figure 4a). Similarly, at the protein level, we detected significant induction of the mesenchymal markers N-cad and vimentin and the downregulation of the epithelial marker E-cad when cells were grown on fibronectin compared to cells grown on control plates coated with poly-D-lysine (PDL; Figure 4b).

We next looked to see if cellular contact with the fibronectin observed in AECII cells could also contribute to the progression of EMT in HBECs. In contrast to what we observed in alveolar type II epithelial cells, fibronectin on its own did not seem to have a significant effect on EMT in HBECs. Remarkably, however, when applied in combination with TGF-β1, fibronectin significantly enhanced TGF-β1-induced EMT in HBECs, as demonstrated by both the induction of mesenchymal and the downregulation of epithelial markers (Figure 4c and 4d).

The collagen receptor DDR1 is downregulated in bronchial epithelial cells during EMT

Since the collagen receptors, DDRs, have been associated with remodelling and fibroproliferative diseases, we addressed the question of whether DDR expression
was altered during EMT in bronchial epithelial cells. Although no DDR expression changes were detected in A549 epithelial cells during EMT (data not shown), DDR1 was consistently downregulated in HBECs at both the RNA and the protein level during TGF-β1-induced EMT (Figure 5a and 5b). In contrast, the other discoidin domain collagen receptor DDR2 expression did not show significant changes under any of the conditions tested.

Discussion
The majority of the work on EMT in pulmonary fibrosis has so far been focused on AECs and it is less clear whether bronchial epithelial cells are able to undergo EMT. Further understanding of the EMT mechanisms in fibrosis is potentially of great importance as it could lead to approaches which impede or reverse the increase in the number of fibroblasts in fibrotic diseases.

Our work reveals that, in addition to AECII, primary HBECs are also able to convert to mesenchymal phenotypes in a Smad-dependent manner. While this work was in progress, other studies have recently also reported EMT in human airway epithelial cells. Zhang and colleagues have reported EMT-related changes in the polarized layer forming airway epithelial cell line 16HBE-14o [48] and our study extends these findings to primary HBECs. Borthwick and colleagues also support these findings [49]. In their study they demonstrated...
EMT of airway epithelial cells in vitro and mesenchymal cell marker expression in the epithelial cells in biopsies from obliterative bronchiolitis patients using immunohistochemistry. Furthermore, in a very recent study, airway epithelial cells isolated from asthmatics have been shown to be sensitized to TGF-β induced EMT [50]. These studies and our present work together provide supportive evidence for the occurrence of EMT in airway epithelium in addition to the more extensively described process in the alveoli. Interestingly, it has been very recently reported that mouse tracheal epithelial cells can be induced to undergo EMT with TGF-β1 in vitro [51]. Whether this is the case with human tracheal cells remains to be determined. Nevertheless, a number of specialized airway and lung epithelial cell types appear to maintain an intrinsic plasticity that allows them to transdifferentiate into mesenchymal cells in pathological settings.

Our results also demonstrate that fibronectin alone is able to promote EMT in A549 epithelial cells and therefore extends the findings of Kim et al. [12] to the human setting, confirming an important role for fibronectin in the induction of EMT in human AECII. While fibronectin alone shows no significant effect in HBECs, EMT in these cells is potentiated in a pathological environment where fibronectin and excess TGF-β1 co-exist, further strengthening the relevance of our findings in disease.

Our study also gives new insight into some of the mechanisms of EMT in bronchial epithelial cells. We show that DDR1 is downregulated during EMT in bronchial epithelium, whereas DDR2 is unaffected. Given that DDR1 is mostly expressed by epithelial, and not mesenchymal tissue, the reduction of its expression during EMT correlates with a decrease in epithelial identity. Remarkably, in a study comparing rat skin fetal fibroblasts of scar-free or scar-forming gestational ages, DDR1 was found to be downregulated at stages where scarring was no longer possible and collagen I was produced in excess. DDR2 levels were similar throughout gestation [52]. An analogy can be drawn with fibrosis in the adult where collagen I is overproduced and fibroblast numbers increase partly due to EMT and scarring of the tissue occurs. It therefore seems that, in situations of excess collagen and fibrosis, DDR1 can be downregulated in epithelial cells. Our findings also support recent reports on SLUG-induced EMT in Madin-Darby canine kidney cells with reduced DDR1 expression when induced to undergo EMT [53]. Shintani and colleagues (2008) [54] showed that collagen I-mediated upregulation of N-cadherin required cooperative signals from DDR1 and integrin β1 in pancreatic cells. Since we and others have shown that N-cadherin is upregulated in bronchial EMT, it will be interesting to evaluate in future studies whether integrins also play a role and whether there is any interplay with DDR1 in EMT in HBECs.

We have also investigated the effect of BMP4 during EMT of primary HBECs. Our data suggest a possible dual and context-dependent role for this growth factor. While it induced morphological EMT-like changes in HBECs, and was also able to upregulate collagen I expression, our data demonstrate that when applied in combination, BMP4 also inhibited TGF-β1-induced epithelial cell differentiation as demonstrated by its effect on MMP expression. Two epithelial cell lines were previously shown to have opposite EMT responses to BMP mediators. BEAS-2B, an airway epithelial cell line, was induced to undergo EMT when exposed to recombinant BMP4 [26,47]. On the other hand, the A549 AEC cell line overexpressing the BMP inhibitor Gremlin also showed enhanced EMT [26,47]. Furthermore, whereas Gremlin is over-expressed in the lungs of IPF patients and the responsiveness of IPF fibroblasts to BMP4 in culture is impaired, BMP4 and BMP7 activity is not reduced in IPF biopsies or in asbestos-exposed mouse lungs, as would be predicted by the increased expression of the BMP inhibitor Gremlin in IPF [47,55]. The BMP4 induced mechanisms thus appear to be complex as BMP4 activity can be modulated depending on the cell type and the cytokine milieu. It is interesting to speculate that the downregulation of MMPs, after BMP treatment, may not be a direct EMT effect but a parallel mechanism caused by the inhibitory effect of BMP signalling upon proteasome function [56]. In previous studies both MMP-2 and MMP-9 have been found to be downregulated by proteasome inhibition and the proteasome pathway has previously been linked to BMP signalling [56,57]. However, further studies are needed in order to unravel the complexities of BMP-TGFβ signalling interactions in EMT in bronchial epithelium.

The induction of the expression of MMPs, including MMP-2 and MMP-9 is well demonstrated in the context of cancer metastasis and is purported to play a role in both angiogenesis and cellular invasiveness [58] by contributing to the enhanced migratory phenotype of the cancer cell. Similarly, it was shown that, in vitro injury models of repairing HBECs, MMP-9 accumulated in migrating HBECs at the leading edge of the wound [59]. Given the dramatic increase of MMP-9 expression that we have observed in HBECs undergoing EMT, it is reasonable to speculate that HBECs also gain a migratory phenotype during fibrosis pathogenesis in the lung. Our planned investigations will address this intriguing possibility in more detail.

Important recent advances demonstrate that several lung epithelial cell types can undergo EMT in vitro and suggest that the mechanism may also play a role in vivo...
during the development of various fibroproliferative diseases. Recent data from two groups using different detection methods demonstrated mesenchymal marker expression in airway epithelial cells of lung transplant patients with bronchiolitis obliterans [48,60]. Nevertheless, it is yet to be shown conclusively that EMT of bronchial epithelial cells plays a significant role in airway tissue remodelling and peri-bronchial fibrosis in the pathogenesis of lung diseases.

Conclusions

Our results provide an additional insight into EMT, a potentially very important mechanism in fibrogenesis. We demonstrate that TGF-β1 can drive EMT in primary HBECS in vitro. We also show that this effect is primarily mediated via a Smad 2/3 dependent mechanism and is potentiated by cell-fibronectin interaction, in the presence of TNF-α, and can be further modulated by BMP pathway activation with BMP4. The results provided in this study establish the basis of future investigations into the mechanism of bronchial epithelial cell to mesenchymal cell differentiation, which, in turn, can contribute to a better understanding of lung fibrosis and to the development of new therapeutic approaches.

Abbreviations

α SMA: α-smooth muscle actin; AOK: activin receptor-like kinase; AECs: alveolar epithelial cells; BMP: bone morphogenetic proteins; COPD: chronic obstructive pulmonary disease; CTGF: connective tissue growth factor; DDR: discocidin domain receptor; ECM: extracellular matrix; EMT: epithelial mesenchymal transition; FBS: fetal bovine serum; FMT: fibroblasts to myofibroblasts transition; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HBEC: human bronchial epithelial cells; IL: interleukin; IPF: idiopathic pulmonary fibrosis; MAF: mitogen-activated protein kinase; MMP: matrix metalloprotease; PDR: polymerase chain reaction; PDL: Poly-D-Lysine; RT: room temperature; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TGF: transforming growth factor; TNF: tumour necrosis factor; TBS: Tris-buffered saline; TBST: TBS tween.

Acknowledgements

We are grateful to Professor John Westwick, Dr Christoph Walker and the Postdoctoral Program of the Novartis Institutes for Biomedical Research for their support of this work. We would also like to thank Dr Christoph Walker for his critical review of the manuscript and members of the laboratory for helpful discussions.

Authors’ contributions

JC carried out the experiments and data analysis. GJ and JC designed the experiments, interpreted the data and wrote the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 30 September 2009
Accepted: 5 January 2010 Published: 5 January 2010

References

1. Zawadzki J, Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. Oncogene 2005, 24:5764-5774.
2. Hay ED. The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev Dyn 2005, 233:706-720.
3. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002, 2:442-454.
4. Wilson MS, Wynn TA. Pulmonary fibrosis: pathogenesis, etiology and regulation. Mucosal Immunol 2009, 2:103-121.
5. Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL. Epithelial-mesenchymal interactions in the pathogenesis of asthma. J Allergy Clin Immunol 2000, 105:193-204.
6. Flanders KC. Smad3 as a mediator of the fibrotic response. Int J Exp Pathol 2004, 85:47-64.
7. Willis BC, Barok Z. TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol 2007, 293:L525-L534.
8. Lee G, Walser TC, Dubinett SM. Chronic inflammation, chronic obstructive pulmonary disease, and lung cancer. Curr Opin Pulm Med 2009, 15:303-307.
9. Yao HW, Xie QM, Chen JQ, Deng YM, Tang HF. TGF-β1 induces alveolar epithelial to mesenchymal transition in vitro. Life Sci 2004, 76:39-37.
10. Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z. TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). Respir Res 2005, 6.
11. Willis BC, Liebler JM, Luby-Peihls K, Nicholson AG, Crandall ED, du Bois RM, Barok Z. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. Am J Pathol 2005, 166:1321-1332.
12. Kim KK, Kugler MC, Wolters Pj, Robillard L, Galvez MG, Brunwell AN, Sheppard D, Chapman HA. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc National Acad Sci USA 2006, 103:13180-13185.
13. Sheppard D. Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. Proc Am Thorac Soc 2006, 3:413-417.
14. Khalil N, Parekh TV, O’Connor R, Antman N, Kepron W, Yehaulaeshet T, Xu YD, Gold Li. Regulation of the effects of TGF-beta 1 by activation of latent TGF-beta 1 and differential expression of TGF-beta receptors (T beta R-I and T beta R-II) in idiopathic pulmonary fibrosis. Thorax 2001, 56:907-915.
15. Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z. TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). Respir Res 2005, 6.
16. Matthey DL, Dawes PT, Nixon NB, Slater H. Transforming growth factor beta 1 and interleukin 4 induced alpha smooth muscle actin expression and myofibroblast-like differentiation in human synovial fibroblasts in vitro: modulation by basic fibroblast growth factor. Ann Rheum Dis 1997, 56:426-431.
17. Allen JT, Knight RA, Bloor CA, Spiteri MA. Enhanced insulin-like growth factor binding protein-related protein 2 (connective tissue growth factor) expression in patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. Am J Respir Cell Mol Biol 1999, 21:693-700.
18. Grotendorst GR. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. Cytokine Growth Factor Rev 1997, 8:171-179.
19. Derynck R, Zhang Y. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003, 425:577-584.
20. Liese D, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. Activation of the Erk pathway is required for TGF-beta-induced EMT on fibroblasts. J Biol Chem 2003, 278:25138-25147.
21. Caraci F, Gili E, Calaforesi M, Falla M, La Rosa C, Crimi N, Sorrentino MA, Nicolleto F, Copani A, Vancrehi C. TGF-beta1 targets the GSK-3beta/beta-catenin pathway via ERK activation in the transition of human lung fibroblasts into myofibroblasts. Pharmacol Res 2005, 52:274-282.
22. Thannickal VI, Lee DY, White ES, Gui Z, Lakos JM, Charon R, Horowitz JC, Day RM, Thomas PE. Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signalling via focal adhesion kinase. J Biol Chem 2003, 278:12384-12389.
23. Belluscio S, Henderson R, Winner G, Okawa T, Hogan BL. Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. Development 1996, 122:1693-1702.
24. Selman M, Pardo A, Kaminski N. Idiopathic pulmonary fibrosis: aberrant recapitulation of developmental programs? PLoS Med 2008, 5:e62.
25. Feng XR, Derynck R. Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol 2005, 21:659-693.
26. Molloy EL, Adams A, Moore JB, Masterson JC, Madrigal-Estebas L, Mahon BP, O’Dea S. BMP4 induces an epithelial-mesenchymal transition-like response in adult airway epithelial cells. Growth Factors 2008, 26:12-22.

27. Zeisberg M, Hanai J, Sugimoto H, Mammoth T, Chaytan D, Strutz F, Kalluri R: BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 2003, 9:964-968.

28. Miner JH, Nguyen NM. Extracellular matrix. Basement membranes. Encyclopedia of Respiratory Medicine London: Academic Press/Geoffrey Lauret, Steven Shapiro 2009, 157-162.

29. Raghu G, Striker LJ, Hudson LD, Striker GE. Extracellular matrix in normal and fibrotic human lungs. Am Rev Respir Dis 1985, 131:281-289.

30. Limper AH, Broekelmann TJ, Colby TV, Malizia G, McDonald JA: Analysis of local mRNA expression for extracellular matrix proteins and growth factors using in situ hybridization in fibroproliferative lung disorders. Chest 1991, 99:555-565.

31. Kuhn C III, Boldt J, King TE Jr, Crouch E, Varto T, McDonald JA: An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. Am Rev Respir Dis 1989, 140:1693-1703.

32. Leitinger B, Hohenester E. Mammalian collagen receptors. Matrix Biol 2007, 26:146-155.

33. Vogel WF, Abdullhussein R, Ford CE. Sensing extracellular matrix: an update on discoidin domain receptor function. Cell Signal 2006, 18:1108-1116.

34. Alves F, Vogel W, Mossie K, Millauer B, Hofler H, Ullrich A: Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer. Oncogene 1995, 10:69-78.

35. Chua HH, Yeh TH, Wang YP, Huang YT, Yeh TC, Tsai CH. Upregulation of discoidin domain receptor 2 in nasopharyngeal carcinoma. Head Neck 2008, 30:427-436.

36. Yano H, Iemura A, Fukuda K, Mizuguchi A, Haramaki M, Kajo M. Establishment of two distinct human hepatocellular carcinoma cell lines from a single nodule showing clonal dedifferentiation of cancer cells. Hepatology 1993, 18:320-327.

37. Ford CE, Lau SK, Zhu CQ, Andersson T, Tsao MS, Vogel WF: Expression and mutation analysis of the discoidin domain receptors 1 and 2 in non-small cell lung carcinoma. Br J Cancer 2007, 96:808-814.

38. Oggoni T, Martinis P, Inghilieri S, Palladini G, Tozza R, Vitolo P, Fagiolo G, Perlini S, Pozzi E: Time course of matrix metalloproteases and tissue inhibitors in bleomycin-induced pulmonary fibrosis. Eur J Histochem 2006, 50:317-325.

39. Yu Q, Stamenkovic I: Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000, 14:161-176.

40. Nicholson AS, Fuller LG, Colby TV, du Bos RM, Hansell DM, Wells AU: The relationship between individual histologic features and disease progression in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2002, 166:173-177.

41. Ward C, Foresti WA, Murphy DM, Johnson GE, Robertson H, Callahan JF, Olson BA: Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. Mol Pharmacol 2002, 62:58-64.

42. Zeisberg M, Shah AA, Kalluri R: Bone morphogenetic protein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. J Biol Chem 2005, 280:8094-8100.

43. Murray LA, Hackett TL, Warner SM, Shaheen F, Argenti RL, Dudas P, Farell FX, Knight DA: BMP-7 does not protect against bleomycin-induced lung or skin fibrosis. PLOS ONE 2008, 3:e0439.

44. Kali K, Mylläriemi M, Vuorinen K, Salmenkivi K, Rynänen MJ, Kinnula VL, Keski-Oja J: Bone morphogenetic protein-4 inhibitor gremlin is overexpressed in idiopathic pulmonary fibrosis. Am J Pathol 2006, 169:61-71.

45. Bonniaud P, Martin G, Marrett P, Ask K, Robertson J, Gauldie J, Kolb M: Connective tissue growth factor is crucial to inducing a profibrotic environment in ‘fibrosis-resistant’ BALB/c mouse lungs. Am J Respir Cell Mol Biol 2004, 31:50-516.

46. Zhang Y, Lee TC, Guillemin B, Yu MC, Rom WH: Enhanced IL-1 beta and tumor necrosis factor-alpha release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis or after asbestos exposure. J Immunol 1993, 150:418-4196.

47. Zhang M, Zhang Z, Pan HY, Wang DX, Deng ZT, Ye XL: TGF-beta1 induces human bronchial epithelial cell-to-mesenchymal transition in vitro. Lung 2009, 187:187-194.

48. Borthwick LA, Parker SM, Brougham KA, Johnson GE, Gorowiec MR, Ward C, Lordan JL, Corris PA, Kirby JA, Fisher AJ: Epithelial to mesenchymal transition (EMT) and airway remodeling after human lung transplantation. Thorax 2009, 64:770-7.

49. Hackett TL, Warner SM, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, Argenti RL, Kicic A, Stick SM, Bai TR, Knight DA: Induction of epithelial-mesenchymal transition in primary airway epithelial cells from asthmatic patients by TGFbeta(1). Am J Respir Crit Care Med 2009, 180:122-33.

50. Kuroki S, Suda T, Fujisawa T, Ide K, Insu N, Nakamura Y, Nakamura H, Chida K: Epithelial-mesenchymal transition induced by transformed growth factor-beta1 in mouse tracheal epithelial cells. Respirology 2009, 14:828-37.

51. Chin GS, Lee S, Hsu M, Liu W, Kim WJ, Levinson H, Longaker MT: Discoidin domain receptors and their ligand, collagen, are temporally regulated in fetal rat fibroblasts in vitro. Plast Reconstr Surg 2001, 107:769-776.

52. Maeyama M, Koga H, Selvendran K, Yanagimoto C, Hanada S, Taniguchi E, Kawaguchi T, Harada M, Ueno T, Sata M: Switching in discoid domain receptor expressions in SLUG-induced epithelial-mesenchymal transition. Cancer 2008, 113:283-291.

53. Shintani Y, Fukamoto Y, Chaika N, Svoboda R, Wheelock MJ, Johnson KR: Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1. J Cell Bio 2008, 180:1277-89.

54. Mylläriemi M, Lindholm P, Ryynänen MJ, Klement CR, Salmenkivi K, Keski-Oja J, Kinnula VL, Oury TD, Koli K: Gremlin-mediated decrease in bone morphogenetic protein signaling promotes pulmonary fibrosis. Am J Respir Crit Care Med 2007, 177:321-329.

55. Lin Y, Martín J, Gruender C, Farley J, Meng X, Li BY, Lechleider R, Huff C, Kim RH, Grasser WA, Parakar V, Wang T: A novel link between the protesome pathway and the signal transduction pathway of the bone morphogenic proteins (BMPs). BMC Cell Biol 2002, 3:15-36.

56. Awasthi N, Wang-Su ST, Wagner BJ: Downregulation of MMP-2 and -9 by proteasome inhibition: a possible mechanism to decrease LEC migration and prevent posterior capsular opacification. Invest Ophthalmol Vis Sci 2008, 49:1998-2003.

57. Derugina IB, Ofugui JP: Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 2006, 25:9-34.

58. Legrand C, Gilles C, Zahm JM, Polette M, Buisson AC, Kaplan H, Birembaut P, Tournaik JM: Airway epithelial cell migration dynamics: MMP-9 role in cell-extracellular matrix remodelling. J Cell Biol 1999, 146:517-529.

59. Hodge S, Holmes M, Barjee P, Musik M, Kicic A, Waterer G, Reynolds PN, Hodge G, Chambers D: Posttransplant bronchiolitis obliterans syndrome is associated with bronchial epithelial to mesenchymal transition. Am J Transplant 2009, 9:727-733.