The role of epithelial-fibroblast communication in asthma and COPD

Osei, Emmanuel

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 3

Interleukin-1α drives the dysfunctional cross-talk of the airway epithelium and lung fibroblasts in COPD

Eur Respir J 2016; 48: 350–358

Emmanuel T. Osei¹,²,³
Jacobien A. Noordhoek¹,²,⁴
Tillie L. Hackett³
Anita I.R. Spanjer²,⁵
Dirkje S. Postma²,⁴
Wim Timens¹,²
Corry-Anke Brandsma¹,²,⁶
Irene H. Heijink¹,²,⁴,⁶

¹These two authors contributed equally to this work

¹University of Groningen, University Medical Center Groningen, Dept of Pathology and Medical Biology, Groningen, The Netherlands.
²University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Groningen, The Netherlands.
³University of British Columbia, Centre for Heart Lung Innovation, Dept of Anesthesiology, Pharmacology and Therapeutics, Vancouver, BC, Canada.
⁴University of Groningen, University Medical Center Groningen, Dept of Pulmonology, Groningen, The Netherlands.
⁵University of Groningen, Dept of Molecular Pharmacology, Groningen, The Netherlands.

“Nkyinkyim” (Twisting)
Adinkra symbol of initiative, dynamism and versatility
ABSTRACT
Chronic obstructive pulmonary disease (COPD) has been associated with aberrant epithelial–mesenchymal interactions resulting in inflammatory and remodelling processes. We developed a co-culture model using COPD and control-derived airway epithelial cells (AECs) and lung fibroblasts to understand the mediators that are involved in remodelling and inflammation in COPD.

AECs and fibroblasts obtained from COPD and control lung tissue were grown in co-culture with fetal lung fibroblast or human bronchial epithelial cell lines. mRNA and protein expression of inflammatory mediators, pro-fibrotic molecules and extracellular matrix (ECM) proteins were assessed.

Co-culture resulted in the release of pro-inflammatory mediators interleukin (IL)-8/CXCL8 and heat shock protein (Hsp70) from lung fibroblasts, and decreased expression of ECM molecules (e.g. collagen, decorin) that was not different between control and COPD-derived primary cells. This pro-inflammatory effect was mediated by epithelial-derived IL-1α and increased upon epithelial exposure to cigarette smoke extract (CSE). When exposed to CSE, COPD-derived AECs elicited a stronger IL-1α response compared with control-derived airway epithelium and this corresponded with a significantly enhanced IL-8 release from lung fibroblasts.

We demonstrate that, through IL-1α production, AECs induce a pro-inflammatory lung fibroblast phenotype that is further enhanced with CSE exposure in COPD, suggesting an aberrant epithelial–fibroblast interaction in COPD.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is mainly caused by exposure to noxious particles, of which cigarette smoking is the major risk factor. Presently, no cure exists for COPD, and current pharmacological treatments can only partly suppress symptoms and exacerbations. The disease is characterized by chronic inflammation and defective tissue repair leading to irreversible chronic airflow limitation as a result of destruction of the gas-exchanging surface of the lung (emphysema), remodelling and narrowing of the small airways.

When inhaled, cigarette smoke first encounters the airway epithelium, which normally forms a continuous and highly regulated structural barrier that is part of the innate immune defense. We have previously shown that cigarette smoke inhibits epithelial barrier function. This damage can also cause the release of pro-inflammatory mediators (interleukin (IL)-8/CXCL8, IL-6) and danger signals known as damage-associated molecular patterns (DAMPS), such as IL-1α and heat shock protein (Hsp70). Additionally, airway epithelium is a source of growth factors (e.g. transforming growth factor (TGF)-β1) that can act on the underlying mesenchymal cells in the lamina propria to induce repair. Mesenchymal fibrocytes, fibroblasts and smooth muscle cells are essential structural cells that produce various extracellular matrix (ECM) proteins within the lung, including collagens and decorin. We have previously demonstrated decreased decorin production by primary lung fibroblasts from severe (Global Initiative for Chronic Obstructive Lung Disease (GOLD) 4) COPD patients compared with mild (GOLD 1) COPD patients. Other groups have also demonstrated that lung fibroblasts respond to IL-1β, IL-1α and prostaglandin PGE2 stimulation by releasing IL-8/CXCL8 and IL-6, and specifically in the case of IL-1β, by also downregulating their ECM protein production. Epithelial–fibroblast communication has been shown to be involved in the pathogenesis of asthma and has also been proposed to contribute to idiopathic pulmonary fibrosis. However, detailed knowledge on the direct interaction between airway epithelial cells (AECs) and the underlying pulmonary fibroblasts in COPD is limited, also with respect to the effects of cigarette smoke.

We hypothesize that dysfunctional epithelium–fibroblast communication through the release of mediators plays a key role in the chronic inflammation and remodelling processes in COPD, and that cigarette smoke exposure contributes to this aberrant process. The objective of the study was to develop a co-culture cell model to investigate the effect of cross-talk between AECs and lung fibroblasts from severe COPD patients and control subjects on pro-inflammatory mediator release and ECM expression. Furthermore, we investigated the role of cigarette smoke exposure on the cross-talk between AECs and fibroblasts in COPD.

METHODS AND MATERIALS

Human airway epithelial and lung fibroblasts

Human bronchial epithelial 16HBE14o- cells (kindly donated by Dr D.C. Gruenert, University of California, San Francisco, CA, USA) were cultured in Eagle's minimal essential medium (EMEM)/10% fetal calf serum (FCS) as described previously. Primary AECs were isolated as described previously from tracheobronchial tissue of 13 COPD patients with severe disease undergoing lung transplantation and from leftover tracheobronchial tissue of 16 non-COPD control donor lungs, for whom no further information was available. Subject characteristics of the donors are given in
Primary AECs were cultured in hormonally supplemented bronchial epithelium growth medium (Lonza, Basel, Switzerland) and used at passage 3 as described previously\textsuperscript{18}.

Fetal lung fibroblast cells (MRC-5; BioWhittaker, Walkersville, MD, USA) were cultured in EMEM/10\% FCS. Primary human lung fibroblasts (PHLFs) were derived from nine COPD patients with severe disease undergoing lung transplantation and five non-COPD controls undergoing tumour resection surgery. Fibroblasts were isolated from lung parenchyma using the explant technique as described previously\textsuperscript{11,20} grown in Ham’s F12 medium/10\% FCS (Lonza, Basel, Switzerland) and used for experiments at passage 5. The full protocol of PHLF isolation can be found in the online supplementary material. Subject characteristics of PHLF donors are available in table 2. The study protocol was consistent with the Research Code of the University Medical Center Groningen (www.rug.nl/umcg/onderzoek/researchcode/ index), and national ethical and professional guidelines (www.federa.org).

### Table 1 Characteristics of severe chronic obstructive pulmonary disease patients from whom primary airway epithelial cells (AECs) were obtained

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age years</th>
<th>Sex</th>
<th>Smoking status</th>
<th>Pack-years</th>
<th>FEV\textsubscript{1} % pred</th>
<th>FEV\textsubscript{1}/FVC %</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>53</td>
<td>Male</td>
<td>Ex</td>
<td>40</td>
<td>25</td>
<td>25</td>
<td>Co-culture and conditioned medium</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>Female</td>
<td>Ex</td>
<td>38</td>
<td>60</td>
<td>46</td>
<td>Co-culture</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>Male</td>
<td>Ex</td>
<td>30</td>
<td>11</td>
<td>31</td>
<td>Co-culture</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>Male</td>
<td>Never</td>
<td>0</td>
<td>39</td>
<td>53</td>
<td>Co-culture</td>
</tr>
<tr>
<td>5</td>
<td>44</td>
<td>Male</td>
<td>Ex</td>
<td>25</td>
<td>60</td>
<td>50</td>
<td>Co-culture</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>Male</td>
<td>Ex</td>
<td>23</td>
<td>16</td>
<td>29</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>Male</td>
<td>Ex</td>
<td>25</td>
<td>17</td>
<td>21</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>8</td>
<td>57</td>
<td>Female</td>
<td>Ex</td>
<td>45</td>
<td>23</td>
<td>24</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>9</td>
<td>57</td>
<td>Female</td>
<td>Ex</td>
<td>40</td>
<td>18</td>
<td>25</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>10</td>
<td>61</td>
<td>Female</td>
<td>Ex</td>
<td>35</td>
<td>19</td>
<td>23</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>11</td>
<td>49</td>
<td>Male</td>
<td>Ex</td>
<td>11</td>
<td>20</td>
<td>22</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>12</td>
<td>55</td>
<td>Female</td>
<td>Ex</td>
<td>72</td>
<td>14</td>
<td>29</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>13</td>
<td>62</td>
<td>Male</td>
<td>Ex</td>
<td>44</td>
<td>22</td>
<td>19</td>
<td>Conditioned medium</td>
</tr>
</tbody>
</table>

FEV\textsubscript{1}: forced expiratory volume in 1 second; FVC: forced vital capacity. Primary AECs were either used for co-culture experiments with MRC-5 or conditioned medium experiments.

### Co-culture Model

16HBE14o- and MRC-5 cells were initially used to develop the model. Significant observations were replicated using: 1) primary AECs from severe COPD patients or controls with MRC-5 fibroblasts and 2) 16HBE14o- cells with PHLFs from COPD patients or controls to assess disease-specific effects in each cell type separately. Briefly, AECs were plated on 0.4-\textmu M pore 6.5-mm transwell membranes (Costar; Corning, New York, NY, USA) and fibroblasts were seeded on 24-well plates. When both cell layers were confluent, the transwell insert with AECs was placed in co-culture with the fibroblasts and left for 72 h in the appropriate medium (see online supplementary material).

### Conditioned Medium experiments and neutralizing antibody experiments

16HBE14o- cells or primary AECs when confluent were serum/hormone-deprived overnight and stimulated with or without 20\% cigarette smoke extract (CSE) for 6 h. The CSE was thoroughly
Table 2 Characteristics of control donors and severe chronic obstructive pulmonary disease patients from whom primary human lung fibroblasts (PHLFs) were obtained

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age years</th>
<th>Sex</th>
<th>Smoking status</th>
<th>Pack-years</th>
<th>FEV₁ % pred</th>
<th>FEV1/FVC %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>Female</td>
<td>Never</td>
<td>0</td>
<td>101</td>
<td>81</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>Female</td>
<td>Current</td>
<td>38</td>
<td>98</td>
<td>76</td>
</tr>
<tr>
<td>3</td>
<td>74</td>
<td>Male</td>
<td>Ex</td>
<td>50</td>
<td>100</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>Male</td>
<td>Ex</td>
<td>40</td>
<td>97</td>
<td>76</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>Male</td>
<td>Ex</td>
<td>31</td>
<td>97</td>
<td>78</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>Male</td>
<td>Ex</td>
<td>38</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>58</td>
<td>Female</td>
<td>Ex</td>
<td>30</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>60</td>
<td>Male</td>
<td>Ex</td>
<td>30</td>
<td>37</td>
<td>49</td>
</tr>
<tr>
<td>9</td>
<td>66</td>
<td>Male</td>
<td>Ex</td>
<td>NA</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td>62</td>
<td>Male</td>
<td>Ex</td>
<td>44</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>11</td>
<td>57</td>
<td>Female</td>
<td>Ex</td>
<td>40</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>12</td>
<td>48</td>
<td>Male</td>
<td>Ex</td>
<td>27</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>13</td>
<td>57</td>
<td>Female</td>
<td>Ex</td>
<td>33</td>
<td>25</td>
<td>33</td>
</tr>
<tr>
<td>14</td>
<td>44</td>
<td>Male</td>
<td>Ex</td>
<td>25</td>
<td>60</td>
<td>50</td>
</tr>
</tbody>
</table>

Control-derived PHLFs

COPD-derived PHLFs

FEV₁: forced expiratory volume in 1 second; FVC: forced vital capacity; NA: not available.

washed of and cells were incubated for another 24 h prior to the CSE-free conditioned medium being collected. Fibroblasts that had been serum-deprived overnight were then treated for 24 h with the CSE-free conditioned medium that had been pre-incubated for 1 h with or without 4 μg·mL⁻¹ IL-1α neutralizing antibody (AB-200-NA) or IL-1β neutralizing antibody (MAB601) (R&D Systems, Europe, Abingdon, UK). Cell-free supernatants were collected and analyzed by ELISA, and cell lysates were harvested for RNA and protein examination. Refer to the online supplementary material for full protocols of experiments.

Statistics

Data were analyzed using SPSS (IBM, Armonk, NY, USA). The Mann–Whitney U-test was used for comparison between subject groups and the Wilcoxon signed-rank test for paired comparisons within groups of primary cells. We tested for normal distribution on the outcomes of the experiments with cell lines and used the t-test for paired differences accordingly. p<0.05 was considered to be statistically significant.

RESULTS

Increased inflammatory mediator release in lung fibroblasts when in co-culture with airway epithelial cells

When 16HBE14o- and MRC-5 cells were placed in co-culture, we found a significant increase in basolateral IL-8/CXCL8 (figure 1a) and Hsp70 (figure 1b) secretion compared with epithelial and fibroblast mono-cultures. IL-1β protein levels were undetectable (data not shown). Subsequent mRNA analyses on the epithelial and fibroblast cell fractions demonstrated that fibroblasts are the main source of secreted IL-8/CXCL8 (figure 1c). Similarly, IL-1β mRNA levels were increased in fibroblasts, but not in epithelial cells, when placed in co-culture (figure 1d). To determine if the
results obtained from the cell lines in co-culture were representative of primary cells, we paired the 16HBE14o- cells with PHLFs derived from COPD and control subjects, and also paired primary AECs from COPD and control donors with MRC-5 cells. As in the co-culture cell line model, we found that co-culture of PHLFs with 16HBE14o-cells also induced a significant increase in basolateral IL-8/CXCL8 (figure 1e) and Hsp70 (figure 1f), without differences in the IL-8/CXCL8 response of PHLFs from ex-smokers, the never-smoker and the current smoker. Furthermore, we confirmed that the mRNA for IL-8/CXCL8 (figure 1g) and IL-1β (figure 1h) was only upregulated in the PHLFs with co-culture. Additionally, combining primary AECs with MRC-5 fibroblasts resulted in increased basolateral IL-8/CXCL8 and Hsp70 levels as well as a trend towards an increase of IL-6 protein levels (online supplementary figure 1), while levels of granulocyte-macrophage colony-stimulating factor
Interleukin-1α drives the dysfunctional cross-talk of the airway epithelium and lung fibroblasts in COPD

and IL-33 were undetectable. Hsp70 and IL-6 levels correlated strongly with IL-8/CXCL8 release in co-culture (online supplementary figure 2). We found no significant difference in the release of mediators between control and COPD-derived AECs and PHLFs.

**Decreased expression of ECM molecules and pro-fibrotic proteins in lung fibroblasts in co-culture with airway epithelial cells**

In contrast to the increased pro-inflammatory response, co-culture of PHLFs from COPD and control subjects with 16HBE14o- cells resulted in a significant downregulation in the mRNA expression of α-smooth muscle actin (α-SMA) (figure 2a), TGF-β1 (figure 2b), and the ECM molecules decorin (figure 2c), fibulin-5 (figure 2d), collagen-Iα1 (figure 2e) and fibronectin (figure 2f) compared with mono-culture conditions. The downregulation of fibronectin and α-SMA was confirmed on the

**Figure 2 Decrease in the expression of extracellular matrix molecules and structural proteins in primary human lung fibroblasts (PHLFs) after co-culture with 16HBE14o- cells.** mRNA expression levels (6 h) (with median) of a) α-smooth muscle actin (α-SMA), b) transforming growth factor (TGF)-β1, c) decorin, d) fibulin-5, e) collagen-Iα1 and f) fibronectin in PHLFs from control donors (open triangles) and chronic obstructive pulmonary disease (COPD) patients (filled triangles) comparing co-culture with 16HBE14o- and mono-cultures. mRNA levels were related to the housekeeping genes β2-microglobulin and protein phosphatase 1α, and expressed as 2−ΔCt. g, h) Relative protein expression levels (with median) and representative blots for g) fibronectin and h) α-SMA in PHLFs from control donors (open triangles) and COPD patients (filled triangles) comparing co-culture with 16HBE14o- and mono-cultures. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. *: p<0.05; **: p<0.01; ***: p<0.001 between the indicated value
protein level using Western blotting (figure 2g and h). Neither the baseline expression nor the decrease of these ECM and structural proteins upon co-culture was significantly different between COPD and control-derived PHLFs.

**Epithelium-derived IL-1α is responsible for pro-inflammatory phenotype switch in lung fibroblasts**

Next, we used conditioned medium from 16HBE14o- cells to investigate whether the observed effects could be due to a soluble factor. Indeed, we observed that epithelial conditioned medium also induced an increase in IL-8/CXCL8 secretion by MRC-5 fibroblasts (figure 3a). Since IL-1β and PGE2 have been shown to induce similar phenotype switches in fibroblasts\(^{12-14}\), we first studied the effect of an IL-1β neutralizing antibody (figure 3b) and inhibition of the downstream effect of PGE2 by the use of an adenylate cyclase inhibitor, MDL-12,330A hydrochloride (online supplementary figure 4). Both did not prevent the IL-8/CXCL8 release from MRC-5 cells. The other agonist of the IL-1R1 receptor is IL-1α. Interestingly, we found that the use of a neutralizing antibody against IL-1α completely abrogated IL-8/CXCL8 secretion by MRC-5 fibroblasts (figure 3c).

To determine if the results obtained using 16HBE14o- conditioned medium were representative of primary cells, we conducted the same experiments using conditioned medium from primary AECs from COPD and control donors on MRC-5 fibroblasts. As shown in figure 4, neutralization of IL-1α also abrogated primary AEC conditioned medium-induced IL-8/CXCL8 secretion (figure 4a) as well as IL-8/CXCL8 and IL-1β mRNA expression (figure 4b and c) in MRC-5 fibroblasts.

Furthermore, we assessed the effect of the neutralizing antibody in the co-culture model itself. As shown in figure 4d, the addition of IL-1α neutralizing antibody in the basal compartment also inhibited the basolateral release of IL-8/CXCL8 upon co-culture of primary AECs from COPD and control subjects with MRC-5. There was also a strong trend towards the downregulation of basolateral IL-6 release (online supplementary figure 1). Moreover, IL-1α neutralization in the co-culture also prevented the downregulation of TGF-β1, decorin, fibulin-5 and collagen-Ia1 mRNA expression in MRC-5 cells when co-cultured with primary AECs (figure 5).
Interleukin-1α drives the dysfunctional cross-talk of the airway epithelium and lung fibroblasts in COPD

Figure 4 Interleukin (IL)-1α from primary airway epithelial cells (AECs) is responsible for a pro-inflammatory phenotype switch in MRC-5 fibroblasts. MRC-5 cells were grown to confluence, serum-deprived overnight and subsequently incubated without (MRC-5 basal) or with conditioned medium from primary AECs (PAEC CM) of control donors (open triangles) and chronic obstructive pulmonary disease (COPD) patients (filled triangles) in the presence or absence of 4 μg·mL$^{-1}$ IL-1α neutralising antibody (NAb). a) IL-8/CXCL8 concentration (with median), and b) IL-8/CXCL8 and c) IL-1β mRNA expression (with median) in MRC-5 cells. d) IL-8/ CXCL8 concentration (with median) in cell-free supernatants (24 h) of the basolateral compartment comparing co-culture and monocultures of primary AECs from control donors (open triangles) and COPD patients (filled triangles) and MRC-5 cells in the presence or absence of 4 µg·mL$^{-1}$ IL-1α NAb. mRNA levels were related to the housekeeping genes β$_2$-microglobulin and protein phosphatase 1α, and expressed as $2^{-ΔΔCt}$. **: $p<0.01$; ***: $p<0.001$ between the indicated values.

Cigarette smoke exposure increases IL-1α expression in airway epithelial cells and subsequent IL-8/CXCL8 production in lung fibroblasts

To evaluate if epithelial exposure to cigarette smoke alters communication with lung fibroblasts, we exposed fibroblasts to conditioned medium from 16HBE14o- cells pre-treated with CSE. Exposure of epithelial cells to CSE significantly increased the release of IL-1α protein (figure 6a) and mRNA (figure 6b) compared with basal levels. Subsequent exposure of MRC-5 fibroblasts to conditioned medium from CSE-treated 16HBE14o- cells induced a significantly stronger increase in IL-8/CXCL8 production than stimulation with basal 16HBE14o- conditioned medium (figure 6c). Similarly, CSE exposure significantly increased IL-1α mRNA expression in our primary AECs (figure 6d). Interestingly, COPD-derived primary AECs showed a stronger increase in IL-1α mRNA expression after CSE exposure than control-derived primary AECs (figure 6d). In line with this increase in IL-1α expression, conditioned medium from CSE-exposed COPD-derived primary AECs caused a significantly stronger increase in IL-8/CXCL8 release from MRC-5 fibroblasts than conditioned medium from CSE-exposed control primary AECs (figure 6e).
Figure 5 Interleukin (IL)-1α is responsible for the decrease in expression of extracellular matrix molecules and structural proteins of lung fibroblasts after co-culture with epithelial cells. mRNA expression levels (with median) of a) transforming growth factor (TGF)-β1, b) decorin, c) fibulin-5 and d) collagen-Iα1 in MRC-5 cells comparing mono-culture and co-culture with primary airway epithelial cells from control donors (open triangles) and chronic obstructive pulmonary disease patients (filled triangles) in the presence and absence of 4 µg·mL⁻¹ IL-1α neutralising antibody (NAb). mRNA levels were related to the housekeeping genes β₂-microglobulin and protein phosphatase 1α, and expressed as 2⁻ΔCt. *: p<0.05 between the indicated values.
DISCUSSION

We demonstrate that lung fibroblasts are directly regulated by AECs to release pro-inflammatory mediators and downregulate ECM synthesis and pro-fibrotic responses. Our data indicate that this regulation is driven by epithelium-derived IL-1α, as neutralizing IL-1α in epithelial conditioned media completely reversed the release of inflammatory mediators by lung fibroblasts. Additionally, we demonstrate that cigarette smoke exposure induces higher IL-1α levels in AECs, particularly in epithelial cells from severe COPD patients. Moreover, cigarette smoke exposure may contribute to an aberrant cross-talk between epithelial cells and the underlying fibroblasts in COPD, as we observed that CSE-exposed epithelium derived from COPD patients induces a stronger increase in IL-8/CXCL8 secretion by lung fibroblasts than CSE-exposed control-derived epithelium.

In the lung, fibroblasts are located within the interstitium in close proximity to the airway epithelium, and hence can be easily influenced by the release of several factors by the epithelium in normal repair and disease states. In COPD, however, the interaction between AECs and the underlying fibroblasts may be increased, and cells may be in closer contact because of the observed fragmentation of the basement membrane in the mucosa. Thus, the epithelium may exert stronger pro-inflammatory effects on fibroblasts in COPD. Fibroblasts in the lung have been.
shown to not only contribute to repair processes through contraction, synthesis and remodelling of granulation tissue, but also through the production of cytokines to aid the normal immune defense mechanisms within the lung\textsuperscript{23}. IL-8/CXCL8 is a chemoattractant for neutrophils in the lungs\textsuperscript{24,25}, and chronic neutrophilic inflammation may contribute to abnormal tissue repair, remodelling and destruction in COPD\textsuperscript{4}. Increased IL-8/CXCL8 release in the lungs has been associated with the pathogenesis of COPD\textsuperscript{26}. Thus, higher IL-8/CXCL8 secretion by lung fibroblasts upon epithelial exposure to cigarette smoke may play a role in the pathogenesis of COPD\textsuperscript{7}. Similarly, the increased release of Hsp70 and IL-6, which are both inflammatory mediators, has been implicated in chronic inflammatory processes in COPD\textsuperscript{8,27}. The airway epithelium has been reported to be the source of mediators that drive chronic inflammation and structural changes in COPD. Among others, IL-1β, IL-1α, PGE\textsubscript{2}, tumour necrosis factor-α, IL-6 and various matrix metalloproteinases are increased in pulmonary epithelial cell supernatants from COPD patients compared with healthy controls\textsuperscript{28}. PGE\textsubscript{2} and IL-1β as well as IL-1α have been shown to induce an increase in IL-8/CXCL8 secretion from fibroblasts\textsuperscript{12,13}. Our results highlight that neutralization of IL-1α completely blocked the production of IL-8/CXCL8 by lung fibroblasts upon stimulation with epithelial conditioned media. Thus, our data provide strong support for a role of epithelium-derived IL-1α in the cross-talk between epithelial cells and fibroblasts during both normal immune defense and aberrant repair in COPD. IL-1α is a member of the IL-1 superfamily of inflammatory cytokines. It plays a crucial role in normal immune responses \textit{in vivo} and is constitutively expressed in lung epithelium\textsuperscript{29}. IL-1α and its agonist IL-1β bind to the IL-1R1 receptor and illicit a similar downstream response with the subsequent activation of transcription factors such as nuclear factor-κB and activator protein-1\textsuperscript{29}. While the activity of IL-1β is dependent on the activation of the NLRP3 inflammasome and subsequent cleavage by caspase-1, IL-1α is both active in its pro-form and upon cleavage by caspases\textsuperscript{30}. Our findings suggest that cigarette smoke may increase the release of IL-1α from the airway epithelium in severe COPD, which subsequently causes an additional increase in IL-8/CXCL8 release from fibroblasts, contributing to neutrophilic inflammation \textit{in vivo}. Relatively little work has been done on the role of IL-1α in COPD and our data suggest the need for a closer look at the role of IL-1α in the pathogenesis of COPD. In line with our findings, Pauwels et al.\textsuperscript{30} showed that cigarette smoke-induced inflammation in mice is dependent on the IL-1R1 receptor, and both IL-1α and its agonist IL-1β are involved in neutrophilic inflammation. Furthermore, Botelho et al.\textsuperscript{31} showed that smoke-induced neutrophilic inflammation in mice was dependent on IL-1α, but not IL-1β. They also measured IL-1α protein in bronchial biopsies and sputum, and showed an increased expression in the inflammatory infiltrate and epithelium in biopsies from COPD patients compared with controls\textsuperscript{31}. Suwara et al.\textsuperscript{12} used another model of COPD where human AECs were injured \textit{in vitro} with hydrogen peroxide and thapsigargin to induce reactive oxidative species injury and endoplasmic reticulum stress, respectively. They found increased levels of IL-1α and IL-1β in the conditioned medium from these cells, which induced the release of IL-8/CXCL8 and IL-6 in MRC-5 fibroblasts\textsuperscript{12}. This effect was completely blocked using IL-1α neutralizing antibody and only partially by using the IL-1β neutralizing antibody. This is in line with our study, demonstrating a crucial role for epithelium-derived IL-1α in regulating fibroblasts to become pro-inflammatory in a co-culture model representative of \textit{in vivo} conditions. Our data further indicate that COPD-derived epithelial cells are more prone to release IL-1α upon CSE exposure. It will be worthwhile to further study
Interleukin-1α drives the dysfunctional cross-talk of the airway epithelium and lung fibroblasts in COPD

the mechanism of IL-1α release in order to understand why COPD epithelial cells express more IL-1α upon CSE exposure, e.g. whether this involves endoplasmic reticulum stress or an oxidant/antioxidant imbalance as shown previously\(^2\).

In addition to the production of inflammatory mediators in our co-culture model, AECs via IL-1α also reduced the expression of the pro-fibrotic cytokine TGF-β1, the structural molecule α-SMA and various ECM molecules, including decorin, fibulin-5 and collagen-Iα1. The exact meaning of this finding needs to be investigated further, as the lack of demonstrating differences between COPD and control fibroblasts could also be due to power limitation. Nevertheless, our current findings support a mechanism whereby small airways may be lost due to defective tissue repair, which would be in line with the findings of McDonough et al,\(^3\) who demonstrated up to 90% loss of small airways in end-stage (GOLD 4) COPD patients. Although our epithelium–fibroblast model reflects the in vivo situation more closely than mono-cultures, our model may not fully reflect the in vivo situation, where other cell types are present as well. Also in our model, we compared control-derived primary AECs from first-generation tracheobronchial tissue to COPD-derived primary AECs from the third to fifth generation of the bronchial tree. Although these cells are not from the exact same site in the lung, various studies have shown similar genomic and epigenomic similarities in AECs from the tracheal and bronchial origin\(^3\)\(^3\)\(^4\). We used cells derived from lung tissue of COPD patients undergoing transplantation for end-stage disease. Further experiments will be required to assess if the observed aberrant cross-talk between epithelial cells and fibroblasts is also present in early, mild and moderate stages of the disease. Future work will need to assess if the increased IL-8/CXCL8 secretion observed in our model additionally promotes neutrophil chemotaxis.

In conclusion, our data show that lung fibroblasts are regulated by AECs to become pro-inflammatory in their function. This regulation is driven by epithelial-derived IL-1α, further enhanced by CSE exposure and stronger in epithelial cells from severe COPD patients than from healthy individuals. Our study offers novel insights into the role of epithelial cells and fibroblasts in the pathogenesis of chronic remodelling and inflammation seen in COPD.

REFERENCES


SUPPLEMENTARY INFORMATION AND DATA

Isolation and culture of primary human lung fibroblasts

Fibroblasts were isolated from lung parenchyma using the explant technique as previously described. Lung tissue was cut into 1 mm³ 26 cubes and put in a 12-well culture plate (Corning Costar Europe Ltd., Badhoevedorp, the Netherlands). The tissue was left for 10 minutes at 20°C and allowed to adhere after which 1.5ml of Ham’s-F12 medium/10% FCS (Lonza) was added. This was placed in the incubator under standard conditions and medium was replaced once a week. Tissue explants were usually removed after four weeks when primary fibroblast outgrowth contained enough cells. Primary fibroblasts were then trypsinized (0.05% trypsin/0.02% EDTA; BioWhittaker) and cells were transferred into a 25cm² culture flask. Primary fibroblasts were grown till confluence, frozen in liquid nitrogen and used for experiments at passage 5.

Full protocol of co-culture model

Primary airway epithelial cells (AECs) and 16HBE14o- cells were plated at 20,000 cells hormonally-supplemented bronchial epithelium growth medium (BEGM, Lonza) or Eagle’s minimal essential medium (EMEM)/10% FCS (Lonza) respectively on coated 0.4μm polyester transwell membrane inserts (upper compartment). Primary human lung fibroblasts (PHLFs) or MRC-5 cells were plated at 20,000 cells in EMEM medium/10% FCS in a 24 well culture plate. Confluent layers of epithelial cells and fibroblasts were obtained after one week of culturing in standard conditions. After this, MRC-5 cells and PHLFs were placed on EMEM/10%FCS when co-cultured with 16HBE14o- cells or BEGM when co-cultured with primary AECs. Mono-culture controls consisted of either epithelial cells on inserts placed in wells without fibroblasts, or confluent fibroblast wells with empty inserts on top in the same medium as used for co-culture. The cell inserts were carefully placed in fibroblast wells and left in co-culture for 72 hours in standard conditions. The cells were placed on serum/hormone free conditions before experimentation. For IL-1α neutralization experiments, the cells were placed in co-culture with or without 4μg/ml IL-1α neutralizing antibody (AB-200-NA, R&D Systems) for 72 hours and the antibody was present until cells were harvested. Epithelial cells and fibroblasts were harvested separately with TRIreagent for RNA isolation, Laemmlli buffer for cell lysate preparation and cell-free supernatants were collected for ELISA.

Conditioned medium (CM) experiments

16HBE14o- cells or primary AECs were plated in their appropriate medium on coated 24 well plates. When confluent, cells were serum/hormone-deprived overnight and subsequently stimulated with 20% cigarette smoke extract (CSE) for 6 hours. CSE was thoroughly washed off and cells were incubated for another 24 hours. The epithelial cells and CSE-free conditioned medium (CM) were collected and stored at -80°C until use. CSE was made as previously described. Smoke of 2 Kentucky 3R4F research21 reference cigarettes without filter (The Tobacco Research Institute, Lexington, Kentucky, USA) were bubbled through 25ml BEBM (Lonza) to make 100% cigarette smoke extract. For each experiment fresh CSE was prepared and diluted in serum/hormone-free medium to 20%. Viability experiments showed that a concentration of 20% CSE did not cause epithelial cell death (online supplement figure S3).
Neutralizing antibody experiments

Fibroblasts were serum-deprived overnight after which they were stimulated with CM for 24 hours. Epithelial CM was pre-incubated for 1 hour with or without 4μg/ml IL-1α neutralizing (AB-200-NA) or IL-1β neutralizing antibody (MAB601, R&D Systems, Europe, Abingdon, UK). Cell-free supernatants were collected and fibroblasts were harvested for RNA isolation or cell lysate preparation. In the co-culture model, cells were placed together for 72 hours with or without 4μg/ml IL-1α neutralizing antibody (R&D Systems) and the antibody was present until the cells were harvested. Epithelial cells and fibroblasts were harvested separately with TRIreagent for RNA isolation and subsequent qRT-PCR, Laemmli buffer for cell lysates preparation for western blot and cell-free supernatants were collected for ELISA. An LDH assay, (G7890, Promega, Southampton, UK) was done to assess cell viability after co-culture and in the presence of the IL-1α neutralizing antibody. This showed no significant changes in cell viability (data not shown).

Measurement of protein levels by ELISA

IL-8/CXCL8, IL-1α, Hsp70 (R&D Systems) and IL-6 (Sanquin, Pelikine, Amsterdam) were measured with sandwich ELISAs in cell-free culture supernatants and performed according to the manufacturer’s instructions.

RNA Isolation and qRT-PCR for structural proteins

RNA was harvested using the standard TRIreagent method. cDNA synthesis was done with iScript cDNA synthesis kit (BioRad, Herts, UK) according to the manufacturer’s instructions. Taqman® Gene expression assays were used for IL-8/CXCL8 (Hs00174103_m1), IL-1α (Hs00174092_m1), IL-1β (Hs01555410_m1), decorin (Hs00754870_s1), fibulin-5 (Hs00197064_m1), collagen-1α1 (Hs00264051_m1), fibronectin (Hs00365052_m1), α-smooth muscle actin (Hs00426835_g1), periostin (Hs01566734_m1), MMP-2 (Hs01548727_m1) and TGF-β (Hs00998133_m1) according to the manufacturer’s instructions. qRT-PCR was performed on the Taqman® or LightCycler 480 II (Roche). Expression of the genes of interest was normalized to expression of the housekeeping genes protein phosphatase 1, catalytic subunit, alpha isoenzyme (Hs00267568_m1) and β2-microglobulin (Hs00984230_m1) with approximately equal amplification efficiency.

Western blotting for ECM molecules

Total cell lysates were obtained by harvesting epithelial cells and fibroblasts separately in 1X Laemmli buffer (containing 2% SDS, 10% glycerol, 2% β-mercapto, 60mM Tris-Hcl (pH 6.8) and bromophenol blue) and boiled for 5 minutes. Samples were subjected to SDS-PAGE and blotted on a nitrocellulose membrane (Schleider and Schuell GmbH, Einbeck, Germany). Expression of fibronectin and α-smooth muscle actin was analyzed using mouse anti-human fibronectin (Sigma, F6140) and mouse anti-human α-smooth muscle actin (Sigma, A5228) respectively, with anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) as loading control as previously described, after which visualization was done with the ODYSSEY® infra-red imaging system (LiCOR®, Lincoln, NE).
Interleukin-1α drives the dysfunctional cross-talk of the airway epithelium and lung fibroblasts in COPD

Figure S1 IL-8/CXCL8, Hsp70 and IL-6 in co-culture of airway epithelial cells and lung fibroblasts. Primary airway epithelial cells (AECs) derived from control (open triangles) and COPD patients (filled triangles) were cultured alone or with MRC-5 cells. A) IL-8/CXCL8 concentration (with median) in cell-free supernatants (24 hours) of the baso-lateral compartment comparing co-culture and mono-cultures. B) IL-8/CXCL8 mRNA expression levels (with median) in MRC-5 cells (6 hours) harvested separately comparing co-culture and mono-cultures. C) Hsp70 concentration (with median) in cell-free supernatants (24 hours) of the baso-lateral compartment comparing co-culture and mono-cultures. D) IL-6 concentration (with median) in cell-free supernatants (24 hours) of the baso-lateral compartment comparing co-culture and mono-cultures in the presence or absence of 4 μg/ml IL-1α Nab mRNA levels were related to the housekeeping genes (β2μG and PP1α) and expressed as 2-ΔCt. **=p<0.01 and *=p<0.05 between the indicated values.

Figure S2 Correlation of IL-8/CXCL8 secretion to Hsp70 and IL-6 secretion in co-culture. IL-8/CXCL8 concentration from baso-lateral part of co-cultures of primary airway epithelial cells (AECs) and MRC-5 cells was correlated with A) Hsp70 and B) IL-6 concentrations measured in the baso-lateral part of the same experiments. Concentrations are shown in a Log scale.
Figure S4 The adenlylate cyclase inhibitor (ACI) MDL-12330A hydrochloride does not block the epithelial conditioned medium-induced IL-8 release.

Conditioned medium was harvested from 16HBE14o- cells grown to confluence. IL-8 concentration released from confluent MRC-5 fibroblasts incubated with 16HBE14o- CM in the presence or absence of 10µM adenylate cyclase inhibitor MDL-12330A hydrochloride. Data is presented as mean±SEM of 3 independent experiments. *=p<0.05 between the indicated values.

Figure S3 Assessment of epithelial cell death after cigarette smoke extract stimulation. 16HBE14o- cells were grown to 90% confluence and then serum-deprived overnight before being stimulated with 0-60% CSE for 6 hours. Cell death was analyzed using the Annexin-V-PI staining for flow cytometry as described previously [32]. Percentage cell death of 16HBE14o- cells after CSE stimulation. Data is presented as mean±SEM of 3 independent experiments. *=p<0.05 & **=p<0.01 between the indicated values.