



Smoke Induced Changes in Epithelial Cell Gene Expression: Development of an *In Vitro* Model for COPD

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Summary

Chronic obstructive pulmonary disease (COPD) includes emphysema and chronic bronchitis, which are characterised by a progressive airflow limitation and chronic inflammation. The pathogenesis of COPD involves different cells, mainly epithelial cells, macrophages, neutrophils, and CD8 lymphocytes. Bronchial epithelium lines the mucosal surface of the airways, forming a mechanical barrier that separates the external environment from the internal milieu. Recently, substantial evidence has emerged indicating that airway epithelial cells are able to liberate a number of chemokines fundamental to both inflammatory and immune responses. Therefore, we established an in vitro model by showing that cigarette smoke is able to induce the release of chemokines by lung epithelial cells. Furthermore, we show that cigarette smoke induced chemokine expression is resistant to dexamethasone, mimicking the clinical situation. In contrast, pyrrolidinedithiocarbamic acid, an experimental antioxidant compound, inhibited smoke induced chemokine expression. These results suggest that this epithelial cell culture model may allow the evaluation of novel anti-inflammatory compounds for the treatment of COPD directly on the relevant target cells in vitro. This approach may result in the replacement of animal experimentation in screening of new therapeutics for COPD.

Zusammenfassung: Veränderung in der Genexpression epithelialer Zellen durch Rauch: Entwicklung eines *in vitro* Modells für COPD

Emphysem und chronische Bronchitis gehören zu den chronisch obstruktiven Lungenerkrankungen (COPD), die durch eine progressive Minderung des Luftflusses und chronische Entzündung gekennzeichnet sind. Verschiedene Zelltypen, vor allem Epithelzellen, Makrophagen, Neutrophile und CD8 Lymphozyten sind an der Pathogenese von COPD beteiligt. Bronchiales Epithel kleidet die Oberfläche der Schleimhaut der Luftwege aus und bildet so eine mechanische Barriere, welche die externe Umgebung vom internen Milieu abtrennt. Kürzlich wurde nachgewiesen, dass die Epithelzellen der Luftwege in der Lage sind, eine große Anzahl von Chemokinen freizusetzen, die eine bedeutende Rolle sowohl in entzündlichen wie auch in Immunprozessen spielen. Deshalb haben wir ein in vitro Modell etabliert, in dem wir zeigen, dass Zigarettenrauch die Freisetzung von Chemokinen aus Epithelzellen der Lunge induziert. Weiterhin zeigen wir, dass die durch Zigarettenrauch induzierte Chemokin-Freisetzung resistent gegenüber Dexamethason ist, was die klinische Situation widerspiegelt. Dagegen inhibierte Pyrrolidinedithiocarbamat, ein experimentelles Antioxidans, Rauch induzierte Chemokin-Expression. Diese Ergebnisse weisen darauf hin, dass dieses Epithelzellkulturmodell die Beurteilung neuer antientzündlicher Substanzen für die Behandlung von COPD direkt an der relevanten Zielzelle in vitro erlaubt. Dieser Ansatz könnte zum Ersatz von Tierexperimenten im Screening neuer Therapeutika für die Behandlung von COPD führen.

Keywords: 3R, COPD, cigarette smoke, epithelial cells, chemokines, inflammation

1 Introduction

Chronic obstructive pulmonary disease (COPD) is a respiratory condition characterised by chronic obstruction of expiratory flow affecting peripheral airways, associated with chronic inflammation, mucus hypersecretion, goblet cell and submucosal gland hyperplasia, and, in the final stage, pulmonary remodelling resulting in emphysema together with

fibrosis. Although COPD is a major cause of chronic morbidity and mortality throughout the world, its recognition as a public health problem has been neglected. The most important cause, by far, is cigarette smoking. However, 10 to 20% of COPD patients are lifelong non-smokers. Additionally, COPD worsens with age. As we face an ageing population, we will be confronted with more and more patients with COPD (Calverley and Walker, 2003).

COPD shares some factors with asthma such as familial components, inflammation, progressive changes. However, the pathogenesis of COPD is quite different from asthma and involves different cells mainly epithelial cells, macrophages, neutrophils, and CD8 T-lymphocytes. Unfortunately, much less is known about the pathogenesis of COPD than that of asthma. In the past years we have learned that COPD, like asthma, is an inflammatory disorder of small airways. It begins with biochemical and cellular

events occurring at the tissue level, which quickly attack small airways and surrounding alveoli. The exact mechanism of the orchestration and accumulation of various cells resulting in bronchial hyperresponsiveness, obstruction and finally in a loss of pulmonary surface is not known (Hill et al., 2000).

Without a doubt, tobacco smoking is a major cause of COPD. However, only a minority of smokers develops clinically relevant disease. The current understanding of the pathogenesis includes an "abnormal inflammation" as a response to various noxious agents. Oxidative stress, inflammation, tissue damage and tissue repair (remodelling) are parts of the complex procedure leading to COPD (Siafakas and Tzortzaki, 2002).

From the present pathogenetic point of view, there are several different cell types, which probably play crucial roles in the pathogenesis of COPD. Macrophages are long-lived effector cells within the lung. These cells may be the major source of matrix metalloproteinases (MMP) (Tetley, 2002). It should, however, be emphasised that they also exert defence mechanisms which either neutralise the effects of MMP or repair the lung tissue destruction. Neutrophils are also increased in the lung of smokers with COPD (Nowak et al., 1990). In the airways the number of CD8+ T-lymphocytes is characteristically increased (Saetta et al., 1999). However, as there is increasing evidence that the human bronchial epithelial cell helps regulate immune and inflammatory responses, it is likely that these cells play a pivotal role in the development of chronic airway inflammation and injury induced by tobacco smoke. The entire respiratory tract is lined with epithelial cells, which are continuously exposed to external factors such as bacteria, viruses, various particles, chemicals, etc. Thus, these epithelial cells play an important role in pulmonary host defence mechanisms. Epithelial cells, in addition to acting as a physicochemical barrier to the external environment, are able to synthesise and release a wide variety of mediators which will lead to inflammatory cell recruitment, cell differentiation and activation. Additionally, cigarette smoke may activate epithelial cells and other cells (e.g.

fibroblasts) to release pro-inflammatory cytokines, proteases and free radicals (Rusznak et al., 2000; Knight, 2001). There is also evidence, that other cells lining the alveolar space (e.g. alveolar epithelial cell type II) may release neutrophil chemotactic factors (Masubuchi et al., 1998).

In the present study, we have used epithelial cells to test the possible initiation of a pulmonary inflammation by releasing chemokines. Cultivated epithelial cells were exposed to cigarette smoke and the expression of several chemokines was measured. The aim of these studies was to establish a novel *in vitro* cellular system for studying pathomechanisms underlying the inflammation observed in COPD. Furthermore, this model system should allow for the evaluation of drug candidates without the use of animal experiments.

2 Materials and methods

2.1 Reagents

Oligonucleotides were synthesised by TIB Molbiol (Berlin, Germany) and Big Biotech (Freiburg, Germany). Dexamethasone and pyrrolidinedithiocarbamic acid (PDTC) were purchased from Sigma (Deisenhofen, Germany). IL-1 β was purchased from TEBU (Offenbach, Germany). HEPES Buffered Saline Solution, Trypsin/EDTA Solution and Trypsin Neutralising Solution were from Bio-Whittaker Europe (Verviers, Belgium). DMEM medium was obtained from Life

Technologies (Heidelberg, Germany). Chemokine measurements in culture supernatants were performed with ELISA kits from BD Pharmingen (Heidelberg, Germany). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Co (Deisenhofen, Germany).

2.2 Cell culture

A human lung adenocarcinoma cell line (A549 cells) representative of distal respiratory epithelium was obtained from ATCC. The cells were cultured in DMEM supplemented with 10% heat-inactivated foetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37°C. When confluent, the cells were washed and resuspended in serum-free DMEM at 10⁶ cells per ml and incubated in 500 μ l aliquots in 24-well tissue culture plates (Falcon Becton Dickinson Labware, Heidelberg, Germany).

2.3 Preparation of cigarette smoke conditioned medium

Fresh cigarette smoke conditioned medium (CSCM) was prepared using filtered cigarettes connected to a vacuum pump. The cigarette was lit and the smoke produced was then bubbled through 50ml of sterile DMEM medium. The vacuum was adjusted in a way that one cigarette was smoked over a period of 10 minutes. CSCM was used within 30 minutes. For all of the following experiments freshly made 10% CSCM was used, as this concentration has consistently been shown

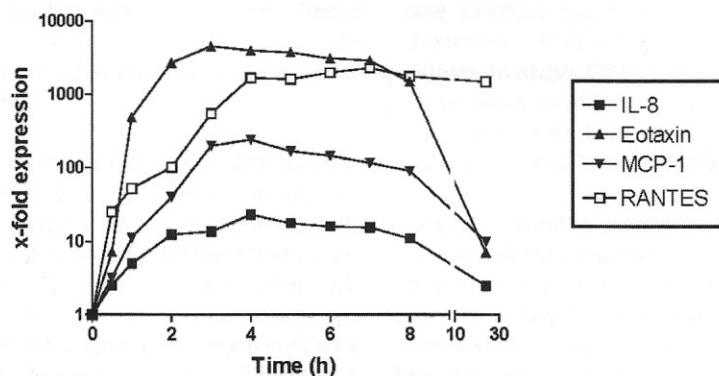


Fig. 1: Induction of chemokines in lung epithelial cells.

A549 cells were stimulated with IL-1 β (10ng/ml) and harvested after different time points. Chemokine mRNA levels were determined using real-time RT-PCR. Levels were normalised to β -actin and unstimulated cells were set to 1. Similar results were obtained in five independent experiments.

to stimulate biological responses without displaying evidence of epithelial cell cytotoxicity.

2.4 Chemokine expression

RNA was prepared from frozen lysates using RNeasy, Qiagen (Hilden, Germany). One-tube RT-PCR was performed using TaqMan EZ RT-PCR Kit from PE Applied Biosystems (Weiterstadt, Germany). Expression of chemokines was determined in relation to β -actin by real time RT-PCR using TaqMan assay on an ABI Prism 7900. Primers and probe sequences are available from the authors. Quantity of mRNA was calculated using the $\Delta\Delta C_T$ method. For each RT-PCR the threshold cycle (C_T) was determined, being defined as the cycle at which the fluorescence exceeds 10 times the standard deviation of the mean baseline emission for cycles 3 to 10. β -actin was used as a housekeeping gene to normalise mRNA levels: $C_T(\text{Parameter}) - C_T(\beta\text{-actin}) = \Delta C_T(\text{Parameter})$. This value was set in relation to the vehicle control, which represents mRNA levels of untreated stimulated cells: $\Delta C_T(\text{Parameter stimulated}) - \Delta C_T(\text{Parameter vehicle}) =$

$\Delta\Delta C_T(\text{Parameter})$. The relative mRNA level was then calculated as $2^{-\Delta\Delta C_T}$ based on the results of control experiments with an efficiency of the PCR reaction of approximately 100% (according to PE Applied Biosystems User Bulletin #2; ABI PRISM 7700 Sequence Detection System, 1997).

3 Results

3.1 Lung epithelial cells are capable of producing various chemokines

A549 lung epithelial cells were treated with IL-1 β , a well-known proinflammatory stimulus. The induction of mRNA of various chemokines was analysed after different times. Expression of mRNA for IL-8, MCP-1, and eotaxin increased rapidly during the first three hours (Fig. 1). After reaching a maximum between three and five hours mRNA levels declined back to baseline. In comparison, the increase of mRNA levels of RANTES was delayed and declined much more slowly (Fig. 1). The increase in mRNA levels was accompanied by

secretion of protein into the cell supernatant as determined by ELISA (data not shown).

3.2 Smoke induces chemokines in lung epithelial cells

Cell culture medium was conditioned by tobacco smoke to produce CSCM. Ten percent of this CSCM was added to A549 lung epithelial cells. The induction of IL-8, MCP-1 and RANTES mRNA could be observed, whereas the expression of eotaxin was not affected (Fig. 2). The induction was not as strong as compared to IL-1 β . However, CSCM and IL-1 β acted synergistically on IL-8 and MCP-1 induction. This effect could not be observed on RANTES induction. In contrast, CSCM inhibited IL-1 β induced eotaxin expression (Fig. 2). The increase in mRNA levels was accompanied by secretion of protein into the cell supernatant as determined by ELISA (Fig. 2).

3.3 Chemokine induction by smoke is resistant to dexamethasone

Current therapy of COPD is limited to very few drugs. No drugs are available to counteract the inflammatory process of

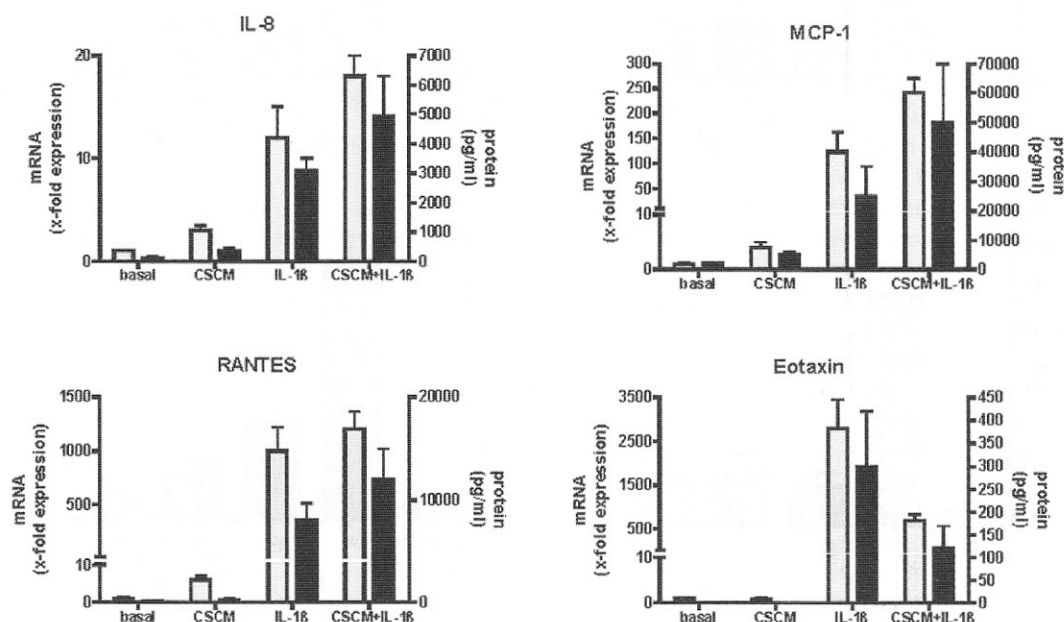


Fig. 2: Smoke conditioned medium induces chemokines in lung epithelial cells.

A549 cells were exposed to CSCM for four or 24 hours, respectively. Chemokine mRNA level were determined using real-time RT-PCR after four hours (gray bars). Levels were normalised to β -actin and

unstimulated cells were set to 1. Chemokine protein levels were determined by ELISA after 24 hours (black bars). Similar results were obtained in four independent experiments.



COPD. We investigated the effects of dexamethasone on chemokine gene expression. Preincubation of A549 lung epithelial cells with dexamethasone almost completely abolished IL-1 β induced IL-8 expression (Fig. 3A). In contrast, CSCM induced IL-8 expression was resistant to dexamethasone. In addition we analysed the effects of an experimental antioxidant compound. PDTc abolished the IL-1 β as well as the CSCM induced expression of IL-8 gene transcription (Fig. 3B). The changes in mRNA levels were mirrored by changes in secreted protein levels (Fig. 3C and D). The effects of dexamethasone and PDTc on MCP-1 and RANTES expression did not differ qualitatively from the effects shown on IL-8 expression (data not shown).

4 Discussion

COPD is a widely prevalent condition, usually associated with cigarette consumption. Besides the successful cessation of cigarette smoking, the future therapy of COPD will be directed toward a better, longer lasting bronchodilation, suppression of the underlying inflammation, improvement of the mucociliary clearance and repair of lung damage. The focuses of our interest are inflammatory mechanisms in COPD and their manipulation by different drugs.

The exact mechanisms leading to smoke-induced changes in the lung are not known. Many different cell types such as macrophages and neutrophils play major roles in the pathogenesis of COPD. However, their precise role needs to be clarified. For example, the cells and

proteases that mediate smoke-induced inflammation are discussed controversially: One favours neutrophils and their serine proteinases, and others favour macrophages with MMPs (Hill et al., 2000). It is, however, very likely, that epithelial cells which act as a barrier and are the first cells, exposed to environmental factors, may play an important role in the early stage of neutrophil inflammation in COPD. It has been proposed, that they exert their effects via chemokine release. Chemokines are a group of small molecules that act on different types of leucocytes via a chemotactic gradient. They have a wide range of effects, mainly cellular recruitment, activation and differentiation. They are fundamental regulators of leukocyte homeostasis and inflammation (Sabroe et al., 2002).

In the present study we have clearly

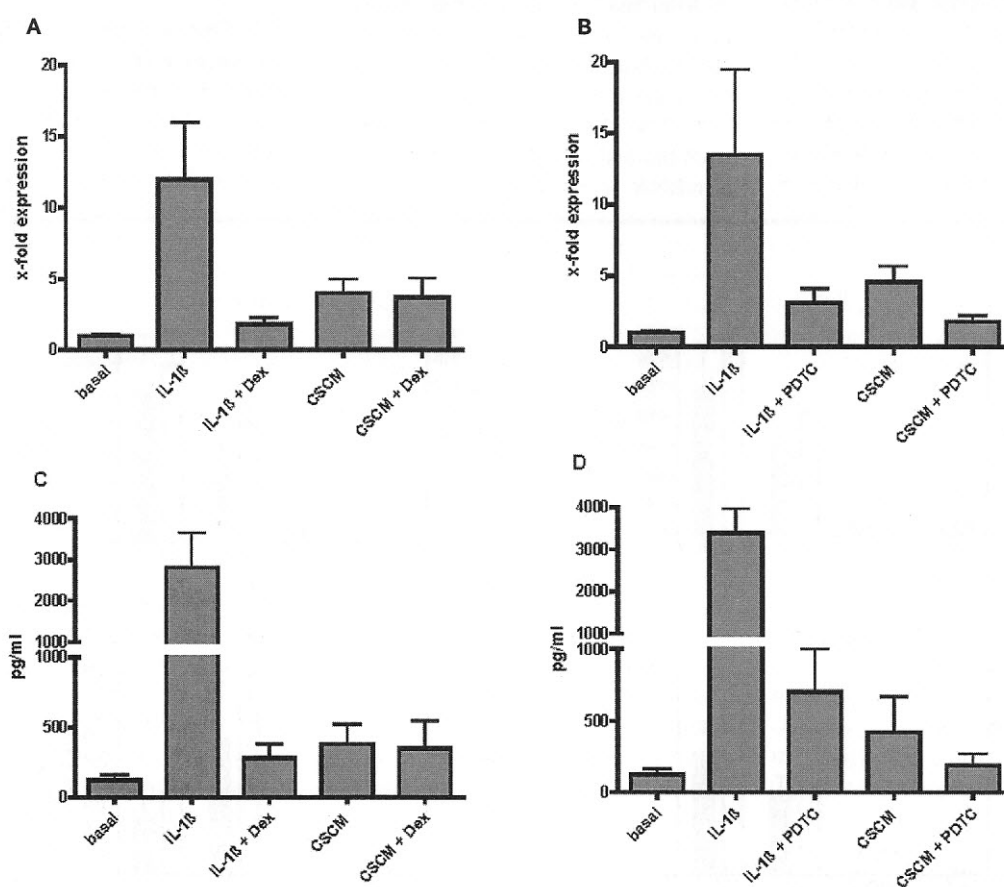


Fig. 3: Inhibition of IL-8 by dexamethasone.

A549 cells were preincubated with vehicle or 1 μ M dexamethasone (A, C) or 100 μ M PDTc (B, D). After 30 minutes cells were either left unstimulated, stimulated with IL-1 β (10 ng/ml) or exposed to CSCM for four hours (A, B) or 24 hours (C, D). RNA levels were

determined by quantitative real-time RT-PCR and IL-8 mRNA levels were referenced to β -actin and unstimulated cells (A, B). IL-8 protein levels in the supernatant were determined by ELISA (C, D). This is one experiment representative of three.

demonstrated that lung epithelial cells are able to synthesise chemokines. A proinflammatory stimulus like IL-1 β induces a strong upregulation of gene expression of several chemokines. Similar findings have been reported by others (Pechkovsky et al., 2000). It has also been shown that cigarette smoke stimulates lung fibroblasts to release neutrophil and monocyte chemotactic activities (Sato et al., 1999). Based on this, we hypothesised, that cigarette smoke might stimulate lung epithelial cells to release chemokines. In order to mimic the situation during inhalation of cigarette smoke into the lung, we exposed lung epithelial cells to cigarette smoke conditioned medium (CSCM). We observed a marked induction of several chemokines under these conditions. The induction was lower as compared with IL-1 β . Interestingly however, eotaxin, a chemokine mainly attracting eosinophils, was not induced, whereas IL-8, a chemokine mainly attracting neutrophils, was induced by CSCM. Since this is reminiscent of the inflammatory situation in COPD, we asked whether dexamethasone is able to inhibit the synthesis of chemokines. It potentially inhibited the IL-1 β induced chemokine synthesis; whereas the CSCM induced synthesis was resistant. Again this reflects very well the clinical situation, where steroids are almost ineffective in the treatment of COPD patients (Keatings et al., 1997). Oxidative stress in COPD has been shown to occur (MacNee, 2001). Therefore, we tested whether an experimental antioxidant compound might inhibit the CSCM induced chemokine expression. PDTC has been shown to inhibit the hypoxia induced activation of NF- κ B and TNF- α in macrophage cells (Chandel et al., 2000). In our experimental system, PDTC was also active in inhibiting CSCM induced chemokine gene expression. This reinforces the idea of using antioxidants to treat the inflammatory process going on in COPD (Rahman, 2002).

The cell culture model presented here may provide an *in vitro* system to investigate the mechanism by which cigarette smoke induces inflammation. These preliminary results suggest, that this model can be further developed to predict the effectiveness of novel anti-inflammatory

compounds for COPD treatment. However, additional data from other cells, especially human primary bronchial epithelial cells, are needed to support the usefulness of this model. By using human lung epithelial cells the experiments can target the relevant cells directly. Animals are not required as direct objects of such experiments or as donors of tissue and cells.

Altogether, our present data suggest that cultured epithelial cells treated with smoke-conditioned medium represent an interesting *in vitro* model for investigating the pathomechanism of COPD and for seeking novel compounds suitable for the treatment of inflammation in COPD.

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