Effects Of Cigarette Smoke Extract On Human Airway Smooth Muscle Cell From COPD

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Take home message

Cigarette smoke extract (CSE) induces changes associated with airway remodelling in airway smooth muscle cells from people with COPD.

ABSTRACT

We hypothesised that the response to cigarette smoke in airway smooth muscle (ASM) cells from people with chronic obstructive pulmonary disease (COPD) would be intrinsically different from people without COPD, producing greater pro-inflammatory mediators and factors relating to airway remodelling.

ASM cells were obtained from smokers with or without COPD, and then stimulated with cigarette smoke extract (CSE) or TGF-β1. The production of chemokines and matrix metalloproteinases (MMPs) were measured by ELISA, and the deposition of collagens by extracellular matrix (ECM) ELISA. The effects of CSE on cell attachment and wound healing were measured by toluidine blue attachment and cell tracker green wound healing assays.

CSE increased the release of CXCL-8 and CXCL-1 from human ASM cells, and cells from COPD produced more CSE induced CXCL-1. The production of MMP-1, -3, -10 and the deposition of collagen VIII α 1 (COL8A1) were increased by CSE, especially in the COPD group which had higher production of MMP-1 and deposition of COL8A1. CSE decreased ASM cell attachment and wound healing in the COPD group only.

ASM cells from smokers with COPD were more sensitive to CSE stimulation, which may explain, in part, why some smokers develop COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide and results in an economic and social burden that is both substantial and increasing [1, 2]. In COPD a number of changes occur in the lungs, namely the development of persistent inflammation and irreversible airflow limitation[3].

Airflow limitation is caused by three interrelated processes: thickening (remodelling) of the small airway walls, loss of small airways, and emphysema. However, small airway remodelling is considered to have the greatest influence on airflow limitation [4-6]. Furthermore, it is likely that the small airway remodelling is the primary pathological insult in COPD. In a study using micro-CT analysis of COPD lung tissue, it was found that remodelling and loss of terminal bronchioles preceded emphysematous changes microscopically [7]. The small airway remodelling in COPD consists of folded mucosa, thickening of basement membrane and deposition of connective tissue, as well as increased airway smooth muscle (ASM) mass, especially in severe COPD [6, 8, 9]. The connective tissue consists of an intertwined framework of extracellular matrix (ECM) proteins, and the specific ECM proteins are known to be altered in the airways of patients with COPD [10, 11].

In the developed world the main risk factor for the development of COPD is cigarette smoking, and through the use of both in-vivo and in-vitro models the effects of smoking upon the aetiology of COPD is beginning to be understood. Most studies address the paradigm that the aetiology of COPD is cigarette smoke induced inflammation leading to tissue damage, however our previous research suggested that airway remodelling may be induced independently of inflammation [12]. We previously have found that fibroblasts from patients with COPD produced an excessive amount of fibronectin and perlecan or reduced proteoglycans (decorin and biglycan) in response to cigarette smoke extract (CSE), in comparison to cells from people without COPD [12, 13]. Epithelial cells in

COPD also respond differently to CSE [14], but whether COPD ASM cells respond differently to CSE is not known.

In this study we hypothesised that the response to cigarette smoke in ASM cells from people with COPD would be intrinsically different to that in ASM cells from people without COPD, specifically in the production of pro-inflammatory mediators and factors relating to airway remodelling.

MATERIAL AND METHODS

Study subjects

Subject information was obtained regarding diagnosis, smoking history, and lung function. Subjects with a diagnosis of asthma, infectious diseases or interstitial lung disease were not included. Samples were obtained from subjects who were classified as follows according to severity of airflow limitation [15]. 1) non-COPD (n=21); forced expiratory volume in one second (FEV_1)/ forced vital capacity (FVC) $\geq$ 70% and $FEV_1 \geq$ 80%. 2) COPD (n=20); $FEV_1/FVC < 70\%$. Full details are provided in the online supplement (supplementary table. S1). All study subjects or their next of kin provided written informed consent. Approval of all the experiments using human lung tissues was provided by the Ethics Review Committee of the South West Sydney Area Health Service, St Vincent's Hospital Sydney, and the University of Sydney Human Research Ethics Committee.

Cell culture and sample preparation

Human ASM cells were obtained from human lung by a method modified from that described previously [16]. Human ASM cells were micro-dissected from approximately sixth-order or greater bronchi, and were initially cultured in growth medium made up of Dulbecco's modified eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum (FBS) (DKSH, Melbourne, AUS), 1% antibiotics (Invitrogen) and 25mM Hepes (Invitrogen). All the cells tested negative for the presence of mycoplasma before they were set up for experiments, and were used between passages 2 and 7. ASM cells were seeded in 6-well or 96-well culture plates (BD Biosciences, North Ryde, Australia) at a density of $1x10^4/cm^2$ in growth medium and incubated at 37°C/ 5% CO_2 for 72 hours, and cells were starved in quiescing medium consisting of DMEM supplemented with 0.1% FBS, 1% antibiotics and 25mM Hepes for 24 hours before stimulation with CSE or 10ng/ml of TGF-β_1 (R&D Systems, Minneapolis, MN, USA) in quiescing medium. After stimulation, supernatants from human ASM cells (in 6-well plates) were collected, and cells (in 96-well plates) were lysed with 0.016mM hydroxylamine (NH_4OH) at 37°C for 20 minutes then washed with 0.05% phosphate buffered saline (PBS)-Tween 20 (vol/vol). These supernatants and cell free ECM plates were stored at -20°C until analysis.

Cigarette smoke extract

We used Marlboro Red cigarettes, and each cigarette contained 1.1mg of nicotine, 15mg of TAR, and 15mg of CO. Cigarette smoke extract (CSE) was prepared by a method modified from that previously described [12]. Briefly, one Marlboro cigarette was bubbled through 25ml of DMEM at a constant rate and this solution was regarded as 100% concentration CSE. The 100% CSE was freshly generated for each experiment, and diluted to final working concentration and used within 30 minutes.

Cell viability and toxicity assays

Human ASM cells were seeded in 96-well plates as described above, and cells were stimulated with serial dilutions of CSE from 0.05% to 50%. The mitochondrial activity of living cells was tested by Thaizolyl blue tetrazolium bromide (MTT) (Sigma Aldrich, St Louis, MO, USA) assay. The membrane integrity of cells was tested by a lactate dehydrogenase (LDH) (Sigma Aldrich) assay which is based on the amount of cytoplasmic LDH released into the medium. After stimulation for 72 hours, MTT and LDH release were measured using a spectrophotometer (Spectramax M2, Molecular Devices, Sunnyvale, CA, USA) setting with absorbance 570nm/690nm and 490nm/690nm respectively.

Chemokine enzyme-linked immunosorbent assay (ELISA)

Human ASM cells were seeded in 6-well plates as described previously, and cells were stimulated with different concentration of CSE or 10ng/ml of TGF-β_1 for 72 hours. The concentrations of α-chemokine-8 (CXCL-8) (interleukin (IL)-8), α-chemokine-1 (CXCL-1) (growth related oncogene-alpha (Groα)), chemokine (c-c motif) ligand 2 (CCL-2), chemokine (c-c motif) ligand 5 (CCL-5), and c-x-c motif chemokine 10 (CXCL-10) in the supernatants from human ASM cells were measured by using commercial human CXCL8/IL-8, CXCL1/GROα, CCL2/MCP-1, CCL5/RANTES, and CXCL10/IP-10 ELISA kits (R&D) according to the manufacturer's instructions. The absorbance was read at 450nm/570nm using a spectrophotometer (Spectramax M2).

Transcription factor NF-κB and AP-1 activity assay

Human ASM cells were seeded in 6-well plates as described previously, and cells were stimulated with CSE (5% and 10%) or 10ng/ml of TGF-β_1 for 60 minutes and then nuclear extracts collected. The activities of NF-κB and AP-1 of each sample were assessed using the TransAM ELISA kits according to the manufacturer's instructions (Active Motif,Carlsbad, CA, USA). The absorbance was read at 450nm/655nm using a spectrophotometer (Spectramax M2).

Real time polymerase chain reaction (PCR) array

Human ASM cells obtained from smokers with (n=3) and without COPD (n=3) were stimulated in-vitro with 5% CSE, 10% CSE or 10ng/ml of TGF-β_1. RNA was collected at 48 hours then purified using the Isolate RNA mini kit (Bioline, London, UK), and mRNA was converted to cDNA using M-MLV reverse transcriptase (Invitrogen). Equal amounts of cDNA of each sample from the same group were pooled; the gene expression was then tested using TaqMan array human extracellular matrix & adhesion molecules 96-well plates according to the manufacturer's instructions (Invitrogen). Real time PCR was performed using the StepOne Plus detection system, data were collected and analysed by StepOne software (Applied Biosystems, Melbourne, AUS). The relative abundance of mRNA was calculated using the ΔΔCt method [17], and results were normalized to 18S rRNA.

Matrix metalloproteinase (MMP) enzyme-linked immune sorbent assay (ELISA)

Human ASM cells were seeded in 6-well plates as described previously, and cells were stimulated with different concentration of CSE or 10ng/ml of TGF-β_1 for 72 hours. The concentrations of total MMP-1, -2, -3, -10 and -12 were measured using human MMP ELISA kits (R&D) according to the manufacturer's instructions, and the reading was performed using a Luminex analyzer (Luminex 200 System, Luminex, Brisbane, AUS). MMP-1 enzyme activity was measured using human active MMP-1

fluorescent assay kit (R&D), and the relative fluorescence units were determined using a fluorescence plate reader (Spectramax M2) setting with excitation wavelength 320nm and emission wavelength 405nm.

ECM enzyme-linked immune sorbent assay (ELISA)

Human ASM cells were seeded in 96-well plates as described previously, and cells were stimulated with different concentration of CSE or 10ng/ml of TGF-β_1 for 72 hours. Cell free ECM plates were used to measure the deposition of protein in the ECM by ELISA according to the method previously modified [16]. Primary antibodies used for detecting ECM proteins were rabbit polyclonal anti-human collagen V (COL5) antibody (2µg/ml) (Abcam, Cambridge, UK), rabbit polyclonal anti-human collagen VIII alpha 1 (COL8A1) antibody (2µg/ml) (Novus Biologicals, Littleton, CO, USA), mouse monoclonal anti-human fibronectin antibody (2µg/ml) (Millipore, Billerica, MA, USA), and mouse monoclonal anti-human perlecan antibody (2µg/ml) (Invitrogen). Rabbit IgG (Dako Cytomation, Glostrup, CA) isotype control antibody and mouse IgG1,κ isotype control antibody (BD) were used at the same concentration as the primary antibodies. For measurement of COL8A1 and perlecan, the biotinylated goat anti-rabbit antibody 0.5µg/ml and biotinylated chicken anti-mouse antibody 0.8µg/ml were used respectively.

Western blots

Human ASM cells were seeded in 6-well plates as described previously, and cells were stimulated with CSE (5% or 10%) or TGF-β_1 for 72 hours. The supernatants from each sample were collected to assess the soluble collagen type 1 (COL1) and soluble fibronectin (FN) using western blots. Proteins were size fractionated on 10% polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes, and blocked in 5% (wt/vol) skim milk solution for 1 hour. The membranes were incubated with primary antibody (8.8 µg/ml of mouse monoclonal anti-COL1 antibody (Sigma) or 1 µg/ml of mouse monoclonal anti-human FN antibody (Millipore) in 2% BSA/TBS-Tw) for 2 hours, followed by incubation with secondary antibody (2.6 µg/ml of rabbit anti-mouse Ig-HRP antibody (Dako) in 2% BSA/TBS-Tw) for 1 hour. Immunoblot detection was performed using Immobilon Western Chemluminescent HRP Subtrate (Millipore) and bands were analysed by using Kodak image station 4000 MM, and the amount of protein present in each sample was determined as the densitometric density.

Immunohistochemistry (IHC)

The preparation of immunohistochemical samples and immunohistochemical methods were as previously described [18]. The airway sections were treated to minimize non-specific background staining, and incubated with primary rabbit polyclonal anti-human COL8A1 antibody 1 µg/ml (Abcam) and rabbit IgG isotype control antibody 1 µg/ml (Dako). The conjugated secondary antibody was labelled polymer anti-rabbit (EnVision+System-HRP) (Dako) and the tissue staining was visualized with substrate chromogen, liquid 3,3'-diaminobenzidine (DAB) (Dako). 10 images of each section (one section per subject) were taken and immunostaining was quantified using Fiji software (ImageJ) [19]. Full details are provided in the online supplement.

Cell attachment assay

96-well culture plates were exposed to growth medium for 72 hours and to quiescing medium for 24 hours then exposed to quiescing medium with different concentration of CSE (0.05% to 10%) for 72 hours. Human ASM cells were seeded on these treated plates at a density of $5x10^4/cm^2$ in quiescing medium for 2 hours. Cell attachment was detected by a toluidine blue attachment assay as previously described [20]. The relative number of attached cells was measured using spectrophotometry at an absorbance of 595 nm (Spectramax M2). Full details are provided in the online supplement.

Wound healing assay

An Oris cell migration assembly kit (Platypus Technologies, Madison, WI, USA) was used to perform the wound healing assay. The wound was created on the treated 96-well black plate and cells were labelled with cell tracker green (CMFDA) (Invitrogen). The labelled human ASM cells were seeded on the wounded black plate at a density of $5x10^4/cm^2$ in growth medium. After adhesion for 24 hours the stoppers were removed, and incubation was continued for 4 hours. The wound healing value was measured using a fluorescence plate reader (Wallac VICTOR^2, Perkin Elmer, Waltham, MA, USA) read from the bottom with excitation wavelength set at 485 nm and emission wavelength at 535 nm. Full details are provided in the online supplement.

Statistical analysis

Data analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). All the data were presented as mean ± standard error of the mean (SEM). One-way analysis of variance (one-way ANOVA), two-way ANOVA plus Bonferroni post test or Mann Whitney test were as appropriately used to determine the statistical significance. A p value of less than or equal to 0.05 ($p \leq 0.05$) was considered significant.

RESULTS

Cytotoxicity of CSE on ASM cells

As high concentrations of CSE are known to be cytotoxic we used two toxicology assays, assessment of mitochondrial activity via MTT and membrane integrity via LDH release, in order to ensure that the concentrations of CSE we used had no toxic effects on human ASM cells. CSE at a concentration greater than or equal to 15% substantially reduced viability (fig. 1), therefore, we used 0.05% to 10% CSE as stimulation in subsequent experiments.

CSE induces chemokines

As CSE is known to induce CXCL-8 release from human ASM cells [21], we used this output to validate our in-vitro model. CXCL-8 release was increased by 10 %CSE and 10ng/ml of TGF-β_1 in both non-COPD and COPD groups (fig. 2 a,b). The release of CXCL-1 was increased by 10% CSE in the non-COPD group and increased by both 5% and 10% CSE in the COPD group, however TGF-β_1 did not induce the release of CXCL-1 in either group (fig. 2 c,d). There were no differences in basal or maximum production of CXCL-8 and CXCL-1 between ASM cells from these two groups. Our results show that the release of CCL-5 and CXCL-10 from ASM cells were too low to be detected (the

detection limit of these assays are 15.625pg/ml and 31.35pg/ml respectively). We found that CCL-2 was produced by ASM cells, but this was not induced by CSE or TGF-β_1, and there were no differences between the non-COPD and COPD groups (data not shown).

CSE effects transcription factors DNA-binding activity

Our results show that neither CSE nor TGF-β_1 increased the activity of transcription factor NF-κB (supplementary fig. S1 a,b), however, 5% CSE increased the activity of transcription factor AP-1, in only the non-COPD group (supplementary fig. S1 c,d).

Gene expression of ECM and adhesion molecule related genes

To investigate the potential effects of CSE on airway remodelling we used a PCR based array as a screening tool. The gene expression of 70 ECM proteins, adhesion molecules and MMPs were assessed in response to CSE and TGF-β_1 stimulations (supplementary fig. S2). CSE up-regulated more ECM and adhesion molecule associated genes than TGF-β_1, and there were differences in MMPs gene expression between CSE and TGF-β_1 stimulations. Using a ≥2 cut off line, and/or over 1.5 fold differences between COPD and non-COPD group, MMP-1, 2, 3, 10 and 12, and COL5, 7 and COL8A1 were further investigated.

CSE induces MMPs

To verify the MMP changes, we measured the release of MMP-1, 2, 3, 10 and 12 from human ASM cells. After stimulation with either CSE or TGF-β_1, the concentration of total MMP-12 in the supernatants from human ASM cells was too low to be detected (data not shown). The concentration of total MMP-1 was increased by 10% CSE in the non-COPD group and increased by 5% and 10% CSE in the COPD group (fig.3 a,b). Neither CSE nor TGF-β_1 affected on the production of MMP-2 (fig. 3 c,d). 10% CSE increased the release of MMP-3 and MMP-10 in both groups, whilst TGF-β_1 increased the release of MMP-3 and MMP-10 in the COPD group only (fig. 3 e-h).

The production of active MMP-1

To further investigate the effect of CSE on the activity of MMP-1, we measured the active form of MMP-1 from human ASM cells. The concentration of active MMP-1 was increased by 10% CSE in both groups, while TGF-β_1 decreased the release of active MMP-1 in non-COPD group only (fig. 4).

The deposition of ECM proteins

To verify the alteration of ECM proteins after stimulation with CSE or TGF-β_1, we assessed the deposition of COL5, 7 and COL8A1 in the ECM. The deposition of COL7 was too low to be detected (data not shown). CSE did not alter the deposition of COL5 (supplementary fig.S3). The deposition of COL8A1 was increased by 0.5%, 1%, 5%, and 10% CSE only in the COPD group, and there was significantly more COL8A1 induced by 1% and 5% CSE from the COPD cells compared to the non-COPD cells (fig. 5 a). TGF-β_1 increased the deposition of COL8A1 in the COPD group only (fig. 5 b). CSE did not alter the deposition of fibronectin from either group which in stark contrast to our previous findings in fibroblasts [12], and CSE inhibited the deposition of perlecan in human ASM cells from both groups (supplementary fig.S4).

The release of ECM proteins

To verify the alteration of soluble ECM proteins after stimulation with CSE or TGF-β_1, we assessed the soluble COL1 and soluble FN in the supernatants using western blots. Our results show that neither CSE nor TGF-β_1 significantly increased the release of COL1 (supplementary fig. S5). Our results also show that CSE did not induce the release of soluble FN, however, TGF-β_1 significantly increased the release of FN (supplementary fig. S6).

Collagen VIII alpha 1 in airway bronchus

As we had found greater CSE induced COL8A1 by the COPD ASM cells, we next investigated if our in-vitro findings were reflective of COPD in-vivo. Immunohistochemistry (IHC) revealed COL8A1 was expressed in airway tissue from patients with (n=10) and without COPD (n=7). In the airways from both groups the COL8A1 appeared to be localised in the basement membranes, vascular walls and ASM bundles. The positive staining was controlled by threshold and isotype control staining. Using densitometric analysis (quantification of staining area) we found there was higher overall expression of COL8A1 in the COPD group (fig. 6).

CSE inhibits Cell attachment and wound healing

To further investigate the effect of CSE on the function of human ASM cells, we assessed cell attachment of ASM cells from people with (n=5) and without (n=5) COPD. CSE significantly decreased the attachment of ASM cells to culture plates from the COPD group only (fig. 7). Wound healing assays showed that high concentration of CSE significantly decreased the rate of wound healing in human ASM cells (n=6) (fig. S7).

DISCUSSION

We have found differential responses to CSE in ASM cells from smokers with and without COPD. Specifically MMP-1 and the deposition of COL8A1 in ASM cells were increased by CSE, and these increases were higher in the COPD group. Our results also showed that CSE decreased ASM cell attachment to culture plates and wound healing specifically in cells isolated from smokers with COPD. These findings suggest that ASM cells from smokers with COPD are more sensitive to CSE stimulation which may explain, in part, the development of COPD in some smokers.

COPD is an inflammatory disease characterised by increased number of neutrophils [9, 22], and increased amount of neutrophil chemokines (such as CXCL-8 and CXCL-1) in bronchoalveolar lavage fluid (BALF) [23, 24]. CSE is a potent inducer of CXCL-8 in ASM cells [21, 25], however whether hypersecretion of CXCL-8 occurs in ASM cells isolated from patients with COPD as occurs in other airway cells [26, 27] was not known. Therefore, we also measured CSE-induced CXCL-8, and found CSE increased the release of CXCL-8 from ASM cells yet without differences between cells from people with and without COPD. We also measured CSE-induced CXCL-1, and found CSE increased the release of CXCL-1 from ASM cells, and furthermore ASM cells from COPD patients were more sensitive to CSE stimulation for the production of CXCL-1. Both CXCL-8 and CXCL-1 have similar biological properties, in that they both have effects on the recruitment of neutrophils [28-30]. In our study we used ASM cells from smokers with and without COPD. The induction of CXCL-8 and CXCL-1 by CSE in ASM cells from both groups may reflect the observation that neutrophils are increased in COPD patients [31] and smokers without COPD [32]. Furthermore, as low concentration of CSE

induced CXCL-1 in the COPD cells only, this suggested that these cells were hyperresponsive to CSE, and may explain why some smokers develop COPD and others do not.

Our results showed that high concentrations of CSE increased the release of both pro and active MMP-1 (interstitial collagenase) from human ASM cells, and this seemed more pronounced in smokers with than without COPD. When we measured only active MMP1 we found similar production between cells from both groups. This indicates that production of active MMP-1 in ASM may not be a key determinant of lung pathology in COPD, but may be related to processes common to both smokers with and without COPD. In other cells, CSE increased the production of MMP-1 from human epithelial cells and human lung fibroblasts which appear to be driven primarily through the extracellular regulated kinase-1/2 (ERK1/2) mitogen activated protein kinase pathway [33, 34]. We also found human ASM cells constitutively produced high levels of MMP-2 (gelatinase A) and this was not increased by CSE, which is in contrast to findings in cigarette smoke exposed fibroblasts [35].

Compared to other MMPs, MMP-3 (stromelysin 1) and -10 (stromelysin 2) have not been extensively studied so far. Our study showed that CSE increases the gene expression of MMP-3 and -10 in human ASM cells, and that 10% CSE increases production of MMP-3 and -10. These results indicate that both MMP-3 and -10 may lead to different progression in smokers with COPD. Two genotyping studies indicated that MMP-3 polymorphisms associate with disease progression in COPD [36, 37]. Another study showed the expression of the MMP-10 gene was increased in both small airways and the parenchyma surrounding small airways in association with progression of COPD [38]. Our results additionally found MMP-12 (macrophage elastase) gene expression to be increased by CSE in-vitro. However, the release of MMP-12 protein from CSE stimulated ASM was lower than the detection limit of the assay (9.2 pg/ml).

We have previously shown that CSE increased the deposition of fibronectin and perlecan from COPD fibroblasts [12], so in this study we also measured their production by ASM cells. However, CSE did not affect the production of fibronectin in COPD ASM cells, and decreased the production of perlecan indicating that responses to CSE are cell type specific. Our array data showed that several collagens also changed in response to CSE stimulation, so we chose to evaluate COL5, 7 and 8. The protein level of COL5 did not change in response to CSE, and the tools to measure COL7 were unreliable. However, CSE induced greater COL8 production from COPD than from control ASM cells. There is little known about COL8 in COPD, especially not on the amount of COL8 in airways of people with and without COPD. In this study we found that the expression of COL8 was increased in COPD airways, particularly in and around the smooth muscle bundles, suggesting that the smooth muscle produces COL8 in-situ and indicating that the deposition of COL8 from smokers with COPD is likely to contribute to the airway pathology. COL8 has a short triple helix and contains α1 and α2 chains, and each α chain contains a collagenous domain, a short N-terminal non-triple-helical region (NC2) and a longer C-terminal non-triple-helical domain (NC1). As is known for other collagens, different regions of COL8 can have opposing biological effects. For example, the entire COL8 molecule increased aortic smooth muscle cell proliferation and migration [39], whilst the NC1 domain of COL8A1 inhibited the mitochondrial activity of bovine aortic endothelial cells [40]. COL8 has not been reported previously as a determinant of ECM in COPD, but exposure of pregnant mice to pollution resulted in increased COL8 in the tubular cells in the kidney of offspring [41]. This raises the question as to whether similar hereditary effects could occur in the offspring of pregnant mothers who smoke. In addition, the functional and long-term outcomes of such exposures have not been examined.

We have not investigated if any interaction between MMP-1 and Collagen VIII occurs, and have not been able to find specific examples in the literature. It would be tempting to speculate that the increased MMP could degrade the collagen, however we found that they both increased at the same time, showing that the net effect is collagen deposition

In COPD there is impaired repair in the small airway walls and alveolar walls [6, 7]. Our results show that CSE only reduced cell attachment in human ASM cells from patients with COPD, and higher concentration of CSE also decreased the wound-healing rate of ASM cells. Those results may indicate an innate difference of ASM cells from smokers with and without COPD. One study about the effect of CSE on the function of the human lung has shown that CSE reduces the migration and contractile activity of normal human bronchial smooth muscle cells[42]. In another study it was shown that CSE impairs the wound healing of bovine bronchial epithelial cells via a reactive oxygen species dependent mechanism [43]. Another study has shown CSE inhibits the proliferation of human lung fibroblasts [44], but whether these fibroblasts were derived from patients with COPD or not was not clear.

To investigate if differences in transcription factor activity could account for the increased responsiveness of the COPD ASM cells to CSE we chose to measure the activity of the transcription factors NF-κB and AP-1, as we have previously shown these be involved in the release of MMPs and cytokines from ASM cells [45][46]. We found no evidence of increased transcription factor activity in COPD ASM cells. This suggests that the increased response to cigarette smoke in the COPD cells may occur due to epigenetic changes, as we have found in other cells in COPD [47]

In conclusion, we showed the differential production of chemokines, MMPs and collagens by human ASM cells from people with and without COPD in response to cigarette smoke stimulation. ASM cells isolated from subjects with COPD showed a higher response to cigarette smoke in the release of inflammatory mediators and factors associated with airway remodelling, and cell behaviour. Our data from this manuscript suggests that the ASM in COPD is capable of responding to the soluble components of cigarette smoke. This would act to recruit neutrophils (through the release of chemokines) and potentially affect other airway cells through the altered deposition of ECM. Since the amount of smooth muscle is positively correlated with COPD severity these effects may be amplified in severe COPD. Therefore, our findings suggest that ASM cells from smokers with COPD contribute to the pathological development of this disease.

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FIGURE 1. The effect of CSE on cell viability. The cytotoxicity of cigarette smoke extract (CSE) a) on the mitochondrial activity and b) lactate dehydrogenase (LDH) release of human ASM cells was measured by MTT and LDH assays. Human ASM cells were stimulated with serial dilution of CSE for 72 hours (n=4). Data were presented as mean ± SEM, one-way ANOVA plus Bonferroni post test, *P < 0.05, **P < 0.01, ***P < 0.001, compared with control.

FIGURE 2. The release of α-chemokine-8 (CXCL-8) and α-chemokine-1 (CXCL-1) from human ASM cells. The concentrations of a,b) CXCL-8 and c,d) CXCL-1 in the supernatants from human ASM cells from subjects with (n=9, black bar) and without (n=9, white bar) COPD after 72 hours stimulation with cigarette smoke extract (CSE) or TGF-β_1 were measured by ELISA. Data were presented as mean ± SEM, two-way ANOVA plus Bonferroni post test, **p<0.01, ***p<0.001, compared with control.

FIGURE 3. The production of matrix metalloproteinases (MMPs) from human ASM cells. The concentrations of a,b) MMP-1, c,d) MMP-2, e,f) MMP-3, and g,h) MMP-10 in the supernatants from human ASM cells from subjects with (n=8, black bar) and without (n=7, white bar) COPD after 72 hours stimulation with cigarette smoke extract CSE or TGF-β_1 were measured by ELISA. Data were presented as mean ± SEM, two-way ANOVA plus Bonferroni post test, *p<0.05, **P<0.01, ***p<0.001, compared with control; #p<0.05, comparison between two groups.

FIGURE 4. The production of active matrix metalloproteinase (MMP)-1 from human ASM cells. The concentrations of active MMP-1 in the supernatants from human ASM cells from subjects with (n=5, black bar) and without (n=5, white bar) COPD after 72 hours stimulation with cigarette smoke extract (CSE) or TGF-β_1 were measured by ELISA. Data were presented as mean ± SEM, two-way ANOVA plus Bonferroni post test, *p<0.05, **P<0.01, ***p<0.001, compared with control

FIGURE 5. The deposition of collagen VIII α 1 (COL8A1) from human ASM cells. The deposited COL8A1 in the ECM from human ASM cells from subjects with (n=7, black bar) and without (n=7, white bar) COPD after 72 hours stimulation with cigarette smoke extract (CSE) or TGF-β_1 was measured by ECM ELISA. Data were presented as mean ± SEM, two-way ANOVA plus Bonferroni post test, *p<0.05, ***p<0.001, compared with control; #p<0.05, comparison between two groups.

FIGURE 6. Collagen VIII α 1 (COL8A1) airway tissue staining (20X magnification). a) COL8A1 in airway bronchus from subjects with (n=10) and without (n=7) COPD was measured by immunohistochemistry. Specific staining was detected by using a chemical chromophore DAB (brown) and cell nucleus was counterstained with haematoxylin (blue). Tissue structure was stained by haematoxylin and eosin (H&E) staining. The scale represents 100μm. b) Immunostaining of COL8A1 in non-COPD and COPD groups were quantified by positive staining area and corrected with isotype control. Data were presented as median, Mann Whitney test, *p<0.05.

FIGURE 7. The effect of cigarette smoke extract (CSE) on cell attachment. 2 hours cell attachment on CSE treated plate of human ASM cells from subjects with (n=5, black bar) and without (n=5, white bar) COPD was measured by a toluidine blue assay. Data were presented as mean ± SEM, two-way ANOVA plus Bonferroni post test, *p<0.05, **P<0.01, ***p<0.001, compared with control.