The role of accelerated ageing in aberrant lung tissue repair and remodelling in COPD

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CHAPTER 7

E-cigarette vapour induces cellular senescence in lung fibroblasts and may contribute to lung pathology

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Research letter submitted

To the editor,

INTRODUCTION

COPD is a progressive inflammatory lung disease caused by exposure to noxious gases, in particular cigarette smoke. Chronic exposure to cigarette smoke (CS) causes chronic inflammation and eventually leads to lung tissue damage. Ultimately, these pathologic events lead to airway fibrosis and airflow limitation, i.e. chronic bronchitis, and the alveolar breakdown and lack of tissue repair in the parenchyma, i.e. emphysema (1).

Cellular senescence has been recognized to play a role in the pathophysiology of COPD (2). Cellular senescence is defined as an irreversible cell cycle arrest to prevent cell death or abnormal growth. Induction of cellular senescence can be caused by multiple mechanisms, including oxidative stress and DNA damage, both known to result from chronic CS exposure (3). Accumulation of senescent cells in lung tissue can result in chronic inflammation and tissue dysfunction (4) and as such contribute to COPD pathology.

Electronic cigarettes have been proposed as a safer alternative to cigarettes. Evidence of the harms related to E-cigarette use is growing. Similar to CS exposure, E-cigarette exposure causes a pro-inflammatory response after acute and chronic exposure in multiple structural lung cells *in vitro*, *in vivo* in mouse lungs and in human clinical studies (5, 6), suggesting prolonged use may also contribute to COPD. Furthermore, E-cigarette vapour exposure has been shown to induce DNA damage and reduce DNA damage repair in lung epithelial cell lines (5). Oxidative stress from E-cigarette vapour exposure has been studied to a greater extent *in vitro* and *in vivo,* with the majority demonstrating a high oxidative burden from E-vapour exposure. Although E-cigarette use may upregulate known senescence inducers, DNA damage and oxidative stress, it is unknown whether E-vapour can actually induce cellular senescence. Therefore, we investigated whether E-vapour exposure induces cellular senescence in primary lung fibroblasts and whether this affects their tissue repair function.

METHODS

Primary parenchymal lung fibroblasts (n=11) were grown in DMEM (Gibco) supplemented with 5% foetal bovine serum (FBS) as described previously (7). At passage 5-6, cells were seeded on 6 well plates (for collection of supernatants, RNA & SA-β-gal staining) and on 12 well plates (for wound healing assay). Cells were serum-starved in 0.5% FBS DMEM after 48 hours, and 24 hours later cells were stimulated with 250 µM Paraquat (PQ; positive control for senescence induction), 5% cigarette smoke extract (CSE), 1.5% (Lo) or 2% (Hi) nicotine-containing (18mg/ml) tobacco-flavoured E-cigarette Vapour extract (EV), or 1.5% (Lo) or 2% (Hi) nicotine-free tobacco-flavoured E-cigarette Vapour extract (NF EV) as described previously (8). A cytotoxic dose for CSE (10%) and both EV (5%) was used as a positive control for the stimuli (data not shown). Supernatants (for IL-8 ELISA) and RNA extracts were

collected after 24 hours of stimulation, whilst remaining plates were refreshed to 5% FBS DMEM to enable cell proliferation for 3 days. Cellular senescence was assessed by SA-β-gal staining, cell proliferation inhibition, and p16 and p21 gene expression as described previously (9). Wound healing assays were performed 4 days after stimulation as described in the figure legend.

RESULTS

To confirm the stimulatory response of the different stimuli on primary lung fibroblasts, IL-8 secretion was measured, which was significantly induced by all stimuli compared to untreated fibroblasts (Fig. 1A). Cellular senescence was induced by PQ and CSE with an increase in p21 expression (Fig. 1B) and percentages of SA-β-gal positive cells (Fig. 1C), and reduced cell proliferation (Fig. 1D). For both no increase in p16 expression was found (data not shown). CS is a known risk factor for COPD and both stimuli are known to induce senescence, confirming our model's validity.

Upon stimulation with nicotine-containing E-vapour extract (EV), cellular senescence was induced with significant differences in the same senescence markers as PQ and CSE, which was dose-dependent (Fig. 1B-D). This induction in cellular senescence by E-vapour appeared to be nicotine-independent as stimulation with nicotine-free E-vapour extract (NF EV) also increased p21 expression and the percentages of SA-β-gal positive cells, and reduced cell proliferation. Only upon the low dose of NF EV stimulation, no significant p21 increase was observed (Fig. 1B). Similar to PQ and CSE, no increase in p16 expression was found after NF EV and EV stimulations (data not shown).

Upon senescence induction by PQ and CSE, impaired repair in a wound healing model occurred after 48 hours and 72 hours (Fig.2). Low dose EV impaired wound healing capacity with reduced wound closure after both 48 and 72 hours, which again appeared to be nicotine-independent as similar results were found in NF EV treated cells. No significant reduction in wound closure was found upon EV Hi stimulation, due to one donor with improved wound closure upon stimulation

To confirm our *in vitro* results *in vivo*, gene expression of p21 was measured in a previously performed mouse model (10), where p21 expression seemed higher upon exposure compared to non-exposed controls (mean fold change + SEM: 1.3+0.09 (EV) and 1.2+0.2 (NF EV) vs SHAM). However, no significant differences were found as this pilot study was underpowered, thus larger *in vivo* studies should be done to confirm our *in vitro* findings.

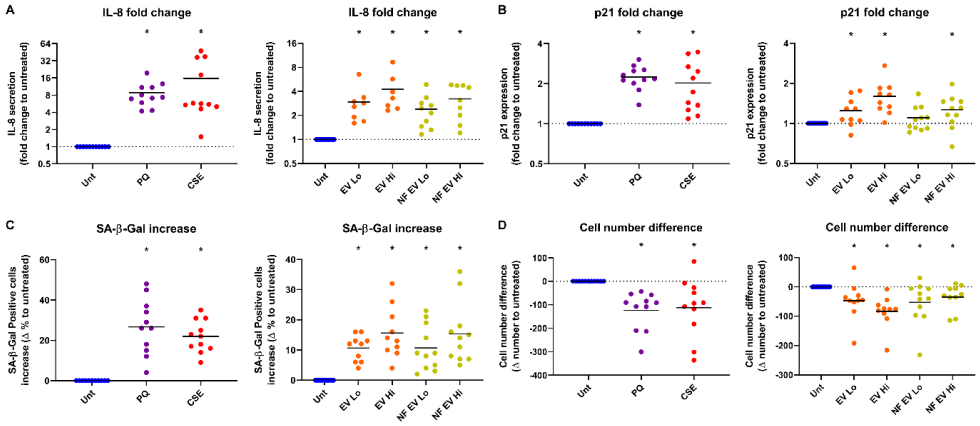

Figure 1: Cellular senescence induction upon stimulation with known senescence-inducers and upon E-vapour extract stimulation. *Differences in IL-8 secretion (A) in cell culture supernatants, p21 gene expression (B), percentages of SA-ß-gal positive cells (C) and total cell numbers (D) are shown compared to untreated (Unt, blue) upon stimulation with 250µM paraquat (PQ, purple) 5% cigarette smoke extract (CSE, red) and high and low doses of nicotine-containing (EV Lo and Hi, orange) and nicotine-free E-vapour (NF EV Lo and Hi, yellow) extracts. N=11 per group. Lines represent means and dotted lines represent levels of untreated. Lo= low dose: 1.5% and Hi= high dose: 2%. Significant differences between stimulated and untreated fibroblasts were tested using One-Way ANOVA with Fisher's LSD tests, * means P-value < 0.05 compared to untreated.*

DISCUSSION

This study is the first to identify E-cigarette vapours' potential to induce cellular senescence in primary lung cells, which is a known contributing factor to disease in COPD (2). The findings of our study further add to the identified risks of E-cigarette use (5). E-cigarette harms are often compared to cigarettes in relation to harm reduction, but this study focused on the standalone risk for E-cigarette users. These risks are not isolated to COPD patients with other E-cigarette users like young never-smokers, more likely to develop lung pathology from long-term use.

In the current study, we did not investigate the mechanisms of senescence induction by E-vapour extract, but we hypothesize that DNA damage and oxidative stress may be involved as previous studies demonstrated that E-cigarette vapour exposure can result in DNA damage and oxidative stress (5). Future studies should elucidate these mechanisms involved in EV-induced senescence and whether particular components of E-liquids are directly causing up-regulation of pathological processes in the lung.

E-cigarettes' potential to induce cellular senescence, alongside other previously identified risks, should serve as a warning to avoid use as a safe alternative to cigarette smoking or as a cessation device. Considering senescence induction was dose-dependent indicates excessive and long-term use should be avoided.

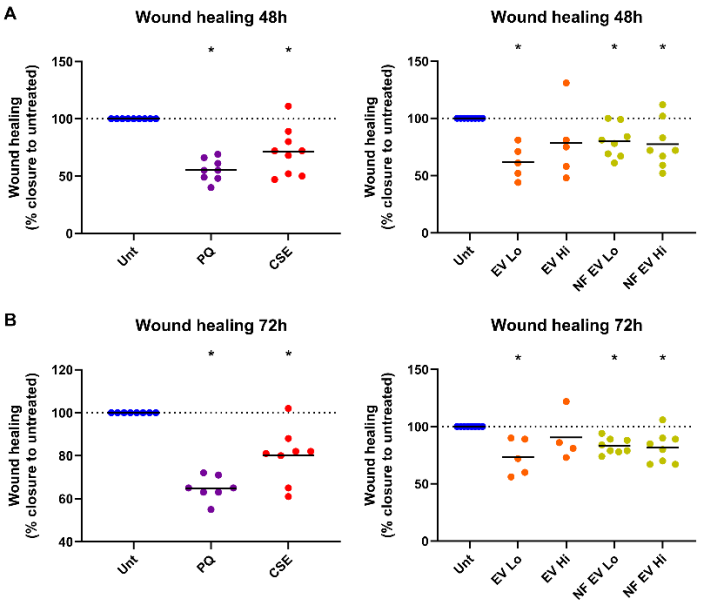

Figure 2: Cellular senescence induction resulted in an impaired wound healing capacity. *Wound healing capacity was assessed 4 days after stimulation using the scratch assay by measuring wound closure after 48 and 72 hours of scratching stimulated and untreated fibroblasts. A wound in the cell layers was made by scratching with a p200 pipet tip in the middle of the well from top to bottom, wells were washed twice with Hanks buffer and DMEM + 0.5% FBS was added to enable wound closure, which was captured on a Nikon Eclipse Ti microscope at total magnification of 40x. Percentages of wound closure after 48 hours (A) and 72 hours (B) compared to untreated (Unt, blue) are shown upon stimulation with 250µM paraquat (PQ, purple) 5% cigarette smoke extract (CSE, red) and high and low doses of nicotine-containing (EV Lo and Hi, orange) and nicotine-free vapour (NF EV Lo and Hi, yellow) extracts. N=11 per group. Lines represent means and dotted lines represent levels of untreated. Lo= low dose: 1.5% and Hi= high dose: 2%. Significant differences between stimulated and untreated fibroblasts were tested using One-Way ANOVA with Fisher's LSD tests, * means P-value < 0.05 compared to untreated.*

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REFERENCES

1. Hogg JC, Timens W. The pathology of chronic obstructive pulmonary disease. Annu Rev Pathol 2009; 4: 435-459.
2. Barnes PJ, Baker J, Donnelly LE. Cellular Senescence as a Mechanism and Target in Chronic Lung Diseases. Am J Respir Crit Care Med 2019; 200: 556-564.
3. Birch J, Anderson RK, Correia-Melo C, Jurk D, Hewitt G, Marques FM, Green NJ, Moisey E, Birrell MA, Belvisi MG, Black F, Taylor JJ, Fisher AJ, De Soyza A, Passos JF. DNA damage response at telomeres contributes to lung aging and chronic obstructive pulmonary disease. Am J Physiol Lung Cell Mol Physiol 2015; 309: L1124-1137.
4. Munoz-Espin D, Serrano M. Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol 2014; 15: 482-496.
5. Bozier J, Chivers EK, Chapman DG, Larcombe AN, Bastian NA, Masso-Silva JA, Byun MK, McDonald CF, Crotty Alexander LE, Ween MP. The Evolving Landscape of e-Cigarettes: A Systematic Review of Recent Evidence. Chest 2020; 157: 1362-1390.
6. Stratton KK, L.Y.; Eaton, D.L. National Academies of Sciences, Engineering, and Medicine. In: Eaton DL, Kwan LY, Stratton K, editors. Public Health Consequences of E-Cigarettes. Washington (DC): Public Health Consequences of E-Cigarettes. Washington, DC; 2018.
7. Rutting S, Zakarya R, Bozier J, Xenaki D, Horvat JC, Wood LG, Hansbro PM, Oliver BG. Dietary Fatty Acids Amplify Inflammatory Responses to Infection through p38 MAPK Signaling. Am J Respir Cell Mol Biol 2019; 60: 554-568.
8. Bozier J, Rutting S, Xenaki D, Peters M, Adcock I, Oliver BG. Heightened response to e-cigarettes in COPD. ERJ Open Res 2019; 5.
9. Woldhuis RR, de Vries M, Timens W, van den Berge M, Demaria M, Oliver BGG, Heijink IH, Brandsma CA. Link between increased cellular senescence and extracellular matrix changes in COPD. Am J Physiol Lung Cell Mol Physiol 2020; 319: L48-L60.
10. Chen H, Li G, Chan YL, Chapman DG, Sukjamnong S, Nguyen T, Annissa T, McGrath KC, Sharma P, Oliver BG. Maternal E-Cigarette Exposure in Mice Alters DNA Methylation and Lung Cytokine Expression in Offspring. Am J Respir Cell Mol Biol 2018; 58: 366-377.