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Metaplastic phenotype in human primary bronchiolar epithelial cells after repeated exposure to native mainstream smoke at the air-liquid interface

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ABSTRACT

3D constructs composed of primary normal differentiated human bronchiolar epithelial (NHBE) cells as mono- or co-culture in combination with normal human lung fibroblasts were exposed repeatedly at the air-liquid interface with non-lethal concentrations of mainstream cigarette smoke (4 cigarettes a day, 5 days/week, 13 times repetition in total) to build up a permanent burden on the cells. Samples were taken after 4, 8 and 13 times of repeated smoke exposure and the cultures were analyzed by histopathological methods In comparison with the clean air exposure (process control) and incubator control cells the cigarette smoke exposed cultures showed a reduction of cilia bearing as well as mucus producing cells. In both mono- as well as co-cultures, hyperplasia was induced showing different histological cell types (undifferentiated secretory and squamous cell types). At the end of the exposure phase, we observed the development of non-hyperplastic areas strongly positive to CK13 antibody, commonly seen in squamous cells as a marker for non-cornified squamous epithelium, thus suggesting a transition of the normal bronchial epithelial cells towards metaplastic cells. The control cultures (clean air exposed and incubator cells) showed no comparable phenotypic changes. In conclusion, our *in vitro* model presents a valuable tool to study the induction of metaplastic alterations after exposure to airborne material.

1. Introduction

Studies of the effects of cigarette smoke (CS) have been carried out mainly in a large scale of animal experiments and resulted in hyperplasia, squamous cell metaplasia, reduced number of ciliated cells, and increased number of goblet cells (Dye and Adler, 1994). In their review Dye and Adler (1994) pointed out the importance of the role of effector cells, which produced after cigarette smoke contact, various kinds of cytokines, enzymes, growth factors, extracellular matrices and so on, and influenced or changed the state of physiology of target epithelial cells. The effector cells may be epithelial or other resident stromal cells including nerve cells and leukocytes. A decade later many people have already embarked on the work with *in vitro* model systems, partly stimulated by an artificial tissue engineering concept based on the development of stem cell technology (Rock et al., 2010) and partly forced by increasingly strong animal protection movements. Initially it appeared that most of the people completely forgot what was advocated by Dye and Adler (1994) with regard to 'effector cells'. Nevertheless, although there is a certain beneficial aspect in using *in vitro* models that practically any types of direct experiments can be applied to human cells and tissues *in vitro*, some people have attempted to incorporate some kinds of effector cell types in their model, and interesting results have been presented.

For example, normal human airway epithelial cells were grown as 3D constructs with (co-cultures, CC) or without (mono-cultures MC) fibroblasts attached underneath the porous membrane under ALI conditions for 48 days for comparing the impact of direct mainstream cigarette smoke exposure on the types of organotypic models. The exposure of the differentiated cultures was carried out onto their luminal side with 0 (air), 8 and 15% of CS generated from 3R4F research cigarettes smoked for 28 min to a standard butt for a 50 mL puff over 2 s twice per min according to the Health Canada smoking regimen (Iskandar et al.,

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2015). A variety of parameters were evaluated and the results indicated that in the co-culture models the impact related to senescence, apoptosis and cell proliferation was stronger than in the mono-culture models. In contrast, the effects related to DNA damage, inflammation and cell stress were stronger in the mono- than in the co-cultures.

For studying "chronic" diseases like asthma, COPD or even cancer human cells and tissues are currently less favored. Keeping *in vitro* reconstructed tissues in a good condition for such a long time as several months is still a very difficult task, although quite recently a new technical improvement has been attempted and the first results have been published and appear promising (Aufderheide et al., 2016). At any rate, this technical limitation has been one of the main reasons why *in vitro* studies in these areas are still missing. Nevertheless, some attempts have recently been made using ALI techniques with a cultivation period limited within a month or so. In some of these works for example, the respiratory epithelia, reconstructed from *in vitro* extracted human cells, were exposed to cigarette smoke extracts or one of their chemical components, and mucus cell hypersecretion (Carson et al., 2014; Schamberger et al., 2015) and basal as well as Club cell activation (Schamberger et al., 2015) were observed. In both studies, cilia underwent growth retardation.

In a similar work, *in vitro* reconstructed airway epithelium was affected from the apical side (mimicking the smoker situation) within a specific exposure system (CULTEX® RFS) with the whole gas phase of cigarette smoke. After cigarette exposure, cilia showed a statistically significant reduction in number and size (Aufderheide et al., 2015).

Mebratu et al. (2011) observed in ALI cultures of normal human airway epithelial cells (HAECs) after exposure to cigarette smoke extract (CSE at a concentration of 1,000 ng/mL for 24 h) about 3-fold more Muc5AC+ mucous cells accompanied by an increase of approximately 1.4-fold in Muc5AC mRNA. Simultaneously, a Bik protein, one of pro-apoptotic Bcl-2 family proteins, was significantly reduced to about 0.4-fold of the control. Restoration of Bik protein expression significantly suppressed the mucous cell hyperplasia induced by CSE.

The treatment with nicotine and/or interleukin (IL)-13 led to hyperplasia and metaplasia of mucus cells mediated by nicotinic acetylcholine receptors (nAChRs) (Gundaravapu et al., 2012). Interestingly, in another similar *in vitro* system, inactivation of nAChR has been shown to lead to basal cell hyperplasia and squamous metaplasia of respiratory epithelia (Mouche et al., 2009).

The negative effects of cigarette smoke on various tissues and organs cannot to be overlooked and the question arises whether it is possible to image pre- and neoplastic phenotypic changes by *in vitro* methods as a model system for the detection of the carcinogenic potential not only for cigarette smoke, but also other airborne substances. We now have or are going to develop powerful *in vitro* systems, with which we can detect more of such "chronic" effects based on repeated exposure studies at non-toxic concentrations of the test atmosphere. Such human *in vitro* models offer the possibility to develop and establish pharmacological agents for aspects of chronic diseases. An example of a classical agent, non-polar arotinoid (Ro 15-0778), as Lasnitzki and Bollag (1987) reported as clinically and experimentally interesting because this compound exerted both *in vivo* and in organculture antagonizing effects on CSE- or BaP-induced hyperplasia and squamous metaplasia. This compound is devoid of hypervitaminosis unlike retinoids (Nettesheim, 1980).

In the present paper we describe the induction of hyperplasia and squamous metaplasia in our 3D ALI culture of reconstructed human airway epithelium, which was exposed to the whole cigarette smoke from the apical side using a smoking machine, the CULTEX® RFS exposure module, and will discuss about the future applicability of the system to the search of protecting agents/measures against the adverse effects of cigarette smoking.

2. Material and methods

2.1. Normal human bronchial epithelial cells (NHBE cells)

Normal human bronchial epithelial (NHBE) cells were isolated from inconspicuous distal bronchus samples with a normal morphology of different patients (NHNBE011: 67 old male with a lung adenocarcinoma in the right upper lobe) obtained from the KRH Klinikum Oststadt-Heidehaus (Hannover, Germany). In accordance with the Declaration of Helsinki, the subjects gave their informed consent to the research use of the lung tissue samples removed. In our studies, we were especially interested in the anatomical region of bronchioles which, in comparison to other regions of the airways, is characterized by a greater amount of stem/progenitor cells differentiating to Club cells with potent drug-metabolizing capacity (Emura et al., 2015).

Upon arrival in our laboratory, the tissue samples were incubated for 24 h at 4 °C on a rocking platform in incubation medium (MEM medium containing dithiothreitol (0.5 mg/mL), DNase (10 μg/mL) and antibiotics (40 μg/mL tobramycin, 50 μg/mL vancomycin, 50 μg/mL ceftazidime, 2.5 μg/mL amphotericin B, 50 U/mL penicillin/streptomycin)). Afterwards, the samples were transferred into a PBS containing petri dish, isolated from residual parenchyma and cut into smaller pieces of approximately 8 × 5 mm. The tissue pieces were then placed into cryovials, containing DMEM with 10% FCS and 10% DMSO and frozen to −80 °C. After storage at −80 °C overnight, the vials were moved to a liquid nitrogen tank and stored until needed.

For cell isolation, the samples were thawed in a water bath at 37 °C, transferred into petri dishes and rinsed with PBS after removal of the freezing medium. Incubation medium containing 0.05% protease XIV was added and the samples were incubated for 2 h at 4 °C on a rocking platform. Afterwards, bronchial epithelial cells were isolated by thoroughly scraping the luminal surface of the bronchus pieces with a scalpel.

The cell suspensions were homogenized, pipetted into centrifugation tubes and centrifuged for 10 min at 170 x *g*. The resulting cell pellets were suspended in 4.5–9 mL BEGM medium. The cell suspensions of each sample were then equally divided to two collagen IV coated wells of a 6-well plate to grow in culture.

After the first passage, NHBE cells were cultivated in collagen IV coated culture flasks using AEGM Medium. After reaching 80–90% confluence, the cells were seeded on collagen IV coated cell culture inserts (seeding density: 1–1.5 × 105/cm2). The cells were cultivated under submerged conditions and supplied with AEGM medium until reaching 100% confluency (3 days) before the apical medium was removed and the basal medium was replaced by differentiation medium (PneumaCult™ – Maintenance, STEMCELL Technologies SARL, Köln, Germany). After 10 days of cultivation at the air-liquid interface, the exposure period started (see Chapter "Cigarette smoke exposure") and the cells were transferred to the CULTEX® RFS (age of the culture: 13 days). The exposure experiments were performed with cells in passage 2. In total, the cells were cultivated for 27 days at the air-liquid interface with an exposure phase of 17 days.

PBS, Penicillin/Streptomycin, DMEM from Biochrom (Cambridge, UK) and AEGM medium from Promocell (Heidelberg, Germany). All other cell culture reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).