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Human Reconstituted Nasal Epithelium, a promising *in vitro* model to assess impacts of environmental complex mixtures

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ABSTRACT

Considering the impact of respiratory diseases around the world, appropriate experimental tools to help understand the mechanisms involved in such diseases are becoming essential. Our aim was to investigate the cellular and morphological reactivity of a human Reconstituted Nasal Epithelium (hRNE) to evaluate the impact of environmental complex mixture (ECM), with tobacco smoke as a model, after three weeks of repeated exposures.
Staining of hRNE showed a multilayered ciliated epithelium, with a regular cilia beats, and a mucus production. When hRNE was exposed to ECM for 5 min once or twice a week, during 3 weeks, significant changes occurred: IL-8 production significantly increased 24 h after the first exposure compared with Air-exposure and only during the first week, without any loss of tissue integrity. Immunostaining of F-actin cytoskeleton showed a modification in cellular morphology (number and diameter).
Taken together our results indicate that hRNE is well suited to study the cellular and morphological effects of repeated exposures to an environmental complex mixture. Human reconstituted epithelium models are currently the best *in vitro* representation of human respiratory tract physiology, and also the most robust for performing repeated exposures to atmospheric pollutants.

1. Introduction

Concerns about the expansion of atmospheric pollution worldwide and its impact on human health have increased over the past few years. Recent evidence has revealed associations between atmospheric pollution and the emergence or exacerbation of airway diseases such as asthma and chronic obstructive pulmonary disease (Annesi-Maesano and Dab, 2006; Guarnieri and Balmes, 2014). Humans are constantly in contact with inhaled outdoor and indoor pollutants, and airway epithelium is a strong barrier which protects the human respiratory tract against this pollution during the respiratory process (Ganesan et al., 2013). Current findings highlight the pivotal role that airway epithelial cells play in the onset of airway diseases (Kumar et al., 2011; Proud, 2011).

Considering the impact of airway respiratory diseases around the world, appropriate experimental tools to help understand the mechanisms involved in such diseases are becoming essential. Modern experimental policy and the 3Rs principle (Reduce, Refine, Replace) advocate using *in vitro* studies rather than animals. EU chemical policy REACH (Registration, Evaluation, Authorization and Restriction of Chemicals directive) supports this trend by promoting *in vitro* tools able to mimic physiological conditions (BeruBe et al., 2009; Buckland, 2011).

Over the past twenty years the design of airway epithelial cell culture has evolved, and new realistic *in vitro* models have emerged. These have progressed from cell monolayers, immortalized cell lines, and primary cells grown in submerged conditions, to multilayered reconstituted epithelium maintained in air–liquid interface that mimics the *in vitro* respiratory tract physiological barrier (Pampaloni et al., 2007; Fulcher and Randell, 2013). These three-dimensional models have many of the characteristics of human epithelium, such as different epithelial cell types: ciliated, goblet and basal cells, and operational tight junctions (Whitcutt et al., 1988; Rothen-Rutishauser et al., 2005; BeruBe et al., 2009).

Up until now the airway reconstituted epithelium model is considered to be one of the best *in vitro* representations of the human respiratory tract. While it has been used over the past decade in different

Abbreviations: ECM, environmental complex mixture; ELISA, Enzyme-linked immunosorbent assay; FOV, field of view; hRNE, human Reconstituted Nasal Epithelium; LDH, Lactate dehydrogenase; PBS, Phosphate buffered saline; SSS, sterile saline solution; TEER, trans-epithelial electrical resistance; WGA, Wheat Germ Agglutinin.

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research areas, such as drug delivery, impact of biological contaminants, and airway repair and remodeling after injury (Huang et al., 2013; Crespin et al., 2014), only few teams have used it to investigate the effect of atmospheric pollutant exposure. Furthermore, most studies have used experimental procedures that last less than one week after reaching full differentiation, and only very few studies have used procedures lasting several weeks, even several months (Anderson et al., 2013; Boublil et al., 2013).

The aim of the present work was to investigate the cellular and morphological reactivity of a human Reconstituted Nasal Epithelium (hRNE) to evaluate the impact of atmospheric pollutants after several weeks of repeated exposures. For this purpose, we tested the feasibility of repeated hRNE tissue exposures to an environmental complex mixture. Tobacco smoke was used as a model of environmental complex mixture because of its well-known respiratory health effects.

2. Experimental procedures

2.1. Chemicals and reagents

Calcium chloride, goat serum, glycine, bovine serum albumin, glycerol, eosin, sodium azide, Eukitt®, sodium cacodylate, glutaraldehyde, hexamethyldisilazane, osmium tetroxide, hydrochloric acid, and propylene oxide were purchased from Sigma-Aldrich (MO, USA); and Hepes and Penicillin–Streptomycin, from Invitrogen (WI, USA). Acetic acid, Hemalun and Triton X100, from Merck (Darmstadt, Germany); paraffin and ethanol, from VWR International (PA, USA); xylene and paraformaldehyde (32%),from Euromedex (France) and sodium chloride, from Minisol (France). Alcian blue 8GX was obtained from RAL diagnostics, VWR (France). Phosphate buffered saline (PBS) was provided by Biosolve Chimie (France). The cytokine DuoSet kits was produced by R&D Systems (MN, USA), and the LDH assay kit (K2054), by Siemens (France).

2.2. hRNE tissue: origin and culture conditions

The study was conducted with a hRNE tissue, known commercially as HF-MucilAir™ and purchased from Epithelix SARL (Geneva, Switzerland). This three-dimensional model is based on primary culture of human nasal epithelial cells differentiated at the air–liquid interface (MucilAir™) and co-cultured with Human airway Fibroblasts (HF) on Costar Transwell insert (0.33 cm^2, pore size of 0.4 μm, Corning, NY, USA). Nasal epithelial cells were obtained from a pool of donors, non-smokers and without respiratory pathologies. Signed informed consent and ethical approval were obtained by the supplier. All batches of hRNE tissue were determined to be negative by the supplier for mycoplasma, human immunodeficiency virus 1 (HIV-1), human immunodeficiency virus 2 (HIV-2), and hepatitis B and C.

hRNE tissues were maintained at the air–liquid interface in an incubator at 37 °C (5% CO_2 and 95% constant relative humidity) with 700 μL of MucilAir™ culture medium (Epithelix, Geneva, Switzerland) in the basal side. To preserve tissue homeostasis, the culture medium was changed every two days and the apical side was washed with sterile saline solution (SSS) (NaCl 0.9%, $CaCl_2$ 1.25 mM, Hepes 10 mM), to remove mucus and surface dead cells, once a week according to the supplier's instructions.

Morphology of hRNE tissue was observed directly using a light inverted microscope (magnification 100×, Nikon Eclipse Ts100) on the insert before and after exposure throughout the experiment period.

2.3. Exposure protocol

All experiments started one week after hRNE delivery. Environmental complex mixture (ECM) was generated from smoking of a French brand cigarette, and was delivered on the apical face of hRNE tissue using a static system adapted from the Vitrocell Cloud chamber. Briefly, before exposure an uncovered culture plate containing hRNE tissues, with 700 μL of medium on the basal side, was placed on the bottom of the exposure chamber, a 3 L tightly closable glass container. 250 mL of ECM, composed by gas and particulate matter (PM), was introduced into the exposure chamber using a syringe. To cover hRNE tissue in a homogeneous manner, ECM was delivered progressively in 10 samples of 25 mL (corresponding to 10 puffs, as an equivalent of one smoked cigarette) in less than 5 min. Then the entire device was placed at 37 °C for 5 min.

During tobacco smoke generation, on average 200 $\mu g/cm^2$ of particulate matter (PM) was deposited on each insert. Deposition did not vary from one generation to another as shown by repeatability and reproducibility experiments.

PM levels deposited on the apical side of the tissues have been estimated as follows. Pre-weighed filters were placed at the bottom of the wells of a 24 wells culture plate and exposed to 250 mL of ECM as described above. After 5 and 15 min at 37 °C, filters were weighed again and the PM levels estimated. The results were respectively, 203 and 200 $\mu g/cm^2$ with a coefficient of variation close to 10% ($n = 4$). The deposition time of 5 min was chosen because it was not accompanied of a cytotoxic effect as observed after 15 min.

After the exposure time, the culture medium was removed and replaced with fresh medium, and hRNE tissues were returned to the incubator.

hRNE tissues were exposed to ECM for 5 min once or twice a week at 48 h intervals over three weeks as described in the exposure protocol design (Fig. 1). At the same time, two controls were considered: Air-exposure performed in the same conditions but without ECM, and an Incubator Control corresponding to hRNE tissues not leaving the incubator.

Twenty four hours after the first exposure (Air-exposure or ECM-exposure) and every day over three weeks, culture medium was collected to assess LDH release and cytokine production. Each week, four days after the first exposure, the apical side was washed with SSS to remove mucus, and trans-epithelial electrical resistance (TEER) was measured. Histological assessment was performed every week, one week after the first exposure (Fig. 1, day 7).

2.4. Biological activity assessment

2.4.1. Trans-epithelial electrical resistance (TEER) measurement

In order to assess the tissue integrity (tight junctions and ionic fluxes), TEER measurement ($\Omega.cm^2$) was performed on tissue with 700 μL of SSS on the basal side and 200 μL on the apical side using electrodes connected to the EVOM^2 epithelial Voltohmmeter (World Precision Instruments, England). Values between 250 and 700 $\Omega.cm^2$ corresponded to an undamaged tissue.

TEER was calculated as following: $TEER = (TEER_{hRNE} - TEER_0) * A$, with $TEER_{hRNE}$ for the experimental value measured of hRNE tissue, $TEER_0$ for the background value of the porous membrane of the insert without tissue (100 $\Omega.cm^2$), A: surface area of the porous membrane of the insert (0.33 $.cm^2$).

Days	0	1	2	3	4	5	6	7
One exposure	X	+	+	+	O+	+	+	H +
Two exposures	X	+	X+	+	O+	+	+	H+

Fig. 1. Weekly exposure protocol. hRNE tissue was exposed at air–liquid interface (**X**) to Air (Air-exposure) or environmental complex mixture (ECM-exposure) during 5 min once or twice a week at 48 h intervals during three weeks. Culture medium was harvested every day (+) to perform cytokine measurements and LDH activity. Four days after the first exposure of the week, TEER was evaluated (**O**). Histological observations (**H**) were performed one week after the first exposure of the week.