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Repeated whole cigarette smoke exposure alters cell differentiation and augments secretion of inflammatory mediators in air-liquid interface three-dimensional co-culture model of human bronchial tissue

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In vitro models of human bronchial epithelium are useful for toxicological testing because of their resemblance to *in vivo* tissue. We constructed a model of human bronchial tissue which has a fibroblast layer embedded in a collagen matrix directly below a fully-differentiated epithelial cell layer. The model was applied to whole cigarette smoke (CS) exposure repeatedly from an air-liquid interface culture while bronchial epithelial cells were differentiating. The effects of CS exposure on differentiation were determined by histological and gene expression analyses on culture day 21. We found a decrease in ciliated cells and perturbation of goblet cell differentiation. We also analyzed the effects of CS exposure on the inflammatory response, and observed a significant increase in secretion of IL-8, GRO-α, IL-1β, and GM-CSF. Interestingly, secretion of these mediators was augmented with repetition of whole CS exposure. Our data demonstrate the usefulness of our bronchial tissue model for *in vitro* testing and the importance of exposure repetition in perturbing the differentiation and inflammation processes.

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1. Introduction

The development of *in vitro* tissue engineering is contributing to the advancement of regenerative medicine, drug development, and chemical risk assessment, since *in vitro* tissue models constructed by tissue engineering display similar morphology and character to *in vivo* tissue (Griffith and Naughton, 2002; Elliott and Yuan, 2011). *In vitro* three-dimensional (3D) culture models are gaining ground in toxicological testing as alternatives to animal experiments.

Human bronchial epithelial cells (HBECs) are known to differentiate into bronchial tissue such as columnar respiratory epithelium with ciliated, goblet, clara, and basal cells when cultured under air-liquid interface (ALI) conditions. ALI-cultured HBECs have been used as an *in vitro* model of human bronchial tissue (BéruBé et al., 2010; Emura et al., 2015). Since the respiratory epithelium plays a role as a defensive barrier against inhaled chemicals (BéruBé et al., 2009), this model is applied to investigate the biological effects of aerosol chemicals such as cigarette smoke (CS). In biological experiments, CS exposure is usually carried out with cigarette smoke extract (CSE) prepared by eluting the filter-trapped CS particles or by bubbling the generated CS through aqueous liquid (Mizusaki et al., 1977; Nakayama et al., 1985). Exposure by adding CSE to the basolateral medium of ALI-cultured HBECs has been reported to affect respiratory defense mechanisms (Glader et al., 2006). However, advancements in exposure methods and instruments have enabled the direct exposure of cultured cells to whole CS (Bakand and Hayes, 2010; Thorne and Adamson, 2013). When the apical surface of ALI-cultured HBECs is directly exposed to whole CS, the exposure mimics the exposure process and conditions in the human respiratory tract. Kuehn et al. (2015) recently applied this exposure method to analyze the effects of whole CS on cilia beating, barrier function of tight junctions, and xenobiotic response in ALI-cultured HBECs. As Kuehn et al. used a co-culture of HBECs with fibroblasts in their study, we can surmise that a co-culture of HBECs with fibroblasts is a useful model to investigate the biological effects of CS *in vitro*, since human bronchial tissue *in vivo* has a collagen matrix layer with fibroblasts under the ciliated epithelial layer; interaction between epithelial cells and fibroblasts is reported to play an important role in epithelial differentiation and functioning (Vermeer et al., 2006; Myerburg et al., 2007). In a recent report, a transcriptomic profile of HBECs co-cultured with fibroblasts showed a higher correlation with transcriptomic data of *in vivo* bronchial brushing samples from a smoker after exposure to whole CS than a mono-culture of HBECs (Iskandar et al., 2015).

Abbreviations: 3D, three-dimensional; AEGM, Airway Epithelial Cell Growth Medium; ALI, air-liquid interface; CS, cigarette smoke; CSE, cigarette smoke extract; COPD, chronic obstructive pulmonary disease; CYP, cytochrome P450; FBS, fetal bovine serum; HBECs, human bronchial epithelial cells; ISO, International Organization for Standardization; LDH, lactate dehydrogenase; MEM, minimum essential medium; OECD, Organization for Economic Co-operation and Development.

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In this study, we report the results of a whole-CS exposure to our ALI 3D co-culture model of human bronchial tissue. We developed a bronchial tissue model that has fibroblasts embedded in a collagen matrix layer directly below the ALI-cultured HBECs; a previously reported co-culture model did not include a collagen matrix layer (Iskandar et al., 2015). Because our fibroblast culture mimics *in vivo* conditions and enables direct contact between HBECs and the fibroblasts layer (Griffith and Swartz, 2006), we believe that our model is suitable for replicating the effects of CS on bronchial tissue *in vivo*. We carried out a whole CS exposure repeatedly during the differentiation process of our bronchial tissue model, and examined the effects on the differentiation process of HBECs by histological and gene expression analyses. CS, bacteria, viruses, and air pollutants are reported to be risk factors for chronic obstructive pulmonary disease (COPD), and many other environmental and genetic risk factors appear to interact and combine to trigger the pathogenesis (Eisner et al., 2010). Because the activation of inflammatory networks and the secretion of inflammatory mediators from human bronchial tissue are reported to be important in the early development of COPD (Barnes, 2013), we also investigated the effects of repeated whole CS exposure on the inflammatory process by gene and protein expression analysis with our bronchial tissue model.

2. Materials and methods

2.1. Cell culture

Human fetal lung fibroblasts (IMR-90) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in minimum essential medium (MEM) (Life Technologies, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (MP Biomedicals, Santa Ana, CA, USA). Normal HBECs (Lonza, Walkersville, MD, USA) were cultured in Airway Epithelial Cell Growth Medium (AEGM) with SupplementPack (Promocell, Heidelberg, Germany).

2.2. Three-dimensional co-culture

For the 3D co-culture, 12-well plates (BD, Franklin Lakes, NJ, USA) and cell culture inserts (1.0 μm pore size, BD) were used. The three-dimensional co-culture model of human bronchial tissue was composed of a collagen matrix layer and an epithelial cell layer. The collagen matrix layer consisted of a base layer and a fibroblast layer. Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan), 10 × MEM (Nihon Seiyaku, Tokyo, Japan) and reconstitution buffer (Nitta Gelatin) were mixed with 8:1:1 by volume ratios and applied to the cell culture insert in 100 μL aliquots as the base layer. The collagen gel base layer was gelled by placing in an incubator at 37 °C with a 5% CO_2 atmosphere for more than an hour. Cellmatrix type I-A, Cellmatrix type I-P (Nitta Gelatin), 10 × MEM, reconstitution buffer and an IMR-90 cell suspension (approximately 2.5×10^6 cells/mL in FBS) were mixed with 4:4:1:1:1 by volume ratios and poured onto the base layer in 250 μL aliquots as the fibroblast layer; they were gelled by placing in an incubator at 37 °C with a 5% CO_2 atmosphere for more than an hour. After the fibroblast layer was formed, 500 μL of MEM supplemented with 10% FBS was added to the cell culture insert as the apical medium, and 1 mL of MEM supplemented with 10% FBS was added to the bottom well as the basolateral medium. The collagen matrix layer was cultured for 2 days before applying the epithelial cell layer. The apical medium and the basolateral medium of the collagen matrix layer were removed, and 500 μL of the HBECs suspension were applied onto the collagen matrix layer (approximately 3.0×10^5 cells/mL in AEGM with SupplementPack). One mL of MEM supplemented with 10% FBS was added to the bottom well as the basolateral medium. The apical and basolateral media (AEGM with SupplementPack and MEM supplemented with 10% FBS) were changed every other day. Three-dimensional co-cultured HBECs were cultured under submerged conditions until they reached semi-confluence.

2.3. ALI culture

PneumaCult–ALI medium (Stemcell Technologies, Vancouver, BC, Canada) was mixed with hydrocortisone (Stemcell Technologies) and heparin (Stemcell Technologies) following the manufacturer's instructions. Thirty nM GM6001 (Sigma-Aldrich, St. Louis, MO, USA) and 1% FBS were also added for preparation of ALI culture medium. The metalloprotease inhibitor GM6001 was added to prevent contraction of the fibroblast-embedded collagen matrix layer (Jeng et al., 2006). The apical and basolateral media were removed, and 600 μL of ALI culture medium were added to the bottom well. The ALI culture medium was changed every other day. As a positive control for goblet cell hyperplasia, 5 ng/mL of IL-13 (Wako Pure Chemical Industries, Osaka, Japan) were added to the ALI culture medium (Yasuo et al., 2006). IL-13 stimulation was carried out from the ALI culture between day 0 and day 21.

2.4. Whole CS exposure

Whole CS exposure was conducted on ALI culture day 7 for a single exposure experiment (Fig. 1A). Cells were exposed to CS from 1, 2, 4, 6, or 8 cigarettes. Whole CS exposure was conducted on ALI culture days 7, 9, 11, 13, 15, 17 and 19 for a repeated exposure experiment (Fig. 1B). Cells were exposed to CS from 1, 2, or 4 cigarettes. The experimental protocol for repeated exposure was based on that published by Aufderheide et al. (2015). The ALI culture medium was changed or sampled before the exposure on culture days 7, 9, 11, 13, 15, 17 and 19. The experimental setup for the whole CS exposure experiments consisted of a VC 10 smoking robot (Vitrocell Systems, Waldkirch, Germany), a dilution system, and a CULTEX RFS module (Cultex Laboratories, Hannover, Germany) (Aufderheide et al., 2011, 2013). The Kentucky reference cigarette 3R4F (University of Kentucky, Lexington, KY, USA), conditioned at 22 ± 2 °C and 60 ± 5% relative humidity for 48 h before use, was smoked in accordance with the International Organization for Standardization protocol (35-mL puffs of 2 s in duration each minute) (ISO, 2000). Whole CS, generated by the smoking robot, was released into a mixing device in 2.8 s exhaust, and was diluted with humidified clean air (>90% relative humidity) at 1.0 L/min dilution flow. Diluted smoke was introduced into the RFS module and guided into the exposure chamber (5 mL/min) using a vacuum pump. The exposure efficiencies of smoke particles and gas molecules in our experimental condition replicated *in vivo* conditions as reported previously (Ishikawa et al., 2016).

2.5. Cytochrome P450 and lactate dehydrogenase assay

The cytochrome P450 (CYP) assay was performed with P450-Glo CYP1A1 Assay (Promega, Fitchburg, WI, USA) in a single exposure experiment on ALI culture day 9 (Fig. 1A). Luciferin-CEE solution (100 μM) was prepared following the manufacturer's protocol, and 250 μL of luciferin-CEE solution were applied to the apical surface of the bronchial tissue after the removal of the basolateral medium from the bottom well. Bronchial tissues were incubated with luciferin-CEE solution in an incubator at 37 °C with a 5% CO_2 atmosphere for 3 h, and luminescence was measured using a GloMax-Multi Detection System (Promega). Basolateral medium removed for the CYP assay was used for lactate dehydrogenase (LDH) analysis using the CytoTox-One Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer's protocol; fluorescence was measured using the GloMax-Multi Detection System.

2.6. Histological analysis

Bronchial tissues were fixed in 4% paraformaldehyde at 4 °C after removing the basolateral medium and washing the apical surface twice with 200 μL of PBS. After fixation, bronchial tissues were embedded in paraffin, and sections of 5 μm thickness were prepared using a microtome. Sections were deparaffinized and hematoxylin and eosin staining or immunostaining was carried out. Immunostaining was performed

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