Toxicology in Vitro 29 (2015) 864–875

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Targeted omics analyses, and metabolic enzyme activity assays demonstrate maintenance of key mucociliary characteristics in long term cultures of reconstituted human airway epithelia

Andrew Baxter [a], Simon Thain [b], Anisha Banerjee [a], Linsey Haswell [a], Aleesha Parmar [a], Gary Phillips [a], Emmanuel Minet [a,*]

[a] BAT, Group Research and Development, Regents Park Road, Southampton SO15 8TL, UK
[b] BAT, Group Research and Development Cambridge, Milton Road, Cambridge CB4 0WA, UK

ARTICLE INFO

Article history:
Received 31 December 2014
Accepted 3 March 2015
Available online 30 March 2015

Keywords:
MucilAir™
Respiratory epithelium
Air liquid interface (ALI) culture
Metabolism
Protein expression
Gene expression

ABSTRACT

3D reconstituted respiratory epithelia have emerged as better *in vitro* models for toxicological testing compared to cell lines due to the conservation of key morphological features and functions. MucilAir™ is a commercially available human airway epithelia system that can potentially maintain functional attributes for up to a year, however, detailed mucociliary characteristics and xenobiotic metabolism relevant to inhaled pro-toxicant bioactivation is lacking. Here, we assessed in MucilAir™ some key biomarkers that are characteristic of the respiratory epithelia including morphology, function and xenobiotics metabolism. The end points that were measured included targeted proteomics using a panel of 243 airway surface liquid (ASL) proteins, cilia beat frequency (CBF), a qRT-PCR screen of xenobiotic metabolizing enzymes, and CYP2A6/13, CYP1A1/1B1 activity. Comparison of ASL proteomics with human sputum identified key proteins common to both matrices, but present at different levels. Xenobiotic metabolism gene profiling demonstrated strong similarities with the normal human lung and did not reveal any consistent changes when assessed over a 6 month period. Inducibility and activity of CYP1A1/1B1 and activity of CYP2A6/2A13 were present at one month in culture and maintained in one tested MucilAir™ donor for several months. In conclusion, MucilAir™ presented important morphological and metabolic characteristics of a mucociliary epithelium in short and long term culture. MucilAir™ is therefore a potentially useful model to test repeated sub-cytotoxic doses of toxicants.

1. Introduction

The respiratory epithelium is in direct contact with the environment and plays a double role as a permeable membrane to facilitate gaseous exchanges and as a barrier to protect against exposure to germs, allergens, and chemical pollutants (Spina and Page, 1992). Tobacco smoke, occupational and environmental exposure to chemicals are major driving factors in the development of respiratory diseases such as lung cancer and chronic obstructive pulmonary disease (COPD) (Mitchell et al., 1999). The respiratory tract is also used as a route for drug delivery for which safety evaluation is required. The metabolism of inhaled chemicals can contribute to their toxic activity; this process of metabolic transformation of a given chemical, also named pro-toxicants, into a reactive species is known as bioactivation (Castell et al., 2005). Well documented examples of enzyme-driven bioactivation of inhaled pro-toxicants include the formation of DNA reactive species such as:

(i) diol-epoxides from CYP1A1-dependent oxidation of benzo[a]pyrene (B[a]P) found in tobacco smoke and exhaust fumes (Uppstad et al., 2010),
(ii) diazo groups resulting from the α-hydroxylation of tobacco nitrosamines by the CYP2A enzyme family (Brown et al., 2007),
(iii) formaldehyde hydrate as a by-product from the glutathione conjugation through inhalation of dichloromethane vapors (Hashmi et al., 1994).

The toxicological assessment of inhaled chemicals is limited however by the availability of physiologically relevant and robust *in vitro* models. Two key requirements for such models are (i) to maintain normal airway mucociliary epithelia characteristics to

* Corresponding author. Tel.: +44 (0)2380 588 997.
E-mail address: emmanuel_minet@BAT.com (E. Minet).

http://dx.doi.org/10.1016/j.tiv.2015.03.004
0887-2333/

allow for air–liquid interface exposure, and (ii) retain metabolic enzyme activity over time, allowing both the assessment of chemicals for acute and repeat exposure. We and others have previously shown that the columnar epithelia morphology and the expression of the xenobiotic metabolizing enzymes, CYPs, is either lost or limited in lung derived cell lines such as BEAS-2B, A549, Calu-1, HAEO, and NCI-H292 (Courcot et al., 2012; Garcia-Canton et al., 2013). This could possibly lead to false negative results when assessing the toxicity of inhaled chemicals. In contrast, 3D cultures of primary lung epithelial cells have shown greater potential to retain normal morphology, metabolic gene expression and metabolic competency (Newland et al., 2011; Reus et al., 2014). The culture of differentiated human bronchial epithelial cells (HBECs) surgically resected from bronchial tissue were first described in 1996 and showed; (i) polarized mucociliary differentiation with airway surface liquid (ASL) mucus secretion, and (ii) good similarity for metabolic gene expression compared to normal lung tissue (Gray et al., 1996). Furthermore, mixed oxidase metabolic activity was detected in this cell model at 7 and 28 days in culture (Newland et al., 2011). There are currently different commercially available respiratory epithelia cultures emulated from the original HBECs model described by Gray and colleagues, notably EpiAirway™ from MatTek and MucilAir™ from Epithelix. These systems form a polarized columnar epithelia when maintained at the air liquid interface (ALI) on transwell porous membranes, in which basal, mucus producing, and ciliated cells can be distinguished (Chemuturi et al., 2005; Crespin et al., 2011; Gray et al., 1996).

EpiAirway™ are tracheobronchial cells that can be maintained at the ALI for up to a month according to the manufacturer (MatTek Corporation, 2014). The mucociliary characteristics of the EpiAirway™ model have been studied to some extent and reported in the literature. Proteomic analysis of the airway surface liquid of EpiAirway™ has revealed that the major functional class of protein secreted were involved in immunity, signaling, cell adhesion and structure and included key markers of mucociliary phenotype such as the MUC proteins (Ali et al., 2011). Some xenobiotic metabolizing enzyme activity has also been reported for EpiAirway™. For instance, CYP1A1 activity was detectable in EpiAirway™ after induction but no activity was reported when CYP2D6, 3A4, 2C9, and 2E1 were tested (Bolmarcich et al., 2010; Hayden et al., 2006).

MucilAir™ is reconstituted from human nasal or bronchial biopsies (Epithelix Sarl, 2006). It is claimed that MucilAir™ can remain in a homeostatic culture state for a long period of time, possibly up to a year, potentially allowing repeated exposures (Epithelix Sarl, 2006). The metabolic capability of this cell system however has never been evaluated either in short or long term culture. Therefore, it is unclear whether MucilAir™ would adequately model adverse biological effects associated with exposure to toxicants requiring bioactivation.

Adequate characterization of an *in vitro* system is a key requirement to evaluate to what extent such a system is relevant for toxicological testing. Our objectives are therefore to characterize:

(i) the mucociliary phenotype of MucilAir™ using a targeted proteomics approach and a microscope-based measurement of cilia beat frequency.
(ii) the metabolic competency of MucilAir™ for key enzymes known to be involved in the bioactivation of inhaled protoxicants tested in short term cultures and in cells maintained in culture for up to 6 months by qRT-PCR and activity assays.

Here we report our findings and contrast the results with normal human sputum and *in vitro* systems including HBECs, EpiAirway™ and the cell line H292.

2. Materials and methods

2.1. Cell culture

The MucilAir™ cell inserts were purchased from Epithelix Sarl (Plan-Les-Ouates, Switzerland). The cells were maintained in proprietary MucilAir™ culture medium at 37 °C and 5% CO_2 in a humidified incubator. The EpiAirway™ cell inserts were purchased from MatTek Corporation (Ashland, MA, USA). Cells were maintained in proprietary EpiAirway™ assay medium at 37 °C and 5% CO_2 in a humidified incubator. Human bronchial epithelial cells (HBECs) from three non-smokers males were obtained from Lonza (Basel, Switzerland) and cultured in 50% BEBM (Lonza Group Ltd, Basel, Switzerland), 50% DMEM with supplements as described previously (Maunders et al., 2007). Each cell donor for each primary cell system used in this study were labeled with individual numbers. Culture time points mentioned in this study refer to days post-delivery of the cells for MucilAir™ and EpiAirways™. HBECs were used at 28 days after seeding into transwells which is the required time to reach full mucociliary differentiation for this airway model. The human hepatocarcinoma-derived cell line (HepaRG) was purchased as vials of cryopreserved differentiated cells from Biopredic International (Saint-Gregoire, France). The cells were maintained on 24 well culture plates (Corning, NY, USA) coated with 0.03 mg/ml PureCol® bovine collagen solution (Nutacon, The Netherlands) with the media supplied by the manufacturer. The human pulmonary mucoepidermoid carcinoma cell line NCI-H292 was purchased from ATCC (Manassas, VA, USA) and seeded onto 12 well plates (Costar 3513) (Corning incorporated, NY, USA) at 1×10^5 cells/ml. The cells were maintained in RPMI cell culture medium (ATCC, Manassas, VA, USA) supplemented with 10% foetal calf serum and 50 U/ml penicillin streptomycin.

2.2. Proteomics of the airway surface liquid (ASL)

The MucilAir™ cell inserts were washed with 200μL phosphate buffered saline (PBS) pH = 7.4 approximately every 4 weeks. The PBS was collected and stored at −80 °C. Sputum from 10 non-smokers (Sofia Bio, Sofia, Bulgaria, study code SB-SC_v1) and MucilAir™ ASL were thawed, centrifuged and filtered using a 0.2 μm filter plate (PALL Corporation, Port Washington, NY, USA). Total protein in the samples was quantified using the bicinchoninic acid (BCA) microassay. 50 μg of protein was prepared for mass spectrometry analysis. The samples were denatured in ammonium bicarbonate and tetrafluoroethylene (TFE) and then digested overnight with trypsin (1:10, Promega, Southampton, UK). After the digestion was complete, the samples were desalted using 96-well C18 desalting plates (Empore, St Paul, MN, USA), transferred into 96-well plates and vacuum evaporated. The trypsin-digested samples were resolubilized with 30 μL of a reconstitution solution including 5 internal standard (IS) peptides. Prior to sample analysis, a Multiple Reaction Monitoring (MRM) mass spectrometry assay was developed using synthetic peptides covering an array of 243 proteins selected from a discovery step. 5 μL (~2.5 μg) of material was injected per sample onto a NanoAcquity UPLC (Waters, Elstree, UK) coupled to a 5500 QTRAP mass spectrometer (AB Sciex, Framingham, MA, USA). For the MRM assay, the optimal 2 transitions per peptide were acquired using selected reaction monitoring (SRM)-triggered MS/MS on a QTRAP 5500 instrument.

2.3. Statistical analysis of MRM protein data

All statistical analysis and graphical plotting was undertaken using Gene Spring 13 (Agilent, Santa Clara, CA, USA) with Mass