Toxicology in Vitro 28 (2014) 812–821

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Isolation and cultivation of metabolically competent alveolar epithelial cells from A/J mice

Tanja Hansen [a], Anil Chougule [b], Jürgen Borlak [b,*]

[a] Fraunhofer Institute of Toxicology and Experimental Medicine, Nikolai-Fuchs-Str. 1, 30625 Hannover, Germany
[b] Centre for Pharmacology and Toxicology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

ARTICLE INFO

Article history:
Received 19 September 2013
Accepted 18 March 2014
Available online 27 March 2014

Keywords:
A/J mice
AECs isolation and cultivation
Characterization of AECs
Cytochrome P450 monooxygenases
Gene expression
EROD and testosterone metabolism

ABSTRACT

The A/J mouse strain is used in lung cancer studies. To enable mechanistic investigations the isolation and cultivation of alveolar epithelial cells (AECs) is desirable. Based on four different protocols dispase digestion of lung tissue was best and yielded 9.3 ± 1.5 × 10^6 AECs. Of these 61 ± 13% and 43 ± 5% were positive for AP and NBT staining, respectively. Purification by discontinuous Percoll gradient centrifugation did not change this ratio; however, reduced the total cell yield to 4.4 ± 1.1 × 10^6 AECs. Flow cytometry of lectin bound AECs determined 91 ± 7% and 87 ± 5% as positive for *Helix pomatia* and *Maclura pomifera* to evidence type II pneumocytes. On day 3 in culture the ethoxyresorufin-O-demethylase activity was 251 ± 80 pmol/4 h × 1.5 × 10^6 and the production of androstenedione proceed at 243.5 ± 344.4 pmol/24 h × 1.5 × 10^6 AECs.

However, 6-α, 6-β and 16-β-hydroxytestosterone were produced about 20-fold less as compared to androstenedione and the production of metabolites depended on the culture media supplemented with 2% mouse serum or 10% FCS. Finally, by RT-PCR expression of CYP genes was confirmed in lung tissue and AECs; a link between testosterone metabolism and CYP2A12, 3A16 and 2B9/10 expression was established. Taken collectively, AECs can be successfully isolated and cultured for six days while retaining metabolic competence.

1. Introduction

The physiological role of the lung is gas exchange and oxygen supply to the body. Because of its large surface area and extensive vascularization administration of drugs via inhalation opens new possibilities for drug entry into systemic circulation particularly for drugs with pharmacokinetics defects, i.e. those that suffer from presystemic metabolic inactivation at the intestinal barriers or due to large hepatic extraction that will result in minimized drug delivery into systemic circulation. In the past, we demonstrated that pulmonary delivery of verapamil abrogated extensive first pass metabolism (Borlak et al., 2005, 2003; Walles et al., 2003, 2002a, 2002b; Koch et al., 2001) with liquid chromatography–tandem mass spectrometry identifying 25 phase I and 14 phase II metabolites of verapamil in cultures of rat hepatocytes, respectively (Walles et al., 2003). Notably, for medications to be delivered via inhalation drug induced pulmonary toxicity need to be considered. The lung is also a major organ exposed to airborne chemicals to possibly cause organ specific toxicity.

Entry into systemic circulation requires the passage of inhaled drugs and/or chemicals across the alveolar barrier. In the case of human lung about 500 million alveoli are present with the alveolar space being composed of type I squamous and type II cuboidal alveolar epithelial cells to account for 95 and 5% of the surface area, respectively (Crapo et al., 1982). Next to alveolar macrophages the tracheobronchial airway is lined with Club (formerly called Clara cells), goblet and serous cells. Alveolar epithelial type I and II cells are morphologically and functionally distinct in structural integrity (cellular thickness, composition, absorptive volume) as well in biochemical function and exhibit different behavior (Steimer et al., 2006) with alveolar type II cells to synthesize and secrete

Abbreviations: AECs, alveolar epithelial cells; AP, alkaline phosphatase; B[a]P, benzo[a]pyrene; CYP, cytochrome P; DMEM, Dulbecco's modified Eagle Medium; EROD, ethoxyresorufin-O-deethylase; FCS, Fetal Calf Serum; FITC, fluorescein isothiocyanate; FMO, flavine monooxygenases; GSH, glutathione S-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; i.p., intraperitoneal; NBT, Nitro Blue Tetrazolium; NNK, 4-(methylnitrosamino)-l-(3-pyridyl)-l-butanone; PAH, polycyclic aromatic hydrocarbon; SD, Sprague Dawley.

* Corresponding author. Tel.: +49 511 532 7250.
E-mail addresses: tanja.hansen@item.fraunhofer.de (T. Hansen), Chougule.Anil@mh-hannover.de (A. Chougule), Borlak.Juergen@mh-hannover.de (J. Borlak).

http://dx.doi.org/10.1016/j.tiv.2014.03.009
0887-2333/

pulmonary surfactant as to regulate surface tension, alveolar fluid balance and repair. Furthermore, there is evidence for alveolar epithelial stem and progenitor cells to replace injured cells thereby contributing to regeneration of the lung (Barkauskas et al., 2013).

To enable mechanistic studies metabolically competent alveolar epithelial cells (AECs) are required. Therefore, researchers will benefit from an isolation and cultivation protocol to investigate mechanistic endpoints in toxicology. Furthermore, an assessment of organ specific toxicity in organotypic cultures derived from animals is complex and hurdled by cell heterogeneity and inter/intraspecies variation (Bhogal et al., 2005). Owing to the 3R principle (Reduce, Replace and Refine) in the use of animals for toxicity testing, alternative testing approaches are needed. An establishment of a cell-culture system consisting of a population of target cell enables researchers to design superior mechanistic toxicological assays; for example, AECs are predominantly involved in B[*a*]P metabolism (Kuriharal et al., 1993). Moreover, the distribution of xenobiotic-metabolizing CYP enzymes in lungs is cell type specific.

The pulmonary expression of CYP monooxygenases and of other biotransformation enzymes was reported for laboratory animals and humans and was shown to encompass glutathione S-transferases (GST), esterases, peptidases, cyclooxygenases and flavine monooxygenases (FMO). Moreover, it was shown that CYP enzymes are mostly expressed in Club cells (Patton et al., 2004), type II pneumocytes and macrophages (Hukkanen et al., 2002). We previously characterized drug metabolism enzymes of AECs isolated from rats and demonstrated AECs to express and to retain a range of CYP monooxygenases (1A1, 1A2, 3A2, 1B1, 2B1, 2E1 and 2J3) when cultured under optimal conditions (Hansen et al., 2006).

Most protocols for an isolation of murine AECs stem from the earlier work of Corti et al. (1996) and Harrison et al. (1995). The principle differences among the applied methods are types and concentration of enzymes used for lung tissue digestion during cell isolation and includes enzyme preparations based on non-specific protease type I, dispase, elastase, trypsin and collagenase. An elastase based digestion of lung tissue and panning of cell suspension on plates coated with IgG was reported by Dobbs et al. (1986) and was found to provide high yield and purity of AECs. Except for rat the method is suitable for many species.

While methods developed for a particular species can be employed as guidance these need to be adopted when used across species and require the lung anatomy to be considered. Specifically, the airway immediately proximal to the bronchiole–alveolar duct junctions in the mouse differs from other species with fewer respiratory bronchioles and airway generations (Irvin and Bates, 2003). Because of these anatomical features the mouse lung has a smaller alveolar surface than rat and human; an absence of submucosal glands is noted but the proportion of Club cells is higher as compared to other rodents. Thus, enzymatic digests of lung tissue contain mostly Club cells. To enrich for AECs flow cytometry based sorting of CD31 and CD45 was reported (Driscoll et al., 2012). However, cell sorting is a significant stress to cells. IgG depletion, magnetic bead isolation and density gradient as well as counterflow centrifugation elutriation techniques enabled separation and enrichment of alveolar epithelial and Club cell fractions isolated from lung tissue (Reddy et al., 2004).

In the present study, we report a protocol for an efficient isolation and cultivation of differentiating AECs from the A/J mice suitable for studies over 6 days. This strain of mice has been used successfully in carcinogenesis studies and was shown to develop lung adenomas within 6–8 month following treatment with PAHs or tobacco carcinogens, such as NNK or B[*a*]P (Witschi, 1998).

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle Medium (DMEM), Fetal Calf Serum (FCS) and phosphate buffered saline solution (PBS) were purchased from Biochrom (Berlin, Germany). The medium to culture primary alveolar epithelial was supplemented with 6.3 μg/ml insulin (Insuman® Rapid, Hoechst Marion Roussel, Frankfurt/Main, Germany), 0.67 μg/ml Prednisolone, 0.016 μg/ml glucagon (Novo, Mainz, Germany), 200 U/ml penicillin and 200 μg/ml streptomycin (Biochrom, Berlin, Germany). Trypsin–EDTA solution (0.25%) was obtained from Sigma (Deisenhofen, Germany). Dispase grade II was obtained from Roche Diagnostics (Berlin, Germany). Mouse serum was prepared from A/J mice using standard procedure. Collagen was prepared from A/J mouse tails according to the method of Elsdale and Bard (1972).

Testosterone, 11-α-hydroxyprogesterone, 2-α-hydroxytestosterone, 6-α-hydroxy-testosterone, 6-α-hydroxytestosterone, 16-α-hydroxytestosterone, androstendione, ethoxyresorufin and resorufin were obtained from Sigma (Deisenhofen, Germany).

2.2. Animals

A/J mice and SD rats ($n = 5$) were purchased from Charles Rivers Laboratory (Sulzfeld, Germany). All animal work followed strictly the Public Health Service (PHS) policy on Human Care and Use of Laboratory Animals of the National Institutes of Health, USA. Formal approval to carry out animal studies was granted by the animal welfare ethics committee of the State of Lower Saxony, Germany ('Lower Saxony State office for Consumer Production and Food Safety', LAVES). The approval ID is Az: 33.9-42502-04-06/1087.

2.3. AECs isolation and culture

A total of $n = 4$ different protocols (A–D) were tested and initially explorative studies were done with $n = 10$ animals. Subsequently, and for each protocol $n = 5$ A/J mice were used for AECs isolation and cultivation. In the case of rat AECs, a total $n = 5$ SD rats were used.

Male A/J mice weighting approximately 30 g were anesthetized by i.p. injection of Ketamin® and Rompun®. After tracheotomy the trachea was cannulated with a bulb head cannula. Following the midline incision and removal of the rib cage, a Luer cannula was inserted into the right ventricle. The pulmonary circulation was perfused with ice cold PBS using a peristaltic pump at a flow rate of 4 ml/min. Then, the perfusate was removed by incision of the left atrium and perfusion with the buffer was continued for 2–3 min until the lungs were completely free of blood. During the perfusion, the lungs were manually ventilated via the tracheal cannula. Then the lungs were explanted and transferred to a sterile flow cabinet. Lungs were lavaged three times with 1 ml PBS with a 22 G needle and 10-cc syringe followed by a 1 ml Trypsin–EDTA solution that was instilled via the trachea and incubated in a shaking water bath at 37 °C for 15 min. Lungs were treated separately with trypsin at two different concentrations of 0.25% and 0.025%, respectively, as depicted in Fig. 1 for protocol A and B. The incubation was stopped by instillation of 10 ml ice cold trypsin-inhibitor solution and the lungs were stored on ice for tissue dissection.

For protocol C and D the lungs were initially filled with 1.5 ml warm dispase solution (>2.4 U/ml) without previous bronchoalveolar lavage at room temperature for 45 min followed by instillation of 0.55 ml of 45 °C warm agarose (1% w/v, low melting). Immediately afterwards, the lungs were covered with crushed ice and