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Primary rat alveolar epithelial cells for use in biotransformation and toxicity studies

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Abstract

The alveolar epithelium may function as a barrier for airborne xenobiotics, and in vitro models mimicking this barrier are useful for metabolism and toxicity studies. To gain insight into the metabolic competence of alveolar epithelial cells (AECs), we investigated transcript expression of 10 different cytochrome P450 monooxygenases as well as expression of surfactant proteins A to D. We also investigated gene expression of the transcription factors PCNA, TTF-1, HNF3β , GATA-6, C/EBPα and C/EBPδ which drive, at least in part, development and differentiation of alveolar epithelium. We further studied the metabolism of testosterone, a substrate for cytochrome P450 (CYP) monooxygenases, in cultures of AECs. Essentially, medium supplementation with 5% rat serum, as opposed to 10% FCS, promoted a high level of differentiation, as judged by the mRNA expression of CYP monooxygenases, e.g. 1A1, 1A2, 2B1 and 2J3, the expression of the surfactant proteins A, B, and C, the immunohistochemical staining for surfactant protein C, and staining for alkaline phosphatase activity. Further, AECs, when cultured in the presence of 5% rat serum, promoted metabolic competence, as evidenced by the fingerprinting of individual testosterone metabolites. We thus characterized AECs in culture and found these respiratory epithelial cells to express an array of differentiation markers and showed these cultures to be metabolically competent under optimized culture conditions.

Keywords: Alveolar epithelial cells; Differentiation markers; Cytochrome P450 monooxygenases; Testosterone metabolism

1. Introduction

The alveolar epithelium consists of two cell types: thin, expansive type I cells, forming the gas exchange surface, and alveolar type II cells, which are cuboidal in shape and located in the alveolar corners. Alveolar epithelial type II cells (AECs) in particular serve two major functions, both of which are essential for maintaining the normal lung physiology. Firstly, they synthesize and secrete pulmonary surfactant (Kasper and Singh, 1995), and secondly, they function as progenitors of newly formed pulmonary alveolar epithelium after lung injury and during normal lung cell turnover (Uhal, 1997). Furthermore, AECs have been reported to express cytochrome P450 (CYP) monooxygenases (Baron and Voight, 1990) and therefore play an important role in the pulmonary metabolism of foreign compounds.

Indeed, airborne xenobiotics have to pass the alveolar epithelium before entering the systemic circulation, and enzymes expressed in the alveolar epithelium may lead to metabolic inactivation, thus providing protection. Controversially, metabolic activation may lead to reactive intermediates to cause cellular damage (Borlak et al., 2000). Knowledge on the activity and regulation of xenobiotic-metabolizing enzymes within the alveolar epithelium is therefore desirable to help understand pulmonary responses to airborne xenobiotics.

Furthermore, drugs may be administered by inhalation to reach systemic circulation. This route of drug application has become increasingly important and has been reported

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for the immunosuppressive agent cyclosporin, several interferons and α1 antitrypsin (McElvaney et al., 1991; Keenan et al., 1992; Halme et al., 1994). Moreover, drugs with pharmacokinetic defects resulting in high first-pass metabolism may be administered by inhalation, as recently reported by us (Borlak et al., 2005).

Although methods for the isolation and culture of AECs from the rat lung have been reported (Dobbs et al., 1986; Richards et al., 1987; Viscardi et al., 1992; Murphy et al., 1999, for review see Dobbs, 1990), AECs are known to lose many of their specific abilities, such as surfactant protein expression and the ability to synthesize surfactant phospholipids, when maintained under standard culture conditions, i.e. adherent to plastic and cultured with medium containing FCS (Shannon et al., 1992). Further, cells undergo a series of characteristic morphologic changes including cell spreading and flattening and the loss of surfactant-storing lamellar bodies. It is desirable to maintain the type II phenotype in culture, which can be promoted by various conditions including high-density seeding (Paine et al., 1995; Reynolds et al., 1999), choice of extracellular matrix substrata (Sugahara et al., 1998), air exposure (Kohsa et al., 1996) and various medium supplementations (Borok et al., 1995, 1998).

Notably, we view expression of CYP monooxygenases and of certain transcription factors, as well as immunohistochemical staining of surfactant protein C (SP-C) and of alkaline phosphatase as hallmarks of differentiated AECs. Thus, the study of these cellular markers guided the development of a protocol for culturing alveolar epithelial cells.

Overall, we investigated the aforementioned markers in cultures of AECs and studied the effects of medium supplemented with 10% FCS or 5% rat serum on cellular phenotype. We used testosterone to probe for CYP enzyme activity and investigated induction of individual CYP isoforms after treatment with Aroclor 1254, a known inducer of CYP monooxygenases (Borlak et al., 1996). We therefore aimed for an improved understanding of regulation of cellular differentiation markers in cultures of primary AECs, to enable its reliable use in experimental pharmacology and toxicology studies.

2. Materials and methods

2.1. Cell culture media 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 used for the cultivation of AECs 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). Trypsin–EDTA solution (0.25%) and rat serum were obtained from Sigma (Deisenhofen, Germany). Rat tail collagen was prepared according to the method of Elsdale and Bard (1972).

2.2. Cell isolation and culture

AECs were isolated as originally described by Richards et al. (1987), with the following modifications: Briefly, male Sprague Dawley rats weighing approximately 120 g were anesthetized by intraperitoneal injection of Ketamin® (100 mg/kg) and Rompun® (10 mg/kg). After tracheotomy the trachea was cannulated with a bulb head cannula. Following midline incision and removal of the rib cage, a Luer cannula was inserted via the vena cava cranialis into the right atrium. The pulmonary circulation was perfused with ice cold PBS using a peristaltic pump at a flow rate of 4 ml/min. 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. The lungs were explanted, placed in a 50 ml Falcon tube containing 30 ml ice cold PBS and transferred to a sterile flow cabinet. The lungs were then lavaged four times with 6 ml PBS, and subsequently 6 ml Trypsin–EDTA solution (0.25%) was instilled into the trachea. The lungs were incubated with trypsin at 37 °C for a total of 45 min. Every 15 min, 4 ml trypsin solution was pumped via the tracheal cannula.

The trachea and main bronchi were removed, and the parenchymal lung tissue was dissected into 1- to 2-mm pieces in a petri dish containing 10 ml ice cold PBS with Ca^{2+}. The lung tissue suspension was incubated for 10 min with 10 ml DNase solution at a concentration of 250 μg/ml DNase and then filtered through a nylon mesh (100 and 60 μm pore size). The alveolar type II pneumocytes were prepared by discontinuous percoll gradient centrifugation (heavy density 1.089 and low density 1.040). Following centrifugation for 30 min at 250*g* and 4 °C, the cell fraction at the interface between the heavy and low-density gradient was removed and mixed with DNase solution containing 50 μg/ml DNase. The cell suspension was again centrifuged at 140*g* at 4 °C for 6 min. The resulting cell pellet was then resuspended in DMEM supplemented with 10% FCS or 5% rat serum and incubated in a 25 cm^2 tissue culture flask at 37 °C for 60 min. The non-adherent cells were removed and plated on collagen-coated 12-well plates. Approximately 2×10^6 cells were seeded per cm^2 in 1 ml culture medium.

2.3. Histochemistry

For histochemistry, cytospots of freshly isolated cells and cells on day 3 and 6 in culture were prepared at an approximate density of 1×10^6 cells per slide and frozen at −80 °C. Staining of type II cells for alkaline phosphatase (AP), a marker for epithelial cells, originally described by the method of Edelson et al. (1988), was performed using 2 mg naphthol-AS-MX-phosphate (Sigma), 200 μl *N*,*N*-dimethylformamide (DMF, Merck, Darmstadt, Germany), 9.8 ml 0.1 m pH 8.2 Tris–HCl buffer and 10 mg fast-red salt