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Characterisation of the R3/1 cell line as an alveolar epithelial cell model for drug disposition studies

Lenka Horálková[a,b], *Aneta Radziwon*[a], *Sibylle Endter*[a], *Rikke Andersen*[c], *Roland Koslowski*[d], *Marek W. Radomski*[a], *Pavel Doležal*[b], *Carsten Ehrhardt*[a,*]

[a] *Trinity College Dublin, School of Pharmacy and Pharmaceutical Sciences, Westland Row, Dublin 2, Ireland*
[b] *Charles University in Prague, Faculty of Pharmacy, Hradec Králové, Czech Republic*
[c] *Department of ADME & Assay Technology, Novo Nordisk A/S, Måløv, Denmark*
[d] *Dresden University of Technology, Institute of Physiological Chemistry, Medical Faculty Carl Gustav Carus, Dresden, Germany*

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ABSTRACT

The rat cell line R3/1 displays several phenotypical features of alveolar epithelial type I cells. In order to evaluate this cell line as potential *in vitro* model for drug disposition studies, R3/1 cells were cultured on Transwell filters and the transepithelial electrical resistance (TEER) was measured to test the integrity of cell layers. The mRNA expression of cell junctional components including E-cadherin, occludin, ZO-1 and ZO-2 was studied using reverse transcriptase-polymerase chain reaction (RT-PCR) and the corresponding proteins by immunofluorescence microscopy (IFM). Moreover, the expression pattern of catabolic peptidases, carboxypeptidase M, aminopeptidases (AP): A, B, N and P, γ-glutamyltransferase (GGT), dipeptidylpeptidase IV, angiotensin-converting enzyme (ACE), and endopeptidases (EP) 24.11 and 24.15 was analysed in R3/1 cells and compared to rat alveolar epithelial I-like cells in primary culture.

TEER peaked at $99 \pm 17\,\Omega\,cm^2$ after 5 days in culture. Addition of 0.1 μM dexamethasone (DEX) with 20% foetal bovine serum further increased TEER by 65%. However, none of the culture conditions used in our study yielded monolayers with TEER values comparable to those of primary cultures of rat pneumocytes. No transcripts encoding for E-cadherin and occludin were detected by RT-PCR. However, ZO-1 and -2 mRNA transcripts were found. IFM using a monoclonal antibody against occludin confirmed the absence of the protein in R3/1 cells. Of the investigated proteolytic enzymes, mRNA transcripts encoding APA and APB as well as EP 24.11 and EP 24.15 were detected; a pattern similar to that of rat alveolar epithelial I-like cells in primary culture.

Thus, although R3/1 cells express certain markers typical for type I pneumocytes (e.g., T1α, ICAM-1, connexin-43, caveolins-1 and -2) they do not form electrically tight monolayers. This excludes R3/1 cells from being used as an *in vitro* model for alveolar absorption. However, the cell line may be suitable to study stability of inhaled and endogenous proteins.

* *Corresponding author*. Tel.: +353 1 896 2441; fax: +353 1 896 2783.
E-mail address: ehrhardc@tcd.ie (C. Ehrhardt).
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1. Introduction

Drug delivery via the pulmonary route represents an attractive avenue for the non-invasive delivery of many clinically relevant compounds. However, limitations need to be acknowledged, among which are the potential impact of active transport mechanisms (e.g., membrane transporters and vesicular transport) upon the epithelial absorption. Moreover, the influence of metabolic enzymes and catabolic peptidases on the drug disposition process (particularly of biopharmaceuticals) is poorly understood (Patton, 1996).

Cell-based *in vitro* models are useful tools for investigations of drug transport and stability at the various epithelia of the lung (Sporty et al., 2008; Forbes and Ehrhardt, 2005). Due to the lack of availability of human lung tissue and ethical constrains pertaining to use of human tissues, most studies have been based on isolation and culture of alveolar epithelial cells (AECs) from the lungs of animals including mouse (Corti et al., 1996), rat (Goodman and Crandall, 1982), rabbit (Shen et al., 1999) and pig (Steimer et al., 2007). Since species differences between human and rodents might be more significant than once assumed (King and Agre, 2001), confirmation of relevance of rodent data to human using human pneumocyte cultures is crucial (Wang et al., 2007; Bur et al., 2006; Ehrhardt et al., 2005).

Primary culture techniques of AECs which involve isolation, purification, and culture of alveolar epithelial type II (ATII) cells from tissues obtained after lung resections or from isolated perfused lungs, are used for most *in vitro* studies of alveolar epithelial function due to the paucity of appropriate alveolar epithelial cell lines that form functional tight junctions (Kim et al., 2001). These ATII cells, when plated on permeable supports or plastic under appropriate culture conditions, acquire type I cell-like phenotypes and morphologies (Demling et al., 2006; Fuchs et al., 2003; Wang et al., 2007). Although isolation of alveolar epithelial type I (ATI) pneumocytes from rat lungs has recently been reported with some success (Borok et al., 2002; Johnson et al., 2002; Chen et al., 2004), development of confluent ATI cell monolayers with electrically tight characteristics has not been reported thus far. It should be noted that unlike many other cells in primary culture, AEC generally exhibit a very limited proliferation profile and are therefore not suitable for passaging (Sporty et al., 2008). Thus, a new preparation of cells must be generated and used for each data set which is tremendously costly and time consuming.

While a number of immortalised cell lines emanating from different cell types of the airway (i.e., tracheo-bronchial) epithelium of lungs from various mammalian species are available (Sporty et al., 2008; Kemp et al., 2008), reliable and continuously growing cell lines that possess alveolar epithelial cell morphology and phenotype have not been reported to date. Most studies have relied on the use of cell lines of alveolar epithelial origin, e.g., A549 cells, for drug absorption studies with observations that are meaningless or hard to extend to humans (Sporty et al., 2008; Lieber et al., 1976; Foster et al., 1998; Elbert et al., 1999).

The R3/1 cell line was established from pulmonary tissue of foetuses of Han-Wistar rats on day 20 of gestation by explant-replica techniques (Knebel et al., 1994). It has been reported to display several phenotypical features of alveolar epithelial type I cells (Koslowski et al., 2004; Barth et al., 2005; Reynolds et al., 2008). In order to characterise the cell line as an *in vitro* model for drug disposition studies, R3/1 cells were cultured on filter inserts at different seeding densities, in various cell culture media, as well as under liquid-covered *vs.* air-interfaced conditions. The transepithelial electrical resistance (TEER) was measured as a parameter for the integrity of cell layers. Presence of cell junctional proteins (E-cadherin, occludin, ZO-1, ZO-2) in R3/1 cells was studied as well as the expression pattern of catabolic peptidases. The following peptidases were investigated: carboxypeptidase M (CPM); aminopeptidases (AP): A, B, N, P; γ-glutamyl transpeptidase (GGT); angiotensin-converting enzyme (ACE); endopeptidases (EP): 24.11 (neprilysin), 24.15 (thimet oligopeptidase 1). Results were compared with data obtained from rat alveolar epithelial type I-like and type II cells in primary culture.

2. Materials and methods

2.1. Cell culture of AEC

2.1.1. Continuous cell line

R3/1 cells of passage numbers 40–59 were grown in an 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (Sigma, Dublin, Ireland) at 37 °C in 5% CO_2 atmosphere. The DMEM/Ham's F-12 was supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES. Moreover, RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin was used as an alternative medium. The media were changed every other day.

To study the influence of media composition on R3/1 cell monolayer integrity, cells were seeded on Transwell Clear permeable filter inserts (Fisher Scientific, Dublin, Ireland) at densities of 0.5–8 × 10^5 cells/cm^2 and cultured under liquid-covered culture (LCC) or air-interfaced culture (AIC) conditions. The effect of dexamethasone (DEX, 0.1 μM) and FBS (0–20%) in the culture medium on the cell function was assessed.

2.1.2. Primary cells

The use of animals for these experiments was concordant with the Declaration of Helsinki and the protocol was approved by the local ethical committees. Specific pathogen-free adult Sprague–Dawley male rats weighing 120–150 g were euthanised with sodium pentobarbital (2.5 mg/kg, i.p.). Rat lungs were perfused *in situ* with 0.9% NaCl solution and alveolar type II (ATII) cells were isolated from the lungs following *ex vivo* elastase digestion (Kim et al., 1991). The crude cell mixture was filtered sequentially through 100, 40 and 10 μm meshes, followed by plating onto IgG-coated bacteriological plates. After 1 h incubation, ATII cells were collected and centrifuged at 150 × *g* for 10 min for further enrichment. Purified rat type II pneumocytes were resuspended and either used directly or seeded onto Transwell filter inserts at 1.2 × 10^6 cells/cm^2. Culture medium consisted of DMEM/Ham's F-12 1:1 sup-