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The human bronchial epithelial cell line 16HBE14o− as a model system of the airways for studying drug transport

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Abstract

The 16HBE14o− cell line, which forms polarised cell layers in vitro, provides a promising opportunity to develop a convenient epithelial cell culture model in which respiratory drug transport can be evaluated in vitro. This study investigated the effect of cell seeding density, collagen substratum and time in culture on the development of barrier properties in this cell line, after which the permeability of the 16HBE14o− cell layers to a series of solutes was studied. Seeding cells at a density of 2.5×10^5 cells per cm^2 on a monofibrillar Vitrogen-100 collagen substratum, followed by culture at an air–liquid interface for 6 days resulted in cell layers with a transepithelial electrical resistance (TER) of $247 \pm 47\,\Omega\,cm^2$ and an apparent permeability coefficient of $2.5 \times 10^{-6}\,cm\,s^{-1}$ for mannitol. The permeability of the 16HBE14o− cells to hydrophilic molecules ($\log P < 1.9$) was of an order of magnitude greater than that of typical alveolar cell cultures, possibly reflecting barrier properties more representative of the airways. More lipophilic drugs showed higher permeabilities indicating a sigmoidal relationship between permeability and lipophilicity similar to that observed for solute transport across primary cultured epithelial cell layers. These results indicate that under appropriate culture conditions, 16HBE14o− cell layers provide a discriminatory barrier to solute transport.

Keywords: Biopharmaceutics; Cell culture; Air-interface; Permeability; Drug absorption; Lung

1. Introduction

In vitro cell culture models of absorptive epithelia based on immortalised cells have proved extremely useful for the study of epithelial permeability and drug absorption. The advantages of cell culture models based on continuous cell lines are well documented and their utility is exemplified by the popularity of the Caco-2 model of the gastrointestinal epithelium (Artursson and Borchardt, 1997; Bailey et al., 1996). However, at present there is no lung equivalent of the Caco-2 cell line to serve as a well-established in vitro model of the respiratory epithelium.

Research into utilising the lung for systemic absorption has tended to focus on the alveolar epithelium, attracted by the large surface area, thin airway to blood barrier and high rate and volume of blood flow in this region. Unfortunately, there are no immortalised epithelial cell lines that represent adequately the alveolar epithelial transport barrier in vitro (Kim et al., 2001). The airways, although anatomically less favourable for airway-blood transport, are more readily targeted by inhaled aerosols than the peripheral lung. It has also been suggested that the importance of

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absorption from the airways has been underestimated (Widdecombe, 1997).

In 2000, two cell lines were identified as promising candidates for culture as drug absorption models of the airway; 16HBE14o− and Calu-3 cells (Forbes, 2000). Although reports of the development of Calu-3 cells as a drug absorption model were quick to appear (Foster et al., 2000; Hamilton et al., 2001; Mathias et al., 2002), progress with the 16HBE14o− cell line has only recently been reported (Ehrhardt et al., 2002). In contrast to methods previously used to culture 16HBE14o− cells for biopharmaceutical purposes (Forbes and Lansley, 1998a,b), submerged rather than air–liquid culture was favoured (Ehrhardt et al., 2002). The effects of different cell culture methods on the properties of cell layers will have important implications for the development of the 16HBE14o− cell line as a drug absorption model.

The 16HBE14o− cell line was originally developed from human bronchial epithelium to study the cystic fibrosis transmembrane conductance (CFTR) regulator (Cozens et al., 1994) and retains many features of differentiated bronchial epithelial cells. 16HBE14o− cells have a cobblestone appearance in culture, form tight junctions and display vectoral ion transport (Cozens et al., 1994). The use of defined culture methods is critical for airway epithelial cell cultures. Factors such as seeding density, collagen substratum and time in culture profoundly affect the growth and differentiation of primary airway epithelial cell cultures (Yamaya et al., 1992; Kaartinen et al., 1993; De Jong et al., 1994; Mathias et al., 1995). The primary objectives in establishing culture conditions for a drug transport model are to promote both the differentiation of the cells and the formation of a suitable epithelial barrier.

Observations made using the model must correlate accurately with measurements of pulmonary absorption if 16HBE14o− cells are to provide the opportunity to use human cells as a predictive in vitro model of the airway permeability. A variety of in vivo and in vitro models have been used to evaluate drug transport in the lung (Mobley and Hochaus, 2001; Schanker et al., 1986), the most directly comparable of which with 16HBE14o− cell culture are primary cell cultures (Mathias et al., 1996; Saha et al., 1994). In this paper, we provide methodological details of the air–liquid culture conditions used in previous reports (Forbes and Lansley, 1998a,b) and report the effect of seeding density, collagen substratum and time in culture on the permeability of 16HBE14o− cells cultured as a drug absorption model. The barrier properties of the model were initially evaluated using mannitol transport and transepithelial electrical resistance (TER). The permeability of 16HBE14o− cells to a series of solutes was then measured and compared to the barrier presented by primary airway cells in culture.

2. Materials and methods

2.1. Chemicals and reagents

^{14}C-Mannitol was obtained from New England Nuclear (Amersham, UK). *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) was obtained from Sigma Chemicals Ltd. (Poole, UK). All other reagents were of analytical grade and obtained from BDH Ltd. (Poole, UK).

2.2. Cell culture materials

16HBE14o− cells were a gift from Dr. Dieter Gruenert (formerly of the University of California, San Francisco, USA). [^{3}H]-Propranolol was supplied by New England Nuclear (Amersham, UK) and [^{3}H]-atenolol was a generous gift from GlaxoSmithKline (Ware, UK). Metoprolol and salbutamol were obtained from Sigma Chemicals Ltd. Dulbecco's minimum essential medium:Ham's F12 1:1 (DMEM:F12), phosphate buffered saline (PBS), 10,000 U ml^{-1} penicillin/10 mg ml^{-1} streptomycin, glutamine, and Hank's balanced salt solution (HBSS; product no. H8264 containing calcium and magnesium) were obtained from Sigma Chemicals Ltd. Ultroser G was obtained from Life Technologies (Paisley, UK). Vitrogen-100 was obtained from Celtrix Laboratories (Palo Alto, USA). Transwell culture chambers (1 cm^2, 0.4 μm pore size, polycarbonate) were obtained from Costar (High Wycombe, UK).

2.3. Cell culture methods

Mycoplasma-free 16HBE14o− cells (passage 15–48) were cultured on Transwell cell culture supports. The supports were used uncoated, coated with

0.2 ml monofibrillar collagen (Vitrogen-100 dried overnight under laminar air flow and neutralised by washing with PBS before seeding with 16HBE14o− cells) or coated with 0.2 ml collagen gel. The gel coating was performed by adding a freshly prepared chilled solution (8 ml Vitrogen-100, 1 ml 10× PBS, 1 ml 0.1 M sodium hydroxide, adjusted to pH 7.4) to the Transwell surface then incubating at 37 °C for 60 min to induce in situ gel formation. Cells were seeded at a range of seeding densities (10^3 to 10^6 cells cm^{-2}) and incubated at 37 °C in 5% CO_2–95% air. Medium (DMEM:F12 with Ultroser G (2%, v/v), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), glutamine (2 mM)) was removed from the apical chamber after 2 days in culture and cells were then maintained at an air–liquid interface. Medium in the basolateral chamber (2 ml) was replaced every 24 h.

2.4. Assessment of permeability barrier

TER was measured across the cell layers using an epithelial voltohmmeter (World Precision Instruments, Stevenage, UK) and silver chloride 'chopstick' electrodes. Measurements were made at 37 °C after a 30 min equilibration period with fresh cell culture medium (or HBSS when part of a transport experiment) in the apical chamber (1 ml) and basolateral chamber (2 ml). The resistance across a cell-free culture support was subtracted from the resistance measured across each cell layer to yield the TER of the epithelial cells.

The apical to basolateral flux of ^{14}C-mannitol (4.6 kBq ml^{-1}), salbutamol (10^{-3} M), ^{3}H-propranolol (55.6 kBq ml^{-1}), metoprolol (10^{-3} M) and ^{3}H-atenolol (8.0 kBq ml^{-1}) across cell layers was measured. The culture medium was removed from the basolateral chamber of Transwell culture well and the cells were washed twice using HBSS and allowed to equilibrate at 37 °C for 30 min with HBSS in the apical chamber (1 ml) and basolateral chamber (2 ml). TER was measured immediately before and after each transport experiment to check that cell layer integrity was maintained during the course of the experiment. To initiate the transport experiment, solution in the donor chamber was replaced by test solution (0.6 ml HBSS containing radiolabelled mannitol ± β-receptor antagonist or salbutamol). The solution in the receiver chamber was replaced by 1.5 ml HBSS to provide a level of solution equivalent to that in the apical chamber, thereby avoiding a hydrostatic pressure gradient between the chambers. The entire experiment was conducted at 37 °C and samples (200 μl) were removed from the receiver chamber at 15, 30, 60, 90 120, 240 min and replaced with an equivalent volume of HBSS. At 240 min a sample was removed from the donor chamber to allow mass balance to be calculated. Radiolabelled compounds were analysed by liquid scintillation counting, after addition of 5 ml of scintillant (Ready Protein®) using a Beckman dual scintillation counter (1209 Rackbeta 'Primo'). Non-radiolabelled compounds were analysed by HPLC. Apparent permeability coefficients (Papp) were calculated using the equation:

$$\text{Papp} = \left(\frac{dQ}{dt}\right) AC_0 \quad (1)$$

where dQ/dt is the transport rate, A is the surface area of the Transwell culture support and C_0 is the initial concentration of the transport compound in the donor chamber.

2.5. Data analysis

The effect of filter coatings was compared using two-way ANOVA. Results were regarded as being significantly different when $P < 0.05$.

3. Results

Preliminary studies were performed to evaluate the use of the collagen substratum on the barrier properties of the cell layers. No difference ($P > 0.05$) in mannitol permeability was measured after 6 days regardless of whether cell culture supports were uncoated or coated with either monofibrillar or gelled collagen ($n = 6$). However, when uncoated supports were used the cells appeared more squamous in shape and were observed to grow down into the pores of the filter inserts. For all further experiments a coating of monofibrillar collagen was used. After preliminary experiments, the effect of seeding cells at densities of 1×10^5, 2.5×10^5 and 5.0×10^5 cells cm^{-2} on TER was investigated (Fig. 1). If the seeding density was too low (10^3 cells per cm^2), complete monolayers did not form whereas if seeding densities were too

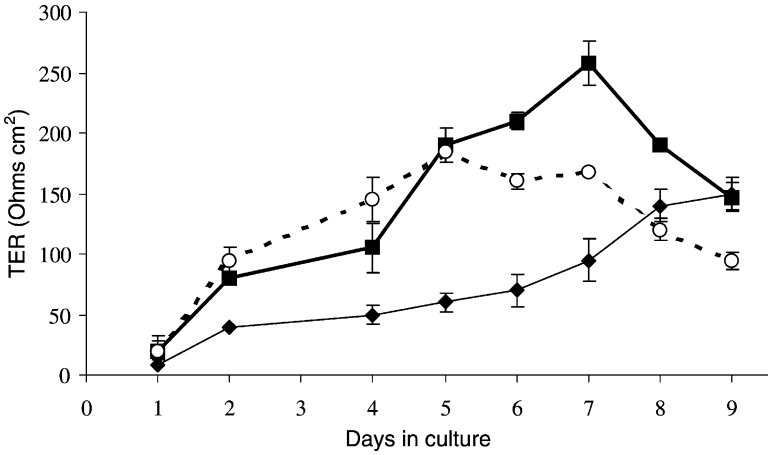

Fig. 1. The variation of transepithelial electrical resistance (TER) with days in culture for 16HBE14o− cells cultured on monofibrillar Vitrogen-100 coated filters using initial seeding densities of 1×10^5 cells cm^{-2} (◆), 2.5×10^5 cells cm^{-2} (■), and 5×10^5 cells cm^{-2} (○). Data represent mean ± S.D., $n = 6$.

high (10^6 cells per cm^2) multilayers of cells formed by day 3 in culture. Maximum TER values (in excess of 200 Ω cm^2) were achieved between days 6 and 7 by cells seeded at a density of 2.5×10^5 cells cm^{-2}, after which the TER fell through to day 9.

Daily measurement of mannitol flux was used to measure the development of the barrier to solute permeability during culture of the 16HBE14o− cell model under the optimised culture conditions (cells seeded at 2.5×10^5 cells cm^{-2} on Vitrogen-100 monofibrillar coated culture supports and cultured for 6 days). The permeability of mannitol decreased with time in culture as the cell layers developed a concomitant increase in TER (Fig. 2). By day 6, the

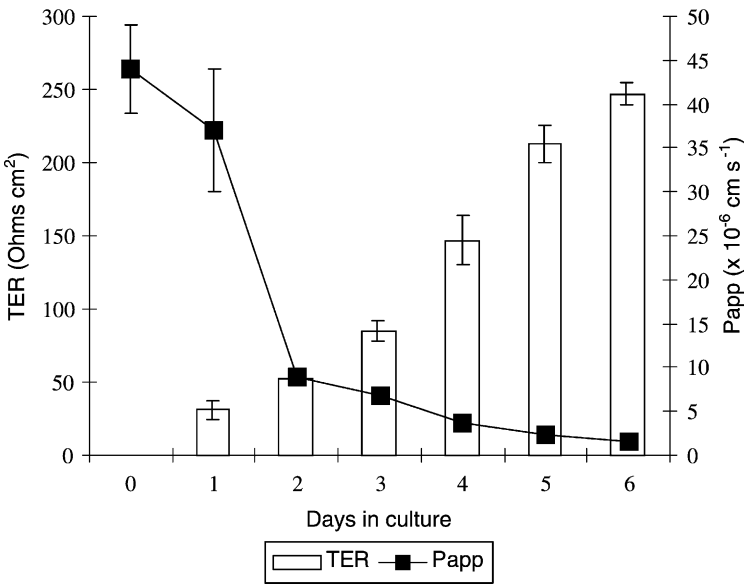

Fig. 2. The variation of the apparent permeability coefficient (Papp) of mannitol and transepithelial electrical resistance (TER) across 16HBE14o− cells cultured on monofibrillar Vitrogen-100 coated filters using an initial seeding density of 2.5×10^5 cells cm^{-2}. Data represent mean ± S.D., $n = 6$.

permeability of mannitol was reduced by greater than 10-fold to 2.53×10^{-6} cm s^{-1}, with a corresponding almost 10-fold increase in TER to 240 Ω cm^2. After further experiments of solute permeability using cells of several passages (15–48), the mean Papp of mannitol was recalculated as $2.36 \pm 0.66 \times 10^{-6}$ cm s^{-1} with a mean TER of 247 ± 47 Ω cm^2 in a larger number of cell layers ($n = 75$).

The transport of three β-receptor antagonists and salbutamol across 16HBE14o− cells was measured. The mean TER and Papp values of mannitol across the cells used for the transport of the solutes were not affected by the transport of the drugs. All solute fluxes were linear over 240 min and mean Papp for each compound ($n = 6$) was salbutamol $1.42 \pm 0.28 \times 10^{-6}$ cm s^{-1}, atenolol $1.52 \pm 0.23 \times 10^{-6}$ cm s^{-1}, metoprolol $2.94 \pm 0.63 \times 10^{-6}$ cm s^{-1} and propranolol $28.6 \pm 0.1.00 \times 10^{-6}$ cm s^{-1}.

4. Discussion

HBE14o− cell layers were grown using the culture conditions reported by the originators of the cell line (Cozens et al., 1994). These conditions are based on those under which primary airway cells display phenotypic properties most characteristic of the native epithelium. Using these conditions, 16HBE14o− cells have been found to stain positively for the tight junction proteins ZO-1 and occludin and intercellular proteins E-cadherin and desmoplakin (Wan et al., 2000). In order to establish conditions for the culture of 16HBE14o− cells as a drug absorption model, we have identified a support coating and seeding density to generate cell layers with suitable barrier properties by day 6 in culture. A seeding density of 2.5×10^5 cells cm^{-2} resulted in cell layers that had optimal barrier properties after 6 days. 16HBE14o− cells grown on monofibrillar collagen coating had similar appearance and barrier properties to cell layers grown on collagen gel. Monofibrillar collagen was preferred for its ease of application, and the uniformity and reproducibility of the collagen layer formed.

As well as promoting the differentiated properties of airway epithelial cells, an important objective was that the cells mimic the barrier function of the airway epithelium in vivo. Although the barrier properties of excised airways (Wangensteen et al., 1993) and the lung in vivo (Schanker et al., 1986) are species-dependent, the TER of the 16HBE14o− cells approximates to those reported for airway epithelia (Rojanasakul et al., 1992; Bhat et al., 1993). For example, rabbit airway has typical TER of 260–300 Ω cm^2 compared to ∼250 Ω cm^2 in the 16HBE14o− cell layers. In contrast, cell models such as Calu-3 cells and primary tracheal cells often display much higher resistances, 800–1000 Ω cm^2 (Foster et al., 2000; Hamilton et al., 2001; Mathias et al., 1996, 2002; Meaney et al., 2002).

Restriction of paracellular transport of large hydrophilic compounds, mannitol (MW 184), inulin (MW 5200) and dextran (MW 77,000) has been demonstrated in 16HBE14o− cell layers (Man et al., 2000). In this study, we have evaluated the permeability of the air–liquid interfaced 16HBE14o− cells to a series of compounds which have been tested in primary cell culture models of the respiratory epithelium (Mathias et al., 1996; Saha et al., 1994). The compounds were of similar molecular weight but with a broad range of lipid solubilities and a sigmoidal relationship was found between Papp and lipophilicity. Compounds with a log $P < 1.9$ exhibited permeabilities similar to the paracellular marker, mannitol. The permeability of the 16HBE14o− model to hydrophilic drugs was greater than that of the primary culture tracheal (Mathias et al., 1996) and alveolar model (Saha et al., 1994), which corresponds to the lower resistance of the 16HBE14o− model (Fig. 3). More lipophilic drugs (log $P > 2$) showed similar high transcellular permeabilities of $\sim 20 \times 10^{-6}$ cm s^{-1} across 16HBE14o−, tracheal and alveolar cell layers. A similar relationship between lipophilicity and drug transport has been observed in Caco-2 cells (Artursson, 1990) and the value of using organ-specific epithelial cells remains to be demonstrated. Interestingly, two of the solutes used in our study were used to characterise the liquid-covered 16HBE14o− cultures of Ehrhardt et al. (2002) with similar results (Papp $\times 10^{-6}$ cm s^{-1}: atenolol 1.5 and 3.9; propranolol 28.6 and 26.6, respectively). In contrast the permeabilities reported for air–liquid cultures by Ehrhardt et al. differed markedly.

In summary, we have reported culture conditions that result in suitable cell layers to evaluate passive absorption. Although it remains to be ascertained how culture conditions can effect the expression of any airway-specific transport mechanisms, established

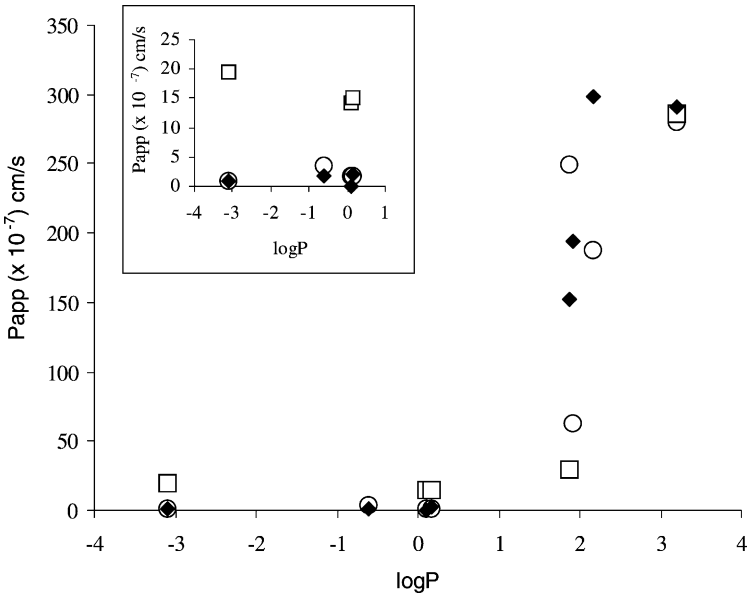

Fig. 3. Comparison of the permeability of cultured respiratory cells to solutes. Data include permeability of 16HBE14o− cells (□) compared to primary cultured rabbit tracheal cells (○; Mathias et al., 1996) and primary cultured rat alveolar cells (◆; Saha et al., 1994). Transported compounds were mannitol ($\log P = -3.1$), sotalol ($\log P = -0.62$), salbutamol ($\log P = 0.11$), atenolol ($\log P = 0.16$), metoprolol ($\log P = 1.88$), timolol ($\log P = 1.91$), betaxolol ($\log P = 2.17$), propanolol ($\log P = 3.21$).

advantages of this cell line over primary cell models are the human origin of the cells, physiological barrier properties and convenience of culture.

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