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Optimization of human nasal epithelium primary culture conditions for optimal proton oligopeptide and organic cation transporters expression *in vitro*

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ABSTRACT

Aim: To investigate the effect of key tissue culture conditions on cell growth, gene expression and functional uptake of peptide and organic cation transporter substrates in the human nasal epithelium (HNE).
Methods: HNE were cultured on different growth surfaces (polystyrene plastic, collagen film, and hydrated collagen gel) and were maintained with three popular nasal tissue culture media supplements [DMEM/F12 supplemented with Ultroser® G (2%), FBS (10%) and NuSerum® (10%)], respectively. The expression of gene transcripts for organic cation and peptide transporters were screened using qPCR and substrate uptake studies.
Results: Cell growth surface (polystyrene plastic surface, dried collagen film and hydrated collagen gel) did not significantly alter gene expression levels. However, Ultroser® G and FBS caused significant increase in PEPT1, PEPT2, PHT1, OCT3, and OCTN1 levels (≅2–5-fold for FBS and 2–8-fold for Ultroser® G). In terms of the degree to which the supplements affected gene expression, the following observations were made: effect on OCTN1 > PEPT2 > OCT3 > PHT1 > PEPT1. Functional uptake of organic cation (4-Di-1-ASP) and peptide [β-Ala-Lys (AMCA)] transporter substrates was significantly lower in cells cultured with NuSerum® compared to Ultroser® G and FBS cultured cells ($p > 0.05$).
Conclusions: Tissue culture media had a major effect on SLC gene expression levels of the human nasal epithelium in primary culture. Ultroser® G was identified as the most efficient culture supplement in maintaining SLC transporter expression under most culture conditions, whereas FBS appears to be an economical choice. We do not recommend the use of NuSerum® as a supplement for growing HNE for transport studies involving SLC transporters.

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1. Introduction

A few *in vitro* nasal models are currently employed for assessing solute transport mechanisms and novel strategies for enhancing nasal drug absorption. These experimental tools include excised nasal tissues, nasal homogenates, and primary cell culture models (Agu et al., 2009, 2011a, 2011b; Cho et al., 2011). The non-availability of suitable nasal cell lines implies that biopsy-based primary nasal cell cultures as well as excised tissue models are the major *in vitro* preclinical models for nasal drug absorption studies using viable cells (Lin et al., 2005). In 1995 Werner and Kissel compared Bovine turbinate cells, human nasal septum tumor cells, and RPMI 2650 with primary human nasal epithelium for *in vitro* nasal drug transport studies (Werner and Kissel, 1995, 1996). Based on better epithelial cell differentiation and consistency in developing measurable transepithelial electrical resistance, the primary culture was recommended as a model for drug delivery studies. In later publications cells grown according to the method described by Werner and Kissel (1996) and later by Agu et al. (2001) as well as Yoo et al. (2003) were used to grow cells for nasal absorption and metabolism studies.

Differentiation of epithelial cells in primary culture can be affected by media supplement, cell processing method, and type of cell growth surface. The level of differentiation can in-turn affect drug transporter expression and ultimately drug transport data generated with the cells. The optimal culture conditions for the growth and differentiation of human nasal epithelial cells remain a subject of contention as researchers have different opinions. Many disagree on the need for cell-adherent surfaces (e.g. collagen supports vs. plastics). Others disagree on whether serum or serum substitutes (e.g. Ultroser® G, NuSerum®) should be used. For instance whereas Wu et al. (1985) reported the inhibition of

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cell growth by fetal bovine serum (FBS) and cell growth supplements, Werner and Kissel reported that FBS was at least similar to the effect of growth supplements – if not even better (Werner and Kissel, 1995). Furthermore, while Lee et al. (2005) suggested that epithelial cells grow poorly on collagen film, and that collagen gel should be used for better differentiation; other researchers found no difference between collagen gel, film and even untreated plastic (Werner and Kissel, 1996; Wu et al., 1985). In our previous work, we discussed the morphological, biochemical, and functional effects of different cell growth substrata on human nasal cells and their suitability for drug delivery studies (Agu et al., 2001). Based on reproducibility and cell morphology, we suggested that nasal transport studies be conducted with special collagen membranes (Cellagen®). Although these membranes work perfectly with nasal epithelial cells, they are very expensive, making them unsuitable for routine studies. Furthermore, their molecular weight cut-off (4000 Da) is considerably lower that the cut-off of most therapeutic proteins making them unsuitable for transport studies with drugs of protein origin.

In order to routinely use the human nasal primary culture as a model for investigating transporter-facilitated drug transport across the nasal epithelium, it is important to optimize the conditions necessary for growing well differentiated cells with high transporter expression. SLC and other transporters affect the pharmacokinetics of drugs *in vivo*. It is therefore important to optimize the tissue culture conditions for cell culture models intended to be used to investigate the absorption of drugs that are substrates of various drug transporters. This way, the *in vivo* expression levels of the transporters may be simulated as much as possible under *in vitro* conditions. There are about 300 solute carrier (SLC) super-family membrane transporters. We chose five representative transporters that we have identified in the human nasal epithelium (PEPT1, PEPT2, PHT1, OCT3, OCTN1) to explore the effect of tissue culture variables on functional and molecular expression of organic cation and peptide transporters in human nasal epithelium.

2. Materials and methods

2.1. Chemicals

4-(4-(Dimethylamino)styryl)-*N*-methylpyridinium iodide (4-Di-1-ASP), Triton X-100®, bovine serum albumin (BSA), pronase, isopropanol, and Hanks' balanced salt (HBSS) were supplied by Sigma (St. Louis, MO, USA). DMEM-F12 1/1, Oligo dT primer, M-MVL reverse transcriptase, cDNA buffer, fetal bovine serum (FBS), Penicillin/Streptomycin and dNTPs were purchased from Invitrogen (Burlington, ON, Canada). SYBR green mix was from Qiagen (Mississuaga, ON, Canada). Ultroser® G, NuSerum® and Bicinchoninic acid (BCA) protein assay kit were purchased from Biosepra (St-Germain-en-Laye Cedex, France), BD Biosciences (Mississauga, ON, Canada) and Millipore (Billerica, MA, USA), respectively. Human bronchial/sub-bronchial gland cell line (Calu-3) was purchased from Cedarlane (Burlington, ON, Canada). β-Ala-Lys (AMCA) was custom-made by Biotrend GmbH (Cologne, Germany).

2.2. Cell culture

The cell culture method used for the study has been described in detail elsewhere (Agu et al., 2001). Human nasal epithelial tissues (without secondary ultra-structural abnormalities) were obtained during elective surgery. The use of human biopsies was approved by QEII Regional Hospital Research Ethics Board. The tissues were transported to the lab in DMEM-F12 1/1 culture medium supplemented with streptomycin 100 μg/ml and penicillin 100 IU/ml. During processing, the tissues were washed three times with physiological saline solution supplemented with antibiotics. The cells were dissociated enzymatically over a period of 16–24 h at 4 °C using 0.1% pronase. The pronase was deactivated with either 10% NuSerum® or FBS prior to cell washing with DMEM-F12 1/1. The washing solution was removed after centrifugation at 70 g for 5 min on each occasion. The resulting suspension of cells was filtered through a 70 μm pore size polycarbonate filter (Pall, Portsmouth, UK) and pre-plated on plastic for 1 h at 37 °C in a 95% O_2 and 5% CO_2 environment to reduce fibroblast contamination. Subsequently, the cells were counted and seeded at defined densities on wells coated with collagen gel, film and on uncoated plates. The cells were incubated at 37 °C in a 95% O_2 and 5% CO_2 environment using DMEM F12 supplemented with Ultroser® G (2%), FBS (10%) and NuSerum® (10%), respectively. The media were changed every other day. The cells were used for experiments upon confluency. For cells that involved passaging, the nasal cells that were grown on plastic surfaces were washed with PBS twice, and then trypsinized for 5 min for detachment. An appropriate amount of medium containing serum was added to dilute the trypsin. Cells were collected following centrifugation, re-suspended and aliquotted into new flasks. For the effect of seeding density on gene expression, cells were seeded at varying densities on plastic.

2.3. Preparation of collagen coating

Rat tail collagen (type I) solution was prepared from rat tendons according to the method described by LeBaron et al. (1989). Briefly, frozen (−20 °C) rat tails were thawed by soaking in 70% ethanol for 30 min. The tendon fibers were removed by breaking off 1-inch segments of the tail (working from the proximal end of the tail) with pliers, thus exposing the fibers in that segment. The fibers were cut off with a razor blade and were weighed and then soaked in 70% ethanol for 30 min for sterilization. Sterilized tendon fibers (4 g/l) were added to sterile 0.017 M acetic acid and stirred for 48 h at 4 °C. The collagen solution was centrifuged at 10,000 × *g* for 1 h at 4 °C. The supernatant fluid was collected into sterile media bottles and store at 4 °C. Two collagen coating surfaces were used for the studies. Hydrated rat tail collagen gel was prepared by placing 0.5 ml of the collagen suspension in culture dishes and exposing the uncovered dishes to 25% ammonia vapor. The ammonia vapor exposure was performed by placing the plates under an inverted baking dish or aluminum tray along with a small beaker containing 25% ammonium hydroxide. Gel formation usually occurred within 20 min. The solidified gel was washed with sterile water several times to remove ammonia residue. Dry rat tail collagen film was prepared by transferring 0.5 ml/well of collagen suspension to 6-well plates. The collagen suspension was air-dried at room temperature in biosafety hood overnight. The collagen films were rinsed three times with PBS before use.

2.4. Cell staining and imaging

The cells were visualized either directly or, after staining with diluted neutral red using a Hund Wetzlar inverted bright field light-microscope (Fisher Scientific Ottawa, ON, Canada). The staining was achieved using 20× diluted neutral red and incubated for 4 h followed by repeated PBS wash. Images were collected using a CCD digital camera attached to the microscope eyepiece.

2.5. Quantitative PCR

Cells grown on various growth surfaces (gel, film and plastic) using Ultroser® G (2%), NuSerum® (10%) and FBS (10%) were subjected to TriZol® extraction and cDNA synthesis. The cells were lysed in 1 ml TriZol® containing 200 μl of chloroform per 1 ml of TriZol® and vortexed. Three phases developed and were

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