EXPERIMENTAL CELL RESEARCH ▮ (▮▮▮▮) ▮▮▮–▮▮▮

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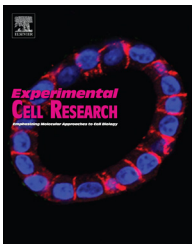

Research Article

Basement membrane influences intestinal epithelial cell growth and presents a barrier to the movement of macromolecules

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ARTICLE INFORMATION

Article Chronology:
Received 6 December 2013
Received in revised form
14 January 2014
Accepted 18 February 2014

Keywords:
Basement membrane
Cell adhesion
Drug delivery
Epithelial cells
Extracellular matrix
Laminin-5

ABSTRACT

This work examines the potential drug delivery barrier of the basement membrane (BM) by assessing the permeability of select macromolecules and nanoparticles. The study further extends to probing the effect of BM on intestinal epithelial cell attachment and monolayer characteristics, including cell morphology. Serum-free cultured Caco-2 cells were grown on BM-containing porous supports, which were obtained by prior culture of airway epithelial cells (Calu-3), shown to assemble and deposit a BM on the growth substrate, followed by decellularisation. Data overall show that the attachment capacity of Caco-2 cells, which is completely lost in serum-free culture, is fully restored when the cells are grown on BM-coated substrates, with cells forming intact monolayers with high electrical resistance and low permeability to macromolecules. Caco-2 cells cultured on BM-coated substrates displayed strikingly different morphological characteristics, suggestive of a higher level of differentiation and closer resemblance to the native intestinal epithelium. BM was found to notably hinder the diffusion of macromolecules and nanoparticles in a size dependent manner. This suggests that the specialised network of extracellular matrix proteins may have a significant impact on transmucosal delivery of certain therapeutics or drug delivery systems.

Introduction

Cell culture-based in vitro intestinal epithelial models are used in a wide variety of research disciplines ranging from study of oral drug delivery [1–3] and nutrient transport [4,5] to investigations into entry mechanisms of infectious agents [6,7] and intestinal disease processes [8,9]. Whilst these models offer advantages, including controlled conditions and reduced animal experiments, current epithelial models fail to accurately represent the native intestinal epithelium. Intestinal epithelial models are currently based on culture of a suitable cell type, predominantly Caco-2, directly on flat, porous supports. However, such culture conditions fail to accurately reflect the environment of the native intestinal epithelium, where epithelial cells are supported by the

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http://dx.doi.org/10.1016/j.yexcr.2014.02.022
0014-4827 © 2014 Published by Elsevier Inc.

basement membrane (BM) as a specialised form of extracellular matrix (ECM).

The intestinal BM is a thin network of ECM, composed of a number of proteins, particularly laminins, type IV collagen and fibronectin [10–12]. These proteins play an important role in biological processes of intestinal cell migration, proliferation, and differentiation [13,14]. The response and behaviour of intestinal epithelial cells following direct culture on flat, polymer-based supports may therefore not be reproduced due to the lack of the natural interaction between the cells with the natural microenvironment in the form of the BM. Consequently, the resulting in vitro model may fail to achieve appropriate characteristics, including cell morphology, expression of relevant proteins and barrier properties, which clearly presents reliability issues for the model. Employing more biologically relevant conditions for culture of intestinal epithelial cells is therefore desirable as the resulting model may express a more relevant phenotype and characteristics.

At the same time, the extent to which the BM contributes to the overall mucosal barrier in terms of mucosal absorption of material is unclear, in contrast to the drug delivery barrier of the components of the mucosa other than the BM, including mucus and the epithelial cell layer, which have been relatively well researched [15,16]. This is particularly important considering the increasing efforts to enable effective non-invasive, mucosal delivery of macromolecular biotherapeutics as BMs have been shown to hinder the diffusion of macromolecules as small as 5000 Da in the non-keratinized oral mucosal epithelium [17]. With an increasing proliferation of biotherapeutics and nanomedicines, coupled with the desire to achieve oral delivery in particular, there is a need to study the drug delivery barrier of the intestinal mucosal BM.

Here we set out to investigate the barrier property of the BM with respect to the movement of macromolecules and nanoparticles as increasingly relevant carriers of therapeutics [18–20]. In doing so, we employed a simple method to obtain BM coated supports on which we cultured Caco-2 cells. This modification resulted in a significant difference on cell attachment, morphology and barrier characteristics of these epithelial monolayers. In this paper we report both the characterisation of these effects on the intestinal epithelial Caco-2 cells and also on the barrier characteristics of both the BM and the epithelial monolayer.

Materials and methods

Materials

Calu-3 bronchial adenocarcinoma cells (used between passages 19–48) and Eagle's Minimal Essential Medium (EMEM) were obtained from the American Type Culture Collection (ATCC)-LGC Promochem (USA). Caco-2 colorectal adenocarcinoma cells (passages 44–58) were obtained from European Collection of Cell Cultures (ECACC). Ham's F-12 medium, Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced Salt Solution (HBSS, with sodium bicarbonate and without phenol red), non-essential amino acids, L-glutamine, antibiotic/antimycotic solution (10–12,000 U/ml penicillin, 10–12 mg/ml streptomycin, 25–30 μg/ml amphotericin B), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid solution (HEPES), non-animal recombinant trypsin solution (TrypZean®), soybean trypsin inhibitor, and fluorescein isothiocyanate (FITC)-labelled dextran of approximate molecular weight of 4 and 10 kDa (FD4 and FD10, respectively) were all supplied by Sigma-Aldrich (UK). Transwell® permeable inserts of 12 mm diameter and 0.4 μm pore size (referred to as 'porous supports' or simply, 'substrates') were purchased from Corning (USA). Mouse, anti-human laminin α3/laminin-5 (α3Aβ3γ2) antibody (monoclonal mouse IgG1), derived from an established in vitro hybridoma, was obtained from R&D Systems (USA). Mouse, anti-human zonula occludens-1 (ZO-1, tight junction protein) and Epilife® Calu-3 serum free (and chemically defined) medium were purchased from Life Technologies Corp. (UK). Goat, anti-mouse IgG-Atto 488 (secondary antibody) was obtained from Sigma-Aldrich (UK). FITC-labelled human serum albumin was supplied by Abcam (UK). Recombinant human insulin solution, human transferrin, sodium selenite, human serum albumin (HSA) were all purchased from Sigma-Aldrich (UK). Fluorescent, carboxylate-modified polystyrene nanoparticles of 20 nm (FluoSpheres®; 505 nm excitation, 515 nm emission) and 50 nm diameters (Fluoresbrite®; 441 nm excitation, 486 nm emission) were purchased from Life Technologies Corp. (UK) and PolySciences Inc. (Germany). Collagenase from *Clostridium histolyticum*, QuantiPro™ high sensitivity BCA (bicinchoninic acid assay) kit, IgG-FITC from human serum, human placenta type IV collagen, human plasma fibronectin and all other chemicals were supplied by Sigma-Aldrich (UK).

Cell culture

Calu-3 and Caco-2 cells were gradually transferred from serum-containing media to serum-free, chemically defined media by reducing the proportion of serum-containing medium and increasing that of the serum-free medium at each subculture point. Caco-2 cells were eventually transferred from 10% v/v DMEM to serum-free and chemically defined Ham's F-12, whilst Calu-3 cells were gradually converted from EMEM to serum free, chemically-defined Epilife® (with added supplement S7). Ham's F-12 was supplemented with 6.25 μg/ml human recombinant insulin, 6.25 μg/ml human transferrin, 1.25 mg/ml human recombinant albumin, 6.25 ng/ml selenium and 5.35 μg/ml linoleic acid, which is a slightly modified culture medium 'recipe' to that previously reported to allow serum-free culture of Caco-2 cells [21].

Unless otherwise stated, culture on permeable supports was conducted using serum-free media for both airway Calu-3 and intestinal Caco-2 cell lines. Both cell lines were seeded on permeable supports at 10^5 cells/cm^2. For Calu-3 cells, air-interface culture (AIC) conditions were created on day 2 post-seeding (culture medium removed from the apical side) and cells were thereafter cultured using AIC conditions. Culture medium was replaced every 2–3 days for both cultures.

Expression of laminin-5 by Calu-3 cells

Calu-3 cells were cultured on permeable supports as described above. Prior to the immunostaining procedure, the resulting cultures were tested for formation of confluent and polarised layers by measurement of transepithelial electrical resistance (TEER). Only cells displaying a TEER of $>500\ \Omega cm^2$ were included in the immunostaining studies. Culture medium was removed and

Please cite this article as: D. Vllasaliu, et al., Basement membrane influences intestinal epithelial cell growth and presents a barrier to the movement of macromolecules, Exp Cell Res (2014), http://dx.doi.org/10.1016/j.yexcr.2014.02.022