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Dissecting stromal-epithelial interactions in a 3D *in vitro* cellularized intestinal model for permeability studies

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ABSTRACT

Absorption evaluation plays an increasingly important role at the early stage of drug discovery due to its potential to scan the ADME (absorption, distribution, metabolism and excretion) properties of new drug candidates. Therefore, a new three-dimensional (3D) *in vitro* model replicating the intestinal functioning is herein proposed aiming to dissect the stromal-epithelial interactions and evaluate the permeation of a model drug, insulin. Inspired on the intestinal mucosal architecture, the present model comprises intestinal myofibroblasts (CCD18-Co cells) embedded in Matrigel, onto which epithelial enterocytes (Caco-2 cells) and mucus-producing cells (HT29-MTX cells) were seeded. CCD18-Co myofibroblasts showed to have a central role in the remodeling of the surrounding matrix confirmed by the production of fibronectin. Subsequently, this matrix revealed to be essential to the maintenance of the model architecture by supporting the overlying epithelial cells. In terms of functionality, this model allowed the efficient prediction of insulin permeability in which the presence of mucus, the less tight character between Caco-2 and HT29-MTX epithelial cells and the 3D assembly were critical factors. Concluding, this model constitutes a robust tool in the drug development field with potential to bridge the traditional 2D cell culture models and *in vivo* animal models.

1. Introduction

The growing interest in predicting the properties of incoming drugs in the market has increasingly valuing *in vitro* tools as they may encompass many characteristics of the tissue in question, and also benefit from standardized conditions and lack of ethical considerations [1]. Caco-2 cell model is the gold standard of intestinal *in vitro* permeability models [2], since after 21 days in culture, these cells acquire a differentiated and polarized phenotype that, morphologically and functionally, resembles the absorptive enterocytes. Besides many of features found in enterocytes are also characteristic of Caco-2 cells presence of microvilli, tight junctions (TJ"), the expression of enzymes (alkaline phosphatase, sucrase) and transporters (P-glycoprotein (P-gp)) [3,4], the value of Caco-2 model has been mitigated. In particular, the tighter TJ tremendously underestimate the paracellular transport [5] and the efflux transport is overestimated due to P-gp up-regulation [6]. Added to that, in order to overcome the lack of mucus layer that extensively influences absorption, the new cellular intestinal models include HT29-MTX cells that are responsible for mucus secretion [7–10].

However, the existing *in vitro* intestinal models are restricted to 'petri-dish'-based cell cultures that do not replicate the entire architecture and mechanisms occurring in a living tissue [11]. In turn, the striking similarity between the morphology and behavior of cells *in vivo* and in 3D culture conditions empower the importance

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of matrix dimensionality [12]. Moreover, intestinal absorption is not only regulated by the intestinal epithelium and epithelial–stromal interactions have been recognized as important players in the maintenance of intestinal mucosal architecture [13]. In particular, intestinal subepithelial myofibroblasts (ISEMFs) constitute a cellular network that sheaths the lamina propria of the intestinal villi. ISEMFs, defined by phenotypic characteristics of both fibroblasts and smooth muscle cells [14], were originally believed to be restricted to a two dimensional network along the villi. In fact, they are connected to α-smooth muscle actin negative fibroblasts-like cells and pericytes (mural cells that surround the capillaries) located in the lamina propria forming a 3D network [15].

The present study extends a traditional standard intestinal model to a deeper layer of the small intestine - the intestinal mucosa. To faithfully capture the *in vivo* scenario, the herein model focus on the first intestinal barrier to absorption - the epithelial layer – but simultaneously centered in the crosstalk with the 3D network of the myofibroblats in the lamina propria. To this end, CCD18-Co intestinal fibroblasts were embedded in Matrigel onto which lay Caco-2 and HT29-MTX cells. The aim of this work was to characterize the model architecture and functioning in view to establish a tool feasible to study the permeability of novel drugs. For this, an extensive characterization of the fibroblasts behavior within the Matrigel matrix was performed, following the characterization of the epithelial layer. Permeability studies were further carried out to confirm the efficiency of the model in predict the permeability of a model drug.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), DMEM without phenol red, non-essential aminoacids (NEAA), 0.05% tripsin-EDTA, 0.4% trypan blue and HBSS (Hank's Balanced Salt Solution) were purchased from Gibco. Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from BioWest. Resazurin, human insulin, DAPI (4′,6-diamidino-2-phenylindole), fluoroshield™ mounting medium, hexamethyldisilazane (HMDS), triton X-100 and 4 kDa fluorescein isothiocyanate-dextran molecule were purchased from Sigma. 6-well Transwell permeable support (PET membrane and 3 μm pore size) and Matrigel® basement membrane matrix were purchased from BD Biosciences. ClearRite 3 and paraffin were purchased from Thermo Scientific. Sodium-cacodylate was purchased from Fluka. Paraformaldehyde (PFA) was purchased from Merck. Glutaraldehyde was purchased from AGAR Scientific. Mouse anti-human vimentin antibody was purchased from Santa Cruz Biotecnology. Donkey anti-mouse Alexa-Fluor 488®, goat anti-mouse Alexa-Fluor 594®, goat anti-rabbit Alexa-Fluor 594®, goat anti-rabbit Alexa-Fluor 488® secondary antibodies and quant-iT PicoGreen dsDNA kit were purchased from Molecular Probes®. Rabbit anti-fibronectin polyclonal antibody was purchased from Sigma.

2.2. Cell lines and cell culture conditions

Caco-2, Raji B and CCD18-Co cell lines were purchased from American type culture collection (ATCC, USA). HT29-MTX cell line was kindly provided by Dr. T. Lesuffleur (INSERM U178, Villejuif, France). CCD18-Co (passage 8–13), Caco-2 (passage 22–40) and HT29-MTX (passage 25–40) cells were grown separately in tissue culture flasks (Orange Scientific), in DMEM basal medium supplemented with 10% (v/v) of inactivated FBS, 1% (v/v) of NEAA and 1% of antibiotic/antimitotic mixture (final concentration of 100 U/mL Penicillin and 100 U/mL of Streptomycin). Cells were maintained in an incubator (CellCulture® CO_2 incubator, ESCO) at 37 °C and 5% CO_2 in water saturated atmosphere. All the cell culture related procedures were done in a Labculture® class II, type A2 biological safety cabinet, from ESCO.

2.3. 2D in vitro models

Matrigel/CCD18-Co monoculture was prepared by seeding CCD18-Co cells (1×10^4 cells/cm^2) onto a thin layer (200 μL/6-well insert) of Matrigel (1:3 (v/v) in serum-free DMEM) polymerized during 30 min at 37 °C. Matrigel/Caco-2 was established as aforementioned by seeding Caco-2 cells (1×10^5 cells/cm^2).

To embedded CCD18-Co cells in Matrigel, CCD18-CO cells were suspended in a thick layer (400 μL/6-well insert) of Matrigel (1:3 (v/v) in serum-free DMEM) and evenly spread on the Transwell. After polymerization (30 min at 37 °C) fresh DMEM was gently added to both sides of the insert.

The stromal-epithelial co-culture of CCD18-Co and Caco-2 cells was prepared by seeding CCD18-Co at a density of 1×10^4 cells/cm^2 onto. On the following day, Caco-2 cells (1×10^5 cells/cm^2) were seeded on top of CCD18-Co cells.

Epithelial co-culture of Caco-2 and HT29-MTX cells was prepared by seeding Caco-2 and HT29-MTX cells to a final density of 1×10^5 cells/cm^2 (90:10 proportion), as previously optimized [16,17].

All the models were established on the Transwell permeable support (3 μm) and maintained for 21 days. A density of 1×10^4 cells/cm^2 was selected based on additional experiments. To monitor models' integrity, transepithelial electrical resistance (TEER) was measured (volt-ohm meter Millicell® ERS-2 (Millipore, USA)). The values were normalized by subtracting the resistance value of the empty insert and expressed in ohm per area of the insert (Ω.cm^2).

2.4. 3D in vitro models

In the attempt of study the influence of CCD18-Co cells assembly on the barrier formation were established two different configurations: (1) CCD18-Co cells embedded in thick layer of Matrigel with Caco-2 cells seeded on top and (2) CCD18-Co and Caco-2 cells separated by a thin layer of Matrigel.

In the first configuration, CCD18-Co cells (1×10^4 cells/cm^2) were entrapped in a thick layer of Matrigel as previously described. After 24 h, Caco-2 cells (1×10^5 cells/cm^2) were added over CCD18-Co cells embedded in Matrigel. In the second configuration, CCD18-Co cells (1×10^4 cells/cm^2) were seeded on the Transwell membrane and DMEM was gently added to both sides of the Transwell. After 24 h, the culture medium was removed and a thin layer (200 μL) of Matrigel (1:3 (v/v)) was added on top of the CCD18-Co cells. After 30 min of polymerization, Caco-2 cells were carefully seeded over the Matrigel layer.

The first configuration was the one adopted to establish the triple-culture (CCD18-Co + Matrigel/Caco-2/HT29-MTX) 3D model, which further included HT29-MTX cells in a proportion of 90:10.

Cultures were maintained during 21 days, periodically measuring TEER values, and the density of 1×10^4 cells/cm^2 of CCD18-Co cells was based on additional experiments.

2.5. Metabolic activity and proliferation of CCD18-Co cells in 2D vs. 3D conditions

Metabolic activity of CCD18-Co cells in 2D and 3D was determined using the resazurin assay. Resazurin pre-warmed to 37 °C was added to the plates in a 10% concentration of the initial volume in the plate and cells in culture for 7 days were incubated at 37 °C. After incubation, a sample was collected and immediately quantified by measuring the relative fluorescence units (RFU) using a microplate reader Synergy™ Mx HM550 (BioTek® Instruments, USA) set at 530/590 nm (excitation/emission wavelength, respectively). The results were normalized by subtracting the negative control (without cells). The assay was repeated for 14 and 21-days cultures.

For the total DNA quantification, cells were recovered by centrifugation (10 000 rpm, 5 min), washed with PBS, centrifuged again and the pellet was stored at −20 ºC until quantification. Cells were lysed with 1% (v/v) triton X-100 during 1 h under agitation at 4 ºC. Since 1% (v/v) Triton X-100 interferes with the measurement, samples were diluted to a final concentration of 0.4% (v/v) Triton X-100 with PBS and the double-stranded DNA (dsDNA) was quantified using the Quant-iT PicoGreen dsDNA kit, according to manufacturer's instructions. Briefly, 10 μL of each sample was transferred to a black 96-well plate with clear bottom (Falcon) and diluted with 90 μL of TE (1x) buffer (200 mM Tris–HCl, 20 mM EDTA, pH 7.5). To each well was added 100 μL of PicoGreen dsDNA reagent and incubated for 5 min at RT, protected from light. Samples were quantified using a microplate reader Synergy™ Mx HM550 (BioTek® Instruments, USA) set at 480/530 nm (excitation/emission wavelength, respectively). The dsDNA concentration (ng/mL) was determined using a standard curve of lambda DNA in a range of 0.0250–2.50 ng/mL.

2.6. Production of ECM components by CCD18-Co cells

The expression of ECM components, namely fibronectin, and the morphology of CCD18-Co cells in 2D vs. 3D conditions was assessed. Samples were fixed with 1% (v/v) glutaraldehyde in 0.1 M sodium cacodylate for 15 min at room temperature (RT), following permeabilization with freshly prepared solution of 0.2% (v/v) Triton X-100 in PBS for 9 min at RT. The blocking step was performed with 1% BSA in PBS during 30 min at RT. Cells were incubated during 2 h at RT with both mouse anti-human vimentin primary antibody (1:100) and rabbit anti-human fibronectin primary antibody (1:400) following incubation with goat anti-mouse Alexa-Fluor 594 and goat anti-rabbit Alexa-Fluor 488 secondary antibodies (1:1000) for 1 h at RT and protected from light. Cell nucleus was counterstained with DAPI (1:1000). With exception of the blocking step, 5 min washes with PBS were performed between fixation, permeabilization and incubation steps.

2.7. Fibroblast-epithelial interactions in CCD18-Co/Caco-2 co-cultures

Monocultures of Caco-2 and CCD18-Co cells and co-cultures of CCD18-Co and Caco-2 were established to study the interaction between fibroblasts and epithelial cells. After 7 days, cells were washed twice with PBS and detached from substrates with 0.05% trypsin-EDTA. Cell suspensions were mixed with 0.4% (v/v) trypan blue