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Research Article

Effect of differentiation on endocytic profiles of endothelial and epithelial cell culture models

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ABSTRACT

Understanding mechanisms of endocytosis and trafficking of nanoparticles through endothelial and epithelial barriers leads potentially to improved efficacy of nanoparticulate drug delivery systems. Detailed characterizations of cell models with respect to endocytic pathway expression and activity (endocytic profiling) should facilitate data interpretation. We performed endocytic profiling of CaCo-2 and hCMEC/D3 cell lines, widely used as human intestinal and blood–brain barrier permeability models, respectively, during cell differentiation. Furthermore, we compared endocytic profiles of cell lines with those of primary cells. Expression of genes involved in specific endocytic pathways was analyzed at mRNA levels by quantitative real time PCR. Where possible, the respective protein levels were analyzed by Western blotting, and endocytic activities of cells were analyzed by flow cytometry. We showed that differentiated CaCo-2 cells formed tight, well polarized monolayers with reduced endocytic activity accompanied by reduced mRNA expression of most of the endocytosis-related genes. In contrast, hCMEC/D3 cells formed a leaky, less polarized barrier, and *in vitro* differentiation had little effect on either the expression of endocytosis-related genes or endocytic activity of these cells. Endocytic profiling of *in vitro* models and comparison with primary cells is an important measure to avoid misleading conclusions in nanoparticle permeation studies.

Abbreviations: NPs, nanoparticles; CME, clathrin-mediated endocytosis; BBB, blood–brain barrier; FBS, fetal bovine serum; TEER, transepithelial electrical resistance; P_{app}, apparent permeability coefficient; ALP, alkaline phosphatase; LacCer, lactosylceramide; PBS, phosphate buffered saline; BSA, bovine serum albumin; ICC, immunocytochemistry; CHC, clathrin heavy chain; qRT-PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CLIC, clathrin-independent carrier; GEEC, GPI-anchored protein enriched early endosomal compartment; CavME, caveolin-mediated endocytosis; FME, flotillin-mediated endocytosis

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Introduction

Nanoparticles (NPs) are prospective tools for the delivery of drugs and genes due to their potential to improve the pharmacokinetic properties of therapeutic molecules [1,2]. Endocytosis is known to be a main pathway for NPs to enter cells. Specific endocytic pathways define the intracellular routing of NPs, as well as defining transcytosis to the other side of a polarized cell [3,4]. Endocytic pathways are typically classified as phagocytosis or pinocytosis. Phagocytosis pertains to the uptake of large particles and is restricted primarily to specialized mammalian cells. Pinocytosis is used for the uptake of fluids and small particles and occurs in all cell types. Pinocytosis can be further subdivided into macropinocytosis, clathrin-mediated endocytosis (CME) and diverse clathrin-independent pathways, the majority of which have recently been discovered and, therefore, are relatively poorly characterized [5]. The studies exploring the mechanisms of NP uptake and transport have been performed typically *in vitro* and utilizing non-polarized cells [3]. For all systemic administration routes, as opposed to local administration to the target cells, successful NP-mediated delivery of a drug requires crossing epithelial/endothelial barriers, composed of either single or multiple layers of polarized cells [6,7]. Therefore, polarized cell models, including intestinal and blood–brain barrier (BBB) permeability models, have attracted more attention over the last few years [8–12]. However, in most of cases it is unknown whether the *in vitro* models utilized are representative of the endocytic pathways *in vivo*. Moreover, endocytic mechanisms are often simplified by assuming only a few of the pathways are active in the studied cell type. The characterization of cell culture models in terms of expression and activity of individual endocytic pathways (endocytic profiling) should facilitate the interpretation of results obtained in NP permeation studies.

Late maturational lineage stages of epithelial and endothelial cells *in vivo* are highly differentiated. Cell differentiation induces a reduction of endocytic activity, at least in some cell types. For example, the reduced endocytosis of cell penetrating peptides and fluid-phase uptake markers has been shown in differentiated MDCKII epithelial cells [13]. The slow-down of NP uptake in differentiated airway epithelial cells [14] and colorectal adenocarcinoma cells [15,16] has been demonstrated. However, the impact of cell differentiation on the activity of individual endocytic pathways has not been specifically and systematically addressed.

In the current work, we studied the effect of differentiation on the tightness and endocytic profiles of two most relevant human intestinal and BBB *in vitro* cell models. CaCo-2 cell line is the most common intestinal model of human origin. Among only a few human *in vitro* BBB models reported in the literature [17], hCMEC/D3 model is the best characterized. Both models have been widely used in nanoparticle uptake and permeability studies. The endocytic profiling performed for each cell line included (1) characterization of the tightness of the cell layer during *in vitro* cell differentiation; (2) evaluation of expression of endocytosis-related genes at mRNA and protein levels during differentiation in comparison with primary cells; and (3) determination of the activity of the endocytic pathways by using either specific markers of endocytosis or model polystyrene NPs.

Materials and methods

Cell culture and differentiation

The human brain endothelial cell line (hCMEC/D3) was kindly provided by Dr. Couraud (Institute of Cohin, Paris, France). The cells were maintained in an endothelial basal medium, EBM-2 (Lonza), supplemented with 5% fetal bovine serum (FBS) (PAA The Cell Culture Company), 1% penicillin–streptomycin (Invitrogen, Gibco), 1.4 μM hydrocortisone (Sigma), 5 μg/ml ascorbic acid (Sigma), 1% chemically defined lipid concentrate (Invitrogen, Gibco), 10 mM HEPES (Invitrogen, Gibco) and 1 ng/ml human basic fibroblastic growth factor. For differentiation the cells were seeded to Rat Collagen I (R&D Systems, Trevigen) thin-coated (150 μg/ml, 2 h at +37 °C) polyester transwell permeable supports (Costar) at a cell density of 50,000 cells/cm^2 and maintained in culture for 1, 3, 7 and 10 days. The culture medium was changed every third day. The cells at passage 30–35 were used in this study.

The human colorectal adenocarcinoma cell line (CaCo-2) was purchased from ATCC and maintained in an ATCC-formulated Eagle's Minimum Essential medium supplemented with 20% FBS (Invitrogen, Gibco) and 1% penicillin–streptomycin (Invitrogen, Gibco). For differentiation the cells were seeded to polycarbonate transwell (Costar) permeable supports thin-coated with Rat Collagen I (150 μg/ml, 2 h at +37 °C; R&D Systems, Trevigen) at a cell density of 60,000 cells/cm^2 and maintained in culture for 1, 3, 7, 14 and 21 days. The culture medium was changed every third day. The cells at passage 30–40 were used in this study.

Evaluation of the tightness/differentiation of the cells

The tightness of the endothelial and epithelial layers was determined by measuring the transepithelial electrical resistance (TEER) and their permeability to Lucifer Yellow. The TEER value was measured by using an Endohm cup (ENDOHM-24SNAP, World precision instruments) with an epithelial voltohmmeter (EVOM, World precision instruments). A filter without cells was used as a background control. To measure the Lucifer Yellow permeability, the culture medium in the inserts was replaced by a 50 mM Lucifer Yellow solution (Sigma) in a transport buffer, composed of 10 mM of HEPES and 1 mM of sodium pyruvate in 1XHBSS (all from Invitrogen, Gibco). The inserts were transferred at 10, 25 and 45 min to a new well containing the transport buffer. The receiver solutions from the inserts were collected in each time point and the luminal solutions were collected at the end of the experiment. The Lucifer Yellow fluorescence in each sample was determined by a plate reader (Varioskan Flash 2.4.3, Thermo Scientific; Ex/Em 425/535 nm). A calibration curve was generated, and the concentration of Lucifer Yellow was calculated. Apparent permeability coefficients (P_{app}) were calculated by using the following formula: $P_{app} = dM/dt \times 1/(A \times C_0)$, where dM/dt is the efflux rate or the slope of the cumulative amount transported *vs.* time plot, C_0 is the initial concentration in the donor compartment, and A is the surface area of the monolayer (=4.67 cm^2).

The activity of alkaline phosphatase (ALP) known to be a marker for differentiation was evaluated. The cells for each timepoint were lysed with a 1X reporter lysis buffer (Promega). A 5 μl sample was transferred as a duplicate to a 96-well plate,

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