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Calibrated flux measurements reveal a nanostructure-stimulated transcytotic pathway

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ABSTRACT

Transport of therapeutic agents across epithelial barriers is an important element in drug delivery. Transepithelial flux is widely used as a measure of transit across an epithelium, however it is most typically employed as a relative as opposed to absolute measure of molecular movement. Here, we have used the calcium switch approach to measure the maximum rate of paracellular flux through unencumbered intercellular junctions as a method to calibrate the flux rates for a series of tracers ranging in 0.6–900 kDa in size across barriers composed of human colon epithelial (Caco-2) cells. We then examined the effects of nanostructured films (NSFs) on transepithelial transport. Two different NSF patterns were used, Defined Nanostructure (DN) 2 imprinted on polypropylene (PP) and DN3 imprinted on polyether ether ketone (PEEK). NSFs made direct contact with cells and decreased their barrier function, as measured by transepithelial resistance (TER), however cell viability was not affected. When NSF-induced transepithelial transport of Fab fragment (55 kDa) and IgG (160 kDa) was measured, it was unexpectedly found to be significantly greater than the maximum paracellular rate as predicted using cells cultured in low calcium. These data suggested that NSFs stimulate an active transport pathway, most likely transcytosis, in addition to increasing paracellular flux. Transport of IgG via transcytosis was confirmed by immunofluorescence confocal microscopy, since NSFs induced a significant level of IgG endocytosis by Caco-2 cells. Thus, NSF-induced IgG flux was attributable to both transcytosis and the paracellular route. These data provide the first demonstration that transcytosis can be stimulated by NSFs and that this was concurrent with increased paracellular permeability. Moreover, NSFs with distinct architecture paired with specific substrates have the potential to provide an effective means to regulate transepithelial transport in order to optimize drug delivery.

1. Introduction

Epithelia are composed of polarized cells that enable organs to compartmentalize. Although each epithelium is specialized, they all serve the common function of forming barriers that share common regulatory mechanisms and molecular constituents. There are two major pathways that extracellular material can use to move across an epithelial monolayer: the paracellular route which is the movement through intercellular junctions and the transcellular route which is the movement through cells [1].

The paracellular pathway involves passive diffusion between cells that is regulated by structures known as tight junctions. Tight junctions link adjacent cells and occlude the paracellular space, thus presenting a significant obstacle to delivery of macromolecules. Tight junctions are composed of transmembrane proteins (claudins, occludin) linked to the actin cytoskeleton through scaffold proteins (including zonula occludens (ZO)-1, ZO-2, and ZO-3) [2–4]. Although tight junctions can act as ion and water channels [5,6], the paracellular permeability of larger molecules requires tight junctions to be actively opened and remodeled [7].

By contrast, the transcellular route is a barrier selective pathway that, for ions and small molecules, is mediated by specific transporters

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or channels located on the apical or basolateral plasma membrane [8,9]. In the case of macromolecules, transcellular transport occurs through a process known as transcytosis that represents the coordinated internalization of ligands by receptor-mediated endocytosis followed by transit through the cell and eventual delivery to the basolateral membrane via exocytosis [10,11].

Techniques to augment the transepithelial passage of drugs would be of great utility and are an active area of research [10,12–17]. Measuring the flux rates of fluorescent or radiolabeled tracers is a commonly employed method to assess the ability of molecules to traverse across epithelial barriers. As generally applied, flux rates provide a relative measure of how different treatments can affect transepithelial transport [1]. Here we have extended the utility of this approach by using the calcium switch method, where cells are cultured in medium containing low calcium and normal magnesium, as a means to fully disassemble epithelial junctions while maintaining cell attachment [18,19]. Measuring transepithelial flux in calcium-depleted cells reflects the maximum level of paracellular flux unencumbered by intercellular junctions that was then used to calibrate the relative enhancement of transepithelial transport induced by other treatments.

In this study, we examined two specific nanopatterns, Defined Nanostructure 2 (DN2) and Defined Nanostructure 3 (DN3), that were nanoimprinted on either polypropylene (PP) or polyether ether ketone (PEEK) generated from molds using electron-beam lithography [16,17]. We previously discovered that nanostructured surfaces have the capacity to enhance the delivery of macromolecules across epithelial monolayers by an integrin-dependent pathway that activates myosin light chain kinase to increase tight junction permeability [16,17]. Here the effect of NSFs on transport of substrates ranging from less than 1 kDa to 900 kDa across Caco-2 human intestinal epithelial cells in vitro was assessed and compared to the maximum flux rate for these same substrates through fully disassembled tight junctions in calcium-depleted cells. We unexpectedly found that NSFs specifically stimulated the transport of fluorescently tagged Fab and IgG at a significantly higher rate of flux than would have been predicted based on measurements obtained with the calcium switch method, suggesting that NSFs induced an active transepithelial transport pathway. Using confocal immunofluorescence microscopy, we found that in addition to increasing paracellular flux, NSFs also stimulated transcellular transport of IgG via transcytosis. To our knowledge, this represents the first demonstration of transcytosis being stimulated through contact with an nanostructured surface. Induced transcytosis has ramifications for the use of NSFs in drug delivery devices designed to enable macromolecules to cross epithelial barriers.

2. Materials and methods

2.1. Nanostructured thin film (NSF) fabrication

Molds for NSFs were fabricated using electron beam lithography followed by anisotropic reactive ion etching to generate small features on the nanometer length scale. Next, nanoimprint lithography was employed to imprint the nanofeatures from the nanofeatured mold onto unstructured thin films (UFs) composed of either polypropylene (PP) or polyether ether ketone (PEEK) through a stamping process [17].

2.2. Cell culture

Caco-2 cells were seeded at an initial density of 200,000 cells/cm^2 in the upper chamber of a 6.5 mm insert Transwell (Costar; Corning Incorporated, Kennebunk, ME.) in 0.25 ml of MEM (Minimum Essential Medium Eagle with Earle's salts and L-glutamine, Corning Cellgro #10-010-CV) containing 20% Fetal Bovine Serum (FBS; Premium Select, Atlanta Biologicals), 100 mM Sodium Pyruvate (ThermoFisher), Pen/Strep (Hyclone), Amphotericin B (Sigma) and Gentamicin (Sigma). The bottom well contained 0.5 ml MEM. The cells were incubated in a CO_2 incubator at 37 °C for 5 days, with a change of medium every second day until they formed a high transepithelial resistance (TER) monolayer of 500 Ωxcm^2 or higher. TER was measured using a voltohmmeter (World Precision Instruments) where the measured resistance in Ohms was multiplied by the area of the Transwell filter (0.33 cm^2).

2.3. Assessment of cell viability

Cell viability was measured using the Live/Dead viability/cytotoxicity kit (ThermoFisher, #L3224) [20]. Cells were washed twice with HEPES-buffered Ringer's solution (140 mM NaCl, 2 mM $CaCl_2{\cdot}2H_2O$, 1 mM $MgCl_2{\cdot}6H_2O$, 10 mM glucose and 10 mM NaHEPES, pH 7.3). Each well was then incubated with 100 μL of a solution containing 2 μM calcein-AM and 4 μM ethidium homodimer, in Ringer's, for 45 min at RT. The cells were then mounted on slides and immediately analyzed by fluorescence microscopy using an Olympus IX70 microscope with a U-MWIBA filter pack (BP460–490, DM505, BA515–550) or U-MNG filter pack (BP530–550, DM570, BA590–800). Alternatively, we used the MTT assay to measure cell viability. A 12 mM stock solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen) was prepared by adding 1 ml of sterile PBS to one 5 mg vial of MTT. Caco-2 cells were seeded as described above were washed twice with phenol free media and then further incubated for 2 h as controls or in contact with either unstructured PEEK or DN3. The cells where then harvested and placed in a 96-well plate followed by the addition of 10 μL of 12 mM MTT stock to each well and incubated at 37 °C for 4 h. Next, 50 μL of DMSO was added to each well and mixed thoroughly with a pipette then incubated for 10 min at 37 °C. Sample absorbance was read at 540 nm.

2.4. Dye flux permeability assay

Probes for dye flux included: calcein (ThermoFisher # C481; 2 μg/ml); Texas Red-labeled 10 kDa dextran (ThermoFisher # D1863; 10 μg/ml); Alexa Fluor 488-labeled Donkey IgG (Jackson ImmunoResearch #711-545-152; 8.3 μg/ml); Alexa Fluor 594-labeled Donkey Fab fragment(Jackson ImmunoResearch #711-587-003; 75 μg/ml); Cy3-labeled Human IgG (Jackson ImmunoResearch #009-160-003; 8.3 μg/ml); Cy2-labeled Human serum IgA (Jackson ImmunoResearch #009-220-011; 29.4 μg/ml); and Alexa Fluor 488-labeled Human IgM (Jackson ImmunoResearch #009-540-012; 29.4 μg/ml).

Caco-2 cells were equilibrated with Ringer's solution for 30 min at 37 °C and then the upper buffer was replaced with Ringer's containing the probe of interest. In some cases, probes with compatible fluorophores were co-incubated to simultaneously measure flux of two different markers. Cells were then unstimulated (control) or stimulated and placed in contact with either an UF (PP, PEEK) or a NSF (DN2, DN3) as previously described [17]. Over a two hour time-course, aliquots were withdrawn from the lower chamber every 30 min and measured using a multichannel plate fluorimeter (BioTek-Synergy H Microplate Reader, Winooski, VT) set to the appropriate excitation and emission wavelengths. Absolute amounts of flux were determined using a standard curve, and plotted as the amount of material appearing in the lower chamber of the Transwell vs. time (e.g. Fig. 4). The apparent rate of diffusion was calculated using the slope of the flux curve [16,17].

2.5. Scanning electron microscopy and atomic force microscopy

DN2 and DN3 NSFs were directly imaged using a Phenom scanning electron microscope (SEM) and Bruker Scan Assist atomic force microscope (AFM). In addition, DN2 and DN3 NSFs in contact with Caco-2 cells on Transwells for 2 h were removed and immediately analyzed by SEM.

2.6. Calcium depletion

To deplete extracellular Ca^{2+}, confluent Caco-2 monolayers were