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Influence of micro-well biomimetic topography on intestinal epithelial Caco-2 cell phenotype

Lin Wang [a], Shashi K. Murthy [a], William H. Fowle [b], Gilda A. Barabino [c], Rebecca L. Carrier [a,*]

[a] Chemical Engineering Department, Northeastern University, 360 Huntington Ave., 342 Snell Engineering, Boston, MA, 02115, USA
[b] Biology Department, Northeastern University, Boston, MA, 02115, USA
[c] Biomedical Engineering Department, Georgia Institute of Technology, Atlanta, GA, 30332, USA

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ABSTRACT

A microfabrication approach was utilized to create topographic analogs of intestinal crypts on a polymer substrate. It was hypothesized that biomimetic crypt-like micro-architecture may induce changes in small intestinal cell (i.e. Caco-2 cell) phenotype. A test pattern of micro-well features with similar dimensions (50, 100, and 500 μm diameter, 50 μm spacing, 120 μm in depth) to the crypt structures found in native basal lamina was produced in the surface of a poly(dimethylsiloxane) (PDMS) substrate. PDMS surfaces were coated with fibronectin, seeded with intestinal-epithelial-cell-like Caco-2 cells, and cultured up to fourteen days. The cells were able to crawl along the steep side walls and migrated from the bottom to the top of the well structures, completely covering the surface by 4–5 days in culture. The topography of the PDMS substrates influenced cell spreading after seeding; cells spread faster and in a more uniform fashion on flat surfaces than on those with micro-well structures, where cell protrusions extending to micro-well side walls was evident. Substrate topography also affected cell metabolic activity and differentiation; cells had higher mitochondrial activity but lower alkaline phosphatase activity at early time points in culture (2–3 days post-seeding) when seeded on micro-well patterned PDMS substrates compared to flat substrates. These results emphasize the importance of topographical design properties of a scaffolds used for tissue engineered intestine.

1. Introduction

The inner surface of the intestine contains a monolayer of enterocytes resting on a basement membrane; this epithelial layer has a convoluted topography consisting of finger-like projections (villi) with deep well-like invaginations (crypts) between the villi. The dimensions of crypts and villi are on the order of hundreds of microns (100–700 μm in height, and 50–250 μm in diameter) [1,2]. The migration, proliferation, differentiation, and function of small intestinal epithelial cells vary with position relative to these structures. The differentiation of enterocytes into absorptive cells takes place as they migrate upward from the crypt to the villus [3], suggesting that the crypt-villus micro-environment may play an important role in cellular differentiation in the intestine. Thus far, the function of the crypt-villus micro-environment in regulating intestinal cell proliferation and differentiation has been poorly understood. The unique geometry of the crypt-villus micro-environment may play a role in controlling intestinal cell function. Specifically, the crypt-villus topography may lead to different mechanical stress distributions within the epithelial cell layer covering the extracellular matrix (ECM). Topography also may affect the distribution of chemical factors, such as cell growth hormones and cell signaling molecules along the crypt-villus axis on the apical side of the intestinal epithelium. The tight spacing within crypts and between villi likely creates an environment of diffusion-limited transport with associated concentration gradients of chemical factors, and proximity of apical cell surfaces may facilitate cell–cell signaling. Therefore, we hypothesized that crypt micro-architecture (micro-wells) may induce changes in intestinal epithelial cell phenotype *in vitro*.

It is generally accepted that cultured mammalian cells respond to topographical cues, and their response depends on cell type, feature size, shape, and physical and chemical properties of substrate material [4–6]. A number of researchers have found that cells (e.g. fibroblasts, macrophages, mesenchymal tissue cells, epithelial cells) will align with multiple grooves on a substrate [7–11]. The degree of cell alignment depends on the cell type, the surface material, groove density, groove width, and groove depth. In some cases, response to grooves has been quantified, for example, it was found that cells responded to grooved surface topography as an automatic controller, with cell migration on a microgroove surface

* Corresponding author. Tel.: +1 617 373 7126; fax: +1 617 373 2209.
E-mail address: r.carrier@neu.edu (R.L. Carrier).

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directly proportional to the square of the product of groove height and spatial frequency, when groove height was in the range of 25–200 nm and spatial frequency was between 100 and 500 mm^{-1} [10]. Pillar and well/pit structures have been much less studied compared to groove/ridge structures [4,6,12]. Green et al. [13] found that fibroblasts cultured on silicone substrates patterned with micro-pillar and micro-well arrays (2 and 5 μm in diameter 0.5 μm in height) showed an increased rate of proliferation and cell density. Chehroudi et al. [14] studied bonelike tissue formation on subcutaneous titanium-coated epoxy implants patterned with either groove or pit structures ranging from 30 to 120 μm in depth. It was reported that both pit and groove patterns promoted bonelike tissue formation, however, pitted surfaces enhanced bone formation as their depth increased, while bone formation on grooved surfaces followed the opposite trend, suggesting shape of surface pattern might significantly alter cell behavior.

Relative to nano-scale or single-cell-scale topography, there have been few attempts to study the effect of topography on the scale of functional subunits of tissue, around 100 μm in size, on cell behavior. As noted above, the intestine has particularly interesting topography on this length scale. Reports on subunits scale topography are limited to groove/ridge structures, and tissues such as bone and skin, but not intestinal tissues [4,14,15]. The present study addresses the influence of micron-scale (~100 μm) crypt-like micro-well topography on intestinal cell behavior to determine whether the unique topography of intestinal crypt-villus unit plays an important role in regulating intestinal cells. Caco-2, a human colon carcinoma cell line which exhibits features similar to human intestinal epithelium, was cultured on PDMS substrates with biomimetic topography (micro-well arrays). It was found that intestinal crypt-like topography affect Caco-2 cell spreading, migration, metabolic activity, as well as cell differentiation. The broad significance of this work lies in understanding critical design elements for biomimetic scaffolds that can ultimately be used in intestinal tissue engineering.

2. Materials and methods

2.1. Fabrication of micro-patterned poly(dimethylsiloxane) (PDMS) substrates

To fabricate a patterned PDMS replica, a master SU-8 mold was created via photolithography. Briefly, 3 square test regions, each containing an array of circles corresponding to the designed diameters, were drawn using AutoCAD software and printed with high resolution on a transparency. The transparency was utilized as a photomask to create an SU-8 master mold with micro-pillar features using contact print UV lithography at G.J. Kostas Micro and Nanofabrication Facility at Northeastern University [16]. The height of the columns on the SU-8 molds was measured by a DEKTAK 3ST profilometer. A patterned PDMS replica with micro-well features was fabricated by pouring liquid PDMS (Sylgard 184®, Dow Corning) pre-polymer (10:1 base to curing agent weight ratio) onto the SU-8 master mold. After degassing for 30 min and curing at 65 °C for 2 h, the solidified PDMS replica was peeled off from the SU-8 mold, producing the final patterned PDMS cell culture substrates. Micro-well depth was again measured by profilometry.

Patterned PDMS substrates were affixed to the bottom of wells of either 12 or 96 well plates using uncured PDMS. Plates were then heated in oven at 65 °C for 2 h to allow scaffolds to completely adhere to the bottoms of the wells. For all the tests, flat PDMS substrate and cell culture treated polystyrene (PS) substrate were used as controls. All cell culture substrates were sterilized by immersing in 70% (v/v) overnight, followed by washing extensively with phosphate buffered saline (PBS, Sigma). PDMS has very poor cell adhesion properties due to its hydrophobicity [17]. Our previous study has found presorption of 50 μg/ml fibronectin (Fn) is sufficient to promote Caco-2 adhesion on PDMS to a level equivalent to that observed on cell culture treated PS. Therefore, before seeding cells, all substrates were coated with 50 μg/ml fibronectin in PBS for 2 h at room temperature.

2.2. Cell culture

A human colon carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC) and cultivated in Eagle's minimum essential medium (ATCC) supplemented with 20% fetal bovine serum (FBS, ATCC) and 1% antibiotic antimycotic solution (containing 10,000 units/ml penicillin G, 10 mg/ml streptomycin sulfate and 25 μg/ml amphotericin B; Sigma). Confluent monolayers were subcultured by incubating with 0.05% trypsin (Sigma) and 0.2% ethylenediaminetetraacetic acid (EDTA, Sigma) in Ca^{2+}- and Mg^{2+}-free PBS. Cultures were incubated at 37 °C in a humidified atmosphere of 95% air, 5% CO_2. For all experiments, cells were seeded at low density (2×10^4 cells/cm^2) onto test surfaces and cultured for specified time periods. Medium was aspirated and replaced after 2 days of seeding and every 2 days in culture.

2.3. F-actin staining (cytoskeletal observation)

Caco-2 cell morphology was assessed by staining with fluorescein phalloidin (Molecular Probes). Caco-2 cells were fixed in 3.7% formaldehyde (Sigma) in PBS for 10 min at room temperature, washed twice with PBS, and permeabilized by 0.1% Triton X-100 in PBS for 5 min. After two washes with PBS, cells were incubated with 0.16 μm phalloidin in 1% BSA/PBS for 20 min at room temperature. Cells were counterstained with 5 μg/ml Hoechst 33 258 (Molecular Probes) in PBS for another 20 min at room temperature. Cell cytoskeleton was observed using a fluorescence microscope (Olympus ×51).

2.4. Image analysis

ImageJ software (http://rsb.info.nih.gov/ij/) was utilized to determine cell coverage on flat and patterned PDMS substrates, and cell culture treated PS surfaces. Cell coverage was determined from F-actin stained images by measuring stained area and dividing by the entire area in a given field of view. It is acknowledged that method does not consider the cell coverage on side walls of well structures.

2.5. Scanning Electron Microscopy (SEM)

Cells were washed twice with Hank's Balanced Salt Solution (HBSS, Sigma) at 37 °C, and then fixed with 3% glutaraldehyde (Sigma) in 0.1 M sodium cacodylate (Sigma), pH 7.4, containing 0.1 M sucrose (Sigma) for 45 min at room temperature. After fixation, the cells were washed in 0.1 M sodium cacodylate, pH 7.4, containing 0.1 M sucrose and dehydrated through graded changes of ethanol solutions (35%, 50%, 70%, 95%, and 100%). Cells were then dried using a Tousimis PVT-3 critical point dryer. Dried samples were mounted on aluminum stubs and sputter coated with gold-palladium. The cells were examined using a Hitachi S4800 field emission SEM.

2.6. MTT assay (metabolic activity)

The MTT assay is used to estimate cellular metabolic activity; NADH- or NADPH-dependent mitochondrial dehydrogenases inside viable cells are able to reduce the yellow MTT tetrazolium salt to blue-purple formazan crystals. The amount of the crystals formed reflects metabolic activity of cells at the end of essay period [18,19]. For each sample, after the cells were incubated on patterned PDMS for 3 or 6 days, the medium was gently removed and replaced with 0.09 ml of phenol red-free Eagle's minimum essential medium (Sigma). 0.01 ml of 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution (Sigma) was then added to each sample located in each well of a 96 well plate. The cell cultures were incubated at 37 °C for 3 h. The formazan crystals generated during the incubation period were dissolved by adding 0.1 ml of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol, Sigma) and gently mixing the solution by trituration. After the crystals were fully dissolved, the absorbances of the solutions at 570 nm (OD_{570}) were measured using a spectrophotometer. Cell culture medium was used as a control. The MTT results are presented below as optical density at 570 nm relative to total number of cells, which was measured by a CyQUANT® cell proliferation assay kit from Molecular Probes, as described below.

2.7. Cell counting assay

A CyQUANT® cell proliferation assay kit was utilized to count total cell number in each sample. Briefly, cells were detached from each PDMS substrate by incubating with 0.05% trypsin and 0.2% EDTA in Ca^{2+}- and Mg^{2+}-free PBS for 20 min. An excess culture medium was added to stop the trypsin reaction, and PDMS substrates were gently removed from the bottom of cell culture plate, inverted, and immersed in medium for 10 min, ensuring the cells trapped inside wells to settle into medium by gravity. The resulting cell suspension was then collected, washed twice with PBS, and concentrated by centrifuging for 5 min at 200 × g. 1 mL of the CyQUANT® GR dye/cell-lysis buffer was added to the cell pellet and incubated for 5 min at room temperature. Fluorescence of the samples was measured using a fluorometer with 480 nm excitation and 520 nm emission. A reference standard curve for converting sample fluorescence values into cell numbers was prepared by counting cells using a hemacytometer and measuring fluorescence intensity of known cell density samples.

2.8. Alkaline phosphatase activity assay

The alkaline phosphatase activity assay was performed on cell lysates. Just before conducting the assay, samples were washed twice with PBS and lysed by