Biochemical and Biophysical Research Communications 457 (2015) 363–369

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

3D-fibroblast tissues constructed by a cell-coat technology enhance tight-junction formation of human colon epithelial cells

Michiya Matsusaki [a], Daichi Hikimoto [a], Akihiro Nishiguchi [a], Koji Kadowaki [a], Kayoko Ohura [b], Teruko Imai [b], Mitsuru Akashi [a, *]

[a] Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka, 565-0871, Japan
[b] Department of Pathopharmacology, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto, 862-0973, Japan

ARTICLE INFO

Article history:
Received 16 December 2014
Available online 8 January 2015

Keywords:
Caco-2
3D-cell culture
Cell-coat technology
Tight-junction protein
Barrier function
Pharmaceutical application

ABSTRACT

Caco-2, human colon carcinoma cell line, has been widely used as a model system for intestinal epithelial permeability because Caco-2 cells express tight-junctions, microvilli, and a number of enzymes and transporters characteristic of enterocytes. However, the functional differentiation and polarization of Caco-2 cells to express sufficient tight-junctions (a barrier) usually takes over 21 days in culture. This may be due to the cell culture environment, for example inflammation induced by plastic petri dishes. Three-dimensional (3D) sufficient cell microenvironments similar to *in vivo* natural conditions (proteins and cells), will promote rapid differentiation and higher functional expression of tight junctions.

Herein we report for the first time an enhancement in tight-junction formation by 3D-cultures of Caco-2 cells on monolayered (1L) and eight layered (8L) normal human dermal fibroblasts (NHDF). Trans epithelial electric resistance (TEER) of Caco-2 cells was enhanced in the 3D-cultures, especially 8L-NHDF tissues, depending on culture times and only 10 days was enough to reach the same TEER value of Caco-2 monolayers after a 21 day incubation. Relative mRNA expression of tight-junction proteins of Caco-2 cells on 3D-cultures showed higher values than those in monolayer structures. Transporter gene expression patterns of Caco-2 cells on 3D-constructs were almost the same as those of Caco-2 monolayers, suggesting that there was no effect of 3D-cultures on transporter protein expression. The expression correlation between carboxylesterase 1 and 2 in 3D-cultures represented similar trends with human small intestines. The results of this study clearly represent a valuable application of 3D-Caco-2 tissues for pharmaceutical applications.

1. Introduction

Permeability characteristics of small intestines have been studied widely to characterize the pharmacokinetic properties of drug candidates using human colon carcinoma cell line Caco-2 [1–3]. Caco-2 cells express tight-junctions, microvilli, and a number of enzymes and transporters that are characteristic of enterocytes: peptidases, esterase, P-glycoprotein, uptake transporters for amino acids, bile acids and carboxylic acids [4]. Artursson and coworkers reported a good correlation between oral drug absorption in humans and apparent drug permeability coefficients in Caco-2 cells [5]. Although a relatively long culture period is required for permeability experiments to obtain sufficient barrier function in Caco-2 cells, until now, the Caco-2 cell monolayer system was the gold standard in pharmaceutical applications. The tight-junction formation (barrier function) of Caco-2 monolayers, which can be detected by trans epithelial electric resistance (TEER) measurements, increase gradually with increasing culture times and reach sufficient barrier function (TEER of over 300 $\Omega^{*}cm^{2}$) after 21–29 days of incubation [2]. This long-term culture of Caco-2 cells before any experiments is labor intensive and time consuming, which limits versatility and thus limits applicability to pharmaceutical applications [6]. If culture times of Caco-2 cells were shorter, then drug permeability assays using Caco-2 cells would be more widespread.

The necessity of long culture times of Caco-2 cells required to obtain enough barrier functions may be due to traditional cultures on “artificial” plastic substrates. We reported high expressions of heat shock protein 70 (Hsp70) and an inflammatory cytokine 6 (IL-6) from monolayers of human umbilical vein endothelial cells

* Corresponding author. Fax: +81 6 6879 7359.
E-mail address: akashi@chem.eng.osaka-u.ac.jp (M. Akashi).

http://dx.doi.org/10.1016/j.bbrc.2014.12.118
0006-291X/

(HUVEC) on tissue culture polystyrene dishes [7]. Hsp70 families are expressed in response to different types of cellular stress, such as elevated heat, mechanical trauma, chemical reagents, and heavy metals [8]. Thus, when cells suffer physical and physicochemical stress from the environment, the production of Hsp70 increases to serve as molecular chaperones in protein folding and transport [9]. IL-6 plays an essential role in intercellular communication, and especially in the inflammatory response [10]. Increase of IL-6 production clearly indicates an inflammatory response in cultured cells on plastic dishes, similar to other reports which represent an induction of inflammation on plastic dishes during the cell culture process [11].

A significant difference between *in vitro* 2D-cultures and *in vivo* tissues or organs is the existence of components surrounding cells, such as extracellular matrices (ECM). In the body, nearly all tissue cells reside in the fibrous nano-meshwork of the ECM, which is typically composed of fibronectin (FN) and collagen, and provides complex biochemical and physical signals [12,13]. Furthermore, *in vivo* tissues or organs are three-dimensional (3D) structures. The 3D-microenvironments consisting of the surrounding ECM and neighboring cells and the signaling of cytokines and growth factors play a significant role in the maturation of cells. Accordingly, 3D-structures of Caco-2 cells consisting of ECM and cells are expected to enhance tight-junction formation (barrier function) during short culture periods.

We developed a simple and unique bottoms-up approach, “a hierarchical cell manipulation technique”, which employs nanometer-sized LbL films consisting of FN and gelatin (G) as a nano-ECM to fabricate 3D-tissue constructs [14–20]. The FN-G nanofilms were prepared directly on cell surfaces, and we discovered that at least 6 nm thick FN-G films acted as a stable adhesive surface for adhesion of the second cell layer. Various 3D-layered constructs consisting of single or multiple types of cells were successfully fabricated such as blood vessel wall structures [19,20]. Recently, we also developed a rapid bottoms-up approach “a cell accumulation technique” by employing a single cell coating using FN-G nanofilms, because the fabrication of two layers (2L) per day is difficult with the technique described above due to the time required for stable cell adhesion [21]. The rapid approach could be used to easily create 3D-tissues over 100 μm thickness after only one day of incubation. Moreover, fully and homogeneously vascularized tissues of 1 cm width and 100 μm height were obtained by a sandwich culture of endothelial cells for one or two days of incubation [21–25]. Notably, we found higher cellular activities and lower inflammatry responses induced from 3D-structures consisting of HUVEC and fibroblast layers as compared to those of monolayer structures [25]. These results motivated us to fabricate 3D-Caco-2 tissue constructs in order to enhance tight-junction formation of Caco-2 cells during short culture periods.

In this study, 3D-multilayered structures consisting of normal human dermal fibroblast (NHDF) and HUVEC were constructed by a cell accumulation technique and their tight-junction formation was evaluated via TEER measurements and messenger RNA (mRNA) expression in relation to the layer number of underlying NHDFs.

2. Materials and methods

2.1. Materials

Fibronectin (FN) from bovine plasma (M_w 4.6×10^5) was purchased from Sigma-Aldrich (MO, USA). Gelatin (G) (M_w 1.0×10^5), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 10% formalin solution, L-Glutamine, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Wako Pure Chemical Industries (Osaka, Japan). ZO-1 mouse monoclonal antibody, Triton X, Goat anti-mouse Alexa Fluor 546-conjugated IgG, 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI), fetal bovine serum (FBS), and MEM Non-Essential Amino Acid (NEAA) were purchased from Life Technologies (CA, USA). The 12 well and 24 well cell culture inserts with 0.4 μm pore size were purchased from BD bioscience (NJ, USA) and Corning Inc. (MA, USA). Normal human dermal fibroblast (NHDF) was purchased from Lonza (NJ, USA). Caco-2 was kindly donated by Prof. Imai from Kumamoto University. All of the chemicals were used without further purification.

2.2. Construction of Caco-2 3D-tissues

The NHDFs after trypsinization were suspended in 0.04 mg/ml of FN and G/Tris-HCl solution (50 mM, pH = 7.4), and alternately incubated for 1 min using a MicrotubeRotator (MTR-103, AS ONE, Japan) with a washing step. The centrifugation was performed at 200 × g for 1 min at each step. After 9 steps of coating, about 10 nm of the FN-G nanofilms were coated onto single cell surfaces. The cells were suspended in 1 ml and 0.3 ml of DMEM with 10% FBS, and were seeded onto 12 well and 24 well trans-well inserts with a semipermeable membrane, and 1.5 ml and 1 ml of media was added into the microplates. After 1 h of incubation, another 1 ml of media was added to connect the inner and outside media of the inserts when 24 well inserts were used. The cells were then incubated in 5% CO_2 at 37 °C. After 1 day, NHDF tissues from a monolayer to 8 layers thickness were constructed. A 1×10^5 cells/layer aliquot was used for the 24 well inserts, and 2.5×10^5 cells/layer for the 12 well inserts. In the same manner, 2.5×10^5 Caco-2 cells were coated with FN-G nanofilms, and were seeded onto each NHDF tissue. The Caco-2 cells adhered within 1 day to form a monolayer Caco-2-3D-NHDF tissues. The medium was changed every other day.

2.3. TEER profiles of Caco-2 3D-tissues

TEER of Caco-2 monolayer and 3D-tissues were measured using MILICELL electrical resistance system-2 (Millipore Corp., Bedford, MA). Resistances of blank membrane were subtracted from those of membranes with cells before final resistances were (in Ω cm^2) calculated.

2.4. RNA isolation and reverse transcription-PCR (RT-PCR)

Total RNA was isolated from cell monolayers using RNAiso reagents (Takara Bio Inc., Shiga, Japan) according to the manufacturer's specifications. First-strand cDNA was synthesized using Oligo(dT) with ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). The Real-time quantitative PCR reaction was performed in Bio-Rad iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR Premix Ex Taq II (Takara Bio Inc.) and primers reported previously [26–28]. Thermal cycling conditions were 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 45 s. Reactions were carried out in triplicate. Relative mRNA levels were calculated using the $2^{-\Delta Ct}$ method.

2.5. Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student's *t*-test. All data are represented as means ± SD. *, $P < 0.05$; **, $P < 0.01$. N.S. means no significant difference.