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A new cell-to-cell interaction model for epithelial microfold cell formation and the enhancing effect of epidermal growth factor

Puwich Chaikhumwang[a], Dachrit Nilubol[b], Angkana Tantituvanont[a,d,*], Pithi Chanvorachote[c,d,**]

[a] Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
[b] Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand
[c] Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
[d] Cell-Based Drug and Health Product Development Research Unit, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

ABSTRACT

The formation of epithelial microfold (M) cells is mediated through cell-to-cell interactions between enterocytes and lymphocytes. Based on this concept, we developed a cell-to-cell model by encouraging interactions between enterocyte C2BBe1 and Raji B cells through a preincubation approach. Raji B cells and C2BBe1 cells were allowed to interact in detached condition for 2 h at ratios of 1:1, 1:2 and 1:4 and then plated in culture plates. Monocultured C2BBe1 cells were used as the control. Flow cytometric analysis of the M cell-specific marker clusterin revealed that the optimum ratio of Raji B to C2BBe1 cells to obtain the maximum number of M cells was 1:1. Scanning electron micrographs exhibiting the lack of microvilli with complete tight junctions and Western blot analysis showing intense expression of clusterin confirmed the unique phenotypes of the formed M cells. Fluosphere® transport studies showed a 7-fold increase in the cell-to-cell model compared to the monoculture control. Importantly, we found that the induction of M cells could be enhanced by the effect of epithelial growth factor (EGF). C2BBe1 cells were pretreated with EGF at 10, 25 and 50 ng/mL before co-culturing with Raji B cells. Flow cytometric analysis of clusterin revealed that EGF significantly increased the formation of M cells. From mechanistic studies, we found an increase in the number of M cells involved the induction of stemness by EGF indicated by a dramatic increase in β-catenin, Nanog, and Oct-4, which in turn up-regulated the cell-to-cell interacting protein Integrin β-1. Furthermore, we confirmed the transport functions of the conventional, cell-to-cell, and cell-to-cell with EGF models using a Fluosphere® transport assay. Overall, we demonstrated an effective novel protocol for the formation of M cells as well as the effect of EGF on enhancing cell-to-cell interaction, which may benefit transport studies in M cells and promote better understanding of the biology of M cells.

1. Introduction

Epithelial cells provide barrier surfaces to protect human hosts from the external environment and microorganisms. In the gastrointestinal (GI) tract, the interaction of commensal microbes and the host immune system helps to maintain the protective capacity of the epithelium (Henriques-Normark and Normark, 2010; Zeissig and Blumberg, 2014). However, in the case of harmful microbes, specialized intestinal epithelial microfold (M) cells residing in the follicle associated epithelium (FAE) of Peyer's patch have the ability to sense toxic stimuli from the microbes, activate the barrier function and participate in the coordination of the appropriate immune response (Williams and Owen, 2015). By engulfing antigens from the mucosal epithelium and transferring them to the underlying immune cells, M cells can facilitate further immune induction (Kerneis, 1997; Masuda et al., 2011; Van der Lubben et al., 2002). These uptake and transcytosis abilities have identified M cells as an important target site for vaccine delivery and an ideal tool for the study of antigen uptake (Casteleyn et al., 2013; Martinez-Argudo and Jepson, 2008; Park et al., 2015; Van der Lubben et al., 2002).

The small number of M cells (Giannasca et al., 1999) in the intestinal tract and the diverse phenotypes of M cells among different species (Clark et al., 1993; Clark et al., 1994; Gebert et al., 1994; Jepson et al., 1993b) have limited their use as a potential tool for uptake studies *in vivo*. *In vitro*, however, preliminary investigations of the influence of M cells on the transport of antigens across epithelial cells

* Correspondence to: A. Tantituvanont, Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences and Cell-Based Drug and Health Product Development Research Unit, Chulalongkorn University, Bangkok, Thailand.
** Correspondence to: P. Chanvorachote, Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences and Cell-Based Drug and Health Product Development Research Unit, Chulalongkorn University, Bangkok, Thailand.
E-mail addresses: angkana.T@pharm.chula.ac.th (A. Tantituvanont), pithi_chan@yahoo.com (P. Chanvorachote).

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are more feasible. M cells are generally established *in vitro* by using human enterocyte Caco-2 cells or C2BBe1 cells (Caco-2 cell subclones) and human B lymphocytes (des Rieux et al., 2007; des Rieux et al., 2005; Gullberg et al., 2000; Masuda et al., 2011; Schimpel et al., 2014). The two common approaches for the generation of M cells are the normally oriented (Gullberg et al., 2000) and inverted models (des Rieux et al., 2005). The normally oriented approach generates M cells based on the concept that lymphocytes introduced from the basolateral compartment will migrate up into the epithelial monolayer in the apical compartment to produce cells of M cell-like morphology. This technique is simple and easy but lacks reproducibility and produces low numbers of M cells. In the inverted approach, lymphocytes are directly added on an inverted epithelial monolayer to encourage closer contact between the two cell types, thereby making the enterocytes more accessible to the lymphocytes. The inverted approach is more efficient in terms of the number of M cells generated, but the culture step is more complicated (des Rieux et al., 2007). Recent evidence shows that the differentiation of epithelial cells into M cells requires signals from the immune cells beneath the FAE in the subepithelial dome (SED). These signals include the cytokine TNF superfamily member receptor activator of nuclear factor kappa-B ligand (RANKL) that relays the signals through its receptor (RANK), which is expressed by epithelial cells residing along the intestine. It has been found that high levels of RANKL-RANK signaling were achieved by the intact transmembrane cytokine after direct cell-to-cell contact, rather than systemic diffusion of soluble RANKL just as in the case of the normally oriented model. Therefore, encouraging direct cell-to-cell contact between enterocytes and lymphocytes could facilitate RANKL-RANK signaling, leading to the increase in the formation of M cells (Knoop et al., 2009; Mabbott et al., 2013). This study discovered a cell-to-cell interaction approach, which is easy and highly efficient in generating M cells *in vitro*. The approach established M cells based on the assumption that the formation of these cells can be enhanced by inducing close cell-to-cell contact through a preincubation technique.

Moreover, an increasing amount of evidence has indicated that stem cells found in several parts of the body regulate and facilitate tissue development (Ayala et al., 2015; Laird et al., 2008; Molina et al., 2015). Not only do the stem cells proliferate and differentiate to substitute damaged cells, but the cytokines and growth factors produced by these cells also play critical roles in the development and homeostasis of tissues (Auricchio et al., 1994; Biteau and Jasper, 2011; Suzuki et al., 2010). Stem cell-related cellular signals have been found to be induced in fully differentiated cells (Paul et al., 2013; Zhang et al., 2014). In particular, several studies demonstrated that epidermal growth factor (EGF) can increase stemness or stem cell-like phenotypes in differentiated cell lines, such as the murine gastric epithelial cells (GIF-11) (Voon et al., 2013), human gallbladder cancer cells (GBh3, HU-CCT-1, FU-GBC-2 and GBd15) (Sasaki et al., 2012) and human colon carcinoma cells (HCT-15, HCT-116 and HT-29) (Ju et al., 2014). The induction of stemness involves an up-regulation of several stem cell-associated transcription factors, such as Nanog and Oct-4 (Takao et al., 2007). Nanog and Oct-4 are the down-stream mediators of the Wnt/β-catenin signaling pathway. In this pathway, GSK3β is phosphorylated in response to the binding of Wnt to the receptor Frizzled resulting in the stabilization of β-catenin (Hu and Li, 2010; Merrill, 2012). Subsequently, the accumulated β-catenin migrates into the nucleus and induces expression of the transcription factors mentioned above (Krausova and Korinek, 2014; Paul et al., 2013).

As EGF is frequently found at epithelial crypts of the GI tract (Bedford et al., 2015; Zhang et al., 2014) and is speculated to play a regulatory role in the maintenance of stem cells (Suzuki et al., 2010; Voon et al., 2013), we hypothesize that this endogenous molecule may increase epithelial stemness and facilitate the formation of M cells. The possible link between stemness and M cell formation could be as follows: (i) Integrin β-1 has been found to be abundant in both stem cells and M cells (Jinka et al., 2011; Weitzman et al., 1995); (ii) Integrin β-1 has been shown to be a key protein through which epithelial cells interact with immune cells (Leoni et al., 2015); and (iii) during the induction of stemness, Integrin β-1 has been found to be significantly up-regulated (Adelsman et al., 1999; Yu et al., 2000).

The goal of this study was to develop a cell-to-cell interaction (cell-cell) model for the induction of M cells *in vitro* by enhancing the interaction of C2BBe1 and Raji B cells *via* two possible approaches: 1) preincubation of C2BBe1 cells and Raji B cells to encourage close cell-to-cell contact and 2) the induction of stemness in C2BBe1 cells by EGF. Accordingly, this study evaluated the effect of EGF on the regulation of stemness of C2BBe1 cells and the interaction of C2BBe1 cells with Raji B cells, which are a type of immune cells. Moreover, we demonstrated the formation of M cells *in vitro* and characterized these cells by immunocytochemistry and scanning electron microscopy (SEM). The knowledge gained from this study may improve the understanding of the biology of M cell formation and facilitate the development of the method of generation of M cells for *in vitro* uptake studies.

2. Materials and methods

2.1. Antibodies, chemicals and nanoparticles

Goat anti-clusterin (sc-6420) (Santa Cruz Biotechnology, CA, USA), fluorescein-conjugated donkey anti-goat (R & D Systems, MN, USA) and horseradish peroxidase (HRP)-conjugated mouse anti-goat (Thermo Fisher Scientific, MA, USA) antibodies were used. Rabbit anti-β-catenin (ab16051), rabbit anti-Nanog (ab80892), rabbit anti-Oct-4 antibody (ab19857), rabbit anti-Integrin β-1 (ab134179), rabbit anti-Actin antibody (ab1801) and HRP-conjugated mouse anti-rabbit HRP conjugated (ab6728) antibodies were obtained from Abcam (Abcam, Cambridge, UK). Transwell® polycarbonate inserts with a pore diameter of 3 μm (Sigma-Aldrich, MO, USA) and yellow-green carboxylated latex particles (Fluospheres®) with a mean diameter of 0.2 μm (Thermo Fisher Scientific, MA, USA) were used for the transport studies.

2.2. Cell culture

C2BBe1 cells, cloned from of the human adenocarcinoma cell line Caco-2 (American Type Culture Collection, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, MD, USA), 0.01% Antibiotic Antimycotic Solution (Gibco™ Invitrogen Corporation, CA, USA), 1% GlutaMAX, 1% nonessential amino acids (Thermo Fisher Scientific, MA, USA) and 0.01% human transferrin (Sigma-Aldrich, MO, USA). Raji B cells, a human B cell Burkitt's lymphoma cell line (American Type Culture Collection, VA, USA), were cultured in Advanced Roswell Park Memorial Institute (RPMI) 1640 (Thermo Fisher Scientific, MA, USA) supplemented with 10% FBS, 0.01% Antibiotic Antimycotic Solution and 1% GlutaMAX. Cells were grown and maintained in T25 tissue culture flasks at 37 °C in 5% CO_2-containing humidified atmosphere. The cells were used for experiments between passages 10 to 33 for C2BBe1 cells and passages 15 to 37 for Raji B cells.

2.3. Construction of the in vitro M cells model

C2BBe1 cells were cultured in DMEM culture medium until they reached 70–80% confluence. Raji B cells were grown in RPMI-1640 culture medium until a cell density of 5×10^6 cells/mL was obtained. To establish the *in vitro* M cells model using the preincubation approach (cell-cell model), 50 μL of C2BBe1 cells were first incubated with 50 μL of Raji B cells in suspension in 1.5 mL microcentrifuge tubes at 37 °C in 5% CO_2-containing humidified atmosphere for 2 h. The ratio of Raji B cells to C2BBe1 cells was varied at 1:1, 1:2, or 1:4 to identify the optimal condition for the formation of M cells. The total number of cells was maintained at 9×10^4 cells per well. The mixture of C2BBe1 and