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Research Article

EXPRESSION OF THE MICROFOLD CELLS IN THREE-DIMENSIONAL COCULTURE SYSTEM FOR *IN VITRO* CULTIVATION OF HUMAN *NOROVIRUS*

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ABSTRACT

Introduction: Human *Norovirus* (HuNoV), a food-borne virus is the leading cause for acute gastroenteritis. However, its inability to propagate *in vitro* persists as major challenge in understanding HuNoV biology.

Objective: This study aims to determine an effective culture system for HuNoV.

Methods: The Caco-2 cells were cocultured with Raji B cells on alginate hydrogel beads. Scanning electron microscopy (SEM) was performed to confirm the three-dimensional (3D) cells morphology. Western blot (WB) analysis was performed to detect protein markers expressed by Microfold (M) cells.

Results: Optimization of Caco-2 cells monoculture in the alginate hydrogel beads showed optimum number of cells of 1×10^6 cells/ml, indicated by the intact structure of the beads. Result of SEM showed clear structure of monoculture in the alginate hydrogel beads indicated by the presence of smooth and regular apical surface while the coculture showed reduced apical surface of M cells. The result of WB showed downregulation of *Ulex europaeus* antibody expression.

Conclusion: It is evident that the expression of M cells grown in 3D alginate hydrogel beads was successful, indicated by the structural morphology seen under SEM as well as expression of protein marker by M cells. This established *in vitro* system is highly potential for cultivation of HuNoV.

Keywords: Human *Norovirus*, Microfold cells, Three-dimensional culture, Alginate hydrogel.

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INTRODUCTION

Human *Norovirus* (HuNoV) is currently the primary cause for non-bacterial endemic acute gastroenteritis. This food-borne virus belongs to the family Caliciviridae [1], having a single-stranded positive-sense RNA as its genome. Virus transmission is mainly through fecal-oral route, giving symptoms of HuNoV infection include severe vomiting and diarrhea [2]. Although it is estimated that HuNoV infection leads to 200,000 deaths annually worldwide, HuNoV remains as the most poorly characterized virus, mainly due to lack of a reliable and reproducible cell culture system for its propagation. This major setback is also hindering studies toward virus-host interactions as well as its pathogenesis.

It has been suggested that on host cells entry, the HuNoV binds to the glycans known as histo-blood group antigens (HBGA) [3]. Other studies reported the role of HBGA in modulating HuNoV pathogenesis in human host [4]. In addition, the binding of HuNoV to the related receptors expressed on the surface of human gastrointestinal epithelium, most likely the Microfold (M) cells have also been reported [5-7]. As an enteric virus, it was proposed that HuNoV enters the host cells by exploiting the formation of M cells while breaching the human intestinal epithelium [8,9]. The M cells, also known as microfold cells, are defined as specialized epithelial cells found in follicle-associated epithelia of the Peyer's patches [10]. The role of M cells includes induction of immune responses within mucosa-associated lymphoid tissue and transcytosis; a process of particles such as antigen and bacteria is transported from the apical side into lymphocytes dome-like structure at the basolateral side of the intestinal epithelium [10,11].

Advances in the application of alginate hydrogel as biopolymer vehicle for controlled delivery system provided new exploratory potential in biomedical, engineering, and pharmaceutical research [12]. Formation of alginate hydrogel proved to be a beneficial three-dimensional (3D) structure of a scaffold, resembling the tridimensional cells present in human. Moreover, the ease of gelation, biocompatibility, low cost, and low toxicity properties are convenient advantages in utilizing this type of biomaterial for wound healing therapy, drug delivery, and tissue engineering [13].

As lack of culture system for HuNoV a major challenge in the study of HuNoV biology, it is imperative to overcome this problem. Thus, this study aims to develop a 3D culture system expressing the M cells to support HuNoV replication. The 3D culture system will compose of colon carcinoma cells and lymphoma cells, cocultivated on the alginate hydrogel beads, which is proven to support the expression of M cells, indicated by the result obtained in this study.

METHODS

Cell culture

The Caco-2 human colorectal adenocarcinoma cells and Raji B human Burkitt's lymphoma cells were purchased from American Type Culture Collection. The Caco-2 cells were grown and maintained in a 1 × concentration of Dulbecco's Modified Eagle's Medium (DMEM) of high glucose supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (1% pen-strep) (Nacalai Tesque, Japan). The Raji B cells were maintained in a 1 × concentration of Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (1%

pen-strep) (Nacalai Tesque, Japan). All cells were maintained at 37°C in a humidified incubator with the presence of CO_2 at 5% and regularly observed using inverted microscope (Olympus IX81, Japan).

Coculture of Caco-2 and Raji B cells in alginate hydrogel beads

The construction of the 3D alginate hydrogel beads was performed according to the method described by Ab-Rahim *et al.* [14]. Method for the coculture of Caco-2 and Raji B cells was adapted from procedures as described by Kleiveland [15]. Briefly, Caco-2 cells were resuspended in 1 ml of 1.2% (w/v) alginate dissolved in 0.15 M sodium chloride (NaCl) to achieve the final cells concentration of 1×10^6 cells/ml. Next, using a 25 G syringe needle, the cells suspension was dropped into a 24 well culture plate, whereby each well contained 1 ml of 102 mM calcium chloride ($CaCl_2$) solution. The cells were left for 10 min to allow polymerization of the beads before the $CaCl_2$ solution was replaced with 1 × DMEM growth media. The beads entrapping Caco-2 cells were maintained for 14 days and culture media were changed at every 2 days interval. Optimization for the suitable Caco-2 cells density to be encapsulated in the alginate hydrogel beads was performed at three concentrations of 4.77×10^5 cells/ml, 1.19×10^6 cells/ml, and 2.385×10^6 cells/ml, respectively. On day 14 of Caco-2 cells culture in alginate hydrogel beads, Raji B cells with the concentration of 1×10^6 cells/ml in the RPMI-1640 media were added to the wells followed by incubation for another 7 days. The 1 × RPMI-1640 growth media were changed at every 2 days interval.

At post 21 days incubation, the coculture alginate hydrogel beads were rinsed with phosphate-buffered saline (PBS), before solubilization steps. The beads were solubilized through immersion into 55 mM sodium citrate in 0.9% NaCl. Next, solution containing the cells was centrifuged for 10 min at 600 × g for cells recovery. The supernatant was discarded, and the cell pellets were resuspended in 1 ml PBS followed by centrifugation for 10 min at 250 × g. Then, the cell pellets were dissolved in PBS and kept at −20°C until further used.

Scanning electron microscopy (SEM)

The presence of M cells on 3D alginate hydrogel beads was observed through SEM according to the protocol adopted from Straub *et al*. [5]. Briefly, the beads entrapping cells were fixed with 4% glutaraldehyde for 30 min followed by staining with 1% osmium oxide for 60 min before being washed by rinsing for 3 times with PBS. Next, the beads were gradually dehydrated in ethanol at 4°C for 5 min, at concentration of 30, 50, 60, 70, 80, and 90%, respectively. However, for the concentration of 100% dehydration was performed at room temperature. Subsequently, the beads were dried using a mixture of ethanol and hexamethyldisilazane (HMDS) at the ratio of 1:1, 1:3, and finally at 100% HMDS for 10 min. The beads were coated with sputter gold coater (Emitech K550X, UK) to establish the sample structure, before being examined by SEM (Hitachi TM3000, Japan).

Western Blot (WB) analysis of M cells protein expression

Total protein was extracted from cell pellet of the solubilized alginate hydrogel beads using RIPA extraction buffer (Nacalai Tesque, Japan). Concentration of the extracted protein was quantified by Quickdrop (SpectraMax QuickDrop, USA). Total protein was separated on 4% stacking and 12% resolving Bis/Tris polyacrylamide gel electrophoresis (Bio-Rad, USA) with Tris/glycine running buffer (Bio-Rad, USA) at 50 V for 90 min. Subsequently, the gel was transferred into the iBlot Membrane Stacks Nitrocellulose (Invitrogen, USA) for 25 min using Dry Blotting System (iBlot, Invitrogen, USA). Next, the membrane was incubated with *Ulex europaeus* Lectin 1 Antibody (UEA) (Novus Biologicals, USA) with the ratio of 1:1000 antibody to 0.05% Tween 20 in 1 × Tris-buffered saline (TBST). After overnight incubation, the membrane was washed triple times with TBST before incubation for 1 h with Goat anti-rabbit immunoglobulin G secondary antibody (horseradish peroxidase) (Novus Biologicals, USA) with ratio of 1:1000 of antibody to TBST. Finally, the membrane was rinsed triple times with TBST before the detection of specific protein signals using Peroxidase Stain 3,3-diaminobenzidine Substrate Kit (Brown Stain) (Nacalai Tesque, Japan).

Statistical analysis

All experiments were carried out in duplicates. All data were presented as mean ± SD. Differences in the values of two different groups were evaluated using mixed model repeated ANOVA by SPSS analytical (version 23.0, SPSS, USA). $p < 0.05$ indicated a statistically significant difference across the compared group.

RESULTS

Coculture of Caco-2 and Raji B cells in alginate hydrogel beads

The adherent Caco-2 cells presented as monolayer of epithelial-like cells while the suspension Raji B cells were observed to have lymphoblast-like morphology (Fig. 1). Monoculture of Caco-2 cells in alginate hydrogel beads at different starting cell density of 4.77×10^5, 1.19×10^6, and 2.385×10^6 cells/ml was observed on day 0 and day 14 of incubation (Fig. 2). The optimization of Caco-2 cells number was done based on the observation of beads structure integrity. The most suitable starting cell number was found to be 1.19×10^6 cells/ml indicated by the minimal change in number of beads as well as the intact structure of all beads after 14 days of incubation (Fig. 3).

SEM

SEM was used in this study to verify the inducement of Caco-2 cells into M cells by Raji B cells and to compare the differences of 3D culture surface morphology. The apical surface of Caco-2 cells monoculture presented as dense brush border structure, indicating a well-distributed microvilli. The SEM results of beads entrapping coculture showed successful expression of M cells indicated by the appearance of reduced apical surface in comparison with the monoculture (Fig. 4).

WB analysis of M cells protein expression

WB was performed to verify the expression of UAE between the monoculture and the coculture. UEA protein with size of 46 kDa showed the decreased expression in the coculture, as detected by the reduced substrate stain (Fig. 5).

DISCUSSION

Optimization of Caco-2 cells number is important to ensure the development of 3D coculture system as the suitable cells number may assist in sustainability and integrity of the alginate hydrogel beads. Based on the results, the Caco-2 cells at the starting concentration of 1.19×10^6 cells/ml grown in 50 alginate hydrogel beads showed a minimal number of beads disintegration with 45 beads remained intact after 14 days of incubation. In comparison to 4.77×10^5 and 2.385×10^6 cells/ml starting concentration of Caco-2 cells, post 14 days of incubation showed only 40 and 30 beads were remained intact, respectively. It is evident from the results that high starting cell number contributed in reducing the growth surface area which leads to disintegration of the alginate hydrogel beads. Nevertheless, very low number of cells might weaken the alginic-calcium bond that leads to reduced rigidity of the alginate hydrogel beads, similar to as reported

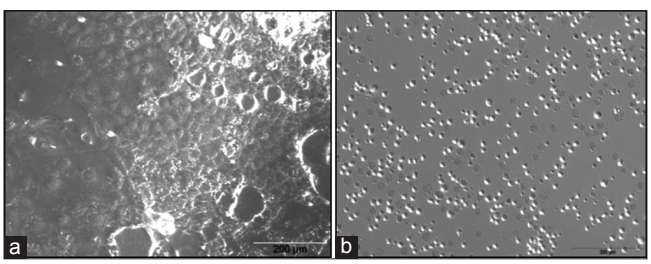

Fig. 1: The morphology of Caco-2 and Raji B cells monoculture. The Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium growth medium while Raji B cells in Roswell Park Memorial Institute 1640. All cells were observed under inverted microscope (×10). (a) Caco-2 cells appeared as polygonal, polarized adherent cells. (b) Raji B cells presented as lymphoblast-like, suspension cells

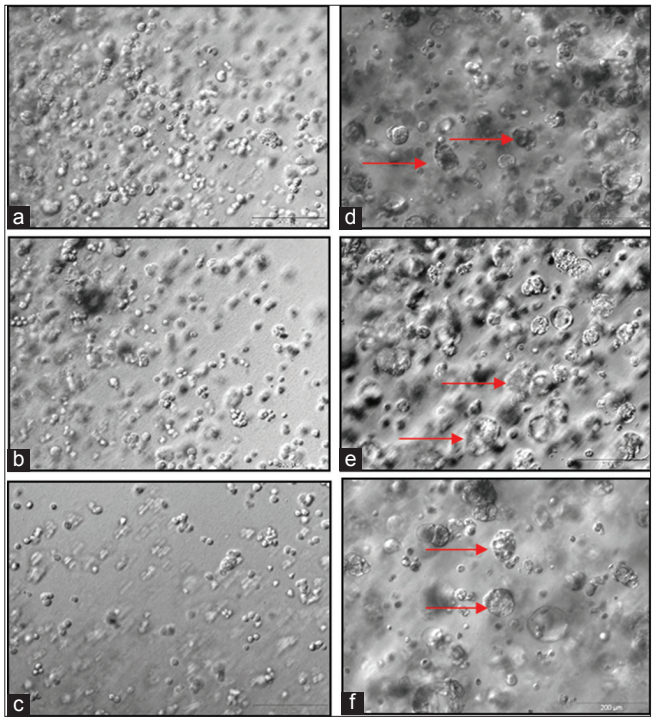

Fig. 2: Formation of Caco-2 cells monoculture in the alginate hydrogel beads under inverted microscope. The Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium at 37°C and observed with inverted microscope (×10). Beads entrapping Caco-2 cells with initial cell number of 2.385 × 10^6, 1.19 × 10^6, and 4.77 × 10^5 cells/ml were observed on day 0 (a, b, and c) and day 14 (d, e, and f), respectively. Red arrow showed enlargement of Caco-2 cells

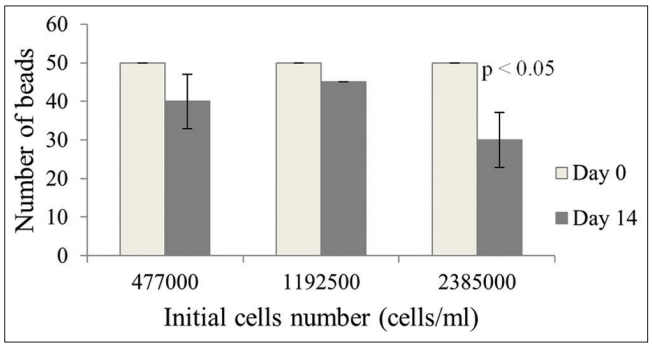

Fig. 3: Optimization of initial Caco-2 cells number relative to number of intact beads. The initial numbers of Caco-2 cells grown in alginate hydrogel beads were tested at three different values; 4.77 × 10^5, 1.19 × 10^6, and 2.385 × 10^6 cells/ml. The most suitable cell number was found to be 1.19 × 10^6 cells/ml relative to the high number of beads remained after 14 days of incubation

previously [12]. Thus, the starting cell number of 1 × 10^6 cells/ml was chosen for this study, which was in line with previous work that used a million number of cells/ml as their seeding density [16].

It was highly suggested that the disorganization of M cells apical surface in human intestinal epithelial facilitated HuNoV host cell entry [9]. Ideally, an established *in vitro* system would have high expression of M cells. The Raji B cells are postulated to induce the expression of M cells from Caco-2 cells [15]. It is evident from the SEM results that the M cells were expressed in 3D coculture alginate hydrogel beads. This is indicated by depressed formation at the surface of apical structure, possibly due to the reduced organization of glycocalyx layer and sparse

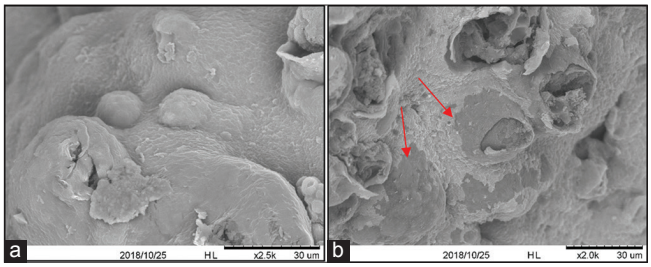

Fig. 4: Scanning electron micrographs of beads entrapping cells at post 21 days of coculture. (a) The apical surface of Caco-2 cells appeared as well-distributed microvilli (×2500). (b) The reduction of apical surface showed by the red arrow indicating the formation of M cells (×2000)

Fig. 5: The western blot results of Microfold cells expression markers. The beads entrapping cells were processed for WB at post 21 days of both monoculture and coculture detected by peroxidase substrate. (a) The *Ulex europaeus* antibody (UEA) was expressed at 46 kDa in the monoculture. (b) The complete reduction of UEA expression in the coculture indicated by absent of the target band. An unidentified protein was expressed as the prominent band

microvilli. In contrast, the morphology of Caco-2 monoculture was observed to have clear, regular brush border and microvilli at the apical surface (Fig. 4). This result was in parallel with the previous work done on established *in vitro* M cells where the coculture of Caco-2 cells with Raji B cells displayed the lack or fewer microvilli formation at the apical surface and presence of sparse, truncated microvillar structures on the edge of M cell membrane [11,17]. Moreover, the evaluation of *in vivo* M cells from mouse Peyer's patches also presented similar results with the appearance of unique morphological structure with lack, blunted and shorter microvilli [6,18,19]. The same characteristic of microvilli was also observed on the small intestine of the newborn piglets, whereby the villous M cells showed depressed, short stub-like microvilli as compared to enterocyte epithelium [20].

In addition to the SEM analysis of the M cells, this study also measured the expression of the M cells marker, UEA through WB. The expression of UEA in monoculture system indicated the binding of UEA associated with alpha-1,2-fucose which is specific for mouse and rabbit M cells [21]. This is in line with our result, whereby the UEA expression was high in the monoculture. More importantly, UEA is a type of lectin that recognizes and binds to carbohydrate moieties of the M cells apical surface. This protein plays a significant role in cell signaling and pathogen recognition molecules [18]. However, inducement of M cells would lead to the relocalization of UEA into the basolateral compartment of the M cells, thus reducing its expression level at the apical surface [22]. This is evident in our result, whereby the UEA expression was completely reduced in the coculture after the formation of M cells. Interestingly, an unidentified protein was found to be prominently expressed at <46 kDa, which required further investigation as a marker for M cells.

CONCLUSION

This study is the first to report the successful use of the alginate hydrogel beads in supporting the coculture of Caco-2 cells and Raji B cells that ultimately induced the formation of M cells. This system is highly potential to be employ as a reliable culture system for HuNoV cultivation.

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AUTHORS' CONTRIBUTIONS

Mr. Mizanurfakhri Ghazali performed all the experiments and obtained all the results presented, as well as drafted the manuscript. Dr. Shariniza Ab-Rahim contributed to the 3D culture work and its analysis. Dr. Mudiana Muhamad conceived the original concept of the work and supervised the findings of this work and contributed immensely to the writing of the manuscript. All authors discussed the results and collectively agreed to the final submitted manuscript.

CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interest.

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