Engineering of a multi-cellular organotypic model of the human intestinal mucosa

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The engineering of multicellular organotypic models of the human intestinal mucosa has wide-ranging potential as a tool for discovery in both health and disease, including interaction with pathogens, antigen trafficking, inflammatory and physiological processes. However, models reported to date remain relatively simple composed of few cell types. Here, we report the development of an organotypic model with close structural and functional resemblance to the human intestinal mucosa; it is comprised of fibroblasts, lymphocytes, epithelial cells, and endothelial cells. Moreover, epithelial cells in our 3-D system can differentiate into multiple lineages (e.g., goblet cells, M cells and differentiated enterocytes). We hypothesized that under both microgravity and proper culture conditions, aggregates of randomly distributed cells will self-organize into a spatial configuration resembling those in native tissues. Our hypothesis was based on recent developments showing that: (1) the culture of epithelial cells as well as endothelial cells under microgravity can be accompanied by a commensurate increase in the cell differentiation[1–3] and (2) gelled collagen-I can constitute a flexible extracellular matrix (ECM) to be used under microgravity.[4] It is known that collagen-I possesses most of the properties of an ideal scaffold, such as specific gravity similar to the culture medium and the capability to incorporate other relevant ECM proteins.[4] In our model, fibroblasts and endothelial cells were embedded in a collagen-I matrix enriched with additional gut basement membrane proteins (i.e., laminin, collagen IV, fibronectin and heparin sulfate proteoglycan) and added to Rotating Wall Vessel (RWV) bioreactors containing epithelial cells (Supplementary Methods). These epithelial cells consisted of a human enterocyte cell line (i.e., HCT-8) that was originally derived from the junction of the small and large bowel.[5] The choice of this cell line was based on its ability to successfully differentiate in a low shear microgravity environment[1] provided by the RWV bioreactor.[6] Activated lymphocytes were added twice to the culture at days 4 (± 1 day) and 9 (± 1 day) (Supplementary Methods).

After 10 to 15 days, histological staining and electron microscopy demonstrated the presence of villus-like structures in the constructs. About 60–80% of these epithelial cells were organized as a monolayer of polarized cells with nuclei located in a basal position near the ECM, a major feature of well-differentiated cells (Figure 1*A*). H&E staining also demonstrated the presence of a brush border, or microvilli, seen collectively as a fuzzy

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fringe in the apical surface of the epithelium (Figure 1*B*). The presence of microvilli was
further evidenced by immunochemical staining using an anti-villin mouse monoclonal
antibody (mAb) or by electron microscopy (Figure 1*C* and *D*, respectively). Assessment by
immunostaining for zonula occludens (ZO)-1, a protein involved in the formation of tight
junctions, and electron microscopy demonstrated that our 3-D organotypic model
recapitulates the formation of tight junctions and desmosomes (Figure 1*E* and *F*). In
addition, because immortalized epithelial cells in 3-D systems may lose the growth pattern
found in the normal epithelium, we assessed the expression of E-cadherin and the Ki-67
proliferation marker in our 3-D model. As expected, HCT-8 cell lines expressed E-cadherin
but only negligible amount of Ki-67 antigen, a pattern consistent with normal highly-
differentiated epithelium (Figures 1*G–I*). Finally, these HCT-8 cells also expressed
numerous markers for multi-lineage differentiation to globet (mucin 2, MUC 2) and M
(Sialyl Lewis Antigen) cell lineages (Figures 1*J* and *K*). Goblet and M cells were distributed
throughout the epithelial layer (Figures 1*J* and *K*, respectively). We also investigated the
presence of the machinery for the specific digestion and transfer of glucose. We observed
apical expression of dissacharidases (i.e., sucrase-isomaltase, SI) and sugar transporters (i.e.,
energy-dependent sodium-glucose co-transporter 1, SGLT1) which strongly suggests that
epithelial cells in our 3-D model are able to absorb and transport glucose (data not shown).
Taken together, these results demonstrate a remarkable degree of differentiation of HCT-8
epithelial cells in our 3-D model.

We also examined the presence and persistence of other cell types by monitoring the
presence of defined cellular markers for each individual cell type (i.e., CD45 for
hematopoietic-derived cells, including lymphocytes; CD31 for endothelial cells and
Vimentin for fibroblasts). These studies were performed by immunochemistry. We observed
that the lymphocytes migrated through the epithelial cell layer and localized to the ECM.
We were able to detect lymphocytes for at least 7 days (last day examined) (Figure 1*L*). As
in the native gut tissue, in our 3-D model fibroblasts and endothelial cells remained
dispersed throughout the ECM. Interestingly, we observed that individual endothelial cells
exhibited cytoplasmic extensions that form bridges between them, suggesting that these
endothelial cells were activated and underwent a remodeling phase (Figures 1*M* and *N*).
However, the vast majority of these endothelial cells do not appear to differentiate towards
the formation of vessel-like conduits. Additional studies are needed to determine the proper
conditions that will further enable vessel-like conduits to form. Interestingly, fibroblasts
showed different shapes, ranging from classical spindle shaped to rounded-fat cell-like
shaped with the nucleus pushed to one side (Figures 1*O* and *P*). These results suggest that
cells were activated and remain able to both receive and respond to differentiation signals.
Finally, several studies have demonstrated that good viability for primary cells lasting more
than 10 days in culture systems is difficult to achieve.[7] Thus, we assessed cell viability by
immunochemical staining using antibodies to cleaved Caspase-3. Up to 20 days after the
initiation of the cultures, few cleaved Caspase-3-positive cells were observed in the ECM,
the major location of primary cells including lymphocytes, fibroblasts and endothelial cells
(data not shown).

Although the combination of the features described above give some insights into the
similarities of our 3-D organotypic model to *in vivo* tissues, we further assessed its
functionality by observing its response to exposure to a human enteric bacterial pathogen.
Specifically, we examined the changes in the epithelial cell morphology, as well as cytokine
production, following exposure to wild-type *Salmonella enterica* serovar Typhi (*S*. Typhi)
strain ISP1820, to evaluate whether it mimicked the reported stages of the infection. The
identification and characterization of *S.* Typhi-infected cells was accomplished using a
polyclonal antibody that specifically detects common *Salmonella* antigens (CSA-1). A
visible feature of the *Salmonella* entry process *in vivo*, i.e., membrane rearrangements or

ruffles, were observed in our system as early as 1 hour after infection (Supplementary Figure 1*B*) and increased over time (Supplementary Figures 1*C* and *D*). Of note, these ruffles were absent in the control (not-infected cells, Supplementary Figure 1*A*). One day after infection, the ruffles fade away and bacteria, when present, was mostly observed on the cell surface/ brush border of healthy looking epithelial cells or intracellular in epithelial cells sloughed from the monolayer which likely died as a result of the infection (Supplementary Figures 1*E* and *F*). We also observed that the exposure to *Salmonella* resulted in production of cytokines, including IL-1β, IL-6, IL-8, IL-11, IL-12p70, IL-17a, IL-21 and TNF-α (Supplementary Figures 1*G, H* and *I*). These results are in agreement, and extend previous studies showing the importance of these cytokines in the control of *Salmonella* infection[8, 9] and further confirming the functional capacity of this 3-D organotypic model to properly react to the presence of an enteric pathogen.

To summarize, although similar approaches have been attempted by other investigators with moderate success,[1, 10] our system provides major innovations and advances such as a matrix enriched with gut basement membrane proteins. Also, to our knowledge, except for our system described above, attempts to integrate multiple cell types in 3-D constructs grown under microgravity have been unsuccessful. Finally, we provided evidence that our 3-D model might be a helpful tool to investigate the early events of the host interaction with gastrointestinal pathogens, including invasion, pathogenesis and immune responses.

Material and Methods

Material and Methods and any associated references are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

CD	cluster of differentiation
CSA-1	common *Salmonella* antigen
DAB	3,3-diaminobenzidine
ECM	extracellular matrix
HUVEC	human umbilical vein endothelial cells
mAb	monoclonal antibody
PBS	phosphate-buffered saline
RWV	rotating wall vessel
S. **Typhi**	*Salmonella enterica* serovar Typhi
3-D	three-dimensional
ZO	zonula occludens

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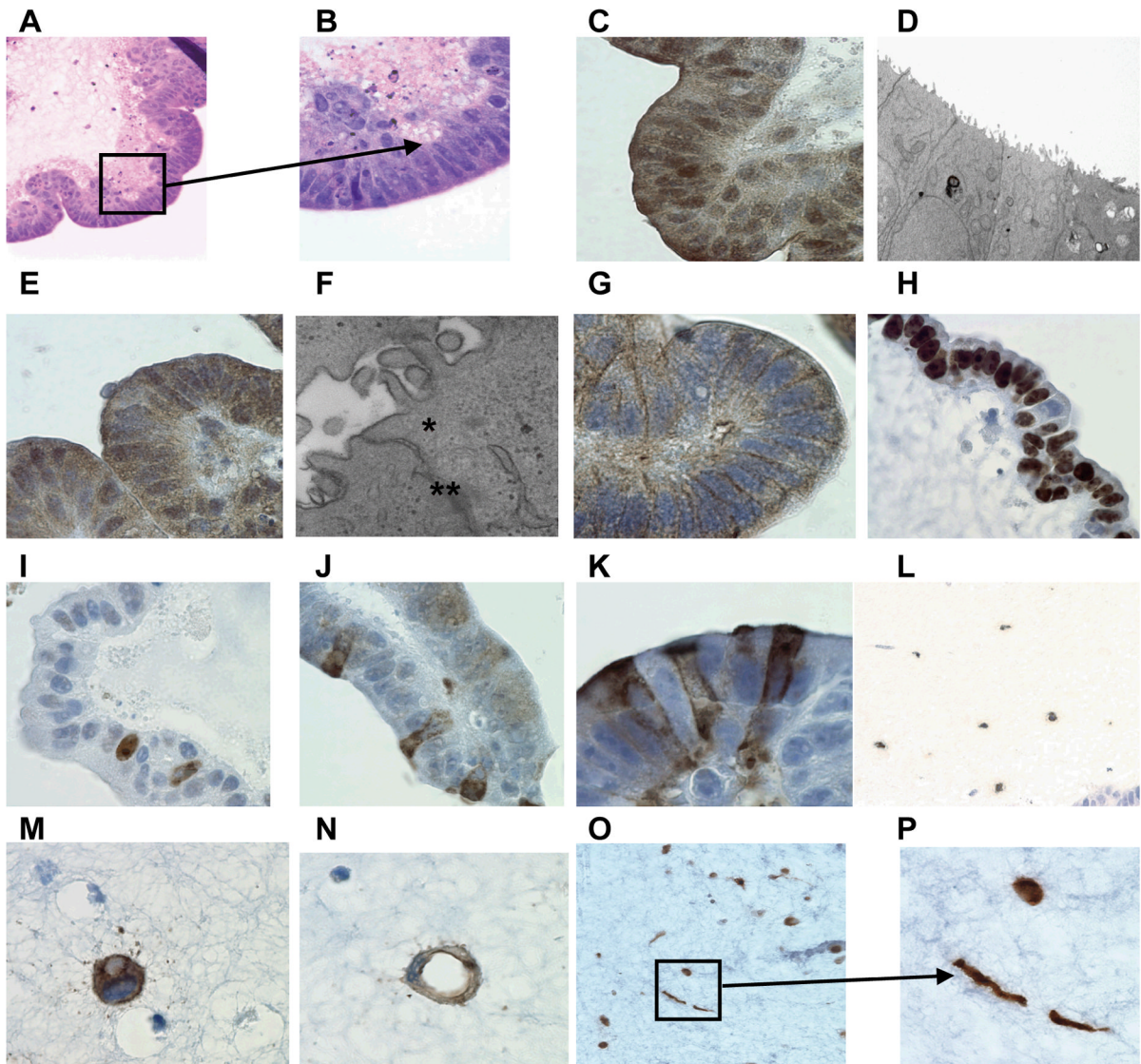
Figure 1.
Epithelial cell differentiation. Hematoxylin and eosin staining of 14 day-cells cultured in the 3-D microgravity model: tissues were stained purple and scaffold stained pink. Lower (**A**) and higher magnification (**B**) shows the presence of polarized epithelial cells with microvilli seen collectively as a fuzzy fringe in the apical surface of the epithelium. The presence of microvilli was further confirmed by immunochemical staining using the anti-villin mouse mAb (**C**) and by electron microscopy (**D**). Presence of tight junctions was detected by Immunochemical staining by using an anti-ZO-1 polyclonal rabbit antiserum (**E**) or electron microscopy (**F**) (tight junctions (*) and desmosome (**)). Immunochemical staining was also used to detect E-cadherin using the anti-E-cadherin mouse mAb (**G**). The presence of proliferating cells was detected at day 5 (**H**) and day 15 (**I**) by using the anti-Ki67 rabbit mAb. The presence of multi-lineage differentiation was also observed: goblet cells using the anti-MUC-2 monoclonal antibody (**J**) and M cells using the anti-Sialyl Lewis Antigen mAb at day 15 (**K**). Cells from a 17-day 3-D organotypic culture were also stained by immunochemistry for lymphocytes using the anti-CD45 mAb (**L**) (low magnification), for endothelial cells using the anti-CD31 mAb (**M**) (sprouting) (**N**) (vessel-like conduit

formation)(high magnification), and for fibroblasts using the anti-vimentin mAb at low (**O**) and (**P**)(spindle shaped rounded-fat cell-like shaped) at high magnification.