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Primary human polarized small intestinal epithelial barriers respond differently to a hazardous and an innocuous protein

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ABSTRACT

An experimental platform employing human derived intestinal epithelial cell (IEC) line monolayers grown on permeable Transwell® filters was previously investigated to differentiate between hazardous and innocuous proteins. This approach was effective at distinguishing these types of proteins and perturbation of monolayer integrity, particularly transepithelial electrical resistance (TEER), was the most sensitive indicator. In the current report, *in vitro* indicators of monolayer integrity, cytotoxicity, and inflammation were evaluated using primary (non-transformed) human polarized small intestinal epithelial barriers cultured on Transwell® filters to compare effects of a hazardous protein (*Clostridium difficile* Toxin A [ToxA]) and an innocuous protein (bovine serum albumin [BSA]). ToxA exerted a reproducible decrease on barrier integrity at doses comparable to those producing effects observed from cell line-derived IEC monolayers, with TEER being the most sensitive indicator. In contrast, BSA, tested at concentrations substantially higher than ToxA, did not cause changes in any of the tested variables. These results demonstrate a similarity in response to certain proteins between cell line-derived polarized IEC models and a primary human polarized small intestinal epithelial barrier model, thereby reinforcing the potential usefulness of cell line-derived polarized IECs as a valid experimental platform to differentiate between hazardous and non-hazardous proteins.

1. Introduction

Most proteins consumed as part of the human diet are not hazardous and are degraded by digestive enzymes into amino acids and small peptides that are absorbed for nutritional purposes (Kiela and Ghishan, 2016). However, there are some proteins such as the kidney bean lectin phytoheamagglutinin E (PHA-E) that produce adverse effects when consumed. These proteins tend to exhibit their effect on the intestinal epithelium as observed from histological evidence of damage in laboratory animals and corresponding clinical gastrointestinal features in humans consuming undercooked kidney beans (Lafont et al., 1988; Rossi et al., 1984; Weinman et al., 1989; Vasconcelos and Oliveira, 2004).

In vitro studies with transformed human intestinal epithelial cell (IEC) lines treated with PHA-E demonstrated direct evidence of adverse effects similar to those observed in laboratory animals treated with PHA-E *in vivo* (Hendriks et al., 1991; Ramadass et al., 2010). Human cell line-derived polarized IEC monolayers have also been used to investigate the potential for adverse effects following exposure to individual chemicals (i.e., not proteins) or pathogenic microbes, with physiologic effects reflective of those observed following *in vivo* exposure to these agents (Hurley and McCormick, 2003; Jensen-Jarolim et al., 1998; Okada et al., 2000). Protein toxins produced by enteropathogens that colonize the intestinal tract have also been examined using the polarized IEC monolayers to gain insight into host responses to enterotoxin exposure (Thorpe et al., 2003). Collectively, these studies support the concept that cell line-derived polarized IEC monolayers contribute toward a better understanding of effects observed following oral exposure to hazardous agents, including proteins.

Based on this premise, an experimental platform built upon aggregate analysis of multiple variables assessing the impact of test proteins on several transformed human polarized IEC monolayers has been explored as a possible alternative to animal studies for identification of hazardous proteins (Hurley et al., 2016a, 2016b).

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Results to date indicate this method is effective at distinguishing between hazardous and non-hazardous proteins and disruption of monolayer integrity was the most sensitive indicator of a potentially adverse effect (Hurley et al., 2016a, 2016b). It is important to note, however, that these previous studies were conducted with multiple distinct yet homogenous populations of transformed enterocytes derived from the human colon. As a model system, cell line-derived IEC monolayers do not fully recapitulate the human small intestinal epithelial barrier that appears to be the primary target for hazardous dietary proteins. The human small intestinal epithelial barrier is comprised of a highly organized integration of diverse polarized cell types including enterocytes, Paneth cells, M cells, tuft cells and intestinal stem cells (Gerbe et al., 2012). An experimental platform built exclusively upon cell line-derived IEC polarized barriers, without additional confirmatory analysis, raises the question of whether such a model system is sufficiently capable of representing human small intestinal responses to hazardous and non-hazardous proteins encountered following ingestion. Both *Clostridium difficile* toxin A (ToxA) and bovine serum albumin (BSA) were extensively analyzed in previous studies for effects following apical exposure to cell line-derived IEC polarized monolayers yielding a clear distinction between the hazardous protein ToxA and the innocuous dietary protein BSA (Hurley et al., 2016a, 2016b). The investigation described herein evaluated the impact of ToxA and BSA on primary human polarized small intestinal epithelial barriers cultured on permeable Transwell filters, a model system that features the appropriate cellular diversity and organization known to be present in the human small intestine.

2. Materials and methods

2.1. Test substances

Bovine serum albumin (BSA) was purchased from Fisher Scientific (Waltham, MA, USA), triton x-100 (TX-100) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and *Clostridium difficile* toxin A (ToxA) was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA). Flagellin (FliC) and TNF-α were obtained from Enzo Life Sciences (Farmingdale, NY, USA) and eBioscience, Inc. (San Diego, CA, USA) respectively. Assay media (DMEM/F12 phenol red free) was purchased from Life-Technologies (Carlsbad, CA, USA).

2.2. Handling of primary human small intestinal epithelial cultures

Normal human 3D Small Intestinal Epithelium (SMI-100) custom grown on 0.33 cm^2, 0.4 μm pore sized Transwell™ inserts (Corning Incorporated/Life Sciences, Tewksbury, MA, USA) were purchased from MatTek Corporation (Ashland, MA, USA). All intestinal tissues for these studies were derived from a single healthy donor. Intestinal tissue cultured on Transwell™ inserts were received in agarose packaging and immediately transferred to a 24-well tissue culture plate containing 550 μl pre-warmed maintenance medium (SMI-100-MM). Following transfer to the 24-well plate, 55 μl SMI-100-MM was added to the apical surface and intestinal cultures, heretofore referred to as primary human small intestinal epithelial barriers, were incubated overnight in a 37 °C, 5% CO_2 incubator. Each primary human small intestinal epithelial barrier was assessed for the development of trans-epithelial electrical resistance (TEER) using a voltmeter (EVOM2, Epithelial Voltohmmeter, World Precision Instruments, Inc., Sarasota, Florida, USA). Intestinal epithelial barriers in this study had initial TEER values in the range of 300–650Ω with an average of 452Ω ± 74Ω and a median of 437Ω.

2.3. Experimental design

Each experiment involved 48 individual primary human small intestinal epithelial barriers that were split into two groups (group #1 and group #2) and analyzed in parallel. Intestinal epithelial barriers were washed two times with assay media and equilibrated for 30 min at 37 °C, 5% CO_2. TEER was measured for each epithelial barrier in duplicate following equilibration as described above (0 h), followed by exposure to differing concentrations of test proteins (BSA or ToxA), negative control (assay media alone) or positive controls (TX-100 for cytotoxicity and disruption of barrier integrity and TNF-α + FliC for inflammatory cytokine release). Group #1 and group #2 were each split into 8 subgroups of three intestinal epithelial barriers apiece and treated with 100 μl assay media alone, 0.1 μg/ml TNF-α/FliC, 100 μg/ml BSA, 1000 μg/ml BSA, 0.02 μg/ml ToxA, 0.2 μg/ml ToxA, 2 μg/ml ToxA, or 0.1% TX-100 on the apical surface. Group #2 epithelial barriers were additionally exposed to 500 μg/ml fluorescein isothiocyanate (FITC)-Inulin (Sigma-Aldrich) and 0.45 μg/ml horseradish peroxidase (HRP; Sigma-Aldrich), included within the 100 μl applied to the apical surface, as these reagents serve as paracellular probes to assess intestinal barrier integrity. Intestinal epithelial barriers were incubated at 37 °C, 5% CO_2 for 22.5 h (group #1) or 24 h (group #2). The level of cytotoxicity (increased lactate dehydrogenase [LDH] release & diminished MTT conversion), disruption of barrier integrity (decreased TEER, increased FITC-inulin flux, & increased HRP flux), and stimulation of inflammatory cytokine production (IL-6 & IL-8) were measured as described briefly below. Intestinal epithelial barriers within group #1 were washed twice and equilibrated for 30 min at 37 °C, 5% CO_2 and subjected to TEER measurement (duplicate measurement/individual barrier) following treatment and incubation (22.5 h). Intestinal epithelial barriers from group #1 were assayed for MTT conversion and supernatant was assessed for the amount of IL-6 and IL-8 release. FITC-inulin and HRP flux were measured across group #2 intestinal epithelial barriers and LDH release was measured in the supernatant of group #2 barriers following 24-h incubation.

2.4. Measurement of FITC-inulin flux

Following 24-h incubation of group #2 primary human small intestinal epithelial barriers, 10 μl from the basolateral well and 10 μl of the start solution were transferred in duplicate to individual wells of a black optical bottom 96-well plate. All samples were diluted 10-fold by adding 90 μl of assay media to 10 μl samples in the black optical bottom 96-well plate and fluorescence intensity was measured at excitation 490 nm and emission 525 nm. The percentage flux of the FITC-inulin was determined by calculating fluorescence intensity measured in the basolateral wells after 24 h divided by fluorescence intensity measured in start solutions originally applied to the apical surface.

2.5. Measurement of HRP flux

Following 24-h incubation of group #2 primary human small intestinal epithelial barriers, 10 μl in duplicate from each basolateral well was transferred to individual wells of a 96-well plate. Additionally, a standard curve of known HRP concentrations was added to the 96-well plate with the highest concentration being 0.5 μg/ml followed by nine 2-fold serial dilutions at a volume of 10 μl/well. 200 μl ABTS peroxidase substrate solution (Sigma-Aldrich) was added to all wells of the plate and incubated at room temperature in the dark for 10 min. Following incubation, the optical density of the plate was read at 405 nm. The standard curve was used to calculate the HRP flux in μg/ml from the optical densities of