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ORIGINAL RESEARCH

Enterohemorrhagic *Escherichia coli* Reduces Mucus and Intermicrovillar Bridges in Human Stem Cell-Derived Colonoids

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SUMMARY

Using a novel human colonoid monolayer model, the earliest targets of enterohemorragic *Escherichia coli* infection by the serine protease EspP have been identified. Mucin-2 and protocadherin-24 are targeted sequentially, leading to bacterial attachment to the epithelium and microvillar effacement.

BACKGROUND & AIMS: Enterohemorrhagic *Escherichia coli* (EHEC) causes over 70,000 episodes of foodborne diarrhea annually in the United States. The early sequence of events that precede life-threatening hemorrhagic colitis and hemolytic uremic syndrome is not fully understood due to the initial asymptomatic phase of the disease and the lack of a suitable animal model. We determined the initial molecular events in the interaction between EHEC and human colonic epithelium.

METHODS: Human colonoids derived from adult proximal colonic stem cells were developed into monolayers to study EHEC-epithelial interactions. Monolayer confluency and differentiation were monitored by transepithelial electrical resistance measurements. The monolayers were apically infected with EHEC, and the progression of epithelial damage over time was assessed using biochemical and imaging approaches.

RESULTS: Human colonoid cultures recapitulate the differential protein expression patterns characteristic of the crypt and surface colonocytes. Mucus-producing differentiated colonoid monolayers are preferentially colonized by EHEC. Upon colonization, EHEC forms characteristic attaching and effacing lesions on the apical surface of colonoid monolayers. Mucin 2, a main component of colonic mucus, and protocadherin 24 (PCDH24), a microvillar resident protein, are targeted by EHEC at early stages of infection. The EHEC-secreted serine protease EspP initiates brush border damage through PCDH24 reduction.

CONCLUSIONS: Human colonoid monolayers are a relevant pathophysiologic model that allow the study of early molecular events during enteric infections. Colonoid monolayers provide access to both apical and basolateral surfaces, thus providing an advantage over three-dimensional cultures to study host–pathogen interactions in a controllable and tractable manner. EHEC reduces colonic mucus and affects the brush border cytoskeleton in the absence of commensal bacteria. *(Cell Mol Gastroenterol Hepatol 2016;2:48–62; http://dx.doi.org/10.1016/j.jcmgh.2015.10.001)*

Keywords: Human Colonoid Monolayers; Intestinal Organoids; Microvillar Effacement; Serine Protease EspP.

Shiga toxin–producing enterohemorrhagic *Escherichia coli* (EHEC) is the major disease-causing food borne *E. coli*, with ~73,000 illnesses, ~3000 hospitalizations, and ~500 deaths annually in the United States.[1] In humans, EHEC colonizes the ascending colon and causes watery diarrhea that can progress into hemorrhagic colitis, and in ~10% of patients causes life-threatening extraintestinal complications, including hemolytic uremic syndrome (HUS).[2] There is currently no treatment available for EHEC infections[3] because drugs commonly used to treat bacterial infections, such as antibiotics, antimotility agents, and narcotics, promote HUS development.[4]

The absence of a specific treatment is partially due to the lack of animal or cell culture models that fully recapitulate the disease. Thus far, studies that have used carcinoma-derived human intestinal epithelial cell lines have had low impact due to their transformed phenotype.[5] Animal models have been developed to study the pathogenesis of EHEC infection

Abbreviations used in this paper: **A/E, attaching and effacing; BB, brush border; CCS, cold chelating solution; CM, complete medium; 3D, three dimensional; EHEC, enterohemorrhagic *Escherichia coli*; EM, expansion medium; HCM, human colonoid monolayers; HUS, hemolytic uremic syndrome; IEC, intestinal epithelial cell; LGR5, leucine-rich repeat containing G protein-coupled receptor 5; MLPCDH, mucin-like protocadherin; MUC2, extracellular mucin 2; NHE2, sodium-hydrogen exchanger isoform 2; NHERF3, sodium-hydrogen exchanger regulatory factor 3; PBS, phosphate-buffered saline; PCDH24, protocadherin 24; PCR, polymerase chain reaction; SPATE, serine protease autotransporters of *Enterobacteriaceae*; Stx, Shiga toxins; TBS, Tris-buffered saline; TER, transepithelial electrical resistance; TJ, tight junction.**

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in vivo but do not mimic all aspects of EHEC-induced disease in humans. For example, rabbits exhibit some of the gastrointestinal characteristics of human EHEC-induced disease, including bloody diarrhea, but do not develop HUS.[6]

Upon interaction with the human intestinal epithelium, EHEC implements two major virulence strategies: production of Shiga toxins (Stx) and formation of attaching and effacing (A/E) lesions on enterocytes.[7] A/E lesions are characterized by extensive actin remodeling of the host cell cytoskeleton, leading to effacement of the microvilli and formation of an F-actin pedestal-like structure beneath the bacteria.[8] Improved understanding of the basis for EHEC pathogenicity is of increasing importance given the growing number of outbreaks worldwide. An infection model that recapitulates the human pathophysiology of EHEC infection is necessary for the development of therapeutic strategies for these currently untreatable conditions.

Stx is the main virulence factor in EHEC infection; however, Stx's effects are most potent in the circulatory and renal systems. Other known EHEC virulence factors that act upon the intestinal epithelium include intimin, tir, and the serine protease autotransporters of *Enterobacteriaceae* (SPATE) family. EspP is a major SPATE secreted by EHEC via the type V secretion system at the early stage of infection.[9,10] Although EspP's primary functions in EHEC-induced disease are not well understood, previous studies on the SPATE family have reported that they cause host cytotoxicity and cleave actin-bound cytoskeletal proteins, causing massive actin rearrangement.[11] Using the intestinal epithelial T84 cell model, we have previously shown that recombinant EspP is sufficient to trigger the described actin remodeling.[12] Therefore, we hypothesized that EspP plays a major role in promoting EHEC pathogenicity.

Recent progress in human stem cell biology, particularly the technology to establish and indefinitely propagate an intestinal epithelial culture,[13] opens new possibilities for studying EHEC interaction with human intestinal epithelium, the first step in disease development. These cultures, termed enteroids or colonoids, typically grow as three-dimensional (3D) spheres with the apical surface facing inward. They are not ideal for studying the interaction between luminal gut bacteria and epithelial cells because the lumen is not easily accessible. We and others have recently pioneered human enteroid monolayer cultures grown on Transwell filters in which the apical surface faces outward and the basolateral surface is attached to the filter.[14,15] These human monolayer cultures provide an advantage in studying luminal infections and testing strategies for luminally delivered pharmacologic agents to interfere with intestinal epithelial infections.

Here we report the method for establishing colonoid monolayers derived from the human proximal colon as a model of EHEC infection. We show that extracellular mucin 2 (MUC2) and the brush border (BB) resident protein protocadherin 24 (PCDH24) are initial targets of EHEC during infection. We determined that the EHEC virulence factor EspP, specifically its protease activity, is responsible for PCDH24 reduction. We conclude that human colonoid monolayers (HCM) are a suitable model to study EHEC intestinal colonization and to characterize the molecular mechanisms of host-EHEC interactions.

Materials and Methods

All authors had access to the study data and reviewed and approved the final manuscript. All human tissue was obtained with informed consent from healthy individuals at the Johns Hopkins Hospital and coded with no patient identifiers. This study was approved by the Johns Hopkins institutional review board protocol (NA_0038329).

Reagents and Antibodies

Advanced Dulbecco's modified Eagle medium/Ham's F12, HEPES, GlutaMAX, B27 supplement minus vitamin A, N2 supplement, epidermal growth factor, Alexa Fluor 568 Phalloidin, 4′,6-diamidino-2-phenylindole, Tris-acetate gradient gels, Tris-glycine gradient gels, and monoclonal antibody against occludin were purchased from Life Technologies (Carlsbad, CA). Penicillin/streptomycin was purchased from Quality Biological (Gaithersburg, MD). Matrigel, Cell Recovery Solution, and the Transwell filter inserts were purchased from Corning (Tewksbury, MA). Jagged-1 and [Leu-15] gastrin were purchased from AnaSpec (Fremont, CA). *N*-acetylcysteine, prostaglandin E_2, collagen IV from human placenta, protease inhibitor cocktail, and polyclonal antibodies against PCDH24, GAPDH, and sodium-hydrogen exchanger regulatory factor 3 (NHERF3) were purchased from Sigma-Aldrich (St. Louis, MO). A83-01 [3-(6-methylpyridin-2-yl)-*N*-phenyl-4-quinolin-4-ylpyrazole-1-carbothioamide], Y-27632 [4-[(1*R*)-1-aminoethyl]-*N*-pyridin-4-ylcyclohexane-1-carboxamide], and CHIR99021 [6-[2-[[4-(2,4-dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl) pyrimidin-2-yl]amino]ethylamino]pyridine-3-carbonitrile] were purchased from Tocris (Bristol, UK). Primocin was purchased from Invivogen (San Diego, CA). Polyclonal antibody against sodium-hydrogen exchanger isoform 2 (NHE2) was kindly provided by Dr. C. Ming Tse (Johns Hopkins University, Baltimore, MD). Polyclonal antibody against EspP was kindly provided by Dr. Harris Bernstein (National Institutes of Health, Bethesda, MD). Monoclonal antibody against Muc2 and polyclonal antibody against phosphorylated ezrin were purchased from Abcam (Cambridge, MA). IRDye-conjugated secondary antibodies were purchased from Rockland (Limerick, PA). Wnt3A (American Type Culture Collection, Manassas, VA), R-spondin1 (kindly provided by Dr. Calvin Kuo, Stanford University, Stanford, CA), and Noggin[16] (kindly provided by Dr. Marcel Bijvelds, Erasmus University, Rotterdam, the Netherlands) cell lines were maintained to produce conditioned media.

Generation and Culturing of Colonoid Monolayers

Proximal colonic crypts isolated from tissue resections or biopsies were processed for colonoid generation as described for small intestinal enteroids.[13,14] Briefly, colonic tissue was collected in sterile phosphate-buffered saline (PBS) and kept on ice. The tissue was then transferred into cold chelating solution (CCS; 5.6 mM Na_2HPO_4, 8 mM KH_2PO_4, 96.2 mM NaCl,