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ORIGINAL RESEARCH

Human Fetal-Derived Enterospheres Provide Insights on Intestinal Development and a Novel Model to Study Necrotizing Enterocolitis (NEC)

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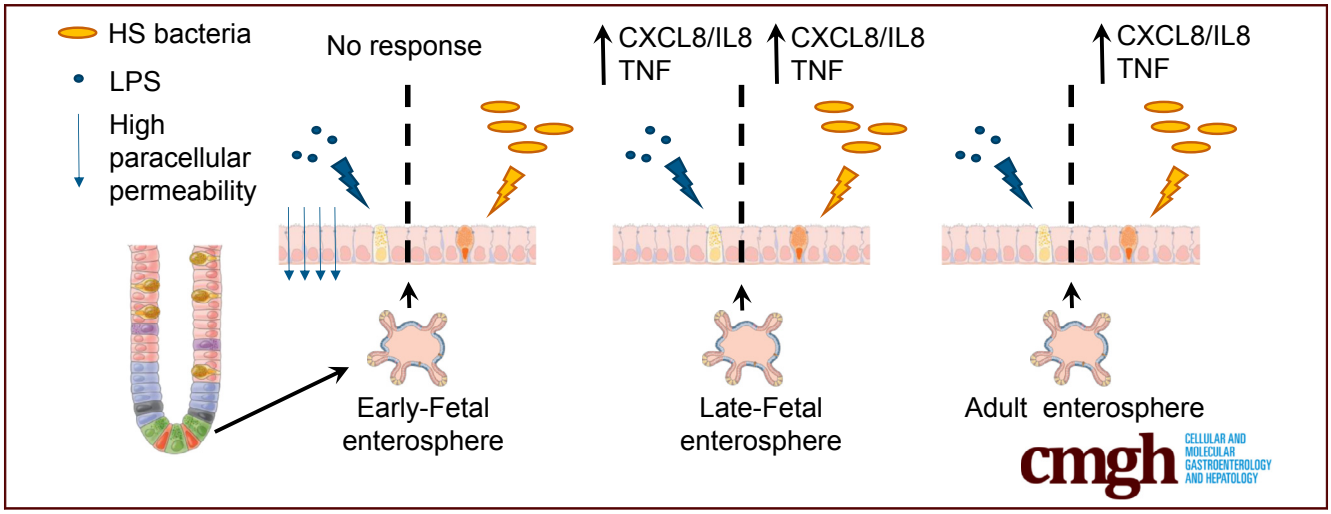

SUMMARY

We established enterospheres from human fetuses ranging from 11 to 22.5 weeks' gestational age. Developmentally regulated differences in maturation, barrier, and innate immunity were found across the samples. Fetal enterospheres can be used as a model for necrotizing enterocolitis pathogenesis.

BACKGROUND & AIMS: Untreated necrotizing enterocolitis (NEC) can lead to massive inflammation resulting in intestinal necrosis with a high mortality rate in preterm infants. Limited access to human samples and relevant experimental models have hampered progress in NEC pathogenesis. Earlier evidence has suggested that bacterial colonization of an immature and developing intestine can lead to an abnormally high inflammatory response to bacterial bioproducts. The aim of our study was to use human fetal organoids to gain insights into NEC pathogenesis.

METHODS: RNA sequencing analysis was performed to compare patterns of gene expression in human fetal-derived enterospheres (FEnS) and adult-derived enterospheres (AEnS). Differentially expressed genes were analyzed using computational techniques for dimensional reduction, clustering, and gene set enrichment. Unsupervised cluster analysis, Gene Ontology, and gene pathway analysis were used to predict differences between gene expression of samples. Cell monolayers derived from FEnS and AEnS were evaluated for epithelium function and responsiveness to lipopolysaccharide and commensal bacteria.

RESULTS: Based on gene expression patterns, FEnS clustered according to their developmental age in 2 distinct groups: early and late FEnS, with the latter more closely resembling AEnS. Genes involved in maturation, gut barrier function, and innate immunity were responsible for these differences. FEnS-derived monolayers exposed to either lipopolysaccharide or commensal *Escherichia coli* showed that late FEnS activated gene expression of key inflammatory cytokines, whereas early FEnS monolayers did not, owing to decreased expression of nuclear factor-κB–associated machinery.

CONCLUSIONS: Our results provide insights into processes underlying human intestinal development and support the use of FEnS as a relevant human preclinical model for NEC. Accession number of repository for expression data: GSE101531. *(Cell Mol Gastroenterol Hepatol 2018;5:549–568; https://doi.org/10.1016/j.jcmgh.2018.01.014)*

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See editorial on page 651.

Necrotizing enterocolitis (NEC) is the most frequent cause of death in premature infants in North America,[1,2] affecting more than 10% of premature babies weighing less than 1500 g, with an average cost of US $500,000 per patient.[3] NEC is characterized by severe inflammation of the gastrointestinal tract, leading to extensive tissue necrosis.[4] Despite several decades of basic and clinical research into NEC, the mortality rate and disease management has not changed appreciably over time. Currently, there is no Food and Drug Administration–approved treatment protocol to manage the disease,[5] with the exception of providing the infant with mother's expressed breast milk.[6] Nonetheless, in the past decade, intensive research efforts using techniques such as animal models, fetal intestinal xenograft transplants, fetal intestinal organ cultures, and a fetal primary intestinal cell line have shown that an abnormal response to gut-colonizing bacteria seems to contribute to NEC susceptibility.[7–9] In particular, the high incidence of NEC among very premature infants implicates intestinal immaturity as an additional risk factor.[10] Studies have shown that the immature human enterocyte reacts to colonizing intestinal bacteria with an enhanced inflammatory response.[11–13] Toll-like receptors (TLRs) have been implicated as key molecules in promoting inflammation.[14] In particular, TLR4 has been found to be up-regulated on the fetal enterocyte surface.[15] Similarly, other signaling factors connecting TLR4 to nuclear factor-κB (NF-κB) and activator protein transcription factor–mediated inflammation were found to be up-regulated as well, whereas genes that inhibited these signaling pathways were down-regulated.[11,16] Together this evidence suggests that an exaggerated innate immune response to colonizing commensal bacteria mediated by TLR activation is mounted by immature intestinal epithelial cells, which could contribute to the pathogenesis of NEC.

A major roadblock in determining the pathogenesis of NEC is limited access to fetal human tissues for experimental studies. Newly established techniques[17–19] creating enteroids from human intestine are a promising tool for the development of a patient-derived in vitro model.[20–22] Enteroids, which are primary cultures generated from intestinal epithelial stem cells, can be used to study the epithelial component of several chronic inflammatory diseases involving the intestinal mucosa.[17,23] In this study, our aim was to generate organoids across the fetal age spectrum to determine specific regulated differences in fetal intestinal development related to the onset of NEC. We compared gene expression of the fetal enteroids (FEnS) with gene expression from enterospheres that we generated from biopsy specimens of adult intestine (AEnS) obtained during clinically indicated endoscopies. Observations made by comparing early and late fetal enterospheres with adult enterospheres are described and the potential for this technique for further studies is discussed.

Materials and Methods

Derivation of FEnS and AEnS From Fetal Intestine

Human sample collection and procedures were approved by institutional review board protocols 1999P003833 (Brigham and Women's Hospital, Boston, MA) and 2016P000949 (Massachusetts General Hospital, Boston, MA) for the derivation of FEnS and AEnS, respectively. Based on these institutional review board–approved protocols, we have pledged not to share the generated material (FEnS and AEnS).

The isolation of intestinal epithelial cells was performed according to previously published protocols[17,24] with minor modifications.

For fetal enterospheres (FEnS), intestinal fragments were collected from aborted fetuses and cut into small pieces. For adult enterospheres (AEnS), 4 biopsy specimens were collected from the duodenum of patients undergoing upper endoscopy (esophagogastroduodenoscopy) for other clinical evaluations who also consented to participate in the study. Both fetal-derived intestinal fragments and biopsy specimens were washed once in cold phosphate-buffered saline (PBS) (ThermoFisher Scientific, Waltham, MA). PBS was replaced with a dissociation buffer containing PBS, penicillin/streptomycin, 1 mmol/L dithiothreitol (Sigma-Aldrich, St. Louis, MO), and 0.5 mmol/L EDTA (Sigma-Aldrich). Intestinal fragments were incubated at 4°C for 30 minutes and then vigorously shaken to promote epithelium dissociation from the basal membrane. This procedure was repeated at least 3 times to collect multiple fractions. Supernatants containing intestinal crypts were processed further and plated in Matrigel as described in previous research.[24] Stem cell media was prepared according to previously published methods with minor modifications.[19,25] Intestinal stem cell media composition was as follows: 500 mL Dulbecco's modified Eagle medium (DMEM)/F12 11330-032, 5 mL penicillin/streptomycin 15140122, 5 mL nonessential amino acids 11140-050, 5 mL sodium pyruvate 11360-070, 5 mL N-2 17502, and 10 mL B-27 17504044 (all purchased from ThermoFisher Scientific); 50 mL fetal bovine serum F4135, 1 mmol/L acetylcysteine A9165, and 10 nmol/L gastrin G9145

§Authors share co-senior authorship.

Abbreviations used in this paper: **AD, adult duodenal; AEnS, adult-derived enterospheres; CLDN, claudin; ΔΔCT, relative threshold cycle; CXCL, chemokine (C-X-C motif) ligand; DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; FDR, false discovery rate; FEnS, fetal-derived enterospheres; FITC, fluorescein isothiocyanate; HIO, human intestinal organoid; HS, *Escherichia coli* human commensal isolate; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide A; MAMP, microbe-associated molecular pattern; NEC, necrotizing enterocolitis; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGE2, prostaglandin E2; RPKM, reads per kilobase of transcript per million; RT-PCR, reverse-transcription polymerase chain reaction; TEER, transepithelial electrical resistance; TLR, Toll-like receptor; TNF, tumor necrosis factor; WAE, wound-associated epithelial cells.**

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