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Genes regulating tight junctions and cell adhesion are altered in early experimental necrotizing enterocolitis

Niclas Högberg[a,*], Anders Stenbäck[a], Per-Ola Carlsson[b,c], Alkwin Wanders[d,1], Helene Engstrand Lilja[a,1]

[a]*Department of Women's and Children's Health, Division of Pediatric Surgery, Uppsala University, Sweden*
[b]*Department of Medical Cell Biology, Uppsala University, Sweden*
[c]*Department of Medical Sciences, Uppsala University, Sweden*
[d]*Department of Immunology, Genetics and Pathology, Uppsala University, Sweden*

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Abstract

Background/purpose: Necrotizing enterocolitis (NEC) represents one of the gravest complications in preterm infants and carries significant morbidity and mortality. Increased intestinal permeability may play an important role in the pathogenesis of NEC. In this study we investigated the genes regulating structural proteins such as tight junctions (TJ) and cell adhesion in a neonatal rat model of early NEC.

Methods: The studies were performed on Sprague-Dawley rat pups. Experimental NEC was induced using hypoxia/re-oxygenation treatment on day 1 after birth. Intestinal specimens from the ileum were obtained, mRNA was purified, and the transcriptome was analyzed using microarray.

Results: We found several TJ genes such as claudins 1, 8, 14, 15, and gap junction protein to be affected. Alterations in genes involved in the inflammatory response was confirmed, along with several genes regulating proteins used as biomarkers for NEC.

Conclusion: This study indicates that tight junctions and cell adhesion may play a critical role in the pathogenesis of early experimental NEC. Better understanding of the pathogenesis of NEC may lead to novel strategies for the prevention and treatment of NEC.

Necrotizing enterocolitis (NEC) is the most serious gastrointestinal disorder in newborns with an incidence of 10–15% in very low birth weight infants (VLBW < 1500 g). More than 90% of the infants who develop NEC are born preterm and the risk is inversely related to gestational age and birth weight [1]. Despite decades of research, NEC remains a major cause of death in neonates with a mortality rate of 15–40% in VLBW infants [2,3]. The morbidity among those infants who require surgical intervention (20–40%) is significant and includes poor neurodevelopmental outcome and complications such as short bowel syndrome [2,4]. Even if the pathophysiology of NEC still remains unclear, current evidence suggests a multifactorial cause [1]. Prematurity is the main risk factor, presumably owing to

* Corresponding author. Division of Pediatric Surgery, Uppsala University Children's Hospital, SE-751 85 Uppsala, Sweden. Tel.: +46 186115901; fax: +46 186115905.
E-mail address: niclas.hogberg@kbh.uu.se (N. Högberg).
[1] These authors contributed equally to this work.

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immaturity of gastrointestinal motility, intestinal barrier function and immune defense. Other contributing factors are thought to be genetic predisposition, enteral feeding, intestinal hypoxia/ischemia and colonization with pathogenic bacteria [2].

The disease is characterized by inflammation of the bowel that can progress to bowel necrosis, sepsis and multiple-organ failure. The inflammatory reactions in NEC have been studied previously [2,3,5].

Tight junctions (TJ) and their structural integrity play a major role in maintenance of the gut barrier and therefore to the defence against microbes. A decreased expression of TJ proteins has been linked to an increased intestinal permeability in NEC and other inflammatory intestinal diseases [6–9].

The main purpose of this study was to investigate genes regulating tight junctions and cell adhesion in a neonatal rat model of early NEC used in our previous work [10]. Genes involved in the inflammatory response and several genes regulating proteins used as biomarkers for NEC were also studied.

1. Materials and methods

1.1. Animals

Time-pregnant Sprague-Dawley rats were obtained on day 15 of gestation. The rats were purchased from Charles River (Charles River GmbH, Sulzfeld, Germany). They were housed in the animal department at Uppsala Biomedical Center, and kept at +22ºC, with a 12-hour light/dark cycle, fed standard pellet food and water ad libitum. On day 21, the rat pups were delivered vaginally. The rat pups were randomized to either induced experimental NEC (N = 5) or to serve as controls (N = 2).

The study was approved by the regional animal ethics committee, and performed in accordance with the *Guide for the Care and use of Laboratory Animals*, published by the National Research Council in 1996.

1.2. Induction of NEC

The rat pups were treated on the first day after delivery. They were stressed with hypoxia/re-oxygenation, breathing 100% carbon dioxide for 10 minutes, followed by 100% oxygen for another 10 minutes. The rats were then returned to their mothers' cages and allowed ad libitum nursing of maternal milk.

1.3. Surgical procedure and RNA isolation

On the second day, all animals were sacrificed by neck dislocation. A laparotomy was performed and intestinal specimens were taken from the distal part of the small bowel. The intestinal specimens were immediately placed in RNAlater solution (Qiagen GmbH, Hilden, Germany) and stored at +6°C over night. Extraction of mRNA was performed using RNeasy Mini kit (Qiagen GmbH) according to the manufacturer's protocol.

1.4. Microarray analysis

RNA concentration was measured with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc., Palo Alto, CA, USA); 250 ng of total RNA from each sample was used to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the Ambion WT Expression Kit (P/N 4425209 Rev B 05/2009) and Affymetrix GeneChip WT Terminal Labeling and Hybridization User Manual (P/N 702808 Rev. 1, Affymetrix Inc., Santa Clara, CA, USA). GeneChip ST Arrays (GeneChip Rat Gene 1.0 ST Array) were hybridized for 16 hours in a 45 °C incubator, rotated at 60 rpm. According to the GeneChip Expression Wash, Stain and Scan Manual (PN 702731 Rev 2, Affymetrix Inc.) the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip® Scanner 3000 7G.

The Affymetrix GeneChip Rat Gene 1.0 ST Array is a whole-transcript high performance assay, providing high resolution and high accuracy, without additional need for technical validation [11,12].

1.5. Microarray data analysis

Subsequent analysis of the gene expression data was carried out in the freely available statistical computing language R (http://www.r-project.org) using packages available from the Bioconductor project (www.bioconductor.org). The raw data was normalized using the robust multi-array average (RMA) method first suggested by Li and Wong in 2001 [13,14]. In order to search for the genes expressed differentially in the NEC samples compared to the control samples, an empirical Bayes moderated t-test was then applied [15], using the "limma" package [16]. To address the problem of multiple testing, the *p*-values were adjusted using the method of Benjamini and Hochberg [17].

$P < 0.01$ was considered statistically significant for all comparisons.

1.6. Morphology

Intestinal specimens were taken from the distal part of the ileum. The specimens were immediately fixed in 4% formaldehyde solution, further processed and embedded in paraffin. Sections of 5 μm were stained with hematoxylin and eosin, and examined by light microscopy. The specimens were evaluated with respect to the degree of ischemic injury and mucosal integrity.