Research Article

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Activated intestinal macrophages in patients with cirrhosis release NO and IL-6 that may disrupt intestinal barrier function☆

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Background & Aims: Bacterial infections commonly occur in decompensated cirrhosis resulting from bacterial translocation from the intestine. We studied the role of intestinal macrophages and the epithelial barrier in cirrhosis.

Methods: Forty-four patients with NASH/ASH cirrhosis (decompensated n = 29, compensated n = 15) and nineteen controls undergoing endoscopy were recruited. Serum was obtained and LPS and LBP levels determined. Intestinal macrophages were characterized by flow cytometry, immunohistochemistry, and nitric oxide (NO) production measured in supernatant of cultured duodenal samples. Quantitative RT-PCR was performed on duodenal biopsies assessing 84 inflammatory genes. Protein levels of cytokines/chemokines were assessed in serum and supernatant. The duodenal wall was assessed by electron microscopy, tight junction protein expression determined by RT-PCR, immunohistochemistry, and Western blot and, functional analysis performed by transepithelial resistance measurement and permeability studies.

Results: Increased plasma LPS, LBP levels and higher numbers of duodenal $CD33^+/CD14^+/Trem\text{-}1^+$ macrophages, synthesizing iNOS and secreting NO were present in decompensated cirrhosis. Upregulation of IL-8, CCL2, CCL13 at the transcriptional level, and increased IL-8, and IL-6 were detected in supernatant and serum in cirrhosis. IL-6 and IL-8 co-localised with $iNOS^+$ and $CD68^+$, but not with $CD11c^+$ cells. Electron microscopy demonstrated an intact epithelial barrier. Increased Claudin-2 was detected by Western blot and immunohistochemistry, while decreased transepithelial resistance and increased duodenal permeability were detected in decompensated cirrhosis.

Conclusions: Our study shows the presence of activated $CD14^{+-}$ $Trem\text{-}1^+iNOS^+$ intestinal macrophages, releasing IL-6, NO, and increased intestinal permeability in patients with cirrhosis, suggesting that these cells may produce factors capable of enhancing permeability to bacterial products.

Keywords: Bacterial translocation; Intestinal macrophages; Epithelial barrier; IL-6; Nitric oxide; Cirrhosis.

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Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; LBP, lipopolysaccharide-binding protein; IHC, immunohistochemical staining; CD14, cluster of differentiation 14; TREM-1, triggering receptor expressed on myeloid cells-1; iNOS/NOS2, inducible nitric oxide synthase 2; IL-8, interleukin 8; CCL2/MCP-1, chemokine (C-C motif) ligand 2; CCL13, chemokine (C-C motif) ligand 13; IL-6, interleukin 6; IBD, inflammatory bowel disease; NEC, necrotising enterocolitis; TNF-α, tumor necrosis factor alpha; TEER, transepithelial resistance; TJ, tight junction.

Introduction

Bacterial infections occur commonly in decompensated cirrhosis, are associated with translocation from the intestine and impact early and late mortality [1]. The natural history of cirrhosis is also altered by circulating bacterial DNA, which even in the absence of culture positive infections, increases the risk of variceal bleeding, hepatic decompensation, and hepatorenal syndrome [2,3].

The reason why the gut epithelial barrier fails in cirrhosis, facilitating translocation of bacterial products and DNA, remains poorly understood. In health, it provides an effective barrier to micro-organisms, but is simultaneously semi-permeable, allowing nutrient absorption [4]. It consists of enterocytes interconnected by tight and gap junctions. Tight junctions (TJ),

composed of various proteins, are essential elements maintaining structural integrity and regulating permeability [5]. The expression and turnover of TJ proteins are influenced by inflammation and oxidative stress [6,7].

Intestinal macrophages localised within the lamina propria provide the first line of defence to micro-organisms breaching the epithelial barrier. In health, these cells are characterized by a specific phenotype, $CD33^{+}CD14^{-}$, are highly anergic and do not produce pro-inflammatory cytokines in response to lipopolysaccaride (LPS) [8,9]. In IBD, however, intestinal macrophages express innate response receptors such as $CD14^{+}$, TREM-1, and release pro-inflammatory cytokines [10]. Activated $CD14^{+}$ macrophages in necrotising enterocolitis produce nitric oxide (NO) that impairs endothelial repair [11,12]. We have shown that macrophages activation in HIV correlated with bacterial translocation and persistent immune activation [13].

We hypothesized that similar to other inflammatory states, intestinal macrophage activation occurs in cirrhosis. The aims of our study were consequently to determine the intestinal macrophage phenotype in decompensated cirrhosis and whether these macrophages are capable of modulating permeability.

Patients and methods

Study population

Patients referred to the Interventional endoscopy unit, Pretoria East, between January 2008 and February 2011, were considered for this study. Cirrhosis was diagnosed by standard clinical, ultrasonographical, and/or histological criteria. Patients with confirmed NASH or alcoholic cirrhosis were included. Decompensated cirrhosis was defined as new onset ascites with or without variceal bleeding, encephalopathy or jaundice. Compensated cirrhosis was defined as patients without ascites, encephalopathy, history of variceal bleeding or a previous episode of decompensation. The protocol was approved by the University of Pretoria, Ethics committee. Written informed consent was obtained from each patient or their legal representative.

Inclusion criteria were age 18–80 years and confirmed NASH or alcoholic cirrhosis. Exclusion criteria were: severe sepsis or SIRS with circulatory dysfunction, hepatocellular carcinoma, portal vein thrombosis, cardiac, renal or respiratory failure, previous luminal gastrointestinal surgery, antibiotic therapy or alcohol use in the preceding 6 weeks. Patients included underwent oesophagogastroduodenoscopy for variceal screening and duodenal biopsies were taken. Patients in the control group underwent endoscopy due to reflux/dyspepsia symptoms. The study population consisted of decompensated (N = 29), compensated (N = 15) NASH/ASH cirrhosis, and controls (N = 19). In addition, 9 patients with ASH/NASH cirrhosis undergoing endoscopy for varices screening were recruited from the liver clinic University hospital, Gasthuisberg, Leuven, (Ethics protocols ML6697, ML8081), for TEER and permeability experiments, and the results compared to a control group.

Biochemistry

Blood samples were collected from a peripheral vein into sterile or endotoxin free tubes, centrifuged, and plasma stored at −80 °C until analysis. Analysis included standard full blood count, liver function tests, INR, CRP.

Plasma LPS and LBP levels

Circulating endotoxin (LPS) and lipopolysaccharide-binding protein (LBP) levels were analysed in duplicate in 96-well plates according to the manufacturer's instructions, Limulus Amoebocyte Lysate (LAL) assay QCL-1000 (Lonza, Valais Switzerland); LBP (Human) Elisa kit (Abnova, Taipei, Taiwan). The lower limit of detection for each assay is LPS = 0.1 EU/ml and LBP = 5 ng/ml (Appendix A).

Tissue samples

Biopsies were obtained from the third part of the duodenum at endoscopy. Biopsies for flow cytometry were placed in cold RPMI 1640. Histological specimens were fixed in 10% formalin for light microscopy and 2.5% glutaraldehyde-formaldehyde for transmission electron microscopy (TEM). Biopsies for gene expression and Western blot were snap frozen and stored at −80 °C. Biopsies for short-term culture studies were placed in cold, sterile PBS, and for TEER and permeability experiments in cold Hank's buffer.

Isolation of mucosal mononuclear cells (MMCs)

A single-cell suspension was obtained by means of GentleMACS dissociator (MiltenyiBiotec, Gladbach, Germany) according to the manufacturer's protocol (Appendix B).

Determination of macrophage phenotype

The phenotype of intestinal macrophages was determined assessing a panel of surface markers, characteristic of monocyte/macrophage lineage (CD33), activation status (CD14, CD16, Trem-1), and co-stimulatory molecules (CD80, CD86). In addition, we assessed the surface expression of the toll-like receptor 2 and 4 (TLR-2, and 4). Single-cell preparations in PEB buffer were stained with 20 μl monoclonal antibodies/100 μl of 10^{6} cells in two-colour combinations (Appendix C).

Gene expression

Following total RNA extraction (RNeasy and RNase-Free DNase kits, Qiagen, Hilden, Germany), RNA quantity and quality were confirmed by Nanodrop ND1000 (Thermo Scientific, DE, USA) and Experion™ (Bio-Rad) analysis, respectively. cDNA was synthesized from 2.0 μg of total RNA using the RT^{2} PCR array first strand kit (SABioscience, Frederick, MD). The expression levels of 84 general inflammatory genes (Appendix D) were assessed by semi-quantitative RT-PCR in 96-well plates using RT^{2} SYBR Green qPCR Master Mix and a CFX96 RT-PCR Detection System (Bio-Rad, Hercules, CA). Fifteen decompensated cirrhotics and five controls were analysed. Data was normalized using five different housekeeping genes and analysed by the comparative cycle threshold method (REST 2009 V2.0.13 software Qiagen, Hilden, Germany).

Quantitative RT-PCR was then performed of 27 upregulated genes and alternative inflammatory pathways not included in the screening assay, in combination with the two most stable housekeeping genes as determined by geNorm software. (Appendix E). Fourteen decompensated cirrhotics, seven compensated cirrhotics and six controls were analysed in triplicate.

Duodenal biopsy cultures

Biopsy specimens were placed in PBS, washed, weighed and incubated in RPMI 1640 containing 10% fetal calf serum (Sigma), 10 μl/ml Pen/Strep Amphotericin B (Cambrex, Walkersville, MD) and 1 μl/ml gentamicin (Genta50, Phenix Pharmaceutials, Belgium) at 37 °C, in humidified 5% CO_2 for 48 h. Supernatants were stored at −70 °C until further analysis.

Nitric oxide determination in culture supernatant

Biopsy samples were incubated with and without LPS (1 μg/ml, Sigma). Total nitrite and nitrate in supernatant were determined by Total Nitric Oxide and Nitrite/Nitrate Assay (R&D Systems, Minneapolis, USA).

Cytokine determination

Cytokine and chemokine levels in plasma and biopsy culture supernatants, were quantified using a customized Bio-Plex Pro™ assay assessing IL-8, CCL2, IL-10, IL-6, TNFα, according to manufacturer's protocol (Bio-Rad, Hercules, CA).

Histopathological and ultrastructural analysis of the duodenal wall

Histological analysis was conducted by a pathologist blinded to patient subgroups. Immunohistochemical staining for iNOS, CD14, CD68, CD11c, IL-6, IL-8, and Claudin-2 was performed. Double staining experiments with dye swap were