Research Article

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Role of interleukin 10 in norfloxacin prevention of luminal free endotoxin translocation in mice with cirrhosis

Isabel Gómez-Hurtado[1,2], Alba Moratalla[1,2], Ángela Moya-Pérez[3], Gloria Peiró[4], Pedro Zapater[1,5], José M. González-Navajas[1,2], Paula Giménez[1], José Such[1,6], Yolanda Sanz[3], Rubén Francés[1,2,6,*]

[1]*CIBERehd, Instituto de Salud Carlos III, Madrid, Spain;* [2]*Unidad Hepática, Hospital General Universitario de Alicante, Alicante, Spain;* [3]*Ecología Microbiana y Nutrición, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (IATA-CSIC), Valencia, Spain;* [4]*Servicio de Anatomía Patológica, Hospital General Universitario de Alicante, Alicante, Spain;* [5]*Unidad de Farmacología Clínica, Hospital General Universitario de Alicante, Alicante, Spain;* [6]*Departamento de Medicina Clínica, Facultad de Medicina, Universidad Miguel Hernández, San Juan de Alicante, Spain*

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Background & Aims: Bacterial endotoxin is present in patients with advanced cirrhosis and can induce an immunogenic response without an overt infection. Norfloxacin is a gram-negative bactericidal drug able to maintain low endotoxin levels and stimulate IL-10 production. We aimed at investigating the role of IL-10 in decreasing endotoxin absorption in cirrhotic mice treated with norfloxacin.

Methods: Cirrhosis was induced by carbon tetrachloride or bile duct ligation in wild type and IL10-deficient mice with or without norfloxacin prior to an intragastrical administration of *E. coli, K. pneumonia* or *E. faecalis*. Spontaneous and induced bacterial translocation, free endotoxin and cytokine levels were evaluated in mesenteric lymph nodes. Intestinal permeability was followed by fluorimetry and barrier integrity markers were measured in disrupted intestinal samples. The inflammatory-modulating mechanism was characterized in purified intestinal mononuclear cells.

Results: Norfloxacin reduced spontaneous and induced MLN positive-cultures in wild type and *IL-10*-deficient animals. However, reduction of free endotoxin levels was associated with norfloxacin in wild type but not in *IL-10*-deficient mice. Wild type but not *IL-10*-deficient mice treated with norfloxacin significantly normalized intestinal permeability and improved gut barrier integrity markers. The toll-like receptor 4-mediated pro-inflammatory milieu was modulated by norfloxacin in a concentration-dependent manner in cultured intestinal mononuclear cells of wild type mice but not of *IL-10*-deficient mice. The restoration of IL-10 levels in *IL-10*-deficient animals reactivated the norfloxacin effect on inflammatory-modulation, gut barrier permeability, and luminal endotoxin absorption.

Conclusion: Norfloxacin not only reduces gram-negative intestinal flora but also participates in an IL-10-driven modulation of gut barrier permeability, thus reducing luminal free endotoxin absorption in experimental cirrhosis.

Keywords: Cirrhosis; Endotoxin; Norfloxacin; Interleukin 10.

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* Corresponding author. Address: CIBERehd-Liver Unit, Hospital General Universitario de Alicante. Avda, Pintor Baeza 12, 03010 Alicante, Spain. Tel.: +34 965 913 928; fax: +34 965 913 922.

E-mail address: frances_rub@gva.es (R. Francés).

Introduction

The process by which bacteria exit the intestinal lumen, access the mesenteric lymph nodes (MLN) and colonize other organs is known as bacterial translocation (BT) [1], and is considered as the key spontaneous bacterial peritonitis (SBP)-leading mechanism in patients with advanced cirrhosis. However, not only viable bacteria but also their products translocate and induce a similar inflammatory response to that developed against SBP in the absence of an overt infection [2]. The presence of these enteric bacterial products such as DNA and lipopolysaccharide (LPS) in the bloodstream represents a threat that may compromise patients' clinical outcome, as reported in studies revealing the association between their presence and a marked hemodynamic derangement [3,4], and others identifying LPS-binding protein and bacterial-DNA as independent predictors of severe bacterial infections [5] and mortality [6], respectively. Therefore, the management of bacterial products trespassing the intestinal barrier to the systemic circulation is of utmost relevance.

Norfloxacin has been shown to effectively reduce the incidence of bacterial translocation when used as secondary prophylaxis of SBP [7,8], and also to reduce non-infectious related clinical complications, such as hepatorenal syndrome, when administered as primary prophylaxis [9]. The selective decontamination of intestinal gram-negative microbiota with norfloxacin generates a temporal overload of free endotoxin in the

intestinal lumen and other bacterial products that may translocate into the blood. Nevertheless, norfloxacin is associated with reduced serum free endotoxin levels in cirrhosis and has been reported to modulate as well patients' pro-inflammatory reaction, showing a direct effect on neutrophil response to oxidative stress, reducing the secretion of reactive oxygen species and increasing the apoptosis rate [10].

IL-10 signalling induces the expression of heme oxygenase (HO)-1, a stress-inducible protein that prevents ethanol-induced inflammation in the intestine [11] and liver [12]. HO-1 correlates with intracellular levels of norfloxacin in cultured cells of cirrhotic patients on selective intestinal decontamination in response to LPS [13]. Therefore, we hypothesized that IL-10 may be involved in the reduced luminal endotoxin absorption induced by norfloxacin.

To investigate this, we studied luminal free endotoxin absorption in spontaneous and induced BT through the intragastrical administration of bacteria in wild type (WT) and IL10-deficient ($IL10^{-/-}$) cirrhotic mice treated with norfloxacin. The results were controlled with the administration of a recombinant IL-10 protein to restore IL-10 levels in $IL10^{-/-}$ mice.

Methods

Animals and study design

Female Balb/c WT and $IL10^{-/-}$ mice (Harlan, Barcelona, Spain) were included in a 16-week study protocol. Mice were caged at a constant room temperature of 21 °C and exposed to a 12:12 light/dark cycle. Adult mice were fed standard rodent chow and treated with 0.25 mmol/L phenobarbital in tap water along study protocol. Animals received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. The study was approved by the Animal Research Committee of Universidad Miguel Hernandez (Alicante, Spain).

After 4 weeks, animals were subjected to experimental cirrhosis induction either with two weekly, weight-controlled doses of CCl_4 intragastrically administered [14], performing laparotomies under anaesthesia with isoflurane at week 16, or with bile duct ligation (BDL) surgery [15], performing laparotomies 4 weeks after BDL.

One week prior to laparotomy, WT and $IL10^{-/-}$ animals received norfloxacin (5 mg/kg intragastrically/daily; Sigma-Aldrich, Madrid, Spain) or placebo. A subgroup of $IL10^{-/-}$ animals also received a non-lytic interleukin-10/Fc Chimera fusion protein (1000 units i.p./every two days; Sigma-Aldrich). Twenty-four hours before laparotomy animals were distributed in spontaneous or induced BT subgroups as shown in Supplementary Fig. 1. Induced BT subgroups received *E. coli* (serotype 0111:B4), *K. pneumoniae* or *E. faecalis* (10^7 cfu/intragastrically). All detectable MLNs were aseptically removed (4–5 MLNs/mouse without differences between groups), sonicated and liquefied for bacterial culture, DNA isolation and sequencing analysis [16,17]. We inoculated 100 μL of liquefied MLNs on MacConkey agar (BioMerieux, Marcy l'Etoile, France). Cultures were incubated in 5% CO_2 at 37 °C and examined daily for visible growth. An additional subset of mice was euthanized at week 0 to serve as naïve controls.

Animals under anaesthesia were euthanized by heart punction and total blood collection. Liver, spleen, intestinal wall from ileum (10 cm) and blood were collected. The liver was perfused *in situ* with HBSS without Ca^{2+} and Mg^{2+} at 37 °C. This was followed by perfusion with HBSS containing 100 mM $CaCl_2$ solution at the same perfusion rate. The liver was then removed and rinsed with HBSS.

Isolation and culture of intestinal mononuclear cells

The whole small intestine was removed from CCl_4 and BDL mice. Intraepithelial and lamina propria cells were isolated as previously described [18]. Resuspended cells (0.5×10^6 cells/well) were cultured for 24 h at 37 °C and 5% CO_2 without stimuli or with norfloxacin 0.5, 1, 2, 4, and 8 μg/μl. After that period, LPS 25 μg/ml (*E. coli* serotype 0111:B4; Sigma) was added to all wells and cells were cultured for an additional 24 h period. Cells and supernatants were collected and stored at −20 °C until use.

Histological analysis

Liver biopsy specimens, 10–15 mm in size, were fixed in buffered formalin and embedded in paraffin. Histological changes were first evaluated by routine H&E in 4 μm thick sections. Hepatic fibrosis and architectural distortion was estimated with the connective tissue stain Masson-trichrome. The amount of fibrosis was blindly assessed semiquantitatively based on the Ishak score [19], using a conventional light microscope (Olympus BX50, Barcelona, Spain). A morphometric analysis of the fibrotic area was blindly performed using the ImageJ software (http://rsbweb.nih.gov/ij/).

Immunohistochemistry

Immunohistochemistry was performed in serial sections of paraffin-embedded liver tissue. We used heat induced antigen retrieval before exposure to the primary antibody (monoclonal mouse anti-human alpha–smooth muscle actin (α-SMA); clone 1A4; ready-to-use) (Dako/Agilent Technologies; Carpinteria, CA). The staining was performed using the EnVision™ Flex detection system and an Autostainer Link 48 (Dako/Agilent Technologies). As a negative control, staining was carried out in the absence of the primary antibody.

FITC-LPS permeability assay

Intestinal permeability was evaluated administering fluorescein isothiocyanate (FITC)-LPS from *E. coli* O111:B4 (Sigma) by gastric gavage 4 h before sacrifice. Whole blood was obtained by cardiac puncture at the time of killing. Dilutions of FITC-LPS in PBS were used as standard curve and FITC-LPS measurements were performed in 100 μl of plasma or standard in a fluorimeter at 488 nm.

Tissue lysates and gene expression analysis

Total cellular RNA was isolated from 20 to 30 mg of liver and 10 cm of intestinal wall disrupted by sonication. Gene expression levels of *collagen α-1*, tumour growth factor beta (*TGF-b*)-1, tissue inhibitor of metalloproteinase (*TIMP*)-1 and matrix metalloproteinase (*MMP*)-2 in liver and *IL10*, *IL10R*, *HO-1*, *Bcl-3*, *TNF-α*, Toll-like receptor (*TLR*)-4, chemokine (C-C Motif) receptor 7 (*CCR7*), chemokine (C-X-C Motif) ligand 11 (*CXCL11*), chemokine (C-C Motif) ligand 18 (*CCL18*) in cultured intestinal mononuclear cells were determined. *Occludin* and *TJP-1* were evaluated in intestinal samples. Primer-pair sequences used are listed in Supplementary Table 1.

ELISAs

The quantitative chromogenic limulus amebocyte lysate test (BioWhittaker, Nottingham, UK) was performed to evaluate endotoxin levels according to the manufacturer's instructions. IL-10 (R&D Systems, Minneapolis, MN), occludin (Antibodies Online, Aachen, Germany) and TJP-1 (BioMedical Assay, Shanghai, China) assays were performed after sonication in disrupted intestinal and liver samples.

Statistical analysis

Continuous variables are reported as mean ± standard deviation and categorical variables as frequency or percentages. Quantitative data were analysed using the Mann-Whitney U test for simple comparisons or the Kruskal-Wallis test followed by pairwise comparisons using the Mann-Whitney U test with the *post-hoc* Bonferroni correction for multiple comparisons. Differences in qualitative variables were analysed using the χ^2 test. Bivariate correlations between continuous variables were calculated using the Spearman test. All reported p values are 2-sided, and p values lower than 0.05 were considered to indicate significance. All calculations were performed using the IBM SPSS Statistics 19.

Results

Characteristics of animals

A total of 100 WT and 170 $IL10^{-/-}$ mice started the protocol for cirrhosis induction with CCl_4. During the 16-week protocol, 22