Research Article

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Prevention of acute kidney injury in a rodent model of cirrhosis following selective gut decontamination is associated with reduced renal TLR4 expression

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Background & Aims: Superimposed infection and/or inflammation precipitate renal failure in cirrhosis. This study aimed at testing the hypothesis that increased gut bacterial translocation in cirrhosis primes the kidney to the effect of superimposed inflammation by upregulating expression of Toll-like receptor 4 (TLR4), NFκB, and cytokines. A well-characterized bile-duct ligated (BDL) model of cirrhosis, which develops renal failure following superimposed inflammatory insult with lipopolysaccharide (LPS), was used and selective gut decontamination was performed using norfloxacin.

Methods: Sprague–Dawley rats were studied: Sham, Sham + LPS; BDL, BDL + LPS; an additional BDL and BDL + LPS groups were selectively decontaminated with norfloxacin. Plasma biochemistry, plasma renin activity (PRA) and cytokines and, protein expression of TLR4, NFκB, and cytokines were measured in the kidney homogenate. The kidneys were stained for TLR4, TLR2, and caspase-3. Endotoxemia was measured using neutrophil burst and Limulus amoebocyte lysate (LAL) assays.

Results: The groups treated with norfloxacin showed significant attenuation of the increase in plasma creatinine, plasma and renal TNF-α and renal tubular injury on histology. The increased renal protein expression of TLR4, NFκB, and caspase-3 in the untreated animals was significantly attenuated in the norfloxacin treated animals. PRA was reduced in the treated animals and severity of endotoxemia was also reduced.

Conclusions: The results show for the first time that kidneys in cirrhosis show an increased expression of TLR4, NFκB, and the pro-inflammatory cytokine TNF-α, which makes them susceptible to a further inflammatory insult. This increased susceptibility to LPS can be prevented with selective decontamination, providing novel insights into the pathophysiology of renal failure in cirrhosis.

Keywords: Acute kidney injury; Hepatorenal syndrome; Toll-like receptor 4; Liver failure.

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Abbreviations: AKI, acute kidney injury; BDL, bile duct ligation; HRS, hepatorenal syndrome; IL-6, interleukin 6; IP, intraperitoneal; LPS, lipopolysaccharide; NFκB, nuclear factor kappa B; Norflox, norfloxacin; SBP, spontaneous bacterial peritonitis; SIRS, systemic inflammatory response; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor α.

Introduction

Hepatorenal syndrome (HRS) is commonly encountered in patients with end stage liver disease. Untreated, HRS has a poor prognosis with 90% mortality at 3 months [1,2]. Our current understanding is that HRS develops secondary to haemodynamic and neurohormonal changes [1,3–6]. Despite the proposed functional nature of the renal dysfunction in HRS, it has been shown that many of the patients have histological evidence of acute kidney injury (AKI) with structural abnormalities on electron microscopy and renal dysfunction of cirrhosis that is associated with infection/inflammation may be pathophysiologically different [7,8]. This may explain the limited response to terlipressin and albumin, improving renal function in only about 45% of patients and with little effect on survival [9].

One common observation is that bacterial infections are often a precipitating factor for HRS [10], suggesting that infection and the subsequent systemic inflammatory response (SIRS) may play a role in the pathogenesis of HRS [11,12]. It is known that anti-inflammatory agents such as pentoxifylline significantly decrease the risk of developing HRS in patients with alcoholic hepatitis [13]. Furthermore, administration of norfloxacin, a selective gut decontaminant, reduces the incidence of renal failure and improves survival [14]. The mechanisms by which infection/inflammation modulates the precipitation of AKI and renal failure in susceptible patients with cirrhosis are not clear.

Toll-like receptors (TLRs) play an important role in the recognition of molecules derived from microbes. Among the TLRs, the role of TLR4 in sepsis and inflammation has been extensively studied [19]. Activation of the TLR4 receptor by lipopolysaccharide (LPS) from Gram-negative bacteria results in an increased

production of pro-inflammatory mediators. Mouse renal tubular epithelial cells express TLR4 receptors *in vitro*. Stimulation of these receptors by LPS results in secretion of cytokines and chemokines [15]. TLR4 has been shown to specifically activate the NFκB pathway which has been implicated in the regulation of multiple biological phenomena including apoptosis [16]. The functional role of TLR4 in AKI of cirrhosis secondary to infection/inflammation is unknown. We hypothesize that ongoing endotoxemia, possibly resulting from increased gut bacterial translocation in cirrhosis, primes the kidneys leading to upregulation of tubular TLR4 which, upon further inflammatory insult, leads to an exaggerated immune response culminating in progressive renal failure. It follows that selective gut decontamination would reduce this increase in TLR4 expression and prevent the deleterious effects of a superimposed inflammatory insult. The specific aims of this study were, therefore, to determine whether cirrhosis is associated with upregulation of TLR4, NFκB, and pro-inflammatory cytokines in the kidney and whether selective gut decontamination using norfloxacin results in reduction in renal TLR4 expression, attenuates NFκB and cytokines and makes the kidneys less susceptible to further endotoxemic insult.

Materials and methods

All experiments were conducted in accordance with local ethical approval and subjected to the UK animals Scientific procedures Act 1986.

Animals

A well-characterized rodent model of advanced fibrosis/cirrhosis induced by bile duct ligation (BDL) was utilized for this study. In this model, administration of LPS leads to the development of acute-on chronic liver failure with evidence of renal dysfunction [17]. Bile duct ligation, housing, and care of the animals were performed as described previously [18,17]. Six groups of adult male Sprague–Dawley rats were studied ($n = 6$ in each group). The animals were studied 4 weeks after either sham-operation or BDL. The animals in each group were terminated 3-h after administration of LPS (1 mg/kg) or saline intraperitoneally under terminal anesthesia. The groups were as follows:

(a) Sham operated controls administered saline (Sham group).
(b) Sham operated controls administered LPS (Sham + LPS group).
(c) BDL animals administered saline (BDL group).
(d) BDL animals administered LPS (BDL + LPS group).
(e) BDL animals treated with oral norfloxacin 20 mg/kg/day (days 18–28) and administered saline (BDL + norfloxacin group).
(f) BDL animals treated with oral norfloxacin 20 mg/kg/day (days 18–28) and administered LPS (BDL + LPS + norfloxacin group).

LPS/saline was administered 3 h prior to termination of the animals. Blood was withdrawn from the descending aorta and immediately placed in ice cold heparin or EDTA-containing tubes (until full exsanguination), centrifuged (4 °C, 3500 rpm, 10 min) and the plasma stored at −80 °C for later use in biochemistry and cytokine measurements. Kidneys were snap frozen in liquid nitrogen for Western blot analyses and a portion of each kidney was harvested in formalin for immunohistochemistry.

Liver and renal biochemistry

Plasma samples were analyzed for ALT, albumin, total protein, bilirubin, creatinine, and electrolytes (Cobas Integra 400, Roche Diagnostics, Burgess Hill, West Sussex, UK).

Plasma and kidney cytokine profile

Kidney samples (100 μg) were homogenized in Tris–HCl cell lysis buffer solution. The protein concentration of the homogenates was determined using the biuret assay. Tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) levels were measured using a commercial cytometric bead assay (Becton Dickenson, UK), quantified using flow cytometry (FACS Canto, Becton Dickenson, UK) and the appropriate software (FCAP array, SoftFlow, Hungary).

Western blotting

TLR4 and NFκBp65 protein expression was determined using Western blot, as previously described [20]. The proteins transferred to the membrane were probed using primary antibodies TLR4 (rabbit polyclonal to TLR4; Abbiotec, UK, 1:1000 dilution for 16 h at 4 °C overnight) and NFκBp65 (rabbit polyclonal to NFκBp65 [ab-536]; Signalway antibody, UK, 1:500 dilution for 16 h at 4 °C overnight) followed by incubation with a peroxidase conjugated anti-rabbit secondary antibody (ProSci, UK, 1:5000 dilution). Protein quantification in both membranes was ascertained using a public domain, Java-based image processing program, Image J. The results were expressed as ratio of either TLR4/β actin or NFκBp65/β actin (rabbit polyclonal to B actin, Abcam, UK, 1:1000 dilution).

Histopathological assessment

Periodic methamine silver stain: Periodic acid silver-methenamine staining was performed according to the method described by Jones [21].

Immunohistochemistry for TLR4, TLR2, and caspase 3: Kidney sections were probed for TLR4, mouse monoclonal anti-TLR4 (eBiosource, UK, 1:100 dilution), TLR2, rabbit polyclonal (Abcam, UK, 1:400 dilution), and caspase-3, rabbit monoclonal to caspase-3 (Abcam, UK, 1:200 dilution) primary antibody. TLR4 and caspase-3 staining was scored by independent histopathologist, blinded to the groups.

Measurement of endotoxemia

Neutrophil burst analysis: Isolated neutrophils from a healthy control individual were resuspended in PBS at a density of 5×10^5 in 50 μl and incubated with 50 μl of rat plasma for 30 min. The Phagoburst kit (Orpegen Pharma, Heidelberg, Germany) was used to determine the percentage of neutrophils producing reactive oxidants via the conversion of dihydrorhodamine (DHR) 123 to the fluorescent Rhodamine 123. Neutrophils were identified using anti-CD16 PE antibody (Immunotools, Germany). The samples were analyzed by fluorescence-activated cell sorting (FACS CANTO II; Becton Dickinson).

Limulus amoebocyte lysate (LAL) assay: The chromogenic kinetic assay (Charles River Laboratories, Charleston, SC, USA) was used for detection of endotoxin. Plasma samples (100 μl) were diluted 1:10 with endotoxin-free water and heat treated for 30 min at 75 °C. Then, 100 μl of sample and 100 μl of LAL reagent were mixed in a 96-well plate and analyzed for change in absorbance at 405 nm with a spectrophotometer using the Endoscan-V software (Charles River Laboratories, Charleston, SC, USA). Results are expressed as EU/ml.

Plasma renin activity

Plasma renin activity (PRA) was measured using the SensoLyte 520 Rat Renin Fluorimetric assay (Cambridge Biosciences, UK). Briefly, 10 μl of plasma samples were incubated with 40 μl of renin substrate solution for 10 min. For kinetic measurement, the fluorescent intensity was measured continuously at 520 nm and the data recorded every 5 min for 60 min. An end point reading was also taken at 60 min. The data was expressed as the rate of change of fluorescence, using the production of 5-carboxyfluorescein (5-FAM) as a marker of plasma renin activity.

Statistical analyses

The data were expressed as mean ± SEM. Determination of normal distribution and significance of difference was tested using one way ANOVA, with Bonferroni's multiple comparison test for selected groups where appropriate, using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Results

Liver and renal biochemistry

Evidence of chronic liver injury with changes consistent with advanced fibrosis was demonstrated in the BDL animals, as