Research Article

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Immunomodulatory and antioxidant function of albumin stabilises the endothelium and improves survival in a rodent model of chronic liver failure

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Background & Aims: Liver failure is characterized by endothelial dysfunction, which results in hemodynamic disturbances leading to renal failure. Albumin infusion improves hemodynamics and prevents renal dysfunction in advance liver failure. These effects are only partly explained by the oncotic properties of albumin. This study was designed to test the hypothesis that albumin exerts its beneficial effects by stabilising endothelial function.

Methods: *In vivo*: systemic hemodynamics, renal function, markers of endothelial dysfunction (ADMA) and inflammation were studied in analbuminaemic and Sprague–Dawley rats, 6-weeks after sham/bile duct ligation surgery. *In vitro*: human umbilical vein endothelial cells were stimulated with LPS with or without albumin. We studied protein expression and gene expression of adhesion molecules, intracellular reactive oxygen species, and cell stress markers.

Results: Compared to controls, analbuminaemic rats had significantly greater hemodynamic deterioration after bile duct ligation, resulting in worse renal function and shorter survival. This was associated with significantly greater plasma renin activity, worse endothelial function, and disturbed inflammatory response. *In vitro* studies showed that albumin was actively taken up by endothelial cells. Incubation of albumin pre-treated endothelial cells with LPS was associated with significantly less activation compared with untreated cells, decreased intracellular reactive oxygen species, and markers of cell stress.

Conclusions: These results show, for the first time, that absence of albumin is characterised by worse systemic hemodynamics, renal function and higher mortality in a rodent model of chronic liver failure and illustrates the important non-oncotic properties of albumin in protecting against endothelial dysfunction.

Introduction

Cirrhosis is characterized by intrahepatic and extrahepatic endothelial dysfunction (ED) [1]. Intrahepatic ED is a key feature of portal hypertension due to hypoactive endothelial cells, excess local vasoconstrictor and inhibitors of nitric oxide production. Additionally, extrahepatic circulation also develops ED, which is associated with splanchnic vasodilation. In advanced cirrhosis, this leads to decreased systemic vascular resistance and activation of compensatory mechanisms to maintain mean arterial pressure (MAP) [2]. Bacterial translocation [3], bacterial infections [4] and oxidative stress [5,6] are common complications that may aggravate this circulatory dysfunction leading to renal failure and high mortality [7].

Endothelial cells regulate blood flow for individual organs and can be activated by bacterial products (i.e., lipopolysaccharide-LPS) [8], inflammatory mediators (i.e., tumour necrosis factor-TNFα [9], IL-1β [10]) and reactive oxygen species (ROS) [11], leading to endothelial dysfunction [12]. Indeed, endothelial activation is thought to play a part in the systemic and splanchnic vasodilation of cirrhosis, partially through the production of endothelial derived microparticles [13]. Likewise, ED has been identified as an important mechanism underlying the circulatory alterations in cirrhosis [14–16].

Inflammation and oxidative stress cause abnormalities in several markers of endothelial function. ADMA (asymmetric dimethylarginine) is an endogenous inhibitor of the nitric oxide

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Abbreviations: ADMA, asymmetric dimethylarginine; BDL, bile duct ligation; COX2, cyclooxygenase2; ED, endothelial dysfunction; FCS, fetal calf serum; FITC, fluorescein isothiocyanate conjugate; HAS, human serum albumin; HSP70, heat shock protein 70; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide; MAP, mean arterial pressure; MFI, mean fluorescence intensity; NAR, nagase analbuminaemic rats; PBMCs, peripheral blood mononuclear cells; PRA, plasma renin activity; ROS, reactive oxygen species; SD, Sprague–Dawley; SDMA, symmetric dimethylarginine; TLR 4, Toll-like receptor 4; TNF, tumour necrosis factor; VCAM-1, vascular cell adhesion molecule-1; vWF, von Willebrand factor.

synthase and a marker of ED in different inflammatory conditions. In particular, ADMA levels correlate with the progression of liver failure and are associated with a worse prognosis [17–19]. Recent evidence also suggests that ADMA is an independent vascular risk factor in patients with cardiovascular diseases [20] and in the healthy population [21]. Moreover, endothelial activation is characterized by the expression of adhesion molecules of which E-Selectin and vascular cell adhesion molecule-1 (VCAM1) are most commonly described. Circulating von-Willebrand factor (vWF), a further marker of endothelial activation, has been associated with progression of liver failure and mortality [22,23].

Human serum albumin (HSA) is a multifunctional protein capable of binding LPS [24], which expands intravascular volume and has antioxidant properties [25]. Both albumin concentration and function are reduced in liver failure [26]. Albumin infusion decreases the incidence of renal failure and improves survival in cirrhotic patients with spontaneous bacterial peritonitis [27]. Albumin is also useful in the management of hepatorenal syndrome [28] and in preventing post-paracentesis circulatory dysfunction [29]. However, the mechanism through which albumin exerts its beneficial effects is not well understood. A potential protective effect on endothelial function has been suggested [30]. Indeed, HSA, but not colloid, decreased plasma levels of vWF in spontaneous bacterial peritonitis [30]. Understanding these mechanisms may enable better management of cirrhotic patients and possibly identify new pharmacological approaches. A model of analbuminaemia may be useful in elucidating the functional roles of albumin. Nagase analbuminaemic rats (NAR) have an inherited defect in the albumin gene leading to a state where these animals are albumin deficient [31]. These rats compensate for the lack of albumin by increasing other plasma globulins thus maintaining oncotic pressure [32]. Their phenotype and survival under normal conditions are not different from the normal Sprague–Dawley (SD) rats [33].

This study was designed to test the hypothesis that albumin exerts its beneficial effects in cirrhosis by stabilising endothelial function through its action on inflammation, oxidative and cellular stress.

Materials and methods

Detailed methods are available in Supplementary material.

In vivo experiments: rodent analbuminaemic model of chronic liver failure

Animals
This study was done in male SD (Charles River, UK) and male NAR rats obtained from the comparative biological unit at University College London.

Animal model
SD and NAR rats (250–300 g) where randomized to a bile duct ligation (BDL) or sham surgery. Four groups of rats were studied: SD Sham (n = 13), NAR Sham (n = 14), SD BDL (n = 16), NAR BDL (n = 15). The animals were followed up to 6 weeks and then each rat underwent a hemodynamic assessment. Rats were then euthanized by exsanguination, plasma collected and stored at −80 °C until assayed.

ADMA/SDMA
Plasma levels of ADMA and SDMA (symmetric dimethylarginine) were measured using liquid chromatography-tandem mass spectrometry.

Pro-inflammatory cytokines, vWF, plasma renin activity (PRA)
Plasma levels of TNF, IL-6 and vWF were measured using commercial ELISA sets. PRA was measured using the Sensolyte 520 Rat Renin Fluorimetric assay.

Monocyte/macrophage population and functional assay
Peripheral blood mononuclear cells (PBMCs) isolation: For each animal, PBMCs were isolated from total blood by lymphoprep (Axis-Shiel, Norway), resuspended (5×10^5 in 50 μl) and used for further analysis.

PBMCs population analysis: Cell preparations (50 μl of cell suspension) were incubated with mouse anti-rat CD32 for fc receptor blocking. Subsequently, cells were stained with conjugated anti-CD11b antibody and PE conjugated anti-CD68 antibody and analysed by flow cytometry (BD LSR II). Monocytes were defined by CD11b positive mononuclear cells gated by forward and side scatter. Activated monocytes were defined by ED1 positive monocytes as previously described [34]. Quantification of data was performed using FlowJo 5.6.1.

PBMCs phagocytic capacity: PBMCs phagocytic capacity was accessed as previously described [35].

In vitro experiments: human umbilical vein endothelial cells (HUVECs)

Cell culture
HUVECs isolated from the umbilical cord of healthy elective caesarean sections were cultured and used between the second and third passage. For some experiments, proliferating HUVECs cells from pooled donors were purchased. For the different experiments, cells were seeded at a constant density of 26,000 cells/cm^2 and allowed to reach 80–90% confluence before treatment.

HUVECs expression of adhesion molecules
Cells were pre-treated for 1 h with 10% FCS media or 4% HSA in 10% FCS media before being exposed to 10 μg/ml *Salmonella* LPS for 6 h. Expression of adhesion molecules (E-selectin, VCAM-1) was assessed by flow cytometry.

RNA isolation, cDNA synthesis and human endothelial cell biology qPCR array
RNA was isolated from treated (4% HSA for 1 h; 10 μg/ml LPS for 6 h; 4% HSA for 1 h followed by 10 μg/ml LPS for 6 h) and control HUVECs. RNA samples were further purified and qualitatively and quantitatively analysed. Each sample was retro-transcribed and then run on human endothelial cell biology PCR arrays. The raw threshold cycle data were uploaded onto the integrated web-based RT^2 Profiler™ PCR Array Data Analysis software package.

Qualitative albumin uptake and localization in HUVECs
HUVECs were incubated for 1, 3 or 6 h with 1% of fluorescein isothiocyanate conjugate (FITC)-albumin. During the last 30 min, Hoechst 33342 (nuclear staining) was added. Cells were then rinsed, fixed in 2% paraformaldehyde, and rinsed again for 15 min before being observed under a fluorescence microscope.

Qualitative and quantitative ROS production in HUVECs induced by LPS
HUVECs were pre-treated with 4% HSA or media for 1 h prior to exposure to LPS. Intracellular ROS was visualised by staining cells with carboxy-H2DCFDA.

ROS were quantified in four treatment groups: control, 4% HSA pre-treated cells, LPS (10 μg/ml) stimulated without HSA pre-treatment, LPS stimulated with HSA pre-treatment. In the last 30 min of incubation, carboxy-H2DCFDA and Hoechst 33342 were added. Cells were washed before measuring ROS using a fluorescence plate reader. ROS (carboxy-H2DCFDA) signal was normalised according to the number of cells (Hoechst 33342).

Quantification of cell stress response in HUVECs treated with LPS in presence and absence of albumin
Cell stress-related protein expression was assessed from the whole-cell extracts using the Human cell Stress Array Kit.

Results

In vivo studies: rodent analbuminaemic model of chronic liver failure

Impact of analbuminaemia on hemodynamics, renal function, endothelial function and survival
To first gain insights into the potential impact of albumin on endothelium, we studied a previously described rodent model of analbuminaemia [31–33]. We developed a model of chronic liver failure in NAR and SD rats, by performing a surgical BDL. Sham surgery was used in controls and the animals were