Gut-Brain Chemokine Changes in Portal Hypertensive Rats

Joaquin Merino · Maria-Angeles Aller · Sandra Rubio · Natalia Arias · Maria-Paz Nava · Maria Loscertales · Jaime Arias · Jorge-Luis Arias

Abstract

Background Hepatic encephalopathy is a syndrome whose physiopathology is poorly understood; therefore, current diagnostic tests are imperfect and modern therapy is nonspecific. Particularly, it has been suggested that inflammation plays an important role in the pathogenesis of portal hypertensive encephalopathy in the rat.

Aim We have studied an experimental model of portal hypertension based on a triple partial portal vein ligation in the rat to verify this hypothesis.

Methods One month after portal hypertension we assayed in the splanchnic area (liver, small bowel and mesenteric lymph nodes) and in the central nervous system (hippocampus and cerebellum) fractalkine (CX3CL1) and stromal cell-derived factor alpha (SDF1-α) as well as their respective receptors (CX3CR1 and CXCR4) because of their key role in inflammatory processes.

Results The significant increase of fractalkine in mesenteric lymph nodes (P < 0.05) and its receptor (CX3CR1) in the small bowel (P < 0.05) and hippocampus (P < 0.01), associated with the increased expression of SDF1-α in the hippocampus (P < 0.01) and the cerebellum (P < 0.01) suggest that prehepatic portal hypertension in the rat induces important alterations in the expression of chemokines in the gut-brain axis.

Conclusion The present study revealed that portal hypertension is associated with splanchnic-brain inflammatory alterations mediated by chemokines.

Keywords Chemokines · Hepatic encephalopathy · Neuroinflammation · CXCR4/SDF1-alpha · CXCR1/Fractalkine · Rat

J. Merino · M.-A. Aller · J. Arias
Department of Surgery I, School of Medicine, Complutense University of Madrid, Plaza de Ramón y Cajal s.n, 28040 Madrid, Spain
e-mail: maaller@med.ucm.es

J. Merino
e-mail: josem2005@yahoo.es

J. Arias
e-mail: jarias@med.ucm.es

S. Rubio
Department of Psychobiology, School of Psychology, Autonomous University of Madrid, 28049 Cantoblanco, Madrid, Spain
e-mail: sandra.rubio@uam.es

N. Arias · J.-L. Arias
Neurosciences Laboratory, School of Psychology, University of Oviedo, Pza. de Feijoo s/n, 33003 Oviedo, Asturias, Spain
e-mail: UO172871@uniovi.es

M.-P. Nava
Department of Physiology (Animal Physiology II), School of Biology, Complutense University of Madrid, J. A. Novais 2, 28040 Madrid, Spain
e-mail: mpaznava@bio.ucm.es

M. Loscertales
Department of Surgery, Massachusetts General Hospital, 1055 Fruit St., Boston, MA 02114, USA
e-mail: mlescertales@partners.org
Introduction

The physiopathology of Hepatic Encephalopathy (HE) is poorly understood and there are few high-quality diagnostic tests and markers. As a result, its treatment has improved only slightly over the last several decades [1].

There are three categories of HE based on the nature of the hepatic dysfunction. Type A encephalopathy is associated with acute liver failure, type B with portosystemic bypass without intrinsic liver disease and type C with cirrhosis [2]. In addition, HE is subcategorized as episodic, persistent or minimal. Particularly, minimal HE is associated with cognitive dysfunction, namely, without overt symptoms [2, 3].

In humans, prehepatic portal hypertension (PH) by extra-hepatic portal vein obstruction with portosystemic shunting and normal liver function results in minimal type B HE [4, 5]. For this reason, the partial portal vein ligated-rat model could be appropriate for the experimental study of this type of HE because portal-systemic shunting is developed and portal stenosis does not seem to produce liver damage [6–9].

Newly emerging concepts concerning HE imply an inflammatory response in its pathogenesis [10–12]. Inflammation, however, may not only be limited to modulating the type and severity of HE but could also be its own physiopathological mechanism [13]. If so, the basic mechanism that drives the essential nature of minimal type B HE secondary to prehepatic PH could be inflammatory. It has therefore been proposed that the inflammatory response in the CNS has a splanchnic origin [14]. With the aim of verifying this hypothesis we have studied the chemokines (chemotactic cytokines)—fractalkine (CX3CL1) and stromal cell-derived factor alpha (SDF1-x)—as well as their respective receptors (CX3CR1 and CXCR4) because of their key role in inflammatory processes [15–17] in the splanchnic area (liver, small bowel and mesenteric lymph node) and in the CNS (hippocampus and cerebellum) in short-term (1 month) partial portal vein-ligated rats.

Methods

Animals

Male Wistar rats, weighing 250–300 g, were obtained from the Vivarium of the Complutense University of Madrid. The animals were fed a standard laboratory rodent diet (rat/mouse A04 maintenance diet, Panlab, Spain) and water ad libitum. They were housed in a temperature- (22 ± 2°C), humidity- (65–70%) and light-controlled room.


Experimental Design

The animals were randomly divided into two groups. Group I (n = 6) was control rats which did not undergo any intervention and group II (n = 10) was composed of rats which underwent triple calibrated portal vein ligation (TPVL). All the animals were euthanized at 1 month by decapitation.

Surgical Technique of Portal Hypertension

The animals were anesthetized by i.m. injection of Ketamine (100 mg/kg) and Xylacine (12 mg/kg). The surgical procedure used to establish PH by TPVL has been described previously [9, 18]. Although the usual surgical technique employed to obtain a prehepatic portal hypertension model in the rat is the partial portal vein ligation (PVL) [9], we have modified this technique. Thus, we performed three equidistant portal ligations of the portal vein (TPVL) in order to increase the length of the stenosed portal tract, thus increasing the initial resistance to blood flow since, according to the Poiseuille equation (R = 8LZ/π r4), resistance (R) to the flow of a vessel depends on the length (L), on the radius (r) and on the viscosity coefficient of the blood (Z). In brief, after laparotomy, the portal vein was isolated and three ligatures, previously fixed on a sylactic guide, were performed in its upper, middle and lower portions. The stenoses were calibrated by a simultaneous ligation (4-0 silk) around the portal vein and a 20-gauge blunt-tipped needle [9]. The midline incision was closed in two layers with an absorbable suture (Polyglycolic acid) and 3-0 silk. Analgesia was maintained for 24 h with Buprenorphine (0.05 mg/8 h s.c.).

We have shown that using this modified technique of TPVL the evolution of portal hypertensive-rats and, especially the grade of collateral circulation development, is increased and more persistent in relationship with simple PVL. The improvement of the obtained experimental model could be due to the initial increase in portal pressure produced by the triple portal vein stenosis in the rat that could have a permanent effect on the evolution of portal hypertension [9].

Biochemical Methods

Isolation of Synaptosomes from Brain Areas and Preparation of Homogenates from Splanchnic Areas (Liver, Mesenteric Lymph Nodes, and Ileum)

After gently removing the brain from the skull, the hippocampus and cerebellum were dissected out [19]. Brain
homogenates and synaptosomes were stored at −80°C and homogenates for the splanchic areas were also collected. We resuspended the tissue in a volume proportional to the tissue volume in each case. For example, 800 μl were used for synaptosome isolation in brain areas from the cerebellum, whereas 500 μl were selected for synaptosome isolation from the hippocampus and small aliquots of homogenates (30 μl) were stored at −80°C. Synaptosomes were obtained by the modified protocol from Lynch and Voss [20].

Brains were homogenated in lysis buffer containing 0.32 M sucrose, HEPES 50 mM, 1 mM DTT, 1 μg/μl aprotinin, 1 μg/μl leupeptin, 100 mM vanadate and 1 μg/μl peptastine. Homogenates were centrifuged for 5 min at 3,500 g (4°C). The supernatants obtained from this first centrifugation were centrifuged again at 15,000 g, for 15 min in order to separate citosol from the membranes. Therefore, after removing the last supernatant in this second centrifugation, the final pellets containing synaptosomes were re-suspended in PBS 1 X (phosphate buffer saline), containing HEPES 50 mM, 1 μg/μl aprotinin, 1 μg/μl leupeptin, 1 μg/μl peptastine, 100 mM and 1 mM DTT - Dithiotreitol.

Protein Assay (Bradford Method)

Total protein from brain and splanchic samples were assayed by the Bradford method [21] reading absorbance at 595 nm in a microreader ELISA plate (software Digiscan 3.0).

ELISA Method for Chemokines Assay in the CNS and Splanchnic System

Chemokines SDF1-z/CXCR4 and fractalkine/CX3CR1 were evaluated by enzyme-linked immunosorbent assay (ELISA) in areas from the brain and from the splanchic system. Briefly, synaptosomes from brain areas and homogenates from splanchic areas were incubated overnight in the microplates with primary antibodies in a buffer containing PBS 1 X plus 0.05% Tween 20 (25%) at 10 μg/μl of Fusin C-20 (# SC 6190, rabbit polyclonal antibody for CXCR4 from Santa Cruz Biotechnology, Germany) whereas SDF1z antibody from E-Bioscience (# JM-5388-1000 BioNova Company) was added overnight at 1:150 in a washing buffer containing PBS 1 x plus 0.05% Tween 20 (25%). CX3CR1 rabbit polyclonal antibody (H-70 # SC 30030, Santa Cruz Biotechnology, Germany) was incubated overnight at 8 μg/μl and a Fractalkine rabbit polyclonal antibody (H-300, # SC 20730, Santa Cruz Biotechnology, Heidelberg) was added at 10 μg/μl to the plate overnight.

After washing all the microplates three times, they were incubated with citrate buffer containing 50 mM sodium citrate and 25 mM citric acid at pH = 4.5. Then, 1 μg/μl OPD (P-1526, Sigma) was added following previous protocols [22]. Ortophenylenediamine and 0.06% hydrogen peroxide (30%, H-1009, Sigma) were added just to the disclosing of the above-mentioned citrate buffer to detect the signal. After stopping the reaction by adding 50 μl of H2SO4, absorbance was measured by a reading at 492 nm in a Digiscan Software Microplate Reader (Digiscan Reader v3.0 and DigiWin Program: ASYS Hitech GmbH, Austria) out of plate [22, 23].

Statistical Analyses

Data were expressed as mean ± standard deviation (SD) and evaluated by Student t test for unpaired data. Significance was accepted if P was <0.05.

Results

Portal Hypertension Model

Portal pressure (PP) in rats with chronic TPLV (15.15 ± 2.76 mmHg) was higher (P < 0.001) than in SO rats (7.08 ± 0.76 mmHg). All of the TPVL rats showed characteristic features of splanchnic venous congestion with dilation of mesenteric venous circulation and portosystemic collateral circulation development of splenorenal and paraesophageal types. In addition, the animals with TPVL showed spleen enlargement, with a significant increase in spleen weight/body weight ratio (0.27 ± 0.04 vs. 0.17 ± 0.02; P < 0.01). Taken together all these findings support the value of this experimental model of prehepatic portal hypertension in the time of the postoperative evolution in which this study was performed.

Body Weight and Liver Weight

The animals with TPVL showed a body weight decrease (P < 0.05) (102.65 ± 24.78 vs. 153.71 ± 31.75 g) and hepatic atrophy (9.89 ± 1.23 vs. 14.12 ± 1.57 g P < 0.001) with a decrease in liver weight/body weight ratio (2.97 ± 0.27 vs. 3.87 ± 0.11 P < 0.001). The liver atrophy must be related to the portal blood deprivation derived from the portal blood flow decreased after TPVL.

CXCR4/SDF1-alpha and CXCR1/Fractalkine in the Splanchnic System

Fractalkine (CX3CL1) was upregulated in the mesenteric lymph nodes (644.50 ± 46.50 vs. 566.70 ± 53.09; P < 0.01), while its receptor, CX3CR1, showed an increased
expression in the ileum (1,773.86 ± 77.76 vs. 1,541.58 ± 103.42; \( P < 0.01 \)) of TPVL rats (Fig. 1). This alteration was accompanied by a distinct chemokine liver profile with underexpression of CX3CR1 (1,127.08 ± 166.02 vs. 1,481.43 ± 128.32; \( P < 0.01 \)) (Fig. 1). SDF1-\( \alpha \) is also underegulated in the liver (1,583.62 ± 134.32 vs. 1,727.84 ± 31.35; \( P < 0.05 \)) and its receptor (CXCR4) showed a decreased expression in the ileum of TPVL-rats in relation to control rats (695.61 ± 132.24 vs. 978.35 ± 262.50; \( P < 0.05 \)). Lastly, SDF1-\( \alpha \) also tends to decrease in mesenteric lymph nodes of TPVL rats (1,632.03 ± 302.22 vs. 1,874.35 ± 302.21) (Fig. 1).

CXCR4/SDF1-alpha and CXCR1/Fractalkine in the Central Nervous System

The chemokine SDF1-\( \alpha \) (1,751.48 ± 76.18 vs. 1,638.79 ± 127.09; \( P < 0.05 \)) and its corresponding receptor, CXCR4 (1,458.88 ± 99.08 vs. 1,300.57 ± 72.46; \( P < 0.01 \)), were overexpressed in the hippocampus. Similarly, this change in CXCR4/SDF1-alpha levels was associated with an increased expression of CX3CR1 (1,962.78 ± 134.87 vs. 1,719.03 ± 188.19; \( P < 0.01 \)), while fractalkine increased, although the difference was not statistically significant (2,030.89 ± 69.38 vs. 1,999.26 ± 33.06) (Fig. 2). Finally, in the cerebellum there is a significant increase (1,640.67 ± 163.08 vs. 1,341.56 ± 181.07; \( P < 0.01 \)) of SDF1-\( \alpha \) (Fig. 2).

Discussion

The results of this study show that in an early evolutive phase (1 month) of prehepatic portal hypertension in the rat, impairment is developed in the chemokine expression of the splanchnic-brain axis. The distinct chemokine profile that is produced between the splanchnic (liver, small bowel and mesenteric lymph nodes) and brain (hippocampus and cerebellum) studied areas (Fig. 3) is also noteworthy.

Chemokines belong to a superfamily of chemotactic proteins involved in the modulation of many biological functions, including cell adhesion, phagocytosis, cytokine secretion, T cell activation, apoptosis, angiogenesis and proliferation [15, 16]. These diverse biologic activities of chemokines play an important role during homeostasis as well as during inflammation [16] and they are necessary for the linkage between innate and adaptive immunity [24]. Therefore, over-expression of fractalkine and its cognate receptor, CX3CR1, in the mesenteric lymph nodes and small bowel, respectively, of portal hypertensive rats suggests that they can play a physiopathological role in the production of portal hypertensive enteropathy because these pathological entities have been considered as a type of low-grade chronic inflammatory bowel disease [25].

Particularly chemokines, i.e. fractalkine/CX3CR1, could be the key mediators in the process of mast cell splanchnic
recruitment and/or proliferation that characterize prehepatic PH in the rat [25, 26]. It has recently been proposed that gut-barrier dysfunction related to alterations of gut flora (microbiota) could be the cause of the bacterial translocation to mesenteric lymph nodes produced in TPVL-rats at 1 month of evolution [27]. In this experimental model of PH, intestinal mast cells could be stimulated by bacterial pathogens through toll-like receptors (TLRs) resulting in the increased expression of cell surface molecules [28], for example, of CX3CR1. The present results also suggest that there is a complementary function between the mesenteric lymph node overexpression of fractalkine and its cognate receptor CX3CR1 in the intestine. If so, fractalkine from the mesenteric lymph nodes, could modulate the intestinal inflammatory response through its action over CX3CR1 expressed by structural and/or recruited inflammatory cells like mast cells.

However, the increased intestinal CX3CR1 expression in TPVL-rats could have an ambivalent meaning. Thus, it has been proposed that CX3CR1-positive intestinal dendritic cells in the lamina propria of rodents are capable of taking up bacteria by way of transepithelial dendrites in order to defend against pathogenic microorganisms [29]. TPVL in the rat produces liver steatosis, a pathological condition in which hepatocyte apoptosis is produced [30]. In this way, the decreased expression of fractalkine receptor (CX3CR1) in the liver of TPVL rats could favor
and also by multiple factors, including oxidative stress.

In the present study we have also shown increased SDF1-α protein levels in the hippocampus and cerebellum of PH-rats. SDF-1α and its cognate receptor, CXCR4, have been shown to play a central role in the development of the hippocampus and cerebellum [35]. SDF1-α has been demonstrated to exert various functions in the brain [36, 37]. The sequence of this chemokine is highly conserved among species, with only one amino acid difference between murine and human SDF1-α, suggesting that this molecule plays a crucial biological role [36].

Cirrhotic patients with cognitive decline showed an impressive redistribution of flow from cortical regions, including the anterior cingulated cortex, to structures such as the hippocampus and thalamus [40]. A comparable pattern of redistribution of cerebral glucose utilization has been reported and suggests that at least at mild stages of HE, cerebral blood flow is still coupled with the metabolism of glucose [40]. Increased glucose utilization by the basal ganglia, the hippocampus and cerebellum are also associated with increased deposition of manganese [41]. In accordance with these findings a significant correlation between cognitive function and glucose utilization of the cortical regions, even in patients with minimal HE [42], has been detected.

In this way, prehepatic portal hypertensive rats present a deficit in spatial working memory, a test frequently used to evaluate the hippocampus-dependent declarative memory [46]. Also in cirrhotic rats, the spatial working memory impairment could be linked to dysfunction in neuronal activity in the hippocampus as well as prefrontal cortex [47]. Furthermore, portal hypertensive cirrhotic rats showed spatial memory impairment associated with morphometric changes characterized by an increased neuronal and astrocytic nuclear volume in all the mammillary nuclei, structures also involved in spatial working memory, and the CA1 hippocampal region [48]. These findings suggest that in cirrhotic rats the spatial memory impairment could be linked to astrocytes and neuronal functional and structural impairment in mammillary nuclei and the hippocampus [49].

Inflammation could inhibit hippocampal neurogenesis. It has been shown that inflammatory blockade with indomethacin restores hippocampal neurogenesis after endotoxin induced inflammation [52]. Fractalkine has also been shown to alter synaptic activity in hippocampal and/or cerebellar neurons in rodents [39] (Fig. 3). The significantly increased expression of the chemokine fractalkine/CX3CL1 receptor, CX3CR1, in the hippocampus of portal hypertensive rats suggests the upregulation of an inflammatory hippocampal response. Fractalkine is predominantly expressed in neurons and its receptor, CX3CR1, is primarily expressed by microglial cells in the CNS [50]. Fractalkine-activated microglia could form a first line of defense in the CNS injury through their capacity to migrate, proliferate and secrete inflammatory and neurotrophic factors, phagocytized damaged cells and debris as well as present antigens [51]. In addition, the production of chemokines by microglia in response to pro-inflammatory cytokines such as TNF-α might result in further migration of leukocytes into the parenchyma [17].

The hippocampus and the cerebellum interact for normal and pathological functions [55]. Therefore, the hippocampus-cerebellar SDF1-α overexpression in prehepatic portal hypertensive rats suggests a synchronous dysfunction, resulting in training or spatial learning-related chemokine changes. It is accepted that adult hippocampal neurogenesis plays an important role in learning and memory [56]. Particularly in this context, SDF1-α serves as
Chemokines and their receptors play an important role in the determination of mast cell tissue localization and function [61]. At least SDF1-α and fractalkine acting in CXCR4 and CXCR1 mast cell receptors, respectively, could induce mast cell migration through the gut-brain axis in TPVL rats. At the splanchic level, it has been considered that mast cells participate in the pathogenesis of inflammatory bowel syndrome, irritable bowel syndrome and food allergy [62]. Mast cells could also be involved in the production of portal hypertensive enteropathy in the rat mediating, among other actions, angiogenesis [26, 63].

It could be concluded that prehepatic portal hypertension in the rat induces the expression of splanchic (small bowel, mesenteric lymph nodes and liver), and central nervous system (hippocampus and cerebellum) inflammatory changes. The significant chemokine alterations in the gut-brain axis could be involved in the pathogenesis of a portal hypertensive encephalopathy in prehepatic portal hypertensive rats. Thus, the induction of this inflammatory gut-brain axis could be etiopathogenically-related with the development of a minimal hepatic encephalopathy, although in this experimental model of portal hypertension a significant degree of liver dysfunction does not exist. Therefore, this experimental model of prehepatic portal hypertension could be useful to study the inflammatory mechanisms possibly involved in the production of the minimal hepatic encephalopathy. In this way, the name of portal hypertensive encephalopathy would be considered. The results of the present study highlight the important role that splanchic-brain inflammatory axis has in this experimental model.

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