Splanchnic-Brain Crosstalk Mediated By Chemokines In Portal Hypertensive Rats
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Citation

Abstract
In experimental portal hypertension, the inflammatory cerebral-splanchnic axis alterations could be mediated by chemokines. In Wistar rats with partial portal vein ligation, RANTES, CXCR4/SDF-1alpha and CX3CR1/Fractalkine were measured in several brain and gastrointestinal areas. Nestine and Bcl-2 were assayed in the hippocampus, and TNF-α in the liver, ileum and mesenteric lymph nodes. In the CNS of portal hypertensive-rats, SDF-1alpha increased in the hippocampus (p<0.05), and cerebellum (p<0.05), and RANTES (p<0.05) decreased in the striatum. TNF-alpha and CXCR4 increased in the hippocampus and TNF-alpha in the ileum and in mesenteric lymphatic nodes. CX3CR1 increased in the ileum (p<0.05), whereas Fractalkine increased (p<0.05) in the mesenteric lymph nodes. Splanchnic CX3CR1 and fractalkine overexpression may suggest the development of anti-inflammatory and repair mechanisms to balance pro-inflammatory mechanisms. SDF1-alpha upregulation in the CNS could suggest its involvement in neuronal remodeling. The existence of a communication mechanism chemokine-dependent through the splanchnic-brain axis in portal hypertension could be hypothesized.

INTRODUCTION
Prehepatic portal hypertension (PH) in humans is associated with neuropsychological and brain magnetic resonance changes consistent with minimal hepatic encephalopathy (HE). Since intrinsic hepatocellular disease does not exist in this type of PH, the existence of portal-systemic bypass is the principal cause of minimal HE that is categorized as type B . The partial portal vein ligated-rat (PVL) model could be appropriate for the experimental study of the minimal HE secondary to prehepatic PH because portal-systemic shunting is developed and portal stenosis does not seem to produce liver damage . It has recently been highlighted the important role that inflammation has on the modulation of the molecular pathogenesis of HE . Inflammation, however, may not only be limited to modulating the severity of HE but could be indeed its own pathophysiological mechanism . If so, the inflammation of the CNS, when is secondary to PH, could be the basic mechanism that drives the essential nature of the minimal hepatic encephalopathy.

In order to verify this hypothesis we have been measured in PVL-rats the chemokines CXCR4/SDF-1alpha (Stromal Cell-Derivated Factor), CX3CR1/Fractalkine and RANTES (Regulated on Activation, Normal T Expressed and Secreted) in the CNS and in the splanchnic system, which is considered the origin of its most known systemic alteration, that is the hyperdynamic circulatory syndrome.

Chemokines (chemotactic cytokines) and their receptors are widely expressed throughout the body. They play significant roles in normal physiology and in many pathological states . Particularly, the complex and often redundant roles of chemokines in diseases pathophysiology represents a major challenge. Indeed, there is growing evidence showing that brain cytokines/chemokines play crucial roles in the neuro-glio-vascular interaction underlying the pathology of various brain disorders .

Chemokines are a family of small chemotactic cytokines, acting via G proteins in the cell surface. We have studied the chemokine pairs CXCR4/SDF-1alpha and CX3CR1/Fractalkine since alpha chemokine SDF binds to CXCR4 chemokine receptor in neurons and glia , whereas
Fractalkine binds to CX3CR1 chemokine receptor in microglia and neurons.  

**MATERIAL AND METHODS**

**ANIMALS**

Male Wistar rats, weighing 250-300 g were obtained from the Vivarium of the Complutense University of Madrid. The animals were anesthetized by i.m. injection of Ketamine (100 mg/kg) and Xylacine (12 mg/kg). The animals were fed standard laboratory rodent's diet (rat/mouse A04 maintenance diet, Panlab, Spain) and water ad libitum. They were housed in a temperature (22 ± 2°C), humidly (65-70%) and light-controlled room.


**EXPERIMENTAL DESIGN**

The animals were randomly divided into three groups: Group 1 (n=8), control rats which did not undergo any operative intervention; Group II (n=6), sham-operated (SO) rats, in which a midline laparotomy and dissection of portal vein was made, and Group III (n=12), triple calibrated portal vein ligation (TPVL) rats.

All the animals were sacrificed at 1 month by decapitation.

**SURGICAL TECHNIQUE OF PORTAL HYPERTENSION.**

The surgical procedure used to establish portal hypertension by triple partial portal ligation of the portal vein (TPVL) has been described previously. The portal vein was isolated and three equidistant ligatures (4-0 silk) were placed around a 20-gauge (20 G) blunt-tipped needle lying along the portal vein.

**BIOCHEMICAL METHODS**

**SYNAPTOSOME ISOLATION FROM BRAIN AREAS AND HOMOGENATES PREPARATION IN SPLANCHNIC AREA (LIVER, MESENTERIC LYMPH NODES AND ILEUM)**

After gently removing the brain from the skull, the hippocampus, prefrontal cortex, striatum, hypothalamus, cerebellum, mamillary cell bodies and olfactory bulb were dissected out. Half of the brain hemisphere was taken out and stored at -80°C for further CXCR4/SDF1 alpha, CX3CR1/Fractalkine and RANTES evaluation by immunofluorescence. Brain synaptosomes and splanchnic areas homogenates were stored at -80°C.

Synaptosomes were obtained by the modified protocol from Lynch and Voss. Brains were dissected out and 200 µL of homogenates were stored at -80°C. 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 twice, 5 minutes at 1000 g and then, 15 minutes at 15,000g (4 °C in a JA 20.21 rotor). Finally, after removing the last supernant, the final pellets containing synaptosomes were resuspended in PBS 1X –Phospate Buffer Saline-, containing HEPES 50 mM, 1 µg/µl aprotinin, 1 µg/µl leupeptin, 100 mM and 1 mM DTT -Dithiotreitol-.

**PROTEIN ASSAY (BRADFORD METHOD)**

Total protein from brain and splanchnic samples were assayed by the Bradford method reading absorbance to 595 nM in a microreader ELISA plate (software Digiscan 3.0)

ELISA Method for chemokines assay in the Central Nervous System (CNS) and Splanchnic System

Chemokines CXCR4/SDF1 alpha, CX3CR1/fractalkine and beta RANTES were evaluated by enzyme-linked immunosorbsent assay (ELISA) in areas from the brain and from the splanchnic system. Briefly, microplates were incubated overnight with primary antibodies in buffer containing PBS 1X plus 0.05% Tween 20 (5 %) at 10 µg/µl of Fusin C-20, #sc 6190, Rabbit policlonal antiboby for CXCR4 from Santa Cruz Biotechnology, Heidelberg, Germany) whereas SDF1 alpha antibody from E-Bioscience, # JM-5388-100, Bionova Company was added overnight at 1:150 in washing buffer containing PBS 1x plus 0.05% Tween 20 (25 %). Furthermore, CX3CR1 rabbit policlonal antibody (H-70, #sc 30030, Santa Cruz Biotechnology, Germany) was incubated overnight at 8 µg/µl and a Fractalkine rabbit policlonal antibody (H-300, #sc 20730, Santa Cruz Biotechnology, Heidelberg, Germany) was added at 10 µg/µl to the plate overnight.

RANTES levels were measured with sheep policlonal antibody from AbCam (MA, Boston, USA) at 5 µg/µl and with a secondary rabbit Ig G-Biotinilated antibody (# sc 2774 from Santa Cruz Biotechnology), whereas RANTES was incubated for 2 hours with Anti (Ig Y) RANTES at 5
µg/ml overnight diluted at 4°C (Chicken polyclonal to RANTES, #ab14066 from Abcam company, MA, Boston, USA) at 1:300 during 1 hour at room temperature in a humidified chamber. RANTES protein levels were evaluated by incubation with secondary anti chicken Biotinilated Ig Y antibody during 2 hours at room temperature (#ab6752, Abcam). After stopping the reaction by adding 50 µg of HSO₄, 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)

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Half the brain hemisphere was cut and postfixed in 4 % of paraformaldehyde at room temperature for 4 hours. Immediately, after postfixation, all the brains and the splanchnic areas (ileum and liver) were immersed in 30 % sucrose for 24 hours at 4°C. 30 micrometer slices were cut and coded for chemokines immunofluorescence studies. The slices were washed 3 times and incubated for 72 hours in PBS-1x at the accurate concentration chosen for each chemokine primary antibody and at room temperature (CXCR4, at 15 µg/µl or Fusin C-20, #sc 6190 from Santa Cruz Biotechnology, Germany) whereas its ligand, SDF1 alpha, was added at 1:150 (E-bioscience, # 14-7992-81) also for 72 hours at room temperature. Regardless of the delta chemokine system, CX3CR1 (H-70, #sc 30030, Santa Cruz Biotechnology, Germany) was incubated for 72 hours at 12 µg/µl whereas its ligand, fractalkine, (M-18, # sc 7227, Santa Cruz Biotechnology) was added for 72 hours at 15 µg/µl in PBS containing 1 % of Triton X-100. Besides, we used a SDF1 alpha antibody from Santa Cruz Biotechnology (Heidelberg, Germany, # sc 6193), which recognizes its alpha isoform in neurons and beta endothelial isoform .

We also detected CX3CR1 protein levels in 30 micrometer slices from the ileum of portal hypertensive-rats after 72 hours incubation with CX3CR1 antibody from Santa Cruz Biotechnology. # CX3CR1 (H-70, #sc 30030, Santa Cruz Biotechnology, Germany). CX3CR1 microvilli signal was detected after incubation for 2 hours with rabbit Alexa Fluor 555 at 1:500 for 3 hours in a humidified chamber.

For PSA-NCAM identification, a neural plasticity or neurogenesis marker , we use a goat antimouse Ig M monoclonal antibody (1:300) raised against PSA (polisialic acid or neuraminic acid) from NCAM –Neural Cell adhesion molecule- (clone 2-2B, AbC0019 from AbCyS, France). PSA-NCAM was incubated at 1:300 for 72 hours in PBS 1x

buffer containing 0.03 % of BSA at room temperature . We performed PSA-NCAM immunodetection by incubation during 3 hours with a goat antimouse-FTTC Ig M secondary at room temperature. Double immunofluorescence studies were carry out with Neurofilament M antibody (Signet Laboratory, Covance #473-01, Neurofilament M, clone 2F11 monoclonal antibody). After washing 3 times in PBS 1x, the sections were incubated with rabbit Alexa Fluor 555 at 1:500 during 3 hours in a humidified chamber (# A21-088; Alexa Fluor Anti Mouse (H+L) 2 mg/ml, Molecular Probes company) for all the chemokines tested. We also used Alexa Fluor 488 donkey Anti-mouse Ig G (H+L); Molecular Probes; USA # 21.202) for CXCR4 endothelial identification in some brain slides. Immunostaining specificity was confirmed by the absent of signal after primary or secondary antibody omission. Double immunofluorescence studies were carry out with Neurofilament M antibody (Signet Laboratory, Covance #473-01, Neurofilament M, clone 2F11 monoclonal antibody. We also included Nestine, as a neural cell stem marker, at 1:300 during 72 hours (RD company, Rat Nestin Affinity Purified Goat Polyclonal IgG antibody # AF2736).

STATISTICAL ANALYSES

Statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, versión 14.00). The results are expressed as mean ± SD and evaluated by analysis of variance one-tailed ANOVA test. Duncan post-hoc analysis was used when appropriate. Significance was accepted if p was < 0.05

RESULTS

The animals with PH showed a body weight decrease (p<0.05) (106.65 ± 31.74 vs 147.72 ± 36.21g) . hepatic atrophy ( 10.19 ± 1.45 vs 13.50 ± 1.79g; p<0.001), with a decrease in liver weight/body weight ratio ( 3.00 ± 0.32 vs 3.59 ± 0.13; p<0.001), and splenomegaly, with an increase in spleen weight/body weight ratio ( 0.25 ± 0.06 vs. 0.19 ± 0.03; p< 0.05 ).

CX3CR1 is overexpressed in the ileum whereas Fractalkine is upregulated in the mesenteric lymph nodes and Bcl-2 is reduced in the liver of rats with portal hypertension

We have shown upregulated CX3CR1 protein levels in the ileum of portal hypertensive-rats in relationship to control rats (p<0.05) (Table 1, Figure 1).
Splanchnic-Brain Crosstalk Mediated By Chemokines In Portal Hypertensive Rats

Figure 1
Table 1: Chemokine protein levels (optical density, absorbance at 492 nm) of CXCR4/SDF-1α, CX3CR1/Fractalkine (FK) and RANTES in splanchnic system of control (CR), sham-operated (SO) and portal hypertensive (PH) rats at 4 weeks of evolution.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CXCR4</th>
<th>SDF-1α</th>
<th>CX3CR1</th>
<th>FK</th>
<th>RANTES</th>
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<tbody>
<tr>
<td>Control (n=5)</td>
<td>76.17</td>
<td>66.60</td>
<td>80.70</td>
<td>86.78</td>
<td>46.97</td>
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<tr>
<td>SO (n=6)</td>
<td>69.04</td>
<td>61.91</td>
<td>67.11</td>
<td>36.09</td>
<td>36.09</td>
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<td>PH (n=12)</td>
<td>104.00</td>
<td>98.76</td>
<td>84.47</td>
<td>51.99</td>
<td>56.19</td>
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Superior Mesenteric Lymph Complex

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<tr>
<th>GROUP</th>
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<th>CX3CR1</th>
<th>FK</th>
<th>RANTES</th>
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<tbody>
<tr>
<td>Control (n=5)</td>
<td>89.44</td>
<td>176.05</td>
<td>137.63</td>
<td>137.92</td>
<td>239.75</td>
</tr>
<tr>
<td>SO (n=6)</td>
<td>153.32</td>
<td>148.52</td>
<td>279.92</td>
<td>157.10</td>
<td>139.10</td>
</tr>
<tr>
<td>PH (n=12)</td>
<td>123.43</td>
<td>125.63</td>
<td>126.92</td>
<td>132.90</td>
<td>236.50</td>
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Liver

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<tr>
<th>GROUP</th>
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<th>CX3CR1</th>
<th>FK</th>
<th>RANTES</th>
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<tr>
<td>Control (n=5)</td>
<td>951.87</td>
<td>1708.06</td>
<td>1370.63</td>
<td>1379.94</td>
<td>929.75</td>
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<tr>
<td>SO (n=6)</td>
<td>155.45</td>
<td>69.69</td>
<td>369.66</td>
<td>267.40</td>
<td>119.90</td>
</tr>
<tr>
<td>PH (n=12)</td>
<td>812.08</td>
<td>1610.26</td>
<td>1156.53</td>
<td>1347.53</td>
<td>848.04</td>
</tr>
</tbody>
</table>

Means ± SD; *p<0.05: statistically significant value in relation to control rats; **p<0.05: statistically significant value in relation to sham-operated rats; *p<0.05: statistically significant value in relation to portal hypertensive rats.

The immunofluorescence studies display increased CX3CR1 in the ileum microvilli (Figure 2) and RANTES shows a trend of increasing in the ileum (Table 1).

Figure 2
Figure 2: Chemokine values in the splanchnic system of control (CR), sham-operated (SO) and portal hypertensive (PH) rats at 4 weeks of evolution. *p<0.05: statistically significant value in relation to control rats; **p<0.05: statistically significant value in relation to sham-operated rats.

Although significant changes of CXCR4/SDF-1 alpha and CX3CR1/Fractalkine levels were not found in the liver of rats with PH, a slight trend of SDF-1 alpha and Fractalkine reduction was detected compared to both the control and sham-operated rats (Table 1). Bcl-2, an anti-apoptotic
marker, is reduced (p<0.05) in the liver of PH-rats (Table 2). In the mesenteric lymph nodes there was a decrease of SDF-1 alpha levels (p=0.06), associated with a trend to increase in Fractalkine (Table1, Figure 1).

**Figure 4**
Table 2: TNF-α and Bcl-2 levels (optical density, absorbance at 492nm) in liver and ileum of control (CR), sham-operated (SO) and portal hypertensive (PH) rats at 4 weeks of evolution.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TNF-α</th>
<th>Bcl-2</th>
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<tbody>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>CR</td>
<td>362.88±82.36</td>
<td>629.16±69.48</td>
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<tr>
<td>(n=8)</td>
<td>268.92±84.67</td>
<td>790.76±54.49</td>
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<tr>
<td>SO</td>
<td>360.79±98.57</td>
<td>949.71±208.44</td>
</tr>
<tr>
<td>(n=12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=12)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5**
Figure 3: Chemokine values in the Central Nervous System in control (CR), sham-operated (SO) and portal hypertensive (PH) rats at 4 weeks of evolution. *p

CXCR4/SDF-1 alpha are up-regulated in the hippocampus and cerebellum of rats with portal hypertension.

SDF-1 alpha is overexpressed in the hippocampus (p<0.05) and in the cerebellum (p<0.05) of PH-rats (Table 2, Figure 3).

SDF-1 alpha was detected by immunofluorescence in the granular cell layer and in the rest of hippocampus (CA1, CA2 and CA3) (Figure 4).
Figure 4: Immunofluorescence showing neuronal immunolabeling for SDF1 alpha in the granular cell layer from dentate gyrus (20 X). Neurons from granular cell layer are identified using Neurofilament M (NF-M) marker, whereas SDF-1 alpha/NF-M colocalization is shown in yellow color.

No significant changes were found in CX3CR1/Fractalkine or CXCR4/SDF-1 alpha in the rest of the brain areas (Table 3). However, CXCR4 protein levels trends to increase in the hippocampus of rats with PH in relationship to control rats (Table 3).
Figure 7
Table 3: Chemokine protein levels (optical density, absorbance at 492 nm) of CXCR4/SDF-1?, CX3CR1/Fractalkine (FK) and RANTES in diverse areas of Central Nervous System of control (CR), sham-operated (SO) and portal hypertensive (PH) rats at 4 weeks of evolution.

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<th>GROUP</th>
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<th>SDF-1?</th>
<th>FK</th>
<th>RANTES</th>
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<tr>
<td>CR (n=6)</td>
<td>1075.62 ± 113.92</td>
<td>1018.47 ± 89.63</td>
<td>942.4 ± 93.20</td>
<td>894.50 ± 76.16</td>
<td>1023.40 ± 72.10</td>
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<td>SO (n=6)</td>
<td>90.49 ± 370.30</td>
<td>258.12 ± 208.12</td>
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<td>83.63 ± 83.63</td>
<td>1049.32 ± 124.32</td>
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<td>PH (n=12)</td>
<td>1033.39 ± 97.43</td>
<td>1103.80 ± 128.33</td>
<td>1074.53 ± 128.95</td>
<td>903.36 ± 65.29</td>
<td>1003.56 ± 86.10</td>
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<td>CR (n=6)</td>
<td>369.69 ± 335.92</td>
<td>1754.06 ± 214.05</td>
<td>1904.41 ± 38.71</td>
<td>447.70 ± 79.93</td>
<td>1005.00 ± 148.16</td>
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<td>SO (n=6)</td>
<td>343.67 ± 343.67</td>
<td>1732.42 ± 208.12</td>
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<td>PH (n=12)</td>
<td>1538.46 ± 90.71</td>
<td>1999.38 ± 96.76</td>
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<tr>
<td>CR (n=6)</td>
<td>986.91 ± 126.10</td>
<td>1170.10 ± 216.06</td>
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<td>SO (n=6)</td>
<td>983.47 ± 131.40</td>
<td>1295.30 ± 160.60</td>
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<td>1968.67 ± 136.17</td>
<td>393.33 ± 78.21**</td>
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<td>PH (n=12)</td>
<td>1024.11 ± 126.25</td>
<td>1213.00 ± 207.14</td>
<td>1604.27 ± 275.17</td>
<td>1821.78 ± 247.35</td>
<td>217.71 ± 43.90**</td>
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<td>1315.00 ± 212.23</td>
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<td>1492.86 ± 113.06</td>
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<td>PH (n=12)</td>
<td>1518.67 ± 280.96</td>
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<td>1105.45 ± 178.19</td>
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<td>1349.95 ± 175.82</td>
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<td>1453.72 ± 162.41</td>
<td>1270.50 ± 104.26</td>
<td>1177.33 ± 92.97</td>
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<td>PH (n=12)</td>
<td>1529.75 ± 192.25</td>
<td>1580.60 ± 269.89*</td>
<td>1254.97 ± 78.92</td>
<td>1118.17 ± 93.88</td>
<td>1390.20 ± 105.41</td>
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<td>CR (n=6)</td>
<td>1355.34 ± 1355.34</td>
<td>1605.01 ± 203.33</td>
<td>1797.00 ± 203.33</td>
<td>2063.39 ± 123.78</td>
<td>1537.32 ± 137.78</td>
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<tr>
<td>SO (n=6)</td>
<td>1381.76 ± 356.59</td>
<td>1712.17 ± 43.30</td>
<td>1924.42 ± 71.95</td>
<td>1043.79 ± 227.84</td>
<td>1666.56 ± 227.84</td>
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<tr>
<td>PH (n=12)</td>
<td>1441.41 ± 356.59</td>
<td>1706.36 ± 77.26*</td>
<td>1875.65 ± 256.39</td>
<td>1876.52 ± 67.03</td>
<td>1695.59 ± 828.00</td>
</tr>
</tbody>
</table>

Mean ± SD
*p<0.05, statistically significant value in relation to control rats; **p<0.05, statistically significant value in relation to sham-operated rats; * statistically significant value in relation to portal hypertensive rats.
It is also worth showing endothelial immunolabelling for CXCR4 in blood vessels from the hippocampus of PH-rats (Figure 5). RANTES, measured by ELISA in synaptosomes from the striatum was reduced (p<0.05) in relation to control rats (Table 3, Figure 2).

**Figure 8**
Figure 5: Vascular immunofluorescence for CXCR4 in hippocampus of a portal hypertensive rat.

Regardless of the neural expression of chemokines, SDF-1 alpha was overexpressed in the granular cell layer of the hippocampus, where, besides, colocalizes with PSA-NCAM, a neural plasticity or neurogenesis marker, as well as with of Nestine, a neural stem marker, positive cells (data not shown). Neuronal loss in the granular layer was not detected in the dentate gyrus in rats with PH using Nilss staining, which specifically identifies neurons (data not shown).

**DISCUSSION**

In this study we report by the first time that in prehepatic portal hypertension in the rat an alteration in the chemokine expression in the axis splanchnic-brain is produced. This change consists in a significant increase of SDF-1 alpha in hippocampus and cerebellum and a decrease of RANTES in striatum, associated with an increase of CX3CL1/Fractalkine in the mesenteric lymph nodes and of Fractalkine and its chemokine receptor, CX3CR1, and RANTES in the ileum.

Chemokines are a family of small chemotactic cytokines acting via G proteins in the cell surface of glia and microglia and they modulate leukocyte chemotaxis in neuroinflammation. Fractalkine (CX3CL1) is a member of the CX3C chemokine family that binds to its specific receptor-1 (CX3CR1) and plays a crucial role in the initiation and progression of inflammation. In this way, increased levels of Fractalkine in the mesenteric lymph nodes and its receptor (CX3CR1) in the ileum of PH-rats are features that suggest the existence of a splanchnic inflammatory response in this experimental model. It has been proposed that Fractalkine and CX3CR1 fulfill special roles in tethering and rolling, arrest, stable adhesion, and transendothelial migration of CX3CR1-leukocytes at sites of Fractalkine-expressing endothelium. In addition, CX3CR1 acts as a cell adhesion molecule and several studies show changes in alpha chemokines, as IL-8 and Macrophage chemokine protein-1 (MCP-1), in the human colonic epithelial cells during bacterial invasion.

We have shown an increased CX3CR1 protein levels in the ileum in rats with PH. Throught the increased CX3CR1 levels, macrophages and dendritic cells could bind to microvilli, since CX3CR1 is expressed in the surface of intestinal epithelial and endothelial cells. Consequently, CX3CR1 overexpression in ileum of portal hypertensive-rats could induce migration of blood monocytes and macrophage trafficking contributing to intestinal inflammation. Interestingly, CX3CR1 immunolabeling displayed a very strong signal for CX3CR1 in the ileum microvilli of rats with PH (Figure 2). Therefore, an enhanced innate immune response due to increased CX3CR1 could be considered in the injured ileum of these animals.

Fractalkine can also act as a mast cell chemoattractant, and this should explain that the most prominent increase of mast cell infiltration in rats with prehepatic PH occurs exactly in the mesenteric lymph nodes. Since mast cell express CX3CR1 receptor for Fractalkine, and this chemokine has chemotactic activity towards mast cells, the Fractalkine overexpression in the mesenteric lymph nodes of portal hypertensive rats is likely to be responsible for the presence of the highest number of mast cells in this site. Moreover, the recruitment of mast cells in ileum could be due to the shown hyperexpression of RANTES. The excess number of mast cells in the mesenteric lymph nodes of rats with PH could be related to their ability for the rapid and abundant release of the TNF-α stored when the appropriate stimulus is acting, namely bacterial translocation from the gut. TNF-α can also stimulate Fractalkine expression, which would maintain the inflammatory activity mediated by mast cells in the mesenteric lymph nodes of rats with prehepatic PH.

The ileal hyperexpression of CX3CR1 could also be a
pathogenic factor in the enteropathy developed in rats with PH, which is characterized by an increased number and diameter of the mucosa and submucous vessels. Since the basic structural changes found in the gastrointestinal tract are vascular, the very appropriate name of “hypertensive portal intestinal vasculopathy” have been proposed. Since Fractalkine stimulates angiogenesis by activating both Raf-1/MEK/ERK and PI3K/AKT/eNOS/NO signal pathways via the G protein-coupled receptor CX3CR1, the interaction between Fractalkine and CX3CR1 could contribute to the pathogenesis of enteropathy in this experimental model of PH. All these factors taken together allow us to suggest that Fractalkine and its receptor CX3CR1 may play a critical role in modulating the splanchnic inflammation related to partial portal vein ligation in the rat.

In rats with prehepatic PH we have shown a progressive hepatocytic fatty infiltration and changes in the hepatic lipid metabolism. In this way, the reduced Bcl-2 protein levels in the liver of PH rats favours the hepatocytic apoptosis and, therefore the hepatic steatosis. Non-alcoholic fatty liver disease (NAFLD) is characterized by a low grade systemic inflammation with raised levels not only of inflammatory (i.e., CCL2/MCP-1), but also of homeostatic chemokines (i.e. CCL19).

There is not up-regulation of CXCR4/SDF-1α in the liver of the rats with PH. As a consequence of the lack in the modulatory effects of this cytokine, impaired reparative mechanisms could take place since the proposed role of the increased SDF-1α in liver repair through stem cell recruitment.

In the CNS of PH-rats SDF-1α protein levels increased not only in the hypcampus, but also in the cerebellum and RANTES decreases in striatum. In the other hand, absent regulation of Fractalkine and CX3CR1 was observed in all brain studied areas, once could reflect a subclinical pathological manifestation of hepatic encephalopathy in these rats. The endogenous chemokine system in the brain, that includes CCL2/MCP-1, CXCL12/SDF-1α, CX3CL-1/Fractalkine, CXCL10/IP 10, CCL3/MIP-1α and CCL5/RANTES, is considered as the third major system of communication in the brain. It acts in concert with the neurotransmitter and neuropeptide systems to govern brain functions. Although our results do not allow us to explain the precise mechanisms by which SDF-1α is up-regulated in both brain areas of portal hypertensive-rats, several hypotheses could be speculated. Take into account that SDF-1α is a proinflammatory cytokine that regulates neurodevelopmental processes in the CNS, migration and neuronal migration, the increased SDF-1α levels found in the hippocampus and cerebellum could underlie in neuronal rearrangements or neurogenesis and remodeling in both brain areas at 1 month of evolution.

Chemokines have a dual role as neurodegenerative or neuroprotective molecules in the CNS. Nowadays, a role of SDF-1α in “neuronal repair mechanisms” in the hippocampus of rats suffering neurodegeneration is accepted. Interestingly, CX3CR1 was not upregulated in the hippocampus of portal hypertensive-rats and Nils staining did not show neuronal loss in all the hippocampal areas tested, neither apoptotic/antiapoptotic dysbalance, since Bcl-2 levels did not change. Conversely, SDF-1α overexpression has been related to neurogenesis in the subventricular zone and, therefore, it could play remodelatory actions since it is located in the inner granular cell layer connecting with mossy fiber in the hippocampus. All these factors taken together could suggest that SDF-1α overexpression in the hippocampus of portal hypertensive-rats induces neuronal repair mechanisms by activating immune responses.

Interestingly, CXCR4 is highly expressed by blood vessels of hippocampus in portal hypertensive-rats (Figure 5), once could be related to its role as well as the SDF-1α in neovascularization and remodeling in hippocampus and cerebellum in hypoxia and angiogenesis. Reparative mechanisms of ischemic injury of hippocampus and kidney in the rat by SDF-1α overexpression are associated with stem cell recruitment. Interestingly, hepatic chemokines production induces leukocyte recruitment to the hippocampus.

The reduced levels of RANTES found in striatum in rats with PH could impair striatal neurotransmission. Moreover, CCR5 and CXCR4 are expressed by dopaminergic DA1-D2 neurons in the striatal system. In rats with PH changes in catecholamines metabolism, with increases in the uptake and release of Norepinephrine, and in tyrosine hydroxilase activity in diencephalic and telencephalic brain regions and decrease of Prolactin, are developed. In this experimental model of prehepatic PH it has also been shown astrogliosis and angiogenesis in CA1 and CA4 hippocampal fields, a higher power of the delta band in EEG and increased blood-brain barrier permeability.
The CX3CR1/Fractalkine system is involved in paracrine neuron-glia communications. CX3CR1 is expressed in the basal state by microglia and is upregulated in response to neuropathy. This fact could confirm the subclinical neurological impairment of this experimental model, as also has been shown in rats with liver encephalopathy induced by thioacetamide. These rats showed cognitive inflexibility after training in the open field, and hippocampal-dependent paradigm.

The brain changes demonstrated in this experimental model could be related to the development of a minimal hepatic encephalopathy. Recently, we have proposed a hypothesis about the inflammatory etiopathogeny of hepatic encephalopathy. Since mast cells are key inflammatory players involved in the splanchic system as in the CNS, they could establish a connection between both systems. It is now generally accepted that mast cells are present in the normal brain in many mammalian species, including humans and rodents, particularly in the thalamus. In the brain, MCs can release different classes of mediators involved in impairments of neurologic functions. These immuneocytes may act as a mobile single-cell glands that deliver biologically active mediators to specific regions of the brain upon demand.

Mature MC have the ability of migrate from extraneural sources to CNS. Silverman et al. have demonstrated that adult rat peritoneal MCs, labelled with ex vivo with vital dyes (PKH26 and Cell Tracker Green) translocated in only 1 hour to the CNS, close to thalamic blood vessels surrounding astrocyte processes and contacting the neuropil, after intravascular injection into a host rat. So, mature MCs, like other immune system cells, can traffic through the CNS upon demand.

CONCLUSIONS

The brain plasticity would reflect the great ability of the CNS for developing metabolic adaptation strategies when it detects that the homeostasis has been disrupted, as in hepatic disease or stress. Taking together all of these facts, we can hypothesize that mast cells would be involved in a splanchic-brain crosstalk chemokine-mediated. Therefore, a true splanchnic-brain axis would be created in this experimental model of portal hypertension.

LIST OF ABBREVIATIONS


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