Maraviroc, a CCR5 antagonist, ameliorates the development of hepatic steatosis in a mouse model of non-alcoholic fatty liver disease (NAFLD)

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Objectives: Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the general population. The NAFLD spectrum ranges from simple steatosis to cirrhosis. The chemokine CCL5/RANTES plays an important role in the progression of hepatic inflammation and fibrosis. The objective of this study was to examine the effects of maraviroc, a CCR5 antagonist, on liver pathology in a NAFLD mouse model.

Methods: A total of 32 male C57BL/6 mice were randomly assigned to one of four groups: (i) control group (chow diet plus tap water); (ii) maraviroc group (chow diet plus maraviroc in drinking water); (iii) high-fat diet (HFD) group (HFD plus tap water); and (iv) maraviroc/HFD group (HFD plus maraviroc). All mice were sacrificed 16 weeks after the beginning of the experiment. Biochemical analyses and liver examinations were performed.

Results: Mice in the HFD group showed a tendency towards increased body mass gain and liver damage compared with the maraviroc/HFD group. Moreover, liver weight in the HFD group was significantly higher than in the maraviroc/HFD group. Hepatic triglyceride concentration in the maraviroc/HFD group was significantly lower than in the HFD group. Interestingly, the maraviroc/HFD group exhibited a lower degree of steatosis. Furthermore, hepatic CCL5/RANTES expression was significantly lower in the maraviroc/HFD group than in the HFD group. Overall, no differences were observed between the control group and the maraviroc group.

Conclusions: Maraviroc ameliorates hepatic steatosis in an experimental model of NAFLD.

Keywords: chemokine, CCL5, high-fat diet, mice models

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome characterized by triglyceride (TGD) accumulation (hepatosteatosis) in the absence of chronic viral hepatitis infection or alcohol consumption.1 NAFLD includes a wide spectrum of liver diseases with very different natural courses and prognoses, ranging from simple steatosis, which usually has a benign and non-progressive course, to non-alcoholic steatohepatitis (NASH), which may progress to liver fibrosis, cirrhosis and end-stage liver disease, including hepatocellular carcinoma.1-3

Nowadays, NAFLD is considered the most common cause of hepatic dysfunction in the general population worldwide,4,5 and its prevalence has increased markedly among adults and children owing to the rising incidence of diabetes and obesity.5 This frequency is even higher in HIV/HCV-coinfected patients (30%–70%).8-12 In these patients, the interactions among HIV infection, viral hepatitis and steatosis accelerate the progression to hepatic fibrosis.9-11 Thus, a better understanding of NAFLD is crucial.

On a molecular level, chemokines play a major role in the health of the immune system. Some of these chemokines have been implicated in different aspects of murine and human liver disease.13 One of these is the proinflammatory molecule CCL5 (also known as RANTES).14,15 Higher levels of CCL5 mRNA have been observed in the livers of HCV patients with an advanced stage of liver injury.16 Indeed, Seki et al.17 showed that CCL5 promotes hepatic inflammation and fibrosis in two different models of experimental fibrogenesis. Furthermore, increased hepatic CCL5 expression in a murine model of hepatic steatosis18 and in obese patients has been reported.19 CCL5 is a natural ligand for the chemokine receptor CCR5. CCR5 also plays a central role in all the events related to the remodelling of liver matrix, and it...
has been observed that patients with chronic liver disease show high levels of CCR5 and CCL5. In addition, gene targeting or the use of a potent antagonist for the murine CCR5 results in a significant reduction of liver fibrosis and hepatocellular carcinoma.

Several small-molecule antagonists of CCR5 have been evaluated as therapeutic options for the treatment of HIV-infected patients, although only one is currently approved for clinical use: maraviroc. These molecules are also effective in blocking CCR5 signal transduction. In the present study, we aimed to demonstrate whether maraviroc ameliorated the development of NAFLD in an experimental mouse model of high-fat diet (HFD)-induced NAFLD.

Methods

Ethics statement

All procedures were carried out in accordance with the European Communities Council Directive (86/609/CEE) on animal experiments and with approval of the ethics committee on animal welfare of our institution. All procedures were carried out in accordance with the European Communities Council Directive (86/609/CEE) on animal experiments and with approval of the ethics committee on animal welfare of our institution (Comité Ético de Experimentación Animal del Centro de Investigación Biomédica de La Rioja, CEEA-CIBIR).

Animal model

A total of 32 male C57BL/6 mice were purchased from Charles River (Barcelona, Spain). All animals had free access to food and drink during the study. When the animals were about 5 weeks old, they were randomly assigned (n=8) to one of four groups and fed for 16 weeks as follows: (a) control group [fed with a normal chow (standard diet RM1A (P); SDS, Essex, UK) and tap water]; (b) maraviroc group [fed the same diet as the control group but also received maraviroc in the drinking water]; (c) HFD group [these animals were fed a normal chow (standard diet RM1A (P); SDS, Essex, UK) and tap water]; and (iv) maraviroc/HFD group (these animals received an HFD (D12492; Research Diets Inc., NJ, USA) and tap water). The four dietary groups had a similar baseline weight. There was a significant statistical interaction between the control group and the HFD group in inducing weight gain (P<0.0001). No differences were observed when comparing the HFD and maraviroc/HFD groups.

Blood sampling and analysis

Plasma levels of alanine aminotransferase (ALT), glucose, TGD and total cholesterol were measured using an automatic biochemical analyser (Cobas C711, Roche, Madrid, Spain). Insulin levels were determined using a commercial kit (Emd Millipore, MO, USA). Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) using the following formula: fasting blood glucose (mg/dL) x fasting insulin (μU/mL)/405.

Hepatic TGD content

To quantify liver TGD content, 150 mg of liver tissue were homogenized using Ultraturrax (IKA-Werke, Staufen, Germany) in 1.5 mL of buffer (150 mM NaCl, 0.1% Triton X-100 and 10 mM Tris pH 8) at 50°C. After centrifugation at 12,000 g for 10 min, the obtained supernatant was used for the measurement of TGD levels, using an AutoAnalyzer as described elsewhere.

Real-time gene expression analysis

Quantitative real-time PCR (qPCR) was performed using Sybr Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and specific primers were used for CCL5 (forward 5′-ATATGGCTCGACACCACCTC-3′; reverse 5′-GTGACAAAACACGACTGCAA GA-3′), CCR5 (forward 5′-CGAAACACATGGCTCAACG-3′; reverse 5′-TTCTCT CTCACAGCTGCAT-3′); tumour necrosis factor α (TNF-α; forward 5′-ACCGCATGATCTCAAAGAC-3′; reverse 5′-AGATAGCAAAATCGGCTGAAG-3′) and interleukin 6 (IL-6; forward 5′-ATGGATGCTACAAACTGGGAT-3′; reverse 5′-TGAAGGACTCTTGCTTGTGCTT-3′). Amplification and detection of specific products were performed using the ABI PRISM 7300 (Applied Biosystems, Foster City, CA, USA). All procedures were performed according to the manufacturers’ instructions. All qPCR reactions were performed in triplicate, and the β-actin gene was used to normalize gene expression (forward 5′-GGCTGTATTCCTCCCTCATCG-3′; reverse 5′-CCAGTTGGTAACAA TGCCATGT-3′).

Haematoxylin–eosin staining

Following fixation, tissues were dehydrated and embedded in paraffin. Tissue sections (3 μm thick) were rehydrated and stained with haematoxylin–eosin according to standard protocols. One section from different hepatic lobes was analysed for each animal and three random pictures were taken from each section with the x10 objective. A single pathologist who was unaware of the treatments blindly scored all the samples. Specimens were scored for the severity of hepatocellular steatosis (NAFLD activity score). This result represents the sum of scores for steatosis (0–3), lobular inflammation (0–3) and ballooning (0–2). The range of this score is from 0 to 8 (a higher score means a higher risk).

Statistical analysis

All results are expressed as means ± SEM. Body weight data were analysed with analysis of variance followed by the Dunnet post-hoc test. For all other data, the Kruskal–Wallis test was used followed by the Mann–Whitney U-test. Correlation between variables was determined using the Spearman rank-sum test. All data were analysed with GraphPad Prism 5 software and were considered statistically significant when P<0.05.

Results

Maraviroc decreased weight gain

The four dietary groups had a similar baseline weight. There was a significant statistical interaction between the control group and the HFD group in inducing weight gain (P<0.0001). No differences were observed when comparing the HFD and maraviroc/HFD groups (Figure 1a). The greater weight gain of the mice in the HFD groups could not be attributed to greater food intake (data not shown).

Likewise, the increase in the weight of the fat pads was significantly larger in the HFD group when compared with the control group (P<0.0001; Figure 1b). No differences were observed when comparing the HFD and maraviroc/HFD groups.
Maraviroc decreased liver weight

Normal liver weight was observed in the control groups (i.e. the control and maraviroc groups), whereas there was a significant increase in liver weight in the animals of the HFD group when compared with those receiving a control diet ($P$, $0.0001$; Figure 1c). Liver weight was significantly lower in the maraviroc/HFD group than in the HFD group (1.3 versus 1.7 $\pm$ 0.1 g; $P$, 0.001).

Maraviroc reduced lipid accumulation in the liver

Liver TGD content in the HFD group was clearly higher than in the maraviroc/HFD group (59.9 $\pm$ 7 versus 39.9 $\pm$ 3.8 mg/g tissue; $P$, 0.05; Figure 1d). Lipid accumulation in the liver resulted in pale discoloration in the HFD group (not shown). The control and maraviroc groups showed a similar TGD content.

A significant association was observed between liver weight and hepatic TGD content in the HFD group ($r$, 0.95; $P$, 0.001). No significant relationship was observed when the other groups were analysed.

Maraviroc improved glucose plasma levels and lipid metabolism

Glucose levels were statistically higher in the HFD group, but other differences were not observed. When we analysed insulin levels and the HOMA-IR index, they were both higher in the maraviroc group than in the control group ($P$, 0.05), and were also significantly higher in the HFD groups. The levels of insulin and HOMA-IR index did not improve significantly in the maraviroc/HFD group when compared with the HFD group (Figure 2a–c). Cholesterol levels were also significantly higher in the HFD groups ($P$, 0.001; Figure 2d). No differences between groups were observed when plasma TGDs were analysed (Figure 2e).

Maraviroc reduced liver damage and steatosis

Serum ALT level, a marker of hepatic damage, was normal in the control group but higher in the HFD and maraviroc/HFD groups (Figure 3a). Remarkably, there was a statistically significant difference in ALT levels when comparing the HFD and maraviroc/HFD....
groups (66 ± 7 versus 43.7 ± 2.8 IU/L; P < 0.05). The NAFLD activity score was higher in the HFD group than the maraviroc/HFD group (2.6 ± 0.2 versus 1.7 ± 0.2; P < 0.01; Figure 3b). No differences were observed when we compared the control groups (i.e. the control and maraviroc groups).

Finally, these control groups (Figure 3c and d) displayed normal liver morphology. However, the HFD group showed a marked macrovesicular steatosis with focal lymphocytic infiltration, hepatocellular dropout and intense lobular inflammation (Figure 3e). However, the administration of maraviroc (maraviroc/HFD group) mitigated these effects, with the livers showing less steatosis (microvesicular) and less inflammation (Figure 3f). Fibrosis was absent in the four experimental groups.

Maraviroc reduced liver CCL5 mRNA expression

At this early stage of NAFLD, no differences in the expression of TNF-α (Figure 4a), IL-6 (Figure 4b) or CCR5 (Figure 4c) were observed in the liver. However, CCL5 expression was clearly lower in the maraviroc/HFD group than in the HFD group (1.7 ± 0.4 versus 0.6 ± 0.1; P < 0.05; Figure 4d).

Discussion

In this study, we demonstrated that the administration of maraviroc to mice fed an HFD has a protective effect against the development of NAFLD. Animal models of NAFLD give crucial information not only about the pathogenesis of NAFLD but also about the therapeutic effects of different therapies. To our knowledge, this is the first study to evaluate the preventive role of maraviroc in the development of NAFLD.

Decreased hepatic TGD is a target for NAFLD therapy because lipid loss reduces many liver injury mediators, including insulin resistance and proinflammation. In our study, hepatic TGD content was clearly lower in the maraviroc/HFD group than in the HFD group. Indeed, histological examination of the livers revealed a better NAFLD activity score in the maraviroc/HFD group in comparison with the HFD group. Moreover, ALT levels were lower in the maraviroc/HFD group when compared with the HFD group, indicating the presence of reduced hepatocellular injury in these mice. Taken together, this study suggests that maraviroc could prevent or delay the development of NAFLD. Kitade et al. also observed that CCR5−/− mice were protected from insulin resistance, hepatic steatosis and diabetes induced by...
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Figure 3. ALT levels, NASH score index and histological images of liver sections stained with haematoxylin–eosin. (a) A significant increase in ALT levels was recorded in the animals of the HFD group. These levels were significantly lower in the maraviroc/HFD group compared with the HFD group. (b) A significant increase in NASH score index was observed in the groups that received the high-fat diet (the HFD and maraviroc/HFD groups). The score was significantly lower in the maraviroc/HFD group compared with the HFD group. (c–f) Representative haematoxylin–eosin-stained sections of liver from the four experimental groups of mice (original magnification ×10). (c and d) The control groups (i.e. the control and maraviroc groups) displayed a normal liver morphology. (e) The livers of the HFD group revealed important microvesicular steatosis. (f) The livers of the maraviroc/HFD group showed intermediate characteristics between the control groups and the HFD group, and the steatotic pattern was macrovesicular. Each bar represents the mean ± SEM of at least seven animals. **P<0.01 and ***P<0.001 with respect to the control group. &P<0.05 with respect to the HFD group. MVC, maraviroc. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

an HFD. In our model, although not significant, we observed that the maraviroc/HFD group not only had lower insulin levels but also a lower HOMA-IR index, which would suggest a beneficial effect on the initial stages of this liver damage. Previous studies from our group showed a dose-dependent increase in glucose uptake in subcutaneous adipocytes treated with maraviroc.15,17 Indeed, the study of Díaz-Delfín et al.33 also showed that maraviroc might minimize adverse effects on adipose tissue development, metabolism and inflammation. Thus, the actions of maraviroc on glucose uptake and adipocyte metabolism could explain, at least in part, the beneficial effects on glucose/insulin/HOMA-IR parameters observed in this study. Knowledge of the clinical impact of maraviroc on glucose metabolism is more limited, although it has been postulated to have a protective effect on type 1 diabetes mellitus.36,37 However, Bigoloni et al.36 observed, in HIV-infected patients treated with a nucleoside analogue-sparing regimen, more cases of new diabetes mellitus in the group of patients treated with raltegravir plus etravirine plus maraviroc than in those treated with the same regimen but using the protease inhibitor darunavir instead of maraviroc.

Maraviroc is also safe with respect to lipid metabolism, as its use has not been associated with elevations in cholesterol, LDL-cholesterol or TGD and has shown beneficial effects on the lipid profile of dyslipidaemic patients.37 In our case, we did not find any difference either in cholesterol or TGD levels, one might consider that systemic changes may occur prior to hepatic changes. However, in a mouse model of genetic dyslipidaemia (ApoE−/−) accelerated by exposure to ritonavir, the authors observed that the treatment of these mice with maraviroc was able to inhibit the progression of spontaneous atherosclerosis by interfering with inflammatory cell recruitment into plaques.38 In the same way, although dyslipidaemia plays the main pathogenic role in this model, the clinical benefit could not be explained by any change in the lipid profile, since maraviroc failed to counteract the pro-dyslipidaemic effects of ritonavir.

Multiple chemokines and cytokines have been found to be involved in the development of steatosis and the progression to NASH.39,40 The chemokine receptor CCR5 has been identified on hepatic stellate cells, suggesting that these liver cells are both a target for and source of CCL5.15,17 In the same way, lipid accumulation is known to induce, dose dependently, CCL5 expression in primary human hepatocytes in vitro.41 These authors also observed that mice fed an HFD had significant hepatic CCL5 expression, even in the absence of significant hepatic inflammation or fibrosis.41 So, although hepatic steatosis is usually considered a benign and reversible process this might not be the case, since CCL5 production by hepatocytes could be an early event in the natural progression of NAFLD to steatohepatitis.41 In our case, we were also able to identify, at this early stage, a higher (but not statistically significant) CCL5 expression in the HFD group. This expression was significantly reduced in the presence of maraviroc, which certainly is important because there is an association between high levels of CCL5 mRNA expression and the presence of fibrosis in fatty liver disease.42

In relation to cytokines, TNF-α and IL-6 are two of the most important molecules (or factors) in the development of NAFLD.43 Crespo et al.42 observed in obese patients an overexpression of TNF-α mRNA in the liver of NASH patients. In the same way,
there is a relationship between TNF-α expression and insulin resistance in NASH.41 For this reason, mice treated with the anti-TNF-α drug thalidomide showed some improvements in the hepatic alterations mediated by an HFD.44 More complex is the role of IL-6 in patients with NASH, because while some studies have shown a positive correlation between IL-6 expression in hepatocytes and the severity of the NAFLD,45 others have shown that IL-6 could also sensitize the liver to injury by stimulating hepatocyte apoptosis, inducing insulin resistance and so participating in the development of NASH.46 In mice fed with a diet that induced NASH, IL-6 pathway neutralization with specific antibodies against the IL-6 receptor enhanced hepatic steatosis, but improved liver damage.46 Our study showed no differences in TNF-α or IL-6 expression between any of the groups analysed.

Finally, although hepatic steatosis leads to chronic hepatic inflammation and progression to fibrosis,9–11 this study was focused on the early changes of NAFLD prior to the development of hepatic fibrosis, because this is poorly understood. Besides, in a previous study we were able to demonstrate that maraviroc was able to reduce mortality, liver fibrosis and tumorigenesis in a mouse model of hepatocellular carcinoma.22 In the same way, a preliminary clinical study showed that maraviroc treatment reduces liver stiffness in HIV/HCV-coinfected patients.47

**Conclusion**
The findings of our study provide evidence that maraviroc has a protective effect on the development of NAFLD. Owing to the fact that maraviroc has a preventive role in liver damage,22 we believe that maraviroc could be an essential antiretroviral drug in HCV/HIV or HIV-infected patients with liver damage (i.e. hepatic steatosis). These data could justify a randomized, controlled trial in order to determine the beneficial effects of maraviroc on the progression of NAFLD.

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Transparency declarations

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