Research Article

JOURNAL OF HEPATOLOGY

Hepatitis C viral proteins perturb metabolic liver zonation

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Background & Aims: The metabolic identity of a hepatocyte is determined by its position along the porto-centrilobular axis of a liver lobule. Altered patterns of metabolic liver zonation are associated with several pathologies. In hepatitis C, although only a minority of hepatocytes harbour the virus, the liver undergoes major systemic metabolic changes. We have investigated the HCV-driven mechanisms that allow the systemic loss of metabolic zonation.
Methods: Transgenic mice with hepatocyte-targeted expression of all HCV proteins (FL-N/35 model) and needle biopsies from hepatitis C patients were studied with respect to patterns of lipid deposition in the context of metabolic zonation of the liver lobule.
Results: We report that low levels of viral proteins are sufficient to drive striking alterations of hepatic metabolic zonation. In mice, a major lipogenic enzyme, fatty acid synthase, was redistributed from its normal periportal expression into the midzone of the lobule, coinciding with a highly specific midzone accumulation of lipids. Strikingly, alteration of zonation was not limited to lipogenic enzymes and appeared to be driven by systemic signalling via the Wnt/β-catenin pathway. Importantly, we show that similarly perturbed metabolic zonation appears to precede steatosis in early stages of human disease associated with HCV infection.
Conclusions: Our results rationalize systemic effects on liver metabolism, triggered by a minority of infected cells, thus opening new perspectives for the investigation of HCV-related pathologies.

Introduction

Hepatic lobule, the basic unit of the liver architecture, is organised into spatially distinct areas along the porto-centrilobular axis, known as metabolic zones [1]. As a consequence, metabolic liver functions involved in lipid, carbohydrate and ammonia metabolism, as well as detoxifying activities, are carried out by distinct sets of hepatocytes, whose metabolic identity in a healthy liver is determined by their position within the lobule.

Hepatitis C virus (HCV), which is a major causative agent of HCC [2], has a vital requirement on lipid droplets for its replication and spread (reviewed in [3]). Several viral proteins, as well as structural motifs of the viral RNA, have been reported to initiate lipogenic signalling [4,5], leading to hepatic steatosis, which is a frequent complication of chronic hepatitis C [6].

Transgenic mice with liver-targeted expression of the HCV full-length open reading frame (FL-N/35 lineage) develop a spectrum of liver metabolic disorders, including alterations of lipid metabolism [7] and micro- and macro-vesicular steatosis [8]. In addition to reproducing a wide range of pathological features associated with chronic hepatitis C [9,10], males of this lineage are cancer-prone [8]. While this phenotype is perfectly reproducible in different animal colonies [11], some genetic backgrounds, for example the widely used CB57Bl/6 animals, are protected from the HCV viral protein-driven carcinogenesis ([12] and our unpublished data).

Because there is an increasing evidence that steatohepatitis is a risk factor for hepatocellular carcinoma, we set out to examine alterations of lipid metabolism in the tumour-prone HCV transgenic mice. Here we report that FL-N/35 animals maintained in the mixed genetic background (C57Bl/6:C3H, 1:1) develop a striking metabolic phenotype, with a clearly zonated origin of HCV-driven steatosis. Moreover, the viral proteins perturb the overall hepatic metabolic zonation in a pattern similar to that found in hepatitis C patients at early stages of liver pathology.

Materials and methods

Ethics statement

Clinical samples were collected in accordance with the Declaration of Helsinki by the Centre des Collections Biologiques Hospitalières de Montpellier (CCBH-M). The research programme was approved by the CCBH-M coordinator and by the Scientific Council of the Montpellier University Hospital on July 6, 2010. The Montpellier University Hospital received the authorisation for use of the samples for scientific research from the National Ethics Committee under

Keywords: HCV; Metabolic liver zonation; Lipogenesis; Wnt/β-catenin pathway.
Received 28 February 2014; received in revised form 22 August 2014; accepted 1 September 2014; available online 16 September 2014
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Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; PP, periportal; PV, perivenous; FASN, fatty acid synthase; ACSL3, acyl-CoA synthetase long-chain family member 3; SCD1, stearoyl-CoA desaturase; GS, glutamine synthetase.

the reference DC-2010-1185/AC-2010-1200. All samples used in this study were collected previously from adult participants and were anonymized prior to use. Animal care and experiments conformed to the European Council Directive 2010/63/EU and French legislation. The protocols were approved (ID of approval for this study N° F 34-172-16) by the Languedoc-Roussillon ethics committee (CEEA-LR1013).

Mice

Six-month-old FL-N/35 males, transgenic for the entire HCV open reading frame (genotype 1b) and wild type controls, both of the C57Bl/6:C3H genetic background were used.

Patient tissue samples

44 liver biopsies from HCV genotype 1 positive and 6 from HCV-negative patients were performed with a cutting needle (Monopty 18 G, Bard Biopsy Systems, Tempe, USA), formalin-fixed and embedded in paraffin. A biopsy was considered adequate for histological analysis if it was 10 mm or longer. The samples used in this study had a median length of 15 mm (range, 10–45 mm). Patients' clinical characteristics are presented in Table 2. All patients were non-obese and at most slightly overweight (BMI <27; median +/− SD = 23.1 +/− 2.3), non-diabetic and without any previous anti-viral treatment. Patients with known excessive alcohol consumption were excluded from the analysis. Samples were processed for immunohistochemistry and read by two expert pathologists (J. Ramos and B. Rivière), who also scored them for steatosis (negative, centrilobular or diffuse) and fibrosis (semi-quantitative METAVIR analysis).

For biochemical analysis, resected samples of non-tumoral regions of livers from patients suffering from HCV-related hepatocellular carcinoma and, as controls, either hepatocellular adenoma or hepatic metastases of endocrine tumours, were used. Small pieces were snap frozen in liquid nitrogen and stored at −80 °C before RNA extraction. In parallel, samples were processed for immunohistochemistry. Cirrhotic samples were excluded from the analysis.

Immunohistochemistry

Mice were sacrificed by cervical dislocation. Livers were removed, fixed in neutral buffered formalin pH 7.2-7.4 (Diapath, Microm Microtech, France) and embedded in paraffin. Sections of 4 µm were either stained with haematoxylin and eosin (H/E) or subjected to immunohistochemical staining (see Supplementary Materials and methods for detailed protocols).

Sections of human liver biopsies were analysed using an automated immunohistochemical stainer (Ventana HX system BenchMark, Ventana Medical Systems, Tucson, AZ). At least 7 periportal-centrilobular axes per biopsy were scored by two experienced pathologists (B. Rivière and J. Ramos).

FASN, steatosis and glutamine synthetase (GS) score evaluation

Distribution of FASN staining in mouse livers was assessed relative to periportal areas, identified by an experienced veterinary anatomopathologist and delimited as illustrated in Fig. 1B. Lack of periportal staining was arbitrarily set at the limit of <30% of hepatocytes labelled by anti-FASN antibody within the area. 30 lobules, each from 3 FL-N/35 transgenic and 3 age and sex-matched control mice, were analysed. 47 human biopsy samples (42 HCV^+ and 5 HCV^-) were evaluated by two experienced pathologists (B. Rivière and J. Ramos). Normal localization (largely periportal) and diffuse staining patterns are illustrated in Supplementary Fig. 3. Steatosis was estimated on haematoxylin/eosin stained sections (Supplementary Fig. 2). GS expression in human samples was evaluated by an experienced anatomopathologist following immunohistochemical staining. Score 0 corresponds to centrilobular pattern, score 1 shows a limited spread of GS labelling and score 2 corresponds to GS expression occupying the entire lobule (Fig. 3).

Statistical analysis

Statistical analyses were performed using Prism Software (GraphPad Software Inc., La Jolla, CA). Mean +/− SEM between groups were compared by a two-tailed Student's *t* test and distributions by the χ^2 test as indicated.

Results

HCV-driven steatosis is zonated

Tumour-prone animals of the FL-N/35 lineage maintained in the Bl/6:C3H genetic background develop hepatic steatosis at about 6 months of age. Histological analysis of liver sections revealed that lipid accumulation occurred in an exquisitely zonated pattern, with lipid-filled hepatocytes occupying only one to two rows of cells in the midzone of the hepatic lobule (Fig. 1A). Development of steatosis was quite abrupt: while it was absent in 3–4 months old mice, it was present in 75% (n = 12) of animals at 6 months of age.

In agreement with histological analyses, biochemical assays revealed the increased fatty acid content in transgenic livers ($p = 0.016$) (Table 1). The very specific midzone localization notwithstanding, the fatty acid composition of lipids in the livers of transgenic animals was comparable to that previously reported, both in livers of HCV-core transgenic mice and in clinical samples of patients suffering from hepatitis C-related steatosis [13]. The high abundance of palmitate (C16:0), which is the final product of fatty acid synthase (FASN), as well as its elongated forms, stearic (18:0) and oleic (18:1) acid, is suggestive of *de novo* lipogenesis as the main source of lipid accumulation in transgenic livers.

In agreement with this hypothesis and with previously reported data [7], appearance of steatosis was associated with the increased expression of enzymes involved in lipogenesis, such as fatty acid synthase (FASN) and those necessary for further steps of fatty acid metabolism, such as acyl-CoA synthetase long-chain family member 3 (ACSL3); stearoyl-CoA desaturase 1 (*SCD1*) mRNA was also increased, albeit without reaching statistical significance (Supplementary Fig. 1A). In support of this observation, the SCD1 activity index (18:1 n-9/18:0) was increased significantly ($p = 0.024$). Analysis of FASN protein abundance in transgenic livers also suggested a slight increase, without reaching statistical significance (Supplementary Fig. 1B and C).

In addition to these rather modest quantitative changes in expression, the HCV transgenic livers displayed a qualitatively altered distribution of FASN-positive hepatocytes. Immunohistochemical analysis of control livers of 6-month old animals revealed diffuse periportal to midzone staining of FASN (Fig. 1B, left panel). The control animals were either free of steatosis or presented a very mild, diffuse steatosis. In contrast, the FL-N/35 transgenic livers were steatotic and displayed a markedly altered pattern of FASN distribution: the periportal zone was free of labelling, instead the enzyme concentrated in the midzone and extended towards the centrilobular region, (Fig. 1B, right panel). A semi-quantitative analysis, performed on 30 lobules from three wild type and three FL-N/35 age- and sex-matched animals, confirmed a highly significant change of FASN expression pattern (Fig. 1C). Prior to the quantification, the pericentral and periportal regions were identified by a veterinary pathologist (Fig. 1B) and scored as $FASN^+$ if >30% (usually 90–100%) of hepatocytes were positive and $FASN^-$ if <30% cells were labelled. Since biochemical assays suggested a slight increase of FASN level and IHC analysis revealed that it is present in fewer cells in transgenic livers, it would appear that the expression of this lipogenic enzyme is likely to be significantly increased in the region of lipid accumulation, i.e. in zone 2 hepatocytes of the FL-N/35 mice.