Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis

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ABSTRACT Liver steatosis, which involves accumulation of intracytoplasmic lipid droplets, is characteristic of hepatitis C virus (HCV) infection. By use of an in vivo transgenic murine model, we demonstrate that hepatic overexpression of HCV core protein interferes with the hepatic assembly and secretion of triglyceride-rich very low density lipoproteins (VLDL). Core expression led to reduction in microsomal triglyceride transfer protein (MTP) activity and in the particle size of nascent hepatic VLDL without affecting accumulation of MTP and protein disulfide isomerase. Hepatic human apolipoprotein AII (apo AII) expression in double-core/apo AII transgenic mice diminished intrahepatic core protein accumulation and abrogated its effects on VLDL production. Apo AII and HCV core colocalized in human HCV-infected liver biopsies, thus testifying to the relevance of this interaction in productive HCV infection. Our results lead us to propose a new pathophysiological animal model for induction of viral-related steatosis whereby the core protein of HCV targets microsomal triglyceride transfer protein activity and modifies hepatic VLDL assembly and secretion.—Perlemuter, G., Sabile, A., Letteron, P., Vona, G., Topilco, A., Chretien, Y., Koike, K., Pessayre, D., Chapman, J., Barba, G., Brechot, C. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis FASEB J. 16, 185–194 (2002)

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Liver steatosis is an important hallmark of several viral infections, notably that concerning hepatitis C virus (HCV) (1–3). There is a substantial body of evidence to implicate steatosis in the development of hepatic fibrosis (4, 5). The mechanisms of viral-related steatosis, however, are unknown. Persistent HCV infec-

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MATERIALS AND METHODS

Transgenic mice

HCV core transgenic mice (lineage C57BL/6N) were obtained as described previously (14). Full-length HCV core expression is achieved in the liver under the control of the HBx gene promoter of hepatitis B virus (14). Apo All transgenic mice of the same lineage (C57BL/6N) expressing human apo All under the control of the regulatory region of the human apo Al gene have been described (16) and were generously provided by Dr. Rubin (Berkeley, CA). Heterozygous siblings were mated to obtain double transgenic mice expressing both HCV core and apo All. Mice were fed with a chow diet containing 3000 kcal/kg (59% nitrogen-free extracts, 17% proteins, 3% lipids, 12% moisture, 5% minerals, and 4% fibers). Since liver steatosis occurs in 100% and 50% of core-expressing males and females, respectively (14), 6-month-old males (transgenic and control) were used. In all experiments, mice were maintained in a fasting state for 24 h.

Lipid metabolism

β-oxidation

A tracer dose of [U-14C]palmitic acid (150 μCi/kg, 0.16 μmol/kg) was administered by gastric intubation in 0.2 ml of corn oil to label newly exhaled CO2. Mice were immediately placed for 6 h in a small plastic cage swept by an air flow of 50 ml/min. The output was bubbled into 100 ml of ethanolamine/2-methoxyethanol (30/70%, v/v). Each hour, 1 ml was removed and counted for [14C]CO2 activity. Results were calculated by measuring fluorescence at the excitation wavelength of 465 nm and emission wavelength of 598 nm using the Fluoroskan Ascent FL (Labsystems S.A., Paris, France).

Lipid peroxidation

Lipid peroxidation was assessed by measuring ethane exhalation and intrahepatic thiobarbituric acid reactants (TBARS) (19, 20). Groups of five mice were weighed and placed in a closed chamber (2.1 L) where CO2 and H2O were trapped and the partial pressure of O2 was maintained constant. The concentration of ethane in air was measured, as previously reported, by gas-liquid chromatography (19). Liver peroxidation products reacting with thiobarbituric acid in vitro were measured by spectrophotometry (20).

Electron microscopy

Ultrathin sections were examined unstained or slightly counterstained with lead citrate. Morphological examination of randomly chosen fields was performed in a JEOL-JEM 1010 electron microscope.

Microsomal triglyceride transfer protein (MTP) activity assay

MTP activity was measured by using an MTP assay Kit according to the manufacturer’s instructions (Roar Biomedical, New York, NY). The assay is based on a transfer of fluorescence, due to MTP activity, between donor and acceptor particles. Liver samples were homogenized and sonicated in buffer (15 mM Tris pH 7.4, 40 mM NaCl, 1 mM EDTA, and protease inhibitors) (22). The MTP assay was performed by incubating 10 μl (50 μg protein) liver homogenate (MTP source) with 10 μl of donor and 10 μl of acceptor solutions in a total volume of 250 μl buffer (15 mM Tris pH 7.4; 40 mM NaCl; 1 mM EDTA) and incubated for 0 to 24 h at 37°C. MTP activity was calculated by measuring fluorescence at the excitation wavelength of 465 nm and emission wavelength of 538 nm using the Fluoroskan Ascent FL (Labsystems S.A., Paris, France).

Northern blot

Liver samples were homogenized in Trizol reagent and total RNA extraction was performed. Then, poly(A) + RNA extraction was performed using the Oligotex mRNA Kit (Qiagen, Courtaboeuf cedex, France). Ten micrograms mRNA from HCV core-positive and -negative transgenic mice liver were size-fractionated on 1% agarose gel in Mops buffer (20 mM, pH 7.0), transferred to a Hybond-N+ nylon membrane (Amersham, Little Chalfont, UK), and probed with a random-primed mouse MTP cDNA (kind gift from Dr. L. Chan).

Western blot

Liver samples were homogenized at ice-cold temperature in a buffer containing 50 mmol/l NaCl, 0.5% Nonidet P-40, 10 mmol/l Tris-HCl pH 8, and 1× protease inhibitor mixture tablets (Boehringer Mannheim, Paris, France). Western blot was performed as described previously (15), using anti-core polyclonal RR8 antibody (1:2,500) (kind gift from Dr. M. Kohara, Tokyo, Japan), polyclonal antibody anti-MTP (1:1,000) (kind gift from Dr. H. Jamil, Bristol-Meyers Squibb, Syracuse, NY), polyclonal antibody anti-protein disulfide isomerase (PDI) (1:1,000) (kind gift from Dr. L. Aggerbeck), and, as an internal control, a monoclonal anti-β-tubulin antibody (1:2,500) (Boehringer Mannheim, Mannheim, Germany). Band intensities were semiquantified using NIH Image 1.57 software (National Institutes of Health, Bethesda, MD).

TNF-α and IL-6 level determination

Tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) were tested in 24 h fasted mice serum and liver using Quantikine mouse TNF-α and IL-6 Kits (R&D Systems, Abingdon, UK). Liver samples were homogenized at ice-cold temperature in phosphate-buffered saline (PBS).

In situ immunofluorescence analysis

Liver sections from two HCV-positive patients with chronic active hepatitis were placed in OCT compound (Miles Laboratories, Elkhart, IN) and snap frozen in liquid nitrogen for subsequent immunofluorescence analysis. The 5 μm frozen sections were dehydrated in ethanol and embedded in Epon 812. Ultrathin sections were examined unstained or slightly counterstained with lead citrate.
sections were fixed in cold acetone. After permeabilization with 0.5% Triton X100, 0.1% deoxycholate in PBS for 30 min, slides were incubated with primary antibodies (monoclonal anti-core; Euromedex; Souffelweyersheim, France) and polyclonal anti-apo AII (kind gift from Dr. Vu-Dac, Institut Pasteur, Lille, France). Antibodies were diluted in PBS 2% FCS, 0.1% Triton X100 for 48 h at 4°C. After washing and blocking with PBS-2% FCS for 30 min at 37°C, the secondary antibodies coupled to FITC and Cyn-5 for core and apo AII labeling, respectively, were added for 1 h. The slides were mounted in Immunomount (Shandon, Runcorn, Cheshire, UK) and analyzed by confocal laser scanning microscopy.

RESULTS

HCV core protein expression reduces VLDL secretion

Taking advantage of an in vivo model of HCV-related steatosis, we have analyzed the main biochemical pathways potentially implicated in steatosis in transgenic mice with the same genetic background, expressing either the HCV core protein alone or both HCV core protein and apo AII. Hepatic TG accumulation can be driven principally either by fatty acid overload, inhibition of fatty acid β-oxidation, decreased secretion of TG-rich, very low density lipoprotein (VLDL), or a combination of these mechanisms (23, 24). After gastric administration of [U-14C]palmitic acid, no difference in [14C]CO2 exhalation was detected between core-, apo AII-, core/apo AII-expressing mice and control animals (data not shown; P=0.91); thus, although we cannot exclude a partial mitochondrial β-oxidation defect, such a mechanism does not appear to play a central role in liver steatosis. Baseline serum concentration of TG was moderately but not significantly decreased; baseline apo B was unchanged in core-expressing mice (Fig. 1a, b). The secretion of TG and apo B, as measured after inhibition of lipoprotein

Figure 1. Serum TG and apo B concentration and increase before and 4 h after Triton WR 1339 administration. a) Moderate but not statistically significant decrease of baseline serum TG concentration in HCV core transgenic mice (10 mice in each group; global comparison: P=0.39). b) No statistically significant modification of baseline serum apo B in the four mice groups (10 mice in each group; global comparison: P=0.26). c, d) Serum TG and apo B increase 4 h after i.p. Triton WR 1339 injection. The y axis shows the ratio of plasma TG and apo B concentration after and before Triton injection. c) Decreased TG secretion in HCV core, but not core/apo All mice, compared with controls and apo All mice (10 mice in each group; global comparison: P<0.002; multiple comparisons: P<0.05 for all comparisons with HCV core transgenic mice). d) Decreased apo B secretion in HCV core, but not core/apo AII mice, compared with controls and apo AII (10 mice in each group; global comparison: P<0.0003; multiple comparisons: P<0.01 for all comparisons with HCV core transgenic mice). Graphs show median ± percentile; nonparametric variance analysis (Kruskal-Wallis) and multiple comparison test (Games-Howell).
lipase activity by Triton WR 1339 (18), was markedly reduced in core protein-expressing mice (Fig. 1c, d) compared with control nontransgenic and apo AII-expressing mice (global and multiple comparisons with HCV core-expressing mice: \( P<0.002 \) and 0.05 for TG; \( P<0.0003 \) and 0.01 for apo B, respectively). Core/apo AII, double transgenic mice showed normal TG and apo B secretion under the same experimental conditions (Fig. 1c, d); thus, HCV core protein profoundly impairs VLDL-TG and apo B secretion, and this effect is abrogated by hepatic expression of human apo AII.

**HCV core protein expression decreases MTP activity and VLDL particle size**

The above results led us to hypothesize that perturbation of hepatic lipid metabolism resulted from a defect in assembly and/or secretion of nascent VLDL. Electron microscopic analysis of hepatic tissue facilitated determination of VLDL particle size and abundance. Comparison of particle size revealed a marked reduction in the number of normal-sized lipoprotein particles as inferred from plasma-derived VLDL (25) in both the Disse space and Golgi areas of the liver of core-expressing (Fig. 2b, d) vs. control nontransgenic mice (Fig. 2a, c): mean 6.1 ± 2.1 vs. 17.3 ± 1.4 normal-sized particles per 5 \( \mu \text{m}^2 \) in the Disse space (~ 250 particles counted in six independent fields; nonparametric variance analysis, Mann-Whitney: \( P<0.003 \)) and 0.54 ± 0.24 vs. 15.1 ± 4.3 normal-sized particles per 5 \( \mu \text{m}^2 \) in the Golgi area (>150 particles counted in 11 independent fields; nonparametric variance analysis, Mann-Whitney: \( P<0.002 \)). In contrast, there was no evidence for abnormal VLDL particle size in core/apo AII transgenic mice (Fig. 2f). This observation was suggestive of a defect in VLDL assembly.

Based on these findings, we tested the possibility that HCV core protein might impair MTP activity and/or apo B expression. MTP and apo B are major regulators of VLDL assembly: MTP stabilizes apo B by lipidation; lipidated apo B then fuses with TG-rich particles, leading to nascent VLDL formation (26, 27). We observed a significant decrease in MTP activity in core protein-expressing mice compared with controls (\( P<0.0001 \)) (Fig. 3a).

Collectively, therefore, the results of our biochemical and ultrastructural analyses were strictly correlated. They demonstrate that HCV core protein impairs VLDL secretion by decreasing MTP activity and thus VLDL assembly. Despite these effects on MTP activity, there was only a moderate (not statistically significant) decrease in baseline serum TG and no effect in apo B baseline serum concentration in core-expressing mice (Fig. 1a, b). These findings lead us to hypothesize that viral-induced modification in VLDL assembly may impair catabolism of apo B-containing particles in HCV core transgenic mice so that effects on basal levels of serum lipoproteins are absent or mild. In contrast, when lipoprotein lipase is totally inhibited by Triton WR 1339, only the major difference in the hepatic secretion rates remains, and the increase in serum lipoproteins is much lower in core transgenic mice. Increased apo CIII or decreased apo E lipoprotein B contents have been shown to impair the in vivo turnover of VLDL, leading to delayed intravascular catabolism (28–32); thus, the decreased hepatic secretion of triglycerides may be partly compensated by a decreased removal of serum triglycerides in core-expressing mice.

**HCV core does not alter MTP and protein disulfide isomerase (PDI) accumulation**

To explore the mechanisms implicated in the inhibition of MTP by HCV core, we tested whether core
might alter MTP RNA and/or protein accumulation. Figure 3b shows representative results obtained when analyzing poly(A)^+ RNA extracted from core-positive and -negative mice. MTP RNA accumulation varied among mice within each group; there was no difference between core-positive and -negative mice. Western blot analysis (Fig. 3c) also showed absence of any significant difference in hepatic MTP protein accumulation between core-positive and -negative mice.

MTP activity is dependent on a complex consisting of the catalytic unit (MTP) and PDI. We therefore also searched for any effect of core on PDI accumulation. Figure 3d shows that there was no difference in hepatic PDI accumulation between core-positive and -negative mice. Previous reports have shown decreased MTP encoding gene transcription by cytokines such as TNF-α and IL-6 (33). We therefore tested whether HCV core protein expression might modify accumulation of these cytokines in serum and liver samples of transgenic mice. Consistent with the results of our Northern blot and Western blot analyses, there was no difference in TNF-α and IL-6 levels, which were extremely low (<23 pg/ml and <15.6 pg/ml in serum for TNF-α and IL-6, respectively; <6 pg/mg liver proteins in liver samples for both TNF-α and IL-6) in both control and core-expressing mice.

**HCV core protein expression increases lipid peroxidation**

Having dissected the mechanisms of core-related steatosis, we then evaluated the potential effect of core protein on hepatic lipid peroxidation. Evidence is available for a key role of elevated peroxidation in the induction of both liver fibrosis as well as DNA repair abnormalities (19, 34, 35). Indeed, increased peroxidation was observed in core protein-expressing mice compared with those expressing both core and apo AII proteins, as determined by measurement of ethane exhalation and hepatic TBAR content (19, 20) (Fig. 4a, b).
HCV core colocalizes with apo AII in human HCV-infected liver samples

We previously reported colocalization of apo AII and HCV core proteins in vitro in two independent studies performed on CHO and CCL13 cells (13, 15). We could not test for apo AII/HCV core colocalization in transgenic mice liver section, since this assay implies the use of a monoclonal antibody (raised in mice). To circumvent this difficulty and further expand the relevance of our results to productive viral infection in humans, we analyzed two liver biopsies obtained from HCV-infected patients. Figure 5 depicts representative results obtained in liver cells that showed a colocalization between apo AII and core. This experiment was based on frozen sections analyzed by immunofluorescence and confocal microscopy; thus, it was not possible to precisely determine the cellular sublocalization of the two colocalizing proteins. Only a fraction of HCV core colocalized with apo AII. This observation might be related at least in part to the presence of various HCV core forms (including in particular carboxyl-terminally deleted core) in infected cells. Our previous study has led to map the domain of HCV core implicated in binding to apo AII to its carboxyl-terminal moiety; it is therefore plausible that only some HCV core molecules bind apo AII. An additional explanation might relate to the different level of HCV core expression achieved after in vitro ectopic expression and in vivo in infected livers.

DISCUSSION

Considered together, our findings have facilitated identification of a new pathophysiological mechanism of viral-induced liver steatosis whereby a viral protein alters MTP activity, a major regulator of the assembly and secretion of nascent TG-rich VLDL particles. Until now, viral-induced steatosis has been thought to be related to general liver dysfunction (36). Steatosis is a frequent histological hallmark of HCV, but not HBV, infections (2, 37). We now demonstrate that in vivo expression of HCV core protein in transgenic mice decreases hepatic apo B and TG secretion as well as the assembly of intracellular VLDL particles and inhibits MTP activity. Thus, our study offers the first evidence for an effect of a viral protein, namely, the HCV core protein, on liver VLDL assembly and secretion as a result of marked reduction of MTP function.

MTP is present in the ER lumen as the heterodimer of a 97 kDa subunit (MTP) and a 58 kDa subunit (PDI) (38), an abundant ER chaperone involved in MTP translocation into the ER lumen and MTP folding (see review in ref 39). MTP transfers lipids from various donor lipid sites to acceptor sites in vitro and plays a fundamental role in vivo in the cotranslational lipidation of Apo B as it enters the ER lumen, thus preventing Apo B degradation (27). In this way, a precursor particle is formed, which may be converted to VLDL by addition of bulk TG. In the present study, HCV core expression did not modify MTP or PDI expression, but inhibited the MTP-mediated transfer of triglycerides from donor to acceptor vesicles in vitro and the assembly and secretion of TG-rich VLDL particles in vivo, thus indicating that core expression inhibits MTP activity without changing MTP expression. Although the exact mechanism has not been demonstrated, our results may orient toward several plausible hypotheses. Even though core associates with several other cellular proteins, we and others could not demonstrate an association of HCV core protein with
ApoB, MTP, or PDI in a two-yeast hybrid screening system using liver-derived cDNA libraries (15). Consistent with this observation, we failed to show MTP and HCV core protein colocalization in HCV-infected liver sections (data not shown). One possibility, though, is that core-mediated MTP inhibition instead involves the interaction of HCV core with lipids rather than proteins. Core associates with the cytosolic surface of the ER membrane (10, 13) and the periphery of TG-rich lipid droplets (13). The presence of HCV core on lipid surfaces might prevent the interaction of lipids with MTP and thus inhibit lipid mobilization from one donor lipid site to MTP, then from MTP to another lipid acceptor site, including apo B. Although ER HCV core is mainly attached to the cytosolic surface of the ER membrane, there is some indication that a minor fraction of core could also reach the ER lumen. In the present study, we were able to detect small amounts of core protein in the serum of core-expressing mice by enzyme immunoassay (40) in the absence of any liver cell necrosis (data not shown). One possibility is that the interaction of core with the lipids of the ER membrane (and perhaps intraluminal triglycerides) could hamper MTP-mediated transfer of triglycerides to the ER lumen and thus VLDL particle formation. Other possibilities could involve an effect of oxidative stress (discussed later) on MTP folding or function or core-mediated modifications of another protein affecting MTP function.

Whereas HCV core could not be shown to bind to apo B, MTP, or PDI, we previously demonstrated that a fraction of HCV core binds apo AII and is secreted in vitro upon fibrate-mediated apo AII overexpression (15). In the present study, we demonstrate that the hepatic expression of apo AII in double-core/apo AII transgenic mice abrogates the effects of core on VLDL secretion. Although apo AII expression has been reported to induce VLDL secretion in apo E-deficient mice (41), VLDL secretion was in fact unchanged in apo AII-expressing mice compared to nontransgenic mice in the present study. Thus, a more specific mechanism is involved in the protective effects of Apo AII overexpression against the HCV core effects. Indeed, semiquantification showed decreased intracellular HCV core concentration in double-core/apo AII transgenic mice (Fig. 6). This leads us to propose a model reconciling the apparently divergent effects of core, which interacts with lipids and MTP on one hand and with apo AII on the other hand. A major consequence of the apo AII/core interaction could be to drive core into the secretory pathway as an Apo AII/core heterodimer that does not inhibit MTP activity (possibly because the heterodimer, unlike core, does not bind to lipids, as the lipid-interacting surfaces of core are
already involved in Apo AI/core heterodimer formation) (Fig. 7).

Circulation of naked core particles has been reported in HCV-infected patients (42; A. Budkowska, unpublished results). Our present in vivo investigation combined with our previous in vitro data supports the contention that secretion of core might occur during viral infection and might depend on core/apo AI interaction. A major feature of HCV infection is the extremely high rate of progression (70–80%) to chronicity (reviewed in ref 43). Some studies have suggested that core protein might depress T cell response to HCV antigens, particularly by binding the complement receptor gC1qR on T cells; it is thus plausible that HCV core protein might contribute to HCV persistence (44, 45). Our data were obtained from transgenic mice expressing the HCV core protein alone and therefore we do not exclude the potential role of other HCV proteins. We also lack a valid in vivo model of productive HCV infection in order to fully address the relevance of our findings to human HCV infection. It is noteworthy, however, that steatosis is a well-recognized feature of HCV infection and that transgenic mice expressing the full-length HCV polyprotein also show steatosis (46). Moreover, we have demonstrated colocalization of apo AI and HCV core protein in HCV-infected human livers. This finding, therefore, supports our previous observations and further emphasizes the in vivo relevance of this interaction to productive HCV infection.

HCV is a major etiological factor of HCC (6); there is now evidence for a direct role of some HCV proteins (core, NS3, NS5A, and E2) in controlling liver cell proliferation and viability. Some core- or polyprotein-expressing transgenic mice develop HCC; interestingly, in the two independent core-expressing transgenic mice (including our present model) so far reported, liver steatosis precedes HCC development in the absence of chronic hepatitis (46, 47). Elevated lipid peroxidation is a functional consequence of steatosis and involves the generation of reactive oxygen species that have been implicated in DNA damage and carcinogenesis (34). High levels of hepatic lipid peroxidation products were observed on core protein expression and could be involved in part in HCV-mediated liver carcinogenesis.

Regulation of the mechanisms implicated in the assembly and secretion of VLDL is the subject of extensive interest. A human genetic disorder (namely, abetalipoproteinemia) arises as the result of mutations in the gene encoding MTP and is characterized by impaired VLDL assembly (48). Pharmacological inhibition of MTP activity or genetic inactivation of the MTP gene induces defective VLDL secretion and steatosis (49). Our results lead us to propose that this major pathway of lipid metabolism can be altered by viral infection as well as by gene mutation.

**Figure 6.** Liver HCV core concentration. a) 30 and 2 μg total proteins from mouse liver and cell extracts, respectively, were tested with polyclonal anti-core antibody RR8 for core detection and monoclonal anti-β-tubulin antibody for normalization. Lane 1, HepG2 cells stably expressing HCV core; lanes 2a, b, two different HCV core transgenic mice; lanes 3a, b, two different HCV core/apo AI double transgenic mice; lane 4, apo AI transgenic mice; lane 5, nontransgenic mice. Lanes 3a, b show decreased intrahepatic core concentration in core/apo AI-expressing mice, as compared with core-expressing mice (lanes 2a, b). b) Semiquantification of HCV core concentration. Liver immunoblots of 12 core and 12 core/apo AI transgenic mice were scanned and band intensities semiquantified. Graph shows median ± percentile; nonparametric variance analysis, Mann-Whitney test: P < 0.05 (statistically significant difference from control is indicated by an asterisk).

**Figure 7.** Hypothetical model of HCV core-induced steatosis and secretion. In HCV core protein transgenic mice, HCV core inhibits MTP activity, thus inhibiting VLDL assembly and secretion. In HCV core/apo AI double transgenic mice, the association of core protein with apo AI drives core into the secretory pathway. Thus, HCV core protein concentration is decreased in the liver and its effects on VLDL assembly and secretion are abrogated.
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