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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., Chrétien, Y., Koike, K., Pessayre, D., Chapman, J., Barba, G., Bréchot, 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)**

Key Words: HCV · VLDL · MTP

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 infection, which develops in at least 70–80% of infected patients, often progresses to chronic hepatitis and is highly correlated with the development of liver cirrhosis and hepatocellular carcinoma (HCC) (6–8). HCV is an enveloped virus belonging to the Flaviviridae family. The virus genome is a linear, positive-stranded RNA molecule of ~9600 nucleotides that contains a single open reading frame encoding a polyprotein precursor of ~3000 amino acids. The amino-terminal portion of the viral RNA encodes for the structural proteins (C, E1, and E2), followed by the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Both host and viral proteases cleave the polyprotein into at least 10 mature proteins (9, 10). There is now evidence for a key role of HCV core protein in regulating cellular gene expression as well as cell proliferation and viability (see reviews in refs 8, 11, 12). The core protein induces accumulation in liver cells of intracytoplasmic triglyceride-rich droplets in vitro (13) and in vivo in some core-expressing transgenic mice (14). Our earlier studies established that the core protein binds human apolipoprotein AII (apo AII) and that this association results in core protein secretion in vitro (15). Here we have investigated the molecular and cellular mechanisms underlying development of liver steatosis in an in vivo transgenic murine model of HCV core protein expression.

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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 AII transgenic mice of the same lineage (C57BL/6N) expressing human apo AII under the control of the regulatory region of the human apo AI 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 AII. 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-^{14}C]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 CO_2. Mice were immediately placed for 6 h in a small plastic cage swept by an air flow of 50 ml/min. The outflow was bubbled into 100 ml of ethanolamine/2-methoxyethanol (30/70%, v/v). Each hour, 1 ml was removed and counted for [^{14}C]CO_2 activity. Results were expressed as cumulative exhalation of [^{14}C]CO_2 over a 6 h period (17).

Hepatic triglyceride and apolipoprotein secretion

We evaluated the increase in serum triglyceride (TG) and apolipoprotein B (apo B) after administration of Triton WR 1339 (Tyloxapol; Sigma, Paris, France) in 24 h fasted mice (18). Thirty milligrams of Triton from a stock solution of 100 mg/ml were injected i.p. in each mouse. Serum TG and apo B were determined immediately before and 4 h after injection, using commercially available colorimetric assays: triglyceride enzymatic trinder (Biotrol, Paris, France) and kit 357 for apo B (Sigma).

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 CO_2 and H_2O were trapped and the partial pressure of O_2 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

Fragments (~0.5 mm^3) of mouse liver (four mice/group) were prefixed by immersion with 1.6% glutaraldehyde in 0.1 M phosphate pH 7.3 (Sörensen's buffer). After washing in the same buffer, the tissue samples were postfixed in phosphate-buffered osmium tetroxide for morphological analysis or in imidazole-buffered OsO_4 as a stain for lipids (21). All material was dehydrated in ethanol and embedded in Epon 812. 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 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-^{14}C]palmitic acid, no difference in [^{14}C]CO_2 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. 1*a, b*). The secretion of TG and apo B, as measured after inhibition of lipoprotein

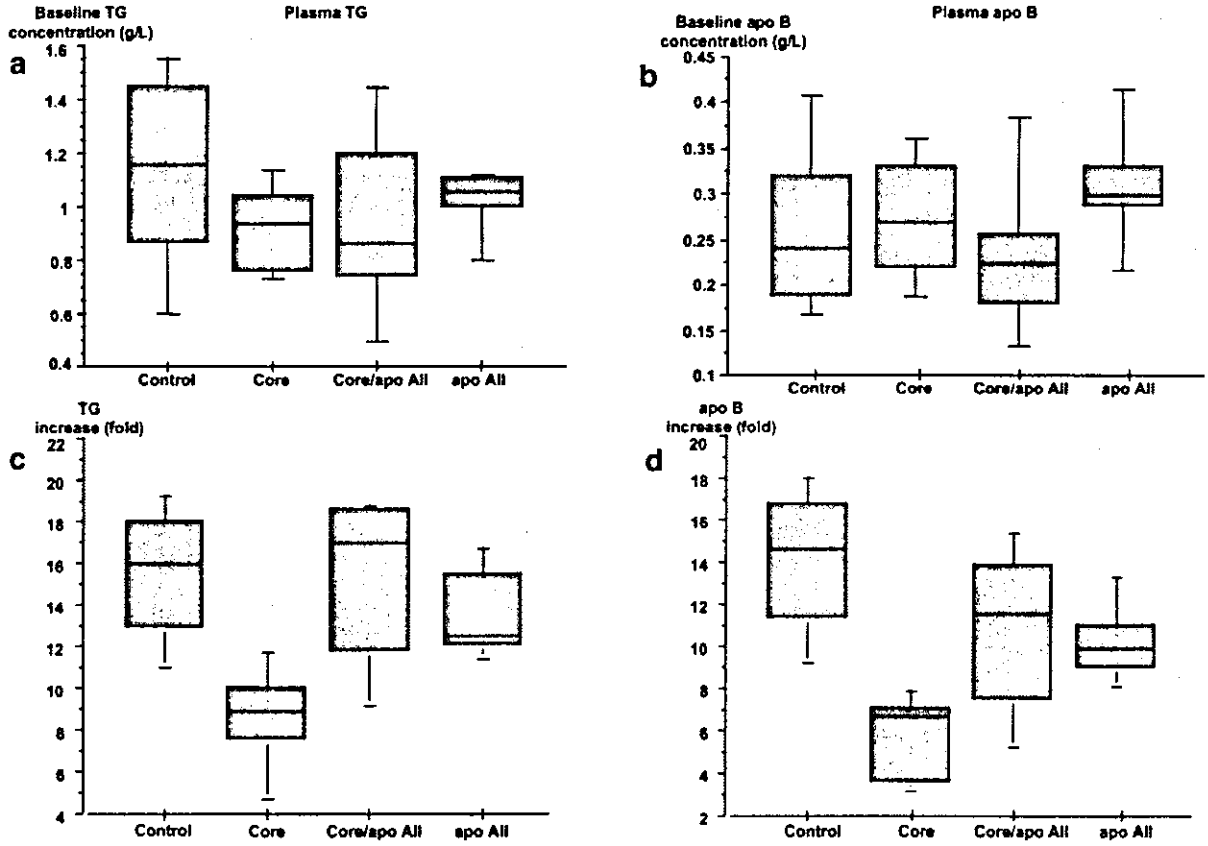

Figure 1. Serum TG and apo B concentration and increase before and 4 h after Triton WR 1339 administration. *a, b)* Serum TG and apo B concentration before Triton WR 1339 injection. *a)* Moderate but not statistically significant decrease of baseline serum TG concentration in HCV core transgenic mice (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 AII mice, compared with controls and apo AII 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. 1*c, 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. 1*c, 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. 2*b, d***) vs. control nontransgenic mice (Fig. 2*a, c*): mean 6.1 ± 2.1 vs. 17.3 ± 1.4 normal-sized particles per 5 μm^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 μm^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. 2*f*). 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. 3*a***).

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. 1*a, 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

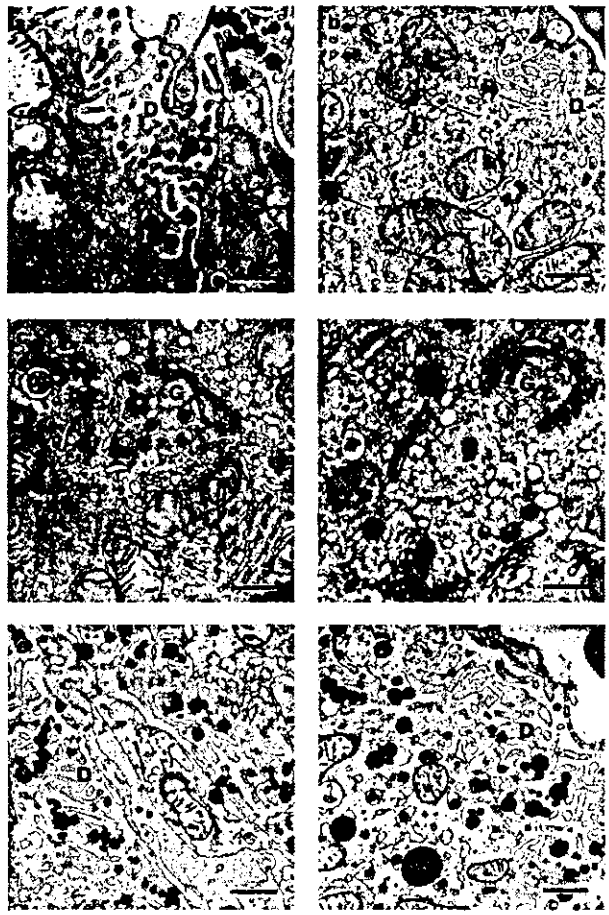

Figure 2. Electron micrograph of the Disse space (D) and the Golgi apparatus (G) of control, HCV core transgenic, apo AII transgenic and HCV core/apo AII double transgenic mice (×10,000). *a)* Disse space of control mice. *b)* Disse space of HCV core transgenic mice. *c)* Golgi apparatus of control mice. *d)* Golgi apparatus of HCV core transgenic mice. *e)* Disse space of apo AII transgenic mice. *f)* Disse space of HCV core/apo AII double transgenic mice. Control, apo AII, and core/apo AII transgenic mice show normal size of VLDL (50) whereas lipoprotein size is decreased both in Disse space and Golgi apparatus of HCV core transgenic mice (arrows). Although a few VLDL of normal size can be observed in the Disse space of HCV core transgenic mice, their number is decreased as compared to that in the other transgenic mice.

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

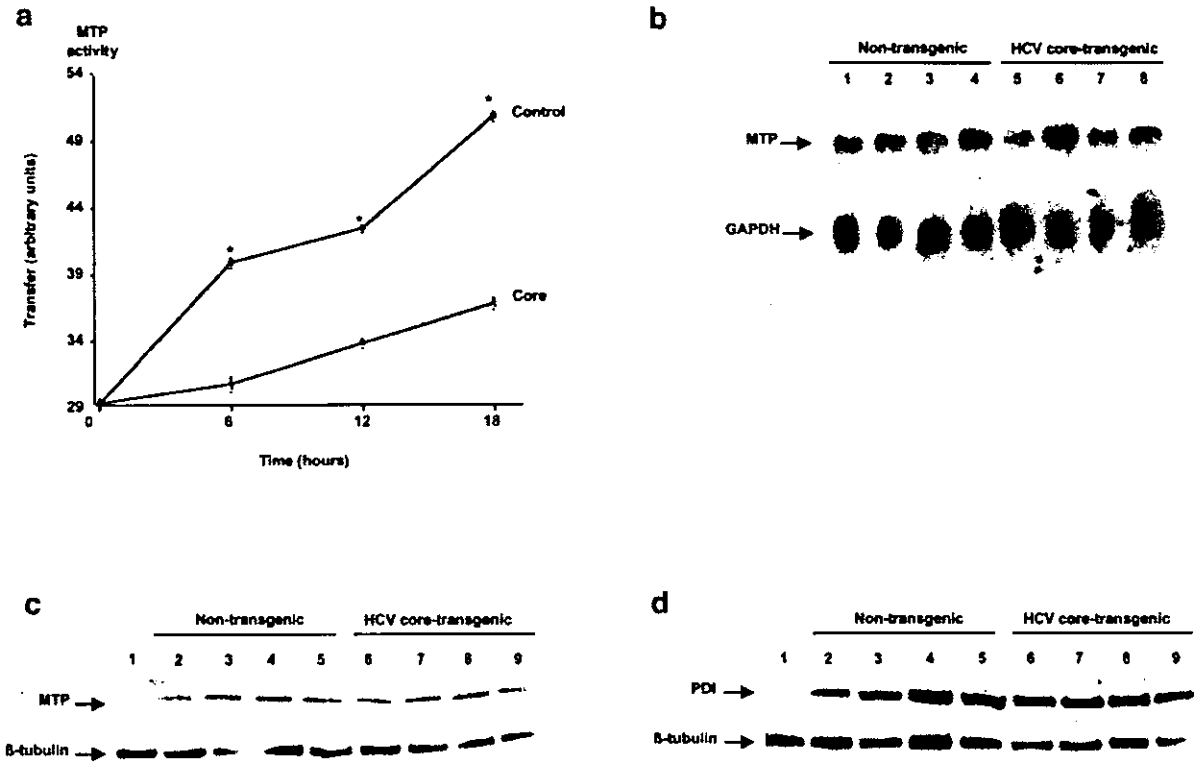

Figure 3. MTP/PDI activity and expression in liver of transgenic mice. *a)* MTP activity was measured using an MTP assay kit (22) in a kinetic assay during 18 h. Decreased MTP activity in HCV core transgenic mice as compared to control nontransgenic mice as early as 6 h after the incubation of liver extract proteins (MTP source) with donor and acceptor solutions. Values correspond to the fluorescence transfer from donor to acceptor particles and are expressed as arbitrary units according to the manufacturer's instructions (Roar Biochemical). They represent mean ± SD (10 mice in each group); variance analysis with repeated measures (ANOVA) $P < 0.0001$; Mann-Whitney test at 6th, 12th, and 18th hour: $P < 0.0001$ (statistically significant differences from control are indicated by an asterisk). *b)* Northern blot analysis of mice liver. Ten micrograms mRNA from HCV core-negative (lanes 1 to 4) and -positive (lanes 5 to 8) transgenic mice liver were size-fractionated, transferred to a nylon membrane and probed with a random-primed mouse MTP cDNA and GAPDH cDNA for normalization. *c, d)* Western blot analysis of mice liver. One hundred micrograms of total proteins from mouse liver were tested with polyclonal anti-MTP (*c*) and anti-PDI (*d*) antibodies and monoclonal anti-β-tubulin antibody for normalization. Lanes 1, negative control; lanes 2 to 5, 4 representative nontransgenic mice; lanes 6 to 10, 4 representative HCV core transgenic mice.

might alter MTP RNA and/or protein accumulation. Figure 3*b* 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. 3*c*) 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 3*d* 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. 4*a, b***).

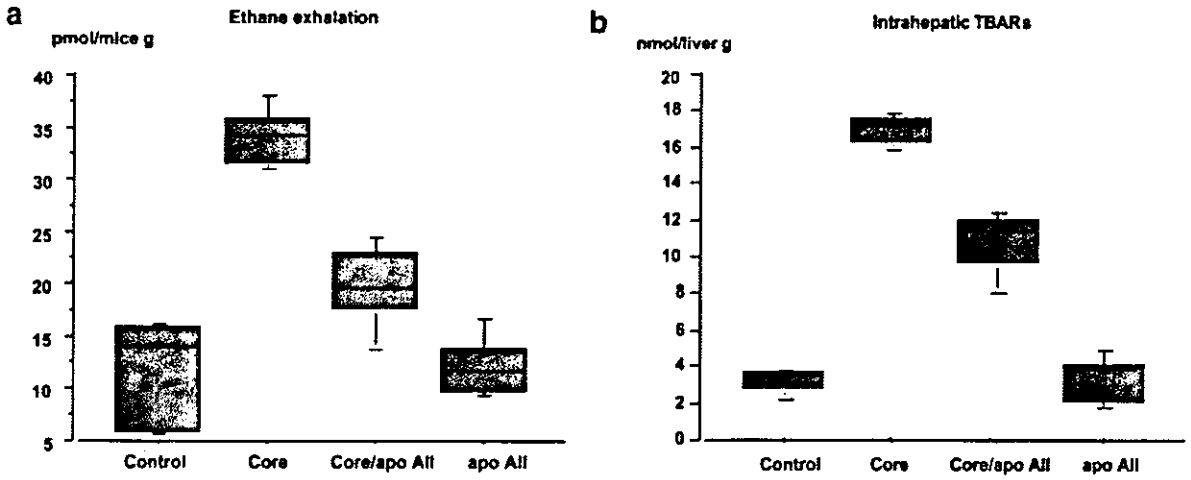

Figure 4. Lipid peroxidation in transgenic mice. *a, b)* Increased lipid peroxidation, measured by ethane exhalation (*a*) and TBARs (*b*) in HCV core, compared with controls, apo AII, and core/apo AII mice. *a)* Ethane exhalation; results are normalized to the mouse body weight (7 experiments with 4 mice in each group; global comparison: $P<0.0001$; multiple comparisons: $P<0.01$ for all comparisons with HCV core transgenic mice). *b)* Liver TBARs values expressed as malondialdehyde equivalents, normalized to liver weight (2 experiments with five mice in each group; global comparison: $P<0.0007$; 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).

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 cotranslocational 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

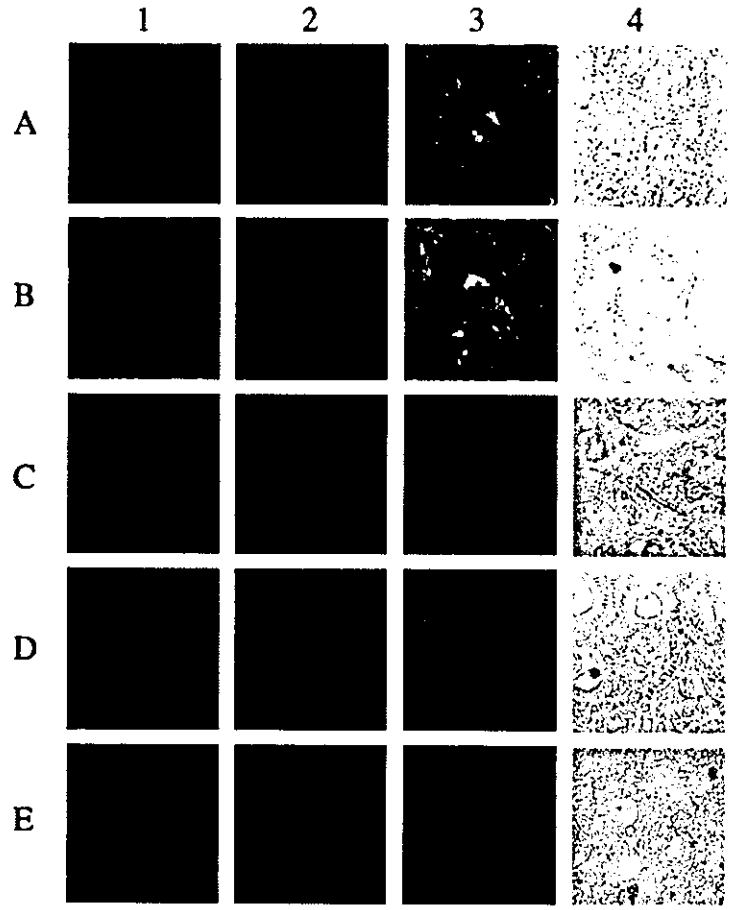

Figure 5. HCV core/apo AII colocalization in HCV-infected human livers. Confocal analysis of double immunofluorescence staining for the HCV core and apo AII proteins. *A, B)* Liver sections from two patients with HCV-related chronic hepatitis. *C)* Negative control corresponding to sample B, tested without primary antibody. *D)* Lung section from an HCV negative subject. *E)* Liver section from an HCV negative subject. Lane 1: apo AII protein stained by cyanin-5; lane 2: HCV core protein stained by fluorescein; lane 3: overlapping of the staining for apo AII and HCV core proteins; lane 4: phase contrast images of the tissue sections. ×63.

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 AII/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 AII 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

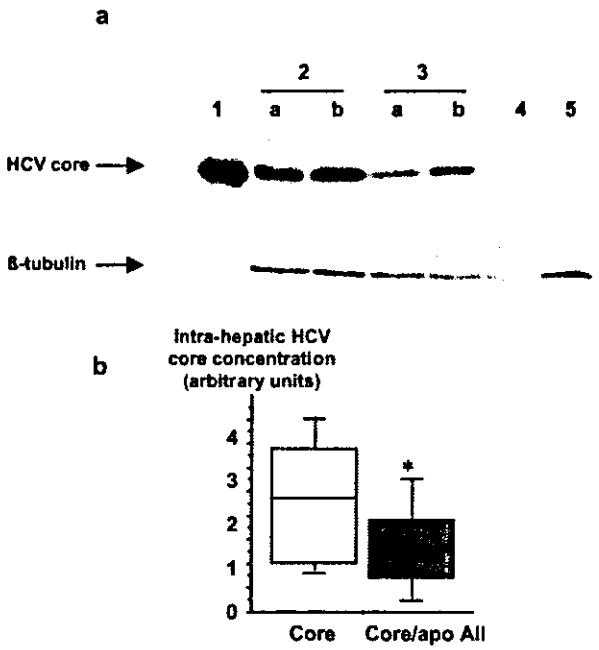

Figure 6. Liver HCV core concentration. *a)* 30 and 2 μg total proteins from mouse liver and cell extracts, respectively, were tested with polyclonal anti-core antibody RR8 for core detection and monoclonal anti-β-tubulin antibody for normalization. Lane 1, HepG2 cells stably expressing HCV core; lanes 2a, b, two different HCV core transgenic mice; lanes 3a, b, two different HCV core/apo AII double transgenic mice; lane 4, apo AII transgenic mice; lane 5, nontransgenic mice. Lanes 3a, b show decreased intrahepatic core concentration in core/apo AII-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 AII 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).

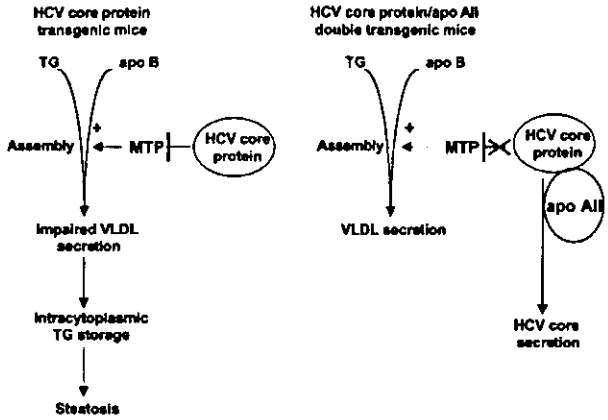

Figure 7. Hypothetical model of HCV core-induced steatosis and secretion. In HCV core protein transgenic mice, HCV core inhibits MTP activity, thus inhibiting VLDL assembly and secretion. In HCV core/apo AII double transgenic mice, the association of core protein with apo AII drives core into the secretory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

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 AII 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. FJ

We would like to thank Lawrence Aggerbeck (Centre de Génétique Moléculaire-CNRS) for helpful discussions and providing anti-MTP and anti-PDI antibodies, Flora Zavala (INSERM U 25) for TNF-α and IL-6 level determinations, Shintaro Yagi (Advanced Life Science Institute Inc., Japan) for performing the serum core assay (40), Gérard Feldman and Marie-Elisabeth Samson-Brouma (INSERM U 327) for helpful discussions. G.P. and A.S. were supported by grants from INSERM and the FRM. These studies were supported by INSERM, ARC, EC, and MNERT.

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Received for publication May 22, 2001.
Revised for publication September 27, 2001.

CYP3A4 Inducible Model for *In Vitro* Analysis of Human Drug Metabolism Using a Bioartificial Liver

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CYP3A is responsible for approximately 50% of the therapeutic drug-metabolizing activity in the liver. The present study was undertaken to establish the CYP3A4 inducible model for analysis of human drug metabolism using a bioartificial liver composed of the functional hepatocellular carcinoma cell (HCC) line FLC-5. A radial-flow bioreactor (RFB), which is a carrier-filled type bioreactor, was used for 3-dimensional perfusion culture of FLC-5 cells. The CYP3A4 messenger RNA (mRNA) expression level 48 hours after rifampicin treatment in the RBF was approximately 100 times higher than that in a monolayer culture. Western blot analysis also demonstrated an increase in expression of the CYP3A protein. When testosterone, a substrate for CYP3A4, was added to the rifampicin-treated cell culture, 6β-hydroxy testosterone as a metabolite was formed. Electrophoretic mobility shift assay (EMSA) with a CYP3A4 ER6 probe demonstrated that relatively high molecular weight complex containing pregnane X receptor (PXR)/retinoid X receptorα(RXRα), compared with that in the monolayer culture, is possibly generated in the RFB culture of FLC-5 treated with rifampicin. Similarly, the assay with a probe of HNF-4α-binding motif indicated the formation of a large protein complex in the RFB culture. Because it is known that PXR transactivates CYP3A4 gene via its response element and expression of PXR is regulated by HNF-4α, the large complexes binding to response elements of PXR or HNF-4α in the RFB culture may contribute to up-regulation of CYP3A4 mRNA. In conclusion, the bioartificial liver composed of human functional HCC cell line was useful in studying drug interactions during induction of human CYP3A4. (HEPATOLOGY 2003;37:665-673.)

Drug-drug interactions can be categorized as mediated by metabolic inhibition and enzyme induction. Numerous studies have been conducted on metabolic inhibition. It is now possible, to some extent, to predict drug-drug interactions *in vivo* based on the results *in vitro*. Enzyme induction has been studied using experimental animals, but, because of the existence of interspecies differences, it is often difficult to extrapolate the results of animal studies directly to humans.[1]

To resolve these problems, enzyme-induction experiments have been performed using human primary cell culture systems, and experimental models established using these cell cultures have been recognized to be useful in the evaluation of enzyme induction.[2] However, long-term primary culture of human hepatocytes can reduce the functions of liver enzymes, as reflected, for example, by a decrease in the level of cytochrome P450 (CYP), an enzyme mainly catalyzing phase-I reactions of drug metabolism, 4 days after isolation, making it difficult to use these cells for prolonged periods of time.[3] Furthermore, because of interracial or sex-related differences and differences in storage periods (cell viability), the drug-metabolizing activity varies greatly among cells, causing problems in reproducibility.[4,5] For these reasons, much has been expected of liver cell lines that have uniform and stable properties, can be used for long-term experiments, are highly differentiated, and retain human liver functions.

Abbreviations: CYP, cytochrome P450; HCC, hepatocellular carcinoma; RFB, radial-flow bioreactor; PXR, pregnane X receptor; RXR, retinoid X receptor; ER, everted repeat; FBS, fetal bovine serum; PB, phosphate buffer; HNF, hepatocyte nuclear factor; FAM, 6-carboxy-fluorescein; JOE, 2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine; RT-PCR, reverse transcription-polymerase chain reaction; mRNA, messenger RNA; HPLC, high performance liquid chromatography; EMSA, electrophoretic mobility shift assay.

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Received June 27, 2002; accepted December 6, 2002.

Supported in part by a grant-in-aid from the Promotion and Mutual Aid Corporation for Private Schools of Japan and the Japan Health Sciences Foundation (Research on Health Sciences Focusing on Drug Innovation, KH71068).

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0270-9139/03/3703-0023$30.00/0

doi:10.1053/jhep.2003.50094

Under these circumstances, studies of drug metabolism in cells derived from humans have been conducted using not only primary liver culture systems but also cell lines established from the liver, such as the human hepatoblastoma cell line HepG2, and the expression and induction of drug-metabolizing enzymes have been examined.[6-8] It has also been reported that their cell line differs from human hepatocytes in terms of cell morphology, protein synthesis, and enzymes involved in the metabolism of the drugs. To date, we have established 7 cell lines derived from Japanese hepatocellular carcinoma (HCC) patients. Of these, one that has relatively well-preserved liver functions and can be cultured in serum-free medium has been named *FLC* (functional liver cell).[9] The FLC-5 cell line was selected for this study because CYP3A4 expression can be induced in it, making it very useful for the study of drug metabolism.

The radial-flow bioreactor (RFB), developed in Japan, is a high-function 3-dimensional culture system, which can be used for high-density culture. This system activates a density 10 times that obtained in hollow-fiber culture systems[10,11] and 100 times that in floating cell culture. The cylindrical reactor is filled with porous hydroxy apatite beads. This bioreactor can be characterized as a system in which the medium flows from the periphery toward the center of the reactor. For obtaining a high-density cell culture, it is essential to ensure that biased distribution of oxygen and nutrients at the inlet and outlet of the culture medium is minimized. If the medium flows from the periphery toward the center, the high perfusion rate at the center would allow adequate supply of oxygen and nutrients to the cells at the center even while oxygen and nutrients are consumed at the periphery, thereby allowing the cells to remain viable. When FLC cells were incubated in this RFB system, they could be cultured at high density and maintained viable for long periods of time.[12] It has also been reported that the ability of FLC cells to produce albumin is higher when cultured in the RFB system than in monolayer culture.[13] These results suggest that this RFB system can be used to obtain cultured hepatocytes with improved cell functions.

Regarding drug-metabolizing functions, a model for analysis of drug metabolism in the human liver could be established if the cells were subjected to 3-dimensional high-density culture in the RFB system, and a bioartificial liver will be created. Of all the human CYP isoforms, CYP3A is responsible for approximately 50% of the therapeutic drug-metabolizing activity in the liver.[14] Particularly, CYP3A4 is the most important subtype of CYP3A in humans. Recently, it has been shown that CYP3A4 inductivity in the liver is regulated by the pregnane X receptor (PXR)/retinoid X receptor (RXR)α heterodimer through a response element containing 2 everted repeats separated by 6 nucleotides (ER6) of CYP3A4 promoter region.[15]

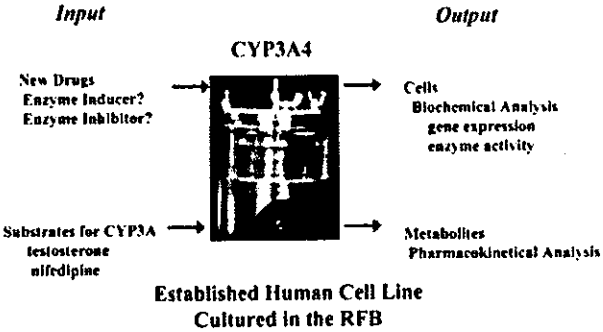

Fig. 1. The simulation system for drug metabolism composed of human functional HCC cell line cultured in RFB. This system is useful in studying drug interactions during induction of human CYP3A4.

In the present study, CYP3A4 inductivity through PXR/RXRα heterodimer in FLC-5 cells cultured in the RFB was compared with that of the cells in monolayer culture, with the goal of establishing a simulation model for the analysis of drug metabolism in the human liver, without recourse to experimental animals (Fig. 1).

Materials and Methods

Human HCC Cell Line. Human HCC cell line FLC-5 is maintained in ASF104 serum-free medium (Ajinomoto Co., Ltd., Tokyo, Japan) in an incubator with a constant temperature of 37°C and a highly humidified atmosphere of 95% air and 5% CO_2. Cell passage was carried out using 25 USP units/mL trypsin (Difco Co., Ltd.) added to 0.02% EDTA in solution to isolate cells. For this experiment, 1 to 2 $\times$ 10^6 cells of FLC-5 were cultured in 4 mL of ASF-104 medium without fetal bovine serum (FBS).

Culture in the Radial Flow Bioreactor. Use of an RFB (RA-15, ABLE Co., Ltd., Tokyo, Japan) and mass flow controller (RAD925, ABLE Co., Ltd.) has previously been reported for the high-density, 3-dimensional mass production of cells, which attach to a matrix.[12,13] The matrix consisted of hydroxy apatite beads (diameter 1-2 mm, pore size $<$200 μm, Asahi Optical Co., Ltd., Tokyo, Japan), with a high pore density, which gave a wide surface attachment area.

Two to 5 $\times$ 10^7 cells of FLC-5 cell were injected in the reservoir of the RFB system, which was filled in ASF104 medium. Isolated cells were loaded in 15 mL volume of the RFB column using a circulation pump at 25 mL/min. Loading cells became trapped and adhered to the porous culture beads at a rate based on medium flow. Although

FLC-5 cells can grow in serum-free medium, 2% FBS and 3 g/L glucose was added to the inoculation medium to facilitate attachment of cells to the matrix. After attachment of cells, the medium was changed to ASF104 without FBS.

Observation for Fine Morphology. For the scanning electron microscopy (SEM), cultured cells were fixed with 1.2% glutaraldehyde in 0.1 mol/L phosphate buffer (PB), pH 7.4 and postfixed with 1% OsO_4 in 0.1 mol/L PB. The fixed cells were rinsed twice with phosphate-buffered saline, subsequently dehydrated in ascending concentrations of ethanol, critical point dried using carbon dioxide, and coated by vacuum-evaporated carbon and ion-spattered gold. Specimens were observed by JSM-35 (JEOL, Tokyo, Japan) at an accelerated voltage of 10 kV. For transmission electron microscopy (TEM), cultured cells were fixed with 2.0% glutaraldehyde in 0.1 mol/L PB and postfixed with 1% OsO_4 in 0.1 mmol/L PB. Specimens were dehydrated in ethanol and embedded in a mixture of Epon-Araldite. Thin sections were made with a diamond knife mounted on a LKB ultratome and stained with aqueous uranyl acetate. Specimens were examined with a JEOL 1200EX electron microscopy.

Real-Time Polymerase Chain Reaction. Based on the DNA sequences in GenBank, primers and the TaqMan probe for CYP3A4, PXR, RXRα, and hepatocyte nuclear factor 4α (HNF-4α) were designed using the primer design software Primer Express TM (Perkin-Elmer Applied Biosystems, Foster City, CA).

AmpliTaq DNA polymerase extended the primer and displaced the TaqMan probe through its 5′-3′ exonuclease activity. The probes were labeled with a reporter fluorescent dye (6-carboxy-fluorescein [FAM] or 2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein [JOE]) at the 5′ end and a quencher fluorescent dye (6-carboxy-tetramethyl-rhodamine [TAMRA]) at the 3′ end.

The primer/probe is as follows: CYP3A4 forward primer: 5′-CTTCATCCAATGGACTGCATAAAT-3′, reverse primer: 5′-TCCCAAGTATAACACTCTACACAGACAA-3′; probe: 5′-(FAM) CCGGGGATTCTGTACATGCATTG (TAMRA)-3′. PXR forward primer: 5′-TCCCCAAATCTGCCGTGTAT-3′, reverse primer: 5′-AGCCCTTGCATCCTTCACAT-3′; probe: 5′-(FAM) ACAAGGCCACTGGCTATCACTTCAATGTCA (TAMRA)-3′. RXRα forward primer: 5′-GCGCTGAGGGAGAAGGTCTAT-3′, reverse primer: 5′-CAGGCGGAGCAAGAGCTTAG-3′; probe: 5′-(FAM) AGGCCTACTGCAAGCACAAGTACCCAGA (TAMRA)-3′. HNF-4α forward primer: 5′-GGTGTCCATACGCATCCTTGA-3′, reverse primer: 5′-TGGCTTTGAGGTAGGCATACTCA-3′; probe: 5′-(FAM) CCTTCCAGGAGCTGCAGATCGATGAC (TAMRA)-3′.

Fifty microliters of reaction mixture were used, containing 10 ng of the extracted total RNA, 0.3 mmol/L forward and 0.9 mmol/L reverse primers, 0.2 mmol/L TaqMan probe, the TaqMan 1-step reverse transcription-polymerase chain reaction (RT-PCR) Master Mix Reagents Kit (4309169; Perkin-Elmer Applied Biosystems Co., Ltd.). The conditions of 1-step RT-PCR were as follows: 30 minutes at 48°C (stage 1, RT), 10 minutes at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and 60 cycles of amplification for 15 seconds at 95°C and 1 minute at 60°C (stage 3, PCR). The assay used an instrument capable of measuring fluorescence in real time (ABI Prism 7700 Sequence Detector; Perkin-Elmer Applied Biosystems Co., Ltd.). Signals were detected according to the manufacturer's instructions.

The calibration curve, covering from 1.000 ng total RNA/50 μL reaction system diluted serially at a common ratio of 1:5 to 0.32 ng total RNA/50 μL, was created using the total RNA collected from monolayer FLC-5 cultures.

The specificity was evaluated using GAPDH messenger RNA (mRNA) as the internal control (4310884E; Perkin-Elmer Applied Biosystems Co., Ltd.). Each test was done in triplicate, and averages were obtained.

Microsome Preparation and Western Blotting. Beads (0.5-1.0 g), to which cells stored at −80°C were attached, were mixed with twice the amount of homogenization buffer (0.25 mol/L sucrose, 50 mmol/L Tris Buffer, pH 7.4). The mixture was agitated on a shaker. After sedimentation of the beads spontaneously, the supernatant containing the destroyed cellular components was transferred to a centrifuge tube for centrifugation at 1,000 rpm for 5 minutes. The supernatant was poured into each ultracentrifuge tube (TL-100, Beckman) for centrifugation at 10,000*g* for 10 minutes, and the supernatant was centrifuged at 105,000*g* for 60 minutes. The microsomal fraction, harvested as a pellet, was suspended in homogenization buffer and stored frozen at −80°C.

Protein in the adjusted microsomal suspension was quantified by Protein Assay Rapid Kit (Wako, Osaka, Japan). Subsequently, 2.5 μg of microsomal protein in each lane was subjected to SDS-polyacrylamide gel electrophoresis, in 4% to 20% gradient gel. Upon completion of electrophoresis, the protein was transferred from the gel onto the polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was immersed in reaction buffer (0.025 mol/L Tris, 0.15 mol/L NaCl, pH 7.4) containing 3% bovine serum albumin and agitated for 30 minutes to effect blocking. The membrane was then exposed to anti-human CYP3A4 antibody, which was made

by Chiba University, at room temperature for 1 hour. Thereafter, after being washed, it was exposed to anti-rabbit-IgG-conjugated alkaline phosphatase (Gentest Corp.) at room temperature for 1 hour. After further washing, it was immersed in alkaline phosphatase buffer (0.1 mol/L NaCl, 0.05 mol/L $MgCl_2$, 0.1 mol/L Tris, pH 9.5) and exposed to substrate solution composed of a mixture of nitroblue tetrazolium (NBT, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) for color development.

Investigation of Testosterone Metabolism Using a Bioartificial Liver. To check for CYP3A activity in the FLC-5 cells incubated in the bioreactor, testosterone (50 μmol/L) was added to the reservoir of the bioreactor system, and the bioreactor was perfused with the culture medium. Six hours later, the medium and cell-affixed beads were harvested and stored frozen at −40°C. To examine CYP3A4 induction, 50 μmol/L of rifampicin (Sigma) was added to the medium 48 hours before the addition of testosterone. Testosterone (Wako, Osaka, Japan) was dissolved in DMSO, and the medium was diluted to obtain a final DMSO concentration of 0.25% or lower. Monolayer cultured FLC-5 cells were also treated with 50 μmol/L of rifampicin for 48 hours.

The concentration of testosterone in the frozen samples was measured by high-performance liquid chromatography (HPLC). As an internal standard for HPLC, nitrazepam (Wako) was added to 0.5 mL of the medium at a concentration of 10 μg/mL methanol. Ethyl acetate (5 mL) was added to the mixture, which was then agitated for 10 minutes and thereafter centrifuged at 3,200 rpm for 5 minutes. A 4.5 mL aliquot of the upper layer (organic layer) was transferred to a glass tube, dried in a centrifuge evaporator, and mixed with the mobile phase for HPLC (250 μL). The mobile phase was composed of a 40%:60% deaired mixture of DW and methanol, whose pH was adjusted to 3.4 with H_3PO_4. The column used was a reversed-phase CAPCELLPAK C18 UG120 (Shiseidou Co., Ltd., Tokyo, Japan). The detector was an SPD-10AVP ultraviolet absorption detector (Shimazu Co., Ltd., Tokyo, Japan). The sample was injected at a volume of 70 μL, and the mobile phase flowed at the rate of 0.7 mL/min. The absorbance at 244 nm was then measured.

Electrophoretic Mobility Shift Assay. For electrophoretic mobility shift assay (EMSA), 20 μL of each sample contained 10 mmol/L Tris (pH 8.0), 40 mmol/L KCl, 0.05% NP-40, 6% glycerol, 1 mmol/L DTT, 0.2 μg of poly (dI-dC), and 20 μg each of FLC-5 nuclear extracts. Competitor oligonucleotides were included at a 5-fold or 10-fold excess. After a 10-minute incubation on ice, 10 ng of [^{32}P]-labeled oligonucleotide was added, and the incubation continued for an additional 30 minutes. DNA-protein complexes were resolved on a 5% polyacrylamide gel in 0.5 × TBE (1 × TBE = 90 mmol/L Tris, 90 mmol/L boric acid, 2 mmol/L EDTA). Gels were dried and determined by a Fujix bioimage analyser BAS2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan). The following oligonucleotides were used as either radiolabeled probes or competitors (sense strand is shown): CYP3A4ER6 binding site: 5′-GATCAATATGAACTCAAAGGAG-GTCAGTG-3′; HNF4α-binding site: 5′-CTCAGCTT-GTACTTTGGTACAACTA-3′; NF-κB binding site: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Anti-PXR (N-16, s.c. 9,690 X; Santa Cruz Biotechnology), anti-HNF-4α (S-20, s.c. 6,557 X; Santa Cruz Biotechnology), and control goat IgG (s.c. 2,028; Santa Cruz Biotechnology) were used in super-shift assays.

Fig. 2. Fine structural observation of FLC-5 cells cultured in a radial-flow bioreactor under an SEM. (A) The FLC-5 cells remain viable and have formed layers on the hydroxyapatite beads. (B) On cross-section, microvilli are distributed densely on the side facing the culture medium flow tract.

Results

Observation With Electron Microscope. FLC-5 cells remain viable and have formed layers on the surface of the hydroxy apatite beads to be present within the pores (Fig. 2A). On cross-section, microvilli had developed