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Hepatitis C Virus Core Protein Modulates Fatty Acid Metabolism and Thereby Causes Lipid Accumulation in the Liver

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We studied the roles of hepatitis C virus (HCV) core protein in hepatic steatosis and changes in hepatic lipid metabolism. HCV core protein expression plasmid was transfected in HepG2 . Triacylglyceride (TG) and mRNA level associated with lipid metabolism were measured. Male C57BL/6 mice were infected with HCV core recombinant adenovirus and used for lipids and mRNA studies. In HCV core protein-expressing cells, peroxisome proliferator-activated receptor (PPAR)α, multidrug resistance protein (MDR) 3, and microsomal triglyceride transfer protein (MTP) were down-regulated 48 hr after transfection. In HCV core protein-expressing mice, hepatic TG content and hepatic thiobarbituric acid-reactive substances increased. PPARα, MDR2, acyl-CoA oxidase (AOX), and carnitine palmitoyl transferase-1 (CPT-1) were down-regulated. HCV core protein down-regulated lipid metabolism-associated gene expression, Mdr2, CPT, and AOX, accompanied by down-regulation of PPARα. There findings may contribute to the understanding of HCV-related steatosis, induction of reactive oxygen species, and carcinogenesis.

KEY WORDS: HCV core protein; steatosis; nuclear receptor; ABC transporter.

Chronic hepatitis C virus (HCV) infection results in necroinflammatory liver disease that is characterized by the insidious progression of hepatic fibrosis and the loss of functioning hepatocytes (1–3). Little is known about the molecular mechanisms underlying liver injury due to infection with this virus, but a cell-mediated immune response associated with prominent lymphocytic infiltration of hepatic tissues is thought to play a major role (4, 5). In addition, various observations have suggested that nonimmune mechanisms may also play an important role. These findings include the frequent presence of hepatic steatosis in patients with chronic hepatitis C, an abnormality that is not often observed in other inflammatory conditions such as autoimmune hepatitis and chronic hepatitis B (6–9). Also, a considerable number of *in vitro* studies have suggested that expression of various HCV proteins may lead to alterations of lipid metabolism and transport, cell cycle dysregulation, increased or decreased susceptibility to apoptosis, and cellular transformation (10–17). In particular, HCV core protein has been suggested to contribute to hepatic steatosis (18–20), induction of reactive oxygen species (ROS) (19–21), and hepatic carcinogenesis (22).

Regarding HCV core protein-induced steatosis, the following findings have been reported: (a) HCV core protein interacts with apoA2, a major component of high-density lipoprotein (10, 23), (b) HCV core protein interferes with the assembly of very low-density lipoprotein (VLDL) by reducing the level of microsomal triglyceride transfer

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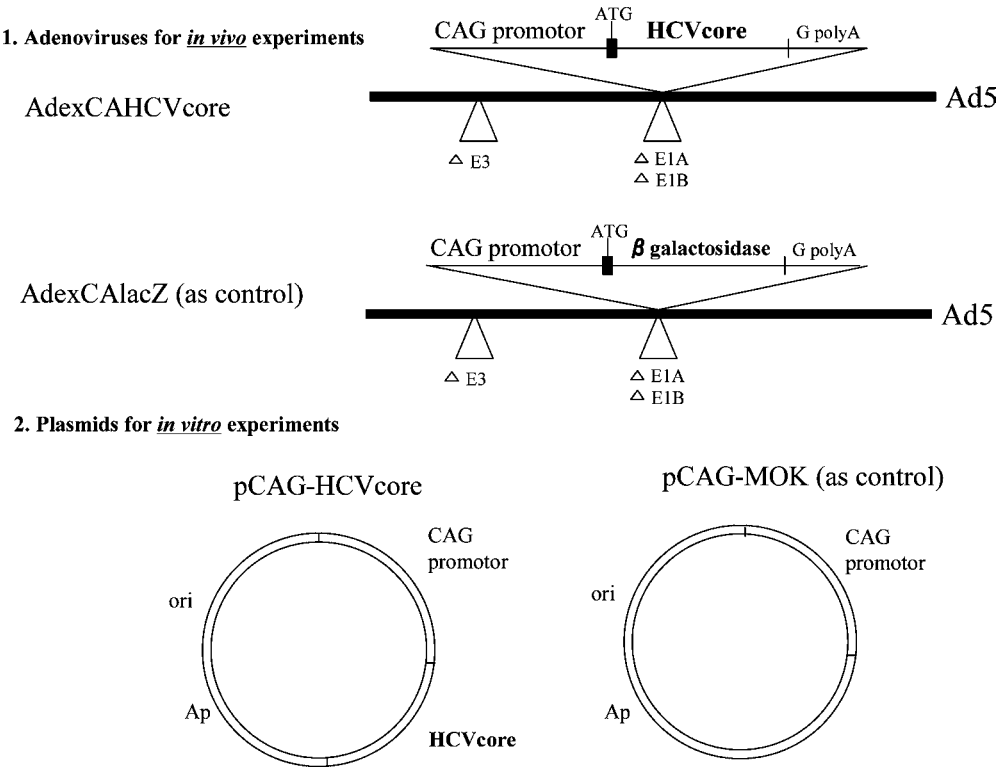

Fig 1. Constructs of recombinant adenoviruses and plasmids employed in this study. See Materials and Methods. ATG, start codon; G poly(A), rabbit β-globin poly(A); CAG promoter; cytomegalovirus enhancer, chicken β-actin promoter, and rabbit β-globin poly(A); Ad5, adenovirus type 5 genome lacking E1A, E1B, and E3.

protein (24), and (c) HCV core protein causes steatosis due to mitochondrial toxicity and production of ROS (19, 20). However, the details of the interaction between HCV and lipid metabolism remain unclear. Hepatocytes represent the crossroads of various metabolic pathways, so HCV may interfere with lipid metabolism via one or several pathways. To investigate the role of HCV core protein in steatosis and the accompanying changes in hepatic lipid metabolism, we focused on fatty acid metabolism-associated proteins, including those involved in fatty acid oxidation and lipid transport into blood and bile, as well as nuclear receptors.

MATERIALS AND METHODS

Plasmid and Recombinant Adenovirus. The complementary DNA clone of the full-length HCV core protein (amino acids [aa] 1–191) was derived from the serum of a patient with HCV 1b by reverse transcription and nested polymerase chain reaction. First-strand primers were 5′-CTGCTAGCCGAGTAGTGTTG-3′ and 5′-CATTGAGGACCACCAGGTTCT-3′, while second-strand primers were 5′-CGGGAATTCTCGTAGACCGTGCACCATG AGC-3′ and 5′-GTTGGGATCCTCCTAAGCGGAAGCTGG GAT-3′. The gene was inserted into pBluescript (Stratagene, La Jolla, CA, USA) and cloned. Then it was made to correspond with HCV 132996 (GenBank) using a QuikChange Site-Directed Mutagenesis kit (Stratagene). The HCV core protein expression plasmid (pCAG-HCVcore), a control plasmid (MOK), and a β-galactosidase expression plasmid (pCAG-LacZ) were prepared using an adenovirus expression vector kit (Takara Biotechnology, Tokyo) (25, 26). The HCV core gene was inserted into the *Swa*I site in cosmid vector pAxCAwt, which is a 44.741-kilobase cosmid containing a 31-kilobase adenovirus type 5 genome lacking the E1A, E1B, and E3 genes, but including the cytomegalovirus enhancer, chicken β-actin promoter, and rabbit β-globin poly(A) signal (pAxCAiHCVcore). The cosmid vector pAxCAiLacZ, with the β-galactosidase gene inserted into pAxCAwt, was included in the adenovirus expression vector kit. These three vectors (pAxCAwt, pAxCAiHCVcore, and pAxCAiLacZ) were digested at the *Sal*I site and ligated, yielding the pCAG-MOK, pCAG-HCVcore, and pCAG-LacZ expression plasmids for cell transfection experiments. The cosmid pAxCAiHCVcore or pAxCAiLacZ was cotransfected into 293 cells with adenovirus DNA by calcium phosphate precipitation. Incorporation of the expression cassette was confirmed by digestion with *Cla*1. Recombinant adenovirus (AdexCAHCVcore or AdexCAlacZ) was propagated in 293 cells and the viral titer was determined as the 50% tissue culture infectious dose using 293 cells. These viruses were used for animal experiments (Figure 1).

Cell Culture. HepG2 cells were seeded into 56-cm^2 tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM)

(Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) and an antibiotic/antimycotic mixture (100 U/ml each) (Gibco) and were cultured in a humidified incubator (5% CO_2) at 37°C. The medium was replaced with fresh medium every 3–4 days. Prior to each experiment, the cells were seeded into 6- or 12-well plates and allowed to attach for at least 24 hr (6-well for triglyceride [TG] assay and 12-well for RNA extraction).

Transfection. Using SuperFect Transfection Reagent (Qiagen, Tokyo), cells were transfected with 4 or 3 μg of pCAG-HCVcore or pCAG-MOK (4 μg for 6-well plates and 3 μg for 12-well plates) and were cultured in DMEM with 10% FBS. After 24 or 48 hr, the cells were harvested for analysis. The efficiency of transfection was investigated using pCAG-LacZ. Cells were washed with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS. Then the cells were stained with X-gal using a β-Gal Staining Set (Roche, Tokyo).

Animals. Adult male C57BL/6 mice (Charles River Laboratories, Yokohama, Japan), which were over 8 weeks old and weighed 21–24 g, were used in this study. All animals were housed in an environmentally controlled facility with a 12-hr lighting time (lights on from 0700 until 1900 hr). They were given free access to standard chow and water. Experiments (intravenous injection and sacrifice) were performed from 0900 to 21 hr. The animals received humane care according to the institutional guidelines for handling experimental animals.

HCV Core Protein Expression in Mice. The animals received an intravenous injection of 1×10^9 pfu (plaque-forming units) of AdexCAHCVcore or AdexCAlacZ and were sacrificed 3 days later. Mice were anesthetized with pentobarbital (100 mg/kg intraperitoneally). Blood was collected by cardiac puncture with a heparinized syringe, after which the liver was rapidly removed, weighed, and perfused with ice-cold PBS (pH 7.4). Part of the liver was fixed in 10% neutral buffered formalin and embedded in paraffin for histologic analysis. Another part was stored in RNA later reagent (Qiagen, Tokyo) at 4°C for extraction of RNA, and the remaining liver tissue was snap-frozen in liquid nitrogen and stored at −80°C until required. Plasma was immediately separated by centrifugation (10,000 rpm at 4°C) and stored at −20°C.

Liver Histology and Serum ALT Level. Sections of liver tissue (4 μm thick) were stained with hematoxylin and eosin for analysis. The serum alanine aminotransferase (ALT) level was measured using an automated technique by SRL Co. (Hiroshima, Japan).

HCV Core Protein Expression in Cells. Proteins were extracted from cells using PRO-PREP protein extraction solution (containing 1.0 mM PMSF, 1.0 mM EDTA, 1 μM pepstatin, 1 μM leupeptin, and 1 μM aprotinin) (Intron Biotechnology, Kyungki-Do, Korea). HCV core antigen levels were measured in cells using an HCV core antigen enzyme-linked immunosorbent assay (ELISA) (Ortho-Clinical Diagnostics K.K., Tokyo).

HCV Core Protein Expression in Mice. We confirmed HCV core protein expression in liver tissue by Western blot analysis. Proteins were extracted using PRO-PREP protein extraction solution. Then 50 μg of protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) using a tank blotting system according to the manufacturer's instructions (Bio-Rad Laboratories). After transfer, the membrane was blocked for 2 hr at room temperature with 5% powdered skim milk dissolved in Tris-buffered saline containing 0.05% between 20 and then incubated overnight at 4°C with a monoclonal mouse antibody to HCV core protein (kindly provided by Ortho-Clinical Diagnostics K.K.). Immune complexes were detected using alkaline phosphatase-conjugated anti-mouse IgG (Cosmo Bio, Tokyo) according to the manufacturer's instructions (Bio-Rad Laboratories). Detection of HCV core protein was performed by comparison with the following standards: myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

Measurement of Triglyceride Content. After the medium was removed, the cells were washed three times with PBS and resuspended in 200 μl of PBS. Then lipids were extracted from 100 μl of PBS by the method of Bligh and Dyer (27) and resuspended in 100 μL of 10% Triton X. The cellular content of TG was measured using enzyme reagents and standards from Wako (Osaka, Japan). The remainder of the PBS suspension was used for the protein assay. In mice experiments, livers were homogenized in PBS and 100 μl of the homogenate was used for extraction of lipids. Total protein was measured with protein assay reagents from Bio-Rad (Richmond, CA, USA).

Hepatic Level of Thiobarbituric Acid-Reactive Substances (TBARS). The hepatic level of TBARS was measured using an OXI-TEK TBARS Assay Kit (Zeptometrix Corporation, New York, USA). Briefly, 100 mg of liver tissue was homogenized in 10 vol of normal saline. Then 100 μl of SDS and 2.5 ml of TBA/buffer reagent were added to 100 μl of this homogenate or the malondialdehyde standard. Samples were incubated at 95°C for 60 min, cooled in an ice bath for 10 min, and centrifuged at 3000 rpm for 15 min, after which the supernatant was analyzed by spectrophotometry (532 nm).

Extraction of RNA and RT-PCR. The medium was removed and the cells were washed twice with PBS. After centrifugation, total RNA was isolated using an RNeasy Mini Kit (Qiagen, Tokyo). From mouse, 20 mg of liver tissue was used for RNA extraction. Then 2 μg of total RNA was employed for reverse transcription using random hexamers (final concentration: 2.5 μM) and murine leukemia virus reverse transcriptase (final concentration: 2.5 U/μl) (Roche, Tokyo). Specific primer sets were synthesized for performance of the PCR (Table 1) and were used for assessment of liver-predominant mitochondrial carnitine palmitoyl transferase-1 (CPT1A in humans and CPT1 in mice; the rate-limiting enzyme of mitochondrial β-oxidation), acyl-CoA oxidase (ACO1 in humans and AOX in mice: the rate-limiting enzyme of peroxisomal β-oxidation), cytochrome P-450 4A11 (CYP4A11; involved in microsomal ω-oxidation), multidrug resistance protein 3 (MDR3 in humans and Mdr2 in mice; an ABC transporter and phospholipid flippase), microsomal TG transfer protein (MTP: a vital protein for TG incorporation into VLDL), and two nuclear receptors (peroxisome proliferator-activated receptor α[PPARα]] and peroxisome proliferator-activated receptor γ (PPARγ). Roles of these genes are summarized in Table 2. Amplification involved 30 cycles of denaturation at 95°C for 60 sec, annealing at each specified temperature (Table 1) for 30 sec, and extension at 72°C for 60 sec. The reaction products were analyzed on a 2% agarose gel and were visualized by ethidium bromide staining. The PCR products were excised from the gel, purified using a gel purification kit (Qiagen), and quantified by spectrophotometry. Dilutions

TABLE 1. PRIMER SETS IN THE EXPERIMENTS

	Forward	*Reverse*	*Annealing temp. (°C)*
Human			
GAPDH	GAACGGGAAGCTCACTGGCATGGC	TGAGGTCCACCCTGTTGCTG	65
PPARα	GGAAAGCCCACTCTGCCCCCT	AGTCACCGAGGAGGGGCTCGA	63
PPARγ	CATTCTGGCCCACCAACTTTGG	TGGAGATGCAGGCTCCACTTTG	63
MDR3 (ABCB4)	GATGAAAAGGCTGCCACTAG	TTGCACTTCTGCTGCTTCAC	62
MTP	GGCTAGCCTATTTCAGACACA	GATGAGCCTGGTAGGTCACT	60
CPT1A	AGACGGTGGAACAGAGGCTGAAG	TGAGACCAAACAAAGTGATGATGTCAG	67
ACO1	GGGCATGGCTATTCTCATTGC	CGAACAAGGTCAACAGAAGTTAGGTTC	60
CYP4A11	GTGGCCCAACCCAGAGGT	TCCCAATGCAGTTCCTTGATC	55
Mouse			
GAPDH	AGAACATCCCTGCATCC	TTGTCATTGAGAGCAATGCC	56
PPARα	TGCAGAGCAACCATCCAG	TAATGGCGAATTATAAAC	50
PPARγ	GGTGAAACTCTGGGAGATTC	CAACCATTGGGTCAGCTCTT	59
Mdr2 (Abcb4)	TATCCGCTATGGCCGTGGGAA	ATCGGTGAGCTATCACAATGG	56
MTP	TGAGCGGCTATACAAGCTCAC	CTGGAAGATGCTCTTCTCGC	60
LCPT	CGCACGGAAGGAAAATGG	TGTGCCCAATATTCCTGG	52
AOX	CTTGTTCGCGCAAGTGAGG	CAGGATCCGACTGTTTACC	56

ranging from 3×10^{-5} to 3×10^{2} pg were prepared in water and used as the standards.

Quantitative PCR. Quantitative PCR was performed using the Light-Cycler Fast-Start DNA Master SYBR Green system (Roche Molecular Biochemicals, Tokyo). PCR was carried out in a final reaction volume of 20 μl using 1 μl of each primer at 10 μM (final concentration: 0.5 μM), 1.6 μl of 25 mM $MgCl_2$(final concentration: 3 mM), 2 μl of the enzyme mix supplied, 12.4 μl of H_2O, and 2 μl of the template. The enzyme mix contained the reaction buffer, Fast-Start Taq DNA polymerase, and DNA double strand-specific SYBR Green I dye for detection of PCR products. PCR was performed in a Light-Cycler (Roche) with preincubation for 10 min at 95°C followed by 40 cycles of denaturation for 15 sec at 95°C, annealing for 5 sec at each specified temperature (see Table 1), and extension for 25 sec at 72°C, with fluorescent detection at the end of extension. Next, the PCR products were subjected to melting curve analysis to exclude the amplification of primer dimmers or other nonspecific products. If primer dimmers and nonspecific bands were detected, fluorescence detection was repeated after extension at each specified temperature for 1 sec. Analysis was carried out with Light-Cycler 3.5 software (Roche). Quantification was done using the "point fitting" mode and baseline adjustment. The standard curve for each gene was created using five different dilutions. The plot of the number of PCR cycles versus log concentration was considered reliable when the error was <0.2.

Statistical Analysis. Results are expressed as the mean $\pm$ SE. Statistical analysis was performed using Student's t-test, and $P < 0.05$ was defined as indicating significance.

RESULTS

HCV Core Protein Expression in HepG2 Cells. The transfection efficiency of pCAG-LacZ was about 20%. HCV core protein expression by the cells was confirmed using the HCV core antigen ELISA. No HCV core antigen was detected in mock-transfected and nontransfected cells. The level of HCV core protein expression showed no difference between 24 and 48 hr after transfection (24 hr,

TABLE 2. ROLES OF ANALYZED GENES IN FATTY ACID METABOLISM

MDR3	Multidrug resistance protein 3
	An ABC transporter and phospholipid flippase
	Role: Phospholipid secretion into bile
MTP	Microsomal triglyceride transfer protein
	A vital protein for TG incorporation into VLDL
	Role: triglyceride secretion into blood
CPT1A	Liver-predominant mitochondrial carnitine palmitoyl transferase-1
	The rate-limiting enzyme of mitochondrial β-oxidation
	Role: Fatty acid β-oxidation in the liver
ACO1	Acyl-CoA oxidase
	The rate-limiting enzyme of peroxisomal β-oxidation
	Role: Fatty acid β-oxidation in the liver
PPARα	Peroxisome proliferator-activated receptor α
	A nuclear receptor
	Role: A nuclear receptor controlling lipid metabolism-associated genes
PPARγ	Peroxisome proliferator-activated receptor γ
	A nuclear receptor
	Role: A nuclear receptor controlling lipid metabolism-associated genes

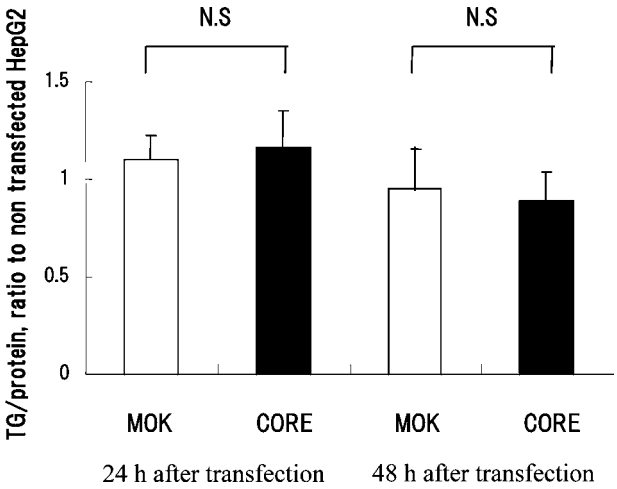

Fig 2. Effect of HCV core protein expression on cellular triglyceride (TG) content. Four micrograms of pCAG-MOK (control) or pCAG-HCVcore was transfected into HepG2 cells cultured in six-well plates by the lipofection method. At 24 or 48 hr after transfection, cells were collected for protein assay and lipid extraction. TG content was measured and expressed as the ratio to the protein content. Data are shown as values relative to those for nontransfected HepG2 cells. Each data point represents the mean ± SD of six individual experiments. P = NS compared with pCAG-MOK (Student's t-test).

1.31 ± 0.20 nmol/mg protein; 48 hr, 1.25 ± 0.16 nmol/mg protein).

TG Content of HepG2 Cells. The cellular TG content at 24 hr after transfection showed no difference between HCV core transfectants (CORE) and mock transfectants (MOK) as control (CORE, 1.16 ± 0.19; MOK, 1.10 ± 0.13; $P = 0.57$). At 48 hr after transfection, the TG content also showed no difference between the groups (CORE, 0.88 ± 0.16; MOK, 0.95 ± 0.18; $P = 0.55$). Data are expressed as the ratio to nontransfected cells (Figure 2).

Expression of Target Genes by HepG2 Cells. At 24 hr after transfection, HCV CORE showed increased expression of mRNA for PPAR γ (CORE, 2.39 ± 0.26; MOK, 1.98 ± 0.28; $P = 0.025$), MDR3 (CORE, 1.30 ± 0.21; MOK, 1.02 ± 0.20; $P = 0.030$), MTP (CORE, 0.37 ± 0.04; MOK, 0.26 ± 0.05; $P < 0.01$), and ACO1 (CORE, 1.11 ± 0.14; MOK, 0.76 ± 0.08; $P < 0.01$)gompared to MOK, while CPT (CORE, 1.18 ± 0.16; MOK, 0.94 ± 0.28; $P = 0.102$) and PPARα (CORE, 0.84 ± 0.14; MOK, 0.69 ± 0.10; $P = 0.055$) expression was normal (Figure 3). At 48 hr after transfection, HCV CORE showed lower expression of mRNA for PPAR α (CORE, 0.89 ± 0.02; MOK, 0.96 ± 0.08;

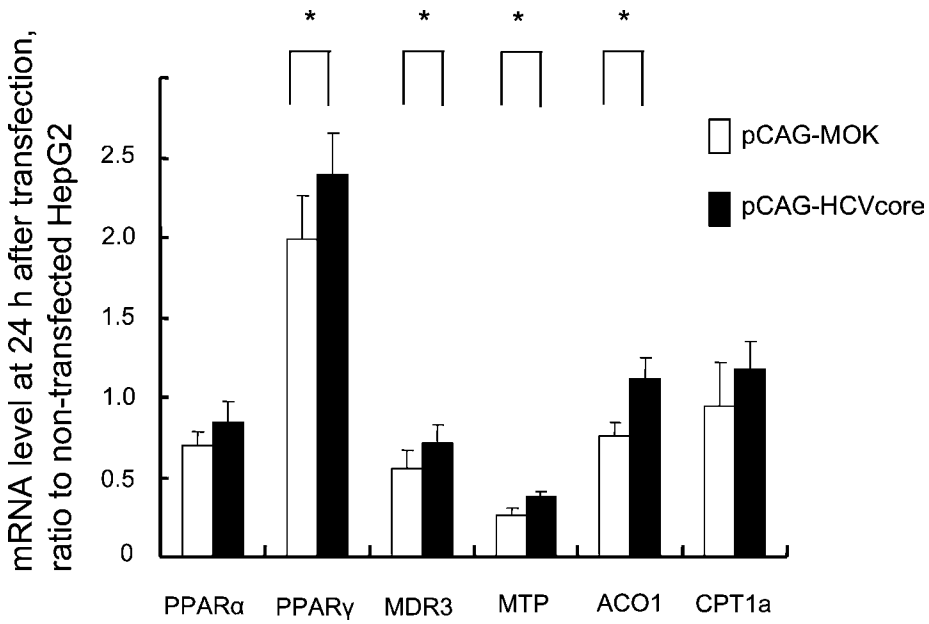

Fig 3. Effect of HCV core protein expression on mRNA levels at 24 hr after transfection. Three micrograms of pCAG-MOK (control) or pCAG-HCVcore was transfected into HepG2 cells cultured in 12-well plates by the lipofection method. At 24 hr after transfection, cells were collected for extraction of RNA. Complementary DNA was synthesized from 2 μg of RNA and used for quantified PCR with the Light-Cycler Fast-Start DNA Master SYBR Green system. GAPDH level was measured as the internal control, and the ratio to GAPDH was calculated for each sample. Data are shown as values relative to those for nontransfected HepG2 cells. Each data point represents the mean ± SD of 6 individual experiments. *$P < 0.05$ compared with pCAG-MOK (Student's t-test).

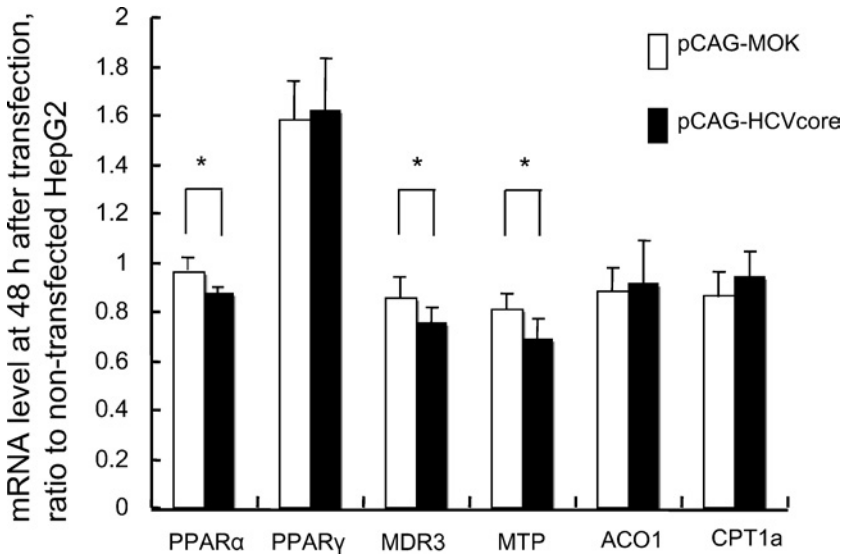

Fig 4. Effect of HCV core protein on mRNA expression at 48 hr after transfection. Three micrograms of pCAG-MOK (control) or pCAG-HCVcore was transfected into HepG2 cells cultured in 12-well plates by the lipofection method. At 48 hr after transfection, cells were collected and used for RNA extraction. Complementary DNA was synthesized from 2 μg of RNA and used for quantified PCR with the Light-Cycler Fast-Start DNA Master SYBR Green system. GAPDH was measured as an internal control, and the ratio to GAPDH was calculated for each sample. Data are shown as values relative to those for nontransfected HepG2 cells. Each data point represents the mean ± SD of 6 individual experiments. $*P < 0.05$ compared with pCAG-MOK (Student's t-test).

$P = 0.048$), MDR3 (CORE, 0.75 ± 0.06; MOK, 0.86 ± 0.08; $P = 0.031$), and MTP (CORE, 0.69 ± 0.08; MOK, 0.81 ± 0.07; $P = 0.016$) compared with MOK, while ACO1 returned to the control level (CORE, 0.91 ± 0.18; MOK, 0.88 ± 0.09; $P = 0.70$) and the CPT level was normal (CORE, 0.94 ± 0.13; MOK, 0.86 ± 0.10; $P = 0.27$). Data are expressed as the ratio to nontransfected cells (Figure 4). Experiments were repeated three times and similar results were obtained, with statistical significance. CYP4A11 was not detected by RT-PCR, so we could not make a standard for the Light-Cycler.

HCV Core Protein Expression in Mice. HCV core protein-expressing mice looked healthy and their body weight (BW) and liver weight remained within the normal range (BW [g]: PBS, 22.5 ± 0.816; AdexCAHCVcore (CORE), 21.7 ± 0.84; AdexCAlacZ (LacZ), as control, 21.5 ± 0.71). Similar mild elevation of ALT and mild hepatic lymphocyte infiltration were observed in both groups of adenovirus-infected mice, showing no differences between Core and LacZ (GPT [IU/ml]: PBS, 65 ± 17.8; CORE, 170 ± 59.4; LacZ, 142.5 ± 82.2). Lipid drops were not observed in either group (data not shown). Western blot analysis revealed the HCV core protein of about 19–20 kDa (Figure 5). In preliminary experiments, animals receiving an intravenous injection of 1×10^9 pfu developed severe hepatitis after 7 days, while animals receiving 1×10^8 pfu showed amild elevation of ALT, but their HCV core protein expression (based on quantification of mRNA and HCV core antigen) was significantly lower at 7 days after injection. Thus, we selected injection of 1×10^9 pfu and sacrifice at 3 days for the study protocol.

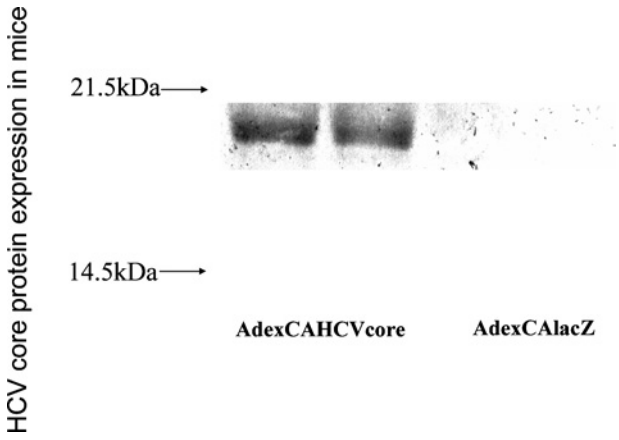

Fig 5. HCV core protein expression in mice. AdexCAlacZ (control recombinant adenovirus) or AdexCAHCVcore was used to infect male C57BL/6 mice (8–10 weeks old) by intravenous administration (1×10^9 pfu). Three days after infection, livers were collected for protein assay. Using 50 μg of protein, HCV core protein expression was confirmed by Western blotting with a mouse monoclonal antibody for HCV core protein (19–20 kDa).

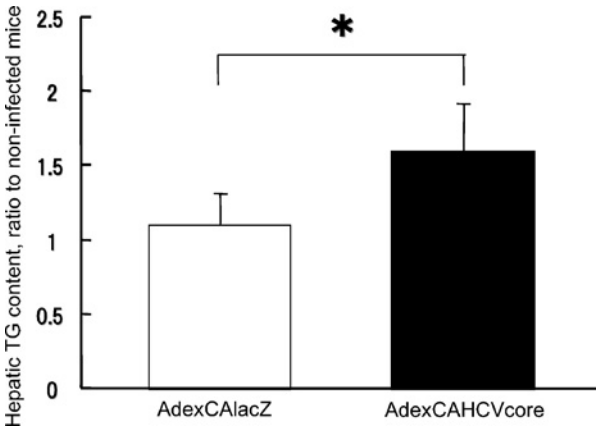

Fig 6. Effect of HCV core protein expression on the hepatic triglyceride content in mice. AdexCAlacZ (control adenovirus) or AdexCAHCVcore was used to infect male C57BL/6 mice (8–10 weeks old) by intravenous administration (1×10^9 pfu). At 3 days after infection, the livers were collected and 100 μl of liver homogenate was used for lipid extraction and for the protein assay. The TG content was measured and expressed as the ratio to the protein content. Data are shown as values relative to those for noninfected mice. Each data point represents the mean ± SD of four individual mice. $^*P < 0.05$ compared with AdexCAlacZ (control adenovirus) by Student's *t*-test.

Hepatic TG Level in Mice. Animals injected with AdexCAHCVcore showed a 1.45-fold increase in hepatic TG content compared to animals injected with AdexCAlacZ (CORE, 1.60 ± 0.33; LacZ, 1.10 ± 0.21; $P = 0.044$; $N = 4$). Data are expressed as the ratio to noninfected mice (Figure 6).

Expression of Target Genes in Mice. In the livers of HCV core protein-expressing mice, PPAR α (CORE, 0.59 ± 0.11; LacZ, 1.33 ± 0.21; $P < 0.01$), PPAR γ (CORE, 1.05 ± 0.10; LacZ, 2.43 ± 0.69; $P < 0.01$), Mdr2 (CORE, 0.85 ± 0.08; LacZ, 1.12 ± 0.12; $P = 0.011$), AOX (CORE, 0.235 ± 0.08; LacZ, 0.401 ± 0.07; $P = 0.02$), and CPT (CORE, 1.14 ± 0.14; LacZ 2.34 ± 0.51; $P < 0.01$) were all down-regulated, while the level of MTP mRNA was unchanged (CORE, 1.37 ± 0.08; LacZ, 1.24 ± 0.17; $P = 0.22$; $N = 4$). Data are expressed as the ratio to noninfected mice (Figure 7).

Hepatic TBARS Level. In the livers of HCV core protein-expressing mice, the TBARS level was increased compared with that in the control group (CORE, 0.84 ± 0.08; LacZ, 0.41 ± 0.01; $P < 0.01$; $N = 4$) (Figure 8).

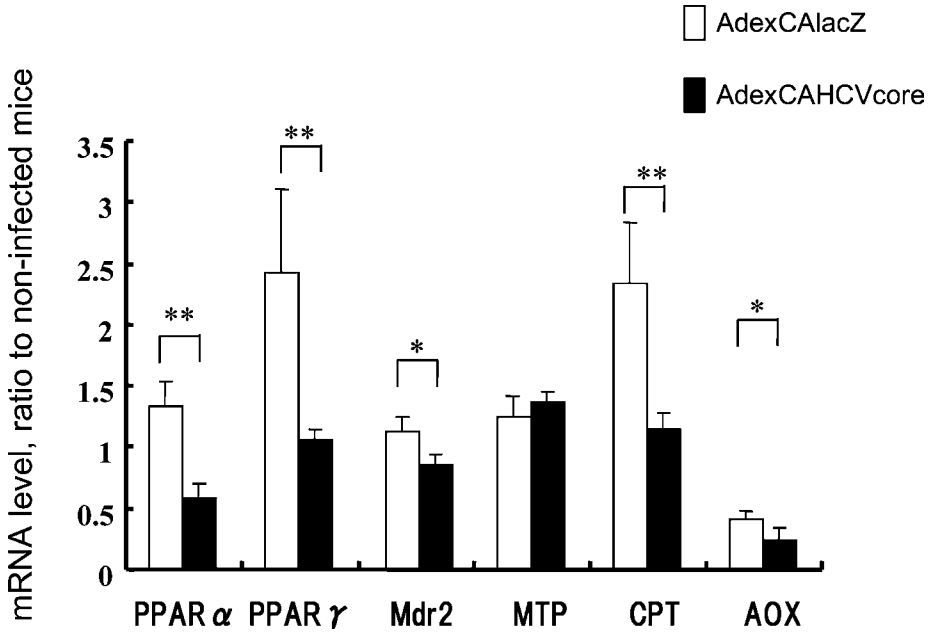

Fig 7. Effect of HCV core protein expression on mRNA levels in mice. AdexCAlacZ (control adenovirus) or AdexCAHCVcore was used to infect male C57BL/6 mice (8–10 weeks old) by intravenous administration (1×10^9 pfu). At 3 days after infection, livers were collected for RNA extraction. Complementary DNA was synthesized from 2 μg of RNA and used for quantified PCR with the Light-Cycler Fast-Start DNA Master SYBR Green system. GAPDH was measured as an internal control, and the ratio to GAPDH was calculated for each sample. Data are shown as relative values to those for noninfected mice. Each data point represents the mean ± SD of four individual mice. $^*P < 0.05$ and $^{**}P < 0.01$ compared with AdexCAlacZ (control adenovirus) by Student's *t*-test.

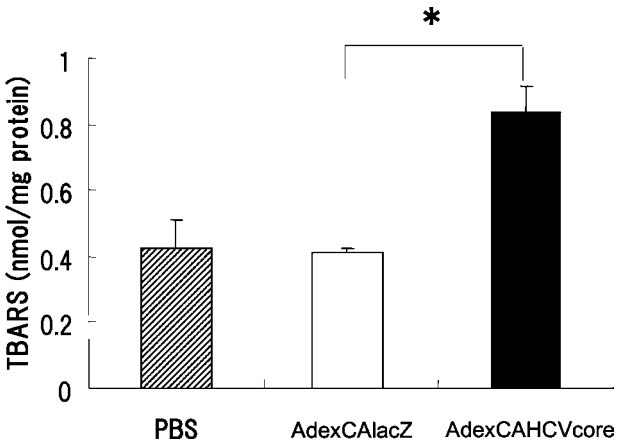

Fig 8. Effect of HCV core protein expression on TBARS in the mouse liver. AdexCAlacZ (control adenovirus) or AdexCAHCVcore was used to infect male C57BL/6 mice (8–10 weeks old) by intravenous administration (1×10^9 pfu). At 3 days after infection, livers were homogenized in 10 vol of normal saline. TBARS and total protein (TP) levels were measured as described under Materials and Methods. Data are expressed as the ratio to the TP level. Each data point represents the mean ± SD of four individual mice. $^*P < 0.01$ compared with AdexCAlacZ (control adenovirus) by Student's *t*-test.

DISCUSSION

HCV core protein was recently reported to cause hepatic steatosis and induction of reactive oxygen species (ROS) in an HCV core protein transgenic mouse model (18–20). In the transgenic mouse model, it was also shown that a decrease in MTP activity contributes to HCV core protein-related steatosis, while β-oxidation is unchanged (24), but the mechanism involved is still unclear. This study was the first investigation of the effect of HCV core protein on the expression of fatty acid metabolism-associated molecules in the acute expression mice model.

Hepatic accumulation of TG is principally driven by the following factors: (a) fatty acid overload (28, 29), (b) inhibition of fatty acid β-oxidation (28, 29), (c) decreased secretion of TG-rich very low density lipoprotein (VLDL) (28, 29), (d) increased de novo fatty acid synthesis, (e) decreased transformation to phospholipids, and (f) a combination of these mechanisms.

In the present study, we initially tested the effect of HCV core protein on a human cell line (HepG2). At 24 hr after transfection, the cellular TG level was unchanged, but the expression of several genes that are thought to promote fatty acid consumption (MTP, ACO1, and MDR3) was up-regulated. At 48 hr after transfection, there was either normal gene expression (ACO1) or a decrease in expression (PPARα, MDR3, and MTP). At 48 hr after transfection, the level of HCV core antigen was still the same as at 24 hr, so it seems possible that HCV core protein may act to down-regulate these genes over a longer period. To further evaluate the effects of HCV core protein, we performed in vivo experiments using transient expression of HCV core protein in mice. Although fatty change of the liver was not seen histologically, the hepatic TG level was increased by transient HCV core protein expression. In addition, expression of mRNA for all of the molecules investigated, except MTP, was down-regulated by HCV core protein expression. The mechanism involved is not understood at present, but reduced expression of these genes might contribute to hepatic TG accumulation.

CPT is the rate-limiting enzyme for mitochondrial β-oxidation (30), which is the main pathway of fatty acid consumption and ROS production. There was a recent report (20) that localization of HCV core protein in the mitochondria led to the increased production of ROS, decreased mitochondrial membrane permeability, and impairment of mitochondrial function. It remains unclear whether ROS induces fat accumulation or whether the accumulation of fat causes an increase in ROS, as well as whether decreased expression of CPT-1 is the first response to HCV core protein expression or follows other earlier changes. However, HCV core expression seems to contribute to hepatic accumulation of lipids and an increase in ROS in mice, along with reduced expression of various fatty acid metabolism-associated genes. AOX is vital for peroxisomal β-oxidation (30) and it has been reported that AOX knockout mice develop steatohepatitis, up-regulation of CYP4A gene expression, and increased production of ROS (31). We were unable to evaluate CYP4A11 in the present study, but the association of HCV-related steatosis with microsomal ω-oxidation is interesting. Mdr2 (Abcb4) is a member of the Abcb subfamily of adenosine triphosphate-binding cassette (ABC) transporter proteins. Mdr2 Pgp is exclusively localized to the canalicular membrane and controls the secretion of phospholipids into the bile (32). We thought that impaired biliary phospholipid secretion might have a role in HCV-related steatosis, based on the fact that phospholipid-associated fatty acid secretion into bile (about 25 μmol per day) is substantial in relation to the hepatic amount of triglyceride-associated fatty acids (about 75 μmol) (33). We found that the expression of MDR3 and Mdr2 was down-regulated, suggesting that reduced expression of these genes could have a causative role in HCV-related steatosis.

Interestingly, down-regulation of Mdr2, AOX, and CPT in the mice was accompanied by down-regulation of PPARα. In mice, the other three genes are thought to undergo transcriptional regulation by PPARα (33, 34), so their expression might be down-regulated secondary to the down-regulation of PPARα. HCV core protein is mainly

localized in the cytosol, but also exists in the nucleus (35, 36), so it is possible that this protein could influence gene transcription. Tsustumi *et al.* (37) used a luciferase assay to show that transcriptional activation of ACO-1 via PPRE is promoted at 24 hr after HCV core protein expression (23). However, we found down-regulation of target gene expression accompanied by decreased PPARα expression after 3 days of HCV core protein expression in mice, as well as at 48 hr after transfection of cells. The expression of PPARα was reported to be under transcriptional regulation by glucocorticoids (38), but the mechanism remains unclear. Accordingly, the mechanism leading to down-regulation of PPARα after HCV core protein expression is also unclear. The lower expression of PPARα and the genes it regulates in human hepatocytes than in mouse hepatocytes (39) could be a reason for the lack of an increase in TG and the small decline in gene expression in our cell experiment. Fibrates that bind with PPARα and increase its activity (although not its expression) might be useful for controlling HCV-related steatosis by increasing the β-oxidation and bliary secretion of fatty acids.

PPARγ improves insulin resistance and is also reported to improve hepatic fibrosis and nonalcholic steatohepatitis (40, 41). Because PPARγ gene expression also showed down-regulation by HCV core protein expression in this study , it may be necessary to examine the role of glucose metabolism, de novo synthesis of fatty acids from glucose, and fatty acid flux through hepatocytes in HCV-related steatosis.

In this study, the increase in TBARS level was found in mice with transient expression of HCV core protein. This suggests that ROS production might be induced by HCV core protein expression, although no mechanistic information for this was provided in this study. It also remains unclear whether intrahepatic fat accumulation enhances ROS production as reflected by an increase in TBARS or, inversely, whether ROS production induces fatty liver change through ROS-associated mitochondrial dysfunction. Certainly, further investigations are needed to clarify this uncertainty, but the fact that HCV core protein expression in mice contributes to the increase in TBARS level may partially characterize the pathogenesis of HCV-related hepatic damage.

In summary, transient expression of HCV core protein in mice down-regulated the expression of various lipid metabolism-associated genes (Mdr2, CPT, and AOX). It also caused down-regulation of PPARα expression and led to the accumulation of TG and the induction of oxidative stress. These findings may provide some clues to the understanding of HCV-related steatosis and to the induction of ROS production and carcinogenesis by infection with this virus.

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