

hashi for sample stock, preparation, and technical support. This study was supported in part by a grant for AIDS Research from the Ministry of Health, Labor, and Welfare of Japan (H15-AIDS-001) and by the Japanese Foundation for AIDS Prevention (X.B.). None of the authors has any conflict of interest.

REFERENCES

- Department of Health and Human Services: Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents—May 4, 2006. <http://aidsinfo.nih.gov/guidelines/>.
- Johnson VA, Brun-Vézinet F, Clotet B, *et al.*: Update of the drug resistance mutations in HIV-1: Fall 2005. *International AIDS Society—USA. Top HIV Med* 2005;13:125–131.
- Meynard J-L, Vray M, Morand-Joubert L, *et al.*: Phenotypic or genotypic resistance testing for choosing antiretroviral therapy after treatment failure: A randomized trial. *AIDS* 2002;16:727–736.
- Wegner SA, Wallace MR, Aronson NE, *et al.*: Long-term efficacy of routine access to antiretroviral-resistance testing in HIV type 1-infected patients: Results of the clinical efficacy of resistance testing trial. *Clin Infect Dis* 2004;38:723–730.
- Tural C, Ruiz L, Holtzer C, *et al.*: Clinical utility of HIV-1 genotyping and expert advice: The Havana trial. *AIDS* 2002;16:209–218.
- Badri SM, Adeyemi OM, Max BE, Zagorski BM, and Barker DE: How does expert advice impact genotypic resistance testing in clinical practice? *Clin Infect Dis* 2003;37:708–713.
- Lorenzi P, Opravil M, Hirschel B, *et al.*: Impact of drug resistance mutations on virologic response to salvage therapy. *AIDS* 1999;13:F17–21.
- Cingolani A, Antinori A, Rizzo MG, *et al.*: Usefulness of monitoring HIV drug resistance and adherence in individuals failing highly active antiretroviral therapy: A randomized study (ARGENTA). *AIDS* 2002;16:369–379.
- Sarmati L, Nicastri E, Uccella I, *et al.*: Drug-associated resistance mutations in plasma and peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected patients for whom highly active antiretroviral therapy is failing. *J Clin Microbiol* 2003;41:1760–1762.
- Bi X, Gatanaga H, Ida S, *et al.*: Emergence of protease inhibitor resistance-associated mutations in plasma HIV-1 precedes that in proviruses of peripheral blood mononuclear cells by more than a year. *J Acquir Immune Defic Syndr* 2003;34:1–6.
- Lawrence J, Mayers DL, Hullsiek KH, *et al.*: Structured treatment interruption in patients with multidrug-resistant human immunodeficiency virus. *N Engl J Med* 2003;349:837–846.
- Izopet J, Massip P, Souyris C, *et al.*: Shift in HIV resistance genotype after treatment interruption and short-term antiviral effect following a new salvage regimen. *AIDS* 2000;14:2247–2255.
- Miller V, Sabin C, Hertogs K, *et al.*: Virological and immunological effects of treatment interruptions in HIV-1 infected patients with treatment failure. *AIDS* 2000;14:2857–2867.
- Deeks SG, Wrin T, Liegler T, *et al.*: Virologic and Immunologic consequences of discontinuing combination antiretroviral-drug therapy in HIV-infected patients with detectable viremia. *N Engl J Med* 2001;344:472–480.
- Boucher S, Recordon-Pinson P, Neau D, *et al.*: Clonal analysis of HIV-1 variants in proviral DNA during treatment interruption in patients with multiple therapy failures. *J Clin Virol* 2005;34(4):288–294.
- Pao D, Andraday U, Clarke J, *et al.*: Long-term persistence of primary genotypic resistance after HIV-1 seroconversion. *J Acquir Immune Defic Syndr* 2004;37:1570–1573.
- Halfon P, Durant J, Clevenbergh P, *et al.*: Kinetics of disappearance of resistance mutations and reappearance of wild-type during structured treatment interruptions. *AIDS* 2003;17:1351–1361.
- Frost SD: Dynamics and evolution of HIV-1 during structured treatment interruptions. *AIDS Rev* 2002;4:119–127.
- Verhofstede C, Wanzeele FV, Van Der Gucht B, De Cabooter N, and Plum J: Interruption of reverse transcriptase inhibitors or a switch from reverse transcriptase to protease inhibitors resulted in a fast reappearance of virus strains with a reverse transcriptase inhibitor-sensitive genotype. *AIDS* 1999;13:2541–2546.
- Devereux HL, Youle M, Johnson MA, and Loveday C: Rapid decline in detectability of HIV-1 drug resistance mutations after stopping therapy. *AIDS* 1999;13:F123–F127.
- Barbour JD, Hecht FM, Wrin T, *et al.*: Persistence of primary drug resistance among recently HIV-1 infected adults. *AIDS* 2004;18:1683–1689.
- Martinez-Picado J, Savara AV, Sutton L, and D'Aquila RT: Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. *J Virol* 1999;73:3744–3752.
- Dykes C, Najjar N, Bosch RJ, *et al.*: Detection of drug-resistance minority variants of HIV-1 during virologic failure of didanosine, lamivudine, and zidovudine. *J Infect Dis* 2004;189:1091–1096.
- Verhofstede C, Noe A, Demecheleer E, *et al.*: Drug-resistant variants that evolve during nonsuppressive therapy persist in HIV-1-infected peripheral blood mononuclear cells after long-term highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* 2004;35:473–483.
- Izopet J, Souyris C, Hance A, *et al.*: Evolution of human immunodeficiency virus type 1 populations after resumption of therapy following treatment interruption and shift in resistance genotype. *J Infect Dis* 2002;185:1506–1510.

Address reprint requests to:

Shinichi Oka

AIDS Clinical Center, International Medical Center of Japan

1-21-1, Toyama

Shinjuku-ku

Tokyo 162-8655, Japan

E-mail: oka@imcj.hosp.go.jp

Involvement of the PA28 γ -Dependent Pathway in Insulin Resistance Induced by Hepatitis C Virus Core Protein[▽]

Hironobu Miyamoto,¹ Kohji Moriishi,¹ Kyoji Moriya,² Shigeo Murata,³ Keiji Tanaka,³ Tetsuro Suzuki,⁴ Tatsuo Miyamura,⁴ Kazuhiko Koike,² and Yoshiharu Matsuura^{1*}

Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka,¹ Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo,² Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Tokyo,³ and Department of Virology II, National Institute of Infectious Diseases, Tokyo,⁴ Japan

Received 4 August 2006/Accepted 16 November 2006

The hepatitis C virus (HCV) core protein is a component of nucleocapsids and a pathogenic factor for hepatitis C. Several epidemiological and experimental studies have suggested that HCV infection is associated with insulin resistance, leading to type 2 diabetes. We have previously reported that HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg) mice develop marked insulin resistance and that the HCV core protein is degraded in the nucleus through a PA28 γ -dependent pathway. In this study, we examined whether PA28 γ is required for HCV core-induced insulin resistance in vivo. HCV core gene-transgenic mice lacking the PA28 γ gene (PA28 $\gamma^{-/-}$ CoreTg) were prepared by mating of PA28 $\gamma^{+/+}$ CoreTg with PA28 γ -knockout mice. Although there was no significant difference in the glucose tolerance test results among the mice, the insulin sensitivity in PA28 $\gamma^{-/-}$ CoreTg mice was recovered to a normal level in the insulin tolerance test. Tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), production of IRS2, and phosphorylation of Akt were suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice in response to insulin stimulation, whereas they were restored in the livers of PA28 $\gamma^{-/-}$ CoreTg mice. Furthermore, activation of the tumor necrosis factor alpha promoter in human liver cell lines or mice by the HCV core protein was suppressed by the knockdown or knockout of the PA28 γ gene. These results suggest that the HCV core protein suppresses insulin signaling through a PA28 γ -dependent pathway.

Hepatitis C virus (HCV) is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (15). Over one-half of patients with the acute infection evolve into a persistent carrier state (24). Chronic infection with HCV frequently induces hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (22) and is known to be associated with diseases of extrahepatic organs, including an essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, and type 2 diabetes (13).

HCV is classified into the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome consisting of a single positive-strand RNA with a nucleotide length of about 9.5 kb. This viral genome encodes a single polyprotein composed of approximately 3,000 amino acids (9). The polyprotein is post-translationally cleaved by host cellular peptidases and viral proteases, resulting in 10 viral proteins (6, 10, 12). The HCV core protein is known to interact with viral-sense RNA of HCV to form the viral nucleocapsid (44). The HCV core protein is cleaved off at residue 191 by the host signal peptidase to release it from the E1 envelope protein and then by the host signal peptide peptidase at around amino acid residues 177 to 179 within the C-terminal transmembrane region (30, 39, 40). The mature core protein is retained mainly on the endoplasmic reticulum, although a portion moves to the nucleus and mitochondria (11, 51).

Recent epidemiological studies have indicated that type 2

diabetes is an HCV-associated disease (7, 29). However, it remains unclear how insulin resistance is induced in patients chronically infected with HCV, since there is no suitable model for investigating HCV pathogenesis. Type 2 diabetes is a complex, multisystemic disease with pathophysiology that includes a high level of hepatic glucose production and insulin resistance, which contribute to the development of hyperglycemia (8, 18). Although the precise mechanism by which these factors contribute to the induction of insulin resistance is difficult to understand, a high level of insulin production by pancreatic β cells under a state of insulin resistance is common in the development of type 2 diabetes. The hyperinsulinemia in the fasting state that is observed relatively early in type 2 diabetes is considered to be a secondary response that compensates for the insulin resistance (8, 18).

The HCV core protein is also known as a pathogenic factor that induces steatosis and hepatocellular carcinoma in mice (33, 35). Previously, we reported that insulin resistance occurs in HCV core gene-transgenic mice due at least partly to an increase in tumor necrosis factor alpha (TNF- α) secretion (47) and that the HCV core protein is degraded through a PA28 γ /REG γ (11S regulator)-dependent pathway in the nucleus (32). It is well known that PA28 γ enhances latent proteasome activity, although the biological significance of PA28 γ is largely unknown, with the exception that PA28 γ is known to regulate steroid receptor coactivator 3 (28). Although several reports suggested that the degradation of insulin receptor substrate (IRS) proteins by a ubiquitin-dependent proteasome activity contributes to insulin resistance (43, 50), the involvement of the HCV core protein in cooperation with PA28 γ in the stability of IRS proteins and in the development of insulin resis-

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.

[▽] Published ahead of print on 29 November 2006.

tance is not known. In this study, we examined the involvement of PA28 γ in the induction of insulin resistance by the HCV core protein *in vivo*.

MATERIALS AND METHODS

Preparation of PA28 γ -knockout HCV core gene-transgenic mice. C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b (PA28 $\gamma^{+/+}$ CoreTg) line C49 and PA28 $\gamma^{-/-}$ mice have been described previously (35, 36). These two genotypes were crossbred to create PA28 $\gamma^{+/+}$ CoreTg mice. PA28 $\gamma^{-/-}$ CoreTg mice were bred to generate PA28 $\gamma^{-/-}$ CoreTg mice (35, 36). The HCV core gene and the target sequence to knock out the PA28 γ gene were identified by PCR. The mice were given ordinary feed (CRF-1; Charles River Laboratories, Yokohama, Japan) and were maintained under specific-pathogen-free conditions.

Glucose tolerance test. The mice were fasted for more than 16 h before glucose administration. D-Glucose (1 g/kg body weight) was intraperitoneally administered to the mice. Blood samples were taken from the orbital sinus at the indicated time points. The plasma glucose concentration was measured by means of a MEDI-SAFE Mini blood glucose monitor (TERUMO, Tokyo, Japan). The serum insulin level was determined by a Mercodia (Uppsala, Sweden) ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA).

Insulin tolerance test. The mice were fed freely and then fasted during the study period. Human insulin (2 U/kg body weight) (Humulin; Eli Lilly, Indianapolis, IN) was intraperitoneally administered to the mice. The plasma glucose concentration was measured at the indicated time and was normalized based on the glucose concentration at the time just before insulin administration.

Histological analysis of pancreatic islets. Pancreas tissues were fixed with paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The relative islet area and islet number were determined with Image-Pro PLUS image analyzing software (NIPPON ROOPER, Tokyo, Japan).

Estimation of tumor necrosis factor alpha and HCV core protein. Mouse TNF- α was measured by using a mouse TNF- α ELISA kit (Pierce, Rockford, IL) and normalized based on the amount of total protein in each sample. The protein concentration was estimated by using a BCA protein assay kit (Pierce). The amount of HCV core protein in the liver tissues was determined by using an ELISA system as described previously (4).

In vivo insulin stimulation and immunoblot analysis. Mice were fasted for more than 16 h before insulin stimulation and then anesthetized with ketamine and xylazine. Five units of insulin were injected into the mice via the interior vena cava. Livers of the mice were collected 5 min after the insulin injection and frozen in liquid nitrogen. Immunoblot analyses of the HCV core protein, PA28 γ , and each of the insulin-signaling molecules were carried out with the liver tissue homogenates prepared in the homogenizing buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na₂VO₄, 100 mM NaF, 50 mM Na₂P₂O₇, 10 mM EGTA, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P40 supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (53). Tissue lysates were subjected to sodium dodecyl sulfate-2% to 15% gradient polyacrylamide gel electrophoresis (PAG Mini DAIICHI 2/15 13W; Daiichi Diagnostics, Tokyo, Japan) and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The protein transferred onto the membrane was reacted with rabbit anti-HCV core (32), rabbit anti-Akt (Cell Signaling, Danvers, MA), rabbit anti-phospho-Ser473-Akt (Cell Signaling), rabbit anti-IRS1 (Upstate, Lake Placid, NY), rabbit anti-phospho-Tyr608 mouse insulin receptor substrate 1 (Sigma, St. Louis, MO), or rabbit anti-IRS2 (Upstate) polyclonal antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody. Blotted protein was visualized using Super Signal FEMTO (Pierce) and an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan).

Quantitative reverse transcription-PCR (RT-PCR). Total RNA was isolated from mouse liver using an RNeasy kit (QIAGEN, Valencia, CA). The RNA preparation was treated with a TURBO DNA-free kit (Ambion, Austin, TX) to remove DNA contamination in the samples. The first-strand cDNAs were synthesized by a first-strand cDNA synthesis kit (Amersham Biosciences, Franklin Lakes, NJ). The targeted cDNA was estimated by using Platinum SYBR Green qPCR Super Mix UDC (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The fluorescent signal was measured by using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The genes encoding mouse TNF- α , IRS1, IRS2, and hypoxanthine phosphoribosyl transferase were amplified with the following primer pairs: 5'-GGTACAACCCATCGGCTGGCA-3' (forward) and 5'-GCGACGTGGAACCTGGCAGAAG-3' (reverse) for TNF- α , 5'-ATAG

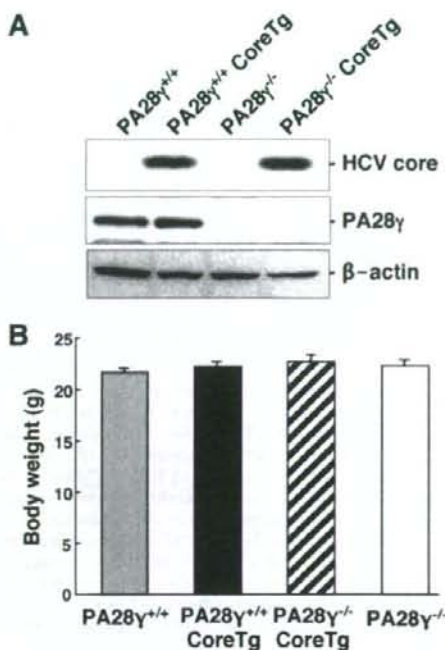


FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28 γ gene. (A) Expression of the HCV core protein and PA28 γ in the livers of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice. Lysates obtained from liver tissues of the mice (100 μ g protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antibodies to the HCV core protein, PA28 γ , and β -actin. (B) Body weights of the mice. Body weights of 2-month-old mice were measured ($n = 7$ in each group). There were no statistically significant differences in body weights among the mice ($P > 0.05$).

CTCTGAGACCTTCTCAGCACCTAC-3' (forward) and 5'-GGAGTTGCCCTCATGTGCTGCCATA-3' (reverse) for IRS1, 5'-AGCCTGGGGATAATGGTGACTATACCGA-3' (forward) and 5'-TTGTGGGCAAAGGATGGGGACACT-3' (reverse) for IRS2, and 5'-CCAGCAAGCTTGAACCTTAACCA-3' (forward) and 5'-GTAATGATCAGTCAACGGGGAC-3' (reverse) for hypoxanthine phosphoribosyl transferase. Each PCR product was found as a single band with the correct size by agarose gel electrophoresis (data not shown).

Reporter assay for TNF- α promoter activity. The promoter region of the TNF- α gene (located from residues -1260 to +140) was amplified from mouse genomic DNA and was then introduced into the KpnI and BglII sites of pGL3-Basic (Promega, Madison, WI) (25). The resulting plasmid was designated as pGL3-tnf- α Pro. The gene encoding the HCV core protein was amplified from HCV strain J1 (genotype 1b) and cloned into pCAG-GS (1, 38). To avoid contamination with endotoxin from *Escherichia coli*, the plasmid DNA was purified by using an EndoFree Plasmid Maxi kit (QIAGEN). The total amount of transfected DNA was normalized by the addition of empty plasmids. Plasmid vector was transfected into hepatoma cell lines by lipofection using Lipofectamine 2000 (Invitrogen). Cells were harvested at 24 h posttransfection. Luciferase activity was determined by using the Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity was normalized to coexpressed *Renilla* luciferase activity. The amount of firefly luciferase activity was presented as the increase (n -fold) relative to the value for the sample lacking the HCV core protein, which was taken to be 1.0. PA28 γ -knockdown cell lines were established by using pSilencer 2.1 U6 Hygro (Ambion) according to the manufacturer's protocol.

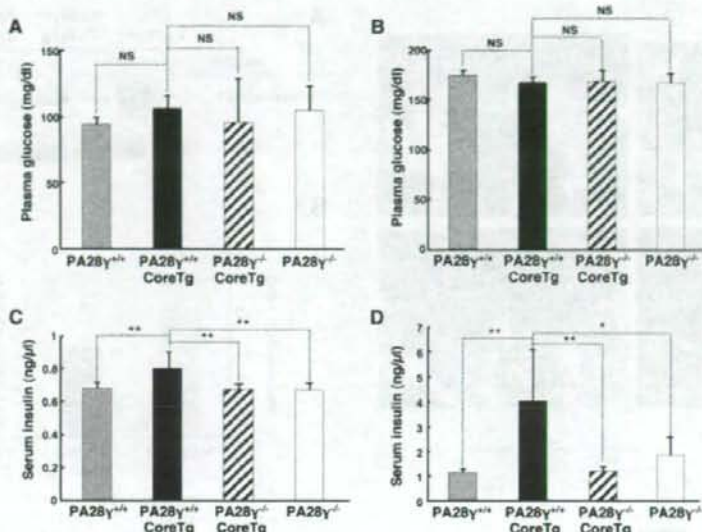


FIG. 2. Knockout of the PA28 γ gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$ mice under fasting (A) or fed (B) conditions ($n = 7$ in each group) are shown. Serum insulin levels in fasting (C) or fed (D) mice ($n = 7$ in each group) are also shown. Values are represented as means \pm standard deviations. * $P < 0.05$; ** $P < 0.01$. NS, not statistically significant.

Statistical analysis. The results are presented as means \pm standard deviations. The significance of the differences was determined by Student's t test. P values of < 0.05 were considered statistically significant.

RESULTS

HCV core gene-transgenic mice deficient in the PA28 γ gene.

To investigate the role of PA28 γ in the development of insulin resistance in HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg)

mice, we generated HCV core gene-transgenic mice deficient in the PA28 γ gene (PA28 $\gamma^{-/-}$ CoreTg). A PA28 $\gamma^{+/+}$ CoreTg mouse expressing an amount of PA28 γ equal to that of its normal littermates (Fig. 1A) was crossbred with a PA28 $\gamma^{-/-}$ mouse to generate a PA28 $\gamma^{+/+}$ CoreTg mouse. PA28 $\gamma^{+/+}$ CoreTg mice were bred with each other, and a PA28 $\gamma^{-/-}$ CoreTg mouse was selected by PCR. The HCV core protein was expressed in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg

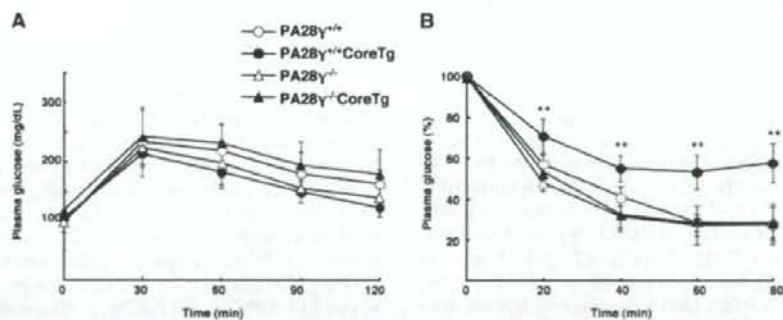


FIG. 3. Knockout of the PA28 γ gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times ($n = 5$ in each group). There were no significant differences in glucose levels among the mice ($P > 0.05$). (B) Insulin tolerance test. Human insulin (2 units/kg body weight) was intraperitoneally administered to the mice, and the plasma glucose levels were estimated at the indicated times. Values were normalized to the baseline glucose concentration at the time of insulin administration ($n = 5$ in each group). The values for the PA28 $\gamma^{+/+}$ (open circles), PA28 $\gamma^{+/+}$ CoreTg (closed circles), PA28 $\gamma^{-/-}$ (open triangles), and PA28 $\gamma^{-/-}$ CoreTg (closed triangles) mice are represented as means and \pm standard deviations. Significant differences in insulin sensitivity ($P < 0.01$) in PA28 $\gamma^{-/-}$ CoreTg mice compared to that in PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, or PA28 $\gamma^{-/-}$ CoreTg mice are indicated by double asterisks (**). There were no significant differences among PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice ($P > 0.05$).

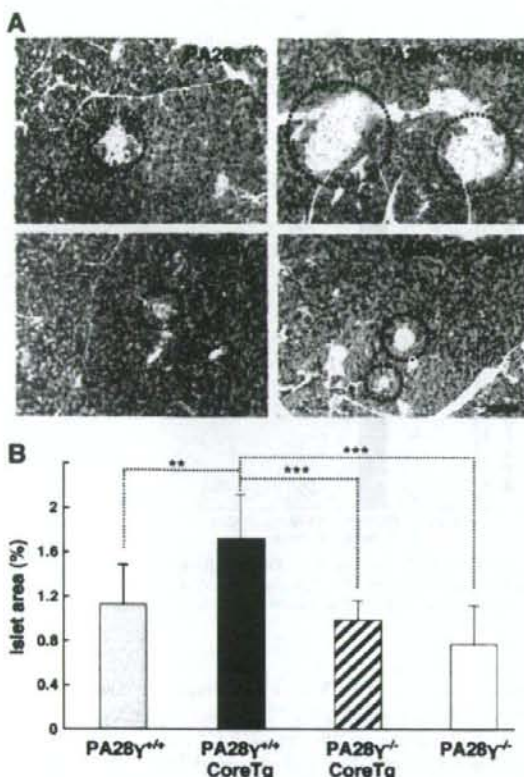


FIG. 4. PA28 γ participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice were stained with hematoxylin and eosin. Dotted circles indicate pancreatic islets. (B) The area occupied by pancreatic islets was measured by computer software in three different fields of every six randomly selected sections of 10 mice per genotype and is represented as a percentage of the total pancreatic area. ** $P < 0.01$; *** $P < 0.001$. The scale bar indicates 100 μ m.

mice but not in PA28 $\gamma^{+/+}$ (normal littermates) or PA28 $\gamma^{-/-}$ mice. PA28 γ was found at a similar level in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{+/+}$ mice but was not present in either PA28 $\gamma^{-/-}$ or PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1A). The expression of the HCV core protein in the livers of 2-month-old male mice was slightly higher in PA28 $\gamma^{-/-}$ CoreTg (1.36 ± 0.44 ng/mg of total protein; $n = 7$) than in PA28 $\gamma^{+/+}$ CoreTg (1.23 ± 0.22 ng/mg of total protein; $n = 7$) mice, but these values were not significantly different ($P > 0.05$). Insulin sensitivity is dependent on several conditions such as body weight, obesity, and liver steatosis (26). PA28 $\gamma^{-/-}$ mice were slightly smaller than their normal littermates (PA28 $\gamma^{+/+}$) at more than 3 months old, as described previously (36), but this was not significantly different in 2-month-old mice (Fig. 1B). PA28 $\gamma^{+/+}$ CoreTg mice exhibited severe hepatic steatosis from 4 months of age (35). To avoid the influence of hepatic steatosis and body weight on the examination of insulin resistance, 2-month-old mice were

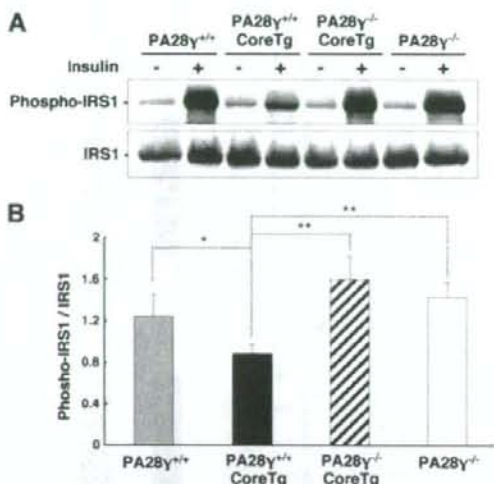


FIG. 5. PA28 γ participated in the inhibition of the tyrosine phosphorylation of IRS1 induced by the HCV core protein. Liver tissues from PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice were prepared after administration of insulin (+) or phosphate-buffered saline (-). The samples (100 μ g of total protein) were examined by immunoblotting with antibodies against IRS1 and phospho-Tyr608 of mouse IRS1 (A). Phosphorylated IRS1 was estimated from the density on the immunoblotted membrane by using computer software (B) ($n = 5$ in each group). The data presented are representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$.

used in this study. Figure 1B shows the body weights of 2-month-old mice. There were no significant differences in body weight among PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{+/+}$ mice. Steatosis was not detected in the livers of the 2-month-old mice (data not shown).

PA28 γ is involved in the development of hyperinsulinemia and insulin resistance in PA28 $\gamma^{+/+}$ CoreTg mice. In our previous study, we found a significant difference in serum insulin levels, but not in plasma glucose levels, between PA28 $\gamma^{+/+}$ CoreTg mice and normal littermates (47). To determine the involvement of PA28 γ in the development of insulin resistance in PA28 $\gamma^{+/+}$ CoreTg mice, we examined here the plasma glucose and insulin levels in the mice under fasting and fed conditions. Although no significant difference in plasma glucose levels was observed in the mice under either fasting (Fig. 2A) or fed (Fig. 2B) conditions, serum insulin levels were significantly higher in PA28 $\gamma^{+/+}$ CoreTg mice than in PA28 $\gamma^{+/+}$ mice under both conditions (Fig. 2C and D), as described previously (47). In contrast, the serum insulin concentration in PA28 $\gamma^{-/-}$ CoreTg mice was recovered to a normal level similar to that of PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ mice under either fasting (Fig. 2C) or fed (Fig. 2D) conditions.

To determine the glucose intolerance among the mice, glucose was administered to the mice after fasting, and the plasma glucose level was then determined. There was no significant difference among the genotypes at any time point in the glucose tolerance test (Fig. 3A), suggesting that the volume of glucose was maintained at a normal level by the higher concentration of insulin in PA28 $\gamma^{+/+}$ CoreTg mice. In our previ-

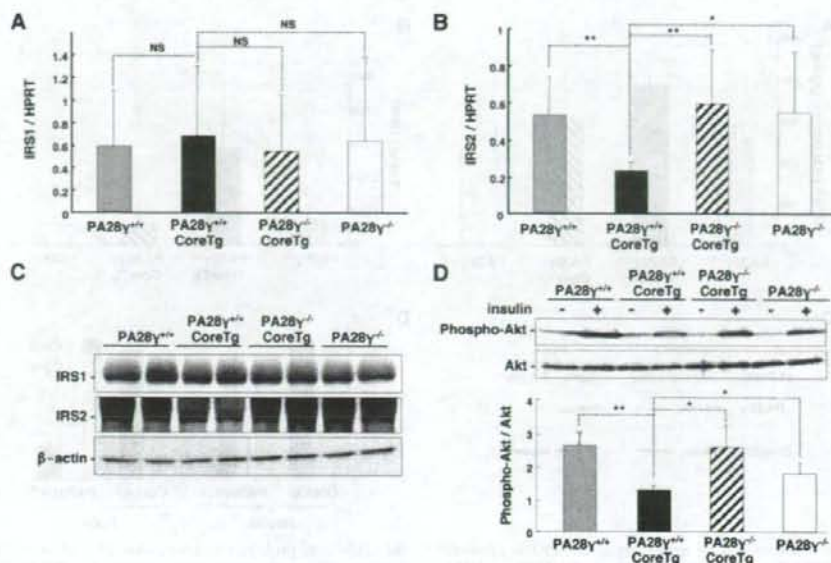


FIG. 6. PA28 γ participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR ($n = 5$ in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were determined by immunoblotting with specific antibodies. (D) Phosphorylation of Akt in the livers of the mice was examined by immunoblotting with antibodies against Akt and phosphorylated Akt. The ratio of Akt phosphorylation was determined by computer software based on the densities of phosphorylated Akt and a total amount of Akt ($n = 3$ in each group). The data presented are representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$. NS, not statistically significant; HPRT, hypoxanthine phosphoribosyl transferase.

ous study, the reduction in the plasma glucose concentration after insulin administration was impaired in PA28 $\gamma^{+/+}$ CoreTg mice (47). In this study, PA28 $\gamma^{-/-}$ CoreTg mice exhibited a normal insulin level comparable to those of PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ mice by an insulin tolerance test, in contrast to PA28 $\gamma^{+/+}$ CoreTg mice, in which a high concentration of plasma glucose was detected at all time points, as previously reported (Fig. 3B). These data suggest that hyperinsulinemia was induced in PA28 $\gamma^{+/+}$ CoreTg mice to compensate for insulin resistance and retain a physiological level of plasma glucose and that PA28 γ participates in the development of hyperinsulinemia and insulin resistance in PA28 $\gamma^{+/+}$ CoreTg mice.

Morphology of pancreatic islets. Hyperinsulinemia and insulin resistance are expected to enlarge the pancreatic islet mass due to the overexpression of insulin. Our previous report showed the enlargement of the pancreatic islets in PA28 $\gamma^{+/+}$ CoreTg mice. To clarify whether a knockout of the PA28 γ gene restores the enlarged pancreatic islets to their normal size, the morphology of the pancreatic islets of the mice was evaluated by histologic examination (Fig. 4A). The relative islet area in the pancreatic cells of the PA28 $\gamma^{-/-}$ CoreTg mice was smaller than that of PA28 $\gamma^{+/+}$ CoreTg mice and comparable to that of PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ mice (Fig. 4B). Infiltration of inflammatory cells within or surrounding the islets was not found in all genotypes of mice. These results suggest that PA28 γ also participates in the enlargement of pancreatic islets induced in PA28 $\gamma^{+/+}$ CoreTg mice.

PA28 γ impairs the insulin-signaling pathway through the suppression of both tyrosine phosphorylation of IRS1 and expression of IRS2. Insulin binds to insulin receptors, resulting in the activation of downstream signaling (26). The activated insulin receptors phosphorylate themselves, IRS1, and IRS2. Phosphorylated IRS1 and IRS2 can activate phosphatidylinositol 3 (PI3)-kinase signaling, leading to the activation of glucose metabolism and cell growth. Our previous report showed that tyrosine phosphorylation of IRS1 is suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice and that the administration of anti-TNF- α antibody restores insulin sensitivity (47). We examined whether a knockout of the PA28 γ gene could restore the tyrosine phosphorylation of IRS1. Tyrosine phosphorylation of IRS1 was suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice in response to insulin stimulation, whereas it was recovered in PA28 $\gamma^{-/-}$ CoreTg mice to levels comparable to those in PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ mice (Fig. 5).

Chronic hyperinsulinemia downregulates the expression of IRS2, which is one of the essential components of the insulin-signaling pathway in the liver (46). However, in our previous study, we showed that there was no significant difference in the phosphorylation of IRS2 between PA28 $\gamma^{+/+}$ CoreTg mice and their normal littermates (47). To gain more insight into the mechanisms of regulation of IRS expression, we determined the transcription and translation of IRS1 and IRS2 in the livers of the mice by real-time PCR and Western blotting, respectively. Although there was no significant difference in IRS1 expression at either the transcriptional or translational level among the mice

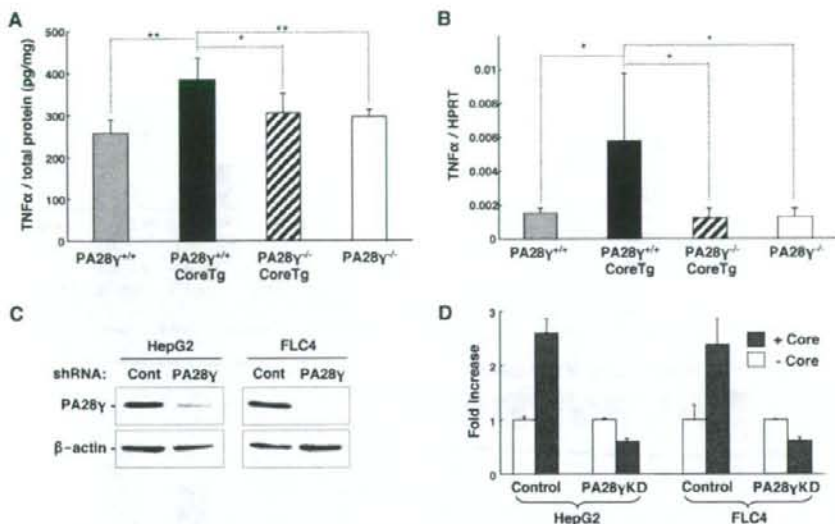


FIG. 7. PA28 γ was required for activation of the TNF- α promoter by the HCV core protein. (A) Expression of TNF- α in the livers of mice was determined by ELISA ($n = 5$ in each group). (B) TNF- α mRNA in the livers of mice was examined by quantitative RT-PCR ($n = 5$ in each group). (C) Knockdown of the expression of PA28 γ in the HepG2 and FLC-4 cell lines by the introduction of a plasmid encoding a short hairpin RNA (shRNA) targeted to the PA28 γ gene. The expression levels of PA28 γ and β -actin were determined by immunoblotting with specific antibodies. (D) Promoter activity of TNF- α in the presence or absence of the HCV core protein was determined by luciferase assay in the PA28 γ -knockdown and control cell lines. The data presented are representative of three independent experiments. HPRT, hypoxanthine phosphoribosyl transferase.

(Fig. 6A and C), the expression of IRS2 was clearly impaired in PA28 $\gamma^{+/+}$ CoreTg mice at both the transcriptional and translational levels compared with that in other mice (Fig. 6B and C). The serine/threonine protein kinase Akt is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) under the activated condition of IRS family proteins (26). The insulin-induced phosphorylation of Akt was suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice but not in those of PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, or PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 6D). These results suggest that the expression of the HCV core protein in the livers of mice in the presence of PA28 γ impairs the insulin-signaling pathway through the suppression of both the tyrosine phosphorylation of IRS1 and the expression of IRS2.

PA28 γ is required for activation of the TNF- α promoter by HCV core protein. TNF- α is an adipokine (54) and suppresses the signaling pathway of IRS1 and IRS2 (14, 42). Several reports suggested that the serum TNF- α level is higher in HCV patients than in healthy individuals (19, 37). Elevations of TNF- α levels have also been demonstrated in the livers of PA28 $\gamma^{+/+}$ CoreTg mice (47). To determine the involvement of PA28 γ in the enhancement of TNF- α expression, the expression of TNF- α in the livers of each genotype was determined by ELISA and real-time PCR (Fig. 7A and B). Transcription and translation of TNF- α were increased in the livers of PA28 $\gamma^{+/+}$ CoreTg mice but were restored in the livers of PA28 $\gamma^{-/-}$ CoreTg mice to levels comparable to those of PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ mice. To determine the effect of PA28 γ expression on the promoter activity of TNF- α in human liver cells, PA28 γ -knockdown human hepatoma cell lines HepG2 and FLC4 were

established by the introduction of a plasmid encoding a short hairpin RNA targeting the PA28 γ gene in the cell lines. The expression of PA28 γ was clearly suppressed in the cell lines (Fig. 7C). The expression of HCV core protein in the hepatoma cell lines potentiated TNF- α promoter activity, whereas the promoter activation by the HCV core protein was suppressed in the PA28 γ -knockdown cell lines (Fig. 7D). These results suggest that PA28 γ is required for the activation of the TNF- α promoter induced by the expression of the HCV core protein in human hepatoma cell lines.

DISCUSSION

HCV infection has a close association with type 2 diabetes, which is a polygenic disease with a pathophysiology that includes a defect in insulin secretion, increased hepatic glucose production, and resistance to the action of insulin (2, 8, 18). Insulin binds to insulin receptors, which exhibit tyrosine kinase activity, leading to the autophosphorylation and phosphorylation of IRS (56). Tyrosine phosphorylation in IRS proteins leads to the interaction between IRS proteins and the regulatory subunit p85 of PI3-kinase, which enhances glucose uptake and inhibits lipolysis (21). Activated PI3-kinase phosphorylates phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3,4,5-triphosphate, which contributes to the activation of PDK1 (55). Activated PDK1 phosphorylates downstream substrates including Akt and other kinases (55). A diabetic phenotype that included insulin resistance was found in IRS2-knockout mice with normal growth (57), although a

knockout of the IRS1 gene has been shown to lead to growth retardation and insulin resistance but not overt diabetes (5, 52). The double knockdown of IRS1 and IRS2 genes in the liver induces hyperinsulinemia and insulin resistance in mice (53). The reduction of both IRS1 and IRS2 under conditions of insulin resistance and hyperinsulinemia (3) and in the livers of *ob/ob* mice, an obese diabetic mouse model (20), has been reported previously. In the present study, the expression of the HCV core protein reduced the phosphorylation of tyrosine on IRS1 and the production of IRS2 in the livers of mice but did not completely abolish the activities of these genes, suggesting that residual activities of IRS transfer a faint signal to the downstream region of IRS. Therefore, PA28 $\gamma^{+/+}$ CoreTg mice may exhibit a milder phenotype than IRS1- and/or IRS2-knockout mice. In this study, knockout of the PA28 γ gene restored the insulin sensitivity and signaling of IRS1 and IRS2 in PA28 $\gamma^{+/+}$ CoreTg mice, suggesting that the expression of the HCV core protein leads to the dysfunction of both IRS1 and IRS2 through a PA28 γ -dependent pathway.

Our previous study suggested that the induction of TNF- α by the HCV core protein plays a role in insulin resistance (47). An increase in TNF- α levels has been correlated with obesity and insulin resistance in animal models and humans (14, 42). However, the mechanism by which TNF- α induces insulin resistance is not completely known. The expression of TNF- α has been shown to be increased in PA28 $\gamma^{+/+}$ CoreTg mice, resulting in the suppression of phosphorylation of IRS1, and insulin sensitivity in PA28 $\gamma^{+/+}$ CoreTg was improved by the administration of an anti-TNF- α antibody (47). In the present study, the expression level of TNF- α in PA28 $\gamma^{+/+}$ CoreTg mice was similar to that in PA28 $\gamma^{-/-}$ mice or their normal littermates. The expression of the HCV core protein enhanced the promoter activity of the TNF- α gene in human liver cell lines but not in those with a knockdown of the PA28 γ gene by RNA interference (Fig. 7D). These data suggest that PA28 γ plays a crucial role in HCV core-induced expression of TNF- α . Sterol regulatory element-binding proteins (SREBPs) were shown to be increased at the stage of viremia in HCV-infected chimpanzees (49). SREBPs are known to regulate not only the biosynthesis of lipid but also the transcription of IRS2 and TNF- α (17, 45). Therefore, it might be feasible to speculate that the HCV core protein may cooperate with PA28 γ to regulate the expression of SREBPs.

Houstis et al. previously reported that reactive oxygen species (ROS) are increased in both cellular and mouse models of insulin resistance induced by treatment with TNF- α or dexamethasone and that insulin sensitivity was restored by treatment with small antioxidant molecules (16). The HCV core protein potentiates ROS production in hepatoma cells and HCV core gene-transgenic mice (23, 34, 41). Accelerated production of ROS results in mitochondrion dysfunction, which contributes to a decrease in fatty acid oxidation. Defects in mitochondrial fatty acid oxidation enhance the production of intracellular fatty acyl coenzyme A (CoA) and diacylglycerol (48, 58). Mitochondrion dysfunction and accumulation of lipid droplets in mice expressing the HCV core or the full-length HCV polyprotein have been reported (27, 34). An increase in lipid droplets also leads to the accumulation of fatty acid CoA and diacylglycerol (48, 58). Fatty acyl CoA and diacylglycerol nonspecifically activate the Ser/Thr kinase cascade, leading to the enhancement of the serine phosphorylation of IRS1 (26). Serine phosphorylation on IRS1 blocks the tyrosine

phosphorylation of IRS1 by insulin receptors (26). In the present study, however, serine phosphorylation of IRS1 in PA28 $\gamma^{+/+}$ CoreTg mice was similar to that in PA28 $\gamma^{-/-}$ CoreTg mice (data not shown). TNF- α signaling pathways other than the accumulation of ROS and fatty acid intermediates may also participate in the inhibition of tyrosine phosphorylation on IRS1 in PA28 $\gamma^{+/+}$ CoreTg mice.

How does the HCV core protein induce TNF- α production? Our previous report suggests that the HCV core protein is degraded through a PA28 γ -dependent pathway (32). Recently, PA28 γ has been shown to participate in the proteasome-dependent degradation of steroid receptor coactivator 3 (28). Degradation products of the HCV core protein via the PA28 γ -dependent pathway may regulate the promoter activity of the TNF- α gene. PA28 proteins are necessary and sufficient to fully reconstitute Hsp90-initiated refolding together with Hsc70 and Hsp40 (31). Therefore, it might also be feasible to speculate that the HCV core protein refolded by an Hsp90/PA28 γ -dependent pathway activates the promoter of the TNF- α gene together with an unknown transcription factor(s) or regulator(s).

In conclusion, the data obtained in this study suggest that the expression of the HCV core protein enhances the production of TNF- α and suppresses the phosphorylation of tyrosine on IRS1 and the production of IRS2 through a PA28 γ -dependent pathway, thereby leading to insulin resistance. PA28 γ may be a novel target for the treatment of HCV-induced diabetes.

ACKNOWLEDGMENTS

We gratefully thank H. Murase for secretarial work.

This study was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); the 21st Century Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

REFERENCES

- Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura. 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. *Hepatology* 27: 621-627.
- Allison, M. E., T. Wreghitt, C. R. Palmer, and G. J. Alexander. 1994. Evidence for a link between hepatitis C virus infection and diabetes mellitus in a cirrhotic population. *J. Hepatol.* 21:1135-1139.
- Anai, M., M. Funaki, T. Ogihara, J. Terasaki, K. Inukai, H. Katagiri, Y. Fukushima, Y. Yazaki, M. Kikuchi, Y. Oka, and T. Asano. 1998. Altered expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. *Diabetes* 47:13-23.
- Aoyagi, K., C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, and S. Yagi. 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. *J. Clin. Microbiol.* 37:1802-1808.
- Araki, E., M. A. Lipes, M. E. Patti, J. C. Brunning, B. Haag III, R. S. Johnson, and C. R. Kahn. 1994. Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* 372:186-190.
- Bukh, J., R. H. Purcell, and R. H. Miller. 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. *Proc. Natl. Acad. Sci. USA* 91:8239-8243.
- Caronia, S., K. Taylor, L. Pagliaro, C. Carr, U. Palazzo, J. Petrik, S. O'Rahilly, S. Shore, B. D. Tom, and G. J. Alexander. 1999. Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 30:1059-1063.
- Cavaghan, M. K., D. A. Ehrmann, and K. S. Polonsky. 2000. Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. *J. Clin. Invest.* 106:329-333.
- Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244:359-362.

10. Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P. J. Barr, et al. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 88:2451-2455.
11. Falcon, V., N. Acosta-Rivero, G. China, J. Gavilondo, M. C. de la Rosa, I. Menendez, S. Bueñas-Carrera, A. Vina, V. Garcia, B. Gra, M. Noa, E. Reytor, M. T. Barcen, F. Alvarez, and J. Morales-Grillo. 2003. Ultrastructural evidences of HCV infection in hepatocytes of chronically HCV-infected patients. *Biochem. Biophys. Res. Commun.* 305:1085-1090.
12. Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice. 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* 67:2832-2843.
13. Gumber, S. C., and S. Chopra. 1995. Hepatitis C: a multifaceted disease. Review of extrahepatic manifestations. *Ann. Intern. Med.* 123:615-620.
14. Hotamisligil, G. S. 1999. The role of TNF α and TNF receptors in obesity and insulin resistance. *J. Intern. Med.* 245:621-625.
15. Houghton, M., A. Weiner, J. Han, G. Kuo, and Q. L. Choo. 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. *Hepatology* 14:381-388.
16. Houtis, N., E. D. Rosen, and E. S. Lander. 2006. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 440:944-948.
17. Ide, T., H. Shimano, N. Yahagi, T. Matsuzaka, M. Nakakuki, T. Yamamoto, Y. Nakagawa, A. Takahashi, H. Suzuki, H. Sone, H. Toyoshima, A. Fukamizu, and N. Yamada. 2004. SREBPs suppress IRS-2-mediated insulin signalling in the liver. *Nat. Cell Biol.* 6:351-357.
18. Kahn, B. B. 1998. Type 2 diabetes: when insulin secretion fails to compensate for insulin resistance. *Cell* 92:593-596.
19. Kallinowski, B., K. Haserath, G. Marinis, C. Hanck, W. Stremmel, L. Theilmann, M. V. Singer, and S. Rossol. 1998. Induction of tumour necrosis factor (TNF) receptor type p55 and p75 in patients with chronic hepatitis C virus (HCV) infection. *Clin. Exp. Immunol.* 111:269-277.
20. Kerouz, N. J., D. Horsch, S. Pons, and C. R. Kahn. 1997. Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. *J. Clin. Invest.* 100:3164-3172.
21. Kido, Y., J. Nakae, and D. Accili. 2001. Clinical review 125: the insulin receptor and its cellular targets. *J. Clin. Endocrinol. Metab.* 86:972-979.
22. Kiyosawa, K., T. Sodeyama, E. Tanaka, Y. Gibo, K. Yoshizawa, Y. Nakano, S. Furuta, Y. Akahane, K. Nishioka, R. H. Purcell, et al. 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 12:671-675.
23. Korenaga, M., T. Wang, Y. Li, L. A. Showalter, T. Chan, J. Sun, and S. A. Weinman. 2005. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J. Biol. Chem.* 280:37481-37488.
24. Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, et al. 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 244:362-364.
25. Kuprash, D. V., I. A. Udolova, R. L. Turetskaya, D. Kwiatkowski, N. R. Rice, and S. A. Nedospasov. 1999. Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J. Immunol.* 162:4045-4052.
26. Lazar, D. F., and A. R. Saltiel. 2006. Lipid phosphatases as drug discovery targets for type 2 diabetes. *Nat. Rev. Drug Discov.* 5:333-342.
27. Lerat, H., M. Honda, M. R. Beard, K. Loesch, J. Sun, Y. Yang, M. Okuda, R. Gosert, S. Y. Xiao, S. A. Weinman, and S. M. Lemon. 2002. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 122:352-365.
28. Li, X., D. M. Lonard, S. Y. Jung, A. Malovannaya, Q. Feng, J. Qin, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley. 2006. The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGgamma proteasome. *Cell* 124:381-392.
29. Mason, A. L., J. Y. Lau, N. Hoang, K. Qian, G. J. Alexander, L. Xu, L. Guo, S. Jacob, F. G. Regenstein, R. Zimmerman, J. E. Everhart, C. Wasserfall, N. K. Maclaren, and R. P. Perrillo. 1999. Association of diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 29:328-333.
30. McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio. 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J.* 21:3980-3988.
31. Minami, Y., H. Kawasaki, M. Minami, N. Tanahashi, K. Tanaka, and I. Yahara. 2000. A critical role for the proteasome activator PA28 in the Hsp90-dependent protein refolding. *J. Biol. Chem.* 275:9055-9061.
32. Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura. 2003. Proteasome activator PA28 γ -dependent nuclear retention and degradation of hepatitis C virus core protein. *J. Virol.* 77:10237-10249.
33. Moriya, K., H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, and K. Koike. 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* 4:1065-1067.
34. Moriya, K., K. Nakagawa, T. Santa, Y. Shintani, H. Fujie, H. Miyoshi, T. Tsutsumi, T. Miyazawa, K. Ishibashi, T. Horie, K. Imai, T. Todoroki, S. Kimura, and K. Koike. 2001. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res.* 61:4365-4370.
35. Moriya, K., H. Yotsuyanagi, Y. Shintani, H. Fujie, K. Ishibashi, Y. Matsuura, T. Miyamura, and K. Koike. 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.* 78:1527-1531.
36. Murata, S., H. Kawahara, S. Tohma, K. Yamamoto, M. Kasahara, Y. Nabeshima, K. Tanaka, and T. Chiba. 1999. Growth retardation in mice lacking the proteasome activator PA28 γ . *J. Biol. Chem.* 274:38211-38215.
37. Nelson, D. R., H. L. Lim, C. G. Marousis, J. W. Fang, G. L. Davis, L. Shen, M. S. Urdea, J. A. Kolberg, and J. Y. Lau. 1997. Activation of tumor necrosis factor- α system in chronic hepatitis C virus infection. *Dig. Dis. Sci.* 42:2487-2494.
38. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193-199.
39. Ogino, T., H. Fukuda, S. Imajoh-Ohmi, M. Kohara, and A. Nomoto. 2004. Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. *J. Virol.* 78:11766-11777.
40. Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura. 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. *J. Virol.* 78:6370-6380.
41. Okuda, M., K. Li, M. R. Beard, L. A. Showalter, F. Scholle, S. M. Lemon, and S. A. Weinman. 2002. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 122:366-375.
42. Ozes, O. N., H. Akca, L. D. Mayo, J. A. Gustin, T. Maehama, J. E. Dixon, and D. B. Donner. 2001. A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. *Proc. Natl. Acad. Sci. USA* 98:4640-4645.
43. Rui, L., T. L. Fisher, J. Thomas, and M. F. White. 2001. Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. *J. Biol. Chem.* 276:40362-40367.
44. Shimoike, T., S. Mimori, H. Tani, Y. Matsuura, and T. Miyamura. 1999. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. *J. Virol.* 73:9718-9725.
45. Shimomura, I., R. E. Hammer, J. A. Richardson, S. Ikemoto, Y. Bashmakov, J. L. Goldstein, and M. S. Brown. 1998. Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev.* 12:3182-3194.
46. Shimomura, I., M. Matsuda, R. E. Hammer, Y. Bashmakov, M. S. Brown, and J. L. Goldstein. 2000. Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. *Mol. Cell* 6:77-86.
47. Shintani, Y., H. Fujie, H. Miyoshi, T. Tsutsumi, K. Tsukamoto, S. Kimura, K. Moriya, and K. Koike. 2004. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 126:840-848.
48. Shulman, G. I. 2000. Cellular mechanisms of insulin resistance. *J. Clin. Invest.* 106:171-176.
49. Su, A. I., J. P. Pezacki, I. Wodicka, A. D. Brideau, L. Supekova, R. Thimme, S. Wieland, J. Bukh, R. H. Purcell, P. G. Schultz, and F. V. Chisari. 2002. Genomic analysis of the host response to hepatitis C virus infection. *Proc. Natl. Acad. Sci. USA* 99:15669-15674.
50. Sun, X. J., J. L. Goldberg, L. Y. Qiao, and J. J. Mitchell. 1999. Insulin-induced insulin receptor substrate-1 degradation is mediated by the proteasome degradation pathway. *Diabetes* 48:1359-1364.
51. Suzuki, R., S. Sakamoto, T. Tsutsumi, A. Rikimaru, K. Tanaka, T. Shimoike, K. Moriishi, T. Iwasaki, K. Mizumoto, Y. Matsuura, T. Miyamura, and T. Suzuki. 2005. Molecular determinants for subcellular localization of hepatitis C virus core protein. *J. Virol.* 79:1271-1281.
52. Tamemoto, H., T. Kadowaki, K. Tohe, T. Yagi, H. Sakura, T. Hayakawa, Y. Terauchi, K. Ueki, Y. Kaburagi, S. Satoh, et al. 1994. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182-186.
53. Taniguchi, C. M., K. Ueki, and R. Kahn. 2005. Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *J. Clin. Invest.* 115:718-727.
54. Uysal, K. T., S. M. Wiesbrock, M. W. Marino, and G. S. Hotamisligil. 1997. Protection from obesity-induced insulin resistance in mice lacking TNF- α function. *Nature* 389:610-614.
55. Vanhaesebroeck, B., and D. R. Alessi. 2000. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* 346:561-576.

56. White, M. F. 1998. The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol. Cell. Biochem.* 182:3-11.
57. Withers, D. J., J. S. Gutierrez, H. Towery, D. J. Burks, J. M. Ren, S. Previs, Y. Zhang, D. Bernal, S. Pons, G. I. Shulman, S. Bonner-Weir, and M. F. White. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900-904.
58. Yu, C., Y. Chen, G. W. Cline, D. Zhang, H. Zong, Y. Wang, R. Bergeron, J. K. Kim, S. W. Cushman, G. J. Cooney, B. Atcheson, M. F. White, E. W. Kraegen, and G. I. Shulman. 2002. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J. Biol. Chem.* 277:50230-50236.

Critical role of PA28 γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis

Kohji Moriishi*, Rika Mochizuki*, Kyoji Moriya[†], Hironobu Miyamoto*, Yoshio Mori*, Takayuki Abe*, Shigeo Murata[‡], Keiji Tanaka[§], Tatsuo Miyamura[§], Tetsuro Suzuki[§], Kazuhiko Koike[§], and Yoshiharu Matsuura*[¶]

*Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; [†]Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan; [‡]Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan; and [§]Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

Edited by Peter Palese, Mount Sinai School of Medicine, New York, NY, and approved December 1, 2006 (received for review August 23, 2006)

Hepatitis C virus (HCV) is a major cause of chronic liver disease that frequently leads to steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC). HCV core protein is not only a component of viral particles but also a multifunctional protein because liver steatosis and HCC are developed in HCV core gene-transgenic (CoreTg) mice. Proteasome activator PA28 γ /REG γ regulates host and viral proteins such as nuclear hormone receptors and HCV core protein. Here we show that a knockout of the PA28 γ gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of CoreTg mice and disrupts development of both hepatic steatosis and HCC. Furthermore, the genes related to fatty acid biosynthesis and *srebp-1c* promoter activity were up-regulated by HCV core protein in the cell line and the mouse liver in a PA28 γ -dependent manner. Heterodimer composed of liver X receptor α (LXR α) and retinoid X receptor α (RXR α) is known to up-regulate *srebp-1c* promoter activity. Our data also show that HCV core protein enhances the binding of LXR α /RXR α to LXR-response element in the presence but not the absence of PA28 γ . These findings suggest that PA28 γ plays a crucial role in the development of liver pathology induced by HCV infection.

fatty acid | proteasome | sterol regulatory element-binding protein (SREBP) | RXR α | LXR α

Hepatitis C virus (HCV) belongs to the Flaviviridae family, and it possesses a positive, single-stranded RNA genome that encodes a single polyprotein composed of \approx 3,000 aa. The HCV polyprotein is processed by host and viral proteases, resulting in 10 viral proteins. Viral structural proteins, including the capsid (core) protein and two envelope proteins, are located in the N-terminal one-third of the polyprotein, followed by nonstructural proteins.

HCV infects >170 million individuals worldwide, and then it causes liver disease, including hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (1). The prevalence of fatty infiltration in the livers of chronic hepatitis C patients has been reported to average \approx 50% (2, 3), which is higher than the percentage in patients infected with hepatitis B virus and other liver diseases. However, the precise functions of HCV proteins in the development of fatty liver remain unknown because of the lack of a system sufficient to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and HCC in transgenic mice (4–6). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and HCC.

Recent reports suggest that lipid biosynthesis affects HCV replication (7–9). Involvement of a geranylgeranylated host protein, FBL2, in HCV replication through the interaction with NSSA suggests that the cholesterol biosynthesis pathway is also important for HCV replication (9). Increases in saturated and monounsaturated fatty acids enhance HCV RNA replication, whereas increases in polyunsaturated fatty acids suppress it (7). Lipid homeostasis is regulated by a family of sterol regulatory element-binding proteins (SREBPs), which activate the expression of >30 genes involved in

the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. Biosynthesis of cholesterol is regulated by SREBP-2, whereas that of fatty acids, triglycerides, and phospholipids is regulated by SREBP-1c (10–14). In chimpanzees, host genes involved in SREBP signaling are induced during the early stages of HCV infection (8). SREBP-1c regulates the transcription of acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, leading to the production of saturated and monounsaturated fatty acids and triglycerides (15). SREBP-1c is transcriptionally regulated by liver X receptor (LXR) α and retinoid X receptor (RXR) α , which belong to a family of nuclear hormone receptors (15, 16). Accumulation of cellular fatty acids by HCV core protein is expected to be modulated by the SREBP-1c pathway because RXR α is activated by HCV core protein (17). However, it remains unknown whether HCV core protein regulates the *srebp-1c* promoter.

We previously reported (18) that HCV core protein specifically binds to the proteasome activator PA28 γ /REG γ in the nucleus and is degraded through a PA28 γ -dependent pathway. PA28 γ is well conserved from invertebrates to vertebrates, and amino acid sequences of human and murine PA28 γ s are identical (19). The homologous proteins, PA28 α and PA28 β , form a heteroheptamer in the cytoplasm, and they activate chymotrypsin-like peptidase activity of the 20S proteasome, whereas PA28 γ forms a homoheptamer in the nucleus, and it enhances trypsin-like peptidase activity of 20S proteasome (20). Recently, Li and colleagues (21) reported that PA28 γ binds to steroid receptor coactivator-3 (SRC-3) and enhances the degradation of SRC-3 in a ubiquitin- and ATP-independent manner. However, the precise physiological functions of PA28 γ are largely unknown *in vivo*. In this work, we examine whether PA28 γ is required for liver pathology induced by HCV core protein *in vivo*.

Results

PA28 γ -Knockout HCV Core Gene Transgenic Mice. To determine the role of PA28 γ in HCV core-induced steatosis and the development of HCC *in vivo*, we prepared PA28 γ -knockout core gene transgenic mice. The PA28 γ -deficient, PA28 γ ^{-/-} mice were born without

Author contributions: K. Moriishi, K.T., T.M., T.S., K.K., and Y. Matsuura designed research; K. Moriishi, R.M., K. Moriya, H.M., Y. Mori, and T.A. performed research; S.M. contributed new reagents/analytic tools; Y. Matsuura analyzed data; and K. Moriishi, K.K., and Y. Matsuura wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Freely available online through the PNAS open access option.

Abbreviations: CoreTg, HCV core gene-transgenic; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LXR, liver X receptor; LXRE, liver X receptor-response element; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; RXR, retinoid X receptor; SRC-3, steroid receptor coactivator-3; SREBP, sterol regulatory element-binding protein.

*To whom correspondence should be addressed. E-mail: matsuura@biken.osaka-u.ac.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0607312104DC1.

© 2007 by The National Academy of Sciences of the USA

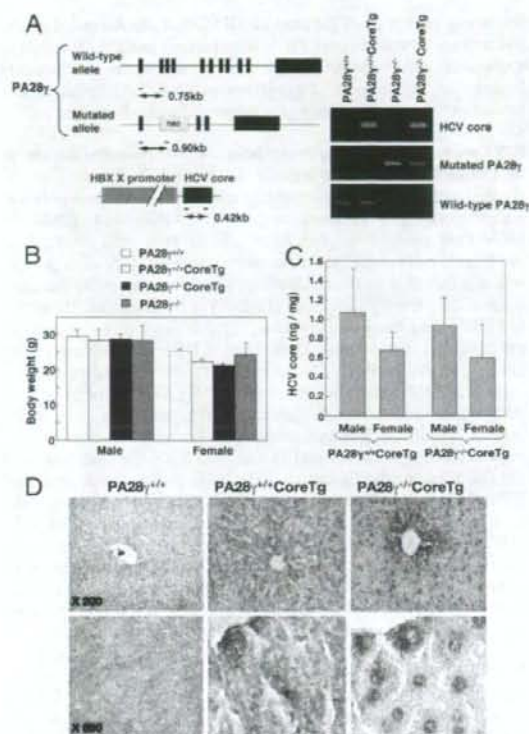


Fig. 1. Preparation and characterization of PA28 γ -knockout HCV core-transgenic mice. (A) The structures of the wild-type and mutated PA28 γ genes and the transgene encoding the HCV core protein under the control of the HBV X promoter were investigated. Positions corresponding to the screening primers and sizes of PCR products are shown. PCR products of the HCV core gene as well as wild-type and mutated PA28 γ alleles were amplified from the genomic DNAs of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice. (B) Body weights of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$ mice at the age of 6 months. (C) HCV core protein levels in the livers of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice were determined by ELISA (mean \pm SD, $n = 10$). (D) Localization of HCV core protein in the liver. Liver sections of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, and PA28 $\gamma^{-/-}$ CoreTg mice at the age of 2 months were stained with anti-HCV core antibody.

appreciable abnormalities in all tissues examined, with the exception of a slight retardation of growth (22). HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg) mice were bred with PA28 $\gamma^{-/-}$ mice to create PA28 $\gamma^{+/+}$ CoreTg mice. The PA28 $\gamma^{+/+}$ CoreTg offspring were bred with each other, and PA28 $\gamma^{-/-}$ CoreTg mice were selected by PCR using primers specific to the target sequences (Fig. 1A). No significant differences in body weight were observed among the 6-month-old mice, although PA28 $\gamma^{-/-}$ mice exhibited a slight retardation of growth (Fig. 1B). A similar level of PA28 γ expression was detected in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{+/+}$ mice (see Fig. 5B). The expression levels and molecular size of HCV core protein were similar in the livers of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1C; see also Fig. 5B).

PA28 γ Is Required for Degradation of HCV Core Protein in the Nucleus and Induction of Liver Steatosis. HCV core protein has been detected at various sites, such as the endoplasmic reticulum, mitochondria, lipid droplets, and nucleus of cultured cell lines, as well as in hepatocytes of PA28 $\gamma^{+/+}$ CoreTg mice and hepatitis C patients

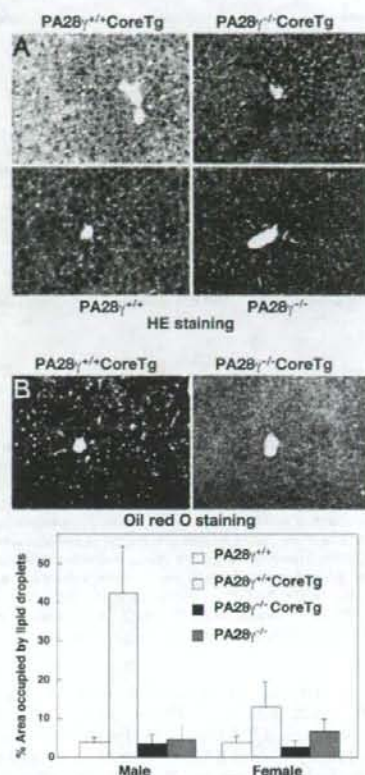


Fig. 2. Accumulation of lipid droplets by expression of HCV core protein. (A) Liver sections of the mice at the age of 6 months were stained with hematoxylin/eosin (HE). (B) (Upper) Liver sections of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice at the age of 6 months were stained with oil red O. (Lower) The area occupied by lipid droplets of PA28 $\gamma^{+/+}$ (white), PA28 $\gamma^{+/+}$ CoreTg (gray), PA28 $\gamma^{-/-}$ CoreTg (black), and PA28 $\gamma^{-/-}$ (dark gray) mice was calculated by Image-Pro software (MediaCybernetics, Silver Spring, MD) (mean \pm SD, $n = 10$).

(6, 23, 24). Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of PA28 $\gamma^{+/+}$ CoreTg mice, as reported in ref. 6, in the present study a clear accumulation of HCV core protein was observed in the liver cell nuclei of PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1D). These findings clearly indicate that at least some fraction of the HCV core protein is translocated into the nucleus and is degraded through a PA28 γ -dependent pathway. Mild vacuolation was observed in the cytoplasm of the liver cells of 4-month-old PA28 $\gamma^{+/+}$ CoreTg mice, and it became more severe at 6 months, as reported in ref. 25. Hematoxylin/eosin-stained liver sections of 6-month-old PA28 $\gamma^{+/+}$ CoreTg mice exhibited severe vacuolating lesions (Fig. 2A), which were clearly stained with oil red O (Fig. 2B Upper), whereas no such lesions were detected in the livers of PA28 $\gamma^{-/-}$ CoreTg, PA28 $\gamma^{+/+}$, or PA28 $\gamma^{-/-}$ mice at the same age. The areas occupied by the lipid droplets in the PA28 $\gamma^{+/+}$ CoreTg mouse livers were ≈ 10 and 2–4 times larger than those of male and female of PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice, respectively (Fig. 2B Lower). These results suggest that PA28 γ is required for the induction of liver steatosis by HCV core protein in mice.

PA28 γ Is Required for the Up-Regulation of SREBP-1c Transcription by HCV Core Protein in the Mouse Liver. To clarify the effects of a knockout of the PA28 γ gene in PA28 $\gamma^{+/+}$ CoreTg mice on lipid

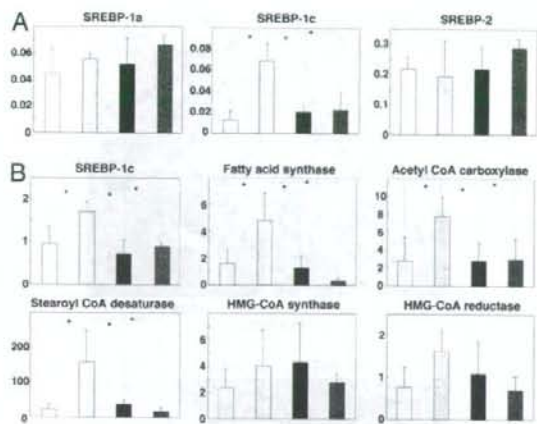


Fig. 3. Transcription of genes regulating lipid biosynthesis in the mouse liver. (A) Total RNA was prepared from the livers of 2-month-old mice; and the transcription of genes encoding SREBP-1a, SREBP-1c, and SREBP-2 was determined by real-time PCR. (B) The transcription of genes encoding SREBP-1c, fatty acid synthase, acetyl-CoA carboxylase, stearyl-CoA desaturase, HMG-CoA synthase, and HMG-CoA reductase of 6-month-old mice was measured by real-time PCR. The transcription of the genes was normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activity ($n = 5$; *, $P < 0.05$; **, $P < 0.01$). The transcription of each gene in PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$ mice is indicated by white, gray, black, and dark gray bars, respectively.

metabolism, genes related to the lipid biosyntheses were examined by real-time quantitative PCR. Transcription of SREBP-1c was higher in the livers of PA28 $\gamma^{+/+}$ CoreTg mice than in those of PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice at 2 months of age, but no such increases in SREBP-2 and SREBP-1a were observed (Fig. 3A). Although transcription of SREBP-1c and its regulating enzymes, such as acetyl-CoA carboxylase, fatty acid synthase, and stearyl-CoA desaturase, was also enhanced in the livers of 6-month-old PA28 $\gamma^{+/+}$ CoreTg mice compared with the levels in the livers of PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice, no statistically significant differences were observed with respect to the transcription levels of cholesterol biosynthesis-related genes that are regulated by SREBP-2 (e.g., HMG-CoA synthase and HMG-CoA reductase) (Fig. 3B). These results suggest the

following: (i) the up-regulation of SREBP-1c transcription in the livers of mice requires both HCV core protein and PA28 γ ; and (ii) the nuclear accumulation of HCV core protein alone, which occurs because of the lack of degradation along a PA28 γ -dependent proteasome pathway, does not activate the *srebp-1c* promoter.

HCV Core Protein Indirectly Potentiates *srebp-1c* Promoter Activity in an LXR α /RXR α -Dependent Manner. LXR α , which is primarily expressed in the liver, forms a complex with RXR α and synergistically potentiates *srebp-1c* promoter activity (16). Activation of RXR α by HCV core protein suggests that cellular fatty acid synthesis is modulated by the SREBP-1c pathway, although HCV core protein was not included in the transcription factor complex in the electrophoresis mobility shift assay (EMSA) (17). To analyze the effect of HCV core protein and PA28 γ on the activation of the *srebp-1c* promoter, we first examined the effect of HCV core protein on the binding of the LXR α /RXR α complex to the LXR-response element (LXRE) located upstream of the SREBP-1c gene (Fig. 4A). Although a weak shift of the labeled LXRE probe was observed by incubation with nuclear extracts prepared from 293T cells expressing FLAG-tagged LXR α and HA-tagged RXR α , a clear shift was obtained by the treatment of cells with 9-*cis*-retinoic acid and 22(*R*)-hydroxycholesterol, ligands for LXR α and RXR α , respectively. In contrast, coexpression of HCV core protein with LXR α and RXR α potentiated the shift of the probe irrespective of the treatment with the ligands. Addition of 500 times the amount of nonlabeled LXRE probe (competitor) diminished the shift of the labeled probe induced by the ligands and/or HCV core protein. Furthermore, coinubation of the nuclear fraction with antibody to FLAG or HA tag but not with antibody to either HCV core or PA28 γ caused a supershift of the labeled probe. These results indicate that HCV core protein does not participate in the LXR α /RXR α -LXRE complex but indirectly enhances the binding of LXR α /RXR α to the LXRE.

The activity of the *srebp-1c* promoter was enhanced by the expression of HCV core protein in 293T cells, and it was further enhanced by coexpression of LXR α /RXR α (Fig. 4B). Enhancement of the *srebp-1c* promoter by coexpression of HCV core protein and LXR α /RXR α was further potentiated by treatment with the ligands for LXR α and RXR α . The cells treated with 9-*cis*-retinoic acid exhibited more potent enhancement of the *srebp-1c* promoter than those treated with 22(*R*)-hydroxycholesterol. HCV core protein exhibited more potent enhancement of the *srebp-1c* promoter in cells treated with both ligands than in those treated with either ligand alone. These results suggest that HCV core protein poten-

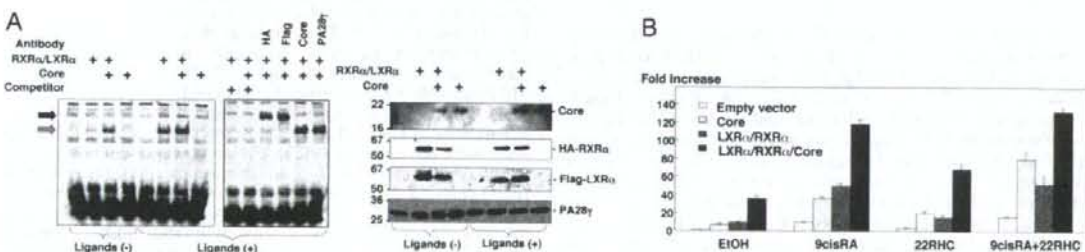


Fig. 4. Activation of the *srebp-1c* promoter by HCV core protein. (A) FLAG-LXR α and HA-RXR α were expressed in 293T cells together with or without HCV core protein. Ligands for LXR α and RXR α dissolved in ethanol [Ligands (+)] or ethanol alone [Ligands (-)] were added to the culture supernatant at 24 h posttransfection. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA in the presence or absence of antibody (100 ng) against HA, FLAG, HCV core or PA28 γ , or nonlabeled LXRE probe (Competitor). (Left) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase/streptavidin. The mobility shift of the LXRE probe and its supershift are indicated by a gray and black arrow, respectively. (Right) Expression of HCV core, HA-RXR α , FLAG-LXR α , and PA28 γ in cells was detected by immunoblotting. (B) Effects of ligands for RXR α , 9-*cis*-retinoic acid (9cisRA), and for LXR α , 22(*R*)-hydroxycholesterol (22RHC), on the activation of the *srebp-1c* promoter in 293T cells expressing RXR α , LXR α , and/or HCV core protein. Ligands were added into the medium at 24 h posttransfection at a concentration of 5 μ M, and the cells were harvested after 24 h of incubation.

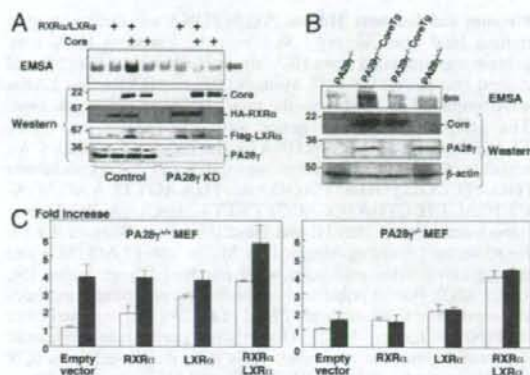


Fig. 5. PA28 γ is required for HCV core-dependent activation of the *srebp-1c* promoter. (A) Effect of PA28 γ knockdown on the LXR α /RXR α -DNA complex. FLAG-LXR α and HA-RXR α were expressed in FLC4 (control) or PA28 γ -knockdown (PA28 γ KD) cells together with or without HCV core protein. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA. (Upper) The resulting mixtures were subjected to EMSA and blotted with horseradish peroxidase-streptavidin. The mobility shift of the LXRE probe is indicated by an arrow. (Lower) Expression of HCV core, HA-RXR α , FLAG-LXR α , and PA28 γ in cells was detected by immunoblotting. (B) Effect of PA28 γ knockout on the LXR α /RXR α -DNA complex in the mouse liver. (Upper) Nuclear extracts were prepared from the livers of 2-month-old PA28 $\gamma^{-/-}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{+/+}$ mice and subjected to EMSA. The mobility shift of the LXRE probe is indicated by an arrow. (Lower) The expression of HCV core, PA28 γ , and β -actin in the livers of the mice was detected by immunoblotting. (C) Effect of HCV core protein on *srebp-1c* promoter activity in PA28 γ -knockout fibroblasts. A plasmid encoding firefly luciferase under the control of the *srebp-1c* promoter was transfected into MEFs prepared from PA28 $\gamma^{+/+}$ (Left) or PA28 $\gamma^{-/-}$ (Right) mice together with a plasmid encoding a *Renilla* luciferase. An empty plasmid or plasmids encoding mouse RXR α or LXR α were also cotransfected into the cells together with (gray bars) or without (white bars) a plasmid encoding HCV core protein. Luciferase activity under the control of the *srebp-1c* promoter was determined, and it is expressed as the fold increase in relative luciferase activity after standardization with the activity of *Renilla* luciferase.

tiates *srebp-1c* promoter activity in an LXR α /RXR α -dependent manner.

HCV Core Protein Activates the *srebp-1c* Promoter in an LXR α /RXR α - and PA28 γ -Dependent Manner. To examine whether PA28 γ is required for HCV core-induced enhancement of *srebp-1c* promoter activity in human liver cells, a PA28 γ -knockdown human hepatoma cell line (FLC4 KD) was prepared. Enhancement of binding of the LXRE probe to LXR α /RXR α by coexpression of HCV core protein and LXR α /RXR α in FLC4 cells was diminished by knockdown of the PA28 γ gene (Fig. 5A). Furthermore, formation of the LXR α /RXR α -LXRE complex was enhanced in the livers of PA28 $\gamma^{+/+}$ CoreTg mice but not in those of PA28 $\gamma^{-/-}$, PA28 $\gamma^{+/+}$, or PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 5B). The expression of the HCV core protein in the mouse embryonic fibroblasts (MEFs) of PA28 $\gamma^{+/+}$ mice induced the activation of the mouse *srebp-1c* promoter through the endogenous expression of LXR α and RXR α (Fig. 5C Left). Further enhancement of the activation of the *srebp-1c* promoter by HCV core protein in PA28 $\gamma^{+/+}$ MEFs was achieved by the exogenous expression of both LXR α and RXR α . However, no enhancing effect of HCV core protein on *srebp-1c* promoter activity was observed in PA28 $\gamma^{-/-}$ MEFs (Fig. 5C Right). These results support the notion that HCV core protein enhances the activity of the *srebp-1c* promoter in an LXR α /RXR α - and PA28 γ -dependent manner.

Table 1. HCC in mice at 16–18 months of age

Mouse and sex	Total no. of mice	No. of mice developing HCC	Incidence, %
PA28$\gamma^{+/+}$CoreTg			
Male	17	5	29.4
Female	28	3	10.7
PA28$\gamma^{-/-}$			
Male	16	0	0
Female	4	0	0
PA28$\gamma^{-/-}$CoreTg			
Male	23	0	0
Female	13	0	0
PA28$\gamma^{+/+}$CoreTg			
Male	15	0	0
Female	21	0	0

PA28 γ Plays a Crucial Role in the Development of HCC in PA28 $\gamma^{+/+}$ CoreTg Mice. The incidence of hepatic tumors in male PA28 $\gamma^{+/+}$ CoreTg mice older than 16 months was significantly higher than that in age-matched female PA28 $\gamma^{+/+}$ CoreTg mice (6). We reconfirmed here that the incidence of HCC in male and female PA28 $\gamma^{+/+}$ CoreTg mice at 16–18 months of age was 29.4% (5 of 17 mice) and 10.7% (3 of 28 mice), respectively. To our surprise, however, no HCC developed in PA28 $\gamma^{-/-}$ CoreTg mice (males, 15; females, 21), although, as expected, no HCC was observed in PA28 $\gamma^{-/-}$ (males, 16; females, 4) and PA28 $\gamma^{-/-}$ mice (males, 23; females, 13) (Table 1). These results clearly indicate that PA28 γ plays an indispensable role in the development of HCC induced by HCV core protein.

Discussion

HCV core protein is detected in the cytoplasm and partially in the nucleus and mitochondria of culture cells and hepatocytes of transgenic mice and hepatitis C patients (6, 23, 24, 26). Degradation of HCV core protein was enhanced by deletion of the C-terminal transmembrane region through a ubiquitin/proteasome-dependent pathway (27). We previously reported (18) that PA28 γ binds directly to HCV core protein and then enhances degradation of HCV core protein in the nucleus through a proteasome-dependent pathway because HCV core protein was accumulated in nucleus of human cell line by treatment with proteasome inhibitor MG132. In this work, accumulation of HCV core protein was observed in nucleus of hepatocytes of PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1D). This result directly demonstrates that HCV core protein migrates into the nucleus and is degraded through a PA28 γ -dependent pathway. However, HCV core protein accumulated in the nucleus because knockout of PA28 γ gene abrogated the ability to cause liver pathology, suggesting that interaction of HCV core protein with PA28 γ in the nucleus is prerequisite for the liver pathology induced by HCV core protein. We have previously shown (18) that HCV core protein is degraded through a PA28 γ -dependent pathway, and Minami *et al.* (28) reported that PA28 γ has a cochaperone activity with Hsp90. Therefore, degradation products of HCV core protein by means of PA28 γ -dependent processing or correct folding of HCV core protein through cochaperone activity of PA28 γ might be involved in the development of liver pathology. We do not know the reason why knockout of the PA28 γ gene does not affect the total amount of HCV core protein in the liver of the transgenic mice. PA28 γ -dependent degradation of HCV core protein may be independent of ubiquitination, as shown in SRC-3 (21), whereas knockdown of PA28 γ in a human hepatoma cell line enhanced the ubiquitination of HCV core protein [supporting information (SI) Fig. 6], suggesting that lack of PA28 γ suppresses a ubiquitin-independent degradation but enhances a ubiquitin-dependent degradation of HCV core protein. Therefore, the total amount of HCV

core protein in the liver of the mice may be unaffected by the knockout of the PA28 γ gene.

Our results suggest that the interaction of HCV core protein with PA28 γ leads to the activation of the *srebp-1c* promoter along an LXR α /RXR α -dependent pathway and the development of liver steatosis and HCC. HCV core protein was not included in the LXR α /RXR α -LXRE complex (Fig. 3A), suggesting that HCV core protein indirectly activates the *srebp-1c* promoter. Cytoplasmic HCV core protein was shown to interact with Sp110b, which is a transcriptional corepressor of RAR α -dependent transcription, and this interaction leads to the sequestering of Sp110b in the cytoplasm, resulting in the activation of RAR α -dependent transcription (29). The sequestration of an unidentified corepressor of the LXR α /RXR α heterodimer in the cytoplasm by HCV core protein may also contribute to the activation of the *srebp-1c* promoter. Although the precise physiological function of PA28 γ -proteasome activity in the nucleus is not known, PA28 γ has previously been shown (21) to regulate nuclear hormone receptors by means of the degradation of its coactivator SRC-3 and to participate in the fully Hsp90-dependent protein refolding (28). It appears reasonable to speculate that degradation or refolding of HCV core protein in a PA28 γ -dependent pathway might be involved in the modulation of transcriptional regulators of various promoters, including the *srebp-1c* promoter. Saturated or monounsaturated fatty acids have been shown to enhance HCV RNA replication in Huh7 cells containing the full-length HCV replicon (7). The up-regulation of fatty acid biosynthesis by HCV core protein may also contribute to the efficient replication of HCV and to the progression of HCV pathogenesis.

Expression of HCV core protein was reported to enhance production of reactive oxygen species (ROS) (30), which leads to carbonylation of intracellular proteins (31). Enhancement of ROS production may trigger double-stranded DNA breaks and result in the development of HCC (30, 32, 33). HCV core protein could enhance the protein carbonylation in the liver of the transgenic mice in the presence but not in the absence of PA28 γ (SI Fig. 7), suggesting that PA28 γ is required for ROS production induced by HCV core protein. Development of HCC was observed in PA28 $\gamma^{+/+}$ CoreTg mice but not in PA28 $\gamma^{-/-}$ CoreTg mice (Table 1). Enhancement of ROS production by HCV core protein in the presence of PA28 γ might be involved in the development of HCC in PA28 $\gamma^{+/+}$ CoreTg mice.

It is well known that resistant viruses readily emerge during the treatment with antiviral drugs targeting the viral protease or replicate, especially in the case of infection with RNA viruses. Therefore, antivirals targeting the host factors that are indispensable for the propagation of viruses might be an ideal target for the development of antiviral agents because of a lower rate of mutation than that of viral genome, if they have no side effects to patients. Importantly, the amino acid sequence of PA28 γ of mice is identical to that of human, and mouse PA28 γ is dispensable because PA28 γ knockout mice exhibit no abnormal phenotype except for mild growth retardation. Therefore, PA28 γ might be a promising target for an antiviral treatment of chronic hepatitis C with negligible side effects.

In summary, we observed that a knockout of the PA28 γ gene from PA28 $\gamma^{+/+}$ CoreTg mice induced the accumulation of HCV core protein in the nucleus and disrupted the development of both steatosis and HCC. Activation of the *srebp-1c* promoter was up-regulated by HCV core protein both *in vitro* and *in vivo* through a PA28 γ -dependent pathway, suggesting that PA28 γ plays a crucial role in the development of liver pathology induced by HCV infection.

Materials and Methods

Histology and immunohistochemistry, real-time PCR, and detection of proteins modified by ROS are discussed in SI Materials and Methods.

Moriishi et al.

Plasmids and Reagents. Human PA28 γ cDNA was isolated from a human fetal brain library (18). The gene encoding HCV core protein was amplified from HCV strain J1 (genotype 1b) (34) and cloned into pCAG-GS (35). Mouse cDNAs of RXR α and LXR α were amplified by PCR from the total cDNAs of the mouse liver. The RXR α and LXR α genes were introduced into pEF-FLAGGspGBK (36) and pcDNA3.1 (Invitrogen, Carlsbad, CA), respectively. The targeting fragment for human PA28 γ knockout (GGATCCGGTGGATCAGGAAGTGAAGTTCAAGAGAGACTTCACTTCTGATCCACCTTTTTGGAAAAGCTT) was introduced into the BamHI and HindIII sites of pSilencer 4.1 U6 hygro vector (Ambion, Austin, TX). Mouse anti-FLAG (M2) and mouse anti- β -actin antibodies were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibody against synthetic peptides corresponding to amino acids 70–85 of PA28 γ was obtained from AFFINITI (Exeter, U.K.). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs were purchased from ICN Pharmaceuticals (Aurora, OH). Rabbit anti-HCV core protein was prepared by immunization with recombinant HCV core protein (amino acids 1–71), as described in ref. 24. Mouse monoclonal antibody to HCV core protein was kindly provided by S. Yagi (37). The plasmid for expression of HA-tagged ubiquitin was described in ref. 27.

Preparation of PA28 γ -Knockout HCV CoreTg Mice. The generation of C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b line C49 and that of PA28 $\gamma^{-/-}$ mice have been reported previously (22, 25). Both strains were crossed with each other to create PA28 $\gamma^{-/-}$ CoreTg mice. PA28 $\gamma^{-/-}$ CoreTg mice were identified by PCR targeted at the PA28 γ or HCV core gene (22, 25). Using 1 μ g of genomic DNA obtained from the mouse tail, the PA28 γ gene was amplified by PCR with the following primers: sense, PA28-3 (AGGTGGATCAGGAAGTGAAGCTCAA); and antisense, PA28 γ -5c (CACCTCACTTGTGATCCGCTCTCTGAAAGAATCAACC). The targeted sequence for the PA28 γ -knockout mouse was detected by PCR using the PA28-3 primer and the PAKO-4 primer (TGCAGTTCATTCAGGGACCGGACAG). The transgene encoding HCV core protein was detected by PCR as described in ref. 25. The expression of PA28 γ and HCV core protein in the livers of 6-month-old mice was confirmed by Western blotting with mouse monoclonal antibody to HCV core protein, clone 11-10, and rabbit antibody to PA28 γ . Mice were cared for according to the institutional guidelines. The mice were given ordinary feed, CRF-1 (Charles River Laboratories, Yokohama, Japan), and they were maintained under specific pathogen-free conditions.

All animal experiments conformed to the Guidelines for the Care and Use of Laboratory Animals, and they were approved by the Institutional Committee of Laboratory Animal Experimentation (Research Institute for Microbial Diseases, Osaka University).

Preparation of Mouse Embryonic Fibroblasts. MEFs were prepared as described in ref. 22. MEFs were cultured at 37°C under an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids.

Transfection and Immunoblotting. Plasmid vectors were transfected into the MEFs and 293T cells by liposome-mediated transfection by using Lipofectamine 2000 (Invitrogen). The amount of HCV core protein in the liver tissues was determined by an ELISA as described in ref. 37. The cell lysates were subjected to SDS/PAGE (12.5% gel), and they were then transferred onto PVDF membranes. Proteins on the membranes were treated with specific antibody and Super Signal FEMTO (Pierce, Rockford, IL). The results were then visualized by using an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan). The method of immunoprecipitation test is described in ref. 18.

Reporter Assay for *srebp-1c* Promoter Activity. The genomic DNA fragment encoding the *srebp-1c* promoter region (located from residues -410 to +24) was amplified from a mouse genome. The fragment was introduced into the KpnI and HindIII sites of pGL3-Basic (Promega, Madison, WI), and it was designated as pGL3-*srebp-1c*Pro. The plasmids encoding RXR α and LXRA were transfected into MEFs together with pGL3-*srebp-1c*Pro and a control plasmid encoding *Renilla* luciferase (Promega). The total DNA for transfection was normalized by the addition of empty plasmids. Cells were harvested at 24 h posttransfection. The ligand of RXR α , 9-*cis*-retinoic acid (Sigma), and that of LXRA, 22(R)-hydroxycholesterol (Sigma) were added at a final concentration of 5 μ M each to the culture medium of 293T cells transfected with pGL3-*srebp-1c*Pro together with expression plasmids encoding RXR α , LXRA, and HCV core protein at 24 h posttransfection. Cells were harvested 24 h after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold increase in relative luciferase units.

Electrophoresis Mobility Shift Assay (EMSA). EMSA was carried out by using a LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's protocol. Nuclear extract of the cell lines and liver tissue was prepared with an NE-PER nuclear

and cytoplasmic extraction reagent kit (Pierce). Briefly, double-stranded oligonucleotides for EMSA were prepared by annealing both strands of each LXRE of the *srebp-1c* promoter (5'-GGACGCCCGCTAGTAACCCCGGC-3') (16). Both strands were labeled at the 5' ends with biotin. The annealed probe was incubated for 20 min on ice with nuclear extract (3 μ g of protein) in a reaction buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.05 μ g/ μ l poly(dI-dC), 2.5% glycerol, 0.05% Nonidet P-40, and 0.1 nM labeled probe, with or without 1 mM nonlabeled probe. The resulting mixture was subjected to PAGE (5% gel) at 120 V for 30 min in 0.5 \times TBE. The DNA-protein complex was transferred to a Hybond N+ membrane (Amersham, Piscataway, NJ), incubated with horseradish peroxidase-conjugated streptavidin, and visualized by using an LAS3000 imaging system.

Statistical Analysis. The results are expressed as the mean \pm SD. The significance of differences in the means was determined by Student's *t* test.

We thank H. Murase for secretarial work and D. C. S. Huang for providing the plasmids. This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the 21st Century Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

- Wasley A, Alter MJ (2000) *Semin Liver Dis* 20:1-16.
- Bach N, Thung SN, Schaffner F (1992) *Hepatology* 15:572-577.
- Lefkowitz JH, Schiff ER, Davis GL, Perrillo RP, Lindsay K, Bodenheimer HC, Jr., Balart LA, Ortigo TJ, Payne J, Dienstag JL, et al. (1993) *Gastroenterology* 104:595-603.
- Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, Eder G, Schaff Z, Chapman MJ, Miyamura T, Brechot C (1997) *Proc Natl Acad Sci USA* 94:1200-1205.
- Hope RG, McLauchlan J (2000) *J Gen Virol* 81:1913-1925.
- Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K (1998) *Nat Med* 4:1065-1067.
- Kapadia SB, Chisari FV (2005) *Proc Natl Acad Sci USA* 102:2561-2566.
- Su AL, Pezacki JP, Wodicka L, Brideau AD, Supkova L, Thimme R, Wieland S, Bukh J, Purcell RH, Schultz PG, Chisari FV (2002) *Proc Natl Acad Sci USA* 99:15669-15674.
- Wang C, Gale M, Jr, Keller BC, Huang H, Brown MS, Goldstein JL, Ye J (2005) *Mol Cell* 18:425-434.
- Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H (1998) *J Clin Invest* 101:2331-2339.
- Pai JT, Guryev O, Brown MS, Goldstein JL (1998) *J Biol Chem* 273:26138-26148.
- Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL (1996) *J Clin Invest* 98:1575-1584.
- Shimano H, Horton JD, Shimomura I, Hammer RE, Brown MS, Goldstein JL (1997) *J Clin Invest* 99:846-854.
- Shimano H, Shimomura I, Hammer RE, Herz J, Goldstein JL, Brown MS, Horton JD (1997) *J Clin Invest* 100:2115-2124.
- Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ (2000) *Genes Dev* 14:2819-2830.
- Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuura T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, et al. (2001) *Mol Cell Biol* 21:2991-3000.
- Tsutsumi T, Suzuki T, Shimoike T, Suzuki R, Moriya K, Shintani Y, Fujie H, Matsuura Y, Koike K, Miyamura T (2002) *Hepatology* 35:937-946.
- Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, Chiba T, Tanaka K, Suzuki R, Suzuki T, et al. (2003) *J Virol* 77:10237-10249.
- Masson P, Andersson O, Petersen UM, Young P (2001) *J Biol Chem* 276:1383-1390.
- Li J, Rechsteiner M (2001) *Biochimie* 83:373-383.
- Li X, Lonard D, Jung SY, Malovannaya A, Feng Q, Qin J, Tsai SY, Tsai M, O'Malley BW (2006) *Cell* 124:381-392.
- Murata S, Kawahara H, Tohma S, Yamamoto K, Kasahara M, Nabeshima Y, Tanaka K, Chiba T (1999) *J Biol Chem* 274:38211-38215.
- Falcon V, Acosta-Rivero N, Chinea G, Gavilondo J, de la Rosa MC, Menendez I, Duenas-Carrera S, Vina A, Garcia W, Gra B, et al. (2003) *Biochem Biophys Res Commun* 305:1085-1090.
- Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, Moriishi K, Iwasaki T, Mizumoto K, Matsuura Y, et al. (2005) *J Virol* 79:1271-1281.
- Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K (1997) *J Gen Virol* 78:1527-1531.
- Yasui K, Wakita T, Tsukiyama-Kohara K, Funahashi SI, Ichikawa M, Kajita T, Moradpour D, Wands JR, Kohara M (1998) *J Virol* 72:6048-6055.
- Suzuki R, Tamura K, Li J, Ishii K, Matsuura Y, Miyamura T, Suzuki T (2001) *Virology* 280:301-309.
- Minami Y, Kawasaki H, Minami M, Tanahashi N, Tanaka K, Yahara I (2000) *J Biol Chem* 275:9055-9061.
- Watahi K, Hijikata M, Tagawa A, Doi T, Marusawa H, Shimotohno K (2003) *Mol Cell Biol* 23:7498-7509.
- Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM (2006) *J Virol* 80:7199-7207.
- Nystrom T (2005) *EMBO J* 24:1311-1317.
- Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr (1999) *Cell* 98:295-303.
- Carballo M, Conde M, El Bekay R, Martin-Nieto J, Camacho MJ, Montecirin J, Conde J, Bedoya FJ, Sobrino F (1999) *J Biol Chem* 274:17580-17586.
- Alzaki II, Aoki Y, Harada T, Ishii K, Suzuki T, Nagamori S, Toda G, Matsuura Y, Miyamura T (1998) *Hepatology* 27:621-627.
- Niwa H, Yamamura K, Miyazaki J (1991) *Gene* 108:193-199.
- Huang DC, Cory S, Strasser A (1997) *Oncogene* 14:405-414.
- Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, Yagi S (1999) *J Clin Microbiol* 37:1802-1808.

DYSFUNCTION OF ENERGY METABOLISM IN HEPATIC CARCINOGENESIS

Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways

Kazuhiko Koike

Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Key words

hepatitis C virus, hepatocarcinogenesis, intracellular signaling transduction, oxidative stress, transgenic mouse.

CorrespondenceDr Kazuhiko Koike, Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
Email: kkoike-ky@umin.ac.jp**Abstract**

Persistent infection with hepatitis C virus (HCV) is a major risk factor for development of hepatocellular carcinoma (HCC). However, it remains controversial in the pathogenesis of HCC associated with HCV as to whether the virus plays a direct or an indirect role. The studies using transgenic mouse models, in which the core protein of HCV has an oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, albeit other factors such as continued cell death and regeneration associated with inflammation would also play a role. The downstream events of the core protein are segregated into two components. One is the augmented production of oxidative stress along with the activation of scavenging system, including catalase and glutathione, in the putative pre-neoplastic stage with steatosis in the liver. Thus, oxidative stress production in the absence of inflammation by the core protein would partly contribute to the development of HCC. The generation of oxidative stress is estimated to originate from mitochondrial dysfunction in hepatocytes by HCV infection. The other component is the alteration of intracellular signaling cascade of mitogen-activated protein kinase and activating factor (AP)-1, leading to the activation of cell cycle control. The combination of these pathways, collective with HCV-associated alterations in lipid and glucose metabolism, would lead to the frequent development of HCC in persistent HCV infection. These results suggest that there would be a mechanism for hepatocarcinogenesis in persistent HCV infection that is distinct from those for the other cancers. Similar to the pathogenesis of other cancers, the accumulation of a set of genetic aberrations may also be necessary for a multistage development of HCC. However, HCV core protein, to which an oncogenic potential is ascribed, may allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore unlike for other cancers, HCV infection may be able to cause HCC in the absence of a complete set of genetic aberrations. Such a scenario, 'non-Vogelstein-type' carcinogenesis, would explain the rare feature of hepatocarcinogenesis in HCV infection, the extraordinarily high incidence and the multicentric nature of HCC development.

Introduction

Hepatitis C virus (HCV) infects hundreds of millions of people persistently, and induces a spectrum of chronic liver disease worldwide.¹ It impacts on society in a number of domains including the medical, sociological and economic. Hepatocellular carcinoma (HCC) has become the major cause of death in individuals persistently infected with HCV. In particular, HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%.² Knowledge on the mechanism of HCC development in chronic HCV infection therefore is urgently required for the prevention of HCC.

Hepatocellular carcinoma frequently develops in persistent HCV infection

How HCV induces HCC is not clear yet, despite the fact that more than 70% of patients with HCC in Japan are infected with HCV.^{1,3,4} Hepatitis C virus infection is also common in patients with HCC in other countries albeit to a lesser extent. These lines of evidence obligate hepatologists to the considerable task of determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting in various forms of hepatitis, should be considered in a study on the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that the necrosis of hepatocytes due to chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis

viruses in HCC via hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation per se result in the development of HCC in such a high incidence or multicentric nature in HCV infection? The secondary role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and reasoning led to the suggestion that HCC may be induced, at least in part, by viral proteins. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. It takes 30–40 years for HCC to develop in individuals infected with HCV. Another constraint common to studies on carcinogenesis is the development of HCC by transformed cells that might have gone out of growth control and escaped surveillance of the host. Should this be the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these points, we chose to investigate carcinogenesis in chronic viral hepatitis using transgenic mouse technology.

HCV core protein has an oncogenic activity in transgenic mouse

Transgenic mouse lines with sections of the HCV genome were engineered by introducing genes excised from the cDNA of the HCV genome of genotype 1b.^{5,6} The mouse lines were from a CS7BL/6 strain, which is known for a rare spontaneous occurrence of HCC.⁷ Three different transgenic mouse lines have been established, which carry the core gene, envelope genes or non-structural genes (Fig. 1), respectively, under the same transcriptional control element. Among these mouse lines, only the transgenic mice car-

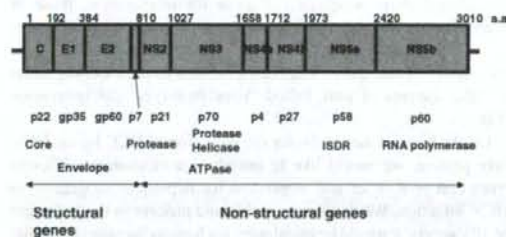


Figure 1 Structure of the hepatitis C virus (HCV) genome. The HCV genome consists of structural and non-structural regions. The structural region consists of the core, envelope and p7 genes. The non-structural region codes enzyme proteins of NS3 to NS5B. Among the three different transgenic mouse lines established, which carry the core, envelope and non-structural region, respectively, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. ISDR, interferon-sensitivity determining region.

rying the core gene develop HCC in two independent lineages.⁸ The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.^{8,9} The transgenic mice carrying the entire non-structural genes have developed no HCC.

The transgenic mice carry the core gene and express the core protein of an expected size, approximately 21 kDa, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.¹⁰ Thus, the core gene transgenic mouse model well reproduces this feature of chronic hepatitis C. Of note, significant inflammation is not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules have a pathology characterized by 'nodule in nodule', and HCC with a low degree of differentiation develops within adenoma as well as within HCC with a higher degree of differentiation.⁵ Although numerous lipid droplets are found in cells forming adenoma, as in non-tumorous cells, they are rarely observed in HCC cells. These histological features closely resemble those observed in HCC developing in chronic hepatitis C patients, in whom prominent lipid droplets are found in small, well-differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.⁵ Notably, the development of steatosis and HCC has been reproduced in other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.¹¹ These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

Sequence to the core protein expression in the liver

It is difficult to clarify the mechanism of carcinogenesis even for our simple model in which only the core protein is expressed in otherwise normal liver tissues. There is a notable feature of the localization of the core protein in hepatocytes: although the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.^{6,12} On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. We note that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.^{13,14} The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, and may, at least in part, contribute to hepatocarcinogenesis in HCV infection (Fig. 2). If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that cannot be scavenged by a physiological antagonistic system. This indicates that the inflammation in chronic HCV infection would be different to that produced in other

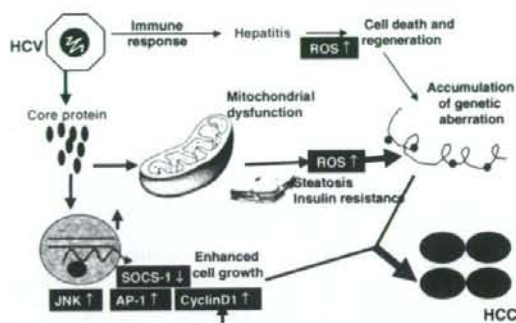


Figure 2 Molecular pathogenesis of liver disease in hepatitis C virus (HCV) infection. Induction of oxidative stress together with hepatic steatosis by the HCV core protein would play a pivotal role in the development of hepatocellular carcinoma (HCC). Alterations in cellular gene expressions, such as tumor necrosis factor- α (TNF- α) or suppressor of cytokine signaling-1 (SOCS-1), and those in the intracellular signaling pathways including c-Jun N-terminal kinase (JNK) would be coaccelerators to hepatocarcinogenesis in HCV infection. ROS, reactive oxygen species.

types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction.^{13,15} The function of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.¹⁶ Hepatic steatosis in hepatitis C may work as fuel for oxidative stress overproduction.^{14,17,18}

Other possible pathways would be the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Fig. 2). For an example, tumor necrosis factor (TNF)- α and interleukin-1 β have been found to be transcriptionally activated.¹⁹ The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)- α , which play pivotal roles in cell proliferation and metabolism.²⁰ The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream of the JNK activation, transcription factor activating factor (AP)-1 activation is markedly enhanced.^{17,21} Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased (Fig. 2). The suppression by HCV core protein of the suppressor of cytokine signaling (SOCS)-1, a tumor suppressor gene, may also contribute to hepatocarcinogenesis. Thus, the HCV core protein modulates the intracellular signaling pathways and gives an advantage to hepatocytes for cell proliferation.

Such an effect of the core protein on the MAPK pathway, combined with that on oxidative stress, may explain the extremely high incidence of HCC development in chronic hepatitis C (Fig. 2).

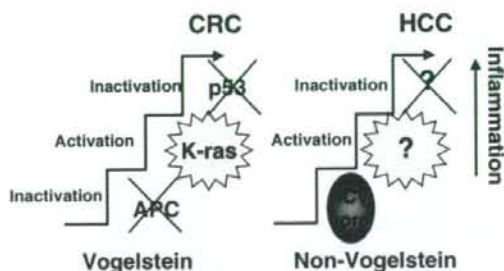


Figure 3 Mechanism of hepatitis C virus (HCV)-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be given for many unusual events occurring in HCV carriers. CRC, colorectal cancer; APC, adenomatous polyposis coli.

Hepatocarcinogenesis in HCV infection: A mechanism distinct from those in other cancers

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV may be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory by Kinzler and Vogelstein has gained popularity.²² They have proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the *adenomatous polyposis coli* gene for inactivation, those in *K-ras* for activation and those in the *p53* gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.²² Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis (Fig. 3).

On the basis of the results for the induction of HCC by the HCV core protein, we would like to introduce a mechanism different from that of Kinzler and Vogelstein for hepatocarcinogenesis in HCV infection. We do allow a multistage process in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (Fig. 3). The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.²³ Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may

also give an account of the non-metastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

References

- Saito I, Miyamura T, Ohbayashi A *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl Acad. Sci. USA* 1990; **87**: 6547-9.
- Ikeda K, Saitoh S, Suzuki Y *et al.* Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J. Hepatol.* 1998; **28**: 930-8.
- Kiyosawa K, Sodeyama T, Tanaka E *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671-5.
- Yotsuyanagi H, Shintani Y, Moriya K *et al.* Virological analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. *J. Infect. Dis.* 2000; **181**: 1920-8.
- Moriya K, Yotsuyanagi H, Shintani Y *et al.* Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.* 1997; **78**: 1527-31.
- Moriya K, Fujie H, Shintani Y *et al.* Hepatitis C virus core protein induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* 1998; **4**: 1065-8.
- Smith GS, Walford RL, Mickey MR. Lifespan and incidence of cancer and other diseases in selected long-lived inbred mice and their F1 hybrids. *J. Natl Cancer Inst.* 1973; **50**: 1195-213.
- Koike K, Moriya K, Ishibashi K *et al.* Expression of hepatitis C virus envelope proteins in transgenic mice. *J. Gen. Virol.* 1995; **76**: 3031-8.
- Koike K, Moriya K, Yotsuyanagi H *et al.* Sialadenitis resembling Sjogren's syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc. Natl Acad. Sci. USA* 1997; **94**: 233-6.
- Bach N, Thung SN, Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. *Hepatology* 1992; **15**: 572-7.
- Lerat H, Honda M, Beard MR *et al.* Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002; **122**: 352-65.
- Moriya K, Fujie H, Yotsuyanagi H *et al.* Subcellular localization of hepatitis C virus structural proteins expressed in transgenic liver. *Jpn J. Med. Sci. Biol.* 1997; **50**: 169-77.
- Moriya K, Nakagawa K, Santa T *et al.* Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocellular carcinogenesis. *Cancer Res.* 2001; **61**: 4365-70.
- Moriya K, Todoroki T, Tsutsumi T *et al.* Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. *Biochem. Biophys. Res. Commun.* 2001; **281**: 1207-12.
- Okuda M, Li K, Beard MR *et al.* Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; **122**: 366-75.
- Moriya K, Tajima A, Tsutsumi T *et al.* Hepatitis C virus core protein insults mitochondrial function through reducing the ETS complex I activity. In: *10th International Meeting on Hepatitis C and Related Viruses, Kyoto, 2003*. Osaka: Japan Linkage, 2003; 73.
- Shintani Y, Fujie H, Miyoshi H *et al.* Hepatitis C virus and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; **126**: 840-8.
- Koike K, Moriya K. Metabolic aspects of hepatitis C: steatohepatitis distinct from NASH. *J. Gastroenterol.* 2005; **40**: 329-36.
- Tsutsumi T, Suzuki T, Moriya K *et al.* Intrahepatic cytokine expression and AP-1 activation in mice transgenic for hepatitis C virus core protein. *Virology* 2002; **304**: 415-24.
- Tsutsumi T, Suzuki T, Shimoike T *et al.* Interaction of hepatitis C virus core protein with retinoid X receptor- α modulates its transcriptional activity. *Hepatology* 2002; **35**: 937-46.
- Tsutsumi T, Suzuki T, Moriya K *et al.* Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice. *Hepatology* 2003; **38**: 820-8.
- Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159-70.
- Koike K, Tsutsumi T, Fujie H *et al.* Role of hepatitis viruses in hepatocarcinogenesis. *Oncology* 2002; **62**: 29-37.