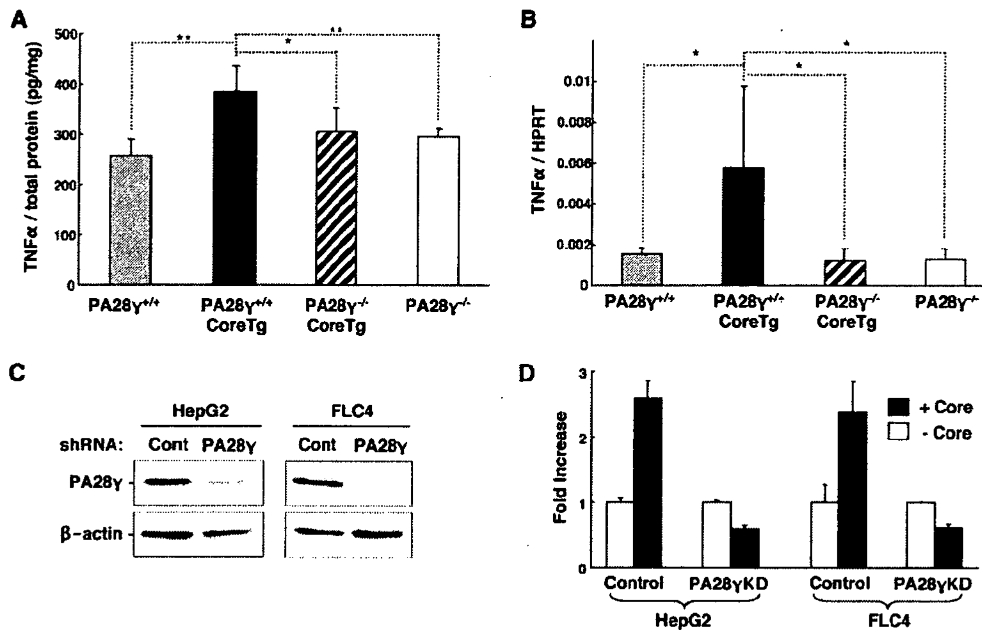

FIG. 7. PA28γ was required for activation of the TNF-α promoter by the HCV core protein. (A) Expression of TNF-α in the livers of mice was determined by ELISA ($n = 5$ in each group). (B) TNF-α mRNA in the livers of mice was examined by quantitative RT-PCR ($n = 5$ in each group). (C) Knockdown of the expression of PA28γ in the HepG2 and FLC-4 cell lines by the introduction of a plasmid encoding a short hairpin RNA (shRNA) targeted to the PA28γ gene. The expression levels of PA28γ 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 PA28γ-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 PA28γ impairs the insulin-signaling pathway through the suppression of both the tyrosine phosphorylation of IRS1 and the expression of IRS2.

PA28γ 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 PA28γ 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 PA28γ expression on the promoter activity of TNF-α in human liver cells, PA28γ-knockdown human hepatoma cell lines HepG2 and FLC4 were established by the introduction of a plasmid encoding a short hairpin RNA targeting the PA28γ gene in the cell lines. The expression of PA28γ 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 PA28γ-knockdown cell lines (Fig. 7D). These results suggest that PA28γ 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 lypolysis (21). Activated PI3-kinase phosphorylates phosphatidylinositol 4,5-biphosphate 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 PA28γ 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 PA28γ-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 PA28γ gene by RNA interference (Fig. 7D). These data suggest that PA28γ 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 PA28γ 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 PA28γ-dependent pathway (32). Recently, PA28γ 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 PA28γ-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/PA28γ-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 PA28γ-dependent pathway, thereby leading to insulin resistance. PA28γ may be a novel target for the treatment of HCV-induced diabetes.

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Critical role of PA28γ 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

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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 PA28γ/REGγ regulates host and viral proteins such as nuclear hormone receptors and HCV core protein. Here we show that a knockout of the PA28γ 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 PA28γ-dependent manner. Heterodimer composed of liver X receptor α (LXRα) and retinoid X receptor α (RXRα) is known to up-regulate *srebp-1c* promoter activity. Our data also show that HCV core protein enhances the binding of LXRα/RXRα to LXR-response element in the presence but not the absence of PA28γ. These findings suggest that PA28γ plays a crucial role in the development of liver pathology induced by HCV infection.

fatty acid | proteasome | sterol regulatory element-binding protein (SREBP) | RXRα | LXRα

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 ≈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 ≈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 NS5A 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 steroid 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 RXRα 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 PA28γ/REGγ in the nucleus and is degraded through a PA28γ-dependent pathway. PA28γ is well conserved from invertebrates to vertebrates, and amino acid sequences of human and murine PA28γs are identical (19). The homologous proteins, PA28α and PA28β, form a heteroheptamer in the cytoplasm, and they activate chymotrypsin-like peptidase activity of the 20S proteasome, whereas PA28γ forms a homoheptamer in the nucleus, and it enhances trypsin-like peptidase activity of 20S proteasome (20). Recently, Li and colleagues (21) reported that PA28γ 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 PA28γ are largely unknown *in vivo*. In this work, we examine whether PA28γ is required for liver pathology induced by HCV core protein *in vivo*.

Results

PA28γ-Knockout HCV Core Gene Transgenic Mice. To determine the role of PA28γ in HCV core-induced steatosis and the development of HCC *in vivo*, we prepared PA28γ-knockout core gene transgenic mice. The PA28γ-deficient, PA28$\gamma^{-/-}$ mice were born without

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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, steroid regulatory element-binding protein.

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

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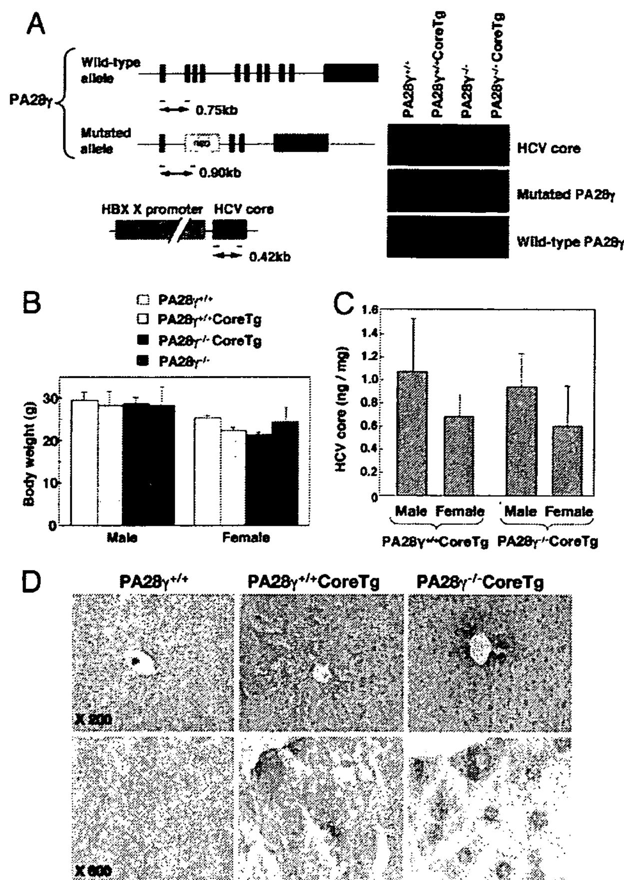

Fig. 1. Preparation and characterization of PA28γ-knockout HCV core-transgenic mice. (*A*) The structures of the wild-type and mutated PA28γ genes and the transgene encoding the HCV core protein under the control of the HBV X promoter were investigated. Positions corresponding to the screening primers and sizes of PCR products are shown. PCR products of the HCV core gene as well as wild-type and mutated PA28γ 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 ± 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.

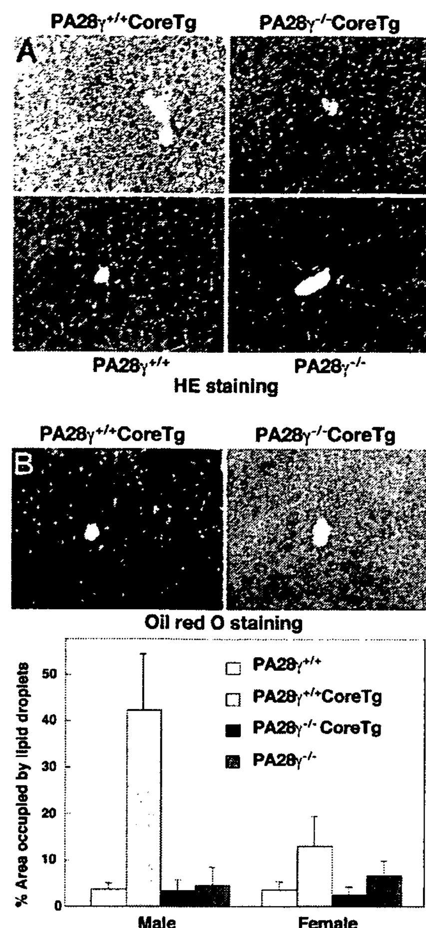

Fig. 2. Accumulation of lipid droplets by expression of HCV core protein. (*A*) Liver sections of the mice at the age of 6 months were stained with hematoxylin/eosin (HE). (*B*) (*Upper*) Liver sections of $PA28\gamma^{+/+}$CoreTg and $PA28\gamma^{-/-}$CoreTg mice at the age of 6 months were stained 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 ± SD, $n = 10$).

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. 1*A*). 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. 1*B*). A similar level of PA28γ expression was detected in $PA28\gamma^{+/+}$CoreTg and $PA28\gamma^{+/+}$ mice (see Fig. 5*B*). 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. 1*C*; see also Fig. 5*B*).

PA28γ 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 (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. 1*D*). These findings clearly indicate that at least some fraction of the HCV core protein is translocated into the nucleus and is degraded through a PA28γ-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. 2*A*), which were clearly stained with oil red O (Fig. 2*B 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. 2*B Lower*). These results suggest that PA28γ is required for the induction of liver steatosis by HCV core protein in mice.

PA28γ 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 PA28γ gene in $PA28\gamma^{+/+}$CoreTg mice on lipid

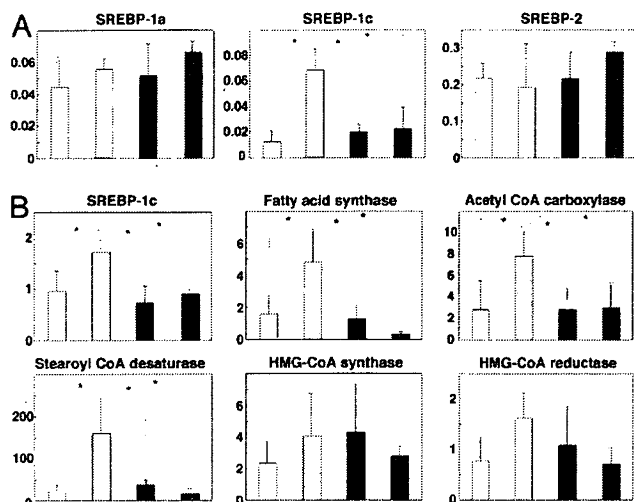

Fig. 3. Transcription of genes regulating lipid biosynthesis in the mouse liver. (*A*) Total RNA was prepared from the livers of 2-month-old mice; and the transcription of genes encoding SREBP-1a, SREBP-1c, and SREBP-2 was determined by real-time PCR. (*B*) The transcription of genes encoding SREBP-1c, fatty acid synthase, acetyl-CoA carboxylase, stearoyl-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. 3*A*). Although transcription of SREBP-1c and its regulating enzymes, such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-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. 3*B*). These results suggest the following: (*i*) the up-regulation of SREBP-1c transcription in the livers of mice requires both HCV core protein and PA28γ; and (*ii*) the nuclear accumulation of HCV core protein alone, which occurs because of the lack of degradation along a PA28γ-dependent proteasome pathway, does not activate the *srebp-1c* promoter.

HCV Core Protein Indirectly Potentiates *srebp-1c* Promoter Activity in an LXRα/RXRα-Dependent Manner. LXRα, which is primarily expressed in the liver, forms a complex with RXRα and synergistically potentiates *srebp-1c* promoter activity (16). Activation of RXRα 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 PA28γ on the activation of the *srebp-1c* promoter, we first examined the effect of HCV core protein on the binding of the LXRα/RXRα complex to the LXR-response element (LXRE) located upstream of the SREBP-1c gene (Fig. 4*A*). Although a weak shift of the labeled LXRE probe was observed by incubation with nuclear extracts prepared from 293T cells expressing FLAG-tagged LXRα and HA-tagged RXRα, a clear shift was obtained by the treatment of cells with 9-*cis*-retinoic acid and 22(*R*)-hydroxylcholesterol, ligands for LXRα and RXRα, respectively. In contrast, coexpression of HCV core protein with LXRα and RXRα 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, coincubation of the nuclear fraction with antibody to FLAG or HA tag but not with antibody to either HCV core or PA28γ caused a supershift of the labeled probe. These results indicate that HCV core protein does not participate in the LXRα/RXRα–LXRE complex but indirectly enhances the binding of LXRα/RXRα 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 LXRα/RXRα (Fig. 4*B*). Enhancement of the *srebp-1c* promoter by coexpression of HCV core protein and LXRα/RXRα was further potentiated by treatment with the ligands for LXRα and RXRα. The cells treated with 9-*cis*-retinoic acid exhibited more potent enhancement of the *srebp-1c* promoter than those treated with 22(*R*)-hydroxylcholesterol. 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-

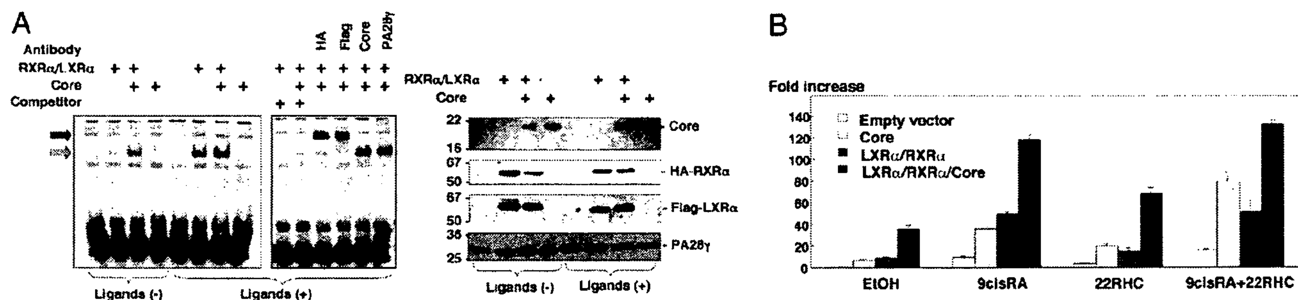

Fig. 4. Activation of the *srebp-1c* promoter by HCV core protein. (*A*) FLAG-LXRα and HA-RXRα were expressed in 293T cells together with or without HCV core protein. Ligands for LXRα and RXRα dissolved in ethanol [Ligands (+)] or ethanol alone [Ligands (−)] were added to the culture supernatant at 24 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 PA28γ, 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-RXRα, FLAG-LXRα, and PA28γ in cells was detected by immunoblotting. (*B*) Effects of ligands for RXRα, 9-*cis*-retinoic acid (9cisRA), and for LXRα, 22(*R*)-hydroxylcholesterol (22RHC), on the activation of the *srebp-1c* promoter in 293T cells expressing RXRα, LXRα, and/or HCV core protein. Ligands were added into the medium at 24 h posttransfection at a concentration of 5 μM, and the cells were harvested after 24 h of incubation.

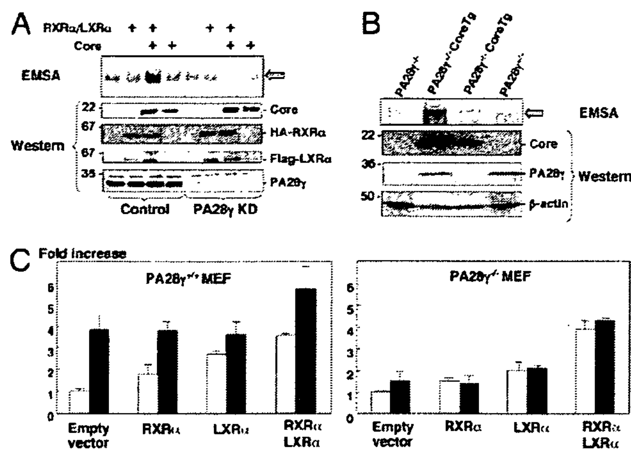

Fig. 5. PA28γ is required for HCV core-dependent activation of the *srebp-1c* promoter. (*A*) Effect of PA28γ knockdown on the LXRα/RXRα–DNA complex. FLAG-LXRα and HA-RXRα were expressed in FLC4 (control) or PA28γ-knockdown (PA28γ KD) cells together with or without HCV core protein. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA. (*Upper*) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase-streptavidin. The mobility shift of the LXRE probe is indicated by an arrow. (*Lower*) Expression of HCV core, HA-RXRα, FLAG-LXRα, and PA28γ in cells was detected by immunoblotting. (*B*) Effect of PA28γ knockout on the LXRα/RXRα–DNA complex in the mouse liver. (*Upper*) Nuclear extracts were prepared from the livers of 2-month-old PA28γ$^{-/-}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$CoreTg, and PA28γ$^{+/+}$ mice and subjected to EMSA. The mobility shift of the LXRE probe is indicated by an arrow. (*Lower*) The expression of HCV core, PA28γ, and β-actin in the livers of the mice was detected by immunoblotting. (*C*) Effect of HCV core protein on *srebp-1* promoter activity in PA28γ-knockout fibroblasts. A plasmid encoding firefly luciferase under the control of the *srebp-1c* promoter was transfected into MEFs prepared from PA28γ$^{+/+}$ (*Left*) or PA28γ$^{-/-}$ (*Right*) mice together with a plasmid encoding a *Renilla* luciferase. An empty plasmid or plasmids encoding mouse RXRα or LXRα 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 LXRα/RXRα-dependent manner.

HCV Core Protein Activates the *srepb-1c* Promoter in an LXRα/RXRα- and PA28γ-Dependent Manner. To examine whether PA28γ is required for HCV core-induced enhancement of *srebp-1c* promoter activity in human liver cells, a PA28γ-knockdown human hepatoma cell line (FLC4 KD) was prepared. Enhancement of binding of the LXRE probe to LXRα/RXRα by coexpression of HCV core protein and LXRα/RXRα in FLC4 cells was diminished by knockdown of the PA28γ gene (Fig. 5*A*). Furthermore, formation of the LXRα/RXRα–LXRE complex was enhanced in the livers of PA28γ$^{+/+}$CoreTg mice but not in those of PA28γ$^{-/-}$, PA28γ$^{+/+}$, or PA28γ$^{-/-}$CoreTg mice (Fig. 5*B*). The expression of the HCV core protein in the mouse embryonic fibroblasts (MEFs) of PA28γ$^{+/+}$ mice induced the activation of the mouse *srebp-1c* promoter through the endogenous expression of LXRα and RXRα (Fig. 5*C Left*). Further enhancement of the activation of the *srebp-1c* promoter by HCV core protein in PA28γ$^{+/+}$ MEFs was achieved by the exogenous expression of both LXRα and RXRα. However, no enhancing effect of HCV core protein on *srebp-1c* promoter activity was observed in PA28γ$^{-/-}$ MEFs (Fig. 5*C Right*). These results support the notion that HCV core protein enhances the activity of the *srepb-1c* promoter in an LXRα/RXRα- and PA28γ-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, %
PA28γ$^{+/+}$CoreTg			
Male	17	5	29.4
Female	28	3	10.7
PA28γ$^{+/-}$			
Male	16	0	0
Female	4	0	0
PA28γ$^{-/-}$			
Male	23	0	0
Female	13	0	0
PA28γ$^{-/-}$CoreTg			
Male	15	0	0
Female	21	0	0

PA28γ Plays a Crucial Role in the Development of HCC in PA28γ$^{+/+}$ CoreTg Mice. The incidence of hepatic tumors in male PA28γ$^{+/+}$ CoreTg mice older than 16 months was significantly higher than that in age-matched female PA28γ$^{+/+}$CoreTg mice (6). We reconfirmed here that the incidence of HCC in male and female PA28γ$^{+/+}$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 PA28γ$^{-/-}$CoreTg mice (males, 15; females, 21), although, as expected, no HCC was observed in PA28γ$^{+/-}$ (males, 16; females, 4) and PA28γ$^{-/-}$ mice (males, 23; females, 13) (Table 1). These results clearly indicate that PA28γ 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 PA28γ 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 PA28γ$^{-/-}$CoreTg mice (Fig. 1*D*). This result directly demonstrates that HCV core protein migrates into the nucleus and is degraded through a PA28γ-dependent pathway. However, HCV core protein accumulated in the nucleus because knockout of PA28γ gene abrogated the ability to cause liver pathology, suggesting that interaction of HCV core protein with PA28γ 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 PA28γ-dependent pathway, and Minami *et al.* (28) reported that PA28γ has a cochaperone activity with Hsp90. Therefore, degradation products of HCV core protein by means of PA28γ-dependent processing or correct folding of HCV core protein through cochaperone activity of PA28γ might be involved in the development of liver pathology. We do not know the reason why knockout of the PA28γ gene does not affect the total amount of HCV core protein in the liver of the transgenic mice. PA28γ-dependent degradation of HCV core protein may be independent of ubiquitination, as shown in SRC-3 (21), whereas knockdown of PA28γ in a human hepatoma cell line enhanced the ubiquitination of HCV core protein [supporting information (SI) Fig. 6], suggesting that lack of PA28γ 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 PA28γ gene.

Our results suggest that the interaction of HCV core protein with PA28γ leads to the activation of the *srebp-1c* promoter along an LXRα/RXRα-dependent pathway and the development of liver steatosis and HCC. HCV core protein was not included in the LXRα/RXRα-LXRE complex (Fig. 3*A*), 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 RARα-dependent transcription, and this interaction leads to the sequestering of Sp110b in the cytoplasm, resulting in the activation of RARα-dependent transcription (29). The sequestration of an unidentified corepressor of the LXRα/RXRα 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 PA28γ-proteasome activity in the nucleus is not known, PA28γ 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 PA28γ-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 PA28γ (SI Fig. 7), suggesting that PA28γ 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 PA28γ 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 replicase, 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 PA28γ of mice is identical to that of human, and mouse PA28γ is dispensable because PA28γ knockout mice exhibit no abnormal phenotype except for mild growth retardation. Therefore, PA28γ 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 PA28γ 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 upregulated by HCV core protein both *in vitro* and *in vivo* through a PA28γ-dependent pathway, suggesting that PA28γ 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*.

Plasmids and Reagents. Human PA28γ 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 RXRα and LXRα were amplified by PCR from the total cDNAs of the mouse liver. The RXRα and LXRα genes were introduced into pEF-FLAGGspGBK (36) and pcDNA3.1 (Invitrogen, Carlsbad, CA), respectively. The targeting fragment for human PA28γ knockdown (GGATCCGGTGGATCAGGAAGTGAAGTTCAAGAGACTTCACTTCCTGATCCACCTTTTTTGGAAAAGCTT) 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 PA28γ 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 PA28γ-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 crossbred with each other to create $PA28\gamma^{-/-}$CoreTg mice. $PA28\gamma^{-/-}$CoreTg mice were identified by PCR targeted at the PA28γ or HCV core gene (22, 25). Using 1 μg of genomic DNA obtained from the mouse tail, the PA28γ gene was amplified by PCR with the following primers: sense, PA28-3 (AGGTGGATCAGGAAGTGAAGCTCAA); and antisense, PA28γ-5cr (CACCTCACTTGTGATCCGCTCTCTGAAAGAATCAACC). The targeted sequence for the PA28γ-knockout mouse was detected by PCR using the PA28-3 primer and the PAKO-4 primer (TGCAGTTCATTCAGGGCACCGGACAG). The transgene encoding HCV core protein was detected by PCR as described in ref. 25. The expression of PA28γ 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 PA28γ. 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% CO_2 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-1cPro. The plasmids encoding RXRα and LXRα were transfected into MEFs together with pGL3-srebp-1cPro 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 RXRα, 9-*cis*-retinoic acid (Sigma), and that of LXRα, 22(*R*)-hydroxylcholesterol (Sigma) were added at a final concentration of 5 μM each to the culture medium of 293T cells transfected with pGL3-srebp-1cPro together with expression plasmids encoding RXRα, LXRα, 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 μg of protein) in a reaction buffer containing 10 mM Tris·HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.05 μg/μl 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× 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 ± SD. The significance of differences in the means was determined by Student's *t* test.

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Review

Drug resistance in antiviral treatment for infections with hepatitis B and C viruses

Hiroshi Yotsuyanagi and 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

Treatments for infections with hepatitis B and C viruses have recently developed markedly, and range from nonspecific interferon-based treatments to specific antiviral treatments, such as those that inhibit hepatitis virus-coded protein production or activity. These developments have contributed to the achievement of excellent enhancement of the antiviral effect. On the other hand, the development of specific antiviral therapies has created unprecedented problems. Antiviral drug-resistant strains of viruses have emerged, leading to a poor prognosis for infected patients. Clarification of the mechanisms underlying the emergence of such resistance to drugs will be useful for the treatment of such patients. In this review, we outline pathological conditions associated with hepatitis B and C viruses and their treatments, and discuss the current situation and mechanisms underlying the emergence of antiviral drug-resistant strains.

Key words: Interferon, lamivudine, entecavir, adefovir, ribavirin

Introduction

Infections with hepatitis B and C viruses (HBV and HCV, respectively) are a problem worldwide. Many patients are infected with HBV and HCV, and these infections are not only a major medical burden but also a socioeconomic burden because of their possible progression from chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC) if left untreated.[1,2] The treatments against these hepatitis viruses have been mainly nonspecific, based on the use of interferon (IFN). Specific antiviral drugs developed recently that inhibit hepatitis viral replication, for example, by inhibition of reverse transcriptase in HBV, have shown remarkable efficacy.[3,4] However, these treatments have also turned out to be a double-edged sword because they have led to the emergence of strains resistant to these drugs. The emergence of antiviral drug-resistant strains of these viruses may hinder the development of treatments against them.

HBV infection

HBV belongs to the family *Hepadnaviridae*. HBV is a DNA virus that has an approximately 3.2-kb circular incomplete double-stranded DNA genome. When it replicates, HBV forms DNA by using its own reverse transcriptase with RNA as a replicative intermediate. Worldwide, the distribution of HBV carriers and the incidence of HCC are closely correlated. The number of HBV carriers in Japan is estimated to be approximately one million, many of whom became infected via perinatal mother-to-child transmission. Hepatitis B e antigen (HBeAg) is a protein produced by the wild-type HBV strain as it replicates that appears in the blood. Generally, the presence of HBeAg indicates an abundance of the virus, and the detection of anti-HBe antibody often indicates a marked decrease in the HBV load.

HBV carriers are HBeAg-positive asymptomatic carriers (AsC) in the early stages of HBV infection. Their blood HBV level is very high in these stages. They can develop symptoms of hepatitis at any time of their life, but generally do between adolescence and their early 30s. At that time, the HBV load decreases markedly, and symptoms of hepatitis improve in 2 to 3 years in 80%–90% of HBV patients. Most of these patients then become negative for HBeAg and positive for the anti-HBe antibody. The annual incidence of seroconversion from being HBeAg-positive to anti-HBe antibody-

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Reprint requests to: H. Yotsuyanagi

positive has been reported to be approximately 5%.[5,6] However, symptoms of hepatitis persist in about 10%–20% of these patients even after seroconversion: such patients often develop cirrhosis and HCC.[7]

Treatment of HBV infection

There are several choices for treatment of chronic hepatitis B, but they all have limited efficacy. HBV elimination [i.e., serum HB surface antigen (HBsAg)-negative status] is difficult in HBV carriers. Seroconversion from HBeAg-positive to anti-HBe antibody-positive is generally the goal of HBV infection treatment. It should also be understood that many HBV AsCs do not need to or often must not undergo treatment with any anti-HBV agent.

In approximately 80% of patients with chronic hepatitis B, seroconversion occurs spontaneously. Consequently, chronic hepatitis B does not progress to cirrhosis in most chronic hepatitis B patients. The 15%–20% of HBV carriers who do not show seroconversion ultimately develop cirrhosis and/or HCC and should undergo antiviral treatment for chronic hepatitis B. IFN and reverse transcriptase inhibitors, including lamivudine, adefovir dipivoxil, and entecavir, have been approved for the treatment of chronic hepatitis B. Among these, lamivudine was the first used for chronic hepatitis B patients.

Lamivudine recipients are more likely than placebo recipients to show a histological response (52% vs. 23%, $P < 0.001$), the disappearance of HBeAg in serum (32% vs. 11%, $P = 0.003$), a sustained decrease in serum HBV DNA level to undetectable levels (44% vs. 16%, $P <$ 0.001), and a sustained normalization of the serum alanine aminotransferase (ALT) level (41% vs. 7 %, $P <$ 0.001) after 52 weeks of treatment.[8]

Entecavir has an antiviral effect comparable to that of lamivudine. In one study of HBeAg-positive patients,[9] histological improvement after 48 weeks occurred in 226 of 314 patients in an entecavir-treated group (72%) and in 195 of 314 patients in a lamivudine-treated group (62%, $P = 0.009$). More patients in the entecavir-treated group than in the lamivudine-treated group had undetectable serum HBV DNA levels according to a polymerase chain reaction (PCR) assay (67% vs. 36%, $P < 0.001$), and normalization of ALT levels (68% vs. 60%, $P = 0.02$). However, the seroconversion rate was not very high in either group.

Emergence of antiviral resistance in HBV infection

The treatment efficacy against HBV infection has improved, as mentioned above. The primary reason for chronic hepatitis B becoming resistant to treatment is the emergence of drug-resistant strains.

Mechanisms underlying development of resistance to IFN-based treatments

IFNs are administered in a wide range of doses according to various protocols for chronic hepatitis B. Such variation in protocols must be taken into account in determining the therapeutic efficacy of such drugs. Viral factors potentially involved in drug resistance include the HBV genotype and the presence of HBeAg, a precore gene mutation, or a core promoter mutation. HBeAg negativity, HBV genotypes C and D, precore gene 1896 mutation, and 1762/1764 mutations in the core promoter have been reported to be responsible for a poor response to IFN treatment. Strictly, these factors do not indicate "drug resistance" to IFN. The acquisition of the precore gene 1896 mutation also decreases the efficacy of IFN treatment.[10]

Mutation of the HBV polymerase domain

The HBV polymerase (*Pol*) gene is shown in Fig. 1. The Pol/RT region of this gene encodes HBV reverse transcriptase (RT). In HBV gene replication, reverse transcription from pregenomic RNA to viral gene DNA occurs, and reverse transcriptase is used in this process. The Pol/RT region is further divided into five domains from A to E. The Pol/RT active center is reported to be in domain C, domains B and E have been reported to be important for RNA template binding, and domains A and D for binding to nucleic acids.

Nucleic acid analog formulations are incorporated into the viral genome involved in reverse transcription instead of nucleic acids to inhibit viral replication competitively. However, if a Pol/RT gene mutation emerges, the nucleic acid analog formulation administered may be unable to inhibit viral replication because of interference with its binding to RT. Acquisition of this mutation of the Pol/RT gene thus results in the emergence of a drug-resistant viral strain. This mutation is shown in Fig. 1.

Resistance to lamivudine

Among nucleic acid analog formulations, lamivudine (LAM) has been used longest for the treatment of chronic hepatitis B , and its use continued until resistance to this drug emerged. Many factors underlying this resistance have been identified.

LAM resistance is caused by a mutation in which the 204th methionine (M) in the polymerase domain is replaced by valine (V) or isoleucine (I) (rtM204V/I). Because the site containing the 204th methionine is referred to as the YMDD motif, this mutation is also called the YVDD mutation or YIDD mutation. Another mutation associated with rtM204V/I is caused by replacement of the 180th leucine (L) by M (rtL180M).

rtM204V/I was detected in 10% of HBeAg-positive patients with chronic hepatitis B 24 weeks after the start

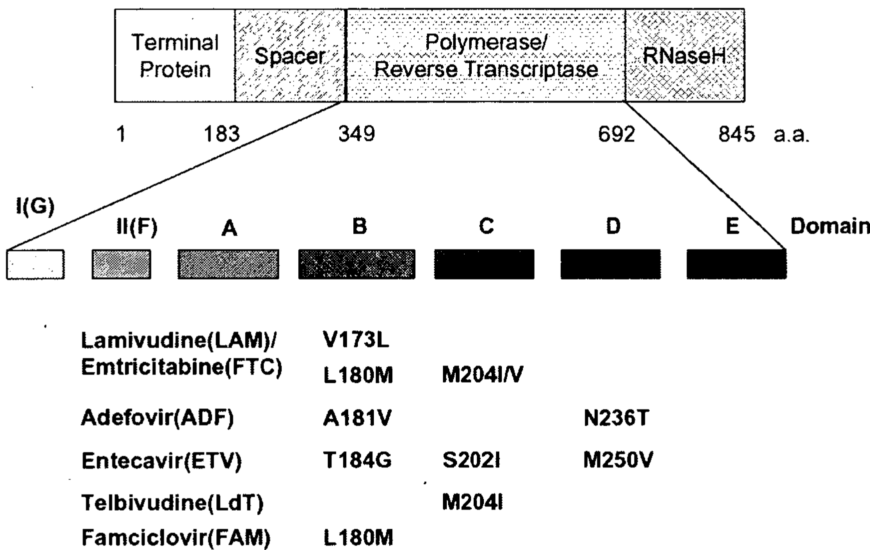

Fig. 1. Structure of the hepatitis B virus polymerase/reverse transcriptase gene and amino acid substitutions associated with resistance to nucleoside analogs. *a.a.*, amino acids

of LAM administration, in 24% after 52 weeks, and in 65% after 5 years.[4] In Japan, in an extensive study on the clinical course of both HBeAg-positive and HBeAg-negative chronic hepatitis B patients, the rate of emergence of LAM-resistant strains was 50%, 5 years after the start of LAM administration.[11] Not all patients having rtM204V/I develop hepatitis. Although the incidence of amino acid mutation of polymerase is high in patients with hepatitis, no specific mutation has been observed.[12]

The case of a patient who was LAM-resistant despite the absence of mutation in the YMDD motif was reported recently. The 181st alanine (A) in the Pol domain was replaced by threonine (T) (rtA181T) in this patient. An experiment using chimeric mice bearing human hepatocytes demonstrated that this mutation causes LAM resistance.[13]

Resistance to adefovir dipivoxil

The use of adefovir dipivoxil (ADV) in combination with LAM has been approved in Japan for patients showing LAM resistance. The emergence of ADV-resistant viral strains was first studied with regard to the administration of ADV alone. ADV resistance occurs in approximately 6% of patients 3 years after the start of ADV administration.[14] The replacement of the 236th asparagine (N) in the Pol domain by T (rtN236T) and that of the 181st A in the Pol domain by V (rtA181V) were observed in ADV-resistant strains.

Following the administration of ADV to patients exhibiting LAM resistance, strains with rtA181V/T or rtN236T emerged in 18% of the patients 1 year after the start of ADV administration.[15] rtA181T was also observed in LAM-resistant strains in the absence of mutation in the YMDD motif. It was reported recently that some patients exhibiting LAM resistance are nonresponders to ADV from the start of ADV administration. These resistant strains had the rtI233V mutation.[16]

Resistance to entecavir

Administration of entecavir (ETV) for more than 52 weeks to patients with LAM-resistant chronic hepatitis B leads to the emergence of resistant viral strains in 1.4% of patients,[17] although no results have been reported for long-term administration of ETV. The amino acid sequence of the RT domain in resistant viral strains was analyzed in two patients, and strains with rtM250V and rtI169T were found in one patient and strains with rtT184G and rtS202I in the other.[21] In Japan, strains with rtS202G and rtL269I have been detected.[22] It is notable that all of the patients who acquired ETV resistance were resistant to LAM. Thus, initial treatment with ETV alone may be less likely to lead to ETV-resistant viral strain emergence, but this hypothesis should be confirmed in future studies (Table 1).

HCV infection

HCV is a positive-strand RNA virus belonging to the family *Flaviviridae*. Approximately 170 million HCV carriers or patients are estimated to be persistently infected with HCV worldwide, and approximately 1.8 million in Japan. HCV is transmitted to humans by direct contact with infected or contaminated blood. The routes of infection include transfusion of contaminated or HCV-tainted blood or blood products, which have now been eliminated in Japan, sharing needles among drug abusers, acupuncture, and tattoos. Acupuncture and

Table 1. Clinical trial of entecavir for the treatment of chronic hepatitis B

No.	Authors	HBeAg	Lam resistance at baseline	ETV resistance at baseline	ETV resistance at end point (1 year)	Biochemical rebound (1 year)	Reference No.
1	Chang et al.	Positive	No		0/339	6/339 (2%)	9
2	Sherman et al.	Positive	Yes	11/186 (6%)	7/134 (5%)		17
3	Colonno et al.	Positive	No		0/354	6/354 (2%)	18
		Negative			1/325 (0.3%)	8/325 (2%)	
4	Lai et al.	Negative	No		0/211	5/211 (2%)	19
5	Chang et al.	Positive	Yes	6/181 (3%)	2/181 (1%)		20
		Negative					

HBeAg, hepatitis B e antigen; LAM, lamivudine; ETV, entecavir

depilation are invasive treatments and should be considered to involve risk for infection with HCV unless disposable medical instruments are used.[23]

HCV infection develops into persistent infection at a very high rate, becoming persistent in 70%–80% of patients with acute HCV infection. Generally, patients develop acute hepatitis 2 to 3 months after their initial infection with HCV. However, many patients are unaware of the onset of hepatitis because of the mild subjective symptoms and mild jaundice, if any. Although 20%–30% of patients developing acute hepatitis recover from the disease spontaneously, acute hepatitis develops into chronic hepatitis (by definition, hepatitis persisting for more than 6 months) in the remaining 70%–80% of patients. Then, chronic hepatitis enters an inactive phase that lasts 10–15 years. Serum ALT level, which indicates the destruction of hepatocytes, is within normal limits during the inactive phase, but viral growth continues.

Chronic hepatitis enters an active phase after 10–15 years in many patients, although there are marked individual differences. Serum ALT level increases to about two to three times the normal level when chronic hepatitis enters the active phase. Once chronic hepatitis C enters the active phase, it will not improve spontaneously. If the disease is left untreated, the risk of progressing from chronic hepatitis to cirrhosis increases. Hepatitis C characteristically progresses gradually but steadily.[24] The risk of developing HCC is high among patients with cirrhosis. The risk of HCC development in cirrhosis patients is 5%–7%.[25] Patients infected with HCV should be diagnosed during the inactive phase of chronic hepatitis C and start treatment for HCV elimination (antiviral treatment) as soon as the hepatitis enters the active phase.

Treatment of HCV infection

HCV infection is treated mainly with IFN-based drugs. The treatment efficacy is evaluated 6 months after the end of IFN-based drug administration. If HCV-RNA is not detected by the sensitive RT-PCR test, the patient is considered to show a sustained virological response (SVR), indicating that HCV has been virtually eliminated.

At present, polyethylene glycol-interferon (Peg-IFN) treatment in combination with ribavirin plays a key role in the treatment of HCV infection. Peg-IFN, an IFN molecule covalently bonded to Peg, is a sustained-release formulation. It needs to be injected only once weekly from the start of treatment, whereas conventional IFN preparations require administration three times weekly. Administration of Peg-IFN alone is more effective than that of a conventional IFN-based drug alone, but the administration of Peg-IFN in combination with ribavirin is even more effective.[26-28] An SVR rate of approximately 50% can be expected even in cases of chronic hepatitis infected with a high viral load of HCV of genotype 1, and an SVR rate of approximately 60% can be generally expected. Peg-IFN is usually administered for 48 consecutive weeks. It is important to continue the treatment for 48 weeks, although the dose may be reduced if adverse drug reactions appear. In addition, extending the administration period to a total of 72 weeks recently proved effective in patients who became HCV-negative after 12 weeks of treatment.[29]

There is a long history of treatment with IFN alone: treatment of non-A, non-B hepatitis with IFN alone dates back to around 1985, before the discovery of HCV. A nationwide survey conducted by the Study Group of the Ministry of Health, Labour and Welfare of Japan in 1995 showed that the SVR rate for treatment with IFN alone for 6 months (administration of 6 to 10 million units) was approximately 30% in all patients. However, in patients with the genotype 1 HCV, which is the major genotype worldwide and in about 70% of Japanese HCV patients, particularly those with high viral loads (determined as an HCV-RNA load of 100 KIU/ml or more), SVR was obtained in only about 2%–7%. The efficacy of treatment with IFN alone is thus low. Hence, Peg-IFN in combination with ribavirin

Table 2. Relationship between the ISDR and the response to interferon treatment for chronic hepatitis patients with genotype 1 hepatitis C virus infection

No.	Authors	Interferon	Ribavirin	Relationship between ISDR and viral load	Relationship between ISDR and response	Ethnicity	Ref. no.
1	Enomoto et al.	α	No	Yes	Yes	Japanese	30
2	Kurosaki et al.	β	No	Yes	Yes	Japanese	31
3	Chayama et al.	α	No	ND	Yes	Japanese	32
4	Zeuzem et al.	α	No	No	No	German	33
5	Squadrito et al.	α	No	No	No	French	34
6	Hofgartner et al.	α	No	ND	No	American	35
7	Khorsi et al.	α	No	ND	No	French	36
8	Saiz et al.	α	No	ND	Yes	Spanish	37
9	Frangeul et al.	α	No	ND	No	French	38
10	Odeberg et al.	α	No	ND	No	Sweden	39
11	Chung et al.	α	No	ND	No	American	40
12	Ibarrola et al.	α	Yes	ND	No	Spanish	41
13	Sarrazin et al.	α	Yes	ND	Yes	German	42
14	McKechnie et al.	α	No	ND	No	English	43
15	Yoshioka et al.	α	No	ND	Yes	Japanese	44
16	Stratidaki et al.	α	No	ND	No	American	45
17	Murphy et al.	α	Yes	ND	No	American	46
18	Cappiello et al.	PEGα	Yes	ND	No	Italian	47
19	Aslan et al.	α	No	ND	No	Turkish	48
20	Murayama et al.	α	Yes	ND	Yes	Japanese	49

ISDR, interferon sensitivity determining region; ND, not described; PEG, pegylated

is the first choice for patients with intractable disease, as mentioned above.

Emergence of antiviral resistance in HCV infection

In the treatment of chronic hepatitis C with IFN alone or IFN (or Peg-IFN) in combination with ribavirin, HCV-RNA does not disappear in some patients, particularly in those with genotype 1 HCV. Approximately 10% of genotype 1 HCV patients with high viral loads never become HCA-RNA-negative during the period of treatment with IFN (or Peg-IFN) in combination with ribavirin.

Not only host factors but also viral factors have been identified as causes for the nonelimination of HCV. The HCV genotype is a typical viral factor, and patients infected with genotype 1 or 4 are more resistant to treatment than those with genotype 2 or 3.

Another reported factor is the interferon sensitivity-determining region (ISDR) in NS5A, a region consisting of 40 amino acids, first reported by Enomoto et al.[30] ISDR is contained in the binding site of interferon α-inducible RNA-dependent protein kinase (PKR). Mutation in ISDR may cause dysfunction in the binding between the NS5A protein and PKR, leading to a decrease in viral protein translation. In Japan, a close correlation between IFN treatment efficacy and mutation in ISDR in genotype 1b HCV patients was found.[31,32] In Europe and the United States, however, the correlation between amino acid mutation in ISDR and IFN treatment efficacy is not clear even in patients infected with HCV genotype 1 (Table 2).

In addition, mutation of the PKR/eIF2α phosphorylation homology domain of the E2 domain has been reported to correlate with IFN-based drug efficacy, but this needs further clarification.

Ribavirin-resistant viral strain

Ribavirin shows low anti-HCV activity in some patients even when it is administered alone, and chronic hepatitis C has been treated with ribavirin alone. The structure of a ribavirin-resistant viral strain that has emerged has been studied. Mutation of the 415th amino acid (F415Y) in the RNA-dependent RNA polymerase (RdRp) domain of NS5B was detected in strains infecting patients treated with ribavirin alone who became ribavirin-resistant.[50] This mutation was considered to be related to IFN treatment efficacy in patients with genotype 1a HCV. In a study using a replicon, mutations of the 404th and 442nd amino acids (G404S and E442G) were detected.[51]

Conclusions

An overview of the mechanisms underlying the emergence of drug-resistant HBV and HCV strains has been given above. The emergence of drug-resistant strains of HBV in particular has posed problems. This resistance has resulted from the development of a wide range of

drugs for HBV, ranging from nonspecific IFN-based drugs to viral protein-specific RT inhibitors. Although no serious problem has arisen to date as regards HCV, specific anti-HCV drugs such as protease inhibitors and RNA polymerase inhibitors are beginning to be developed, so the emergence of drug-resistant viral strains is expected to be a major problem. Indeed, the emergence of a strain resistant to VX950, an HCV protease inhibitor with high antiviral activity, following a short period of administration of this drug has already been reported.[52] HBV and HCV do not seem to be very easy to eliminate.

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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CASE REPORT

Fatal liver failure caused by reactivation of lamivudine-resistant hepatitis B virus: A case report

Yuka Suzuki, Hiroshi Yotsuyanagi, Chiaki Okuse, Yoshihiko Nagase, Hideaki Takahashi, Kyoji Moriya, Michihiro Suzuki, Kazuhiko Koike, Shiro Iino, Fumio Itoh

Yuka Suzuki, Chiaki Okuse, Yoshihiko Nagase, Hideaki Takahashi, Michihiro Suzuki, Fumio Itoh, Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8511, Japan
Hiroshi Yotsuyanagi, Kyoji Moriya, Kazuhiko Koike, Division of Infectious Diseases, Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
Shiro Iino, Center for Liver Diseases, Seizankai Kiyokawa Hospital, 2-31-12 Asagayaminami, Suginami, Tokyo 166-0004, Japan
Correspondence to: Hiroshi Yotsuyanagi, MD, PhD, Division of Infectious Diseases, Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. hyotsu-tky@umin.ac.jp
Telephone: +81-3-5800-8720 Fax: +81-3-5800-8796
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Abstract

We present a case of fetal liver failure caused by the activation of lamivudine-resistant hepatitis B virus (HBV) nine months after lamivudine treatment. A 57-year old man visited our hospital for the treatment of decompensated chronic hepatitis B. Lamivudine was started in December 2001. Subsequently, serum HBV was negative for HBV DNA with seroconversion from HBeAg to anti-HBe and improvement of liver function. However, HBV DNA and HBeAg were again detected in September 2002. He was complicated by breakthrough hepatitis and admitted to our hospital in November for severely impaired liver function. Vidarabine treatment was started and serum HBV DNA and alanine aminotransferase (ALT) decreased transiently. However, after the start of α-interferon treatment, HBV DNA level increased and liver function deteriorated. He died 1 mo after admission. An analysis of amino acid sequences in the polymerase region revealed that rtM204I/V with rtL80I/V occurred at the time of viral breakthrough. After the start of antiviral treatment, rtL180M was detected in addition to rtM204I/V and rtL80I/V, and became predominant in the terminal stage of the disease. HBV clone with a high replication capacity may be produced by antiviral treatment leading to the worsening of liver function. Antiviral therapy for patients with breakthrough hepatitis in advanced liver disease should be carefully performed.

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Key words: Hepatitis B virus; Lamivudine; Polymerase; Interferon; Tyrosine-methionine-aspartate-aspartate

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INTRODUCTION

Lamivudine is a nucleoside analogue that interrupts the reverse transcription of hepatitis B viral (HBV) pregenomic RNA. Lamivudine is effective for controlling chronic hepatitis B and currently recommended as the first line of treatment for chronic active hepatitis B[1,2]. Even for patients with decompensated liver cirrhosis, lamivudine improves liver function and extends transplantation free intervals[3-10]. Since more than 10% of patients with chronic HBV infection are estimated to develop liver cirrhosis and may eventually suffer from decompensated liver cirrhosis or hepatocellular carcinoma, the role of lamivudine in the treatment of advanced liver disease caused by chronic HBV infection is large[11-14].

The major problems concerning lamivudine treatment are the viral and biochemical breakthroughs caused by drug resistance. Amino acid mutation in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif can occur six months after treatment and often increases alanine aminotransferase (ALT) level. Although the increase is usually mild, a marked increase in ALT level leading to fatal hepatic failure has been reported[15-17]. Factors other than the YMDD motif mutation that are associated with the worsening of liver function remain to be clarified.

Here, we report a case of fatal hepatic failure caused by lamivudine-resistant HBV. A serial analysis of viral amino acid sequences indicated that the acquisition of mutations outside the YMDD motif might be related to the deteriolation of the patient's condition.

CASE REPORT

A 57-year old man visited our hospital in September 2001 for the treatment of decompensated chronic hepatitis

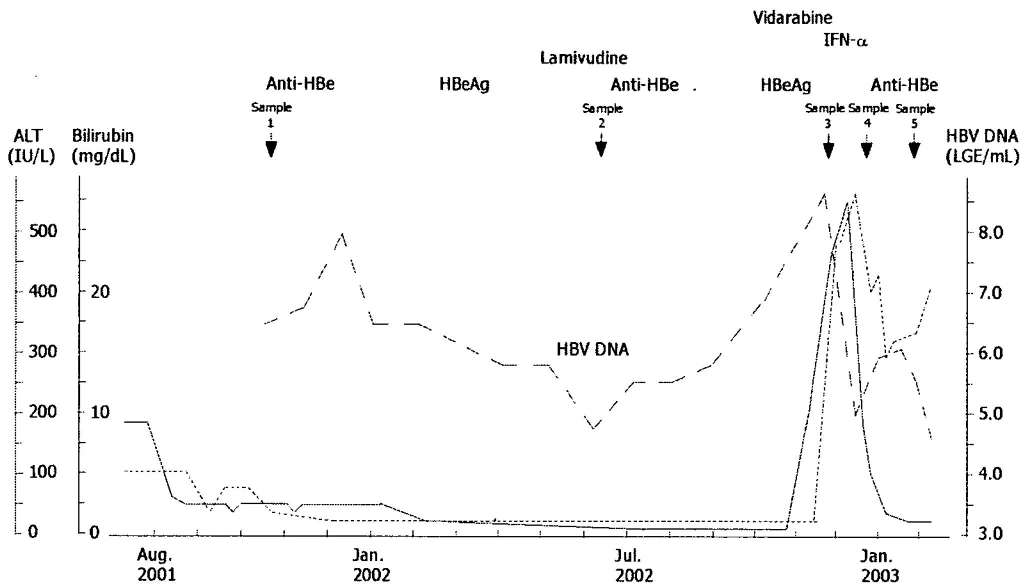

Figure 1 Clinical course of our patient. HBV DNA level was quantified by transcription-mediated amplification assay. The levels of HBV DNA started to increase 8 mo after treatment with reappearance of HBeAg. Breakthrough hepatitis developed 12 mo after treatment. The timing of serum sample analysis for mutations is shown by the arrowhead.

B. In 1978, He was found to be positive for serum HBs antigen (HBsAg). In July 2001, he was admitted to a nearby hospital for ascites where he was diagnosed as having decompensated cirrhosis with exacerbated chronic hepatitis B. The symptomatic control of his ascites improved his general condition. For further treatment, he was referred to our hospital.

On his first visit, he showed no symptoms or signs of worsening hepatic failure or encephalopathy. No ascites or leg edema was observed. His bulbar conjunctiva was slightly jaundiced. Dilated vasculature was observed in his neck and chest. His ALT, total bilirubin and albumin were 50 IU/L, 3.1 mg/dL and 3.7 g/dL, and his prothrombin time was 76%. He was diagnosed as having liver cirrhosis with a Child-Pugh score of 8. He was negative for HBe antigen (HBeAg) and his HBV DNA level measured by transcription-mediated amplification and hybridization protection assay[18] was 106.5 genome copies/mL.

In November 2001, he was found to be positive for HBeAg and showed an increase in HBV DNA level. Because he had a history of decompensated chronic hepatitis B, lamivudine treatment (100 mg/d) was started in December. Figure 1 shows the clinical course. The high serum levels of bilirubin and ALT decreased and normalized within 6 mo after lamivudine treatment was started. The patient became negative for HBV DNA and HBeAg.

However, in September 2002, he was found to be positive for HBeAg again and showed an increase in HBV DNA level. In November 2002, he observed jaundice of his bulbar conjunctiva and was admitted to our hospital. Although he was alert, his bulbar conjunctiva and skin were jaundiced. His ALT, total bilirubin, were 474 IU/L, 11.4 mg/dL and 4.3 g/dL. His HBV DNA level was 108.6 genome copies/mL. He was diagnosed as having breakthrough hepatitis caused by lamivudine-resistant mutants of HBV. HBV with an amino acid substitution in the YMDD motif in the domain C of polymerase region was detected.

Because interferon is not indicated in patients with decompensated cirrhosis, vidarabine, which is effective for the control of active HBV infection[19-21], was administered together with lamivudine under informed consent. Liver function improved transiently with a decrease in HBV DNA within 2 wk. As prolonged vidarabine administration may induce several complications[22], vidarabine was switched to interferon-α. After the start of interferon-α treatment, HBV DNA level increased and liver function worsened. He died of hepatic failure and rupture of esophageal varices 1 mo after his admission.

The histolopathology of the patient's liver after necropsy showed cirrhosis with zonal necrosis. Hepatocyte regeneration was scarce (Figure 2).

To elucidate the viral factors affecting early viral breakthrough and fatal outcome, amino acid sequences of the upstream polymerase region (aa 1-250) of HBV DNA in serum were examined at 5 points as shown in Figure 1. The methods were as follows.

First, DNA was extracted from 100 μL of a serum sample using the QIAamp DNA blood mini kit (Qiagen Inc., Valencia, CA). Three fragments spanning the upper polymerase region of HBV DNA were amplified by nested PCR with the primers shown in Table 1. The first stage of amplification was carried out using a thermal cycler for 40 cycles (94℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min) in 100 μL of reaction mixture containing 200 mmol/L dNTPs, 1.0 mmol/L each of the primers and 1 × PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$ and 0.001% (w/v) gelatin] and 2 units of Ampli-Taq polymerase gold (Perkin Elmer Cetus Corp., CT). Two microliters of the PCR products was subjected to the second stage of amplification under the same conditions as the first stage.

Second, PCR products were purified using Wizard PCR preps DNA purification resin (Promega, WI) and cloned into a plasmid vector using the TA cloning kit (PCR cloning kit Qiagen, CA). Four clones were selected from each plate, from which recombinant plasmid DNA was

Table 1 Primers used for amplification and sequencing of polymerase region of HBV

Region 1		
Outer sense	nt 2222-2241	CTTACTTTTGGAAGAGAAAC
Outer antisense	nt 2490-2509	GGACAGTAGAAGAATAAAG
Inner sense	nt 2222-2241	CTTACTTTTGGAAGAGAAAC
Inner antisense	nt 2478-2497	GAATAAAGCCCAGTAAAGTT
Region 2		
Outer sense	nt 2413-2434	GCGTCGCAGAAGATCTCAATC
Outer antisense	nt 2816-2835	GTTCCCAAGAATATGGTGAC
Inner sense	nt 2434-2452	CTCGGGAATCTCAATGTTAG
Inner antisense	nt 2816-2835	GTTCCCAAGAATATGGTGAC
Region 3		
Outer sense	nt 2490-2509	CTTTATTCTTCTACTGTACC
Outer antisense	nt 3121-3143	CGATTGGTGGAGGCAGGAGGAGG
Inner sense	nt 2637-2656	ATGCCTGCTAGGTTTTATCC
Inner antisense	nt 3121-3143	CGATTGGTGGAGGCAGGAGGAGG

purified using a commercially available kit (Plasmid midi kit, Qiagen, Valencia, CA). Nucleotide sequences were determined bidirectionally using the dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, CA) and the PCR primers. Sequencing was performed using an automated DNA sequencer (ABI 377: PE Applied Biosystems).

The determined amino acid sequences in the polymerase region are shown in Figure 3. No amino acid sequence changes were found at the start of lamivudine treatment. At the time of viral breakthrough, rtM204I with rtL80I became dominant. After the start of interferon treatment, rtM204I was replaced by rtM204V and rtL80I by rtL80V. At the final stage of the disease, mutation rtL180M appeared besides rtM204V and rtL80V.

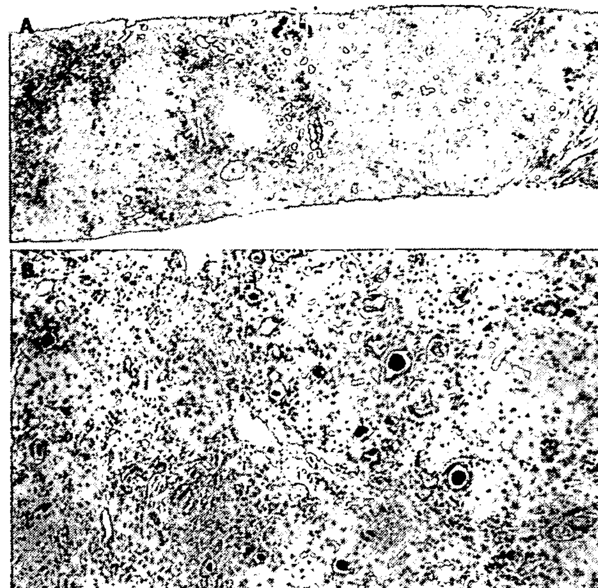

Figure 2 Histopathological findings of liver specimens. Irregularly-shaped parenchymal cells with massive necrosis (**A**) and scarce hepatocyte regeneration (**B**) surrounded by extensive fibrosis (**A**: HE × 20; **B**: HE × 80).

DISCUSSION

Lamivudine monotherapy is effective in suppressing HBV replication and ameliorating liver disease in chronic hepatitis B patients regardless of HBeAg positivity. A one-year study of HBeAg-positive chronic hepatitis B patients showed that 16% of these patients become seroconverted to anti-HBe and 72% of these patients showed normalization of their ALT levels[23]. Furthermore, treatment with lamivudine is associated with histologic improvement not only in terms of necroinflammatory score but also in terms of fibrosis score after long-term treatment[24].

One advantage of lamivudine is that it can be used safely in patients with decompensated cirrhosis[3-10]. In contrast to IFN-α, lamivudine is well tolerated without any significant side effects even in patients with decompensated cirrhosis. Furthermore, lamivudine can improve liver function and survival prognosis.

However, the emergence of a drug-resistant mutant is a big problem in lamivudine treatment. A large-scale Asian study showed that lamivudine resistant HBV infection occurred in 23 % of patients in year one and 65 % of patients in year five. Hepatitis flares, which occurred more commonly in patients with lamivudine resistant mutations, occurred in 10% of patients in year one, and in 18% to 21% of patients in years two to five. Among patients with lamivudine resistant HBV infection, occurrence of hepatic decompensation increased significantly in patients with lamivudine resistant HBV infection for more than 4 years (from 0% to 6%)[25]. In this large-scale Asian study, liver-disease-related death occurred in two patients.

The prognosis of patients with lamivudine-resistant HBV infection, particularly those with advanced liver disease, may be determined by the timing and severity of breakthrough hepatitis. However, the viral factors that may influence the severity of this hepatitis remain to be clarified. A recent study indicated that patients with a normal ALT level even after the emergence of a YMDD motif mutant are characterized by HBeAg negativity during pretreatment, HBeAg loss during therapy, a longer duration from the commencement of therapy until the emergence of YMDD mutant, and lack of mixed-type YMDD mutants[26]. In contrast, patients with severely exacerbated hepatitis after the emergence of a YMDD mutant tend to have more substitutions in the reverse transcriptase (rt) region within the polymerase gene at the time of hepatitis exacerbation than those without hepatitis exacerbation[25].

Our patient acquired amino acid mutations in the polymerase region one after the other. Amino acid changes in rtM204/I appeared at the time of viral breakthrough. After the initial treatment with vidarabine, rtM204/V substituted for rtM204/I in one of the four clones. During the interferon treatment, rtM204/V became predominant.

Another mutation observed in our patient was rtL80I/V. Ogata *et al*[27] showed that rtL180M is accompanied with rtM204I in some patients with resistance to lamivudine. Because the mutation at aa position 80 was found at the same time as that at aa position 204 in our patient, it is not clear whether the mutation at aa position 80 affects the clinical course.

At the final stage of the disease with deterioration of