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. Chinea, J. Gavilondo, M. C. de la Rosa, I. Menendez, S. Duenas-Carrera, A. Vina, W. Garcia, B. Gra, M. Noa, E. Reytor, M. T. Barcelo, 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 TNFalpha 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. **Houstis, 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. Haseroth, G. Marinos, 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. Investig. **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. Udalova, 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 PA28γ-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 PA28gamma. 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-alpha 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. Investig. **106**:171–176.
49. **Su, A. I., J. P. Pezacki, L. 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. Tobe, 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. Investig. **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-alpha 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 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

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 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, PA28γ$^{-/-}$ 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, steroid 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/0607312104/DC1.

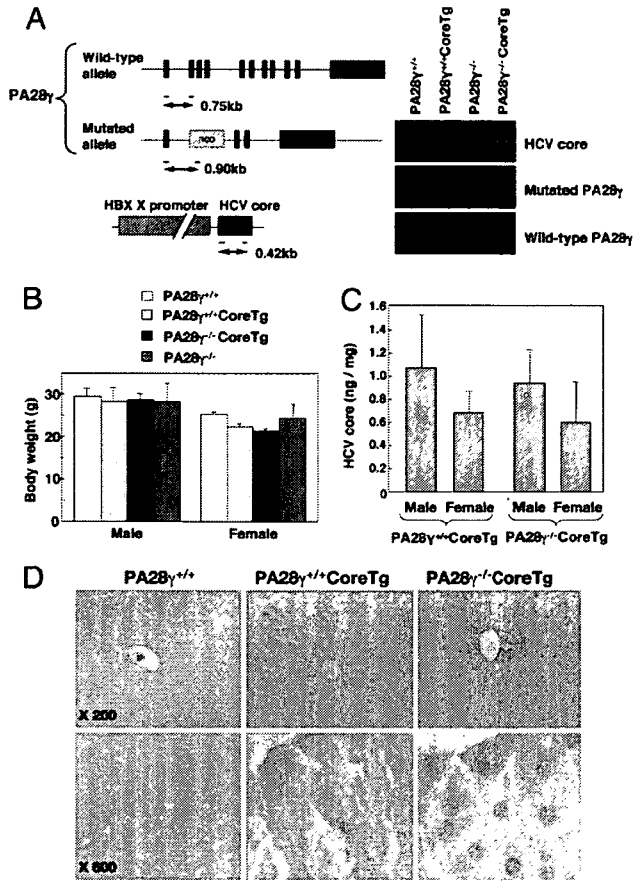

Fig. 1. Preparation and characterization of PA28γ-knockout HCV core-transgenic mice. (*A*) The structures of the wild-type and mutated PA28γ genes and the transgene encoding the HCV core protein under the control of the HBV X promoter were investigated. Positions corresponding to the screening primers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

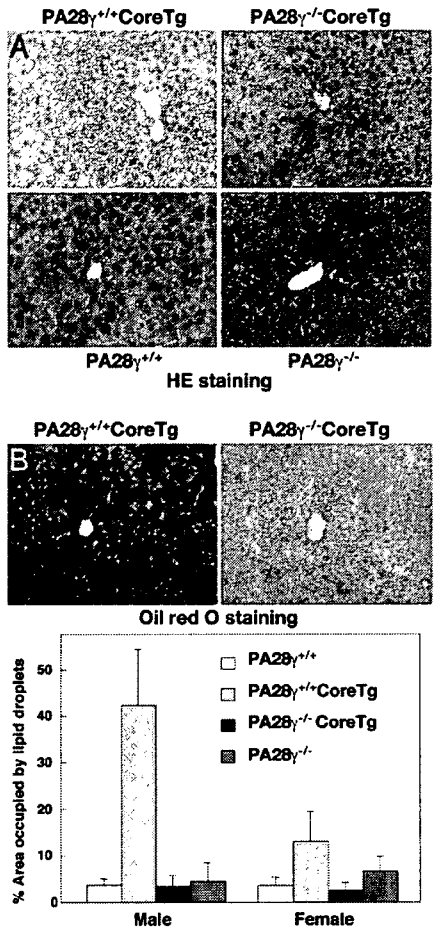

Fig. 2. Accumulation of lipid droplets by expression of HCV core protein. (*A*) Liver sections of the mice at the age of 6 months were stained with hematoxylin/eosin (HE). (*B*) (*Upper*) Liver sections of PA28$\gamma^{+/+}$CoreTg and PA28$\gamma^{-/-}$CoreTg mice at the age of 6 months were stained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

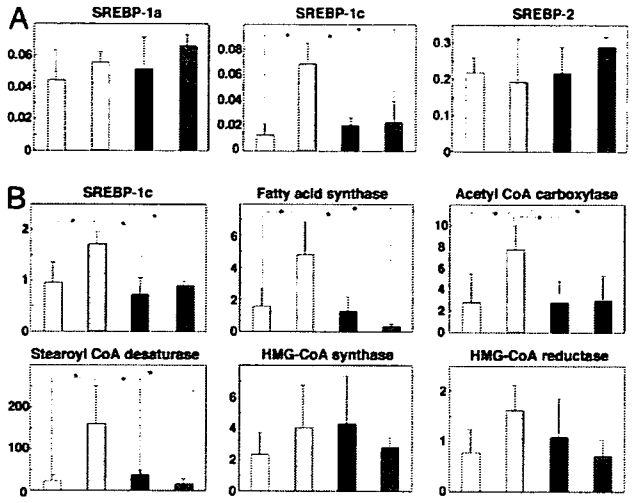

Fig. 3. Transcription of genes regulating lipid biosynthesis in the mouse liver. (*A*) Total RNA was prepared from the livers of 2-month-old mice; and the transcription of genes encoding SREBP-1a, SREBP-1c, and SREBP-2 was determined by real-time PCR. (*B*) The transcription of genes encoding SREBP-1c, 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-

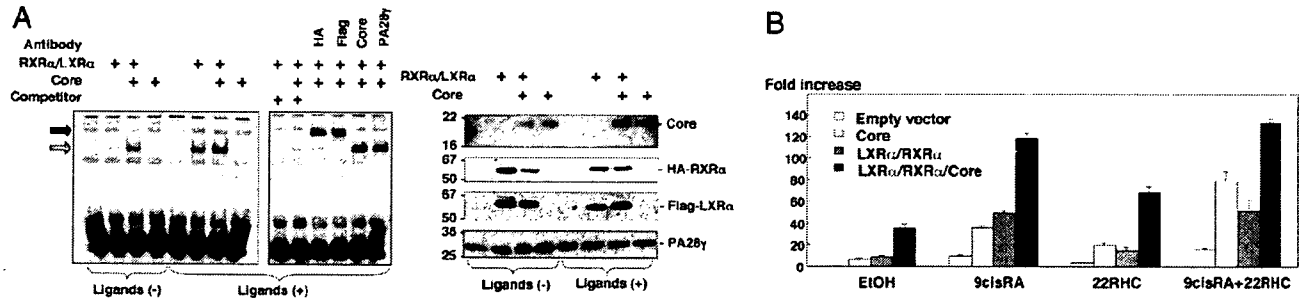

Fig. 4. Activation of the *srebp-1c* promoter by HCV core protein. (*A*) FLAG-LXRα and HA-RXRα were expressed in 293T cells together with or without HCV core protein. Ligands for LXRα and RXRα dissolved in ethanol [Ligands (+)] or ethanol alone [Ligands (−)] were added to the culture supernatant at 24 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.

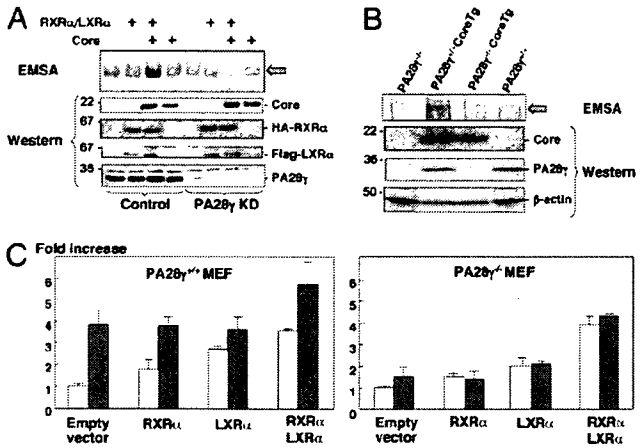

Fig. 5. PA28γ is required for HCV core-dependent activation of the *srebp-1c* promoter. (*A*) Effect of PA28γ knockdown on the LXRα/RXRα–DNA complex. FLAG-LXRα and HA-RXRα were expressed in FLC4 (control) or PA28γ-knockdown (PA28γ KD) cells together with or without HCV core protein. Cells were harvested 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 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, 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 PA28$\gamma^{+/+}$ (*Left*) or PA28$\gamma^{-/-}$ (*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 PA28$\gamma^{+/+}$CoreTg mice but not in those of PA28$\gamma^{-/-}$, PA28$\gamma^{+/+}$, or PA28$\gamma^{-/-}$CoreTg mice (Fig. 5*B*). 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 LXRα and RXRα (Fig. 5*C 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 LXRα and RXRα. However, no enhancing effect of HCV core protein on *srebp-1c* promoter activity was observed in PA28$\gamma^{-/-}$ 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, %
PA28$\gamma^{+/+}$CoreTg			
Male	17	5	29.4
Female	28	3	10.7
PA28$\gamma^{+/-}$			
Male	16	0	0
Female	4	0	0
PA28$\gamma^{-/-}$			
Male	23	0	0
Female	13	0	0
PA28$\gamma^{-/-}$CoreTg			
Male	15	0	0
Female	21	0	0

PA28γ 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 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 PA28$\gamma^{-/-}$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 PA28γ$^{+/+}$CoreTg mice but not in PA28γ$^{-/-}$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 PA28γ$^{+/+}$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 PA28γ$^{+/+}$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 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 (GGATCCGGTGGATCAGGAAGTGAAGTTCAAGAGA-CTTCACTTCCTGATCCACCTTTTTTGGAAAAGCTT) 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 PA28γ$^{-/-}$ mice have been reported previously (22, 25). Both strains were crossbred with each other to create PA28γ$^{-/-}$CoreTg mice. PA28γ$^{-/-}$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 (CACCTCACTTGTGATCCGCTCTCT-GAAAGAATCAACC). The targeted sequence for the PA28γ-knockout mouse was detected by PCR using the PA28-3 primer and the PAKO-4 primer (TGCAGTTCATTCAGGGCACCGGA-CAG). 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.

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.

1. Wasley A, Alter MJ (2000) *Semin Liver Dis* 20:1–16.
2. Bach N, Thung SN, Schaffner F (1992) *Hepatology* 15:572–577.
3. Lefkowitch JH, Schiff ER, Davis GL, Perrillo RP, Lindsay K, Bodenheimer HC, Jr., Balart LA, Ortego TJ, Payne J, Dienstag JL, *et al.* (1993) *Gastroenterology* 104:595–603.
4. 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.
5. Hope RG, McLauchlan J (2000) *J Gen Virol* 81:1913–1925.
6. 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.
7. Kapadia SB, Chisari FV (2005) *Proc Natl Acad Sci USA* 102:2561–2566.
8. Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, Wieland S, Bukh J, Purcell RH, Schultz PG, Chisari FV (2002) *Proc Natl Acad Sci USA* 99:15669–15674.
9. Wang C, Gale M, Jr, Keller BC, Huang H, Brown MS, Goldstein JL, Ye J (2005) *Mol Cell* 18:425–434.
10. Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H (1998) *J Clin Invest* 101:2331–2339.
11. Pai JT, Guryev O, Brown MS, Goldstein JL (1998) *J Biol Chem* 273:26138–26148.
12. Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL (1996) *J Clin Invest* 98:1575–1584.
13. Shimano H, Horton JD, Shimomura I, Hammer RE, Brown MS, Goldstein JL (1997) *J Clin Invest* 99:846–854.
14. Shimano H, Shimomura I, Hammer RE, Herz J, Goldstein JL, Brown MS, Horton JD (1997) *J Clin Invest* 100:2115–2124.
15. 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.
16. Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuzaka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, *et al.* (2001) *Mol Cell Biol* 21:2991–3000.
17. 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.
18. 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.
19. Masson P, Andersson O, Petersen UM, Young P (2001) *J Biol Chem* 276:1383–1390.
20. Li J, Rechsteiner M (2001) *Biochimie* 83:373–383.
21. Li X, Lonard D, Jung SY, Malovannaya A, Feng Q, Qin J, Tsai SY, Tsai M, O'Malley BW (2006) *Cell* 124:381–392.
22. Murata S, Kawahara H, Tohma S, Yamamoto K, Kasahara M, Nabeshima Y, Tanaka K, Chiba T (1999) *J Biol Chem* 274:38211–38215.
23. 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.
24. 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.
25. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K (1997) *J Gen Virol* 78:1527–1531.
26. 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.
27. Suzuki R, Tamura K, Li J, Ishii K, Matsuura Y, Miyamura T, Suzuki T (2001) *Virology* 280:301–309.
28. Minami Y, Kawasaki H, Minami M, Tanahashi N, Tanaka K, Yahara I (2000) *J Biol Chem* 275:9055–9061.
29. Watashi K, Hijikata M, Tagawa A, Doi T, Marusawa H, Shimotohno K (2003) *Mol Cell Biol* 23:7498–7509.
30. Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM (2006) *J Virol* 80:7199–7207.
31. Nystrom T (2005) *EMBO J* 24:1311–1317.
32. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr (1999) *Cell* 98:295–303.
33. Carballo M, Conde M, El Bekay R, Martin-Nieto J, Camacho MJ, Monteseirin J, Conde J, Bedoya FJ, Sobrino F (1999) *J Biol Chem* 274:17580–17586.
34. Aizaki H, Aoki Y, Harada T, Ishii K, Suzuki T, Nagamori S, Toda G, Matsuura Y, Miyamura T (1998) *Hepatology* 27:621–627.
35. Niwa H, Yamamura K, Miyazaki J (1991) *Gene* 108:193–199.
36. Huang DC, Cory S, Strasser A (1997) *Oncogene* 14:405–414.
37. Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, Yagi S (1999) *J Clin Microbiol* 37:1802–1808.

doi:10.1111/j.1440-1746.2006.04669.x

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.

Correspondence
Dr 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-tky@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

Journal of Gastroenterology and Hepatology 22 (2007) Suppl. 1; S108–S111

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 C57BL/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 carrying the core gene develop HCC in two independent lineages.[6] 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.

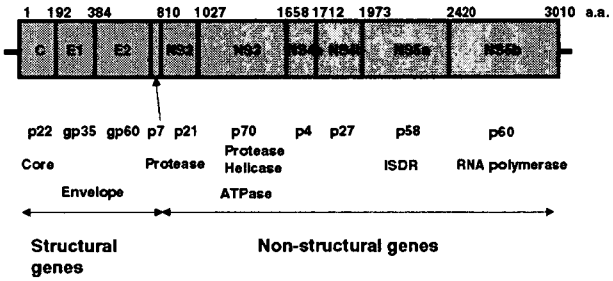

Figure 1 Structure of the hepatitis C virus (HCV) genome. The HCV genome consists of structural and non-structural regions. The structural region consists of the core, envelope and p7 genes. The non-structural region cones enzyme proteins of NS3 to NS5B. Among the three different transgenic mouse lines 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.

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.[6] 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.[6] 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

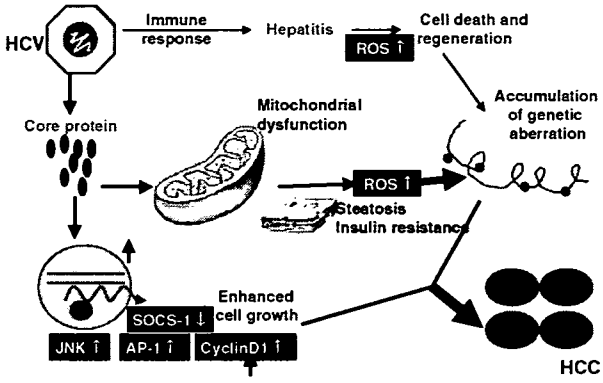

Figure 2 Molecular pathogenesis of liver disease in hepatitis C virus (HCV) infection. Induction of oxidative stress together with hepatic steatosis by the HCV core protein would play a pivotal role in the development of hepatocellular carcinoma (HCC). Alterations in cellular gene expressions, such as 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.

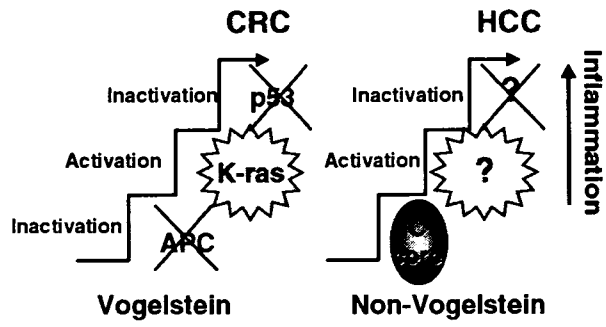

Figure 3 Mechanism of hepatitis C virus (HCV)-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in 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 give for many unusual events occurring in HCV carriers. CRC, colorectal cancer; APC, *adenomatous polyposis coli.*

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-1β 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.[19,21] 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).

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

1 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.
2 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.
3 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.
4 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.
5 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.
6 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.
7 Smith GS, Walford RL, Mickey MR. Lifespan and incidence of cancer and other diseases in selected long-lived inbred mice and their F 1 hybrids. *J. Natl Cancer Inst.* 1973; **50**: 1195–213.
8 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.
9 Koike K, Moriya K, Yotsuyanagi H *et al.* Sialadenitis resembling Sjögren's syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc. Natl Acad. Sci. USA* 1997; **94**: 233–6.
10 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.
11 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.
12 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.
13 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.
14 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.
15 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.
16 Moriya K, Tajima A, Tsutsumi T *et al.* Hepatitis C virus core protein insults mitochondrial function through reducing the ETS complex 1 activity. In: *10th International Meeting on Hepatitis C and Related Viruses, Kyoto, 2003.* Osaka: Japan Linkage, 2003; 73.
17 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.
18 Koike K, Moriya K. Metabolic aspects of hepatitis C: steatohepatitis distinct from NASH. *J. Gastroenterol.* 2005; **40**: 329–36.
19 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.
20 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.
21 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.
22 Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159–70.
23 Koike K, Tsutsumi T, Fujie H *et al.* Role of hepatitis viruses in hepatocarcinogenesis. *Oncology* 2002; **62**: 29–37.

Hepatology Research 2007; 37 (Suppl. 2): S115–S120 doi: 10.1111/j.1872-034X.2007.00173.x

Pathogenesis of HCV-associated HCC: Dual-pass carcinogenesis through activation of oxidative stress and intracellular signaling

Kazuhiko Koike

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

Overwhelming lines of epidemiological evidence have indicated that persistent infection with hepatitis C virus (HCV) is a major risk toward development of hepatocellular carcinoma (HCC). It remains controversial, however, in the pathogenesis of HCC associated with HCV, whether the virus plays a direct role or merely an indirect one. The studies using transgenic mouse models by us and others, in which the core protein of HCV has oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, albeit other factors such as continued cell death and regeneration associated with inflammation would play a role, as well. 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 glutathion (GSH) in the putative preneoplastic 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 is the alteration of intracellular signaling cascade of MAPK (JNK), AP-1, cyclin D1, and CDK4. 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. Our results suggest that there would be a mechanism for hepatocarcinogenesis in persistent HCV infection that is distinct from those for other cancers. Similar to the pathogenesis of other cancers, the accumulation of a set of genetic aberrations may also be necessary for 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 other cancers, HCV infection can elicit HCC in the absence of a complete set of genetic aberrations. Such a scenario, "non-Vogelstein-type" carcinogenesis, would explain the unusually high incidence and multicentric nature of HCC development in HCV infection.

Key words: hepatitis C virus, hepatocarcinogenesis, intracellular signaling transduction, oxidative stress, transgenic mouse

INTRODUCTION

WORLDWIDE, HEPATITIS C virus (HCV) infects hundreds of millions of people persistently, and induces a spectrum of chronic liver diseases.[1] Hence, it affects society in a number of domains including medical, sociological, and economic. Hepatocellular carcinoma (HCC) has become the most frequent 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 of the mechanism of HCC development in chronic HCV infection, therefore, is imminently required for the prevention of HCC.

Correspondence: Professor 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-tky@umin.ac.jp

UNIQUENESS OF HCC DEVELOPMENT IN HCV INFECTION

HOW HCV INDUCES HCC is not yet clear, despite the finding that more than 70% of patients with HCC in Japan are infected with HCV.[1,3,4] HCV infection

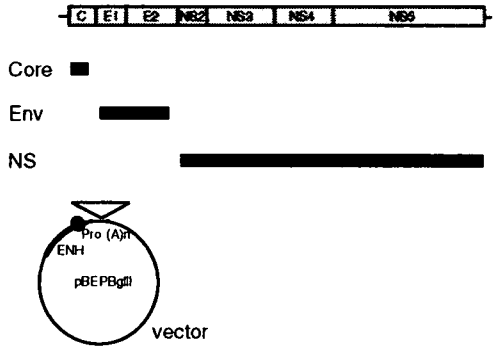

Figure 1 Hepatitis C virus (HCV) transgenic mouse lines. Among the three different transgenic mouse lines established, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. core, core genes, env, envelope genes; NS, non-structural genes.

is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence obligate hepatologists to a 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 caused by 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 lead to a possible activity of viral proteins for inducing HCC. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. A difficulty in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest. 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 viewpoints, we started tackling carcinogenesis in chronic viral hepatitis by transgenic mouse technology.

CORE PROTEIN OF HCV HAS ONCOGENIC ACTIVITY *IN VIVO*

AS ILLUSTRATED IN Figure 1, transgenic mouse lines with parts of the HCV genome were engineered by introducing the genes excised from the cDNA of the HCV genome of genotype 1b.[5,6] The background of the mouse lines is a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] Established are three different transgenic mouse lines, which carry the core gene, envelope genes, or non-structural genes, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages (Fig. 1).[6] 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 not developed HCC.

The transgenic mice carrying the core gene 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 a histologic characteristic 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 disclose 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.[6] Although numerous lipid droplets are found in cells forming adenoma, as in non-tumorous cells, they are rarely observed in HCC cells. These histologic features

closely resemble those observed in HCC developing in chronic hepatitis C patients, in which prominent lipid droplets are found in small differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.[6] Notably, the development of steatosis and HCC has been reproduced by 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 of HCV has an oncogenic potential when expressed *in vivo*.

MECHANISM OF HEPATOCARCINOGENESIS IN MOUSE MODEL FOR HCV-ASSOCIATED HCC

IT IS DIFFICULT to sort out the mechanism of carcinogenesis even for our simple model, in which only the core protein is expressed in otherwise normal liver tissue. There is a notable feature in the localization of the core protein in hepatocytes; while 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 meticulously analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. The production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects a state of an 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. If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that can no longer be scavenged by a physiologically antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic different in quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to 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]

Table 1 Biomolecular alterations with core protein expression observed in the transgenic mouse model

1.	Induction of cytokines including TNF-α and IL-1β[19]
2.	Activation of MAPK pathway and enhancement of AP-1 activation[19,20]
3.	Overproduction of oxidative stress or ROS in the absence of inflammation[13]
4.	Synergy of HCV core and alcohol in inducing oxidative stress and activating MAPK[13,20]
5.	Interaction of HCV core and RXR-α and PPAR-α[21]
6.	Induction of insulin resistance[17]
7.	Development of steatosis by inhibiting MTP activity[5,14,22]
8.	Interaction of HCV core and proteasome activator PA28γ[23]
9.	Inhibition of SOCS-1[24]

AP-1, activated protein-1; HCV, hepatitis C virus; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MTP, microsomal triglyceride transfer protein; PPAR-α, peroxisome proliferator agonist receptor-α; ROS, reactive oxygen species; RXR-α, retinoid X receptor; SOCS-1, suppressor of cytokine signaling; TNF-α, tumor necrosis factor.

Other possible pathways are the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Table 1). For example, tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β) have been found transcriptionally activated.[19] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)-α, that 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 in the JNK activation, transcription factor AP-1 activation is markedly enhanced.[19,21] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes.

Such an effect of the core protein on the MAPK pathway, combined with that on oxidative stress, may

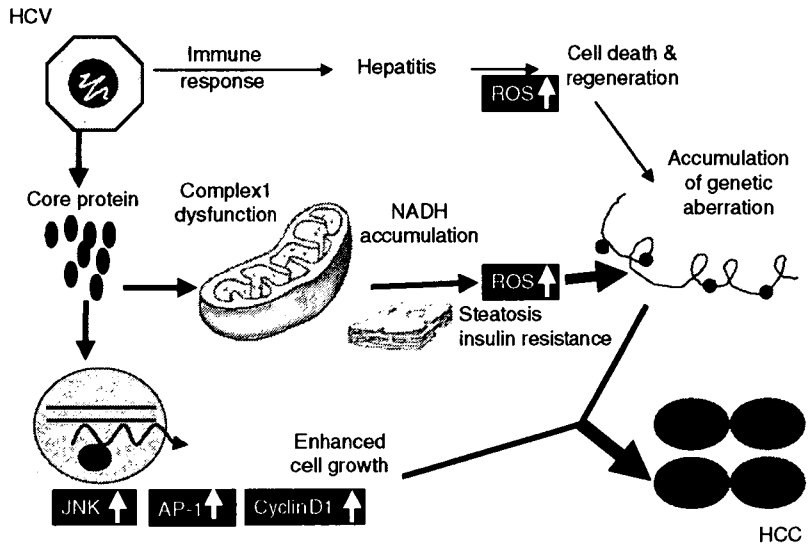

Figure 2 Mechanism of hepatitis C virus (HCV)-associated hepatocarcinogenesis. Inflammation should contribute to hepatocarcinogenesis by producing genetic aberrations via continual cell death and regeneration. In the case of HCV infection, the virus would contribute to hepatocarcinogenesis via two pathways: (i) the core protein acts on the function of mitochondrial electron transfer system, leading to the overproduction of oxidative stress. Inflammation may act synergistically with the core protein in inducing oxidative stress. The presence of steatosis and insulin resistance would enhance the production of oxidative stress; and (ii) modulation of cellular gene expression and signal transduction, which would give a growth advantage to hepatocytes. The combination of these alterations would escalate the development of hepatocellular carcinoma (HCC) in HCV infection. AP-1, activated protein-1; JNK, Jun N-terminal kinase; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species.

explain the extremely high incidence of HCC development in chronic hepatitis C.

HEPATOCARCINOGENESIS INDUCED BY HCV INFECTION: MECHANISM DISTINCT FROM OTHER CANCERS

THE RESULTS OF our studies on transgenic mice 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[25] has gained wide popularity. They proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They deduced that mutations in the *APC* 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.[25] The theory has been extended to the carcinogenesis of other cancers as well, called "Vogelstein-type" carcinogenesis (Fig. 2).

On the basis of results we obtained for the induction of HCC by the HCV core protein, we introduce a mechanism different from that of Kinzler and Vogelstein[25] for hepatocarcinogenesis in HCV infection. We allow multistages 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.[26] 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.

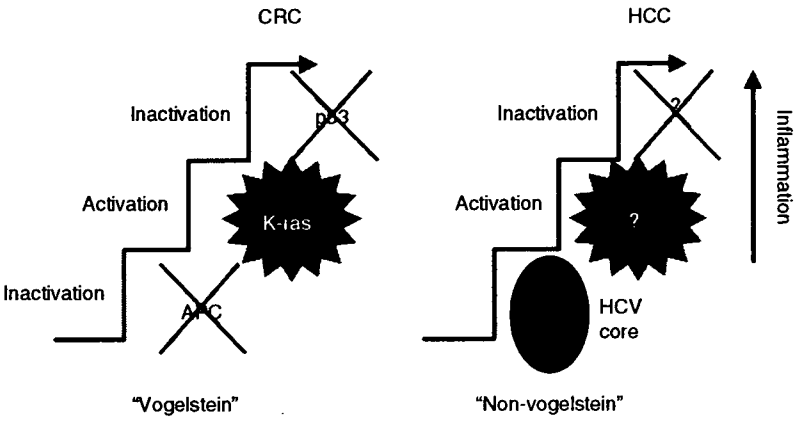

Figure 3 Hepatitis C virus (HCV)-associated hepatocarcinogenesis is a non-Vogelstein-type. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of core protein. Overall effects achieved by the expression of 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 in HCV carriers. CRC, colorectal cancer.

CONFLICT OF INTEREST

NO CONFLICT OF interest has been declared by the author.

REFERENCES

1 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.
2 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.
3 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.
4 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.
5 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.
6 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.
7 Smith GS, Walford RL, Mickey MR. Lifespan and incidence of cancer and other diseases in selected long-lived inbred mice and their F 1 hybrids. *J Natl Cancer Inst* 1973; **50**: 1195–213.
8 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.
9 Koike K, Moriya K, Yotsuyanagi H *et al.* Sialadenitis resembling Sjögren's syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc Natl Acad Sci USA* 1997; **94**: 233–6.
10 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.
11 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.
12 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.
13 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.
14 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. *Biophys Biochem Res Commun* 2001; **281**: 1207–12.

15 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.
16 Moriya K, Tajima A, Tsutsumi T *et al.* Hepatitis C virus core protein insults mitochondrial function through reducing the ETS complex 1 activity. In Miyamura T, Shimotohno K, eds. *Proceedings of the 10th International Meeting on Hepatitis C and Related Viruses, 2–6 December 2003, Kyoto, Japan.* Kyoto: Japan Linkage Service, 2003, 73.
17 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.
18 Koike K, Moriya K. Metabolic aspects of hepatitis C: steatohepatitis distinct from NASH. *J Gastroenterol* 2005; **40**: 329–36.
19 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.
20 Tsutsumi T, Suzuki T, Shimoike T *et al.* Interaction of hepatitis C virus core protein with retinoid X receptor-a modulates its transcriptional activity. *Hepatology* 2002; 35: 937–46.
21 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.
22 Perlemuter G, Sabile A, Letteron P *et al.* Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J* 2002; **16**: 185–94.
23 Moriishi K, Okabayashi T, Nakai K *et al.* Proteasome activator PA28g-dependent nuclear retention and degradation of hepatitis C virus core protein. *J Virol* 2003; 77: 10 237–49.
24 Miyoshi H, Fujie H, Shintani Y *et al.* Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling (SOCS)-1 gene expression. *J Hepatol* 2005; 43: 757–63.
25 Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159–70.
26 Koike K, Tsutsumi T, Fujie H *et al.* Role of hepatitis viruses in hepatocarcinogenesis. *Oncology* 2002; **62**: 29–37.

Hepatology Research 2007; 37: 731–739 doi: 10.1111/j.1872-034X.2007.00120.x

Original Article

Amino acid substitutions in the S region of hepatitis B virus in sera from patients with acute hepatitis

Junko Aono,[1,3] Hiroshi Yotsuyanagi,[1] Hideyuki Miyoshi,[2] Takeya Tsutsumi,[1] Hajime Fujie,[2] Yoshizumi Shintani,[1] Kyoji Moriya,[1] Chiaki Okuse[4], Michihiro Suzuki,[4] Kiyomi Yasuda,[5] Shiro Iino[5] and Kazuhiko Koike[1]

Departments of [1]Infectious Diseases and [2]Gastroenterology, Internal Medicine, University of Tokyo, [5]Center for Liver Diseases, Seizankai Kiyokawa Hospital, Tokyo, [3]Faculty of Pharmaceutical Sciences, Teikyo Heisei University, Chiba, [4]Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki, Japan

Background: An increase in the number of acute hepatitis patients with hepatitis B virus (HBV) of non-indigenous genotypes may reduce the efficacy of vaccination against HBV.

Methods: We have determined the amino acid (aa) sequences in the major hydrophilic region (MHR) in the S region of HBV in patients with acute hepatitis B and compared those with the ones from HBV strains used for the production of HBV vaccines commercially available in Japan.

Results: Of 48 patients studied, 11 were infected with genotype A, 11 with genotype B and 26 with genotype C HBV. The aa sequences of the nine genotype A isolates were the same as the aa sequence of J02205 which is used for the production of one of the commercially available recombinant vaccines. The aa sequences of the 11 genotype B isolates differed from the aa sequence of J02205 in two or three amino acids. Of the 26 genotype C isolates, 22 had the same aa sequence as X01587 which is used for the production of another recombinant vaccine. The remaining genotype C isolates had aa substitutions at aa131, which have a potential to alter the hydropathy and the three-dimensional structure of the MHR. The differences among the three current HBV vaccines in aa sequences in the MHR theoretically alter the hydropathy and three-dimensional structure.

Conclusion: Our results suggest that the transmission of HBV isolates with different genotypes or with aa substitutions in the MHR might reduce the efficacy of currently available HBV vaccines in the protection of HBV infections.

Key words: genotype, hepatitis B virus, major hydrophilic region, vaccine

INTRODUCTION

ABOUT 300 MILLION people in the world are chronically infected with hepatitis B virus (HBV). Chronic infection may eventually lead to liver cirrhosis or hepatocellular carcinoma.[1–4] To prevent the transmission of this virus, vaccination has been introduced in many countries. Indeed, universal vaccination has not only reduced the number of infected individuals, but also the number of deaths related to HBV.[5,6]

Correspondence: Dr Hiroshi Yotsuyanagi, Department of Infectious Diseases, Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan. Email: hyotsu-tky@umin.ac.jp
Received 20 September 2006; revision 31 January 2007; accepted 8 March 2007.

In Japan, in 1985, a national project was started to vaccinate children born to HBV-infected mothers. The chances of vertical transmission from HBV-carrying mothers have decreased. Recently, the prevalence of HBV in Japan has decreased to approximately 0.6%.[7]

Because the number of individuals infected with HBV has decreased, the number of patients with acute hepatitis B, mainly caused by horizontal transmission from HBV carriers, should also have decreased. However, in Japan, the number of patients with acute hepatitis B has recently increased (Yatsuhashi H. *et al.*, 2004, unpubl. data).

The increase in the number of patients with acute hepatitis B may, in part, be the result of patients carrying novel HBV genotypes imported from abroad. For

example, in recent years, genotype A HBV has often been detected in patients with acute hepatitis B.[8,9]

Genotype A HBV is transmitted from individuals who live in or have immigrated from other countries to Japan. Its infection is characterized by a high viral load and a long hepatitis B surface antigen (HBsAg) positivity period. The transition of acute hepatitis B with genotype A HBV infection to the chronic state has been reported recently.[8,10] Decreasing the transmission rate of genotype A HBV is therefore important for the control of the disease. Introducing universal vaccination for adolescents or adults is a measure to be considered.

The effectiveness of universal vaccination depends on the reactivity of vaccines against HBV. HBsAg binds antibody to hepatitis B surface antigen (anti-HBs) produced against HBV vaccines mainly via the 'a' determinant region (aa124–aa149). This region contains common antigenic epitopes of all subtypes (adw, adr, ayw, ayr) of HBsAg and lies in the major hydrophilic region (MHR) between aa99 and aa169. Amino acid (aa) substitutions in the MHR, particularly in the 'a' determinant region, can alter B cell epitopes of HBsAg, leading to immunological escape from the host immunity induced by either vaccination or previous infection.[11] Therefore, if HBV prevalent in Japan has aa substitutions in the MHR, the effect of universal vaccination may be reduced.

In Japan, three types of HBV vaccine (Bimmugen, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan; Heptavax, Merck & Co., Whitehouse Station, NJ, USA; and Meinyu, Meiji Dairies, Tokyo, Japan) are now available. Efficacy and immunogenicity of vaccines are not always comparable or identical.[12,13] Whether giving a single vaccine effectively prevents the transmission of all genotypes of HBV is an important but still unsolved problem. Elucidating the aa substitutions in the MHR may give a clue to this problem.

The purpose of the present study is to determine the difference of the aa sequences in the MHR of HBV among isolates from patients with acute hepatitis and also the difference of the aa sequences among viral strains used for the production of anti-HBV vaccines, and to find ways to use currently available vaccines as effective prophylaxes.

METHODS

Patients

FROM 1992 TO 2001, serum samples were collected from 48 patients diagnosed with acute hepatitis B in our institutions. Only patients whose serum samples were stored at the onset of hepatitis were included in this study. All the 48 patients ran a self-limited clinical course. No patients subsequently developed fulminant hepatic failure or chronic sequelae.

The criteria for the diagnosis of acute hepatitis B were the following: (i) an acute onset of liver injury without a history of liver dysfunction and positivity for HBsAg in serum; and (ii) immunoglobulin M (IgM) antibody to HBV core antigen (anti-HBc) at a titer of more than 2.5 of cut-off index. Coinfection with a hepatitis A virus or a hepatitis C virus was excluded by serological tests. None of the patients had previously received any vaccination against HBV.

Serum samples from the 48 patients with acute hepatitis B were examined virologically, and the results were examined for correlations with clinical characteristics. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

Determination of HBV-DNA

Hepatitis B virus DNA level was determined using transcription-mediated amplification (TMA) and a hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan) using the protocol of Kamisango *et al.*[14] The range of detection using TMA was from 3.7 log genome equivalents (LGE)/mL (i.e. $10^{3.7}$ copies/mL corresponding to 5000 copies/mL) to 8.7 LGE/mL ($10^{8.7}$ copies/mL). In seven of 34 studied serum samples, the level of HBV-DNA was lower than 3.7 LGE/mL and these were categorized as 3.7 LGE/mL.

Genotyping HBV

Hepatitis B virus genotypes were determined using commercial enzyme immunoassay kits (Smitest HBV Genotyping kit; Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by polymerase chain reaction (PCR) using three sense primers (i.e. s1: 5′-ACCAACCCTCTGGGATTCTTTCC-3′, s2: 5′-ACCAATCCTCTGGGATTCTTCCC-3′, and s3: 5′-AGCAATCCTCTAGGATTCCTTCC-3′ [nt 2902–2924]) and an antisense primer (i.e. as1: 5′-GAGCCTGA GGGCTCCACCC-3′ [nt 3091–3073]) biotinylated at the 5′ end; their sequences were deduced from conserved sequences in the pre-S1 region of HBV. The biotin-labeled and amplified HBV-DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one of the seven HBV genotypes (A-G) immobilized on wells of a 96-well