at work to reduce platelet count. Spleen size was well correlated with liver stiffness and was same at all of the strata of liver stiffness between the two groups, suggesting that spleen size is similarly regulated throughout the progression of liver stiffness in CLD-B and CLD-C—unlike platelet count.

In addition to the difference in the degree of thrombocytopenia between CLD-B and CLD-C patients, serum albumin level and prothrombin time (%) were lower in the latter patient group after adjustment for liver stiffness, suggesting that the ability to produce proteins may be impaired more in CLD-C patients than CLD-B patients. Of note, thrombopoietin, an important regulator in platelet production [39–41], is known to be mainly produced in the liver [42]. Thrombopoietin has long been considered to play a role in the progression of thrombocytopenia in patients with CLD [43–46], but its exact role has not yet been clarified. It has been reported that the peripheral thrombopoietin level is correlated not only to the degree of liver fibrosis but also to liver functional impairment in patients with chronic viral hepatitis [10, 47–50], but conflicting findings have also been reported [51–54]. The plasma thrombopoietin level is mainly regulated by binding to platelets and megakaryocytes rather than by the upregulation or downregulation of its production [32–36]. For example, the plasma thrombopoietin level increases in patients with aplastic anemia or idiopathic thrombocytopenic purpura due to the reduced peripheral platelet count, with the increase being greater in aplastic anemia than idiopathic thrombocytopenic purpura because the megakaryocyte number is decreased in aplastic anemia. In our study, the number of immature platelets and the total platelet count were lower in CLD-C than in CLD-B patients at the highest stratum of liver stiffness. However, the plasma thrombopoietin level was not enhanced in any of these patients and was the same in both the CLD-C and CLD-B patients, suggesting that thrombopoietin production may be more severely impaired in CLD-C patients than in CLD-B patients. Collectively, these results suggest that the thrombopoietic activity may be impaired more in patients with CLD-C than in those with CLD-B. Of interest is the recent report that an active thrombopoietin-receptor agonist increased platelet counts in patients with HCV-related cirrhosis, thereby enabling the initiation of antiviral therapy and suggesting an important role of thrombopoietin in thrombocytopenia in advanced CLD-C patients [1].

It has recently been shown that transient elastography is an unreliable tool for the detection of cirrhosis in patients with acute liver damage [55] and that acute viral hepatitis increases liver stiffness values measured by transient elastography. These results suggest that the extent of necroinflammatory activity needs to be carefully considered in terms of validating transient elastography [55, 56]. In our study, the difference in liver damage assessed by ALT or total bilirubin was not determined in any stratum of liver stiffness of CLD-B and CLD-C patients, indicating that the evaluation of liver stiffness values was validated in all the strata of both patient groups. Regarding the potential influence of hepatitis viruses on the estimation of necroinflammatory activity by transient elastography, Marcellin et al. [57] recently reported that the liver stiffness measurement appears to be a reliable marker for the detection of significant fibrosis or cirrhosis in HBV patients and that cut-off values are only slightly different from those observed in HCV patients. In contrast, Ogawa et al. [58] showed that the liver stiffness cut-off value for each fibrosis stage was lower in patients with chronic HBV infection than in those with chronic HCV infection, indicating that the liver fibrosis stage may be higher in chronic HBV infection than in chronic HCV infection at the same stratum of liver stiffness. Whatever the case, there has been no report showing that liver fibrosis stage is more advanced in CLD-C than in CLD-B patients at the same liver stiffness, which could influence the results reported here. Furthermore, among CLD-B patients, the positivity of HBeAg may influence the liver stiffness measurement because patients with HBeAg are shown to have thicker fibrosis in portal area than those without HBeAg [59]. The significance of HBeAg positivity in the liver stiffness measurement should be further elucidated.

In conclusion, the data reported here indicate that the degree of thrombocytopenia is more severe in patients with advanced CLD-C than in those with CLD-B, even at the same grade of liver stiffness and splenomegaly, and that impaired platelet production may underlie the mechanism. The mechanism(s) responsible for thrombocytopenia—in addition to splenomegaly—in advanced CLD should be further elucidated with the aim of developing new therapeutic strategies.

References

1. McHutchison JG, Dusheiko G, Shiffman ML, Rodriguez-Torres M, Sigal S, Bourliere M, et al. Eltrombopag for thrombocytopenia in patients with cirrhosis associated with hepatitis C. N Engl J Med. 2007;357:2227–36.
2. National Institutes of Health. NIH Consensus Development Conference Statement: Management of hepatitis C. Hepatology. 2002;36:S3–20.
3. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. Lancet. 2003;362:2095–100.
4. Liaw YF, Tai DI, Chu CM, Chen TJ. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology. 1988;8:493–6.
5. Fattovich G, Brollo L, Giustina G, Noventa F, Pontisso P, Alberti A, et al. Natural history and prognostic factors for chronic hepatitis type B. Gut. 1991;32:294–8.

6. Aster RH. Pooling of platelets in the spleen: role in the pathogenesis of "hypersplenic" thrombocytopenia. J Clin Invest. 1966;45:645–57.
7. Schmidt KG, Rasmussen JW, Bekker C, Madsen PE. Kinetics and in vivo distribution of 111-In-labelled autologous platelets in chronic hepatic disease: mechanisms of thrombocytopenia. Scand J Haematol. 1985;34:39–46.
8. Peck-Radosavljevic M. Hypersplenism. Eur J Gastroenterol Hepatol. 2001;13:317–23.
9. Bolognesi M, Merkel C, Sacerdoti D, Nava V, Gatta A. Role of spleen enlargement in cirrhosis with portal hypertension. Dig Liver Dis. 2002;34:144–50.
10. Adinolfi LE, Giordano MG, Andreana A, Tripodi MF, Utili R, Cesaro G, et al. Hepatic fibrosis plays a central role in the pathogenesis of thrombocytopenia in patients with chronic viral hepatitis. Br J Haematol. 2001;113:590–5.
11. Karasu Z, Tekin F, Ersoz G, Gunsar F, Batur Y, Ilter T, et al. Liver fibrosis is associated with decreased peripheral platelet count in patients with chronic hepatitis B and C. Dig Dis Sci. 2007;52:1535–9.
12. Lu SN, Wang JH, Liu SL, Hung CH, Chen CH, Tung HD, et al. Thrombocytopenia as a surrogate for cirrhosis and a marker for the identification of patients at high-risk for hepatocellular carcinoma. Cancer. 2006;107:2212–22.
13. Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet. 2001;357:1069–75.
14. Myers R, Tainturier M, Ratziu V, Piton A, Thibault V, Imbert-Bismut F, et al. Prediction of liver histological lesions with biochemical markers in patients with chronic hepatitis B. J Hepatol. 2003;39:222–30.
15. Yeh WC, Li PC, Jeng YM, Hsu HC, Kuo PL, Li ML, et al. Elastic modulus measurements of human liver and correlation with pathology. Ultrasound Med Biol. 2002;28:467–74.
16. Saito H, Tada S, Nakamoto N, Kitamura K, Horikawa H, Kurita S, et al. Efficacy of non-invasive elastometry on staging of hepatic fibrosis. Hepatol Res. 2004;29:97–103.
17. Dienstag J. The role of liver biopsy in chronic hepatitis C. Hepatology. 2002;36:S152–60.
18. Lok A, McMahon B. Chronic hepatitis B. Hepatology. 2001;34:1225–41.
19. Maharaj B, Maharaj R, Leary W, Cooppan R, Naran A, Pirie D, et al. Sampling variability and its influence on the diagnostic yield of percutaneous needle biopsy of the liver. Lancet. 1986;1:523–5.
20. McGill D, Rakela J, Zinsmeister A, Ott B. A 21-year experience with major hemorrhage after percutaneous liver biopsy. Gastroenterology. 1990;99:1396–400.
21. Sandrin L, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, et al. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. Ultrasound Med Biol. 2003;29:1705–13.
22. Castera L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology. 2005;128:343–50.
23. Ziol M, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. Hepatology. 2005;41:48–54.
24. Verveer C, de Knegt RJ. Non-invasive measurement of liver fibrosis: application of the FibroScan in hepatology. Scand J Gastroenterol. 2006;41[Suppl 243]:85–8.
25. Ganne-Carrie N, Ziol M, de Ledinghen V, Douvin C, Marcellin P, Castera L, et al. Accuracy of liver stiffness measurement for the diagnosis of cirrhosis in patients with chronic liver diseases. Hepatology. 2006;44:1511–7.
26. Fraquelli M, Rigamonti C. Diagnosis of cirrhosis by transient elastography: what is hidden behind misleading results. Hepatology 2007;46:282 (author reply, p. 283).
27. Masuzaki R, Tateishi R, Yoshida H, Yoshida H, Sato S, Kato N, et al. Risk assessment of hepatocellular carcinoma in chronic hepatitis C patients by transient elastography. J Clin Gastroenterol. 2008;42:839–43.
28. Lamb PM, Lund A, Kanagasabay RR, Martin A, Webb JA, Reznek RH. Spleen size: how well do linear ultrasound measurements correlate with three-dimensional CT volume assessments? Br J Radiol. 2002;75:573–7.
29. Kurata Y, Hayashi S, Kiyoi T, Kosugi S, Kashiwagi H, Honda S, et al. Diagnostic value of tests for reticulated platelets, plasma glycocalicin, and thrombopoietin levels for discriminating between hyperdestructive and hypoplastic thrombocytopenia. Am J Clin Pathol. 2001;115:656–64.
30. Briggs C, Kunka S, Hart D, Oguni S, Machin SJ. Assessment of an immature platelet fraction (IPF) in peripheral thrombocytopenia. Br J Haematol. 2004;126:93–9.
31. Cobbold JF, Morin S, Taylor-Robinson SD. Transient elastography for the assessment of chronic liver disease: Ready for the clinic? World J Gastroenterol. 2007;13:4791–7.
32. Bartley TD, Bogenberger J, Hunt P, Li YS, Lu HS, Martin F, et al. Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. Cell. 1994;77:1117–24.
33. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature. 1994;369:568–71.
34. Fielder PJ, Gurney AL, Stefanich E, Marian M, Moore MW, Carver-Moore K, et al. Regulation of thrombopoietin levels by c-mpl-mediated binding to platelets. Blood. 1996;87:2154–61.
35. Emmons RV, Reid DM, Cohen RL, Meng G, Young NS, Dunbar CE, et al. Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. Blood. 1996;87:4068–71.
36. Li J, Xia Y, Kuter DJ. Interaction of thrombopoietin with the platelet c-mpl receptor in plasma: binding, internalization, stability and pharmacokinetics. Br J Haematol. 1999;106:345–56.
37. Koike Y, Miyazaki K, Higashihara M, Kimura E, Jona M, Uchihashi K, et al. Clinical significance of detection of immature platelets: comparison between percentage of reticulated platelets as detected by flow cytometry and immature platelet fraction as detected by automated measurement. Eur J Haematol. 2010;84:183–4.
38. Kajihara M, Okazaki Y, Kato S, Ishii H, Kawakami Y, Ikeda Y, et al. Evaluation of platelet kinetics in patients with liver cirrhosis: similarity to idiopathic thrombocytopenic purpura. J Gastroenterol Hepatol. 2007;22:112–8.
39. Kaushansky K. Thrombopoietin: basic biology, clinical promise. Int J Hematol. 1995;62:7–15.
40. Kaushansky K. Thrombopoietin: the primary regulator of megakaryocyte and platelet production. Thromb Haemost. 1995;74:521–5.
41. Eaton DL, de Sauvage FJ. Thrombopoietin: the primary regulator of megakaryocytopoiesis and thrombopoiesis. Exp Hematol. 1997;25:1–7.
42. Jelkmann W. The role of the liver in the production of thrombopoietin compared with erythropoietin. Eur J Gastroenterol Hepatol. 2001;13:791–801.

43. Koike Y, Yoneyama A, Shirai J, Ishida T, Shoda E, Miyazaki K, et al. Evaluation of thrombopoiesis in thrombocytopenic disorders by simultaneous measurement of reticulated platelets of whole blood and serum thrombopoietin concentrations. Thromb Haemost. 1998;79:1106–10.
44. Peck-Radosavljevic M, Wichlas M, Zacherl J, Stiegler G, Stohlawetz P, Fuchsjager M, et al. Thrombopoietin induces rapid resolution of thrombocytopenia after orthotopic liver transplantation through increased platelet production. Blood. 2000;95:795–801.
45. Peck-Radosavljevic M. Thrombocytopenia in liver disease. Can J Gastroenterol. 2000;14[Suppl D]:60D–6D.
46. Wolber EM, Ganschow R, Burdelski M, Jelkmann W. Hepatic thrombopoietin mRNA levels in acute and chronic liver failure of childhood. Hepatology. 1999;29:1739–42.
47. Kawasaki T, Takeshita A, Souda K, Kobayashi Y, Kikuyama M, Suzuki F, et al. Serum thrombopoietin levels in patients with chronic hepatitis and liver cirrhosis. Am J Gastroenterol. 1999;94:1918–22.
48. Espanol I, Gallego A, Enriquez J, Rabella N, Lerma E, Hernandez A, et al. Thrombocytopenia associated with liver cirrhosis and hepatitis C viral infection: role of thrombopoietin. Hepatogastroenterology. 2000;47:1404–6.
49. Giannini E, Borro P, Botta F, Fumagalli A, Malfatti F, Podesta E, et al. Serum thrombopoietin levels are linked to liver function in untreated patients with hepatitis C virus-related chronic hepatitis. J Hepatol. 2002;37:572–7.
50. Giannini E, Botta F, Borro P, Malfatti F, Fumagalli A, Testa E, et al. Relationship between thrombopoietin serum levels and liver function in patients with chronic liver disease related to hepatitis C virus infection. Am J Gastroenterol. 2003;98:2516–20.
51. Stockelberg D, Andersson P, Bjornsson E, Bjork S, Wadenvik H. Plasma thrombopoietin levels in liver cirrhosis and kidney failure. J Intern Med. 1999;246:471–5.
52. Schoffski P, Tacke F, Trautwein C, Martin MU, Caselitz M, Hecker H, et al. Thrombopoietin serum levels are elevated in patients with hepatitis B/C infection compared to other causes of chronic liver disease. Liver. 2002;22:114–20.
53. Aref S, Mabed M, Selim T, Goda T, Khafagy N. Thrombopoietin (TPO) levels in hepatic patients with thrombocytopenia. Hematology. 2004;9:351–6.
54. Ozer B, Serin E, Sezgin N, Cosar A, Guclu M, Gur G, et al. Thrombopoietin or interleukin-6 has no effect on thrombocytopenia of cirrhosis. Hepatogastroenterology. 2007;54:1187–91.
55. Sagir A, Erhardt A, Schmitt M, Haussinger D. Transient elastography is unreliable for detection of cirrhosis in patients with acute liver damage. Hepatology. 2008;47:592–5.
56. Arena U, Vizzutti F, Corti G, Ambu S, Stasi C, Bresci S, et al. Acute viral hepatitis increases liver stiffness values measured by transient elastography. Hepatology. 2008;47:380–4.
57. Marcellin P, Ziol M, Bedossa P, Douvin C, Poupon R, de Ledinghen V, et al. Non-invasive assessment of liver fibrosis by stiffness measurement in patients with chronic hepatitis B. Liver Int. 2009;29:242–7.
58. Ogawa E, Furusyo N, Toyoda K, Takeoka H, Otaguro S, Hamada M, et al. Transient elastography for patients with chronic hepatitis B and C virus infection: non-invasive, quantitative assessment of liver fibrosis. Hepatol Res. 2007;37:1002–10.
59. Hui CK, Leung N, Shek TW, Yao H, Lee WK, Lai JY, et al. Sustained disease remission after spontaneous HBeAg seroconversion is associated with reduction in fibrosis progression in chronic hepatitis B Chinese patients. Hepatology. 2007;46:690–8.

Journal of Medical Virology 82:776–782 (2010)

Hepatitis C Virus Core Protein Compromises Iron-Induced Activation of Antioxidants in Mice and HepG2 Cells

Kyoji Moriya, Hideyuki Miyoshi, Seiko Shinzawa, Takeya Tsutsumi, Hajime Fujie, Koji Goto, Yoshizumi Shintani, Hiroshi Yotsuyanagi, and Kazuhiko Koike*

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

One of the characteristics of hepatitis C virus (HCV) infection is the unusual augmentation of oxidative stress, which is exacerbated by iron accumulation in the liver, as observed frequently in hepatitis C patients. Using a transgenic mouse model, the core protein of HCV was shown previously to induce the overproduction of reactive oxygen species (ROS) in the liver. In the present study, the impact of iron overloading on the oxidant/antioxidant system was examined using this mouse model and cultured cells. Iron overloading caused the induction of ROS as well as antioxidants. However, the augmentation of some antioxidants, including heme oxygenase-1 and NADH dehydrogenase, quinone 1, was compromised by the presence of the core protein. The attenuation of iron-induced augmentation of heme oxygenase-1 was also confirmed in HepG2 cells expressing the core protein. This attenuation was not dependent on the Nrf2 transcription factor. Thus, HCV infection not only induces oxidative stress but also hampers the iron-induced antioxidant activation in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis. ***J. Med. Virol. 82:776–782, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: oxidative stress; transgenic mouse; hepatocellular carcinoma; antioxidant; heme oxygenase-1

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver disease. Persistent HCV infection leads to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC), thereby being a serious problem both in medical and socio-economical aspects [Saito et al., 1990]. Despite overwhelming evidence from epidemiological studies, the precise mechanism of hepatocarcinogenesis in HCV infection is still not fully understood. Recently, it has been shown that the core protein of HCV induces HCC in transgenic mice [Moriya et al., 1998; Naas et al., 2005; Machida et al., 2006]. Augmentation of oxidative stress is implicated in the pathogenesis of liver disease in HCV infection as shown by a number of clinical and basic studies [Farinati et al., 1995; Moriya et al., 2001; Choi and Ou, 2006]. Reactive oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis [Fujita et al., 2008]. Recent studies have shown that oxidative stress is more augmented in hepatitis C than in other types of hepatitis such as hepatitis B [Farinati et al., 1995; Chapoutot et al., 2000]. On the other hand, in chronic hepatitis C, HCC and fibrosis are closely associated with the amount of iron in the liver. Iron depletion both in the form of dietary iron restriction or phlebotomy improved hepatic inflammation and lowered serum aminotransferase levels in hepatitis C patients. Phlebotomy decreases the hepatic content of 8-OH deoxyguanosine, a marker of DNA damage, improved inflammation and fibrosis

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; HO-1, heme oxygenase-1; thiobarbituric acid reactive substances (TBARS); GST, glutathione-*S*-transferase; SOD, superoxide dismutase; GPx, glutathione peroxidase; NQO1, NAD(P)H dehydrogenase, quinone 1; AP-1, activator protein-1; NF-κB, nuclear factor-kappa B; Bach1, BTB and CNC homology 1.

Grant sponsor: Ministry of Education, Culture, Science, Sports and Technology of Japan (Grant-in-Aid for Scientific Research on Priority Area, partly supported); Grant sponsor: Ministry of Health, Labor and Welfare (Health Sciences Research Grants, Research on Hepatitis).

*Correspondence to: Kazuhiko Koike, MD, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: kkoike-tky@umin.ac.jp

Accepted 11 August 2009

DOI 10.1002/jmv.21661

Published online in Wiley InterScience (www.interscience.wiley.com)

scores, and prevented HCC development [Kato et al., 2001].

Thus, a major role in the pathogenesis of HCV-associated liver disease has been attributed to oxidative stress augmentation, in association with iron accumulation, but no underlying mechanism is understood well yet. Hence, it is an important issue to understand the mechanism of oxidative stress augmentation, which may allow us to develop new tools of therapies for chronic hepatitis C. Iron accumulation and oxidant/antioxidant status were assessed with or without iron overloading in the liver of a transgenic mouse model of HCC in HCV infection. The expression levels of genes associated with the antioxidant system were also determined.

MATERIALS AND METHODS

Transgenic Mice and Cultured Cells

The production of HCV core gene transgenic mice has been described previously [Moriya et al., 1998]. Briefly, the core gene of genotype 1b HCV was introduced into C57BL/6 mouse embryos (Clea Japan, Tokyo, Japan). Mice were cared for according to institutional guidelines approved by the institutional review board of the animal care committee, fed an ordinary chow diet (Oriental Yeast Co., Ltd, Tokyo, Japan), and maintained in a specific pathogen-free state. At least five mice were used for each experiment and the data were subjected to statistical analysis.

Determination of Iron in the Liver

Determination of Fe in the liver was performed by Shimadzu Techno-Research, K.K. (Kyoto, Japan) using Inductively Coupled Plasma apparatus, ICP8100 (Shimadzu Corp.). Briefly, samples were resolved by microwave (Microwave Resolution System ETHOS-TC, Milestone General, Inc., Tokyo, Japan) after the addition of nitric acid, and the volume was adjusted by the addition of H_2O. This solution was then subjected to the quantification of iron using ICP8100 (http://www.shimadzu.com/products/lab/ms/glossary/oh80jt00000008w4.html).

Iron Loading Experiments

For the short-term iron loading experiment, three doses of $FeSO_4$ solution (100 mg/kg BW, suspended in dH_2O) or vehicle only were administered to the core gene transgenic or control mice i.p., with intervals of 24 hr at the age of 6 months [Zhu and Miller, 2007]. For the long-term iron loading experiment, 50 mg/kg BW of $FeSO_4$ was administered to the core gene transgenic mice i.p., once a week for 3 months from the age of 3 months. HepG2 cell lines expressing the HCV core protein under the control of the CAG promoter (Hep39J, Hep396, and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously [Ruggieri et al., 2004]. For the iron loading experiments, hemin solution (10 mM in DMSO) was added to the culture medium at the final concentration of 5 μM, and the cells were incubated for 5 or 72 hr.

Evaluation of Oxidant and Antioxidant Systems

Lipid peroxidation in the liver was estimated spectrophotometrically using thiobarbituric acid reactive substances (TBARS) and is expressed in terms of malondialdehyde formed per milligram protein. In the cell culture experiment, the cells were examined for ROS production using chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Inc., Eugene, OR). For the evaluation of DNA damage in cells, apurinic/apyrimidinic (AP) sites were determined using a DNA Damage Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan).

Real-Time PCR and Western Blotting

RNA was prepared from mouse liver tissues using TRIzol LS (Invitrogen, Carlsbad, CA). The first-strand cDNAs were synthesized with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The fluorescence signal was measured using ABI Prism 7000 (Applied Biosystems, Tokyo, Japan). Primers and probes for hepcidin (Unigene ID: Mm. 439939), catalase (Mm. 4215), glutathione-*S*-transferase (GST) (Mm. 1090), superoxide dismutase (SOD)1 (Mm. 01344233), glutathione peroxidase (GPx)1 (Mm. 1090), heme oxygenase (HO)-1 (Mm. 00516004), NAD (P)H dehydrogenase, quinone (NQO) 1 (Mm. 500821), activator protein (AP)-1 (Mm. 275071), nuclear factor-kappa B (NF-κB)1 (Mm. 256765), BTB and CNC homology (Bach)1 (Mm. 26147), and hypoxanthine phosphoribosyltransferase (Mm. 299381) were purchased as assays-on-demand (Applied Biosystems). Each cDNA prepared was used in triplicate for the real-time PCR procedures for each gene tested.

Western blotting was performed with an anti-HO-1 antibody (Stressgen Biotechnologies, Corp., Victoria, BC, Canada) or anti-Nrf2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and Super Signal Femto (Pierce, Rockford, IL).

Statistical Analysis

Data are presented as the mean ± SE. The significance of the difference in means was determined by Mann–Whitney's *U*-test. $P < 0.05$ was considered significant.

RESULTS

Iron Accumulation in Ordinarily Fed Core Gene Transgenic Mice

The core gene transgenic mice develop HCC after an incubation period of approximately 16 months, in the absence of inflammation [Moriya et al., 1998]. During the incubation period, there is augmentation of oxidative stress with a concomitant activation of antioxidants and development of DNA damage in the liver [Farinati

et al., 1995]. For mice fed with normal chow, the concentration of total iron in the liver was higher in the core gene transgenic mice than in the control mice, and the difference became significant after the age of 12 months (Fig. 1A). The level of hepcidin mRNA, the product of which maintains iron homeostasis by a direct inhibition of ferroportin [Muckenthaler, 2008], was significantly higher in the core gene transgenic mice than in the control mice at the age of 3 and 15 months (Fig. 1B).

Iron Overloading to Core Gene Transgenic Mice

When the mice were overloaded with iron, the intrahepatic levels of iron markedly increased. In the short-term iron loading (for three consecutive days at the age of 6 months), the iron concentration in the iron-treated mice was more than twofold higher than that in the vehicle-treated ones in both the core gene transgenic and control mice, but there was no difference between the core gene transgenic and control mice. In the long-term iron loading (for 3 months from the age of 3 months), the iron concentration became significantly higher in the core gene transgenic mice than in the control mice (Fig. 1C). The hepcidin mRNA level was proportionally higher in the long-term iron-loaded mice than in the vehicle-treated mice and was significantly higher in the core gene transgenic than in the control mice ($P < 0.05$, Fig. 1D), suggesting that the positive feedback from iron to hepcidin is instrumental.

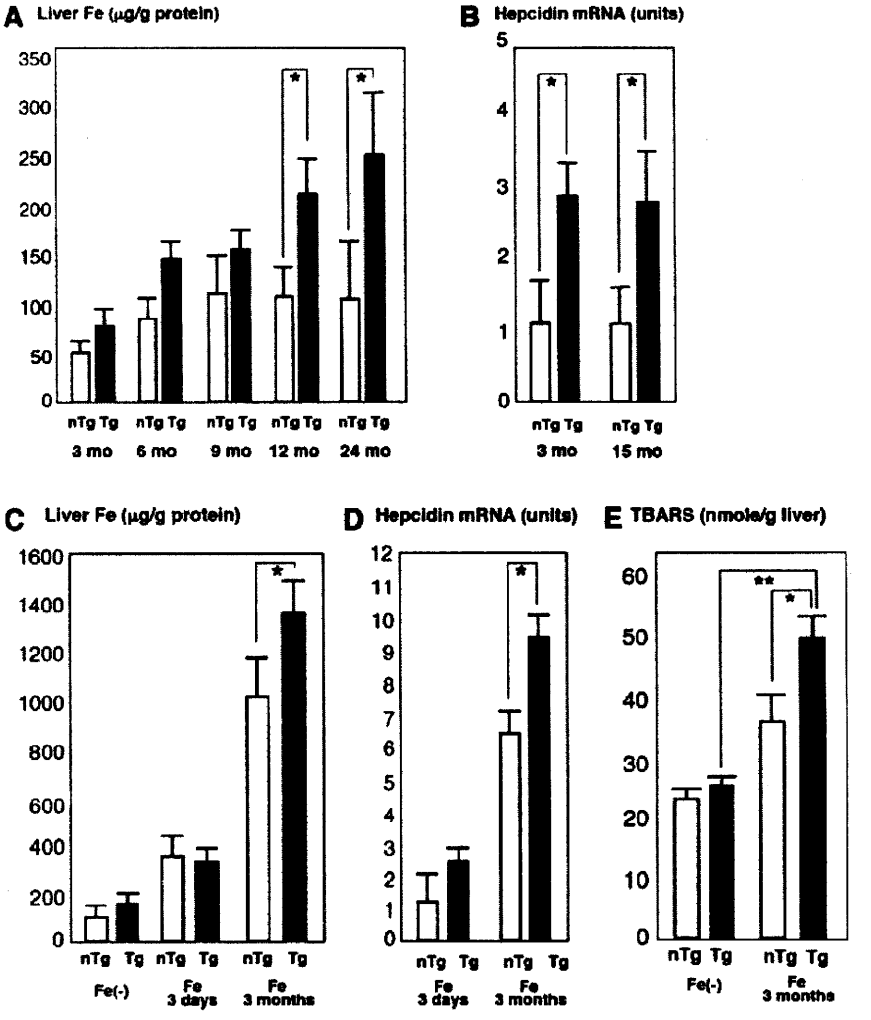


Fig. 1. A: Amounts of iron accumulated in the liver of mice fed with normal chow. B: Hepcidin mRNA level in the liver of mice fed with normal chow at 3 and 15 months. C: Amounts of iron in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. D: Hepcidin mRNA level in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. E: Oxidative stress in the liver of mice subjected to iron overloading for 0 or 3 months. The data represent means ± SE, n = 5 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$. nTg, nontransgenic control mice; Tg, transgenic mice; TBARS, thiobarbituric acid reactive substances.

Oxidative Stress and Iron Overloading in Mice

As described previously, ROS production in the liver of the core gene transgenic mice is already augmented at the young age, but the lipid peroxidation level is not higher than that in the control mice due to the concomitant activation of antioxidant system [Moriya et al., 2001]. This was also the case in the current experiment: there was no significant difference in the ROS level at the age of 6 months between the core gene transgenic and control mice that were treated with the vehicle, as determined by TBARS (Fig. 1E). However, after the long-term iron treatment, ROS levels in the liver of the core gene transgenic mice became significantly higher than that in the control mice (Fig. 1E, $P < 0.05$). After the long-term iron treatment, the AP site index, a marker for nuclear DNA damage, became significantly higher in the core gene transgenic mice than in the vehicle-treated core gene transgenic mice (5.2 ± 0.6 vs. 3.9 ± 0.3 nmol/g liver, $P < 0.05$), showing that iron overloading facilitates nuclear DNA damage through oxidative stress augmentation.

Impact of Iron Overloading on Antioxidants

The effect of long-term iron overloading on antioxidants was evaluated by real-time PCR analysis (Fig. 2). As already reported [Moriya et al., 2001], the levels of antioxidant enzymes, such as catalase, GST, or SOD1, were higher in the core transgenic mice than in the control mice before iron overloading. The levels of antioxidant mRNAs were higher in the long-term iron-treated mice than in the vehicle-treated mice. However, the magnitude of augmentation by iron overloading was different among the antioxidant genes. While catalase and GST genes were significantly more enhanced in the core gene transgenic mice than in the control mice by iron overloading ($P < 0.05$), there was less augmentation in the expressions of HO-1 and NQO-1 genes in the core gene transgenic mice than in the control mice. The level of HO-1 mRNA in the long-term-treated mice was significantly higher in the control mice than in the core gene transgenic mice ($P < 0.05$, respectively), in contrast to that of catalase, GST, or SOD1 gene. The level of NQO-1 mRNA in the liver was lower in the core gene transgenic than in the control mice, although the difference was not statistically significant.

To confirm this observation, the protein level of HO-1 was determined by Western blotting. As shown in Figure 3A, there was only a marginal expression of HO-1 protein in the untreated core gene transgenic and nontransgenic mice. After the short-term iron overloading, there was a marked induction of HO-1 protein but there was no significant difference between these two. However, after the long-term overloading, there was an attenuation in the levels of HO-1 protein in both the core gene transgenic and control mice; in particular, the HO-1 level was lower in the core gene transgenic mice than in the control mice. Thus, the long-term in vivo iron overloading compromised some antioxidants, such as HO-1 and NQO-1, which may lead to an augmentation of oxidative stress in HCV infection.

HO-1 and Iron in Cultured Cells

HepG2 cells expressing the core protein were treated with hemin as described in the Materials and Methods

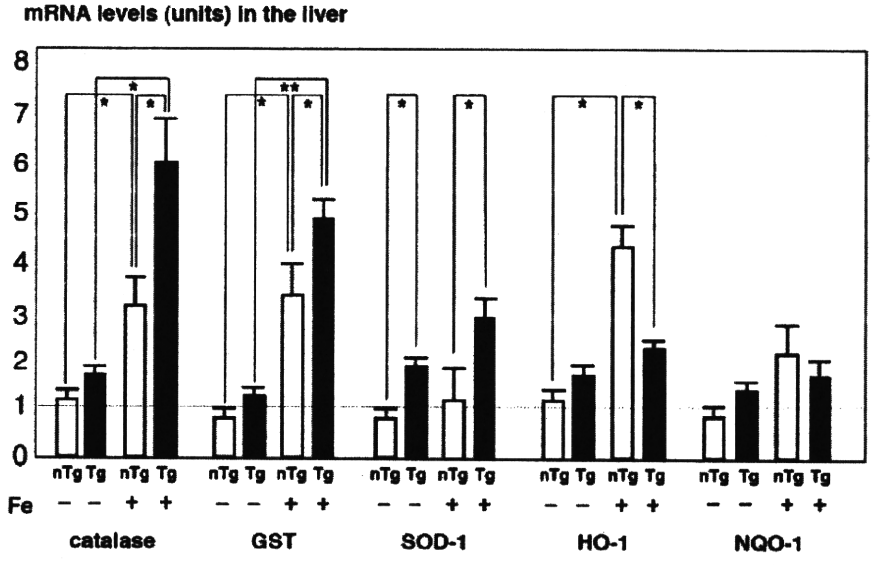


Fig. 2. Levels of antioxidant mRNA in the liver of mice subjected to iron overloading. Levels of mRNA were determined in nontransgenic control mice (open bars) or in transgenic mice (closed bars) with 3-month administration of iron (Fe+) or without it (Fe−). Mice were sacrificed at 6 months and liver tissues were subjected to determination. The data represent means ± SE, n = 5 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$. nTg, nontransgenic control mice; Tg, transgenic mice; GST, glutathione-*S*-transferase: SOD1, superoxide dismutase 1; HO-1, heme oxygenase 1; NQO-1, NAD(P)H dehydrogenase, quinone 1.

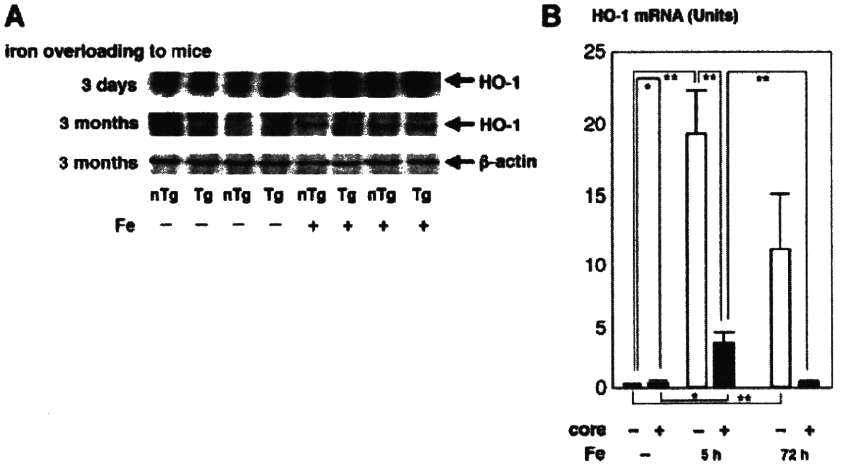


Fig. 3. **A**: Levels of HO-1 protein determined by Western blotting in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. **B**: Levels of HO-1 mRNA in core-protein-expressing or nonexpressing HepG2 cells subjected to iron loading for 0, 5, or 72 hr. nTg, non-transgenic control mice; Tg, transgenic mice; HO, heme oxygenase.

Section. Before the treatment, the HO-1 mRNA level was significantly higher in the core-expressing cells than in the control HepG2 cells (Fig. 3B, 1.00 ± 0.33 vs. 0.19 ± 0.10 arbitrary units, $P < 0.05$). After the short-term iron treatment (for 5 hr), the HO-1 mRNA levels increased in both cell lines but it was significantly higher in the control cells than in the core-expressing cells (Fig. 3B, $P < 0.01$). After the long-term iron treatment (72 hr), there was a decrease in the HO-1 mRNA levels in both cell lines compared with those after the short-term iron treatment, but the magnitude of decrease was marked in the core-expressing cells ($P < 0.05$, 5 hr core (+) vs. 72 hr core (+)). Thus, the core protein compromised the iron-induced enhancement of HO-1, and such an effect of the core is more prominent in the long-term iron overloading than in the short-term one. Thus, both in mice and cultured cells, the iron-induced induction of HO-1 was compromised by the presence of the core protein. Similar to the iron-overloading experiment in mice, iron treatment induced ROS production in cultured cells to a greater extent in the core-expressing cells than in control cells (data not shown).

Absence of Nrf2 Involvement in HO-1 Impairment by the Core Protein

To explore the mechanism underlying the differential responses to iron overloading in antioxidant gene expressions, the intracellular distribution of a transcription factor, Nrf2, which regulates the expression of HO-1 [Srisook et al., 2005; Farombi and Surh, 2006] was examined. For this analysis, the liver tissues from the core gene transgenic and control mice, either iron-overloaded or not, were subjected to subcellular fractionation, followed by detection by Western blotting. However, there was no decrease or rather an increase in the Nfr2 level localized in the nuclear fraction after the iron-overloading treatment in the core transgenic mouse liver (Fig. 4A), indicating that the attenuation of HO-1 expression is not dependent on Nrf2. Finally, no interaction was observed between the core protein and the Nrf2 protein as determined by coimmunoprecipitation using cultured cells (data not shown). Because transcription factors, other than Nrf2, such as AP-1 and NF-κB, may be responsible for the HO-1 gene expression [Ferrándiz and Devesa, 2008], and Bach1, an HO-1 repressor [Shan et al., 2004], may be responsible for the attenuation of HO-1 expression, changes in expression levels of these factors were determined by the real-time PCR. AP-1 was activated by the core protein while NF-κB was not in the liver of transgenic mice [Tsutsumi et al., 2002]. With the administration of iron, mRNA levels of AP-1 and NF-κB1 were increased slightly both in core gene transgenic and control mice (Fig. 4B), thereby not explaining the attenuation of iron-induced HO-1 induction by the core protein. Bach1 expression level was not changed significantly by iron administration in the core gene transgenic mice (Fig. 4B), negating the possibility that this repressor acts to inhibit the induction of HO-1 gene expression by iron.

DISCUSSION

Chronic hepatitis C is characterized by its prominent augmentation of oxidative stress [Choi and Ou, 2006; Fujita et al., 2008; Moriya et al., 2001]. Iron accumulation in the liver has been shown to aggravate the oxidative stress as shown by the increase in the amount of DNA adducts in the liver [Chapoutot et al., 2000; Moriya et al., 2001; Choi and Ou, 2006; Fujita et al., 2008]. In this study, iron was accumulated in the liver of the HCV core gene transgenic mice, which is destined to develop HCC after a certain period with increased

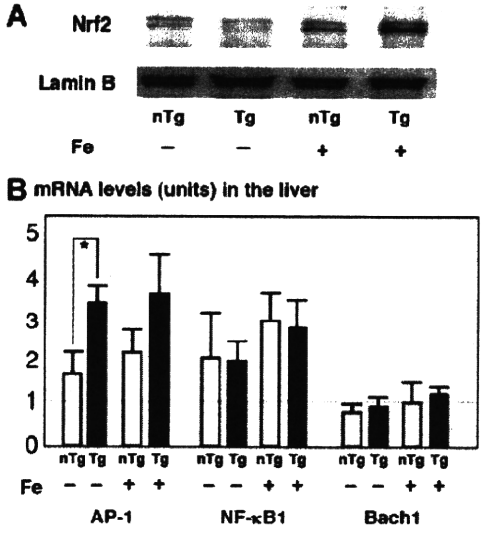


Fig. 4. A: Intracellular localization of Nfr2 in the liver of mice subjected to iron overloading for 0 or 3 months. The nuclear fraction was isolated from liver tissues and subjected to Western blotting with antibody to Nrf2 or lamin B (control). Levels of antioxidant mRNA in the liver of mice subjected to iron overloading. B: Levels of transcription factor mRNA were determined in nontransgenic control mice (open bars) or in transgenic mice (closed bars) with 3-month administration of iron (Fe+) or without it (Fe−). Mice were sacrificed at 6 months and liver tissues were subjected to determination. The data represent means ± SE, n = 5 in each group. *$P < 0.05$. nTg, nontransgenic control mice; Tg, transgenic mice; AP-1, activator protein-1; NF-κB1, nuclear factor-kappa B1; Bach1, BTB and CNC homology 1.

oxidative stress and steatosis [Moriya et al., 1998, 2001]. Some of the key antioxidant enzymes, HO-1 and NQO-1, were not augmented sufficiently by iron overloading, while other antioxidant enzymes such as catalase and GST were augmented more strongly in the iron-overloaded core gene transgenic mice than in the iron-overloaded control or noniron-overloaded core gene transgenic mice.

The accumulation of iron observed in the liver of the core gene transgenic mice fed with normal chow corroborates well the observation in chronic hepatitis C patients [Farinati et al., 1995; Chapoutot et al., 2000; Kato et al., 2001]. Recently, it has been reported that the expression level of hepcidin, which regulates iron metabolism by inhibiting iron absorption from the intestine and utilization from the reticuloendothelial system [Muckenthaler, 2008], is decreased in the liver of mice that are transgenic for the entire HCV genome [Nishina et al., 2008] or hepatitis C patients [Fujita et al., 2007], and is implicated in iron accumulation. ROS overproduction in the liver of the entire HCV genome transgenic mice is suggested to be responsible for the decrease in the hepcidin level [Nishina et al., 2008]. In the current study, however, the level of hepcidin mRNA was significantly higher in the liver of the core gene transgenic mice than that of the control mice despite the augmentation of oxidative stress. In the current model, hepcidin seems to be instructive in the regulation of iron homeostasis and cannot be a cause of hepatitis-C-associated iron accumulation. The reason for this dissociation between the two HCV mouse models is unclear, but the difference in the transgene construct may account for it: the core gene only or the full coding sequence [Moriya et al., 1998; Nishina et al., 2008].

It is interesting that the induction of HO-1 by iron overloading was compromised by the presence of the core protein, while the HO-1 mRNA level was higher in the core gene transgenic mice than in the control mice before iron treatment. This was also observed in the case of another antioxidant, NQO-1 [Nioi and Hayes, 2004], although its induction by iron in the control mice was not as marked as that of HO-1. HO-1 is an inducible cytoprotective enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves pro-oxidant heme to form biliverdin with the release of carbon monoxide. Biliverdin is converted to bilirubin in mammals, both of which have been known to have very strong antioxidant activities [Stocker et al., 1987]. Thus, HO-1 is an antioxidant defense enzyme that converts potentially toxic heme into antioxidants. In addition, HO-1 has been also suggested to be a central antioxidant under the condition of glutathione depletion [Oguro et al., 1998]. Therefore, HO-1 is an essential protective endogenous mechanism against oxidative stress, particularly, in the case of iron overload, although it is hard to compare the abilities of antioxidants quantitatively [Srisook et al., 2005; Farombi and Surh, 2006]. Therefore, it is probable that the attenuation of HO-1 and NQO-1 would hamper the antioxidant system and lead to a robust production of oxidative stress in HCV infection. There have been some contradicting reports on the interaction between HCV and HO-1. Abdalla et al. [2004] reported that the HCV core protein down-regulated HO-1 expression, while Ghaziani et al. [2006] insist that HCV proteins including the core up-regulates HO-1 expression. HO-1 has recently been shown to suppress the propagation of HCV replicons [Zhu et al., 2008] and might be an essential molecule in the pathogenesis of HCV infection.

The mechanism underlying the compromise of iron-induced HO-1 gene augmentation by the core protein is unclear. HO-1 gene has a regulatory element that is controlled by a transcription factor Nrf2 stress [Srisook et al., 2005; Farombi and Surh, 2006]. Therefore, if the subcellular distribution of Nrf2 was altered, that is, nuclear transport were disturbed, it would explain this compromised phenomenon. However, this was not the case in the present experiment. The fact that the GST gene, the expression of which was augmented substantially by the iron overloading, also has a binding site for Nrf2 also negates the involvement of Nfr2 in the compromise by the core protein of iron-induced HO-1 gene augmentation. It has been suggested that complex intracellular signaling cascades mediate the expression of HO-1 in response to external stimuli. Transcription factors, other than Nrf2, such as AP-1 and NF-κB [Ferrándiz and Devesa, 2008] and Bach1, an HO-1 repressor [Shan et al., 2004], may be responsible for the HO-1 gene expression, but these factors did not explain

the current attenuation of HO-1 by the core protein under iron stimulation. Identification of the mechanism may lead to the development of new therapeutic devices with the relief of the core-induced compromise of the iron-induced augmentation of HO-1, which may strengthen the antioxidant system and suppress HCV replication.

ACKNOWLEDGMENTS

This study was supported in part by Grant-in-Aid for Scientific Research on Priority Area from the Ministry of Education, Culture, Science, Sports and Technology of Japan; Health Sciences Research Grants of The Ministry of Health, Labor and Welfare (Research on Hepatitis).

REFERENCES

Abdalla MY, Britigan BE, Wen F, Icardi M, McCormick ML, LaBrecque DR, Voigt M, Brown KE, Schmidt WN. 2004. Down-regulation of heme oxygenase-1 by hepatitis C virus infection in vivo and by the in vitro expression of hepatitis C core protein. J Infect Dis 190: 1109–1118.

Chapoutot C, Esslimani M, Chapoutot C, Esslimani M, Joomaye Z, Ramos J, Perney P, Laurent C, Fabbro-Peray P, Larrey D, Domergue J, Blanc F. 2000. Liver iron excess in patients with hepatocellular carcinoma developed on viral C cirrhosis. Gut 46: 711–714.

Choi J, Ou JH. 2006. Mechanisms of liver injury. III. Oxidative stress in the pathogenesis of hepatitis C virus. Am J Physiol Gastrointest Liver Physiol 290:G847–G851.

Farinati F, Cardin R, De Maria N, Della Libera G, Marafin C, Lecis E, Burra P, Floreani A, Cecchetto A, Naccarato R. 1995. Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. J Hepatol 22:449–456.

Farombi EO, Surh YJ. 2006. Heme oxygenase-1 as a potential therapeutic target for hepatoprotection. J Biochem Mol Biol 39: 479–491.

Ferrándiz ML, Devesa I. 2008. Inducers of heme oxygenase-1. Curr Pharm Des 14:473–486.

Fujita N, Sugimoto R, Takeo M, Urawa N, Mifuji R, Tanaka H, Kobayashi Y, Iwasa M, Watanabe S, Adachi Y, Kaito M. 2007. Hepcidin expression in the liver: Relatively low level in patients with chronic hepatitis C. Mol Med 13:97–104.

Fujita N, Sugimoto R, Ma N, Tanaka H, Iwasa M, Kobayashi Y, Kawanishi S, Watanabe S, Kaito M, Takei Y. 2008. Comparison of hepatic oxidative DNA damage in patients with chronic hepatitis B and C. J Viral Hepat 15:498–507.

Ghaziani T, Shan Y, Lambrecht RW, Donohue SE, Pietschmann T, Bartenschlager R, Bonkovsky HL. 2006. HCV proteins increase expression of heme oxygenase-1 (HO-1) and decrease expression of Bach1 in human hepatoma cells. J Hepatol 45:5–12.

Kato J, Kobune M, Nakamura T, Kuroiwa G, Takada K, Takimoto R, Sato Y, Fujikawa K, Takahashi M, Takayama T, Ikeda T, Niitsu Y. 2001. Normalization of elevated hepatic 8-hydroxy-2'-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 61:8697–8702.

Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. 2006. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. J Virol 80:7199–7207.

Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K. 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 4:1065–1067.

Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Miyazawa T, Ishibashi K, Horie T, Imai K, Miyamura T, Kimura S, Koike K. 2001. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. Cancer Res 61:4365–4370.

Muckenthaler MU. 2008. Fine tuning of hepcidin expression by positive and negative regulators. Cell Metab 8:1–3.

Naas T, Ghorbani M, Alvarez-Maya I, Lapner M, Kothary R, De Repentigny Y, Gomes S, Babiuk L, Giulivi A, Soare C, Azizi A, Diaz-Mitoma F. 2005. Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. J Gen Virol 86:2185–2196.

Nioi P, Hayes JD. 2004. Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. Mutat Res 555:149–171.

Nishina S, Hino K, Korenaga M, Vecchi C, Pietrangelo A, Mizukami Y, Furutani T, Sakai A, Okuda M, Hidaka I, Okita K, Sakaida I. 2008. Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription. Gastroenterology 134:226–238.

Oguro T, Hayashi M, Nakajo S, Numazawa S, Yoshida T. 1998. The expression of heme oxygenase-1 gene responded to oxidative stress produced by phorone, a glutathione depletor, in the rat liver; the relevance to activation of c-jun n-terminal kinase. J Pharmacol Exp Ther 287:773–778.

Ruggieri A, Murdolo M, Harada T, Miyamura T, Rapicetta M. 2004. Cell cycle perturbation in a human hepatoblastoma cell line constitutively expressing hepatitis C virus core protein. Arch Virol 149:61–74.

Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y, Choo Q, Houghton M, Kuo G. 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 87:6547–6549.

Shan Y, Lambrecht RW, Ghaziani T, Donohue SE, Bonkovsky HL. 2004. Role of Bach-1 in regulation of heme oxygenase-1 in human liver cells: Insights from studies with small interfering RNAS. J Biol Chem 279:51769–51774.

Srisook K, Kim C, Cha YN. 2005. Molecular mechanisms involved in enhancing HO-1 expression: De-repression by heme and activation by Nrf2, the "one-two" punch. Antioxid Redox Signal 7:1674–1687.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. 1987. Bilirubin is an antioxidant of possible physiological importance. Science 235:1043–1046.

Tsutsumi T, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Matsuura Y, Kimura S, Koike K, Miyamura T. 2002. Intrahepatic cytokine expression and AP-1 activation in mice transgenic for hepatitis C virus core protein. Virology 304:415–424.

Zhu L, Miller DD. 2007. Tissue iron distribution and urinary mineral excretion vary depending on the form of iron (FeSO4 or NaFeEDTA) and the route of administration in rats given high doses of iron. J Agric Food Chem 55:8793–8799.

Zhu Z, Wilson AT, Mathahs MM, Wen F, Brown KE, Luxon BA, Schmidt WN. 2008. Heme oxygenase-1 suppresses hepatitis C virus replication and increases resistance of hepatocytes to oxidant injury. Hepatology 48:1430–1439.

Oncology

Oncology 2010;78(suppl 1):24–30
DOI: 10.1159/000315226

Published online: July 8, 2010

Lipid Metabolism and Liver Disease in Hepatitis C Viral Infection

Kazuhiko Koike Takeya Tsutsumi Hiroshi Yotsuyanagi Kyoji Moriya

Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Key Words

Hepatitis C · Hepatocellular carcinoma · Transgenic mouse · Core protein · Steatosis · Insulin resistance · Oxidative stress

Abstract

Persistent infection with hepatitis C virus (HCV) is a major risk toward development of hepatocellular carcinoma. A number of transgenic mouse lines carrying the cDNA of HCV genome have been established and evaluated in the study of HCV pathogenesis. Among those, the studies using transgenic mouse lines that carry the HCV genome containing the core gene indicate the direct involvement of HCV in pathogenicity, including that in oncogenesis. Oxidative stress overproduction and intracellular signaling augmentation are shown to be the key events in HCV-associated hepatocarcinogenesis. Besides the data in hepatitis C patients, connecting liver fibrosis progression and the disturbance in lipid and glucose metabolisms, these mouse models also show a close relationship between HCV and metabolic alterations including hepatic steatosis and insulin resistance. Furthermore, the persistent activation of peroxisome proliferator-activated receptor-α has recently been found, yielding dramatic changes in the lipid metabolism and oxidative stress overproduction in cooperation with the mitochondrial dysfunction. These results would provide a clue for further understanding of the role of lipid metabolism in pathogenesis of hepatitis C including liver injury and hepatocarcinogenesis.

Introduction

Hepatitis C virus (HCV) infection frequently evolves into a persistent state, leading to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC). Recently, there have been increasing lines of evidence to indicate metabolic disturbances in HCV infection, which would influence the pathogenesis of chronic hepatitis C. The discovery of HCV in 1989 enabled the comparison between chronic hepatitis C and the other chronic hepatitis, resulting in repeated reports that steatosis is significantly associated with chronic hepatitis C [1, 2]. Steatosis in HCV infection is reproduced in animal models [3] or cultured cells [4], strengthening a pathologic role of HCV in it. Furthermore, patients infected with HCV have abnormalities in serum lipids, such as hypocholesterolemia or abnormal levels of apolipoproteins in serum [5, 6]; they are corrected in sustained virological responders to antiviral treatment [6]. Thus, the association between HCV infection and disturbance in lipid metabolism has become increasingly strong both in patients and experimental systems including animals. Finally, patients with chronic hepatitis C accompanied by severe steatosis develop hepatic fibrosis more rapidly [7]. Thus, abnormal lipid metabolism in HCV infection would be deeply involved in the pathogenesis of hepatitis C.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0030–2414/10/0787–0024$26.00/0

Accessible online at:
www.karger.com/ocl

Kazuhiko Koike, MD, PhD
Department of Gastroenterology
Graduate School of Medicine, University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655 (Japan)
Tel. +81 3 5800 8800, Fax +81 3 5800 8799, E-Mail kkoike-tky@umin.ac.jp

HCV and Hepatocarcinogenesis

The mechanism underlying hepatocarcinogenesis in HCV infection is not fully understood yet, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV [8]. HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence prompted us to seek for determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered, of course, in a study on the hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations 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 context leaves us with a serious question: Can inflammation alone result in the development of HCC in such a high incidence (90% in 15 years) or multicentric nature in HCV infection?

The other role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in which severe inflammation in the liver persists indefinitely, even after the development of cirrhosis. These backgrounds and reasonings lead to a possible activity of viral proteins for inducing neoplasia. 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. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C, in vivo, by transgenic mouse technology.

Transgenic Mouse Lines Carrying the HCV Genome

As described above, the HCV proteins have been characterized chiefly using in vitro translation or cultured cells. Little is known, however, about the role of HCV or its proteins in the pathogenesis of hepatitis and subsequent liver diseases, cirrhosis and HCC. One of the major issues regarding the pathogenesis of HCV-associated liver lesion is whether the HCV proteins have direct effects on pathological phenotypes. Although several strategies have been used to characterize the hepatitis C viral proteins, the relationship between the protein expression and disease phenotype has not been clarified. For this purpose, several lines of mice have been established which were transgenic for the HCV cDNA. They include the ones carrying the entire coding region of HCV genome [9], the core region only [3, 10], the envelope region only [11], the core and envelope regions [12] and the core to non-structural (NS)2 regions [13]. Although detection of mRNA from the NS regions of the HCV cDNA has been reported [9], the detection of HCV NS proteins in the transgenic mouse liver has not been successful. The reason for this failure in detecting NS proteins is unclear, but the expression of the NS enzymes may be harmful to mouse development and may allow the establishment of only low-expression mice.

In terms of expression system, two different ways have been applied: transient and constitutive expression systems. One transgenic mouse line has been reported which expresses the HCV genes using a transient expression system. Wakita et al. [13] utilized the Cre/*lox*P system, by which a gene under silent can be switched on by the introduction of Cre recombinase. They established a transgenic mouse line that had the core, envelopes and NS2 genes of HCV in a silent state. After the injection of the recombinant adenovirus that had *Cre* recombinase in the mice, the HCV genes expressed transiently. These mice developed acute hepatitis, which was blocked by the administration of anti-CD4 and CD8 antibodies. This mouse system would provide a good animal model for acute hepatitis C and be useful for the study of immunological aspects of hepatitis. The possibility, however, that the greatly overexpressed HCV proteins had caused the death of hepatocytes and provoked the immune response thereafter still remains.

We have engineered transgenic mouse lines carrying the HCV genome were by introducing the genes from the cDNA of the HCV genome of genotype 1b [3, 14]. Established are four different kinds of transgenic mouse lines, which carry the core gene, envelope genes, the entire NS genes, or NS5A gene, respectively, under the same transcriptional regulatory element (fig. 1). Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages [14]. The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins [11, 15], and the transgenic mice carrying the entire NS or NS5A gene have developed no HCC.

The core gene transgenic mice express the core protein of an expected size, and the level of the core protein in the liver is similar to that in chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is

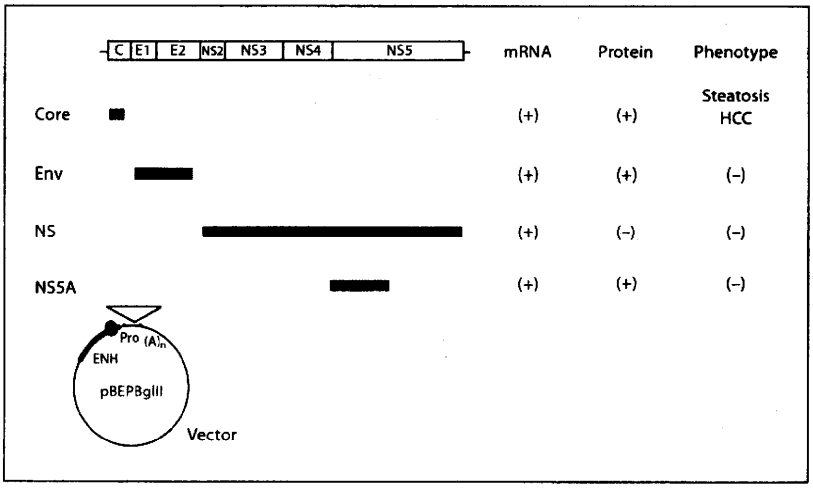


Fig. 1. Transgenic mouse lines carrying the HCV genome. Four different kinds of transgenic mouse lines, carrying the core gene, envelope genes, the entire NS genes, or NS5A of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, only the transgenic mice carrying the HCV core gene develop HCC after an early phase with hepatic steatosis in two independent lineages. HCV = Hepatitis C virus; HCC = hepatocellular carcinoma; Env = envelope genes; NS = non-structural genes.

one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damages [1]. Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. Of note, any pictures of significant inflammation are not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. 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 [9, 10, 12]. These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed in vivo.

Enhancement of Oxidative Stress and Intracellular Signaling in HCV-Associated Hepatocarcinogenesis

It is difficult to elucidate the mechanism underlying the development of HCC, even for our simple model in which only the core protein is expressed in otherwise normal liver. 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 [14]. On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver. 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 [16, 17]. The overproduction of oxidative stress results in the generation of deletions in the mitochondrial and nuclear DNA, an indicator of genetic damage. In addition, analysis of antioxidant system revealed that some antioxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice. These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of antioxidant system, which may explain the mechanism underlying the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

In the absence of inflammation, thus, the core protein induces oxidative stress overproduction, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that cannot be scavenged anymore by a physiological antagonistic system. This suggests that the inflammation in chronic HCV infection would have a characteristic different in its quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction [16, 18]. The dysfunction of the electron transfer system of the mitochondrion is suggested in as-

sociation with the presence of the HCV core protein [18, 19].

Other pathways in hepatocarcinogenesis would be the alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin-1β have been found transcriptionally activated [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. In the downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced [20, 21]. At far downstream, both the mRNA and protein levels of cyclin D1 and cyclin-dependent kinase (CDK)4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes.

Hepatitis C as a Metabolic Disease

Steatosis is frequently observed in chronic hepatitis C patients, and significantly associated with increased fibrosis and progression rate of fibrosis of the liver [22]. A comprehensive analysis of gene expression in the liver of core gene transgenic mice, in which steatosis develops from early in life, revealed that a number of genes related to lipid metabolism are significantly up- or downregulated.

The composition of fatty acids that are accumulated in the liver of core gene transgenic mice is different from that in fatty liver due to simple obesity. Carbon-18 monounsaturated fatty acids (C18:1) such as oleic or vaccenic acids are significantly increased. This is also the case in the comparison of liver tissues from hepatitis C patients and simple fatty liver patients due to obesity [17]. The mechanism of steatogenesis in hepatitis C was investigated using this mouse model. There are, at least, three pathways for the development of steatosis. One is the frequent presence of insulin resistance in hepatitis C patients as well as in the core gene transgenic mice, which occurs through the inhibition of tyrosine phosphorylation of insulin receptor substrate (IRS)-1 [23]. Insulin resistance increases the peripheral release and hepatic uptake of fatty acids, resulting in an accumulation of lipid in the liver. The second pathway is the suppression of the activity of microsomal triglyceride transfer protein (MTP) by HCV core protein [24]. This inhibits the secretion of very-low-density protein (VLDL) from the liver, yielding an increase of triglycerides in the liver. The last one involves by the sterol regulatory element-binding protein (SREBP)-1c, which regulates the production of triglycerides and phospholipids. In HCV core gene transgenic mice, SREBP-1c is activated, while neither SREBP-2 nor SREBP-1a is upregulated [25].

Steatosis, HCV and PA28γ

Interestingly, we found recently that a protein interacting with the core protein, proteasome activator (PA)28γ, is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC [3, 25]. Steatosis is defined as an accumulation of lipid droplets, a majority of which are triglycerides. Biosynthesis of triglycerides is mainly regulated by SREBP-1c. Transcription of SREBP-1c is controlled by a heterodimer of nuclear hormone receptors, liver X receptor (LXR)-α and retinoid X receptor (RXR)-α. Indeed, it has been reported that many genes regulated by SREBPs were induced during the early stage of HCV infection in the livers of chimpanzees. Our study has demonstrated that the core protein enhances the binding activity of the LXR-α–RXR-α complex to the *srebp-1c* promoter in a PA28γ-dependent manner, resulting in upregulation of SREBP-1c and its regulating genes [25]. The activation may be mediated by the direct interaction between the core protein and RXR-α [26] or by suppression of a corepressor such as Sp110b, a negative regulator of RAR-α, by sequestering it in the cytoplasm via interaction with the cytoplasmic core protein [27]. Another mechanism is thought to be suppression of lipid secretion. Reduced serum levels of cholesterol and apolipoprotein B have been reported in patients with severe hepatitis C and the core gene transgenic mice [5]. As stated before, the MTP regulates the assembly and secretion of VLDLs consisting of apolipoprotein B, cholesterol and triglycerides. In the core gene transgenic mice, MTP-specific activity is significantly decreased. Therefore, the downregulation of MTP may be involved in the development of the steatosis cooperating with upregulation of SREBP-1c, although the precise role of HCV core protein is still unclear. Recently, it has been reported that the assembly and budding of HCV occur around the accumulated lipid droplets within the endoplasmic reticulum [28]. Furthermore,

increases in saturated and monounsaturated fatty acids enhance HCV RNA replication. These data suggest that regulation of lipid metabolism by the core protein plays crucial roles in the HCV life cycle. Obesity and hepatic steatosis often result in insulin resistance. However, 1- to 2-month-old core gene transgenic mice, which do not exhibit apparent steatosis and obesity, already exhibit insulin resistance due to a decrease in insulin sensitivity in the liver [23]. Moreover, the core gene transgenic mice have been shown to exhibit overt diabetes when fed a high-fat diet, while control mice do not. Binding of insulin to the insulin receptor triggers tyrosine phosphorylation of the IRS proteins, leading to the following signal transductions to increase glucose uptake and inhibit the net production of glucose in the liver. An inflammatory cytokine, TNF-α, is known to impair the insulin-signaling pathway via inhibition of tyrosine phosphorylation of IRSs. In fact, the overproduction of TNF-α has been reported to reduce the phosphorylation of IRS-1 and Akt in the core gene transgenic mice despite the absence of hepatic. In the latter study, moreover, hyperinsulinemia was cured by depletion of TNF-α, suggesting that upregulation of TNF-α contributes to the core protein-induced insulin resistance [23]. Our previous study has indicated that the core protein-induced overexpression of TNF-α is also dependent on the presence of PA28γ [25].

In relation to lipid metabolism, the core protein has also been found to interact with RXR-α [26]. RXR-α is one of the nuclear receptors which forms a homodimer or heterodimers with other nuclear receptors including peroxisome proliferator-activated receptor (PPAR)-α, and plays a pivotal role in the regulation of the expression of genes relating to lipid metabolism, cell differentiation and proliferation. In fact, the core protein of HCV activates genes that have an RXR-α-responsive element as well as those with a PPAR-α-responsive element, in both mice and cultured cells [26]. Based on these results, we, then, examined the expression and function of PPAR-α in the liver of core gene transgenic mice.

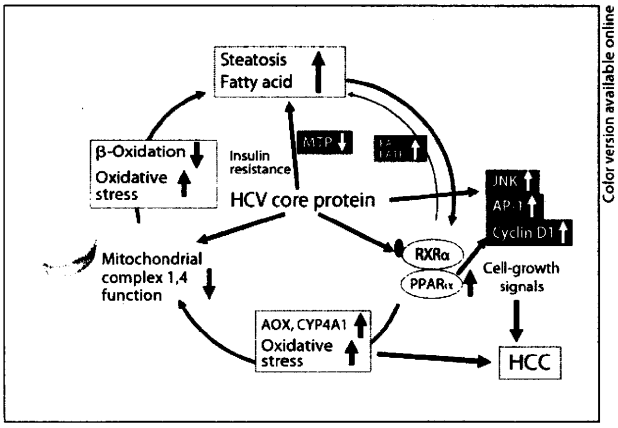
PPAR-α Activation and 'Fatty Acid Spiral' in HCV-Associated Hepatocarcinogenesis

PPAR-α is one of PPAR genes, and plays a central role, as a heterodimer with RXR-α, in regulating fatty acid transport and catabolism. It is also known as a molecular target for lipid-lowering fibrate drugs. On the other hand, a prolonged administration of PPAR-α agonists causes HCC in rodents. Currently, there is little evidence that the low-affinity fibrate ligands are associated with human cancers, but it is possible that chronic activation of high-affinity ligands could be carcinogenic in humans.

The level of PPAR-α protein was increased in the liver of core gene transgenic mice as early as 9 months. PPAR-α protein is accumulated with age in the nuclei of hepatocytes together with cyclin D1 protein. However, the level of PPAR-α mRNA was not increased at any age. By the pulse-chase experiment, the stability of nuclear PPAR-α turned out to be increased in the presence of the core protein. In line with the increase of PPAR-α protein, target genes of PPAR-α were activated in the liver of core gene transgenic mice; these genes include cyclin D1, CDK4, acy-CoA oxidase, and peroxisome thiolase [29]. However, in general, the activation of PPAR-α leads to improvement but not aggravation of steatosis. Then, what is a function of PPAR-α activation that is observed in the core gene transgenic mice?

To clarify the role of PPAR-α activation in pathogenesis of steatosis and HCC, we made mating of core gene transgenic mouse with PPAR-α knockout (KO) mouse, and studied the phenotype. PPAR-α KO mice have reduced expressions of target genes of PPAR-α, and have mild steatosis in the liver as expected. It was unanticipated, however, that steatosis was absent in PPAR-α-null or -heterozygous core gene transgenic mice but present in PPAR-α-intact core gene transgenic mice at the age of 9 or 24 months [29]. 8-Hydroxydeoxyguanosine and peroxylipids, both of which are markers for oxidative stress, were decreased in PPAR-α KO core gene transgenic mice. Mitochondrial dysfunction in the core gene transgenic mice, which contributes to an overproduction of oxidative stress, was also improved in PPAR-α KO core gene transgenic mice.

Finally, PPAR-α KO core gene transgenic mice did not develop HCC at the age of 24 months, while about one-third of PPAR-α-intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for PPAR-α gene did not develop HCC either [29]. When clofibrate, a peroxisome proliferator, was administered for 24 months to PPAR-α-heterozygous mice, either with or without the core gene, HCC developed in a higher rate in the core-gene (+) mice with a greater PPAR-α activation. It should be noted that steatosis was present only in core-gene (+) PPAR-α-heterozygous mice. In summary, steatosis and HCC developed in PPAR-α-intact but not in PPAR-α-heterozygous or PPAR-α-null core gene transgenic mice, indicating that not the presence but the persistent activation of PPAR-α would be important in hepatocarcinogenesis by HCV



Color version available online

Fig. 2. HCV core protein causes 'fatty acid spiral'. In HCV infection, the core protein induces steatosis via several pathways, leading to 'fatty acid spiral' in the presence of the mitochondrial complex-1 dysfunction and PPAR-α activation, both of which are also caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling. HCV = Hepatitis C virus; HCC = hepatocellular carcinoma; JNK = c-Jun N-terminal kinase; AP-1 = activating protein-1; RXRα = retinoid X receptor-α; PPARα = peroxisome proliferator activated receptor-α; AOX = acyl-CoA oxidase; CYP = cytochrome P450; MTP = microsomal triglyceride transfer protein; FAT = fatty acid translocase; FATP = fatty acid transport protein.

core protein. In general, PPAR-α acts to ameliorate steatosis, but with the presence of mitochondrial dysfunction, which is also provoked by the core protein, the core-activated PPAR-α may exacerbate steatosis. A persistent activation of PPAR-α with 'strong' ligands such as the core protein of HCV could be carcinogenic in humans, although the low-affinity fibrate ligands are not likely associated with human cancers.

Figure 2 illustrates our current hypothesis for the role of lipid metabolism in HCV-associated hepatocarcinogenesis. Immune-mediated inflammation should also play a pivotal role in hepatocarcinogenesis in HCV infection. However, in HCV infection, the core protein induces steatosis through the above-mentioned pathways, leading to the 'fatty acid spiral' in the presence of the mitochondrial electron transfer system dysfunction [18, 19] and PPAR-α activation, both of which are caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling.

Conclusion

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory by Kinzler and Vogelstein [30] has gained a wide popularity. 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 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 [30]. Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis.

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for the hepatocarcinogenesis in HCV infection. We do 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. 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. 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.

Disclosure Statement

The authors declare that they have no financial conflict of interest.

References

1 Bach N, Thung SN, Schaffner F: The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. Hepatology 1992;15:572–577.

2 Lefkowitch JH, Schiff ER, Davis GL, Perrillo RP, Lindsay K, Bodenheimer HC Jr, et al: Pathological diagnosis of chronic hepatitis C: a multicenter comparative study with chronic hepatitis B. Gastroenterology 1993; 104:595–603.

3 Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al: Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 1997;78: 1527–1531.

4 Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, et al: Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci USA 1997;94: 1200–1205.

5 Moriya K, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Yotsuyanagi H, et al: Serum lipid profile of patients with genotype 1b hepatitis C viral infection in Japan. Hepatol Res 2003;25:369–374.

6 Naeem M, Bacon BR, Mistry B, Britton RS, Di Bisceglie AM: Changes in serum lipoprotein profile during interferon therapy in chronic hepatitis C. Am J Gastroenterol 2001;96:2468–2472.

7 Lonardo A, Adinolfi LE, Loria P, Carulli N, Ruggiero G, Day CP: Steatosis and hepatitis C virus: mechanisms and significance for hepatic and extrahepatic disease. Gastroenterology 2004;126:586–597.

8 Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, et al: Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 1990;87:6547–6549.

9 Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, et al: Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology 2002;122:352–365.

10 Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM: Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. J Virol 2006;80:7199–7207.

11 Koike K, Moriya K, Ishibashi K, Matsuura Y, Suzuki T, Saito I, et al: Expression of hepatitis C virus envelope proteins in transgenic mice. J Gen Virol 1995;76:3031–3038.

12 Naas T, Ghorbani M, Alvarez-Maya I, Lapner M, Kothary R, De Repentigny Y, et al: Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. J Gen Virol 2005;86:2185–2196.

13 Wakita T, Taya C, Katsume A, Kato J, Yonekawa H, Kanegae Y, et al: Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the Cre/loxP system. J Biol Chem 1998;273: 9001–9006.

14 Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Matsuura Y, et al: Hepatitis C virus core protein induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065–1068.

15 Koike K, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, et al: Sialadenitis resembling Sjögren's syndrome in mice transgenic for hepatitis C virus envelope genes. Proc Natl Acad Sci USA 1997;94:233–236.

16 Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, et al: Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocellular carcinogenesis. Cancer Res 2001; 61:4365–4370.

17 Moriya K, Todoroki T, Tsutsumi T, Fujie H, Shintani Y, Miyoshi H, 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: 1207–1212.

18 Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA: Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology 2002;122:366–375.

19 Tsutsumi T, Matsuda M, Aizaki H, Moriya K, Miyoshi H, Fujie H, Shintani Y, Yotsuyanagi H, Miyamura T, Suzuki T, Koike K: Proteomics analysis of mitochondrial proteins reveals overexpression of a mitochondrial protein chaperone, prohibitin, in cells expressing hepatitis C virus core protein. Hepatology 2009;50:378–386.

20 Tsutsumi T, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, et al: Intrahepatic cytokine expression and AP-1 activation in mice transgenic for hepatitis C virus core protein. Virology 2002;304:415–424.

21 Tsutsumi T, Suzuki T, Moriya K, Shintani Y, Fujie H, Miyoshi H, et al: Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice. Hepatology 2003;38:820–828.

22 Powell EE, Jonsson JR, Clouston AD: Steatosis: co-factor in other liver diseases. Hepatology 2005;42:5–13.

23 Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Kimura S, Moriya K, et al: Hepatitis C virus and diabetes: direct involvement of the virus in the development of insulin resistance. Gastroenterology 2004;126:840–848.

24 Perlemuter G, Sabile A, Letteron P, Vona G, Topilco A, Koike K, 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;16:185–194.

25 Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, et al: Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc Natl Acad Sci USA 2007;104:1661–1666.

26 Tsutsumi T, Suzuki T, Shimoike T, Moriya K, Yotsuyanagi H, Matsuura Y, et al: Interaction of hepatitis C virus core protein with retinoid X Receptor alpha modulates its transcriptional activity. Hepatology 2002;35: 937–946.

27 Watashi K, Hijikata M, Tagawa A, Doi T, Marusawa H, Shimotohno K: Modulation of retinoid signaling by a cytoplasmic viral protein via sequestration of Sp110b, a potent transcriptional corepressor of retinoic acid receptor, from the nucleus. Mol Cell Biol 2003;23:7498–7509.

28 Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, et al: The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 2007;9:1089–1097.

29 Tanaka N, Moriya K, Kiyosawa K, Koike K, Gonzalez FJ, Aoyama T: PPAR-α is essential for severe hepatic steatosis and hepatocellular carcinoma induced by HCV core protein. J Clin Invest 2008;118:683–694.

30 Kinzler KW, Vogelstein B: Lessons from hereditary colorectal cancer. Cell 1996;87:159–170.

Hepatology Research 2010; 40: 69–82 doi: 10.1111/j.1872-034X.2009.00593.x

Review Article

Animal models for hepatitis C and related liver disease

Kazuhiko Koike,[1] Kyoji Moriya[2] and Yoshiharu Matsuura[3]

Departments of [1]Gastroenterology and [2]Infection Control and Prevention, Graduate School of Medicine, University of Tokyo, Tokyo, and [3]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Persistent infection with hepatitis C virus (HCV) is a major risk toward development of hepatocellular carcinoma (HCC). The elucidation of pathogenesis of HCV-associated liver disease is hampered by the absence of appropriate animal models: there has been no animal model for HCV infection/pathogenesis except for the chimpanzee. In contrast, a number of transgenic mouse lines carrying the cDNA of the HCV genome have been established and evaluated in the study of HCV pathogenesis. The studies using transgenic mouse models, in which the HCV proteins such as the core protein are expressed, indicate the direct pathogenicity of HCV, including oncogenic activities. HCV transgenic mouse models also show a close relationship between HCV and some hepatic and extrahepatic manifestations such as hepatic steatosis, insulin resistance or Sjögren's syndrome. A crucial role of hepatic steatosis and insulin resistance in the pathogenesis of liver disease in HCV infection has been demonstrated, implying hepatitis C to be a metabolic disease. Besides the data connecting liver fibrosis progression and the disturbance in lipid and glucose metabolisms in hepatitis C patients, a series of evidence was found showing the association between these two conditions and HCV infection, chiefly using transgenic mouse carrying the HCV genome. Furthermore, the persistent activation of peroxisome proliferator-activated receptor (PPAR)-α has recently been found, yielding dramatic changes in the lipid metabolism and oxidative stress overproduction in cooperation with the mitochondrial dysfunction. These results would provide a clue for further understanding of the role of lipid metabolism in pathogenesis of hepatitis C including liver injury and hepatocarcinogenesis.

Key words: core protein, hepatitis C, hepatocellular carcinoma, insulin resistance, steatosis, transgenic mouse.

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection frequently evolves into a persistent state, leading to the development of chronic hepatitis, cirrhosis and, eventually, hepatocellular carcinoma (HCC). For understanding of the mechanism of entry into hepatocytes, replication and the pathogenesis of HCV, an *in vitro* replication system or animal models for HCV infection/pathogenesis have been eagerly awaited. An *in vitro* HCV replication system was not established until the development of a subgenomic, non-structural region HCV replicon system or an infectious genotype 2a HCV clone, JFH-1.[1] There has been no animal model for HCV infection/pathogenesis except for the chimpanzee.[2] Recently, however, several small animal models for HCV infection have been evaluated, including *Tupaia*[3] and genetically engineered mice that are chimeric for human hepatocytes.[4] On the other hand, a number of transgenic mouse lines carrying the cDNA of HCV genome have been established and evaluated in the study of HCV pathogenesis, as described hereafter. These mice, including those that are transgenic for the core gene of HCV, show the features resembling those of chronic hepatitis C patients, such as hepatic steatosis, insulin resistance and HCC. These animal models provide us a molecular understanding of the pathogenesis of HCV infection and a perspective for the future development of treatment and prophylaxis of liver disease occurring in HCV infection.

Correspondence: Professor Kazuhiko Koike, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: kkoike-tky@umin.ac.jp

Received 19 June 2009; revision 31 July 2009; accepted 31 July 2009.

THE CHIMPANZEE MODEL

AS EARLY AS the discovery of the cDNA clone of HCV, or even before that, the chimpanzee has been known to be susceptible to HCV (or the non-A, non-B

hepatitis agent), and has long been used as a sole animal model for HCV infection.[2] However, due to ethical reasons and vast costs, the use of this animal for HCV research is limited: the data on this animal model were obtained from the studies chiefly conducted in the USA. The serum samples from hepatitis C patients were inoculated to chimpanzees, and the natural course was evaluated in biochemical, virological or histological methods. These studies demonstrated that the course of HCV infection in this animal is similar to that in human beings, warranting the chimpanzee to be a good animal model for HCV infection, albeit HCC being a rare occurrence in chimpanzees.

In 1997, potential infectious HCV clones, which were produced by several study groups, were evaluated for *in vivo* infectivity using chimpanzees. The chimpanzees were also used for the evaluation of a role of cellular immunity in acute HCV infection: intrahepatic $CD4^+$ or $CD8^+$ T-cell response was found to play a crucial role in the eradication of HCV from the liver. Recently, this animal is also used for the evaluation of candidates for HCV vaccines and the assessment of *in vivo* infectivity of JFH-1 HCV viral clone, which shows a robust replication in human HCC-derived HuH-7 cells.[1] Immunization with virus-like particles of chimpanzees induced an HCV-specific immune response of $CD4^+$ or $CD8^+$ T cells, thereby suppressing the development of high viral loads in chimpanzees that were challenged with HCV.[5] Also, inoculation of the non-structural proteins of HCV using recombinant adenovirus vector induced HCV-specific immune T-cell response, leading to a significant suppression of replication of genotype 1a HCV that was challenged after immunization.[6]

In general, the liver lesions observed in HCV-inoculated chimpanzees are milder than those in human chronic hepatitis C patients, for example, cirrhosis or HCC rarely develops, but the morphological changes and inflammatory responses are similar to those in humans.[2] Therefore, the studies using chimpanzees are indispensable now and in the future for the analyses of viral replication, pathogenesis of liver disease and the evaluation of candidates for HCV vaccines.

THE SMALL PRIMATES MODEL

TUPAIA (*TUPAIA BELANGERI chinensis*), a small primate resembling the squirrel, has been reported to be susceptible to hepatitis B virus (HBV) infection in 1996,[3] and was used for the study of HCV infection.[7] However, only a quarter of inoculated individuals contracted HCV infection, and developed only a transient or intermittent viremia with low viral loads. Another study group reported on the usefulness of how a primary culture of hepatocytes from the liver of Tupaia can be infected with serum- or plasma-derived HCV from infected humans, as measured by de novo synthesis of HCV RNA, analysis of viral quasispecies evolution, and detection of viral proteins.[8,9]

While the development of liver disease (a cirrhosis-like lesion) in HCV-infected Tupaia was presented at scientific meetings, a scientific paper describing it has not appeared yet. In conclusion, the value of Tupaia in HCV research is limited, but it may be utilized for the analysis of viral entry or replication when HCV particles other than JFH-1 are used for the study.

HCV

THE DEVELOPMENT OF transgenic mouse technology was a great step forward in biotechnology in that this technology provides opportunities to examine *in vivo* an exceptionally wide variety of biological questions that were previously examined only *in vitro*. The selective addition of defined genes to the genome of a living animal is useful for investigating the consequences of expression of dominant genes, and thus a number of exogenous genes including oncogenes and humoral factor genes have been introduced into mouse eggs. Viral genes have also been transferred to define the complex cascades of events that can be triggered *in vivo* in response to the expression of a viral protein.

Hepatitis C virus is an enveloped RNA virus of the *Flavivirus* family, in which a positive-sense, single-stranded RNA genome of approximately 9600 nucleotides (nt) is contained within the nucleocapsid.[10] The genome consists of a large translational open reading frame (ORF) encoding a polyprotein of approximately 3010 amino acids (aa) (Fig. 1). The ORF is flanked by highly conserved untranslated regions (UTR) at both the 5′ and 3′ termini. The complete 5′ UTR consists of 341 nt and acts as an internal ribosomal entry site. This feature leads to the translation of the RNA genome using a cap-independent mechanism, rather than ribosome scanning from the 5′ end of a capped molecule.

The polyprotein is processed by both the cellular and viral proteases to generate the viral gene products, which are subdivided into the structural and non-structural proteins. The structural proteins, which are encoded by the NH_2-terminal quarter of the genome, include the

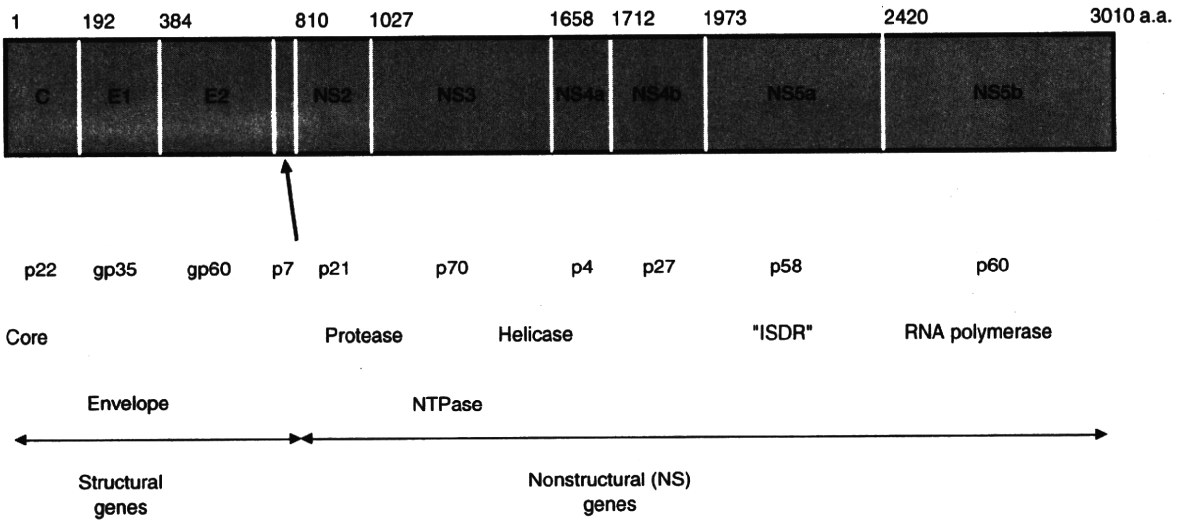


Figure 1 The structure of hepatitis C virus (HCV) genome. The HCV genome RNA encodes a polyprotein of 3010 amino acids (a.a.), which is processed to structural and non-structural proteins by the cellular or viral proteases. ISDR, interferon sensitivity-determining region.

core protein and the envelope proteins, E1 and E2. The E2 has an alternative form, E2-p7, though it is not clear whether or not the p7 composes the viral particle. The NS2, NS3, NS4A, NS4B, NS5A and NS5B are the non-structural proteins that are coded in the remaining portion of the polyprotein. These include serine protease (NS3/4A), NTPase/helicase (NS3) and RNA-dependent RNA polymerase (NS5B).

The core protein of HCV occupies residues 1–191 of the precursor polyprotein and is cleaved between the core and E1 protein by host signal peptidase. The C-terminal membrane anchor of the core protein is further processed by host signal peptide peptidase.[11] The mature core protein is estimated to consist of 177–179 amino acids and shares high homology among HCV genotypes. The HCV core protein possesses the hydrophilic N-terminal region "domain 1" (residues 1–117) followed by a hydrophobic region called "domain 2", which is located from residue 118–170. The domain 1 is rich in basic residues, and is implicated in RNA-binding and homo-oligomerization. The amphipathic helices I and II spanning from residue 119–136 and residue 148–164, respectively, in domain 2 are involved in the association of HCV core protein with lipid.[12] In addition, the region spanning from residue 112–152 is associated with membranes of the endoplasmic reticulum and mitochondria.[13] The core protein is also localized into the nucleus[14,15] and binds to the nuclear proteasome activator PA28γ/REGγ, resulting in PA28γ-dependent degradation of the core protein.[16]

A recent report suggests that ubiquitination and adenosine triphosphate (ATP) are not required for PA28γ-dependent proteasome activity.[17] HCV core protein is also known to be ubiquitinated by E3 ligase E6AP and degraded in the ubiquitin/ATP-dependent pathway.[14] Thus, the HCV core protein is degraded in at least two different ways. To further assess the pathological significance of the interaction of core protein with PA28γ, Core-Tg/PA28γ-knockout mice have been generated and analyzed as described below (section 9).[15]

POSSIBLE ROLE OF HCV IN HEPATOCARCINOGENESIS

THE MECHANISM UNDERLYING hepatocarcinogenesis in HCV infection is not fully understood yet, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV.[18–20] HCV infection is also common in patients with HCC in other countries albeit to a lesser extent. These lines of evidence prompted us to seek to determine the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered, of course, in a study on the hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells,