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Review

Metabolic aspects of hepatitis C viral infection: steatohepatitis resembling but distinct from NASH

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Although the target of hepatitis C virus (HCV) infection is the liver, it has become progressively more evident that HCV can induce diseases in numerous organs. Recently, much attention has been drawn to metabolic disorders in HCV infection. Initially, hepatic steatosis and disturbances in lipid metabolism were found to be characteristic of HCV infection, and, subsequently, a correlation was noted between HCV infection and diabetes. It is now evident that HCV, by itself, can induce insulin resistance by way of disturbing the intracellular signaling pathway of insulin by the function of HCV core protein. Insulin resistance, caused by HCV infection, evolves to type 2 diabetes when superimposed on a high-fat diet and obesity. The fact that HCV infection induces insulin resistance by the virus itself may influence the progression of chronic hepatitis and open up novel therapeutic approaches. When hepatitis C is compared with nonalcoholic steatohepatitis (NASH), there are a number of similarities and several differences. From the metabolic aspect, hepatitis C resembles NASH in numerous features, such as the presence of steatosis, serum dyslipidemia, and oxidative stress in the liver, suggesting that hepatitis C is a steatohepatitis. In contrast, there are noticeable differences between hepatitis C and NASH, in that HCV modulates cellular gene expression and intracellular signal transduction, including the activation of mitogen-activated protein (MAP) kinase and transcription factor activator protein (AP)-1, while such details have not been noted for NASH. This difference may explain the markedly higher incidence of HCC development in chronic hepatitis C compared with that in NASH. HCV infection needs to be viewed not only as a liver disease but also as a metabolic disease, and this viewpoint could open up a novel way to the molecular understanding of the pathogenesis of hepatitis C, as a virus-associated steatohepatitis (VASH).

Key words: diabetes, hepatitis C virus, insulin resistance, steatohepatitis, hepatocarcinogenesis, lipid metabolism

Introduction

Approximately 1.8 million people in Japan and 200 million people in the world are chronically infected with hepatitis C virus (HCV). Chronic HCV infection may lead to cirrhosis and hepatocellular carcinoma (HCC), thereby being a worldwide problem, both from the medical and socioeconomic aspects.[1] In addition, chronic HCV infection is a multifaceted disease, which is associated with numerous clinical manifestations, such as type II mixed cryoglobulinemia, porphyria cutanea tarda, and membranoproliferative glomerulonephritis (Table 1).[2] Furthermore, strong associations of HCV infection with Sjögren's syndrome and lichen planus have been noted, which have been validated in an animal model.[3]

Steatosis and HCV infection

In addition, 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 a comparison between chronic hepatitis C and other types of chronic hepatitis, resulting in repeated reports that steatosis was significantly associated with chronic hepatitis C.[4,5] Steatosis in HCV infection is reproduced in animal models[6] and cultured cells,[7]

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strengthening the idea of a pathologic role of HCV in steatosis. Furthermore, patients infected with HCV have abnormalities in serum lipids, such as hypocholesterolemia or abnormal levels of apolipoproteins in serum;[8,9] these abnormalities are corrected in sustained virological responders to antiviral treatment.[9] Thus, the association shown between HCV infection and disturbances in lipid metabolism has become increasingly stronger both in patients and in experimental systems, including animals. Further, patients with chronic hepatitis C accompanied by severe steatosis develop hepatic fibrosis more rapidly than those without steatosis.[10] Thus, abnormal lipid metabolism in HCV infection could be deeply involved in the pathogenesis of hepatitis C.

Diabetes may also be a manifestation of HCV infection

Another metabolic aspect of HCV infection is type 2 diabetes. In 1994, Allison et al.[11] reported an epidemiological link between diabetes and HCV infection, but in a cirrhotic cohort. This report made little impact, however, in view of the well-known impaired glucose tolerance in advanced chronic liver disease. Several reports followed along this line, from the same group and others. The trend to accept a positive association between diabetes and HCV infection seems to have been triggered by a population-based study in the United States,[12] in which a solid association was found between them. The association between diabetes and HCV infection, however, is confounded by factors such as the development of cirrhosis, obesity, and older age, which are common in patients with hepatitis C; these factors could make it difficult to prove this association to be real. Hence, there is a need to evaluate the association, using experimental systems.

Table 1. Hepatitis C as a multifaceted disease

Hepatitis, cirrhosis and, eventually, HCC
Mixed cryoglobulinemia
MPGN
Sjögren's syndrome
Lichen planus
B-cell lymphoma
Disturbance in lipid metabolism
Diabetes or insulin resistance

HCV infection induces insulin resistance in vivo

We used mice transgenic for the HCV core gene[6,13] to assess the association between HCV infection and diabetes. These mice carry the core gene of genotype 1b HCV, and express HCV core protein of an expected size in the liver, in levels comparable to those in patients with chronic hepatitis C (Fig. 1). They develop HCC late in life.[13] These transgenic mice were maintained and fed together with their normal littermates, and the glucose metabolism was studied.[14] Although the core gene transgenic mice did not develop overt diabetes, they had markedly elevated serum levels of insulin. Plasma glucose levels were somewhat higher in transgenic mice than in their normal control littermates, but there was

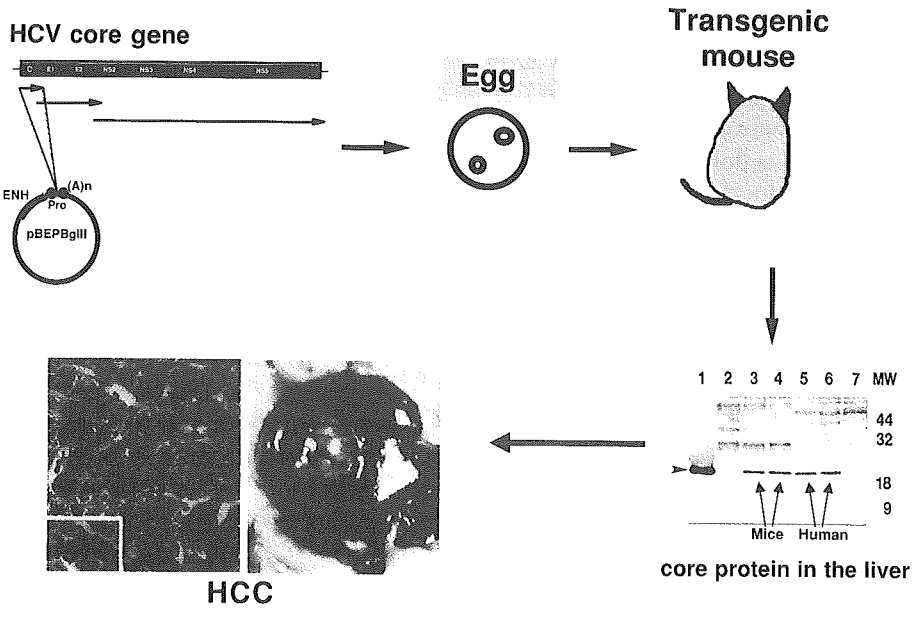

Fig. 1. Mouse model of hepatitis C virus (*HCV*)-induced liver pathogenesis. HCV core gene transgenic mice carry the core gene, alone, of genotype 1b HCV and express the core protein of an expected size in the liver, at levels comparable to those in human patients with chronic hepatitis C. The mice eventually develop hepatocellular carcinoma (*HCC*) late in life. *ENH*, enhancer; *Pro*, promoter; *A(n)*, polyadenylation signal

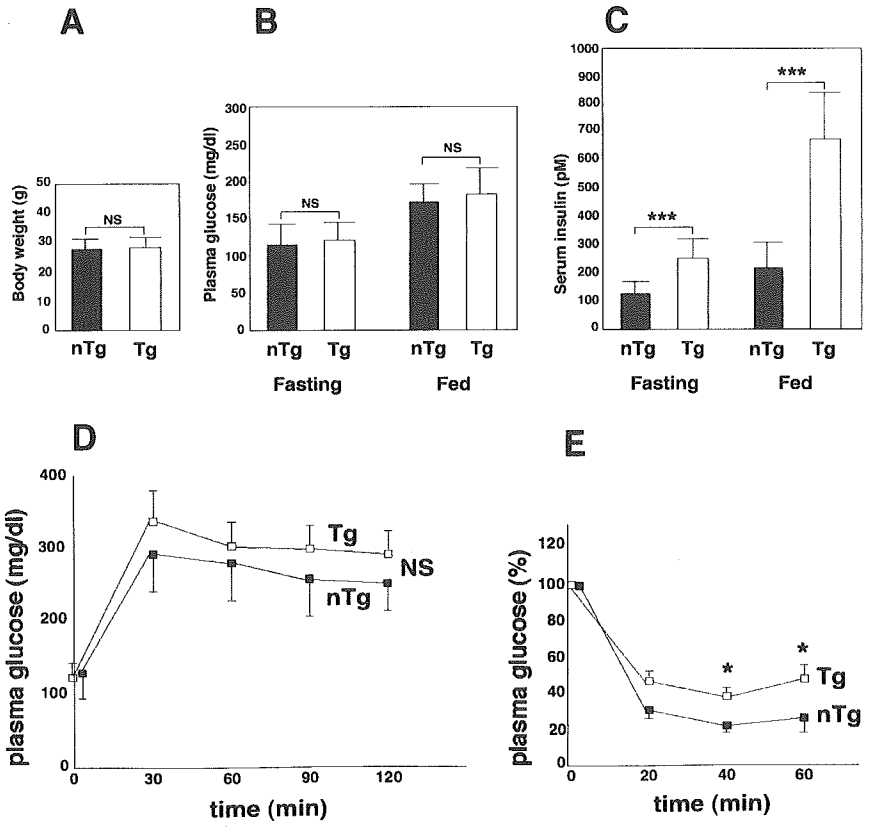

Fig. 2A–E. Altered glucose homeostasis in HCV core gene transgenic mice. **A** Body weights of 2-month-old mice. **B** Plasma glucose levels in fasting and fed mice. **C** Serum insulin levels in fasting and fed mice. The insulin level was significantly higher in the core gene transgenic mice than in control mice. **D** Glucose tolerance test. Animals were fasted overnight. D-Glucose (1 g/kg body weight) was administered by i.p. injection to conscious mice, and plasma glucose levels were determined at the time points indicated. **E** Insulin tolerance test. Human insulin (1 U/kg body weight) was administered by i.p. injection to fasted conscious mice, and glucose concentrations were determined. Values were normalized to the baseline glucose concentration at the time of insulin administration. Values are means ± SE, $*P < 0.05$; $***P < 0.001$, *NS*, statistically not significant; *nTg*, nontransgenic mice; *Tg*, transgenic mice

Table 2. Types of insulin resistance

Peripheral insulin resistance	A shortage of insulin action in the muscle (deficit in the insulin-induced glucose uptake into the muscles)
Central insulin resistance	A shortage of insulin action in the liver (deficit in the insulin-induced suppression of glucose production in the liver)

no significant difference between them (Fig. 2B). In contrast, serum insulin levels were significantly higher in transgenic than in normal control mice in both the fasting and fed conditions (Fig. 2C). Because such a combination of normal glucose levels and hyperinsulinemia points to insulin resistance, we conducted tests to determine glucose levels and insulin resistance. The core gene transgenic mice exhibited glucose levels a little higher than those of their normal littermates, but without any significant differences between them (Fig. 2D). In the insulin resistance tests, glucose levels were significantly higher in the transgenic than in the normal control mice, both 40 and 60 min after injection with insulin (Fig. 2E). These results indicate the presence of insulin resistance in the core gene transgenic mice. Because only the HCV core gene had been incorporated into these transgenic mice, the core protein of HCV would be able to induce insulin resistance in vivo.

By what mechanism, then, would the insulin resistance observed in this animal model arise? Insulin resistance is considered to involve two factors: central and peripheral insulin resistances (Table 2).[15] The hyperinsulinemic-euglycemic clamp method was employed for differentiating between these factors. In this method, hepatic glucose production (HGP) is calculated on the basis of the amount of glucose required for keeping plasma glucose levels within a certain range at serum insulin levels higher than physiological ones. In the normal control mice, HPG was suppressed by 60% by the administration of insulin, in contrast to findings in the core gene transgenic mice, in which there was only marginal suppression of HGP by insulin. These results indicate a hepatic (central) origin of insulin resistance in the transgenic mice. For further confirmation of this, uptake of glucose into the muscle was determined. There was no difference in this uptake in response to the administration of insulin between the transgenic

and normal control mice. The insulin resistance in mice transgenic for the HCV core gene, therefore, is central and hepatic.

The mechanism underlying insulin resistance in HCV infection

Next, we evaluated how insulin resistance emerged in our mouse model. For this purpose, liver homogenate was immunoblotted with anti-phosphotyrosine and anti-phosphoserine antibodies after insulin receptor substrate (IRS)-1 and IRS-2 had been immunoprecipitated. Tyrosines in IRS-1 were weakly phosphorylated in both the normal and transgenic mice before they received insulin, with no differences between them. After the administration of insulin, however, the phosphorylation of tyrosines in IRS-1 increased in the normal, but not in the transgenic mice. The obtained results suggested a disturbance in tyrosine phosphorylation as one of the factors for insulin resistance in the liver. There were no differences in the phosphorylation of serines in IRS-1 or tyrosines in IRS-2 between the transgenic and normal control mice. Overall, these results provided experimental evidence for the development of insulin resistance induced by the presence of HCV in the liver, which would disturb the transduction of insulin signaling in hepatocytes (Fig. 3). There remains a possibility that the HCV core protein could directly prohibit the phosphorylation of tyrosines. Alternatively, this protein may inhibit tyrosine phosphorylation via certain cytokines.

In our extensive search for the expression of cytokines in the liver of the HCV core gene transgenic mice, only tumor necrosis factor (TNF)-α and interleukin (IL)-1β levels were found to be increased.[16] For the purpose of evaluating the role of TNF-α in insulin resistance in transgenic mice, therefore, serum insulin was determined and an insulin resistance test was performed in them after they had received anti-TNF-α intraperitoneally. Pretreatment with anti-TNF-α partially restored insulin sensitivity in the HCV core gene transgenic mice. Although direct anti-insulin activity of the core protein cannot be excluded, high levels of TNF-α in the liver could be one of the factors involved in the induction of insulin resistance in this mouse model.

Pathogenesis of insulin resistance in hepatitis C patients

Simultaneously with our report of experimental systems, Aytug et al.[17] investigated insulin signaling in biopsied liver specimens from patients with chronic hepatitis C. Specifically, they evaluated changes in IRS-1, IRS-2, and phosphatidyl-inositol (PI)3-kinase levels in the livers of the patients. With insulin stimulation of the biopsied liver samples, insulin-receptor proteins and IRS-1 increased, while the phosphorylation of tyrosines in IRS-1 decreased to one-half the baseline value, along with diminished activity for PI3-kinase associated with IRS-1. The results reported by Aytug et al.[17] coincide with ours, in terms of analyzing the mechanism of insulin resistance in our experimental system in mice. Both our findings and theirs implicate the impaired tyrosine phosphorylation in IRS-1 in the induction of insulin resistance by HCV infection. It struck us as a surprise, in a sense, that the mechanism of insulin resistance induced by HCV infection showed agreement between

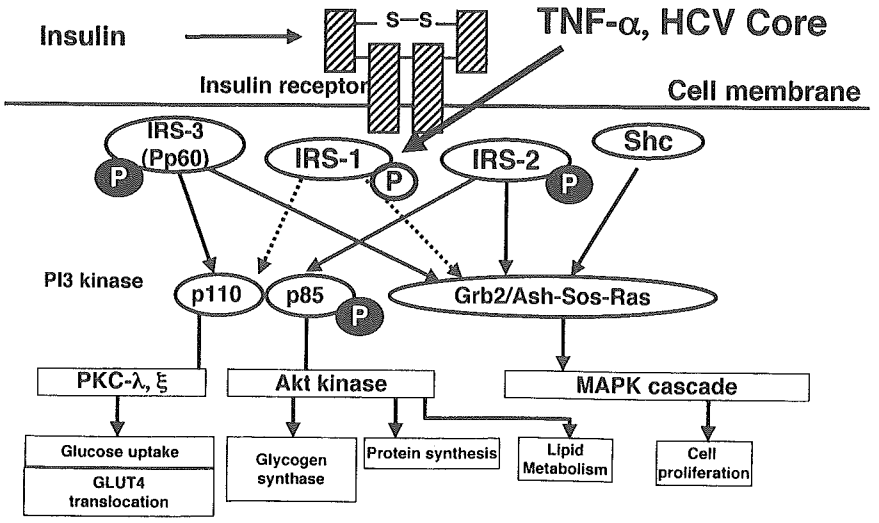

Fig. 3. Insulin resistance and HCV infection. HCV core protein or elevated intrahepatic tumor necrosis factor-α (*TNF-α*) inhibits tyrosine phosphorylation of insulin receptor substrate (*IRS*)-1 in the liver, suppresses insulin intracellular signal transduction, and leads to insulin resistance. *PKC*, protein kinase C; *PI3-kinase*, phosphatidyl inositol 3 kinase; *MAPK*, mitogen-activated protein kinase

clinical samples and experimental animals, although hepatic IRS-2 was reported to be preferred to IRS-1 for a role in the development of insulin resistance in earlier studies.[18] HCV infection could be peculiar, in that IRS-1 is more deeply involved than IRS-2 in the induction of hepatic insulin resistance. Although our data strongly indicate a hepatic characteristic of insulin resistance in HCV infection, they by no means exclude the roles of other factors in the induction of this resistance. There is little expression of the HCV core gene in the muscles of our animal model; it is not known if HCV infects muscle cells in patients with chronic hepatitis C. Factors not intrinsic to the liver would have to be evaluated to sort this out, including mitochondria dysfunction being involved in the induction of insulin resistance.[19]

Insulin resistance as a risk factor for progression of hepatic fibrosis

Insulin resistance in HCV infection may have an additional significant clinical implication. In 260 patients with chronic hepatitis C, Hui et al.[20] tried to establish a relationship between liver histology and indicators of glucose metabolism, as well as insulin resistance, represented by the homeostasis model assessment of insulin resistance (HOMA-IR). They found that insulin resistance already existed in hepatitis C patients with stage 0 or stage 1 fibrosis of the liver. This indicates that insulin resistance in HCV infection is not attributable to advanced liver disease. HOMA-IR was a significant and independent predictor for the stage and velocity of progression of hepatic fibrosis. The results of their study are important, because they implicate a role of hyperinsulinemia, and insulin resistance by inference, in promoting the progression of hepatic fibrosis. Insulin has been proven to be an aggravating factor not only in atherosclerosis but also in systemic inflammation and fibrosis. The liver would not be an exception in this respect.

Similarities and differences between hepatitis C and nonalcoholic steatohepatitis (NASH): hepatitis C could be a virus-associated steatohepatitis

We have demonstrated that HCV per se induces insulin resistance in an animal model. A high-fat diet and obesity superimposed on HCV infection lead to overt diabetes.[14] In view of the progression of chronic hepatitis C accelerated by insulin resistance,[20] insulin resistance would naturally influence the development of HCC. Although the association has not yet been shown to be definite between NASH and the development of HCC, it needs to be pursued energetically, in view of the histological resemblance of NASH to chronic hepatitis C.

When hepatitis C and NASH are compared, there are a number of similarities between these two medical conditions (Table 3). Steatosis, which is one of the definitions of NASH, is a characteristic trait of chronic hepatitis C.[4-6,13] Disturbances in lipid metabolism are present in both conditions, although the phenotypes may be distinct: hypo-β-lipoproteinemia in hepatitis C vs hyperlipidemia in NASH. As described above, insulin resistance often arises in chronic hepatitis C, and it is also a feature frequently observed in NASH; indeed insulin resistance is considered to be a basis for the pathogenesis of NASH.[21] Some cytokines, such as TNF-α, are considered to be critical in the pathogenesis of both conditions. TNF-α levels are increased in patients with chronic hepatitis C and are implicated in insulin resistance. TNF-α is also implicated in the pathogenesis of NASH.[21] The overproduction of oxidative stress or reactive oxygen species (ROS) plays a pivotal role in the progression of hepatitis and the development of HCC in both hepatitis C and NASH: in a mouse model of HCV infection, ROS were overproduced in the liver in the absence of inflammation, contributing, at least in part, to the development of HCC.[13,19,22] Presumably associated with ROS overproduction, a functional abnormality in the mitochondrion is suggested in the pathogenesis of liver diseases, including HCC, in both hepatitis C and NASH. In an HCV mouse model, a functional disorder of the electron transfer system of the mitochondrion was implicated as the origin of ROS overproduction (Table 3).

HCC develops in both chronic hepatitis C and NASH. However, an association between NASH and HCC is not yet conclusive, while there is a well-established connection of HCC with HCV infection.[1,20] Nevertheless, HCC does develop in patients with NASH, although the reported rate of occurrence varies. Hence, the mechanism underlying hepatocarcinogenesis in NASH awaits further investigation. The analogy between chronic hepatitis C and NASH, as described above, may be a clue to solve puzzles in the pathogenesis of NASH, including hepatocarcinogenesis.

Table 3. Comparison of hepatitis C and NASH

Hepatitis C	NASH
Steatosis	Steatosis
Hypo-β-lipoproteinemia	Hyperlipidemia
Insulin resistance	Insulin resistance
Cytokines (TNF-α, etc.)	Cytokines (TNF-α, etc.)
Oxidative stress	Oxidative stress
Mitochondrial abnormality	Mitochondrial abnormality
Obesity?	Obesity
HCC	HCC?

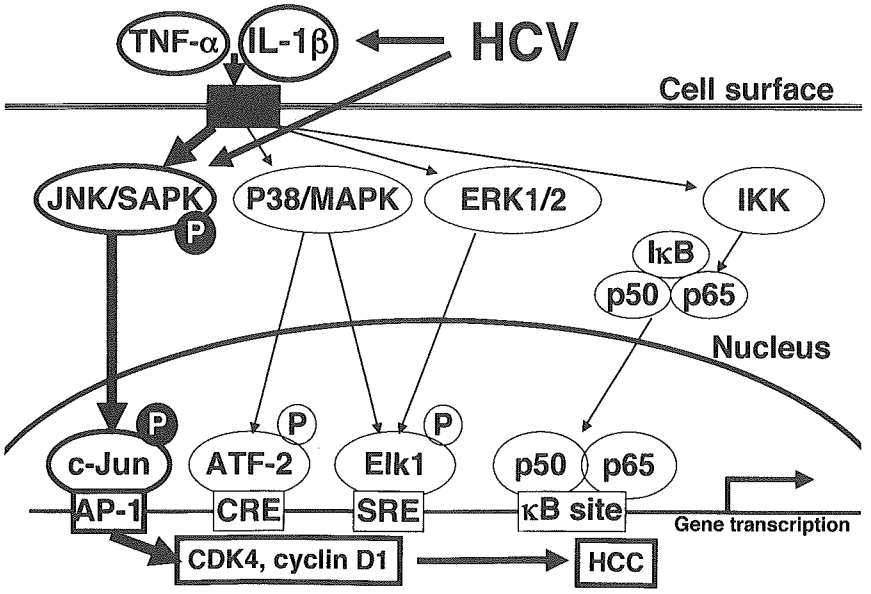

Fig. 4. *TNF*, tumor necrosis factor; *IL*, interleukin

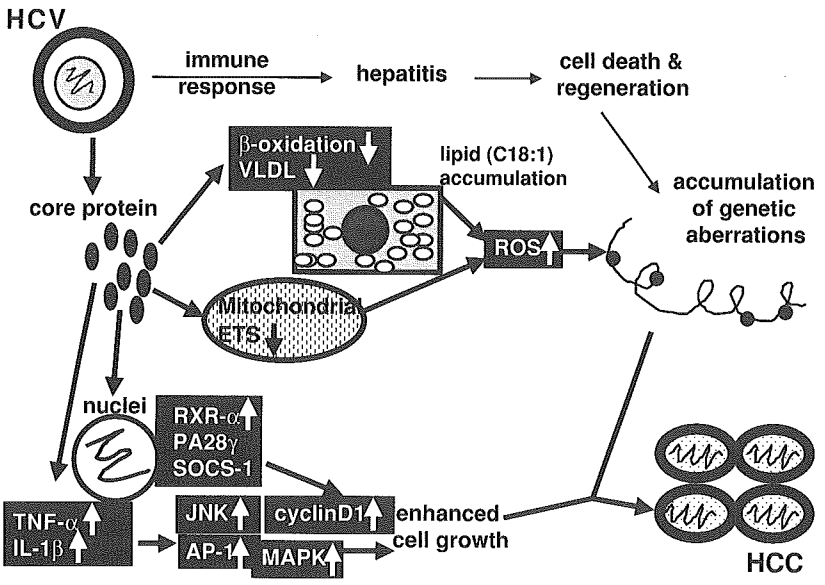

Fig. 5. Molecular pathogenesis of liver disease in HCV infection. The induction of oxidative stress, together with hepatic steatosis induced by the HCV core protein could play a pivotal role in the development of HCC. Alterations in the expressions of cellular genes, such as *TNF*-α or *SOCS-1*, and alterations in the intracellular signaling pathways, including c-Jun N terminal kinase (*JNK*), could be co-accelerators of hepatocarcinogenesis in HCV infection. The activation of intracellular signaling pathway has not been found in nonalcoholic steatohepatitis (NASH), while induction of oxidative stress may be common in the pathogenesis of both hepatitis C and NASH. *HCC*, hepatocellular carcinoma; *TNF*-α, tumor necrosis factor-α; *SOCS-1*, suppressor of cytokine signaling-1; *VLDL*, very low density lipoprotein; *ROS*, reactive oxygen species; *RXR*-α, retinoid X receptor; *PA28γ*, proteasome activator; *API*, activator protein

Though not yet completely elucidated, the pathogenesis of HCC in HCV infection has been substantially understood by the analysis of animal models.[23,24] For instance, in the MAPK intracellular signaling system, c-Jun N-terminal kinase (JNK) is activated in the liver by HCV. Downstream of JNK, a transcription factor, AP1, and cell-cycle molecules, CDK4 and cyclin D1, are subsequently activated, conferring a proliferative advantage to hepatocytes (Fig. 4).[16,23] Such activation of cellular gene expression and signaling systems has not yet been identified for NASH. The overproduction of ROS, together with the presence of hepatic steatosis, may be a common pathway to hepatocarcinogenesis in both hepatitis C and NASH (Fig. 5, upper half). However, the alterations in cellular gene expression and/or intracellular signaling systems occur only in hepatitis C in the presence of the viral protein(s), putting chronic hepatitis C onto the fast track to the development of HCC (Fig. 5, lower half). This aspect, observed in HCV infection, is distinct from NASH, which may explain the difference in the incidence of HCC in the two conditions. Based on the analogous metabolic pathways of hepatitis C and NASH, but taking into account the differences cellular gene expression and/or intracellular signaling systems,

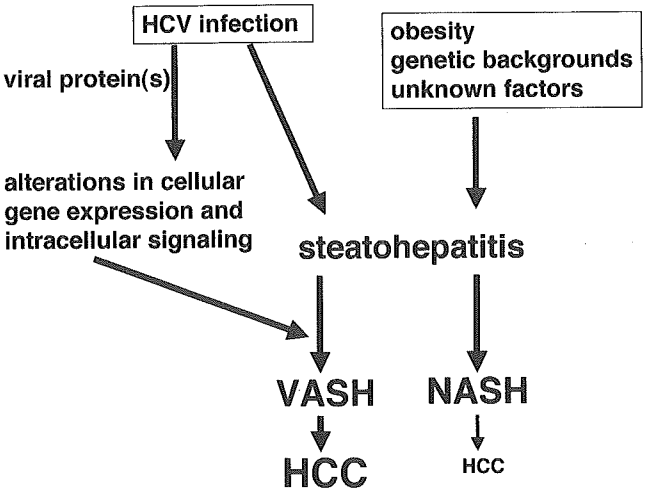

Fig. 6. Virus-associated steatohepatitis (*VASH*). HCV infection and NASH show a similar phenotype, steatohepatitis. However, in HCV infection, the presence of the viral proteins; in particular, the core protein of HCV, confers alterations in cellular gene expression and intracellular signaling systems to hepatocytes, leading to the high incidence of hepatocellular carcinoma (*HCC*). *HCV*, hepatitis C virus; *NASH*, nonalcoholic steatohepatitis

which are induced by the viral protein, we would like to propose the term "virus-associated steatohepatitis (VASH)" for hepatitis C (Fig. 6).

Perspectives for therapeutic strategies

We have demonstrated that HCV per se induces insulin resistance in an animal model. A high-fat diet and obesity superimposed on HCV infection lead to overt diabetes. In view of the progression of chronic hepatitis C being accelerated by insulin resistance, insulin resistance would naturally influence the development of HCC. Although an association between NASH and HCC has not been established, investigation of such an association needs to be pursued energetically, in view of the histological homology of NASH to chronic hepatitis C. Drugs for improving glucose metabolism and reducing insulin resistance need to be kept in mind in the treatment of hepatitis C patients who have failed to respond to antiviral treatment, because such drugs may well prevent the progression of fibrosis and the development of HCC in such patients. The traditional "high-protein and high-calorie" diet, advocated for chronic hepatitis patients in Japan, especially post-World War II, is obviously detrimental, except in some patients with advanced cirrhosis. Because hepatitis C is an infectious disease, the eradication of the virus is, naturally, the most efficient way to cure the disease. However, nearly one-half of chronic hepatitis C patients who were treated with interferon/ribavirin combination therapy did not achieve eradication of the virus.[25] Therefore, besides anti-viral treatment for HCV, consultations with hepatitis C patients on their dietary habits should include recommendations for iron restriction,[26] as well as weight control, because a high-calorie intake is likely to accelerate hepatic fibrosis by aggravating insulin resistance in these patients.

References

1. 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;87:6547–9.
2. Gumber SC, Chopra S. Hepatitis C: a multifaceted disease. Ann Intern Med 1995;123:615–20.
3. 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;94:233–6.
4. Bach N, Thung SN, Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. Hepatology 1992;15:572–7.
5. 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.
6. 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;78:1527–31.
7. 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 U S A 1997;94:1200–5.
8. 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;25:369–74.
9. 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;96:2468–72.
10. 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;126:586–97.
11. Allison ME, Wreghitt T, Palmer CR, Alexander GJ. Evidence for a link between hepatitis C virus infection and diabetes mellitus in a cirrhotic population. J Hepatol 1994;21:1135–9.
12. Mehta SH, Brancati FL, Sulkowski MS, Strathdee SA, Szklo M, Thomas DL. Prevalence of type 2 diabetes mellitus among persons with hepatitis C virus infection in the United States. Ann Intern Med 2000;133:592–9.
13. 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. Nature Med 1998;4:1065–8.
14. 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;126:840–8.
15. Kahn BB. Type 2 diabetes: when insulin secretion fails to compensate for insulin resistance. Cell 1998;92:593–6.

16. 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;304:415–24.
17. Aytug S, Reich D, Sapiro LE, Bernstein D, Begum N. Impaired IRS-1/PI3-kinase signaling in patients with HCV: a mechanism for increased prevalence of type 2 diabetes. Hepatology 2003; 38:1384–92.
18. Suzuki R, Tobe K, Aoyama M, Inoue A, Sakamoto K, Yamauchi T, et al. Both insulin signaling defects in the liver and obesity contribute to insulin resistance and cause diabetes in Irs2(−/−) mice. J Biol Chem 2004;279:25039–49.
19. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, et al. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science 2003;300:1140–2.
20. Hui JM, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, et al. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression. Gastroenterology 2003;125:1695–704.
21. Day CP. The potential role of genes in nonalcoholic fatty liver disease. Clin Liver Dis 2004;8:673–91.
22. 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;61:4365–70.
23. Koike K, Tsutsumi T, Fujie H, Shintani Y, Moriya K. Role of hepatitis viruses in hepatocarcinogenesis. Oncology 2002;62:29–37.
24. 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;122:352–65.
25. Fried MW, Hadziyannis SJ. Treatment of chronic hepatitis C infection with peginterferons plus ribavirin. Semin Liver Dis 2004;24(Suppl 2):47–54.
26. Kato J, Kobune M, Nakamura T, Kuroiwa G, Takada K, Takimoto R, et al. Normalization of elevated hepatic 8-hydroxy-2′-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 2001;61:8697–702.

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Editorial

Steatosis in chronic hepatitis C: fuel for overproduction of oxidative stress?

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In situ detection of oxidized n-3 polyunsaturated fatty acids in chronic hepatitis C: correlation with hepatic steatosis

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Endogenous oxidative stress generated by multiple intracellular pathways is an important class of naturally occurring carcinogens.[1,2] Reactive oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism.[3] ROS include a number of species such as superoxide, hydroxyl, and peroxyl radicals and certain nonradicals such as singlet oxygen and hydrogen peroxide that can be easily converted into radicals. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis.[4,5] Most markers of oxidative injury that are utilized reflect free radical attack on polyunsaturated fatty acids, with the classical route of attack involving lipid peroxidation, which generates hydroperoxides and endoperoxides. The major species of ROS are superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), or hydroxyl radicals (HO·). Lipid peroxide products include 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA), and 4-hydroxy-2-hexenal (HHE), which can be markers for oxidative stress, as 8-hydroxy-deoxy-guanosine (8-OHdG), a DNA adduct, can be.

Such oxidative stress is supposed to play a pivotal role in the development of liver injury or hepatocellular carcinoma (HCC) in chronic hepatitis C virus (HCV) infection.[6,7] In addition, it is notable that in HCV infection the viral proteins themselves as well as inflammation due to hepatitis are regarded as a cause of oxidative stress overproduction.[8,9] Therefore, it is critical to study the state of oxidative stress in the liver of hepatitis C patients.

In the current issue of the *Journal of Gastroenterology*, Kitase et al.[10] studied the peroxidation of n-3 polyunsaturated fatty acids (PUFAs) in the liver of chronic hepatitis C patients and correlated it with steatosis in the liver. They found that the oxidative stress as detected by the marker of HHE was significantly associated with the presence of hepatic steatosis in liver tissues of chronic hepatitis C patients. They also showed that HHE was a better marker of oxidative stress in chronic hepatitis C than MDA or HNE, although that may be due to a better affinity of the antibody than antibodies to other oxidants. They also demonstrated that the n-6PUFA:n-3PUFA ratio is higher in a resected liver tissue sample with steatosis than in one without steatosis, but the relevance of this finding is not yet clear. Their observation is of interest because steatosis is considered to be a significant predictive factor for the progression of fibrosis in chronic hepatitis C patients, as evidenced by recent clinical studies.[11–14] Fartoux et al.[11] reported that steatosis was the only independent predictive factor of progression of fibrosis in multivariate analysis. Collier et al.[12] showed that the rate of progressive fibrosis was predicted by baseline fibrosis, steatosis, and lobular inflammation. In addition, the presence of steatosis is associated with resistance to antiviral therapy for HCV infection,[14] re-emphasizing the importance of steatosis in hepatitis C.

The mechanism underlying lipid accumulation in the liver of hepatitis C patients is not completely clear yet, but the impairment of very low density lipoprotein (VLDL) secretion from the liver[15] and the induction of insulin resistance by HCV[16] are assumed to be major causes. Hepatic steatosis is induced by the action of HCV core protein in both in vivo and in vitro models, and the formula of lipids accumulated in the HCV-related steatosis is distinct from that in HCV-unrelated steatosis.[17] Moreover, oxidative stress, in particular, as a form of lipid peroxide, is increased in HCV infection,[6,7] which is also well reproduced in an animal model.[8] Steatosis and oxidative stress generally seem to appear together in the liver with HCV infection. Hence, one may think that lipids present in liver tissues accelerate

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hepatic fibrosis through increasing lipid peroxidation, acting like fuel on raging flames.

Then, how is steatosis, i.e., accumulation of lipids in the liver, involved in the generation of oxidative stress? Are lipids present in the liver automatically peroxidated, leading to DNA damage? The probable answer is no. In most subjects with simple fatty liver, the liver disease is not progressive, except in those with nonalcoholic steatohepatitis (NASH). It has also been demonstrated that simple fatty liver is not a source for oxidative stress in experimental models.[8,17] Additional factors are apparently necessary for lipids in the liver to be a supply for oxidative stress overproduction.

Because both hepatitis C and NASH have steatohepatitis, in which both steatosis and oxidative stress are present, it may be helpful to compare some aspects of these two medical conditions. There are a number of similarities between hepatitis C and NASH. Hepatic steatosis, disturbances in the lipid metabolism, insulin resistance, and overproduction of oxidative stress are common features of both conditions. Elevated levels in the liver of some cytokines such as TNF-α, or genetic predisposition to their overproduction, may be one of the bases for the pathogenesis of hepatitis C and NASH.[18] Functional abnormalities in the mitochondria, which may be an origin of oxidative stress overproduction in steatohepatitis, are also implicated in both conditions.

This mitochondrial abnormality may be essential in the pathogenesis of HCV infection.[18] The major origin of oxidative stress production in hepatocytes is the mitochondrion. Most of the oxygen consumed by mammalian cells is converted to water via the mitochondrial electron transfer system. However, up to 5% of the oxygen can become uncoupled and singly leak onto oxygen to form superoxide.[1,2] ROS, generated in the cells, is very fragile and hyperreactive and attacks biomolecules such as DNA, lipids, or proteins. The liver produces a large amount of ROS, because hepatocytes contain abundant amounts of mitochondria. Because an impairment of mitochondrial function such as β-oxidation of fatty acids also leads to the accumulation of lipids in the liver, a putative defect in the mitochondrial function in hepatitis C[11,12,18] or NASH[19] may be a key event in the pathogenesis of steatohepatitis. Accumulated lipids may be utilized as fuel to overproduction of oxidative stress, thereby placing patients with chronic hepatitis C or NASH onto the fast track to HCC development. Hepatic steatosis in HCV infection may be a crucial target of pharmaceutical and nutritional approaches for therapeutic development.

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References

1. Cerutti PA, Trump BF. Inflammation and oxidative stress in carcinogenesis. Cancer Cells 1991;3:1–7.
2. Dreher D, Junod AF. Role of oxygen free radicals in cancer development. Eur J Cancer 1996;32:30–8.
3. Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev 1979;59:527–605.
4. Cerutti PA. Oxy-radicals and cancer. Lancet 1994;344:862–3.
5. Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem J 1996;313:17–29.
6. Farinati F, Cardin R, De Maria N, Della Libera G, Marafin C, Lecis E, et al. Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. J Hepatol 1995;22:449–56.
7. Paradis V, Mathurin P, Kollinger M, Imbert-Bismut F, Charlotte F, Piton A, et al. In situ detection of lipid peroxidation in chronic hepatitis C: correlation with pathological features. J Clin Pathol 1997;50:401–6.
8. 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;61:4365–70.
9. Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, et al. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology 2002;122:366–75.
10. Kitase A, Hino K, Furutani T, Okuda M, Gondo T, Hidaka I, et al. In situ detection of oxidized n-3 polyunsaturated fatty acids in chronic hepatitis C: correlation with hepatic steatosis. J Gastroenterol 2005:617–24.
11. Fartoux L, Chazouilleres O, Wendum D, Poupon R, Serfaty L. Impact of steatosis on progression of fibrosis in patients with mild hepatitis C. Hepatology 2005;41:82–7.
12. Collier JD, Woodall T, Wight DG, Shore S, Gimson AE, Alexander GJ. Predicting progressive hepatic fibrosis stage on subsequent liver biopsy in chronic hepatitis C virus infection. J Viral Hepat 2005;12:74–80.
13. Castera L, Hezode C, Roudot-Thoraval F, Bastie A, Zafrani ES, Pawlotsky JM, et al. Worsening of steatosis is an independent factor of fibrosis progression in untreated patients with chronic hepatitis C and paired liver biopsies. Gut 2003;52:288–92.
14. Patton HM, Patel K, Behling C, Bylund D, Blatt LM, Vallee M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol 2004;40:484–90.
15. Perlemuter G, Sabile A, Letteron P, Topilco, Samson-Bouna M-E, Chretien Y, et al. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. FASEB J 2002;16:185–94.
16. 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;126:840–8.
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;281:1207–12.
18. Koike K, Moriya K. Metabolic aspects of hepatitis C: steatohepatitis resembling but distinct from NASH. J Gastroenterol 2005; in press.
19. Fromenty B, Robin MA, Igoudjil A, Mansouri A, Pessayre D. The ins and outs of mitochondrial dysfunction in NASH. Diabetes Metab 2004;30:121–38.

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Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling *(SOCS)-1* gene expression

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***Background/Aims*: Suppressor of cytokine signaling (SOCS)-1, a negative feedback regulator of cytokine signaling pathway, also has a tumor suppressor activity, the silencing of its gene by hypermethylation is suggested to contribute to hepatocarcinogenesis. We studied the effect of the core protein of hepatitis C virus (HCV) on the expression of *SOCS-1* gene.**

***Methods*: HCV core gene transgenic mice, which develop hepatocellular carcinoma late in life, HepG2 cells expressing the core protein, and human liver tissues were analyzed.**

***Results*: The expression of *SOCS-1* gene was significantly suppressed in the liver of core gene transgenic mice and HepG2 cells expressing the core protein, while that of *SOCS-3* gene was conserved. *SOCS-1* expression levels also decreased in HCV-positive human liver tissues. The core protein differentially down-regulated the expression of signal transducer and activator of transcription (STAT) target genes, but rather enhanced STAT1 and STAT3 activation after interleukin-6 stimulation in mouse liver tissues and cells.**

***Conclusions*: HCV core protein down-regulates the expression of *SOCS-1* gene. This is a mechanism leading to *SOCS-1* silencing, an alternative to the hypermethylation of the gene; this effect of the core protein may modulate the intracellular signaling pathway, contributing to the pathogenesis in HCV infection including hepatocarcinogenesis.**

***Keywords*: Tumor suppressor gene; Hepatocellular carcinoma; Transgenic mouse; STAT3**

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis. A substantial proportion of patients with chronic hepatitis C eventually develop hepatocellular carcinoma (HCC), which is one of the leading causes of death worldwide [1,2]. Despite the absence of appropriate in vitro replication systems or practical infectious animal model systems, the mechanism underlying hepatocarcinogenesis in human HCV infection is gradually clarified. Both the direct and indirect effects of HCV on HCC development are demonstrated [3–6]. The accumulation of gene aberrations, such as the inactivation of tumor suppressor genes or the activation of oncogenes, which are induced through the inflammation-mediated continuous death of hepatocytes followed by regeneration, may be one of the mechanisms underlying hepatocarcinogenesis [3]. On the other hand, the viral gene products are suggested to contribute to the development of HCC by their direct effects on hepatocytes [4]. Such direct effects have been demonstrated by the use of model systems including mice

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[7–9]. HCV-infected hepatocytes produce viral structural and nonstructural proteins. Some of these confer certain phenotypes to hepatocytes and may be associated with the pathogenesis of HCV infection including the development of HCC. Among such viral proteins, the core protein of HCV has a variety of biological activities, including oncogenic activity, which substantially affects host cellular functions [7–11].

Suppressor of cytokine signaling (SOCS)-1, also called signal transducer and activator of transcription (STAT)-induced STAT inhibitor-1 or Jak binding protein-1, is a negative feedback regulator of cytokine signaling through the Jak/STAT pathway. SOCS-1 contains the SH2 domain and directly interacts with the kinase domain of Jak to suppress Jak activity. *SOCS-1* gene expression is augmented by various cytokines, such as interferon (IFN)-γ, interleukin (IL)-6 or leukemia inhibitory factor (LIF), resulting in the suppression of the signal transduction downstream pathways of these cytokines [12–14]. Moreover, SOCS-1 has been recently shown to exhibit a tumor suppressor activity. SOCS-1 suppresses the expression of several oncogenes or growth-related genes acting as a negative regulator of cell proliferation: the loss of function of SOCS-1 facilitates tumor progression [15–17]. As a mechanism underlying the loss of function of SOCS-1, a recent study has revealed a frequent silencing of the *SOCS-1* gene by CpG methylation in HCC tissues [18–20]. Alternatively, however, it may be possible that HCV infection, particularly, the proteins that the HCV genome encodes per se, may render the *SOCS-1* gene unable to exhibit its function by gene silencing.

We examined such a possibility using a mouse model for HCV infection that is destined to develop HCC [7,9], as well as cultured cells expressing the HCV core protein [21]. The core protein markedly suppressed the expression of the *SOCS-1* gene in both liver tissues and cultured cells. This silencing of the *SOCS-1* gene may be one of explanations for the pathogenicity of HCV in humans.

2. Materials and methods

2.1. Transgenic mouse and cell lines

HCV core gene transgenic mice have been described previously [7]. These mice develop HCC late in life [7,9]. The mice were cared for according to the institutional guidelines and maintained in a specific pathogen-free state. All the animals received humane care and the study protocol complied with the institution's guidelines for the care and use of experimental animals. HepG2 cell lines expressing the HCV core protein under the control of CAG promoter (Hep39J, Hep396 and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously [21,22].

2.2. IL-6 Stimulation

For the in vivo experiments, 0.05–0.5 μg/g BW murine IL-6 (Diaclone, Besançon, France) was administered into 8 w.o. male mice i.p., and liver tissues were obtained 60 min later. Cultured cells were treated with human IL-6 (Diaclone) at 10–100 ng/ml or IFN-α at 1.0–10.0 ng/ml and then were harvested 60 min later.

2.3. Reverse transcription (RT)-PCR analysis

Total RNA was extracted from liver tissues or cultured cells before and after the treatment with IL-6 using TRIzol (Invitrogen). RNA was reverse-transcribed using oligo(dT) primers and Superscript II (Invitrogen). Equal amounts of cDNA were then subjected to PCR. The primer pairs used were:

5′-CACTCACTTCCGCACCTTCC-3′ (forward) and
5′-TCCAGCAGCTCGAAAAGGCA-3′ (reverse) for murine *SOCS-1*,
5′-CACGCACTTCCGCACATTCC-3′ (forward) and
5′-TCCAGCAGCTCGAAGAGGCA-3′ (reverse) for human *SOCS-1*,
5′-TCACCCACAGCAAGTTTCCCGC-3′ (forward) and
5′-GTTGACAGTCTTCCGACAAAGATGC-3′ (reverse) for murine *SOCS-3*,
5′-CACGCACTTCCGCACATTCC-3′ (forward), and
5′-GTTGACGGTCTTCCGACAGAGATGC-3′ (reverse) for human *SOCS-3*.

For the RT-PCR analysis, the quantity of cDNA template and the number of amplification cycles were optimized to ensure that the reaction was terminated during the linear phase of product amplification, so that the semiquantitative comparison of mRNA abundance between different samples was possible [23]. The intensities of the bands were determined using a densitometer. RT-PCR was also done using GAPDH primers to adjust the amounts of RNA in each experiment.

2.4. Human liver tissue samples and real-time PCR

Nine patients with HCC who had underlying chronic hepatitis C were studied for *SOCS-1* expression in noncancerous tissues. Additional nine patients, who were found to be negative for both HBsAg and anti-HCV at the time of operation, were also studied. The latter patients underwent liver resection for metastatic liver tumors from colon cancer. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethics Review Committee for Human Experimentation. Informed consent was obtained from each patient. The noncancerous liver tissues obtained from these patients were immediately frozen and stored at −80 °C until further use.

Taqman real-time RT-PCR was performed as described previously [24], using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and the TaqMan probe for *SOCS-1* were as follows:

Forward primer:5′-CTGGCCCCGGAGCAT-3′
Reverse primer:5′-GTTGTGTGCTACCATCCTACAGA-3′
Probe:5′-FAM-CCGGACGCTATGGCCCA-MGB-3′

Primers and probes for *SOCS-3*, β-*actin*, interferon regulatory factor (*IRF*)-1, c-*myc* and *bcl*-X_L genes were purchased from ABI by Assays-on-Demand system.

2.5. Methylation status

The methylation status of the *SOCS-1* gene was analyzed by methylation-specific PCR as described previously [20].

2.6. Western blotting and immunoprecipitation

Nuclear and cytoplasmic fractions were prepared from HepG2 cells, and Western blotting was performed as described previously [25]. Anti-STAT1 and anti-STAT3 polyclonal antibodies (Cell Signaling Technology, Inc., Beverly, MA), anti-phosphorylated STAT3 (Tyr705) polyclonal antibody (Cell Signaling Technology), anti-phosphorylated STAT3 monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY), and anti-protein inhibitor of activated *STAT* (*PIAS*)*1*, *anti-PIAS3* and *anti-SOCS-1* antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were

used. Immunoprecipitation was done as described previously using antibodies followed by protein A-Sepharose [26].

2.7. Immunocytofluorescence

HepG2 cell lines with or without the core gene were grown overnight on chamber slides and treated with 10 ng/ml human IL-6 for 60 min. Cells were fixed with 4% paraformaldehyde plus methanol, and reacted with the anti-STAT3 antibody followed by incubation with a FITC-labeled secondary antibody.

2.8. Statistical analysis

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

3. Results

3.1. HCV core protein suppresses SOCS-1 gene expression

To examine the impact of the core protein on *SOCS-1* gene expression, we analyzed mRNA expression levels by semi-quantitative RT-PCR in liver tissues from the HCV core gene transgenic and nontransgenic control mice. *SOCS-1* mRNA expression levels in mouse liver tissues of nontransgenic mice were increased in a dose-dependent manner of IL-6, but were only marginal in the liver tissues from the core gene transgenic mice even in those treated with the maximal dose of IL-6 (0.5 μg/g BW, $n=5$ each) (Fig. 1(A) and (C)). In contrast, the expression levels of *SOCS-3* mRNA in the core gene transgenic mice were comparable to or rather higher than those in nontransgenic mice, before and after stimulation with IL-6 (Fig. 1(A) and (E)) [27,28].

We then examined whether or not this observation in mice is reproducible in HepG2 cell lines that constitutively express the core protein. *SOCS-1* gene expression was suppressed in the core-expressing HepG2 cell lines Hep396, Hep397 and Hep39J, even after stimulation with IL-6, while control bulk HepG2 cells or a control Hepswx cell line expressed *SOCS-1* mRNA at high levels (Fig. 1(B) and (D)). In contrast, the levels of *SOCS-3* gene expression were similar among the core-expressing HepG2 cell lines and control HepG2 cells, and were augmented by stimulation with IL-6 (Fig. 1(B) and (F)). These observations indicate that the core protein selectively suppresses *SOCS-1* gene expression before the translational level. The SOCS-1 protein was not detectable by Western blotting either in the mouse liver or HepG2 cells using currently available anti-SOCS-1antibodies.

These results, obtained in HepG2 cell lines constitutively expressing the core protein, were then evaluated using a transient expression system. In this system, HepG2 cells were infected with baculovirus, expressing the core protein as described previously [29], and *SOCS-1* expression was determined by semiquantitative RT-PCR. The introduction

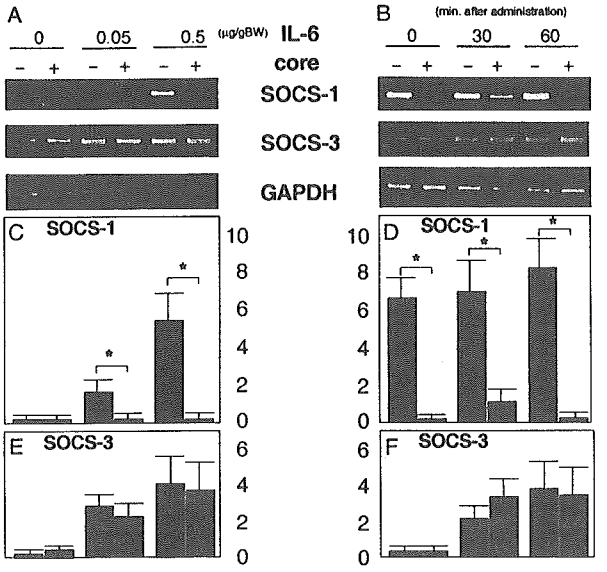

Fig. 1. Suppression of *SOCS-1* gene expression by hepatitis C virus core protein. (A) and (B) RNA from mouse liver tissues (A) or HepG2 cells (B) with or without the core protein was subjected to RT-PCR for the determination of *SOCS-1* and *SOCS-3* gene expression. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g BW of IL-6. HepG2 cells (Hep396 and Hepswx) were treated with 10 ng/ml of IL-6 for 0, 30 or 60 min before RNA extraction. Bottom panels in (A) and (B) show the expression level of the housekeeping gene GAPDH as an internal control. (C) and (D) Represent means± SD. of five independent experiments on *SOCS-1* gene expression corresponding to the lanes in (A) and (B), respectively. *, $P<0.05$. (E) and (F) Represent means±SD. of five independent experiments on *SOCS-3* gene expression corresponding to the lanes in (A) and (B), respectively.

of the core protein selectively suppressed the expression level of *SOCS-1* mRNA even after stimulation with IL-6 (data not shown).

Modulation of expression by the core protein of STAT-target genes other than *SOCS*s was then examined by determining the mRNA levels in mouse liver tissues. Expression of *IRF-1* gene was suppressed in the presence of the core protein under the stimulation with IL-6, while that of c-*myc* was not affected (Fig. 2(A) and (B)). The expression of *bcl*-X_L gene was rather augmented by the core protein although the difference was not statistically significant (Fig. 2(C)).

The methylation status of *SOCS-1* gene was then explored in liver tissues from the core gene transgenic mice by a method described previously [20], to determine whether or not the *SOCS-1* gene expression may be suppressed by hypermethylation. No hypermethylation was observed in the *SOCS-1* gene of the core gene transgenic mice either at the 5′-noncoding region or the CpG island in the coding region (Fig. 3).

In the analysis of *SOCS-1* expression in noncancerous liver tissues from patients with HCV infection, the *SOCS-1* mRNA expression levels were 0.494±0.352 in HCV-positive patients ($n=9$) and 0.862±0.465 in the control subjects without HCV infection ($n=9$) (in arbitrary units,

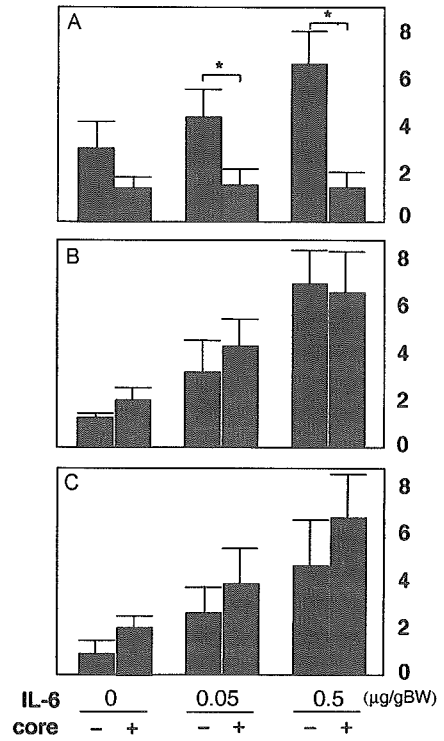

Fig. 2. Effect of hepatitis C virus core protein on the expression of STAT-target genes. RNA from mouse liver tissues with or without the core protein was subjected to RT-PCR for the determination of *IRF1* (A), c-*myc* (B) and *bcl*-X_L (C) gene expressions. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g BW of IL-6. *, $P<0.05$.

$P=0.0345$). Thus, the SOCS-1 levels in the liver tissues of chronic hepatitis C patients were significantly lower than those of subjects without HCV infection.

3.2. The core protein did not suppress phosphorylation of STAT3 or STAT1

The activation of STAT3 enhances *SOCS-1* expression, thereby forming a negative feedback loop to the STAT3 status [17]. To determine whether or not STAT3 activation is involved in the *SOCS-1* gene suppression in this system, the tyrosine phosphorylation of STAT3 in the mouse liver was examined by Western blotting using an anti-phospho-STAT3 (tyrosine (Tyr)^{705}) antibody. At baseline, Tyr^{705} phosphorylation of STAT3 was low in both the core gene transgenic and nontransgenic mice. However, in response to stimulation with IL-6, the levels of Tyr^{705} phosphorylation of STAT3 was higher in the liver tissues from the core gene transgenic mice than that from nontransgenic mice. A representative result is shown in Fig. 4(A). Similarly, the levels of Tyr^{705} phosphorylation of STAT3 were higher in HepG2 cell lines expressing the core protein than those in control cells (Fig. 4(B)). These results observed in HepG2 cells constitutively expressing the core protein was also evaluated in a transient expression system using a recombinant baculovirus, as described above. The Tyr^{705} phosphorylation of STAT3 was enhanced in HepG2 cells infected with baculovirus expressing the core protein compared with mock-infected HepG2 cells (data not shown). The activation of STAT1 was also analyzed using HepG2 cell lines. As shown in Fig. 4(C), the levels of STAT1 phosphorylation was higher in HepG2 cells expressing the core protein than in control cells similar to the result on STAT3.

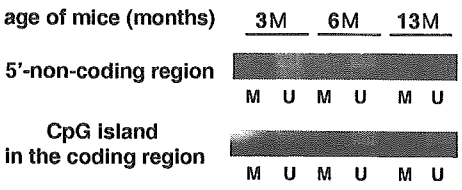

Fig. 3. Methylation status of *SOCS-1* gene in liver from hepatitis C virus core gene transgenic mice. DNA from the liver tissues of core gene transgenic mice at the age of 3 months (3M), 6 months (6M) or 13 months (13M) was subjected to methylation-specific PCR. Only PCR with unmethylation-specific primers yielded bands indicating that the *SOCS-1* gene was unmethylated in the liver tissues of core gene transgenic mice. M, methylation-specific primers; U, unmethylation-specific primers.

3.3. Subcellular localizations of STAT3 and STAT1

STAT activation by tyrosine phosphorylation results in the migration of STAT from the cytoplasm to the nucleus to bind to genomic DNA, modulating of cellular gene expression. We thereby evaluated the subcellular localization of STAT3 and STAT1 by preparing cytoplasmic and nuclear fractions from HepG2 cells followed by Western blotting. The amounts of STAT3 in the nuclei of core-expressing HepG2 cells were similar to or slightly larger

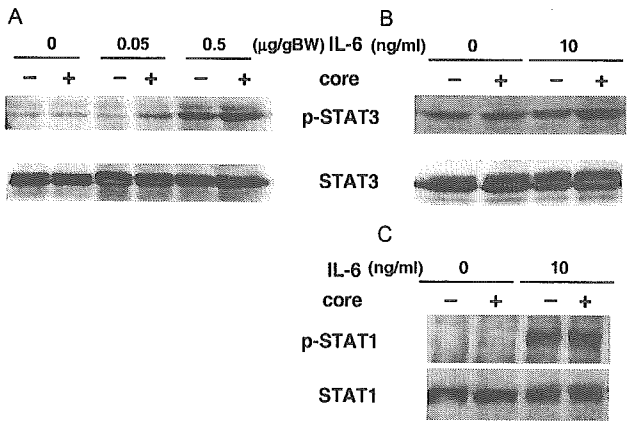

Fig. 4. Increase in the level of tyrosine phosphorylation of STAT3 and STAT1 by hepatitis C virus core protein. Whole cell lysates from mouse liver tissues (A) and HepG2 cells (B) and (C) were subjected to SDS-PAGE followed by Western blotting with anti-STAT3 and anti-*P*-STAT3 (A) and (B) or with anti-STAT1 and anti-*P*-STAT1 (C). Liver tissues were obtained from the mice treated as described in the Fig. 1 legends. HepG2 cells were treated with 10 ng/ml IL-6 or vehicle for 1 h. *P*-STAT3, phosphorylated STAT3; STAT3, total STAT3; p-STAT1, phosphorylated STAT1; STAT1, total STAT1. The experiments were repeated three times.

than those in control HepG2 cells in the presence or absence of IL-6 (Fig. 5(A)). This result was confirmed by an immunofluorescence study (Fig. 5(B)). A similar result was obtained in the analysis of STAT1 subcellular localization (Fig. 5(C)). These observations indicate that the HCV core protein does not inhibit the translocation of STAT3 or STAT1 to the nucleus, and the feedback mechanism is not the cause of *SOCS-1* gene suppression.

Because PIAS3 blocks the nuclear translocation of STAT3 or binding of STAT3 to genomic DNA [30], the expression of PIAS3 was examined by Western blotting. However, there was no significant difference in the levels of PIAS3 between core-expressing HepG2 cells and control HepG2 cells (data not shown). Co-immunoprecipitation analysis was also performed using HepG2 cell lines to know whether or not the core protein affects the association of PIAS1 with STAT1 or PIAS3 with STAT3. However, neither co-immunoprecipitation of STAT1 with anti-PIAS1 antibody nor that of STAT3 with anti-PIAS3 antibody was affected by the presence of the core protein (Fig. 6). We also examined the possibility of the interaction of the core protein with STAT3, which blocks the binding of STAT3 to the promoter of *SOCS-1* gene. For this purpose, a co-immunoprecipitation technique was utilized with whole-cell extracts of core-expressing HepG2 cells. However, no association was observed between these two proteins.

4. Discussion

In the current study, we demonstrated that the core protein of HCV suppresses the expression of *SOCS-1* mRNA in the liver tissues of mice that develop HCC late in their life [4,7]. This observation was reproduced in cultured cells that expressed the core protein. This phenomenon may contribute to the modification of the IFN signaling systems

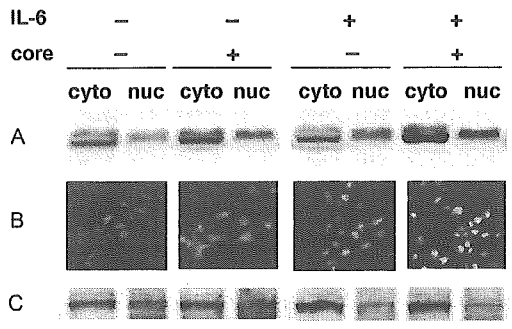

Fig. 5. Hepatitis C virus core protein did not affect subcellular localization of STAT3 or STAT1. Cytoplasmic and nuclear fractions from HepG2 cells with or without the core protein were subjected to Western blotting with the anti-STAT3 antibody (A) or anti-STAT1 antibody (C). HepG2 cells were fixed and an immunocytofluorescence study was performed using the anti-STAT3 antibody (B). Cells were processed before or 60 min after the treatment with 10 ng/ml of IL-6. cyto, cytoplasmic fraction; nuc, nuclear fraction [This figure appears in colour on the web].

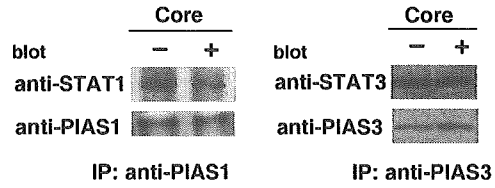

Fig. 6. Effect of the core protein on the interaction of STATs and PIASs. Cell lysates were immunoprecipitated with anti-PIAS1 or anti-PIAS3 antibody, and immunoblotted with anti-STAT1 or STAT3 antibody, respectively. There was no difference in the amounts of STAT1 or STAT3 that were co-immunoprecipitated with anti-PIAS antibodies.

in HCV infection, because SOCS-1 and SOCS-3 play central roles in the Jak/STAT pathway as negative feedback regulators [12–14]. In addition, since SOCS-1 also possesses a tumor suppressor activity [15–17], the down-regulation of *SOCS-1* may contribute to hepatocarcinogenesis in HCV infection. It has been reported that the silencing of the *SOCS-1* gene by hypermethylation is associated with the development of HCC [18–20]. Among patients with HCV infection, a major cause of chronic hepatitis worldwide, HCC develops at a very high incidence [1,2]. Hence, there may be an alternative mechanism of *SOCS-1* silencing to gene methylation in HCV infection. Our current data suggest a possibility of such a mechanism in that HCV per se acts as a negative regulator of SOCS-1, a tumor suppressor. The expression levels of *SOCS-1* mRNA in noncancerous liver tissues in chronic hepatitis C patients were also significantly lower than those in HCV-negative subjects, although the 'shut-off' of the *SOCS-1* gene observed in the experimental systems was not the case. This may be due to the presence of other factors influencing *SOCS-1* gene expression in vivo, including inflammation.

In the exploration of the mechanism underlying the down-regulation of *SOCS-1* expression, we first examined the methylation status of the *SOCS-1* gene in liver tissues from core gene transgenic mice by methylation-specific PCR. Neither the 5′-non-coding region nor the CpG island in the coding region of the *SOCS-1* gene [18–20] was hypermethylated, refuting methylation as a mechanism of SOCS-1 suppression.

We next determined whether or not STAT3, a transcription factor for the *SOCS-1* gene, is involved in the suppression of *SOCS-1* by the core protein: a decreased level or a disturbed phosphorylation of STAT3 may account for the suppression of *SOCS-1*. It was found, however, that STAT3 was rather activated by the core protein, consistent with a previous report [28]. The effect on STAT3 activation by the core protein is yet controversial [31]. Similarly, the activation and nuclear translocation of STAT1 was not disturbed by the presence of the core protein. The core protein differentially affected the expression of STAT-target genes such as *IRF1*, c-*myc* or *bcl*-X_L. The core protein suppressed *IRF1* expression in the mouse liver but did not those of c-*myc* and *bcl*-X_L genes. Regulation of *IRF1*

expression is STAT1-dependent in general, although STAT3 is also involved when stimulated by IL-6 [32]. c-*myc* and *bcl*-X_L inductions by IL-6 are chiefly mediated by STAT3 [33]. Thus, the modulation of expression by the core protein may occur in some other STAT-target genes, suggesting somewhere in Jak/STAT signaling pathway including STAT1 activation is impaired by the core protein. However, no defect was identified in the activation and nuclear translocation of STAT1 and STAT3 in the current study. Thus, although we could not define the precise role of the core protein in *SOCS-1* gene suppression, the direct effect of the core protein on the transcription of the gene is the most likely.

In summary, we found that the HCV core protein selectively suppresses *SOCS-1* gene expression in the liver tissues of animals and cultured cells. These findings may provide a basis for an alternative mechanism of the switch-off of SOCS-1 in the pathogenesis of HCV infection by modulating a tumor suppressor activity or responses to IFNs.

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References

[1] 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.

[2] Simonetti RG, Camma C, Fiorello F, Cottone M, Rapicetta M, Marino L, et al. Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. Ann Intern Med 1992;116:97–102.

[3] Umeda T, Hino O. Molecular aspects of human hepatocarcinogenesis mediated by inflammation: from hypercarcinogenic state to normo- or hypocarcinogenic state. Oncology 2002;62:38–42.

[4] Koike K, Tsutsumi T, Fujie H, Shintani Y, Moriya K. Role of hepatitis viruses in hepatocarcinogenesis. Oncology 2002;62:29–37.

[5] Block TM, Mehta AS, Fimmel CJ, Jordan R. Molecular viral oncology of hepatocellular carcinoma. Oncogene 2003;22: 5093–5107.

[6] Colombo M. Hepatitis C virus and hepatocellular carcinoma. Semin Liver Dis 1999;19:263–269.

[7] Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4: 1065–1067.

[8] 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.

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

[10] Tsuchihara K, Hijikata M, Fukuda K, Kuroki T, Yamamoto N, Shimotohno K. Hepatitis C virus core protein regulates cell growth and signal transduction pathway transmitting growth stimuli. Virology 1999;258:100–107.

[11] Ray RB, Meyer K, Ray R. Hepatitis C virus core protein promotes immortalization of primary human hepatocytes. Virology 2000;271: 197–204.

[12] Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, et al. A family of cytokine-inducible inhibitors of signaling. Nature 1997;387:917–921.

[13] Endo TA, Masuhara M, Yokochi M, Suzuki R, Sakamoto H, Mitsui K, et al. A new protein containing an SH2 domain that inhibits JAK kinases. Nature 1997;387:921–924.

[14] Naka T, Narazaki M, Hirate M, Matsumoto T, Minamoto S, Aono A, et al. Structure and function of a new STAT-induced STAT inhibitor. Nature 1997;387:924–929.

[15] Rottapel R, Ilangumaran S, Neale C, Rose JL, Ho JMY, Nguyen MHH, et al. The tumor suppressor activity of SOCS-1. Oncogene 2002;21:4351–4362.

[16] Ilangumaran S, Rottapel R. Regulation of cytokine receptor signaling by SOCS1. Immunol Rev 2003;192:196–211.

[17] Kishimoto T, Kikutani H. Knocking the SOCS off a tumor suppressor. Nat Genet 2001;28:4–5.

[18] Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, et al. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet 2001; 28:29–35.

[19] Okochi O, Hibi K, Sakai M, Inoue S, Takeda S, Kaneko T, et al. Methylation-mediated silencing of SOCS-1 gene in hepatocellular carcinoma derived from cirrhosis. Clin Cancer Res 2003;9: 5295–5298.

[20] Miyoshi H, Fujie H, Moriya K, Shintani Y, Tsutsumi T, Makuuchi M, et al. Methylation status of suppressor of cytokine signaling-1 gene in hepatocellular carcinoma. J Gastroenterol 2004;39:563–569.

[21] Ruggieri A, Harada T, Matsuura Y, Miyamura T. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology 1997; 229:68–76.

[22] Aizaki H, Harada T, Otsuka M, Seki N, Matsuda M, Li YW, Kawakami H, et al. Expression profiling of liver cell lines expressing entire or parts of hepatitis C virus open reading frame. Hepatology 2002;36:1431–1438.

[23] Tsutsumi T, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, et al. Alteration of intrahepatic cytokine expression and AP-1 activation in transgenic mice expressing hepatitis C virus core protein. Virology 2002;304:415–424.

[24] 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.

[25] Moriya K, Fujie H, Yotsuyanagi H, Shintani Y, Tsutsumi T, Matsuura Y, et al. Subcellular localization of hepatitis C virus structural proteins expressed in transgenic liver. Jpn J Med Sci Biol 1997;50:169–177.

[26] Tsutsumi T, Suzuki T, Shimoike T, Suzuki R, Moriya K, Shintani Y, et al. Interaction of hepatitis C virus core protein with retinoid X receptor-α modulates its transcriptional activity. Hepatology 2002;35: 937–946.

[27] Bode JG, Ludwig S, Ehrhardt C, Erhardt A, Albrecht U, Schaper F, et al. IFN-α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J 2003;17:488–490.
[28] Yoshida T, Hanada T, Tokuhisa T, Kosai K, Sata M, Kohara M, et al. Activation of STAT3 by the hepatitis c virus core protein leads to cellular transformation. J Exp Med 2002;196:641–653.
[29] Shoji I, Aizaki H, Tani H, Ishii K, Chiba T, Saito I, et al. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J Gen Virol 1997;78:2657–2664.
[30] Chung CD, Liao J, Liu B, Rao X, Jay P, Berta P, et al. Specific inhibition of Stat3 signal transduction by PIAS3. Science 1997;278:1803–1805.
[31] Hosui A, Ohkawa K, Ishida H, Sato A, Nakanishi F, Ueda K, et al. Hepatitis C virus core protein differently regulates the JAK-STAT signaling pathway under interleukin-6 and interferon-γ stimuli. J Biol Chem 2003;278:28562–28571.
[32] Kojima H, Nakajima K, Hirano T. IL-6-inducible complexes on an IL-6 response element of the junB promoter contain Stat3 and 36 kDa CRE-like site binding protein(s). Oncogene 1996;12: 547–555.
[33] Kiuchi N, Nakajima K, Ichiba M, Fukada T, Narimatsu M, Mizuno K, et al. STAT3 is required for the gp130-mediated full activation of the c-myc gene. J Exp Med 1999;189:63–73.

Molecular Basis of Hepatitis C Virus–Associated Hepatocarcinogenesis: Lessons From Animal Model Studies

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Despite numerous lines of epidemiologic evidence connecting HCV infection and the development of hepatocellular carcinoma (HCC), it remains controversial whether HCV itself plays a direct role or an indirect role in the pathogenesis of HCC. Through the use of transgenic mice, it has become evident that the core protein of HCV has oncogenic potential. HCV is directly involved in hepatocarcinogenesis, albeit other factors such as inflammation and environmental factors might also play a role. The direct involvement of HCV in hepatocarcinogenesis would be achieved via 2 pathways. In one pathway, the core protein acts on the function of mitochondria, leading to the overproduction of oxidative stress, which yields genetic aberrations in cell growth–related genes. The other pathway involves the modulation of cellular gene expressions and intracellular signal transductions, such as mitogen-activated protein kinase pathway, which results in the activation of transcription factors and cell cycle machineries. The combination of these alterations would be hypothesized to provoke the development of HCC in HCV infection. This would be a mechanism for HCC development in HCV infection that is distinct from those for other cancers. The presence of the HCV core protein, to which an oncogenic potential is ascribed, might allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore, unlike in other cancers, HCV infection can elicit HCC in the absence of a complete set of genetic aberrations. Such a scenario, "non-Vogelstein type" carcinogenesis, may explain the unusually high incidence and multicentric nature of HCC development in HCV infection.

Worldwide HCV chronically infects hundreds of millions of people and induces a spectrum of chronic liver diseases.[1] Hence, it impacts the society in a number of domains including medical, sociologic, and economic. Hepatocellular carcinoma (HCC) has become the most frequent cause of death in individuals persistently infected with HCV. In particular, HCV has received increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC. Once cirrhosis is established in Japanese patients infected with HCV, HCC develops at a yearly rate of 5%–7%.[2] Knowledge about the mechanism of HCC development in chronic HCV infection, therefore, is required for the prevention of HCC.

Pathogenesis of Hepatocellular Carcinoma in Persistent Hepatitis C Virus Infection

How HCV induces HCC is not yet clear, despite the fact that more than 70% of patients with HCC in Japan are infected with HCV.[1,3,4] HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These epidemiologic facts are a stimulus to determine the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting itself in various forms of hepatitis, should be considered in a study of the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that necrosis of hepatocytes as a result of chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, which can culminate in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation per se result in the development of HCC in such a high incidence or multicentric pattern in HCV infection? The secondary role of HCV would have to be weighed against the extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and line of reasoning lead to the hypothesis that viral proteins might play a role in inducing HCC. 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 that the carcinogenic capacity of HCV, if any, appears to be weak and would thus take a long time to manifest itself. Actually, it takes 30–40 years for

Abbreviations used in this paper: HCC, hepatocellular carcinoma; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

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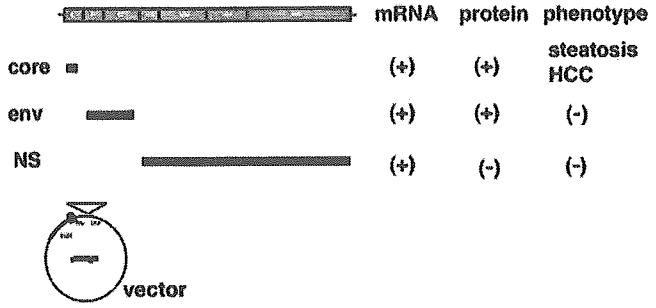

Figure 1. HCV gene transgenic mouse lines. Among the 3 different transgenic mouse lines established, only the transgenic mice carrying the HCV core gene develop HCC after an early phase with hepatic steatosis in 2 independent lineages. The mice transgenic for the envelope (*env*) genes or nonstructural (*NS*) genes do not develop HCC.

HCC to develop in individuals infected with HCV. Another constraint common to studies of carcinogenesis is the development of HCC by transformed cells that might have resulted from uncontrolled growth and escaped surveillance of the host. If this is the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these viewpoints, we initiated a study of carcinogenesis in chronic viral hepatitis by transgenic mouse technology.

Core Protein of Hepatitis C Virus With an Oncogenic Activity in Vivo

As illustrated in Figure 1, transgenic mouse lines with parts of the HCV genome were engineered by introducing genes excised from the cDNA of the HCV genome of genotype 1b.[5,6] The background of the mouse lines is a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] Three different transgenic mouse lines are established, which carry the core gene, envelope genes, or nonstructural genes under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in 2 independent lineages (Figure 1).[6] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[8,9] The transgenic mice carrying the entire nonstructural genes have not developed HCC.

The transgenic mice carry the core gene and express the core protein of an expected size, approximately 21 kd, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[10] Thus, the core gene transgenic mouse model reproduces well this feature of chronic hepatitis C. Of note, evidence of significant inflammation is observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules exhibit a pathology characterized by "nodule in nodule," and HCC with a low degree of differentiation develops within an adenoma as well as within HCC with a higher degree of differentiation.[6] Although numerous lipid droplets are found in cells forming an adenoma, as in nontumorous cells, they are rarely observed in HCC cells. These histologic features closely resemble those observed in HCC developing in chronic hepatitis C patients, in which prominent lipid droplets are found in small, differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.[6] Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[11] These outcomes indicate that the core protein of HCV has an oncogenic potential when expressed in vivo.

Mechanism of Hepatocarcinogenesis in Animal Model for Hepatitis C Virus–Associated Hepatocellular Carcinoma

It is difficult to determine the mechanism of carcinogenesis, even for our simple model in which only the core protein is expressed in otherwise normal liver tissues. There is a notable feature in the localization of the core protein in hepatocytes; whereas the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[6,12] On the basis of this finding, the pathways related to these 2 organelles, the mitochondria and nuclei, were meticulously analyzed.

One activity 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, ie, hepatitis. This reflects a state of an overproduction of reactive oxygen species in the liver or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[13,14] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces excessive oxidative stress in the absence of inflammation and might, at least in part, contribute to hepatocarcinogenesis in HCV infec-

Table 1. Biomolecular Alterations With the Core Protein Expression Observed in the Transgenic Mouse Model

1. Induction of cytokines including tumor necrosis factor–α and interleukin-1β[19]
2. Activation of MAPK pathway and enhancement of AP-1 activation[19,21]
3. Overproduction of oxidative stress or reactive oxygen species in the absence of inflammation[13]
4. Synergy of HCV core and alcohol in inducing oxidative stress and activating MAPK[13,21]
5. Interaction of HCV core and retinoid X receptor–α and peroxisome proliferator activated receptor–α[20]
6. Induction of insulin resistance[17]
7. Development of steatosis by inhibiting microsomal triglyceride transfer protein activity[5,14,24]
8. Interaction of HCV core and proteasome activator PA28γ[25]
9. Inhibition of suppressor of cytokine signaling–1[26]

tion. If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that cannot be further scavenged by a physiologic antagonistic system. This indicates 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 might be ascribed to the mitochondrial dysfunction.[13,15] The function of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[16] Hepatic steatosis in hepatitis C might work as fuel for oxidative stress overproduction.[14,17,18]

Other possible pathways would be alteration in the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Table 1). For example, tumor necrosis factor–α and interleukin-1β have been found to be transcriptionally activated.[19] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor–α, that play pivotal roles in cell proliferation and metabolism.[20] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of 3 routes, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase, is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model before HCC development, only the JNK route is activated. In the downstream of the JNK activation, transcription factor AP-1 activation is markedly enhanced.[19,21] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives an advantage for cell proliferation to hepatocytes.

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

Hepatocarcinogenesis Induced by Hepatitis C Virus: A Mechanism Distinct From Those in Other Cancers

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV might be directly involved in hepatocarcinogenesis.

In research studies of carcinogenesis, the theory of Kinzler and Vogelstein[22] has gained 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 together lead toward the development of colorectal cancer. Their theory has been extended to the carcinogenesis of other cancers as well, so-called Vogelstein-type carcinogenesis (Figure 2).

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a mechanism different from that of Kinzler and Vogelstein[22] for the hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all

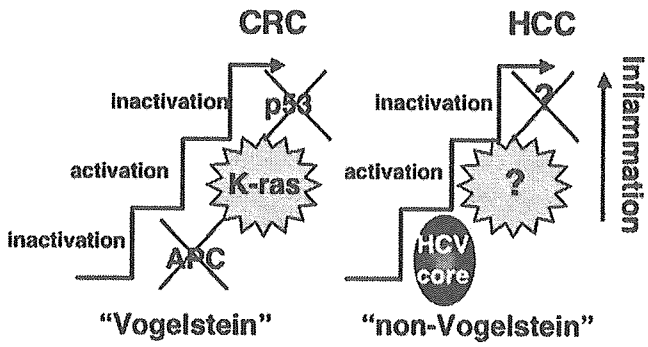

Figure 2. Mechanism of HCV-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps might be skipped in the development of HCC in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a non-Vogelstein–type process for the induction of HCC, a plausible explanation might be given for many unusual events happening in HCV carriers. *CRC*, colorectal cancer.