

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 PA28 γ [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

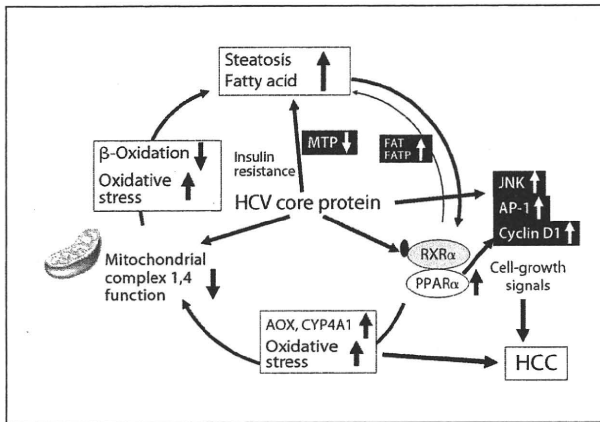


Fig. 2. HCV core protein causes 'fatty acid spiral'. In HCV infection, the core protein induces steatosis via several pathways, leading to 'fatty acid spiral' in the presence of the mitochondrial complex-1 dysfunction and PPAR- α activation, both of which are also caused by the core protein. These intracellular 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; RXR α = retinoid X receptor- α ; PPAR α = 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 fibrates 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.

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Review Article

Animal models for hepatitis C and related liver disease

Kazuhiko Koike,¹ Kyoji Moriya² and Yoshiharu Matsuura³Departments of ¹Gastroenterology and ²Infection Control and Prevention, Graduate School of Medicine, University of Tokyo, Tokyo, and ³Department 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-ky@umin.ac.jp
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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 con-

tracted 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 NH₂-terminal quarter of the genome, include the

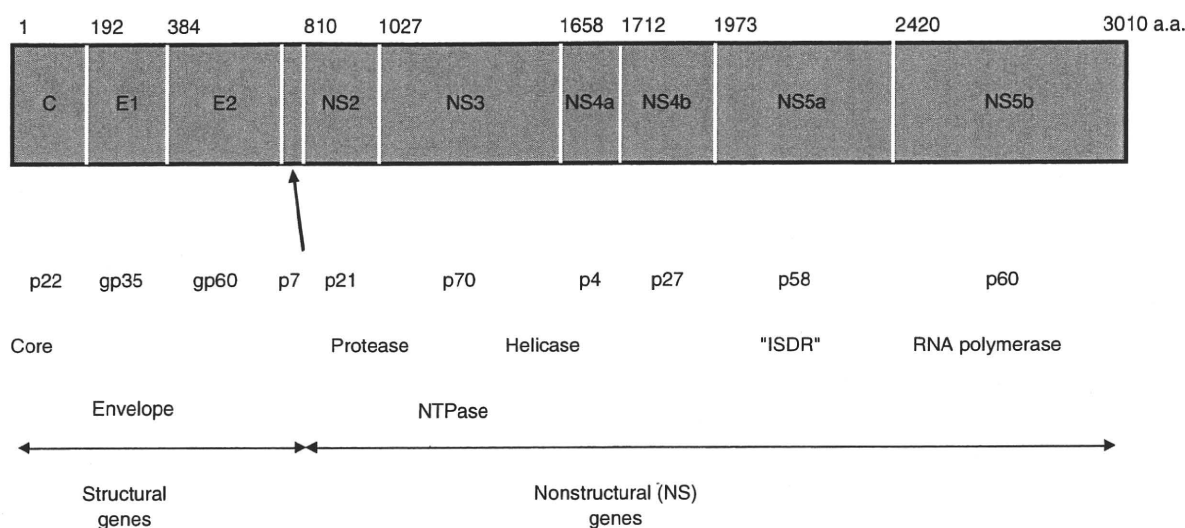


Figure 1 The structure of hepatitis C virus (HCV) genome. The HCV genome RNA encodes a polyprotein of 3010 amino acids (a.a.), which is processed to structural and non-structural proteins by the cellular or viral proteases. ISDR, interferon sensitivity-determining region.

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 PA28 γ /REG γ , resulting in PA28 γ -dependent degradation of the core protein.¹⁶

A recent report suggests that ubiquitination and adenosine triphosphate (ATP) are not required for PA28 γ -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 PA28 γ , Core-Tg/PA28 γ -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,

the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC through 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 the multicentric nature of HCV infection?

The other role of HCV would have to be weighed against a 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 lesions 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 (Table 1). They include the ones carrying the entire coding region of the HCV genome,³³ the core region only,^{21,29} the envelope region only,^{30,31} the core and envelope regions^{33,34} and the core to NS2 regions.⁴¹ Although detection of mRNA from the NS regions of the HCV cDNA has been reported,^{33,37} the detection of HCV NS proteins in the transgenic mouse liver have 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.* utilized the Cre/loxP 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.⁴¹ 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 by introducing the genes from the cDNA of the HCV genome of genotype 1b.^{21,22} Established are three different kinds of transgenic mouse lines, which carry the core gene, envelope genes or non-structural genes, respectively, under the same transcriptional regulatory element. Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages (Fig. 2).²² The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins,^{31,32} and the transgenic mice carrying the entire non-structural genes 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 one of the histological characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.⁴³ 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.^{29,33,34} These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

Table 1 Transgenic mouse lines constitutively expressing hepatitis C virus proteins

HCV gene	Genotype	Promoter	Protein expression	Phenotypes	References
Core	1b	HBV	Similar to patients	Steatosis, HCC, insulin resistance, oxidative stress	Moriya 1997 ²¹ & 1998 ²² Tsutsumi 2002 ²³ & 2003 ²⁴ Moriishi 2003 ¹⁶ & 2007 ²⁵ Shintani 2004 ⁴⁶ Miyamoto 2007 ¹⁵ Tanaka 2008 ^{27,28} Machida 2006 ²⁹
Core	1b	EF-1a	Similar to patients	Steatosis, adenoma, HCC, oxidative stress	
Core, E2 truncated	1b	MUP	(-)	None	Pasquinelli 1998 ³⁰
E1-E2	1b	HBV	Abundant	None in the liver	Koike 1995, ³¹ Koike 1997 ³²
Core-E1-E2	1b	Albumin	Similar to patients	Steatosis, HCC, oxidative stress	Lerat 2003 ³³
Core-E1-E2	1a	CMV	Similar to patients	Steatosis, HCC	Naas 2005 ³⁴
Core or structural proteins	1b	Alb	Low	Larger tumor development with DEN treatment	Kamegaya 2005 ³⁵
Structural proteins	1b	MUP	Low	None	Kawamura 1998 ³⁶
Structural proteins	1b	MHC	Low in the liver	Hepatitis	Honda 1999 ³⁷
Entire polyprotein	1b	Albumin	Only mRNA detectable	Steatosis, HCC	Lerat 2003 ³³
Entire polyprotein	1a	A1-antitrypsin		Steatosis, intrahepatic T cell recruitment	Alonzi 2004 ³⁸
NS3/4A	1a	MUP		None (modulation of immunity)	Frelin 2006 ³⁹
NS5A	1a	apoE		None (resistance to TNF)	Majumder 2002 ⁴⁰

HBV, hepatitis B virus; EF, elongation factor; MUP, major urinary protein; Alb, albumin; CMV, cytomegalovirus; MHC, major histocompatibility complex; AT, anti-trypsin; apo E, apolipoprotein E.

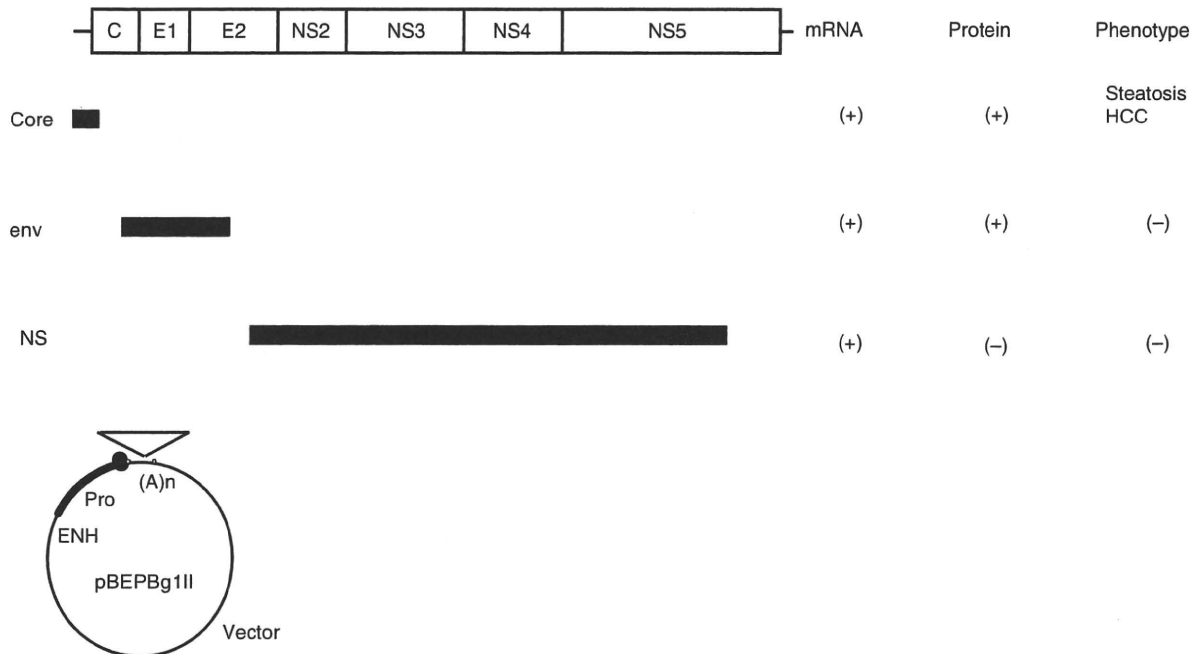


Figure 2 Transgenic mouse lines carrying the hepatitis C virus (HCV) genome.^{21,22,31,32,42} Three different kinds of transgenic mouse lines, carrying the core gene, envelope genes or non-structural genes of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. env, envelope genes; NS, nonstructural genes.

OXIDATIVE STRESS AND INTRACELLULAR SIGNALING IN HCV-ASSOCIATED PATHOGENESIS

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.^{14,22} 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 interven-

ing inflammation.^{44,45} 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 the 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 the antioxidant system, which may explain the mechanism underlying the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

Thus, in the absence of inflammation, 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

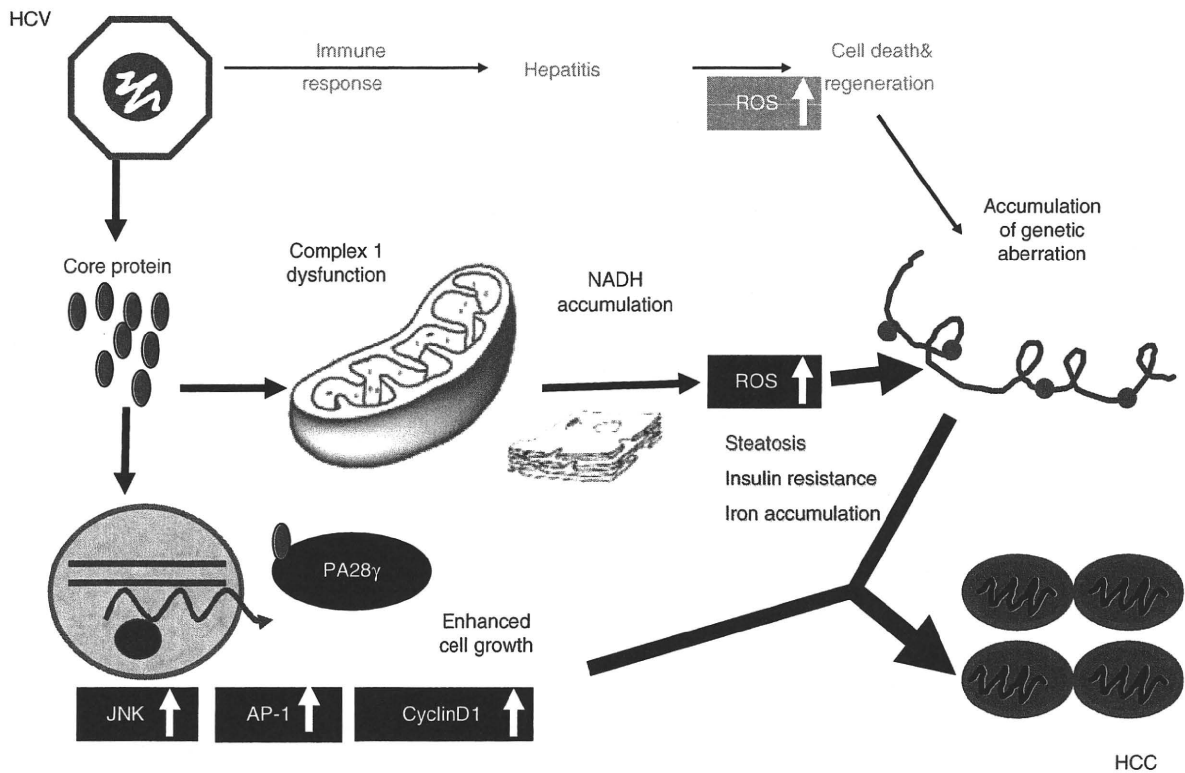


Figure 3 Mechanism of hepatocarcinogenesis in hepatitis C virus (HCV) infection. The core protein is localized in the nuclei of hepatocytes in addition to cytoplasm and may interact with nuclear factors, thereby deregulating the cell growth and death. The core protein may also affect the cell growth by its accumulating lipid in the hepatocytes, because lipid such as triglycerides are now known to be ligands for some nuclear receptors. Accumulated lipid may also cause genetic aberrations through its alteration to peroxylipid. AP-1, activating protein-1; HCC, hepatocellular carcinoma; JNK, c-Jun N-terminal kinase; NADH, nicotinamide adenine dinucleotide; PA28, proteasome activator 28; ROS, reactive oxygen species.

hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction.^{22,44} The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.⁴⁶

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 (IL)-1 β have been found transcriptionally activated.²³ 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.^{23,24} 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 to cell proliferation to hepatocytes (Fig. 3).

METABOLIC ASPECTS OF HCV INFECTION

STEATOSIS IS FREQUENTLY observed in chronic hepatitis C patients, and is significantly associated

Table 2 Cellular genes differentially expressed in hepatitis C virus core transgenic mouse³⁶

Liver	Upregulated	Downregulated
Lipid metabolism	NPC1 Catalase Very long chain acyl-CoA Dehydrogenase Carboxylesterase Selenoprotein P Carbonic anhydrase Adipose differentiation Related protein Bilirubin/phenol family UDP Glucuronosyltransferase	Stearoyl-CoA desaturase Sterol-carrier protein X α -Enolase carnitine acetyltransferase Gal- β 1,4(3) GlcNAc- α 2,3-Sialyltransferase Very long chain acyl-CoA synthetase Liver transferrin 4-Hydroxyphenylpyruvate dioxygenase LAF1 transketolases-adenosylmethionine synthetase Apolipoprotein A-II
Transcription and cell proliferation	Int-6 GCN5L1 <i>Homo sapiens</i> 8.2 kDa differentiation factor USF1 Initiation factor eIF-4AI Human elongation factor-1- δ Suil	Human guanine nucleotide regulatory protein alpha-fetoprotein Retinol binding protein
Inflammation	α -1 Protease inhibitor 3 Hemopexin	α -2-Macroglobulin LMW prekininogen Complement component C3 AHSG (α -2 HS-glycoprotein) homolog
Others	Microvascular endothelial differentiation gene 1 Diazepam-binding inhibitor Argininosuccinate synthetase Skeletal muscle- α tropomyosin Ampd3 gene DNA-binding protein	Vitronectin Epithelin 1 and 2 Murinoglobulin

with increased fibrosis and progression rate of fibrosis of the liver.⁴⁷ 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 (Table 2).

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 mono-unsaturated 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.⁴⁵ 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)-1.²⁶

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.⁴⁸ 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 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 This corroborates the results in *in vitro* studies^{49,50} and a chimpanzee study.⁵¹ In humans, McPharson *et al.* have reported that there was no significant difference in the level of SREBP-1c mRNA in the liver tissues of chronic hepatitis C patients and normal subjects.⁵² However, the number of samples in that study was small, and a larger number must be examined to draw a conclusion in human patients.

PROTEASOME ACTIVATOR 28 γ AND HCV

INTERESTINGLY, WE FOUND recently that a protein interacting with the core protein, proteasome activator (PA)28 γ , is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC.^{15,25} The pathogenic mechanisms underlying the core protein-induced diseases are summarized in Figure 3. Steatosis is defined as an accumulation of lipid droplets, the 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 SREBP were induced during the early stage of HCV infection in the livers of chimpanzees.⁵³ 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 PA28 γ -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 ⁵⁴ or by suppression of a co-repressor such as Sp110b, a negative regulator of RAR- α , by sequestering it in the cytoplasm through interaction with the cytoplasmic core protein.⁵⁵ 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.⁵⁶ As stated before, the MTP regulates the assembly and secretion of VLDL consisting of apolipoprotein B, cholesterol and triglycerides. In the core gene transgenic mice, MTP-specific activity is significantly decreased.⁴⁸ 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.⁵⁷ Furthermore, increases in saturated and monounsaturated fatty acids enhance HCV RNA replication.⁵⁸ 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.^{15,26} Moreover, the core gene transgenic mice have been shown to exhibit overt diabetes when fed a high-fat diet,

while control mice do not.²⁶ 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 through inhibition of tyrosine phosphorylation of IRS. 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 steatosis.^{15,26} Moreover, in the latter study, hyperinsulinemia was cured by depletion of TNF- α , suggesting that upregulation of TNF- α contributes to the core protein-induced insulin resistance.²⁶ Our previous study has indicated that the core protein-induced overexpression of TNF- α is also dependent on the presence of PA28 γ .¹⁵

In relation to lipid metabolism, the core protein has also been found to interact with RXR- α .⁵⁹ 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.⁵⁵ Based on these results, we then examined the expression and function of PPAR- α in the liver of core gene transgenic mice.

PPAR- α AND “FATTY ACID SPIRAL” IN HCV-ASSOCIATED HEPATOCARCINOGENESIS

PEROXISOME PROLIFERATOR-ACTIVATED receptor- α is one of the 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.⁶⁰ 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.⁵⁶

The level of PPAR- α protein was increased in the liver of core gene transgenic mice as early as 9 months old. 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.

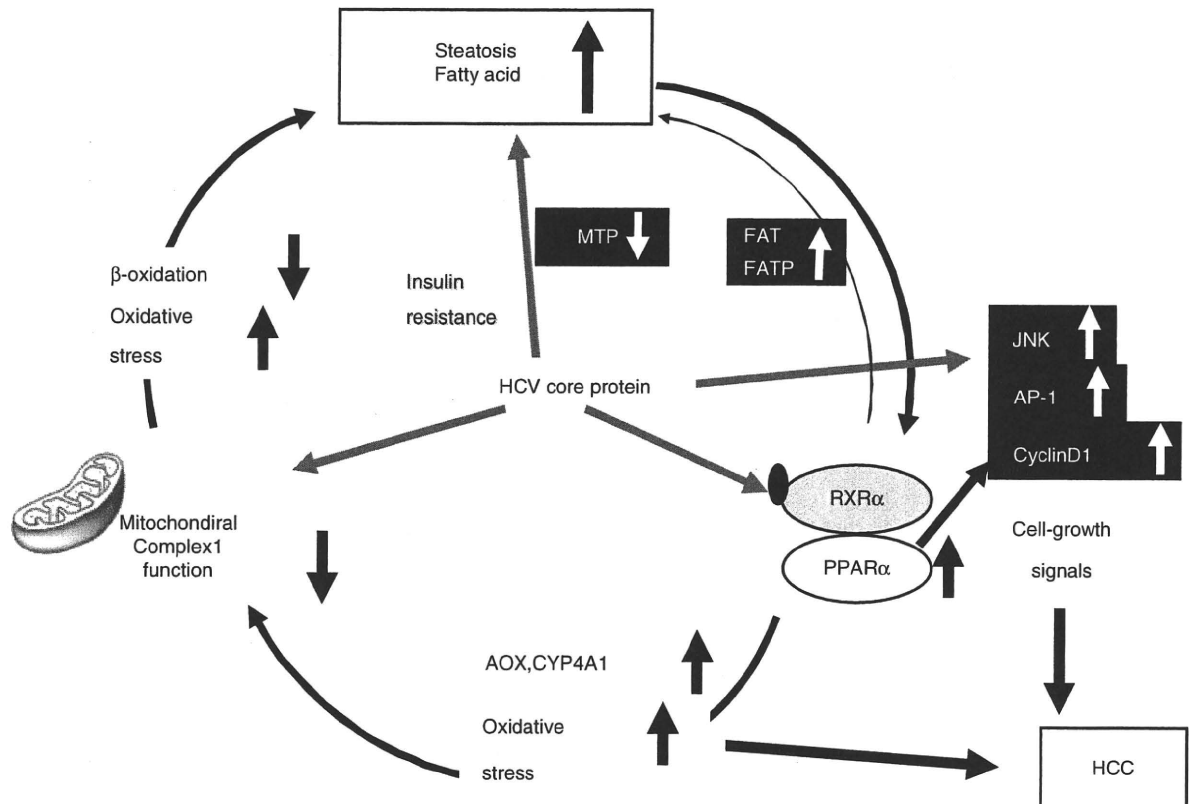


Figure 4 Hepatitis C virus (HCV) core protein causes “fatty acid spiral”. In HCV infection, the core protein induces steatosis through several pathways, leading to “fatty acid spiral” in the presence of the mitochondrial complex 1 dysfunction and peroxisome proliferator-activated receptor- α (PPAR- α) 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. AOX, acyl-CoA oxidase; AP-1, activating protein-1; CYP, cytochrome P450; FAT, fatty acid translocase; FATP, fatty acid transport protein; HCC, hepatocellular carcinoma; JNK, c-Jun N-terminal kinase; MTP, microsomal triglyceride transfer protein; RXR- α , retinoid X receptor- α .

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.²⁷ However, in general, the activation of PPAR- α leads to improvement but not aggravation of steatosis. Then, what is the 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 mated 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.²⁸ 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.²⁷ 8-Hydroxy deoxyguanosine (8-OHdG) and peroxy lipids, 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,^{46,61} 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 approxi-

mately one-third of PPAR- α -intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for the PPAR- α gene neither developed HCC.²⁸ 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 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 4 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 “fatty acid spiral” in the presence of the mitochondrial complex 1 dysfunction 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 Vogelstein⁶² 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

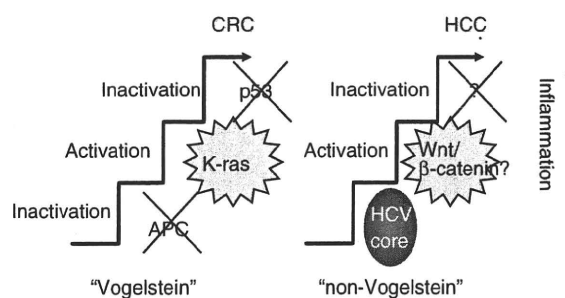


Figure 5 The role of hepatitis C virus (HCV) in hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a “non-Vogelstein-type” process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers. APC, adenomatous polyposis coli; CRC, colorectal cancer.

adenomatous polyposis coli gene for inactivation, those in K-ras for activation and those in the p53 gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.⁶² Their theory has been extended to the carcinogenesis of other cancers as well, called “Vogelstein-type” carcinogenesis (Fig. 5).

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 allow multi-stages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (Fig. 5). 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.⁴² 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.

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