

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

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

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

## INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver disease. Persistent HCV infection leads to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC), thereby being a serious problem both in medical and socio-economical aspects [Saito et al., 1990]. Despite overwhelming evidence from epidemiological studies, the precise mechanism of hepatocarcinogenesis in HCV infection

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

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

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

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

## MATERIALS AND METHODS

### Transgenic Mice and Cultured Cells

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

### Determination of Iron in the Liver

Determination of Fe in the liver was performed by Shimadzu Techno-Research, K.K. (Kyoto, Japan) using Inductively Coupled Plasma apparatus, ICP8100 (Shimadzu Corp.). Briefly, samples were resolved by microwave (Microwave Resolution System ETHOS-TC, Milestone General, Inc., Tokyo, Japan) after the addition of nitric acid, and the volume was adjusted by the addition of H<sub>2</sub>O. This solution was then subjected to the quantification of iron using ICP8100 (<http://www.shimadzu.com/products/lab/ms/glossary/oh80jt00000008w4.html>).

### Iron Loading Experiments

For the short-term iron loading experiment, three doses of FeSO<sub>4</sub> solution (100 mg/kg BW, suspended in dH<sub>2</sub>O) or vehicle only were administered to the core gene transgenic or control mice i.p., with intervals of 24 hr at the age of 6 months [Zhu and Miller, 2007]. For the long-term iron loading experiment, 50 mg/kg BW of FeSO<sub>4</sub> was administered to the core gene transgenic mice i.p., once a week for 3 months from the age of 3 months. HepG2 cell lines expressing the HCV core protein under the control of the CAG promoter (Hep39J, Hep396, and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously [Ruggieri et al., 2004]. For the iron loading experiments, hemin solution (10 mM in DMSO) was added to the culture medium at

the final concentration of 5 μM, and the cells were incubated for 5 or 72 hr.

## Evaluation of Oxidant and Antioxidant Systems

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

### Real-Time PCR and Western Blotting

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

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

### Statistical Analysis

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

## RESULTS

### Iron Accumulation in Ordinarily Fed Core Gene Transgenic Mice

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

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

### Iron Overloading to Core Gene Transgenic Mice

When the mice were overloaded with iron, the intrahepatic levels of iron markedly increased. In the

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

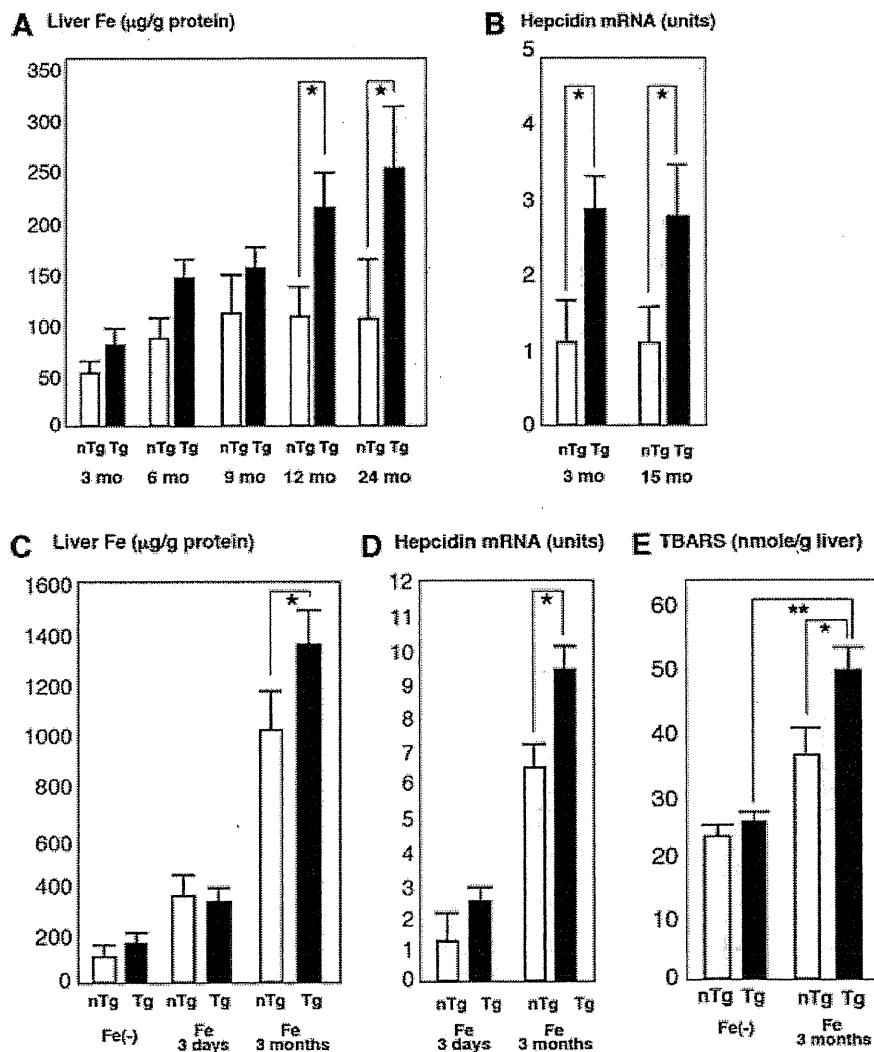


Fig. 1. A: Amounts of iron accumulated in the liver of mice fed with normal chow. B: Hepcidin mRNA level in the liver of mice fed with normal chow at 3 and 15 months. C: Amounts of iron in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. D: Hepcidin mRNA level in the liver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

**Oxidative Stress and Iron Overloading in Mice**

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

**Impact of Iron Overloading on Antioxidants**

The effect of long-term iron overloading on antioxidants was evaluated by real-time PCR analysis (Fig. 2). As already reported [Moriya et al., 2001], the levels of antioxidant enzymes, such as catalase, GST, or SOD1, were higher in the core transgenic mice than in the control mice before iron overloading. The levels of antioxidant mRNAs were higher in the long-term iron-treated mice than in the vehicle-treated mice. However, the magnitude of augmentation by iron overloading was

different among the antioxidant genes. While catalase and GST genes were significantly more enhanced in the core gene transgenic mice than in the control mice by iron overloading ( $P < 0.05$ ), there was less augmentation in the expressions of HO-1 and NQO-1 genes in the core gene transgenic mice than in the control mice. The level of HO-1 mRNA in the long-term-treated mice was significantly higher in the control mice than in the core gene transgenic mice ( $P < 0.05$ , respectively), in contrast to that of catalase, GST, or SOD1 gene. The level of NQO-1 mRNA in the liver was lower in the core gene transgenic than in the control mice, although the difference was not statistically significant.

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

**HO-1 and Iron in Cultured Cells**

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

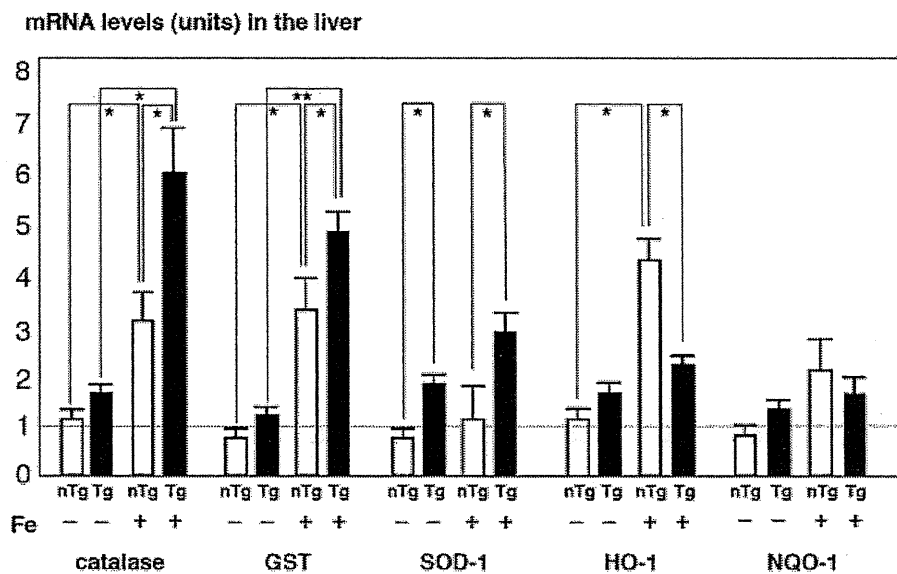


Fig. 2. Levels of antioxidant mRNA in the liver of mice subjected to iron overloading. Levels of mRNA were determined in nontransgenic control mice (open bars) or in transgenic mice (closed bars) with 3-month administration of iron (Fe+) or without it (Fe-). Mice were sacrificed at 6 months and liver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

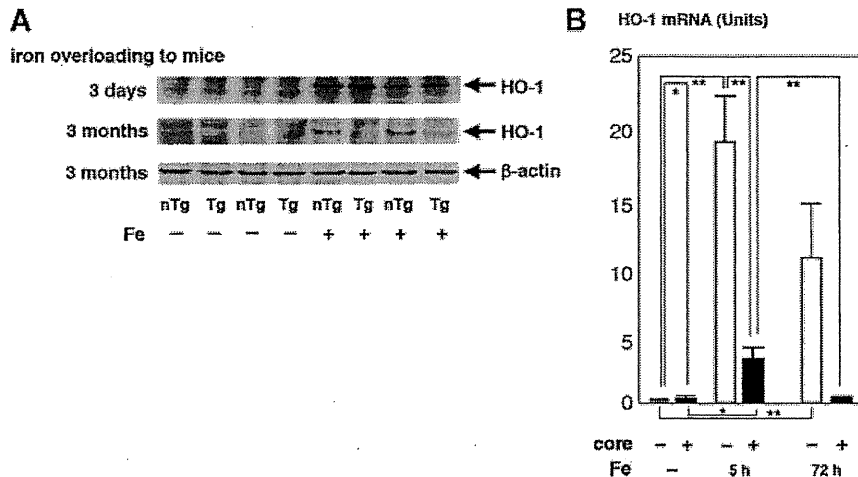


Fig. 3. A: Levels of HO-1 protein determined by Western blotting in the liver of mice subjected to iron overloading for 0, 3 days, or 3 months. B: Levels of HO-1 mRNA in core-protein-expressing or nonexpressing HepG2 cells subjected to iron loading for 0, 5, or 72 hr. nTg, non-transgenic control mice;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

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

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

To explore the mechanism underlying the differential responses to iron overloading in antioxidant gene expressions, the intracellular distribution of a transcription factor, Nrf2, which regulates the expression of HO-1 [Srisook et al., 2005; Farombi and Surh, 2006] was examined. For this analysis, the liver tissues from the core gene transgenic and control mice, either iron-overloaded or not, were subjected to subcellular fractionation, followed by detection by Western blotting. However, there was no decrease or rather an increase

in the Nrf2 level localized in the nuclear fraction after the iron-overloading treatment in the core transgenic mouse liver (Fig. 4A), indicating that the attenuation of HO-1 expression is not dependent on Nrf2. Finally, no interaction was observed between the core protein and the Nrf2 protein as determined by coimmunoprecipitation using cultured cells (data not shown). Because transcription factors, other than Nrf2, such as AP-1 and NF- $\kappa$ B, may be responsible for the HO-1 gene expression [Ferrándiz and Devesa, 2008], and Bach1, an HO-1 repressor [Shan et al., 2004], may be responsible for the attenuation of HO-1 expression, changes in expression levels of these factors were determined by the real-time PCR. AP-1 was activated by the core protein while NF- $\kappa$ B was not in the liver of transgenic mice [Tsutsumi et al., 2002]. With the administration of iron, mRNA levels of AP-1 and NF- $\kappa$ B1 were increased slightly both in core gene transgenic and control mice (Fig. 4B), thereby not explaining the attenuation of iron-induced HO-1 induction by the core protein. Bach1 expression level was not changed significantly by iron administration in the core gene transgenic mice (Fig. 4B), negating the possibility that this repressor acts to inhibit the induction of HO-1 gene expression by iron.

#### DISCUSSION

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

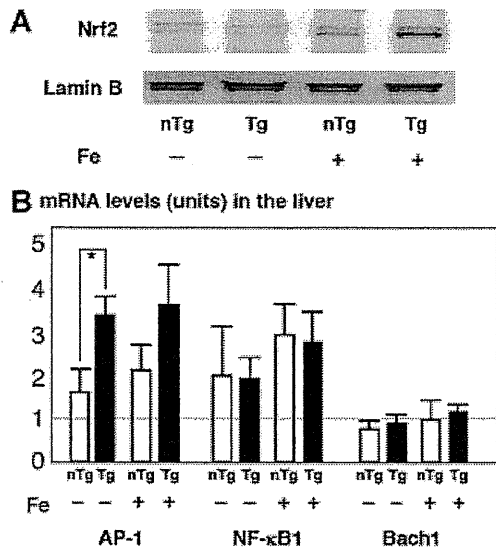


Fig. 4. A: Intracellular localization of Nrf2 in the liver of mice subjected to iron overloading for 0 or 3 months. The nuclear fraction was isolated from liver tissues and subjected to Western blotting with antibody to Nrf2 or lamin B (control). Levels of antioxidant mRNA in the liver of mice subjected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

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

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

hepatitis-C-associated iron accumulation. The reason for this dissociation between the two HCV mouse models is unclear, but the difference in the transgene construct may account for it: the core gene only or the full coding sequence [Moriya et al., 1998; Nishina et al., 2008].

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

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

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

### ACKNOWLEDGMENTS

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## Review Article

## Animal models for hepatitis C and related liver disease

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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)- $\alpha$  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.<sup>1</sup> There has been no animal model for HCV infection/pathogenesis except for the chimpanzee.<sup>2</sup>

Recently, however, several small animal models for HCV infection have been evaluated, including *Tupaia*<sup>3</sup> and genetically engineered mice that are chimeric for human hepatocytes.<sup>4</sup> 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.

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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.<sup>2</sup> 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<sup>+</sup> or CD8<sup>+</sup> 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.<sup>1</sup> Immunization with virus-like particles of chimpanzees induced an HCV-specific immune response of CD4<sup>+</sup> or CD8<sup>+</sup> T cells, thereby suppressing the development of high viral loads in chimpanzees that were challenged with HCV.<sup>5</sup> 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.<sup>6</sup>

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.<sup>2</sup> 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,<sup>3</sup> and was used for the study of HCV infection.<sup>7</sup> 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.<sup>8,9</sup>

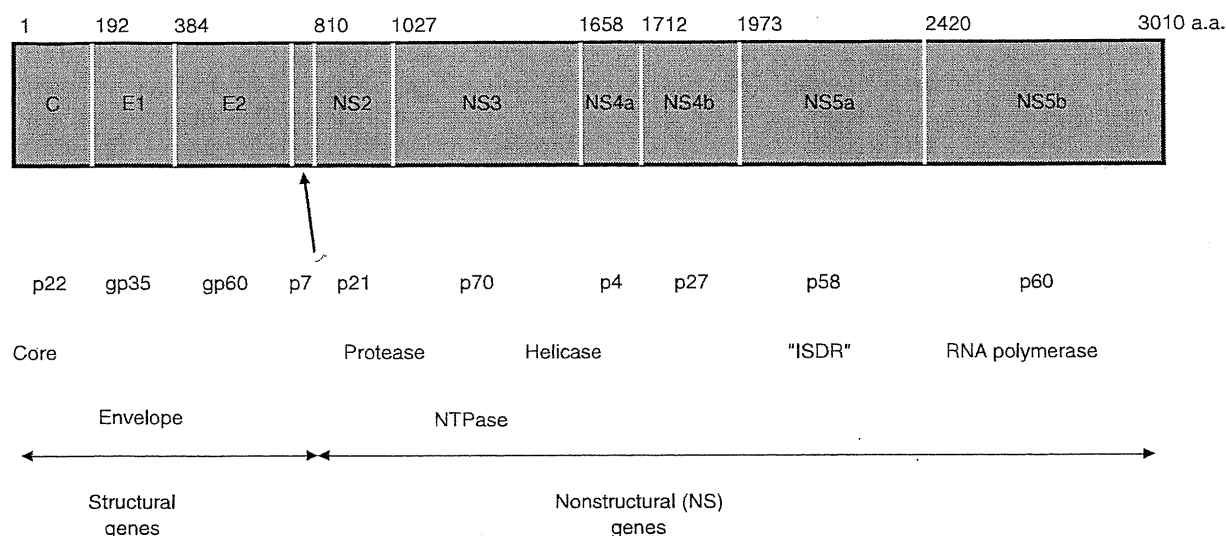
While the development of liver disease (a cirrhosis-like lesion) in HCV-infected Tupaia was presented at scientific meetings, a scientific paper describing it has not appeared yet. In conclusion, the value of Tupaia in HCV research is limited, but it may be utilized for the analysis of viral entry or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

### HCV

**T**HE 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.<sup>10</sup> The genome consists of a large translational open reading frame (ORF) encoding a polyprotein of approximately 3010 amino acids (aa) (Fig. 1). The ORF is flanked by highly conserved untranslated regions (UTR) at both the 5' and 3' termini. The complete 5' UTR consists of 341 nt and acts as an internal ribosomal entry site. This feature leads to the translation of the RNA genome using a cap-independent mechanism, rather than ribosome scanning from the 5' end of a capped molecule.

The polyprotein is processed by both the cellular and viral proteases to generate the viral gene products, which are subdivided into the structural and non-structural proteins. The structural proteins, which are encoded by the NH<sub>2</sub>-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.<sup>11</sup> 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.<sup>12</sup> In addition, the region spanning from residue 112–152 is associated with membranes of the endoplasmic reticulum and mitochondria.<sup>13</sup> The core protein is also localized into the nucleus<sup>14,15</sup> and binds to the nuclear

proteasome activator PA28 $\gamma$ /REG $\gamma$ , resulting in PA28 $\gamma$ -dependent degradation of the core protein.<sup>16</sup>

A recent report suggests that ubiquitination and adenosine triphosphate (ATP) are not required for PA28 $\gamma$ -dependent proteasome activity.<sup>17</sup> HCV core protein is also known to be ubiquitinated by E3 ligase E6AP and degraded in the ubiquitin/ATP-dependent pathway.<sup>14</sup> 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 $\gamma$ , Core-Tg/PA28 $\gamma$ -knockout mice have been generated and analyzed as described below (section 9).<sup>15</sup>

## POSSIBLE ROLE OF HCV IN HEPATOCARCINOGENESIS

**T**HE 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.<sup>18–20</sup> 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,<sup>33</sup> the core region only,<sup>21,29</sup> the envelope region only,<sup>30,31</sup> the core and envelope regions<sup>33,34</sup> and the core to NS2 regions.<sup>41</sup> Although detection of mRNA from the NS regions of the HCV cDNA has been reported,<sup>33,37</sup> 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.<sup>41</sup> 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.

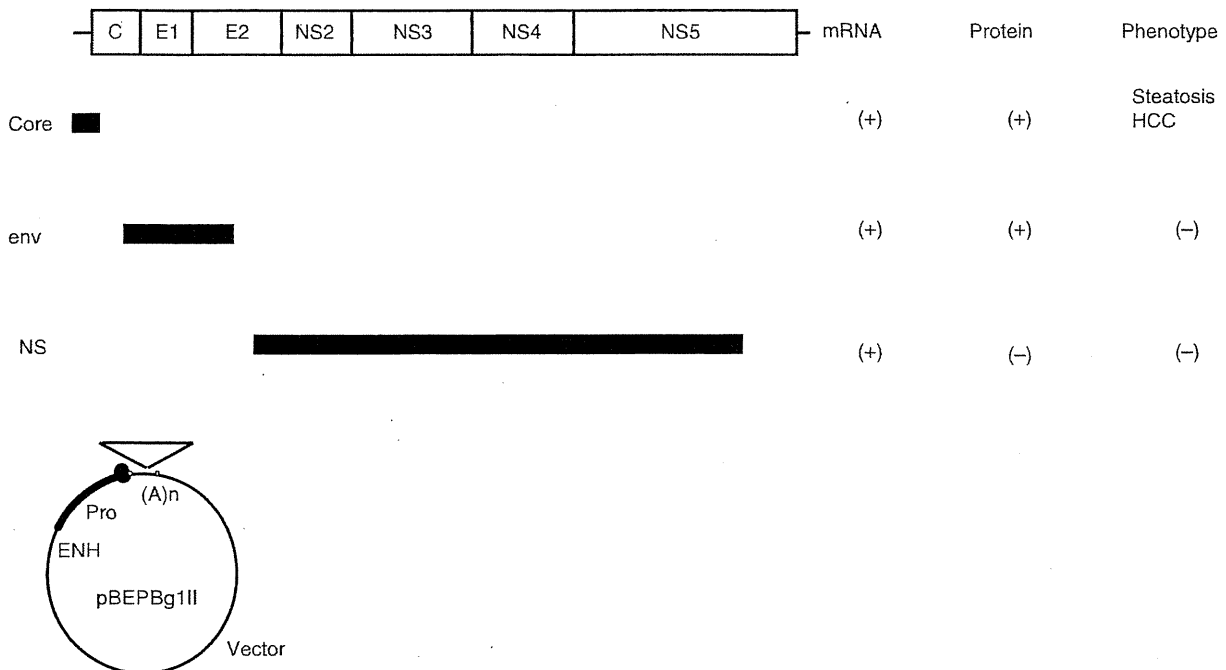
We have engineered transgenic mouse lines carrying the HCV genome by introducing the genes from the cDNA of the HCV genome of genotype 1b.<sup>21,22</sup> Established are three different kinds of transgenic mouse lines, which carry the core gene, envelope genes or non-structural genes, respectively, 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).<sup>22</sup> The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins,<sup>31,32</sup> 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.<sup>43</sup> 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.<sup>29,33,34</sup> 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 <sup>21</sup> & 1998 <sup>22</sup> Tsutsumi 2002 <sup>23</sup> & 2003 <sup>24</sup> Moriishi 2003 <sup>16</sup> & 2007 <sup>25</sup> Shintani 2004 <sup>26</sup> Miyamoto 2007 <sup>15</sup> Tanaka 2008 <sup>27,28</sup> Machida 2006 <sup>29</sup>
Core	1b	EF-1a	Similar to patients	Steatosis, adenoma, HCC, oxidative stress	Pasquinelli 1998 <sup>30</sup>
Core, E2 truncated	1b	MUP	(-)	None	Koike 1995, <sup>31</sup> Koike 1997 <sup>32</sup>
E1-E2	1b	HBV	Abundant	None in the liver	Lerat 2003 <sup>33</sup>
Core-E1-E2	1b	Albumin	Similar to patients	Steatosis, HCC, oxidative stress	Naas 2005 <sup>34</sup>
Core-E1-E2	1a	CMV	Similar to patients	Steatosis, HCC	Kamegaya 2005 <sup>35</sup>
Core or structural proteins	1b	Alb	Low	Larger tumor development with DEN treatment	Kawamura 1998 <sup>36</sup>
Structural proteins	1b	MUP	Low	None	Honda 1999 <sup>37</sup>
Structural proteins	1b	MHC	Low in the liver	Hepatitis	Lerat 2003 <sup>33</sup>
Entire polyprotein	1b	Albumin	Only mRNA detectable	Steatosis, HCC	Alonzi 2004 <sup>38</sup>
Entire polyprotein	1a	A1-antitrypsin		Steatosis, intrahepatic T cell recruitment	Frelin 2006 <sup>39</sup>
NS3/4A	1a	MUP		None (modulation of immunity)	Majumder 2002 <sup>40</sup>
NS5A	1a	apoE		None (resistance to TNF)	

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.<sup>21,22,31,32,42</sup> 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.<sup>14,22</sup> 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.<sup>44,45</sup> 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

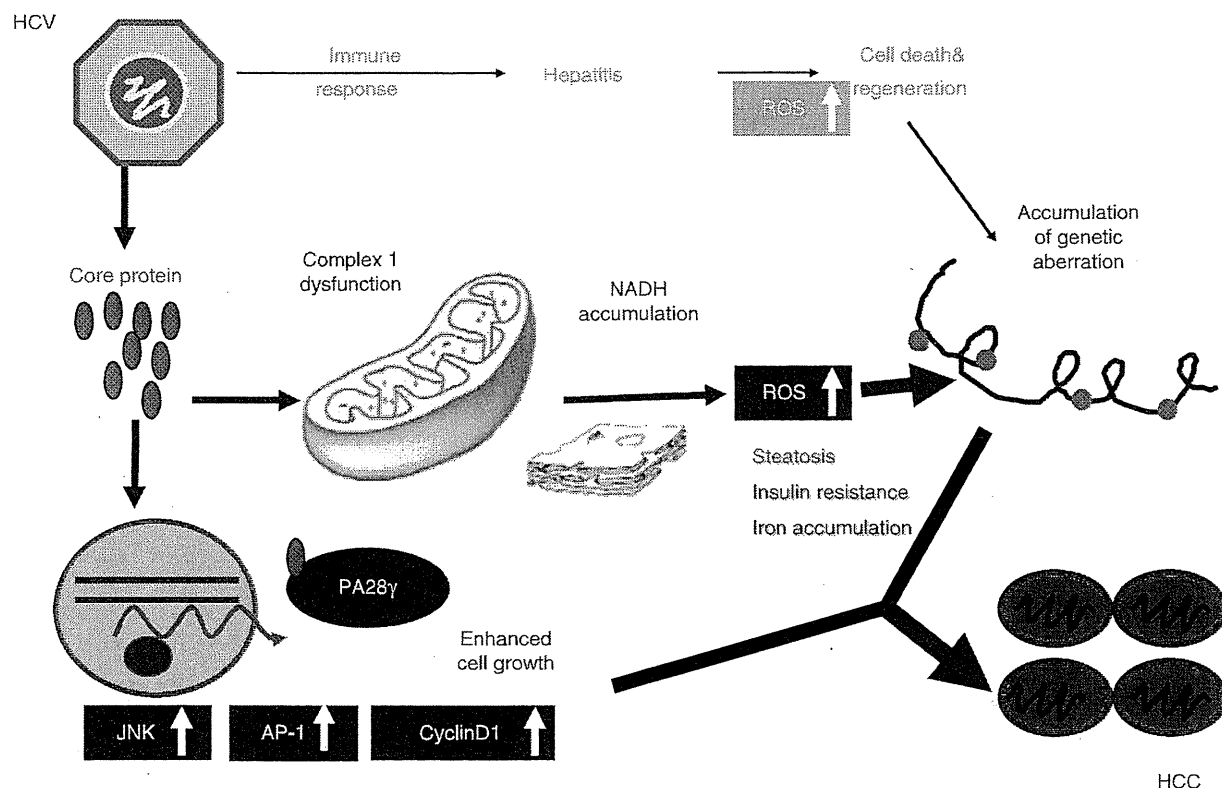


Figure 3 Mechanism of hepatocarcinogenesis in hepatitis C virus (HCV) infection. The core protein is localized in the nuclei of hepatocytes in addition to cytoplasm and may interact with nuclear factors, thereby deregulating the cell growth and death. The core protein may also affect the cell growth 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 peroxy lipid. 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.<sup>22,44</sup> The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.<sup>46</sup>

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)- $\alpha$  and interleukin (IL)-1 $\beta$  have been found transcriptionally activated.<sup>23</sup> 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.<sup>23,24</sup> 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 mouse<sup>3a</sup>

Liver	Upregulated	Downregulated
Lipid metabolism	NPC1	Stearoyl-CoA desaturase
	Catalase	Sterol-carrier protein X
	Very long chain acyl-CoA	$\alpha$ -Enolase carnitine acetyltransferase
	Dehydrogenase	Gal- $\beta$ 1,4(3) GlcNAc- $\alpha$ 2,3-Sialyltransferase
	Carboxylesterase	Very long chain acyl-CoA synthetase
	Selenoprotein P	Liver transferrin
	Carbonic anhydrase	4-Hydroxyphenylpyruvate dioxygenase
	Adipose differentiation	LAF1 transketolases-adenosylmethionine synthetase
	Related protein	Apolipoprotein A-II
	Bilirubin/phenol family UDP	
	Glucuronosyltransferase	
Transcription and cell proliferation	Int-6	Human guanine nucleotide regulatory protein
	GCN5L1	alpha-fetoprotein
	<i>Homo sapiens</i> 8.2 kDa differentiation factor	Retinol binding protein
	USF1	
	Initiation factor eIF-4A1	
	Human elongation factor-1- $\delta$	
	Sui1	
Inflammation	$\alpha$ -1 Protease inhibitor 3	$\alpha$ -2-Macroglobulin
	Hemopexin	LMW prekininogen
		Complement component C3
		AHSG ( $\alpha$ -2 HS-glycoprotein) homolog
Others	Microvascular endothelial differentiation gene 1	Vitronectin
	Diazepam-binding inhibitor	Epithelin 1 and 2
	Argininosuccinate synthetase	Murineoglobulin
	Skeletal muscle- $\alpha$ tropomyosin	
	Ampd3 gene	
	DNA-binding protein	

with increased fibrosis and progression rate of fibrosis of the liver.<sup>47</sup> 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.<sup>45</sup> 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.<sup>26</sup>

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.<sup>48</sup> 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.<sup>25</sup> This corroborates the results in *in vitro* studies<sup>49,50</sup> and a chimpanzee study.<sup>51</sup> 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.<sup>52</sup> 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 $\gamma$ AND HCV

INTERESTINGLY, WE FOUND recently that a protein interacting with the core protein, proteasome activator (PA)28 $\gamma$ , is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC.<sup>15,25</sup> 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)- $\alpha$  and retinoid X receptor (RXR)- $\alpha$ . 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.<sup>53</sup> Our study has demonstrated that the core protein enhances the binding activity of the LXR- $\alpha$ -RXR- $\alpha$  complex to the *srebp-1c* promoter in a PA28 $\gamma$ -dependent manner, resulting in upregulation of SREBP-1c and its regulating genes.<sup>25</sup> The activation may be mediated by the direct interaction between the core protein and RXR- $\alpha$ <sup>54</sup> or by suppression of a co-repressor such as Sp110b, a negative regulator of RAR- $\alpha$ , by sequestering it in the cytoplasm through interaction with the cytoplasmic core protein.<sup>55</sup> 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.<sup>56</sup> 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.<sup>48</sup> 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.<sup>57</sup> Furthermore, increases in saturated and monounsaturated fatty acids enhance HCV RNA replication.<sup>58</sup> 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.<sup>15,26</sup> Moreover, the core gene transgenic mice have been shown to exhibit overt diabetes when fed a high-fat diet,

while control mice do not.<sup>26</sup> 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- $\alpha$ , is known to impair the insulin-signaling pathway through inhibition of tyrosine phosphorylation of IRS. In fact, the overproduction of TNF- $\alpha$  has been reported to reduce the phosphorylation of IRS-1 and Akt in the core gene transgenic mice despite the absence of hepatic steatosis.<sup>15,26</sup> Moreover, in the latter study, hyperinsulinemia was cured by depletion of TNF- $\alpha$ , suggesting that upregulation of TNF- $\alpha$  contributes to the core protein-induced insulin resistance.<sup>26</sup> Our previous study has indicated that the core protein-induced overexpression of TNF- $\alpha$  is also dependent on the presence of PA28 $\gamma$ .<sup>15</sup>

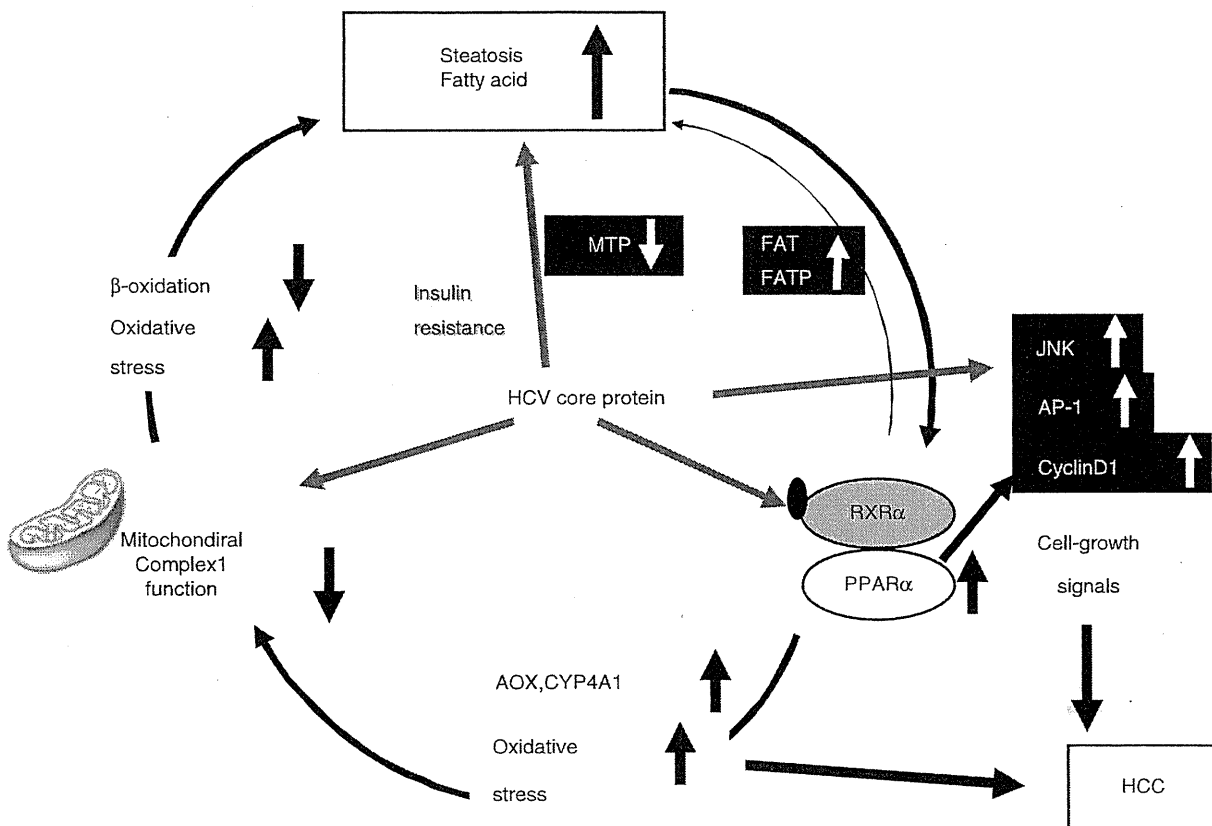
In relation to lipid metabolism, the core protein has also been found to interact with RXR- $\alpha$ .<sup>59</sup> RXR- $\alpha$  is one of the nuclear receptors, which forms a homodimer or heterodimers with other nuclear receptors including peroxisome proliferator-activated receptor (PPAR)- $\alpha$ , 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- $\alpha$ -responsive element as well as those with a PPAR- $\alpha$ -responsive element, in both mice and cultured cells.<sup>55</sup> Based on these results, we then examined the expression and function of PPAR- $\alpha$  in the liver of core gene transgenic mice.

## PPAR- $\alpha$ AND “FATTY ACID SPIRAL” IN HCV-ASSOCIATED HEPATOCARCINOGENESIS

PEROXISOME PROLIFERATOR-ACTIVATED receptor- $\alpha$  is one of the PPAR genes, and plays a central role, as a heterodimer with RXR- $\alpha$ , in regulating fatty acid transport and catabolism. It is also known as a molecular target for lipid-lowering fibrate drugs.<sup>60</sup> On the other hand, a prolonged administration of PPAR- $\alpha$  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.<sup>56</sup>

The level of PPAR- $\alpha$  protein was increased in the liver of core gene transgenic mice as early as 9 months old. PPAR- $\alpha$  protein is accumulated with age in the nuclei of hepatocytes together with cyclin D1 protein. However, the level of PPAR- $\alpha$  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- $\alpha$  (PPAR- $\alpha$ ) 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- $\alpha$ , retinoid X receptor- $\alpha$ .

By the pulse-chase experiment, the stability of nuclear PPAR- $\alpha$  turned out to be increased in the presence of the core protein. In line with the increase of PPAR- $\alpha$  protein, target genes of PPAR- $\alpha$  were activated in the liver of core gene transgenic mice; these genes include cyclin D1, CDK4, acy-CoA oxidase, and peroxisome thiolase.<sup>27</sup> However, in general, the activation of PPAR- $\alpha$  leads to improvement but not aggravation of steatosis. Then, what is the function of PPAR- $\alpha$  activation that is observed in the core gene transgenic mice?

To clarify the role of PPAR- $\alpha$  activation in pathogenesis of steatosis and HCC, we mated core gene transgenic mouse with PPAR- $\alpha$  knockout (KO) mouse, and studied the phenotype. PPAR- $\alpha$  KO mice have reduced

expressions of target genes of PPAR- $\alpha$ , and have mild steatosis in the liver as expected.<sup>28</sup> It was unanticipated, however, that steatosis was absent in PPAR- $\alpha$ -null or -heterozygous core gene transgenic mice but present in PPAR- $\alpha$ -intact core gene transgenic mice at the age of 9 or 24 months.<sup>27</sup> 8-Hydroxy deoxyguanosine (8-OHdG) and peroxy lipids, both of which are markers for oxidative stress, were decreased in PPAR- $\alpha$  KO core gene transgenic mice. Mitochondrial dysfunction in the core gene transgenic mice, which contributes to an overproduction of oxidative stress,<sup>46,61</sup> was also improved in PPAR- $\alpha$  KO core gene transgenic mice.

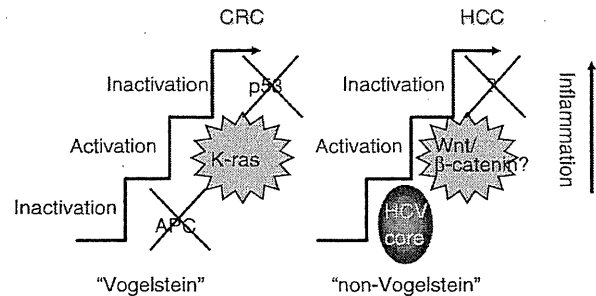
Finally, PPAR- $\alpha$  KO core gene transgenic mice did not develop HCC at the age of 24 months, while approxi-

mately one-third of PPAR- $\alpha$ -intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for the PPAR- $\alpha$  gene neither developed HCC.<sup>28</sup> When clofibrate, a peroxisome proliferator, was administered for 24 months to PPAR- $\alpha$ -heterozygous mice, either with or without the core gene, IICC developed in a higher rate in the core-gene<sup>+</sup> mice with a greater PPAR- $\alpha$  activation. It should be noted that steatosis was present only in core-gene<sup>+</sup> PPAR- $\alpha$ -heterozygous mice. In summary, steatosis and HCC developed in PPAR- $\alpha$ -intact but not in PPAR- $\alpha$ -heterozygous or PPAR- $\alpha$ -null core gene transgenic mice, indicating that not the presence but the persistent activation of PPAR- $\alpha$  would be important in hepatocarcinogenesis by HCV core protein. In general, PPAR- $\alpha$  acts to ameliorate steatosis, but with the presence of mitochondrial dysfunction, which is also provoked by the core protein, the core-activated PPAR- $\alpha$  may exacerbate steatosis. A persistent activation of PPAR- $\alpha$  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- $\alpha$  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

**T**HE 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<sup>62</sup> 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.<sup>62</sup> 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 IICC 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 IICC 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.<sup>42</sup> 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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