

gies, Inc., Tokyo, Japan) following the manufacturer's protocol.

Determination of Reactive Oxygen Species

Cells were plated onto glass coverslips and examined for reactive oxygen species (ROS) production as a marker for oxidative stress. They were loaded for 2 hours with chloromethyl 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes Inc., Eugene, OR) at a final concentration of 10 $\mu\text{mol/L}$.²⁶ Results were expressed as relative fluorescence intensity and normalized to the control cells. In some experiments, ROS was measured after the incubation with tacrolimus or CyA.

Measurement of Ketone Body Ratio

For the determination of ketone body ratio (KBR), cells were cultured to confluence on a 3.5-cm dish, and the medium was replaced with 700 μl of fresh medium. For arterial KBR, the mice were fasted for >16 hours, followed by the drawing of arterial blood. After a 24-hour incubation, acetoacetate and β -hydroxybutyrate in the medium were measured by monitoring the production or consumption of NADH with a Ketorex kit (Sanwa Chemical, Nagoya, Japan).²⁷ The KBR was calculated as the acetoacetate/ β -hydroxybutyrate ratio.

Microarray Analysis

An Affymetrix GeneChip analysis cDNA array system (Mouse Genome 430A 2.0, Kurabo, Osaka, Japan) was used for the analysis. Two thousand species of mouse DNA fragments were spotted on the filter. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice compared with mice treated with vehicle were defined as up-regulated or down-regulated, respectively.

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 fluorescent signal was measured with an ABI Prism 7000 system (Applied Biosystems, Tokyo, Japan).

The genes encoding mouse *tumor necrosis factor (TNF)- α* , *sterol regulatory element binding protein (SREBP)-1c*, *resistin*, *stearoyl-CoA desaturase (SCD)-1*, and *hypoxanthine phosphoribosyltransferase* were amplified with the primer pairs 5'-GACAAGGTGGGCTACGGGCTTG-3' and 5'-TCCCAAATGGGCTCCCTCT-3', 5'-ACGGAGCCATGGATTGCACATTTG-3' and 5'-TACATCTTTAAAGCAGCGG-GTGCCGATGGT-3', 5'-GAAGGCACAGCAGTCTTGA-3' and 5'-GCGACCTGCAGCTTACAG-3', 5'-TTCCCTCTG-CAAGCTCTAC-3' and 5'-CGCAAGAAGGTGCTAAC-GAAC-3', and 5'-CCAGCAAGCTTGCAACCTTAACCA-3' and 5'-GTAATGATCAGTCAACGGGGGAC-3', respec-

tively. The sense and antisense primers were located in different exons to avoid false-positive amplification from contaminated genomic DNA. Each PCR product was confirmed as a single band of the correct size by agarose gel electrophoresis (data not shown).

Reporter Assay for SREBP-1c Promoter Activity

A plasmid encoding firefly luciferase under the control of the *SREBP-1c* promoter (pGL3-srebp-1cPro) and a control plasmid encoding *Renilla* luciferase (Promega, Madison, WI) were transfected into 293T cells. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium of 293T cells transfected with pGL3-srebp-1cPro with or without an expression plasmid of HCV core protein at 24 hours after transfection. Cells were harvested 24 hours after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold-increase in relative luciferase units.

Statistical Analysis

Data are presented as the mean \pm SE. The significance of the difference in means was determined by a Mann-Whitney *U* test wherever appropriate. $P < 0.05$ was considered significant.

Results

Effect of Tacrolimus on Insulin Resistance Induced by HCV

The *core gene* transgenic mice exhibit insulin resistance in the absence of obesity from the age of 2 months.¹⁶ In tacrolimus-treated mice, there was a slight, but not significant, reduction in body weight compared with control mice at the end of tacrolimus administration at 6 months of age (Figure 1A). Tacrolimus administration to the *core gene* transgenic mice restored the plasma glucose levels to within normal limit (Figure 1B) ($P < 0.05$), whereas it caused no significant reduction in the control mice. The plasma glucose levels in the vehicle-treated *core gene* transgenic mice were higher than those in the *core gene* transgenic mice reported previously,¹⁶ probably owing to the older age of mice in the current study than in the previous one. The levels of serum insulin were also significantly reduced by the treatment with tacrolimus for 3 months in the *core gene* transgenic mice, whereas there was no significant change in the control mice (Figure 1C). The reduction in both plasma glucose and serum insulin levels indicates that the administration of tacrolimus restored the resistance to insulin action, which is attributed to the suppression of insulin action in the liver by the *core gene*.¹⁶ Actually, an insulin tolerance test (1 U/kg b.wt.) demonstrated the improvement of insulin action in the tacrolimus-treated *core gene* transgenic mice (Figure 1D).

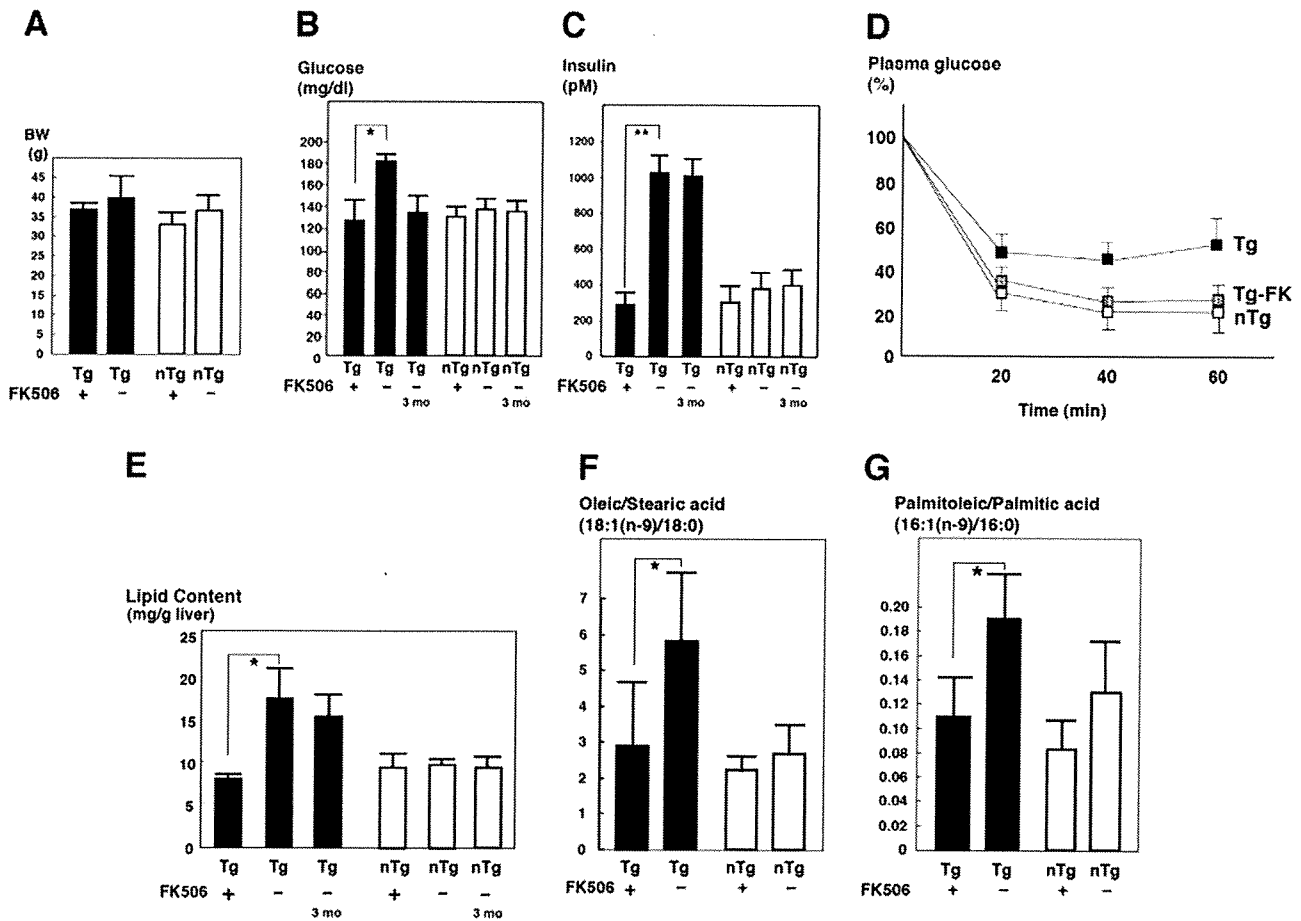


Figure 1. Effect of tacrolimus (FK506) on glucose and lipid metabolism in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle was administered to core gene transgenic or control mice i.p., three times weekly for 3 months beginning at 3 months of age. **A:** Body weight at the baseline and end of treatment. **B:** Plasma glucose level. **C:** Serum insulin level. **D:** Insulin tolerance test. Black boxes represent core gene transgenic mice; white boxes represent control mice; gray boxes represent core gene transgenic mice treated with tacrolimus (Tg-FK). **E:** Total lipid content in the liver. **F:** Ratio of oleic/stearic acid [18:1(n-9)/18:0]. **G:** Ratio of palmitoleic/palmitic acid [16:1(n-9)/16:0]. black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice showing the baseline state just before FK treatment, and Tg indicates 6-month-old transgenic mice, either with or without tacrolimus treatment for 3 months. Values represent the mean \pm SE, $n = 5$ in each group. * $P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. ** $P < 0.01$.

Tacrolimus Improves Lipid Metabolism Disorders in Mice

We then studied whether tacrolimus administration affects lipid metabolism in the mice. The core gene transgenic mice developed a marked hepatic steatosis.^{6,14} In addition, the composition of accumulated lipid was different from that in the fatty liver as a result of simple overnutrition: carbon 18 or 16 monounsaturated fatty acid levels were significantly increased.¹⁵ As shown in Figure 1E, the tacrolimus treatment significantly reduced the lipid content in liver tissues compared with the vehicle treatment of the core gene transgenic mice ($P < 0.05$, $n = 5$ each), whereas there was no change in the control mice. The increased ratios of oleic to stearic acid [18:1(n-9)/18:0] and palmitoleic to palmitic acid [16:1(n-9)/16:0] in the core gene transgenic mice returned to levels similar to those in control mice (Figure 1, F and G) ($P < 0.05$). Thus, the administration of tacrolimus for 3 months restored the abnormalities in lipid metabolism that were induced by the core protein of HCV. Histologically, tacrolimus significantly improved steatosis in the liver of

core gene transgenic mice, in which micro- and macrovesicular lipid droplets were accumulated in hepatocytes, chiefly around the central veins of the liver (Figure 2A). There was no sign of inflammation in the liver with or without the tacrolimus treatment.

Effect of Tacrolimus on Lipid Metabolism in HepG2 Cells Expressing HCV Core Protein

To further prove the ameliorating effect of tacrolimus on lipid metabolism, we then performed experiments using HepG2 cells that express the core protein.^{22,23} HepG2 cells, the lipid metabolism of which is somewhat different from that in normal hepatocytes,²⁸ show a significant increase in the level of 5,8,11-eicosatrienoic acid [20:3(n-9)], as a result of activations of the fatty acid enzymes, Δ^9 -, Δ^6 -, and Δ^5 -desaturases, by the core protein (H. Miyoshi and K. Koike, unpublished data). Incubation of the core-expressing HepG2 cells with tacrolimus at 100 nmol/L and 1 μ mol/L for 48 hours significantly reduced the accumulation of 20:3(n-9), whereas CyA treat-

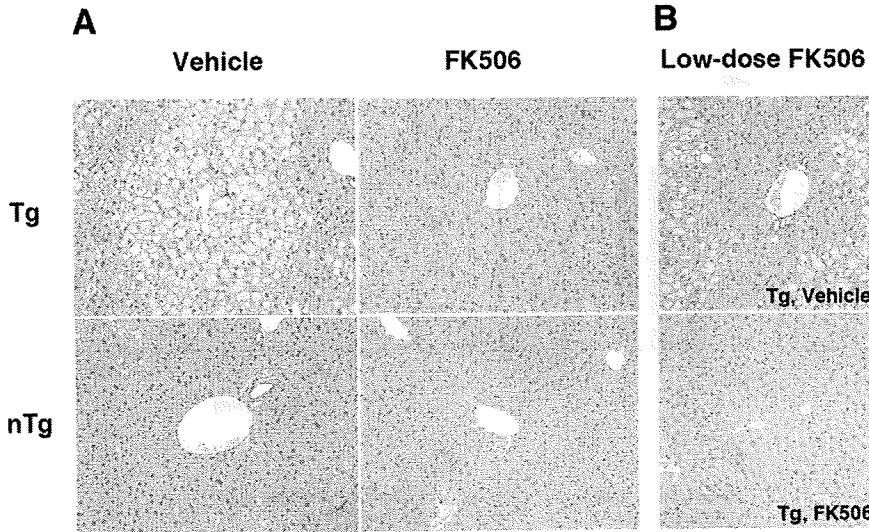


Figure 2. Morphological analysis of the liver of the core gene transgenic mice. Representative cases are shown either treated with tacrolimus (FK506) or vehicle (H&E staining). **A:** There is a prominent improvement of steatosis in the 3-month tacrolimus-treated core gene transgenic mice compared with the vehicle-treated mice. **B:** A prominent improvement in steatosis was also obtained by the administration of one-fifth dose of tacrolimus for 1 month beginning at 3 months of age. For histological analysis, two independent researchers evaluated 40 microscopic fields each, and a representative picture is shown for each category. Original magnification, $\times 125$. Tg, transgenic mice; nTg, nontransgenic control mice.

ment increased the level of 20:3(n-9) in a dose-dependent manner in the core-expressing HepG2 cells (Figure 3, A and B). Neither tacrolimus nor CyA changed the 20:3(n-9) content in HepG2 cells that do not express the core protein.

Low Dose of Tacrolimus Also Ameliorates Steatosis and Insulin Resistance

Because the usual dose of tacrolimus for liver transplantation naturally induces an immunosuppressed state in patients, we conducted a mouse study with a tacrolimus dose lower than that in the aforementioned study. In this low-dose experiment, tacrolimus at 0.02 mg/kg b.wt. (one-fifth of the previous one) was administered to mice for 1 month from the age of 3 months. Similar to the results with the dose of 0.1 mg/kg b.wt., there were significant decreases in the liver (9.5 ± 0.8 [0.02 mg/kg b.wt. tacrolimus] versus 18.7 ± 4.4 [vehicle only] mg/g liver; $P < 0.05$) and serum insulin concentration (96.6 ± 16.9 [0.02 mg/kg b.wt. tacrolimus] versus 1137.1 ± 88.0 [vehicle only] pmol/L; $P < 0.05$) in

the core gene transgenic mice treated with tacrolimus. Histological changes are shown in Figure 2B.

Effect of Tacrolimus on Oxidative Stress and Antioxidative System in Mice

We next examined whether the 3-month administration of tacrolimus affects the redox state in the core gene transgenic mice. In the liver of the core gene transgenic mice, the ROS level was higher than that in the liver of control mice as determined by lipid peroxidation.¹⁰ Treatment with tacrolimus significantly reduced the level of thiobarbituric acid-reactive substances in the liver of the core gene transgenic mice (Figure 4A) ($P < 0.05$). As a result of oxidative stress overproduction, there was damage in the DNA of hepatocytes of the core gene transgenic mice from a young age.¹⁰ To evaluate the effect of tacrolimus on the nuclear DNA damage, the apurinic/aprimidinic site index was determined in liver tissues from the core gene transgenic mice. As shown in Figure 4B, the apurinic/aprimidinic site index in the liver of the core gene transgenic mice, which was significantly higher

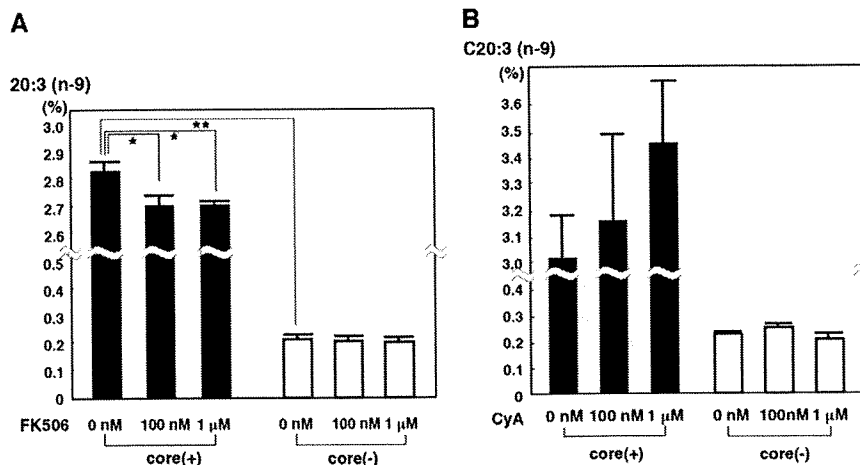


Figure 3. Effect of tacrolimus (FK506) or CyA on fatty acid compositions in HepG2 cells expressing the core protein. The fatty acid compositions of the total cell lipids were analyzed, and the percentage of 5,8,11-eicosatrienoic acid [20:3(n-9)] in the core-expressing and control HepG2 cells was calculated. **A:** Treatment with tacrolimus at 0 nmol/L, 100 nmol/L, or 1 μ mol/L. **B:** Treatment with CyA at 0 nmol/L, 100 nmol/L, or 1 μ mol/L. Black bars represent core-expressing cells; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$ and ** $P < 0.01$.

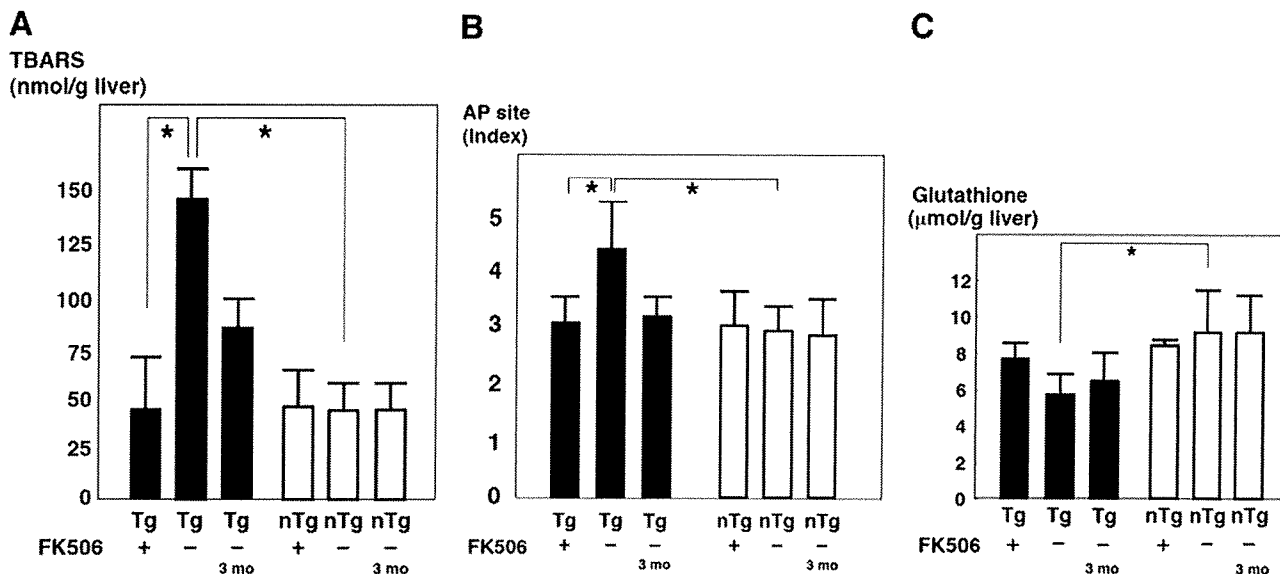


Figure 4. Effect of tacrolimus (FK506) on oxidative stress in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle only was administered to the core gene transgenic or control mice for 3 months. **A:** Lipid peroxidation in the liver. **B:** apurinic/aprimidinic (AP) site in the liver as a marker of nuclear DNA damage; **C:** Total glutathione level in the liver. Black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice, showing the baseline state just before tacrolimus treatment, and Tg indicates 6-month-old transgenic mice, either with or without 3 months of tacrolimus treatment. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. TBARS, thiobarbituric acid-reactive substances.

than that in the control mice, was significantly decreased by the tacrolimus treatment to a level similar to that in the control mice ($P < 0.05$).

The level of glutathione, one of the antioxidant systems, was significantly decreased in the liver of the core gene transgenic mice presumably as a result of oxidative stress overproduction but returned to a level similar to that in the control mice after the 3-month administration of tacrolimus, although the difference was not statistically significant ($P = 0.063$) (Figure 4C). Thus, the oxidative stress augmentation induced by the core protein of HCV was reduced by tacrolimus.

Effect of Tacrolimus on Oxidative Stress in Core-Expressing HepG2 Cells

Evidence for scavenging ROS by the administration of tacrolimus to the mice prompted us to validate this finding using cultured cells. For this purpose, tacrolimus or CyA was added to the culture medium of HepG2 cells that express or do not express the core protein. After 24 hours of incubation, tacrolimus decreased the ROS production level in the core-expressing HepG2 cells in a dose-dependent manner (Figure 5A). In contrast, no decrease but rather an augmentation of ROS production was observed by the treatment with CyA at various concentrations (Figure 5B).

Because dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be the reason for the ROS production associated with HCV infection (H. Miyoshi and K. Koike, unpublished data),^{12,13,17} an increase in the NADH/NAD⁺ ratio, which is caused by the repression of the complex 1 NADH dehydrogenase activity, would be a good marker for the mitochondrial complex 1 dys-

function. Therefore, we evaluated the effect of tacrolimus on the accumulation of NADH in the core-expressing HepG2 cells. The NADH/NAD⁺ ratio, which is strictly estimated from a reciprocal of KBR,^{26,29} was significantly higher in the core gene transgenic mice than in control mice (1/atrial KBR) and in HepG2 cells expressing the core protein than in control cells (1/KBR) (Figure 6A). By the treatment with 1 μ mol/L tacrolimus, the ratio significantly decreased compared with the baseline (Figure 6B), whereas CyA treatment caused no effect in the core-expressing HepG2 cells (Figure 6C), as was the

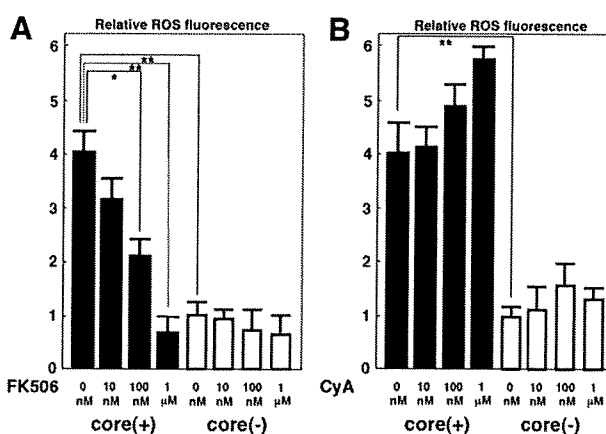


Figure 5. Effect of tacrolimus (FK506) or CyA on ROS production in HepG2 cells expressing the core protein. Results are expressed as relative brightness and normalized to control cells. **A:** Treatment with tacrolimus at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. **B:** Treatment with CyA at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$; ** $P < 0.01$.

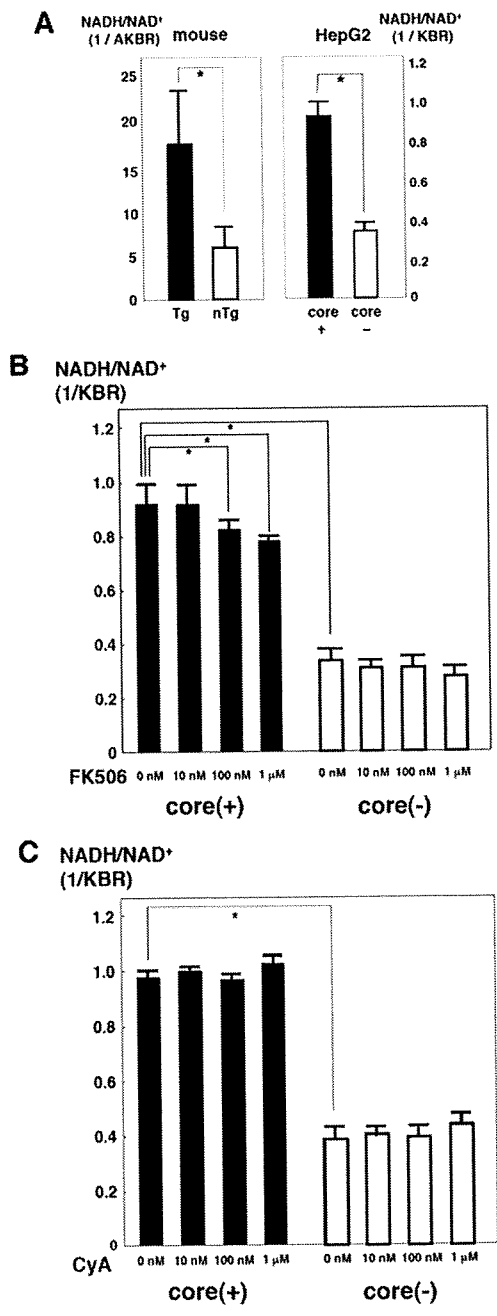
case in the determination of ROS by chloromethyl 2',7'-dichlorodihydrofluorescein diacetate.

Changes in Gene Expression by Tacrolimus Treatment of Mice

We then performed a comprehensive microarray analysis of gene expression in the liver, which was up- or down-regulated by tacrolimus. For this analysis, the tacrolimus-treated mice were compared with the vehicle-treated mice, in two pairs of the core gene transgenic and control mice, respectively. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice

compared with those treated with vehicle were defined as up-regulated or down-regulated, respectively. As shown in Table 1, several genes were found to be up-regulated or down-regulated in both the core gene transgenic and control mice after the treatment with tacrolimus for 3 months. A number of genes including that for TNF- α were up- or down-regulated both in the core gene transgenic and control mice. In contrast, the expressions of some genes including that for resistin were differentially regulated between the core gene transgenic and control mice. The expressions of these genes were confirmed by real-time PCR analysis.

Then, to explore the mechanism by which tacrolimus reverses the pathological effect of the core protein in the liver, we examined, by real-time PCR analysis, the expression of some cellular genes including TNF- α , SREBP-1c, SCD-1, and proteasome activator 28- γ . These genes or gene products have been suggested to play a pivotal role in the pathogenesis of HCV-associated liver disease.^{30,31} TNF- α and SREBP-1c genes have been shown to be up-regulated in the liver of the core gene transgenic mice and considered to play a role in the development of insulin resistance and steatosis.^{30,31} By the treatment of the core gene transgenic mice with tacrolimus for 3 months, there was a significant decrease in the mRNA level of both TNF- α and SREBP-1c (Figure 7, A-C) ($P < 0.05$). The SCD-1 mRNA level was also reduced in the tacrolimus-treated core gene transgenic mice. Because down-regulation of SREBP-1c expression by tacrolimus was observed only in the core gene transgenic mice but not in control mice, it is estimated that tacrolimus antagonizes the action of core protein in its transactivating function of the SREBP-1c promoter. The down-regulation of SREBP-1c, then, would lead to the suppression of SCD-1 expression and amelioration of steatosis. We confirmed this by conducting luciferase assays using cultured cells. As shown in Figure 7D, tacrolimus cancelled the effect of the core protein on the activation of SREBP-1c gene promoter. The level of the proteasome activator 28- γ protein, which is indispensable for the action of the core protein in the pathogenesis of HCV-associated liver lesion,³¹ was determined by Western blotting, but there was no change caused by the tacrolimus treatment (data not shown).



Discussion

Antiviral treatment for chronic hepatitis C has advanced markedly. Nearly 50% of patients with chronic hepatitis C

Figure 6. Effect of tacrolimus (FK506) or CyA on NADH accumulation in HepG2 cells expressing the core protein. **A:** NADH/NAD⁺ was determined in mice (left) or HepG2 cells (right) with or without the core protein. **B:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with tacrolimus for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. **C:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with CyA for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μ mol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396 and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$. AKBR, arterial KBR; Tg, transgenic mice; nTg, nontransgenic mice.

Table 1. Genes Whose Expression Levels in the Mouse Liver Were Altered by the Treatment with FK506

	Up-regulated in Tg	Down-regulated in Tg
Up-regulated in nTg	Nuclear factor, erythroid derived 2 DNA segment, human D6S2654E Fatty acid binding protein 5 epidermal squalene epoxidase Zinc finger protein 69	Resistin Resistin like alpha Nuclear receptor subfamily 4, group A, member insulin-like growth factor binding protein 1 calcium and integrin binding family member 3
Down-regulated in nTg	X-linked lymphocyte-regulated 4 Cytochrome P450, family 2, subfamily b, polypeptide 9 X-linked lymphocyte-regulated 3a Signal sequence receptor, delta	Tumor necrosis factor alpha Cytochrome P450, family 17, subfamily a, polypeptide 1 B-cell leukemia/lymphoma 6

Genes with altered expression in Tg (columns) or in nTg (rows) are described in a 4 × 4 table. Genes that were 1.5-fold increased or decreased in both of the two FK506-treated mice compared with those treated with placebo were defined as up-regulated or down-regulated, respectively. Tg, core gene transgenic mouse; nTg, nontransgenic control mouse.

with HCV genotype 1 and high viral loads achieve a sustained virological response as a result of ribavirin/peginterferon combination therapy.^{32,33} However, the remaining patients who could not achieve sustained virological response continue to experience progression of chronic hepatitis and have a high probability for development of HCC. Although therapies with new agents such as viral protease or RNA polymerase inhibitors are being developed, there is hope for development of the means to retard the progression of chronic hepatitis.

Recently, evidence showing that hepatic steatosis and insulin resistance are crucial determinants of the progression of liver fibrosis has accumulated.^{34–37} Moreover, the importance of oxidative stress, which is closely associated with metabolic disorders such as insulin resistance and steatosis, is implicated in the pathogenesis of HCV-associated liver disease. Given the suggested association of oxidative stress augmentation with the dysfunction of mitochondrial respiration in HCV infection,^{12,13,17} one possibility to ameliorate such a condition is the use of agents that can protect the mitochondrial respiratory function. Tacrolimus is one such agent with evidence of providing protection of the mitochondrial respiratory function,^{18–21} although it does not show an antiviral effect.

In the current study, the administration of tacrolimus significantly improved the disturbances in lipid and glucose metabolism both *in vivo* and *in vitro*. As disorders of

lipid metabolism associated with HCV infection, hepatic steatosis and increases in monounsaturated fatty acid levels have been demonstrated.^{3,4,6,7,15} The latter is caused by the activation of fatty acid enzymes such as Δ^9 - or Δ^6 -desaturase, resulting in increases in 18:1(n-9)/18:0 and 16:1(n-9)/16:0 ratios (H. Miyoshi and K. Koike, unpublished data).¹⁵ Tacrolimus ameliorated these lipid alterations associated with HCV infection with no impact on mouse body weight. Tacrolimus also improved the insulin resistance in the HCV mouse model, in which tyrosine phosphorylation of insulin receptor substrate-1 is impaired by the HCV core protein.¹⁶

Moreover, tacrolimus treatment ameliorated oxidative stress augmentation, which is considered to play a pivotal role in the progression of liver disease or the development of HCC in HCV infection.^{10–13} In mice transgenic for the HCV core gene, in which DNA damage develops because of oxidative stress augmentation,¹³ tacrolimus decreased the levels of peroxylipid and DNA damage formations. Dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be a source of ROS overproduction in HCV infection.^{12,13,17} To assess changes in mitochondrial complex 1 function caused by tacrolimus, the NADH/NAD⁺ ratio, which reflects the complex 1 NADH dehydrogenase activity, was determined in HepG2 cells expressing the core protein. The NADH/NAD⁺ ratio, which is strictly estimated from a reciprocal of KBR (1/atrial KBR),^{26,29} was significantly re-

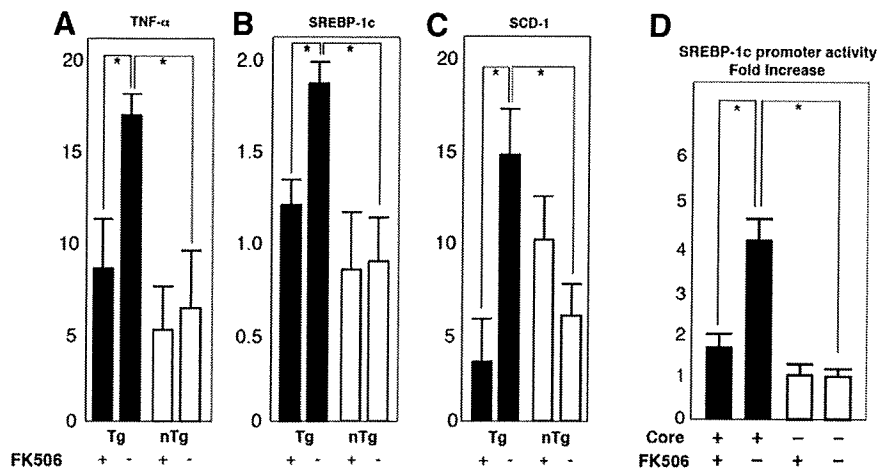


Figure 7. A–C: Effect of tacrolimus (FK506) on mRNA levels of cellular genes. The mRNA levels of TNF- α (A), SREBP-1c (B), and SCD-1 (C) genes were determined by real-time PCR analysis in the tacrolimus- or vehicle-treated mouse livers. The transcriptions of the genes were normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activities. **D:** Effect of tacrolimus on the transactivating function of the core protein on the SREBP-1c promoter. A luciferase assay was performed using a plasmid encoding firefly luciferase under the control of the SREBP-1c promoter with or without the expression of HCV core protein. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium. Black bars represent transgenic mice; white bars represent control cells. Values represent the mean \pm SE; $n = 5$ in each group. * $P < 0.05$. Tg, transgenic mice; nTg, nontransgenic mice.

duced by the addition of tacrolimus but not CyA. Thus, tacrolimus protected the mitochondrial respiratory chain complex 1 function from the impact of the core protein, decreased oxidative stress, and improved steatosis and insulin resistance.

Some of features induced by the core protein including steatosis, insulin, and DNA damage were already present in the core gene transgenic mice at 3 months of age as the baseline, and those were improved by tacrolimus treatment. This fact indicates that tacrolimus is not only preventing the development of core-induced features but also reversing such changes in the mouse liver.

The tacrolimus dose used in the current study was 0.1 mg/kg b.wt. This is the same dose as that used in recipients of liver or kidney transplantation. The result of a subexperiment with a lower tacrolimus dose of 0.02 mg/kg b.wt. was similar to that with the dose of 0.1 mg/kg b.wt. This finding is promising because it indicates that the "anti-core protein effect" may be achievable at such a low dose of tacrolimus without provoking strong immunosuppression. The tacrolimus concentration (100 nmol/L) that caused the anti-core protein effect in the cultured cell study is similar to that in the blood of recipients of liver transplantation and much lower than those used in previous studies.^{19,38} In the current study, tacrolimus was administered only i.p., although it tacrolimus is administered i.v. or p.o. in humans. Therefore, a concern may arise regarding the administration route. Because the bioavailability of tacrolimus is approximately 25% (range from 5 to 93%) in human patients,³⁹ a difference in the concentrations of tacrolimus may be possible between i.p. and p.o. administration. However, in human patients, target levels of tacrolimus concentration are generally achieved by p.o. administration as the maintenance therapy. Therefore, the target concentration would be achieved in mouse models by p.o. administration for 3 months as it is in human patients. Our current results strongly support the notion that tacrolimus can protect the mitochondrial respiratory function, resulting in a reduction of ROS production.

There is also a controversy concerning the effect of tacrolimus on glucose homeostasis. Post-transplantation diabetes is a complication in kidney or liver transplantation.^{40,41} *In vivo* and *in vitro* studies have shown that tacrolimus may inhibit insulin secretion from the pancreatic β -cells.⁴⁰ Thus, tacrolimus may have a potential to induce diabetes. However, there have been no well designed studies on this specific point: in one study, corticosteroid withdrawal from tacrolimus-based immunosuppression reduced insulin resistance without changing insulin secretion.⁴¹ In our study using the HCV mouse model, tacrolimus administration at the dose similar to those in organ transplant recipients decreased serum insulin levels without increasing plasma glucose levels. These results point toward the future use of tacrolimus *in vivo* for the amendment of metabolic abnormalities, such as steatosis and insulin resistance, associated with HCV infection. However, it should be noted that there is a difference between our mouse model and human patients. Organ transplant recipients generally have injury to other bodily organs after a prolonged course of illness,

whereas the mouse model we have exploited does not. In addition, our mouse model originally has insulin resistance with the presence of hyperplasia of Langerhans islands.¹⁶ Therefore, the effect of tacrolimus on glucose homeostasis in the current mouse study may not be exactly applicable to human patients.

The results of the gene expression analysis by microarray and subsequent real-time PCR were of considerable interest. Tacrolimus reduced the mRNA levels of TNF- α , SCD-1, and SREBP-1c genes, which are elevated in both patients with chronic hepatitis C and HCV core gene transgenic mice.^{30,31} The elevation in the TNF- α level causes insulin resistance *in vivo*, which is also observed in HCV core gene transgenic mice.¹⁶ The elevations in SREBP-1c and SCD-1 gene mRNA levels cause the overproduction of triglycerides, leading to the development of steatosis. The reductions in the expression levels of these genes may explain the effect of tacrolimus on the improvement of steatosis, insulin resistance, and oxidative stress in these HCV models. Although recent investigations have shown that the immunosuppressive drugs tacrolimus and rapamycin inhibit the expression of different inflammatory mediators,^{42,43} the anti-inflammatory functions of these drugs are not well established. Our *in vitro* and *in vivo* experiments confirmed that tacrolimus inhibited the induction of ROS generation, which is mediated by the core protein. Our data indicate that the inhibition of ROS formation may explain part of the favorable effect of immunosuppressive agents on inflammatory conditions.

In conclusion, our results demonstrate that tacrolimus has protective potential against damage caused by the HCV core protein including the induction of steatosis, insulin resistance, and oxidative stress, both in mice and cultured cells. Although more studies are required to elucidate the precise mechanism underlying the potential of tacrolimus in reversing the pathogenesis in HCV infection, these results may provide new therapeutic tools for chronic hepatitis C, in which oxidative stress and abnormalities in lipid and glucose metabolism contribute to liver pathogenesis.

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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)- 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.

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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

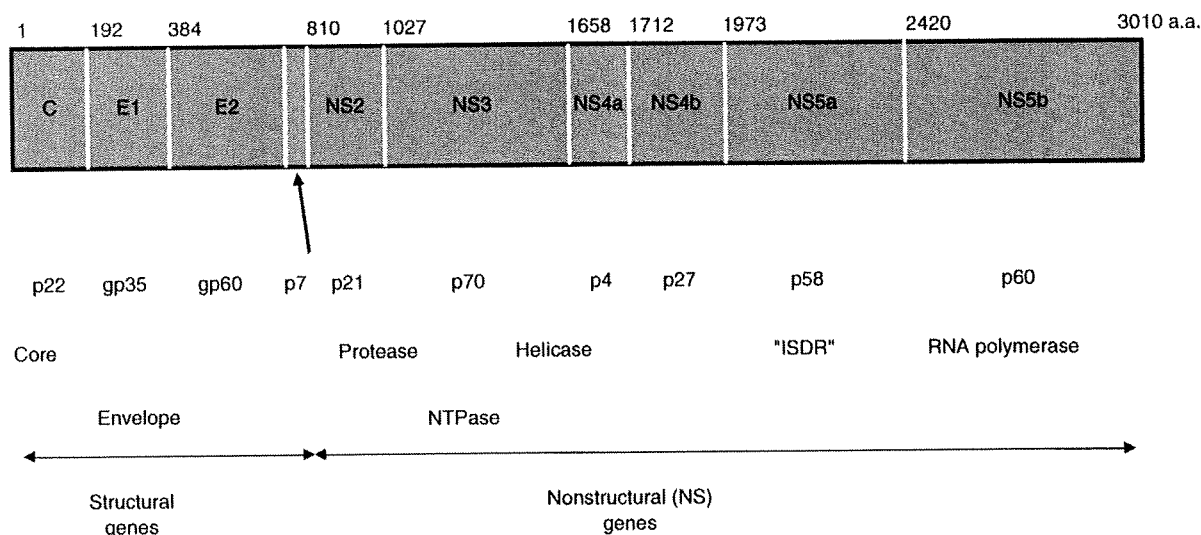


Figure 1 The structure of hepatitis C virus (HCV) genome. The HCV genome RNA encodes a polyprotein of 3010 amino acids (a.a.), which is processed to structural and non-structural proteins by the cellular or viral proteases. ISDR, interferon sensitivity-determining region.

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-Ig/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 ²⁶ 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.

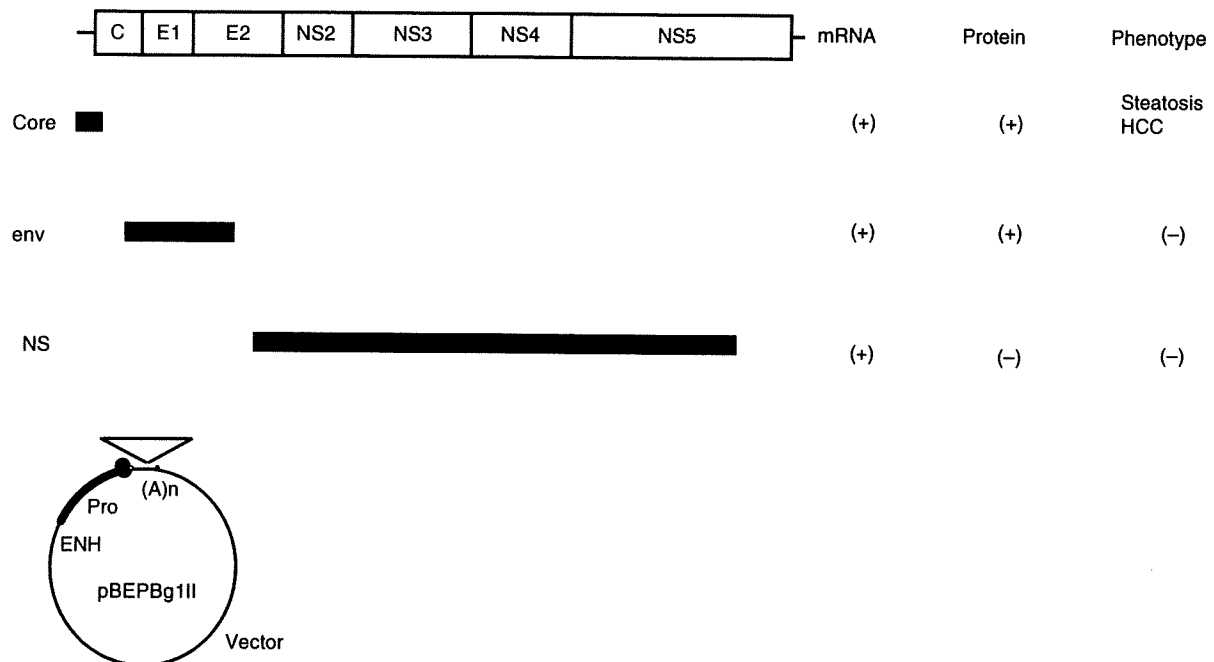


Figure 2 Transgenic mouse lines carrying the hepatitis C virus (HCV) genome.^{21,22,31,32,42} Three different kinds of transgenic mouse lines, carrying the core gene, envelope genes or non-structural genes of HCV, respectively, were established under the control of the same regulatory elements. Among 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

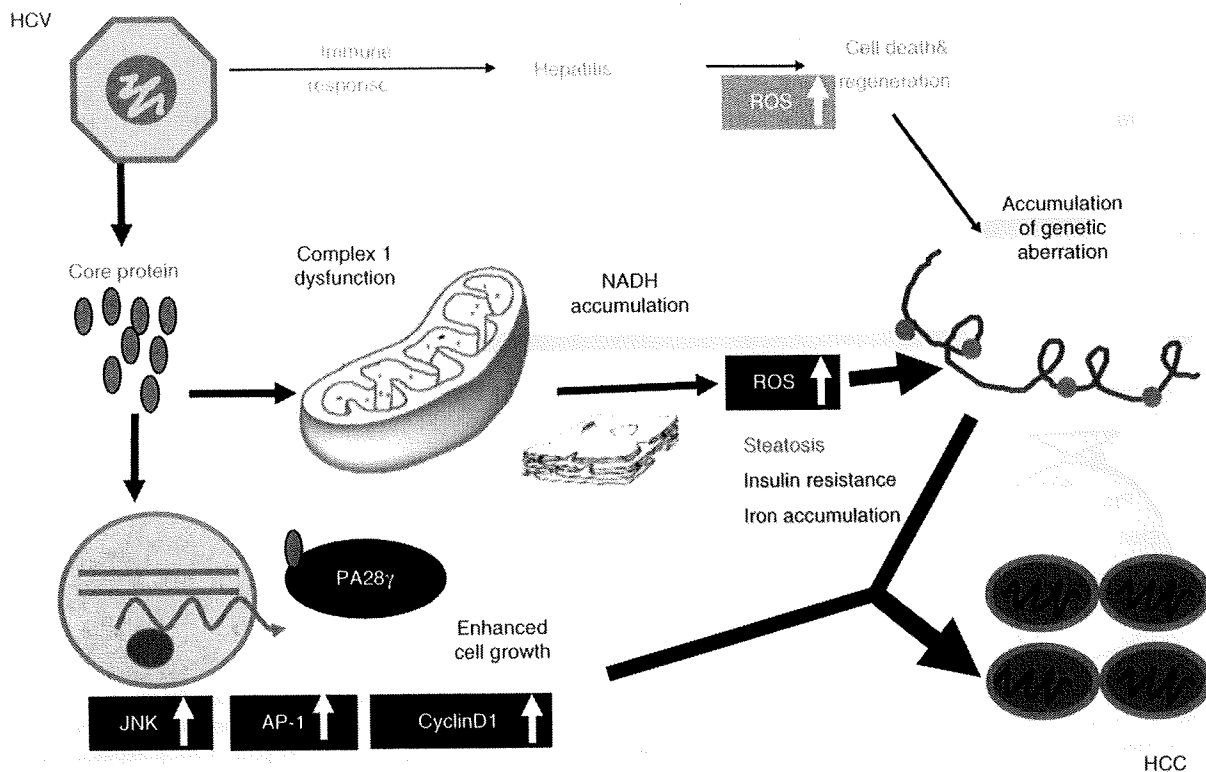


Figure 3 Mechanism of hepatocarcinogenesis in hepatitis C virus (HCV) infection. The core protein is localized in the nuclei of hepatocytes in addition to cytoplasm and may interact with nuclear factors, thereby deregulating the cell growth and death. The core protein may also affect the cell growth 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 Sui1	Human guanine nucleotide regulatory protein α -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, McPherson *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.

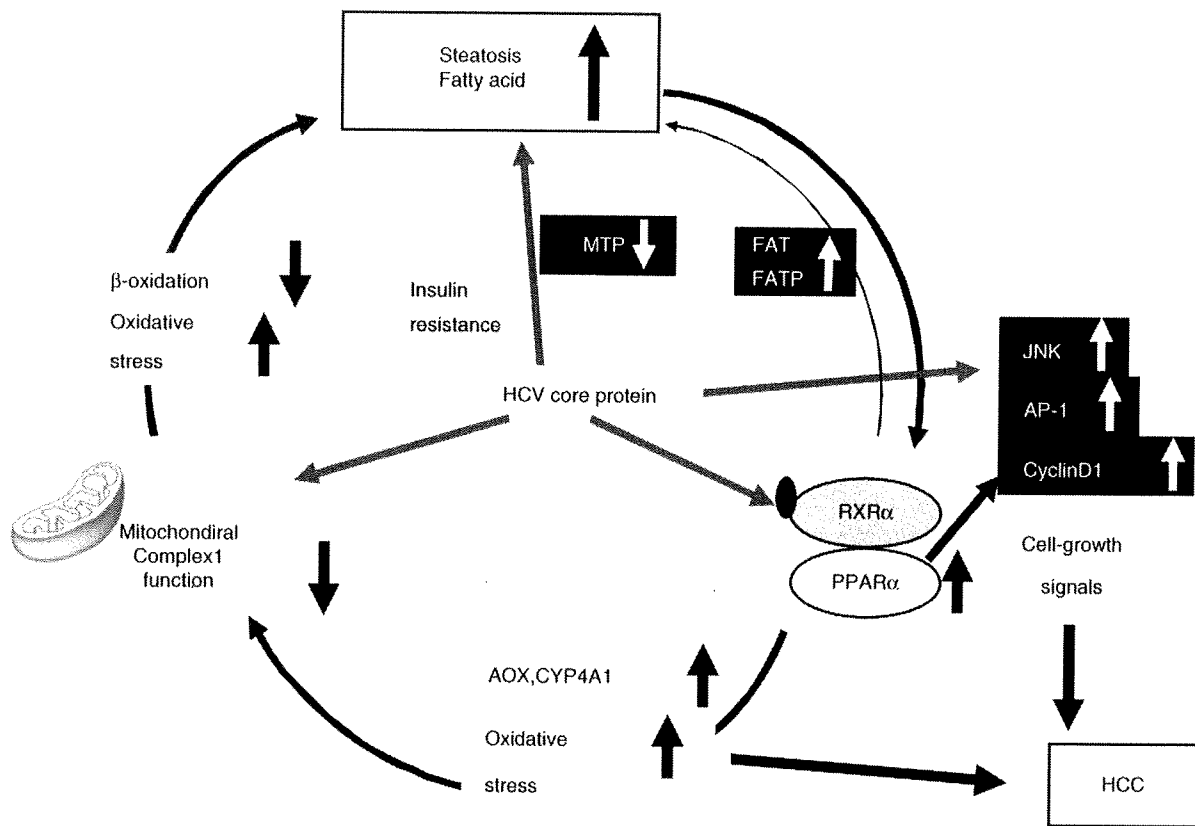


Figure 4 Hepatitis C virus (HCV) core protein causes "fatty acid spiral". In HCV infection, the core protein induces steatosis through several pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and peroxisome proliferator-activated receptor- α (PPAR- α) 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

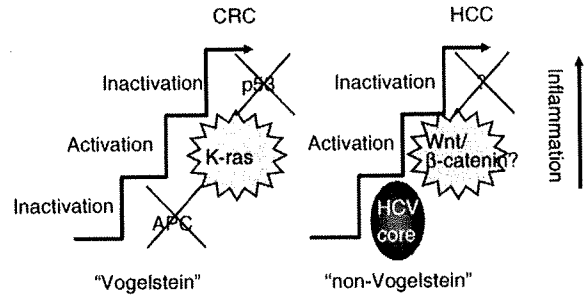


Figure 5 The role of hepatitis C virus (HCV) in hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development 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 give 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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