PPARα activation is essential for HCV core protein–induced hepatic steatosis and hepatocellular carcinoma in mice

Naoki Tanaka,1,2 Kyoji Moriya,3 Kendo Kiyosawa,2 Kazuhiko Koike,3 Frank J. Gonzalez,4 and Toshifumi Aoyama1

1Department of Metabolic Regulation, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine, Matsumoto, Nagano, Japan.
2Division of Gastroenterology, Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto, Nagano, Japan.
3Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.
4Laboratory of Metabolism, National Cancer Institute, NIH, Bethesda, Maryland, USA.

Introduction

HCV is one of the major causes of chronic hepatitis, whereas patients with persistent HCV infection have a high incidence of hepatocellular carcinoma (HCC) (1, 2). Occurrence of HCC associated with chronic HCV infection has increased over the past 2 decades (3–5), and chronic HCV infection is recognized as a serious debilitating disease. However, the mechanism in which chronic HCV infection mediates hepatocarcinogenesis remains unclear.

PPARα activation is essential for the pathogenesis of hepatic steatosis and HCC induced by HCV infection.

Nonstandard abbreviations used: ACC, acetyl-CoA carboxylase; AOX, acyl-CoA oxidase; CDK, cyclin-dependent kinase; CYP4A1, cytochrome P450 4A1; FAS, fatty acid synthase; FAT, fatty acid translocase; FATP, fatty acid transport protein; HCC, hepatocellular carcinoma; HCVcpTg, HCV core protein–expressing transgenic; L-FABP, liver fatty acid-binding protein; MCAD, medium-chain acyl-CoA dehydrogenase; MTP, microsomal transfer protein; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PCNA, proliferating cell nuclear antigen; RXRα, retinoid X receptor α.

Conflict of interest: The authors have declared that no conflict of interest exists.


Transgenic mice expressing HCV core protein develop hepatic steatosis and hepatocellular carcinoma (HCC), but the mechanism underlying this process remains unclear. Because PPARα is a central regulator of triglyceride homeostasis and mediates hepatocarcinogenesis in rodents, we determined whether PPARα contributes to HCV core protein–induced diseases. We generated PPARα-homozygous, -heterozygous, and -null mice with liver-specific transgenic expression of the core protein gene (Ppara+/−:HCVcpTg, Ppara+/+:HCVcpTg, and Ppara−/−:HCVcpTg mice). Severe steatosis was unexpectedly observed only in Ppara−/−:HCVcpTg mice, which resulted from enhanced fatty acid uptake and decreased mitochondrial β-oxidation due to breakdown of mitochondrial outer membranes. Interestingly, HCC developed in approximately 35% of 24-month-old Ppara−/−:HCVcpTg mice, but tumors were not observed in the other genotypes. These phenomena were found to be closely associated with sustained PPARα activation. In Ppara+/−:HCVcpTg mice, PPARα activation and the related changes did not occur despite the presence of a functional Ppara allele. However, long-term treatment of these mice with clofibrate, a PPARα activator, induced HCC with mitochondrial abnormalities and hepatic steatosis. Thus, our results indicate that persistent activation of PPARα is essential for the pathogenesis of hepatic steatosis and HCC induced by HCV infection.
could lead to continuous PPARα activation because of the presence of fatty acid metabolites that serve as natural PPARα ligands. For example, mice lacking expression of the peroxisomal acyl-CoA oxidase (AOX) gene showed massive accumulation of very-long-chain fatty acids in hepatocytes, severe microvesicular steatosis, chronic PPARα activation, and development of hepatic adenoma and HCC by 15 months of age (22). These results suggest a strong contribution of activated PPARα to liver tumorigenesis.

On the basis of several lines of evidence, we hypothesized that PPARα might contribute to hepatocarcinogenesis in HCV core protein–expressing transgenic (HCVcpTg) mice. To explore this possibility, PPARα-homozygous (Ppara+/+), PPARα-heterozygous (Ppara+/-), and PPARα-null (Ppara-/-) mice bearing the HCV core protein gene, designated Ppara+/+;HCVcpTg, Ppara+/-;HCVcpTg, and Ppara-/-;HCVcpTg mice, were generated, and phenotypic changes were examined. Surprisingly, we found that severe hepatic steatosis and HCC induced by HCV core protein developed only in Ppara+/+ mice, which were related to persistent PPARα activation.

Results

Expression of HCV core protein in transgenic mice. Ppara+/-;HCVcpTg and Ppara+/-;HCVcpTg mice appeared healthy, and body weight in both genotypes was similar to that of Ppara+/-;HCVcpTg and Ppara+/- mice without the transgene. When hepatic expression of HCV core protein in 9-month-old transgenic mice was examined by immunoblot analysis, it was similar among Ppara+/-;HCVcpTg, Ppara+/-;HCVcpTg, and Ppara+/-;HCVcpTg mice (Figure 1A) and was also similar to expression in HCVcpTg mice reported previously (7, 9). Age and sex had only a minor influence on the hepatic expression of HCV core protein.

Requirement of homozygous PPARαs for the development of hepatic steatosis in transgenic mice. Livers of 9-month-old male HCVcpTg mice with or without the Ppara allele were examined. Those of Ppara+/-;HCVcpTg mice were soft, slightly enlarged, and light in color and histologically showed macrovesicular and microvesicular steatosis with no apparent inflammation or hepatocyte necrosis (Figure 1B), in agreement with previous reports (7, 9). Biochemical analysis of liver extracts showed marked hepatic accumulation of triglycerides (Figure 1D). In contrast, livers of 9-month-old Ppara+/-;HCVcpTg and Ppara+/-;HCVcpTg mice showed neither histological abnormalities nor accumulation of triglycerides (Figure 1, B and D). Hepatic levels of free fatty acids in Ppara+/-;HCVcpTg mice were approximately 3 times those in Ppara+/-;HCVcpTg and Ppara+/-;HCVcpTg mice or Ppara+/- mice not expressing the HCV core protein.

In 24-month-old Ppara+/-;HCVcpTg mice, hepatic steatosis was found (Figure 1C), and the hepatic levels of triglycerides were further increased (Figure 1D). Apparent inflammation, hepatocyte...
degeneration and necrosis, and fibrosis were not detected. On the other hand, $P_{para}^{+/–}:\text{HCVcpTg}$ and $P_{para}^{–/–}:\text{HCVcpTg}$ mice showed no steatosis (Figure 1, C and D). These results indicate that hepatic steatosis develops in $P_{para}^{+/+}:\text{HCVcpTg}$ mice, but not in $P_{para}^{+/–}:\text{HCVcpTg}$ mice.

**Hepatic fatty acid and triglyceride metabolism in transgenic mice.** To investigate the mechanism responsible for the development of severe steatosis in $P_{para}^{+/+}:\text{HCVcpTg}$ mice, the expression of genes associated with fatty acid and triglyceride metabolism in the livers of 9-month-old mice was analyzed using the quantitative RT-PCR method. As shown in Figure 2A, the mRNA levels of genes related to de novo lipogenesis (fatty acid synthase [FAS] and acetyl-CoA carboxylase [ACC]) and secretion of VLDL (microsomal transfer protein [MTP] and apoB) were constant in all groups. The mRNA levels of fatty acid translocase (FAT) and fatty acid transport protein (FATP), which are associated with the uptake of fatty acids into hepatocytes, were significantly increased only in $P_{para}^{+/+}:\text{HCVcpTg}$ mice, but not in $P_{para}^{+/–}:\text{HCVcpTg}$ mice. Surprisingly, the mRNA levels of AOX and medium-chain acyl-CoA dehydrogenase (MCAD) were not affected.

**Results and Discussion.** To investigate the mechanism responsible for the development of severe steatosis in $P_{para}^{+/+}:\text{HCVcpTg}$ mice, the expression of genes associated with fatty acid and triglyceride metabolism in the livers of 9-month-old mice was analyzed using the quantitative RT-PCR method. As shown in Figure 2A, the mRNA levels of genes related to de novo lipogenesis (fatty acid synthase [FAS] and acetyl-CoA carboxylase [ACC]) and secretion of VLDL (microsomal transfer protein [MTP] and apoB) were constant in all groups. The mRNA levels of fatty acid translocase (FAT) and fatty acid transport protein (FATP), which are associated with the uptake of fatty acids into hepatocytes, were significantly increased only in $P_{para}^{+/+}:\text{HCVcpTg}$ mice, but not in $P_{para}^{+/–}:\text{HCVcpTg}$ mice. Surprisingly, the mRNA levels of AOX and medium-chain acyl-CoA dehydrogenase (MCAD) were not affected.

**Figure 2**
Analyses of factors associated with hepatic fatty acid and triglyceride metabolism. (A) Expression of genes associated with fatty acid and triglyceride metabolism in 9-month-old mouse livers. Total RNA was extracted from each mouse liver, and mRNA levels were determined by RT-PCR. mRNA levels were normalized by those of GAPDH and subsequently normalized by those in $P_{para}^{+/+}$ nontransgenic mice. Results are expressed as the mean ± SD ($n = 6$ group). *$P < 0.05$ compared with $P_{para}^{+/+}$ nontransgenic mice; **$P < 0.05$ compared with $P_{para}^{+/–}:\text{HCVcpTg}$ mice; ***$P < 0.05$ compared with $P_{para}^{–/–}:\text{HCVcpTg}$ mice. (B) Uptake of fatty acids in 9-month-old mouse livers. Liver slices obtained from 3 mice in each group were incubated in medium containing 0.8 mM [1-14C]palmitic acid for 7 h. Fatty acid uptake ability was estimated by the sum of palmitic acid converted to CO$_2$ and ketone bodies with that incorporated into total cellular lipids after incubation. The experiment was repeated 3 times. Results were normalized by those of $P_{para}^{+/+}$ nontransgenic mice and expressed as the mean ± SD. (C) Plasma concentrations of free fatty acids, glucose, and insulin. After an overnight fast, blood was obtained from each mouse and the above variables were determined. Results are expressed as the mean ± SD ($n = 6$ group).
dehydrogenase (MCAD), a rate-limiting enzymes in the peroxisomal and mitochondrial β-oxidation pathways, respectively, were significantly increased in Ppara⁺/-:HCVcpTg mice. When fatty acid uptake ability was measured in fresh liver slices, it was significantly enhanced only in Ppara⁺/-:HCVcpTg mice (Figure 2B). Additionally, plasma free fatty acid levels were higher in these mice than in mice in the other groups. Although there were no differences in fasting plasma glucose levels between the groups, hyperinsulinemia was observed only in Ppara⁺/-:HCVcpTg mice (8). Similar results were obtained from 24-month-old mice (data not shown). These results combined show that the increased plasma fatty acid levels, which were likely due to enhanced peripheral fatty acid release caused by insulin resistance, and the increase in fatty acid uptake ability are consistent with steatogenesis in Ppara⁺/-:HCVcpTg mice.

Decreased mitochondrial β-oxidation in transgenic mice. Although the transcriptional activities of major β-oxidation enzymes were markedly increased, Ppara⁺/-:HCVcpTg mice had severe steatosis. To explore this discrepant result, peroxisomal and mitochondrial β-oxidation activities were measured using lignoceric and palmitic acids as substrates, respectively. The lignoceric acid-degrading capacity was increased only in Ppara⁺/-:HCVcpTg mice, where it corresponded to an increase in AOX expression. However, the capacity for palmitic acid degradation, which occurs particularly in mitochondria, was significantly lower in Ppara⁺/-:HCVcpTg mice than in Ppara⁺/-:HCVcpTg and Ppara⁻/⁻:HCVcpTg mice (Figure 3A). Thus, decreased mitochondrial β-oxidation ability was considered to be another important mechanism for the development of steatosis induced by the core protein.

We further evaluated mitochondrial abnormalities. In electron microscopic examination, discontinuous outer membranes (Figure 3B, arrows) and amorphous inner structures were observed in mitochondria of 9-month-old HCVcpTg mice. Upper and lower rows show a lower and higher magnification, respectively. In Ppara⁺/-:HCVcpTg mice, some mitochondria showing discontinuance of outer membranes (arrows) and amorphous inner structures were observed in Ppara⁺/-:HCVcpTg and Ppara⁻/⁻:HCVcpTg mice, mitochondria appeared normal; the scale bars represent 200 nm (top) and 30 nm (bottom), respectively. Immunoblot analysis of cytochrome c in 9-month-old mice. Whole-liver lysate, mitochondrial fraction, or cytosolic fraction (50 μg protein) was loaded in each well. Results are representative of 4 independent experiments. (D) Immunoblot analysis of representative mitochondrial β-oxidation enzymes using a mitochondrial fraction prepared from 9-month-old mouse livers. The mitochondrial fraction (20 μg protein) was loaded in each well. Results are representative of 4 independent experiments. The band intensity was quantified densitometrically and normalized by that in Ppara⁻/⁻ nontransgenic mouse. The mean value of the fold changes is shown under the representative band. LACS, long-chain acyl-CoA synthase; CPT, carnitine palmitoyl-CoA transferase.

Figure 3
Analyses of mitochondrial abnormalities. (A) Lignoceric and palmitic acid β-oxidation activities in 9-month-old mice. Results are expressed as the mean ± SD (n = 6/group). *P < 0.05 compared with Ppara⁻/⁻ nontransgenic mice; **P < 0.05 compared with Ppara⁺/-:HCVcpTg mice; *P < 0.05 compared with Ppara⁺/+:HCVcpTg mice. (B) Electron microscopic features of hepatic mitochondria of 9-month-old HCVcpTg mice. Upper and lower rows show a lower and higher magnification, respectively. In Ppara⁺/-:HCVcpTg mice, some mitochondria showing discontinuance of outer membranes (arrows) and amorphous inner structures were observed. In Ppara⁺/-:HCVcpTg and Ppara⁻/⁻:HCVcpTg mice, mitochondria appeared normal; the scale bars represent 200 nm (top) and 30 nm (bottom), respectively. (C) Immunoblot analysis of cytochrome c in 9-month-old mice. Whole-liver lysate, mitochondrial fraction, or cytosolic fraction (50 μg protein) was loaded in each well. Results are representative of 4 independent experiments. (D) Immunoblot analysis of representative mitochondrial β-oxidation enzymes using a mitochondrial fraction prepared from 9-month-old mouse livers. The mitochondrial fraction (20 μg protein) was loaded in each well. Results are representative of 4 independent experiments. The band intensity was quantified densitometrically and normalized by that in Ppara⁻/⁻ nontransgenic mouse. The mean value of the fold changes is shown under the representative band. LACS, long-chain acyl-CoA synthase; CPT, carnitine palmitoyl-CoA transferase.
Microscopic examination showed that there were no dysplastic cells consistent with the previous report (9). Surprisingly, nodules (Table 1). Microscopically, these nodules had the appearance of well-differentiated HCC with trabecular features, which was consistent with the previous report (9). Surprisingly, Ppara−/−:HCVcpTg mice had macroscopically evident hepatic nodules (Table 1). Microscopically, these nodules had the appearance of well-differentiated HCC with trabecular features, which was consistent with the previous report (9). Surprisingly, Ppara−/−:HCVcpTg mice and Ppara−/−:HCVcpTg mice of the same ages developed no evidence of hepatic tumors, despite the expression of HCV core protein at all in transgenic mice, whereas, at 24 months, approximately 35% of hepatic tumors, despite the expression of HCV core protein at 24 months of age for analysis. HCC was diagnosed according to histological findings.

Requirement of homozygous PPARα for hepatic tumor development in transgenic mice. At 9 months of age, hepatic nodules were not observed at all in transgenic mice, whereas, at 24 months, approximately 35% of Ppara−/−:HCVcpTg mice had macroscopically evident hepatic nodules (Table 1). Microscopically, these nodules had the appearance of well-differentiated HCC with trabecular features, which was consistent with the previous report (9). Surprisingly, Ppara−/−:HCVcpTg mice and Ppara−/−:HCVcpTg mice of the same ages developed no evidence of hepatic tumors, despite the expression of HCV core protein at similar levels to those found in Ppara−/−:HCVcpTg mice (Table 1). Microscopic examination showed that there were no dysplastic cells or precancerous lesions throughout the livers in Ppara−/−:HCVcpTg and Ppara−/−:HCVcpTg mice (Figure 1C). These results provide strong evidence that homozygous PPARα is essential for hepatic tumorigenesis induced by HCV core protein.

Increased hepatocyte proliferation only in Ppara−/−:HCVcpTg mice. Because sustained acceleration of hepatocyte proliferation relative to apoptosis may promote the development of HCC, these opposing processes were quantified in the livers of 24-month-old mice. Both the liver-to-body weight ratio and the number of hepatocytes expressing proliferating cell nuclear antigen (PCNA) were increased only in Ppara−/−:HCVcpTg mice (Figure 4, A and B). In contrast, the number of TUNEL-positive hepatocytes and the hepatic caspase 3 activity, indicators of hepatocyte apoptosis, remained similar among the 3 mouse strains (Figure 4, C and D). Interestingly, despite the presence of HCV core protein, the amounts of these proliferative and apoptotic markers in Ppara−/−:HCVcpTg and Ppara−/−:HCVcpTg mice were similar to those in Ppara−/−:nontransgenic mice. Expression levels of several proteins, such as protooncogenes (c-Fos and c-Myc), cell-cycle regulators (cyclin D1, cyclin-dependent kinase [CDK] 4, and PCNA), and phosphorylated ERK 1 and 2, all of which are associated with hepatocyte proliferation, were elevated in Ppara−/−:HCVcpTg mice but not in other genotypes (Figure 4, E and F).

Increased oxidative stress and DNA damage only in Ppara−/−:HCVcpTg mice. HCV core protein is associated with increased production of ROS (23). Enhanced ROS production induces nuclear DNA damage, which results in the initiation of hepatocarcinogenesis, and can also injure organelles, which can result in disorders in their

Table 1

<table>
<thead>
<tr>
<th>HCV core protein</th>
<th>Ppara</th>
<th>Mice</th>
<th>Mice with HCC</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>+/+</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>−</td>
<td>−/+</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>−</td>
<td>−/−</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>+/+</td>
<td>17</td>
<td>6</td>
<td>35.3</td>
</tr>
<tr>
<td>+</td>
<td>+/−</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>−/−</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Mice were killed at 24 months of age for analysis. HCC was diagnosed according to histological findings. *P < 0.05 compared with Ppara−/−:nontransgenic mice; **P < 0.05 compared with Ppara−/−:HCVcpTg mice, P < 0.05 compared with Ppara−/−:HCVcpTg mice.

Figure 4

Increased hepatocyte proliferation in Ppara−/−:HCVcpTg mice at 24 months of age. (A) Liver-to-body-weight ratio. Results are expressed as the mean ± SD (n = 6/group). (B) Numbers of proliferating hepatocytes. Two thousand hepatocytes were examined in each mouse, and hepatocyte nuclei positive for anti-PCNA antibody were counted. Results are expressed as the mean ± SD (n = 6/group). For A and B, comparisons are designated as follows: *P < 0.05 compared with Ppara−/−:nontransgenic mice; **P < 0.05 compared with Ppara−/−:HCVcpTg mice; #P < 0.05 compared with Ppara−/−:HCVcpTg mice. (C) Numbers of apoptotic hepatocytes. Liver sections were subjected to TUNEL staining, and TUNEL-positive hepatocytes were counted in 2,000 hepatocytes from each mouse. Results are expressed as the mean ± SD (n = 6/group). (D) Caspase 3 activity. Results are expressed as the mean ± SD (n = 6/group). (E) Immuno blot analysis of oncogene products and cell cycle regulators. The same sample used in Figure 1A (whole-liver lysate, 50 μg protein) was loaded in each well. The band of actin was used as the loading control. Results are representative of 4 independent experiments. The band intensity was quantified densitometrically, normalized by that of actin, and subsequently normalized by that in Ppara−/−:nontransgenic mice. The mean value of the fold changes is expressed under each band. (F) Immuno blot analysis of phosphorylated ERK1/2 and total ERK1/2. The same samples in Figure 4E (50 μg protein) were used.

function. The number of hepatocytes positive for 8-hydroxy-2-deoxyguanosine (8-OHdG), an indicator of oxidative damage to nuclear DNA, was increased only in 24-month-old Ppara+/−: HCVcpTg mice (Figure 5, A and B). Lipid peroxides were slightly increased in the livers of 9-month-old Ppara+/−: HCVcpTg mice (data not shown) and were more abundant in the livers of 24-month-old Ppara+/−: HCVcpTg mice than in those of Ppara+/−: HCVcpTg and Ppara+/−: HCVcpTg mice or Ppara+/+ nontransgenic mice (Figure 5C). Expression of typical ROS-generating enzymes (AOX and cytochrome P450 4A1 [CYP4A1]) and ROS-eliminating enzymes (catalase and Cu, Zn-SOD) was examined. Immunoblot analysis showed marked increases in the expression of AOX and CYP4A1 and mild increases in that of catalase only in Ppara+/−: HCVcpTg mice. No changes in Cu, Zn-SOD were found in the subgroups of transgenic mice (Figure 5D). These results suggest that enhanced oxidative stress causes damage in nuclear DNA and probably in mitochondria in the Ppara+/−: HCVcpTg mice.

Persistent and spontaneous PPARα activation in Ppara+/−: HCVcpTg mice. Liver tumorigenesis induced by long-term exposure to peroxisome proliferators and the related changes, such as sustained hepatocyte proliferation and increased oxidative stress, are associated with persistent PPARα activation (19–21). To examine the activation of PPARα, we quantified the level of PPARα mRNA, which is induced by PPARα activation (24, 25). PPARα mRNA levels were higher in 9-month-old Ppara+/−: HCVcpTg mice than in Ppara+/− nontransgenic mice (Figure 6A). These increases were more pronounced at 24 months of age. However, there were no differences in PPARα mRNA levels between Ppara+/−: HCVcpTg and Ppara+/− nontransgenic mice at either 9 or 24 months of age. The expression levels of typical PPARα target genes (16, 25, 26) — such as FAT, FATP, L-FABP, AOX, and MCAD (Figure 2); c-Myc, cyclin D1, CDK4, and PCNA (Figure 4); and CYP4A1 (Figure 5) — were simultaneously and synchronously increased in Ppara+/−: HCVcpTg mice, but not in Ppara+/−: HCVcpTg or Ppara+/−: HCVcpTg mice. These results confirm that persistent activation of PPARα occurs only in Ppara+/−: HCVcpTg mice. Various changes observed in Ppara+/−: HCVcpTg mice, i.e., increased fatty acid uptake, mitochondrial abnormalities, steatosis, ROS overproduction, accelerated hepatocyte proliferation, and hepatocarcinogenesis, were considered to be closely linked with sustained PPARα activation.

Nuclear PPARα content. The results described above suggest that persistent PPARα activation is critical to the steatogenesis and hepatocarcinogenesis induced by the HCV core protein. A question arises as to why Ppara+/−: HCV cpTg mice with an active Ppara allele do not exhibit the hallmarks of PPARα activation and do not develop HCC. To address this issue, the nuclear PPARα content was analyzed. Immunoblot analysis for PPARα showed that the amount of nuclear PPARα protein in Ppara+/−: HCVcpTg mice was approximately 2- to 3-fold that of Ppara+/− nontransgenic mice, which was disproportionate to the higher PPARα mRNA levels (approximately 1.2- to 1.6-fold) (Figure 6A and B). The level of nuclear PPARα in Ppara+/−: HCVcpTg mice was significantly lower than that in Ppara+/−: HCVcpTg mice and was similar to that in Ppara+/− nontransgenic mice (Figure 6B). Thus, the lower amount of nuclear PPARα in Ppara+/−: HCVcpTg mice than in Ppara+/−: HCVcpTg mice might have heightened the threshold of expression required for long-term spontaneous PPARα activation.

The degree of an increase in nuclear PPARα levels was evidently higher than the degree of an increase in PPARα mRNA levels in HCVcpTg mice (Figure 6A and B). To investigate this disparity, the stability of nuclear PPARα was evaluated by pulse-chase experiments using isolated hepatocytes obtained from these mice. The half-life of nuclear PPARα was significantly longer (P < 0.05) in Ppara+/−: HCVcpTg mice (11.5 ± 2.3 h) than in Ppara+/− nontransgenic mice (5.8 ± 1.4 h) (Figure 6C). The half-life of nuclear PPARα in Ppara+/−: HCVcpTg mice tended to be prolonged compared with that in Ppara+/− nontransgenic mice (Figure 6C). These results suggest that the stability of nuclear PPARα was increased as a result of HCV core protein expression. Because it is known that the core protein interacts with retinoid X receptor α (RXRα) (27) and that...
PPARα influences the stability of RXRα (28), it is plausible that the core protein would affect its action in nuclei through an interaction with the PPARα-RXRα heterodimer and stabilization of PPARα.

Development of hepatic steatosis and HCC with long-term clofibrate treatment in Ppara+/−:HCVcpTg mice. To further confirm the significance of persistent PPARα activation on core protein–induced pathological changes, Ppara+/− and Ppara+/+:HCVcpTg mice were fed a standard diet containing 0.05% clofibrate for 24 months. Interestingly, hepatic steatosis appeared in the clofibrate-treated Ppara+/−:HCVcpTg mice, but not in the Ppara+/− mice under the same treatment conditions (Figure 7, A and B). Similar to our observations in Ppara+/−:HCVcpTg mice not treated with clofibrate, aberrant mitochondria with discontinuous outer membranes and decreased palmitic acid β-oxidation activity were found only in the clofibrate-treated Ppara+/−:HCVcpTg mice (Figure 7, A and C). In addition, levels of MCAD mRNA; AOX, and CYP4A1 proteins; PPARα mRNA; and nuclear PPARα protein were higher in the clofibrate-treated Ppara+/−:HCVcpTg mice than in the clofibrate-treated Ppara+/− mice (Figure 7, D–F), which suggests that the degree of PPARα activation in the former group was greater than that in the latter group and similar to that in Ppara+/−:HCVcpTg mice not treated with clofibrate. Finally, the incidence of HCC after clofibrate treatment was higher in Ppara+/−:HCVcpTg mice (25%; 5 in 20 mice) than in Ppara+/− mice (5%; 1 in 20 mice). Therefore, these results corroborate the importance of constant PPARα activation to the pathogenesis of hepatic steatosis and HCC in the transgenic mice.

Discussion

A novel and striking finding in this study is the absolute requirement of persistent PPARα activation for the development of HCV core protein–induced steatosis and HCC. Our data also show that the HCV core protein alone cannot induce steatosis and HCC in transgenic mice.

Mechanisms of development of steatosis in HCVcpTg mice were previously explained as an enhancement of de novo synthesis of fatty acids (29) and a decrease in MTP expression, the latter of which results in insufficient VLDL secretion from hepatocytes (30). In the present study, we revealed 2 novel mechanisms of steatogenesis in the transgenic mice, i.e., an impairment of mitochondrial β-oxidation due to the breakdown of mitochondrial outer membranes and an increase in fatty acid uptake into hepatocytes, associated with PPARα activation. PPARα activation, mitochondrial dysfunction, and hepatic steatosis appeared in 9-month-old Ppara+/−:HCVcpTg mice and continued until 24 months of age, clearly preceding development of HCC. These findings thereby indicate a correlation between PPARα activation, hepatic steatosis, and HCC.

We obtained the novel and rather paradoxical finding that significant PPARα activation, which generally is expected to reduce hepatic triglyceride levels, is essential for the development of severe steatosis induced by HCV core protein. According to the results of this study, the following hypothesis concerning the development of steatosis in Ppara+/−:HCVcpTg mice is proposed. First, the HCV core protein localizes partly in mitochondria (9). A recent study...
showed that, in isolated mitochondria, the core protein directly increased Ca\(^{2+}\) influx, inhibited electron transport complex I activity, and induced ROS production (31), all of which can increase the fragility of mitochondria and depress mitochondrial function. In addition, the HCV core protein also localizes in nuclei (9) and can coexist in PPAR\(\alpha\)-RXR\(\alpha\) heterodimer through a direct interaction with the DNA-binding domain of RXR\(\alpha\), which enhances the transcriptional activity of PPAR\(\alpha\) target genes, such as AOX, despite the absence of PPAR\(\alpha\) ligands in cultured cells (27). The HCV core protein can also be involved in the PPAR\(\alpha\)-RXR\(\alpha\) complex through a direct interaction with cyclic-AMP responsive element binding protein-binding protein (32), which is able to bind to PPAR\(\alpha\) (33). Thus, the core protein probably serves as a coactivator and stabilizer of PPAR\(\alpha\) in vivo, which was further confirmed in this study. Moreover, because it is also known that the core protein itself activates ERK1/2 and p38 mitogen-activated protein kinase (34), these activations might phosphorylate PPAR\(\alpha\) and thereby transactivate it (35). The core protein–induced PPAR\(\alpha\) activation enhances the basal expression of AOX and CYP4A1, which leads to increased production of ROS and dicarboxylic acids. These toxic compounds can damage mitochondrial outer membranes, which impairs the mitochondrial \(\beta\)-oxidation system. These damages directly induce the accumulation of long-chain fatty acids in hepatocytes. Furthermore, PPAR\(\alpha\) activation increases the expression of FAT and FATP, which promotes the influx of fatty acids from blood. Long-chain fatty acids and their CoA esters accumulated in hepatocytes are likely to act as potent detergents, which further damages the outer membranes of mitochondria. Fatty acids and their derivatives function as natural ligands of PPAR\(\alpha\), which results in the activation of PPAR\(\alpha\) and the induction of FAT, FATP, AOX, and CYP4A1, which further accelerates mitochondrial damage, the reduction of mitochondrial \(\beta\)-oxidation activity, and the accumulation of fatty acids in a vicious cycle.

Persistent PPAR\(\alpha\) activation increases oxidative DNA damage because of a disproportionate increase in ROS-generating enzymes relative to the levels of degrading enzymes such as catalase and SOD, which can predispose hepatocytes to malignant transformation. In addition, persistent PPAR\(\alpha\) activation leads to increased...
from *Ppara*+/−:HCVcpTg mice were similar to those from *Ppara*+/+:HCVcpTg mice, which demonstrates that the presence of functional PPARα itself is not a prerequisite for the occurrence of steatosis and HCC induced by the HCV core protein. Moreover, a comparison between *Ppara*+/−:HCVcpTg and *Ppara*+/−:HCVcpTg mice uncovered an unexpected and important fact that the core protein–dependent pathological changes do not appear without significant activation of PPARα. Thus, it is not the presence of PPARα per se, but rather a high level of PPARα activation that seems to be essential for the development of HCV core protein–induced steatosis and HCC.

To reinforce the abovementioned hypothesis, *Ppara*+/− and *Ppara*+/−:HCVcpTg mice were treated with an exogenous PPAR agonist, clofibrate, for 24 months. In *Ppara*+/− mice, long-term clofibrate treatment caused a certain level of persistent PPARα activation and a low incidence of HCC. Interestingly, in *Ppara*+/+:HCVcpTg mice, clofibrate treatment induced more intensive PPARα activation and HCC at a much higher incidence, accompanied by damaged mitochondrial outer membranes, severe steatosis, and decreased mitochondrial β-oxidation activity. The results from the clofibrate-treated *Ppara*+/−:HCVcpTg mice were similar to those of the *Ppara*+/−:HCVcpTg mice not treated with clofibrate. Therefore, these findings further support the concept that a long-term and high level of PPARα activation is necessary for steatogenesis and hepatocarcinogenesis in HCVcpTg mice and emphasize the significant role of the HCV core protein as a PPARα coactivator in vivo.

A pulse-chase experiment showed that PPARα was stabilized in hepatocyte nuclei in mice expressing the HCV core protein. Many nuclear receptors, including PPARα and RXRα, are known to be degraded by the ubiquitin-proteasome system (38), which plays an important role in modulating the activity of nuclear receptors. Further studies will be needed to clarify whether the core protein influences the ubiquitin-proteasome pathway.

Recent studies have shown conflicting results, i.e., that PPARα was downregulated in the livers of chronic hepatitis C patients (39, 40). Although the association between PPARα function and chronic HCV infection remains a matter of controversy in humans, the changes observed in the transgenic mice resemble in many ways the clinicopathological features of chronically HCV-infected patients; both show a high frequency of accompanying steatosis (10, 40, 41), increased accumulation of carbon 18 mono- and unsaturated fatty acids in the liver (42), mitochondrial dysfunction (43), increased insulin resistance (44) and oxidative stress (45, 46), male-preferential (2) and multicentric occurrence of HCC (47, 48), and the well-differentiated appearance of HCC, including trabecular features and often a “nodule-in-nodule” pattern (47, 48). Thus, it is postulated that the mechanism of steatogenesis and hepatocarcinogenesis we proposed may partially apply to patients with chronic HCV infection. If so, therapeutic interventions to alleviate persistent and excessive PPARα activation might be beneficial in the prevention of HCC. To clarify the exact relationship between PPARα activation and HCV-induced hepatocarcinogenesis in humans, further cell division, as revealed by the expression of cell cycle regulators such as cyclin D1 and CDK4. Furthermore, there is little change in apoptosis, which, under normal circumstances, would remove damaged cells capable of undergoing transformation. Thus, under these conditions, it is plausible that some aberrant hepatocytes do not undergo apoptosis and develop into HCC.

It is well known that chronic activation of PPARα is associated with hepatocarcinogenesis in mice exposed to peroxisome proliferators or in mice lacking AOX expression. The common clinicopathological characteristics of HCC in these mice are multicentric HCC (20, 22, 36, 37), the well-differentiated appearance of HCC including trabecular features and often a “nodule-in-nodule” pattern (22, 36, 37), and no evidence of fibrosis or cirrhosis in the nonneoplastic liver parenchyma (22, 36), similar to that observed in *Ppara*+/−:HCVcpTg mice. However, mice chronically exposed to peroxisome proliferators are clearly distinct from *Ppara*+/−:HCVcpTg mice in that they have normal mitochondrial organization, increased mitochondrial β-oxidation activity, and no steatosis (16, 36). AOX-null mice are also different from *Ppara*+/−:HCVcpTg mice with respect to mitochondrial structure (22). These detailed comparisons between the 3 mouse models reveal the importance of mitochondrial abnormalities in the pathogenesis of HCV-related diseases.

PPARα is known to regulate the hepatic expression of many proteins associated with fatty acid and triglyceride metabolism, cell division and apoptosis, oxidative stress generation and degradation, and so forth (15, 16, 20, 21, 24–26); therefore, complete deletion of the PPARα gene from mice might cause hitherto unknown influences on the pathways involved in the development of hepatic steatosis and HCC. To consider these unknown effects, *Ppara*+/−:HCVcpTg mice were adopted in the current study. Surprisingly, almost all results

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Primer pairs used for RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>GeneBank accession number</td>
</tr>
<tr>
<td>ACC</td>
<td>NM_13360</td>
</tr>
<tr>
<td>ApoB</td>
<td>NM_006993</td>
</tr>
<tr>
<td>AOX</td>
<td>NM_015729</td>
</tr>
<tr>
<td>FAS</td>
<td>NM_007988</td>
</tr>
<tr>
<td>FAT</td>
<td>NM_007564</td>
</tr>
<tr>
<td>FATP</td>
<td>NM_011977</td>
</tr>
<tr>
<td>GAPDH</td>
<td>M32599</td>
</tr>
<tr>
<td>L-FABP</td>
<td>NM_017389</td>
</tr>
<tr>
<td>MCAD</td>
<td>NM_007382</td>
</tr>
<tr>
<td>MTP</td>
<td>NM_008642</td>
</tr>
<tr>
<td>PPARα</td>
<td>NM_011144</td>
</tr>
</tbody>
</table>

F, forward sequence; R, reverse sequence.
experiments using noncancerous liver tissues obtained from HCV-related HCC patients and using mice carrying human PPARα and HCV core protein genes are needed.

In conclusion, we clarified for the first time that persistent and potent PPARα activation is absolutely required for the development of severe steatosis and HCC induced by HCV core protein. In addition, we uncovered paradoxical and specific functions of PPARα in the mechanism of steatogenesis mediated by the core protein. Our results offer clues in the search for novel therapeutic and nutritional management options, especially with respect to neutral lipids, for chronically HCV-infected patients.

**Methods**

**Mice.** The generation of HCVcpTg mice and Ppara+/– mice was described previously (7, 24, 49). Male HCVcpTg mice and female Ppara+/– mice were mated, and F1 mice bearing the HCV core protein gene were intercrossed to produce F2 mice. Ppara+/–, Ppara+/+, and Ppara−/− mice bearing the HCV core protein gene, designated as Ppara−/−:HCVcpTg, Ppara−/−:HCVcpTg, and Ppara−/–:HCVcpTg mice, in the F4 generation were subjected to serial analyses. Because HCC develops preferentially in male HCVcpTg mice (9), male mice were analyzed. Age-matched male Ppara−/− mice without the core protein were used as controls. For identifying genotypes, genomic DNA was isolated from mouse tails and amplified by PCR. Primer pairs were designed as described elsewhere: 5′-GCCCAACGGAGCTTTAGGTCC-3′ and 5′-TAGTTCACGCCGGCTCTCCAG-3′ for the HCV core gene (7) and 5′-AAACGCAACGTAGAGTGCTG-3′ for the Ppara gene (24). Amplified alleles for HCV core and Ppara genes were 460 and 472 base pairs, respectively. Five mice per cage were fed a routine diet and were kept free of specific pathogens according to institutional guidelines. For the clonotrate treatment experiments, 2-month-old male Ppara+/– and Ppara−/–:HCVcpTg mice were randomly divided into 2 groups (n = 20 in each group) and were fed either a routine diet or one containing 0.05% (w/w) clofibrate (Wako Pure Enzyme Co., Ltd.; Tokyo, Japan) and loose-fitting pestle. The homogenate was centrifuged at 500 g for 10 min at 4°C to obtain a crude mitochondria pellet. This pellet was resuspended with isolation buffer and centrifuged at 20,000 × g for 10 min at 4°C, and the resulting pellet was resuspended and centrifuged at 4,500 g for 10 min at 4°C. The pellet fraction was resuspended again and centrifuged at 20,000 × g for 1 h at 4°C, and the resulting pellet was used as the nuclear fraction. The combined supernatant fractions were centrifuged at 7,800 × g for 10 min at 4°C to obtain a crude mitochondria pellet. This pellet was resuspended with isolation buffer centrifuged at 7,800 × g for 10 min at 4°C, and used as the mitochondrial fraction. Finally, all supernatant fractions were collected and centrifuged at 20,000 × g for 30 min at 4°C, and the resulting supernatant was used as the cytosolic fraction.

**Immunoblot analysis.** Protein concentrations were measured colorimetrically with a BCA Protein Assay kit (Pierce). For the analysis of fatty acid-metabolizing enzymes, hepatocyte mitochondrial fractions or whole-liver lysates (20 μg protein) were subjected to 10% SDS-PAGE (16). For analysis of PPARα, nuclear fractions (100 μg protein) were used. For analysis of other proteins, whole lysates or cytosolic fractions (50 μg protein) were subjected to electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were incubated with the primary antibody and then with alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG. Antibodies against HCV core protein, fatty acid–metabolizing enzymes, CYP4A1, catalase, and PPARα were described previously (9, 16, 24, 50). Antibodies against other proteins were purchased commercially: cytochrome c antibody from BD Transduction Laboratories and other antibodies from Santa Cruz Biotechnology. The band of actin or histone H1 was used as the loading control. The band intensity was measured densitometrically, normalized to that of actin or histone H1, and subsequently expressed as fold changes relative to that of Ppara−/– nontransgenic mice.

**Analysis of mRNA.** Total liver RNA was extracted using an RNeasy Mini Kit (Qiagen), and cDNA was generated by SuperScript II reverse transcriptase (Gibco BRL). Quantitative RT-PCR was performed using a SYBR green PCR kit and an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The primer pairs used for RT-PCR are shown in Table 2. The mRNA level was normalized to the GAPDH mRNA level and subsequently expressed as fold changes relative to that of Ppara−/– nontransgenic mice.

**Light microscopy and immunohistochemical analysis.** Small blocks of liver tissue from each mouse were fixed in 10% formalin in phosphate-buffered saline and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin. For immunohistochemical localization of PCNA and 8-OHdG, other small blocks of liver tissue were fixed in 4% paraformaldehyde in phosphate-buffered saline. Sections (4 μm thick) were then affixed to glass slides and incubated overnight with mouse monoclonal antibodies against PCNA (1:100 dilution; Santa Cruz Biotechnology) or 8-OHdG (1:10 dilution; Japan Institute for the Control of Aging). Sections were immunostained using EnVision+ kit, with 3,3′-diaminobenzidine as a substrate (DAKO). Hepatocytes positive for PCNA or 8-OHdG were examined in 10 randomly selected ×400 microscopic fields per section. Two-thousand hepatocytes were examined for each mouse, and the number of immunostained hepatocyte nuclei was expressed as a percentage.

**Assessment of hepatitis apoposis.** TUNEL assay was performed using a MEBSTAIN Apoptosis Kit II (Medical & Biological Laboratories). Two thousand hepatocytes were examined for each mouse, and the number of TUNEL-positive hepatocytes was expressed as a percentage.

**Pulse-label and pulse-chase experiments.** Parenchymal hepatocytes were isolated by the modified in situ perfusion method (51). After perfusion with 0.05% collagenase solution (Wako), the isolated hepatocytes were washed 3 times by means of differential centrifugation and the dead cells were removed by density-gradient centrifugation at 500 g for 3 min at 4°C on Percoll (Amersham Pharmacia Biotech). The live hepatocytes were washed and suspended in William’s E medium containing 5% FBS. When the viability of the isolated hepatocytes exceeded 85% as determined by the trypan blue exclusion test, the following experiments were conducted. The isolated hepatocytes were washed twice and incubated in methionine-free medium containing 5% dialyzed FBS for 1 h at 37°C. The medium was replaced with the same medium containing 300 μCi/ml of 35S)methionine (Amersham Pharmacia Biotech). After a 3-h incubation, the labeled medium was changed for the standard medium, and the preparation was chased for 3, 6, or 12 h. The labeled cells were washed, homogenized, and centrifuged at 800 g for 5 min at 4°C to obtain a crude nucleus pellet. This pellet was resuspended with isolation buffer and centrifuged at 20,000 g for 1 h at 4°C to prepare the nuclear fraction. The levels of radioactivity in the homogenates of the pulse-labeled preparations were similar between the transgenic and nontransgenic mice, which suggested that the 35S)methionine uptake capacity in the former hepatocytes was similar to that in the latter. The nuclear fraction was lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4; 0.2% sodium deoxycholate, 0.2% Nonidet P-40, 0.1% SDS,
Hepatocellular carcinoma: recent trends in Japan.


10. Ito, K., et al. 2000. To determine the hepatic content of lipids and lipid peroxides, mice were fasted overnight. Livers were removed quickly, rinsed in ice-cold saline solution, and cut into 500-μm thick slices with an Oxford Vibratome (Oxford Laboratories). Approximately 150 mg of fresh liver (6–8 liver slices) was placed on stainless steel grids positioned in a 25-ml flask equipped with suspended plastic center wells (Kontes) and incubated in RPMI-1640 medium (Sigma-Aldrich) lacking the peroxisome proliferator-activated receptor α target gene in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor α natural ligand metabolism. J. Biol. Chem. 273:15639–15645.


