Hepatitis C virus (HCV) core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor α in transgenic mice: implications for HCV-associated hepatocarcinogenesis

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Abbreviations: AOX, acyl-CoA oxidase; CDK, cyclin-dependent kinase; DAB, 3,3'-diaminobenzidine; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; L-FABP, liver-type fatty acid-binding protein; NEFA, nonesterified fatty acid; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator-activated receptor; PT, peroxisomal thiolase; RXR, retinoid X receptor; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

Research category: Infectious causes of cancer

Novelity and impact of this paper:

1) We demonstrated for the first time that HCV core protein modulated the function of nuclear receptor PPARα in mice carrying the core gene. Increased stability of PPARα through a possible interaction with the core protein in hepatocyte nuclei and the resultant PPARα activation enhanced transcriptional activity of PPARα target genes, such as cyclin D1, c-Myc, and cyclin-dependent kinase 4, in HCV core gene transgenic mice.

2) Persistent PPARα activation caused by the core protein expression accelerated hepatocyte proliferation over the long term, which may have led to the development of HCC. Thus, activation of PPARα may be one of the possible mechanisms of the hepatocarcinogenesis mediated by the core protein.

Abstract

Persistent infection of hepatitis C virus (HCV) can lead to a high risk for hepatocellular carcinoma (HCC). HCV core protein plays important roles in HCV-related hepatocarcinogenesis, because mice carrying the core protein exhibit multicentric HCCs without hepatic inflammation and fibrosis. However, the precise mechanism of hepatocarcinogenesis in these transgenic mice remains unclear. To evaluate whether the core protein modulates hepatocyte proliferation and/or apoptosis *in vivo*, we examined these parameters in 9- and 22-month-old transgenic mice. Although the numbers of apoptotic hepatocytes and hepatic caspase 3 activities were similar between transgenic and non-transgenic mice, the numbers of proliferating hepatocytes and the levels of numerous proteins such as cyclin D1, cyclin-dependent kinase 4, and c-Myc, were markedly increased in an age-dependent manner in the transgenic mice. This increase was correlated with the activation of peroxisome proliferator-activated receptor α (PPARα). In these transgenic mice, spontaneous and persistent PPARα activation occurred heterogeneously, which was different from that observed in mice treated with clofibrate, a potent peroxisome proliferator. We further demonstrated that stabilization of PPARα through a possible interaction with HCV core protein and an increase in nonesterified fatty acids, which may serve as endogenous PPARα ligands, in hepatocyte nuclei contributed to the core protein-specific PPARα activation. In conclusion, these results offer the first suggestion that HCV core protein induces spontaneous, persistent, age-dependent, and heterogeneous activation of PPARα in transgenic mice, which may contribute to the age-dependent and multicentric hepatocarcinogenesis mediated by the core protein.

Introduction

Hepatitis C virus (HCV) is one of the major causes of chronic hepatitis, and persistent infection with this virus can lead to a high incidence of hepatocellular carcinoma (HCC).[1,2] The prevalence of HCC due to chronic HCV infection has increased over the past two decades,[3,4] and chronic HCV infection has therefore been recognized as a serious disease. However, the precise mechanism of hepatocarcinogenesis during chronic HCV infection remains unclear.

Many experiments using cell culture systems have suggested the possibility that HCV core protein itself can modulate various cellular functions and can be directly linked to the development of HCV-related HCC.[5] For example, HCV core protein transforms rat embryo fibroblasts to a tumorigenic phenotype in cooperation with the H-*ras* oncogene,[6] suppresses *c-myc*-related apoptosis[7] and transcription of the *p53* gene,[8] interacts with a variety of proteins, including helicase, lymphotoxin-β receptor, or dead box protein, and modulates their functions.[9] We further established transgenic mouse lines carrying the HCV core gene, in which the core protein is constitutively expressed in the liver at levels similar to that found in chronic hepatitis C patients.[10] These mice exhibited multicentric hepatic adenomas, and developed HCCs in an age-dependent manner.[11] The livers of these mice were almost free of inflammation, necrosis, and fibrosis,[10,11] suggesting that the core protein itself has a hepatocarcinogenic potential *in vivo*. However, the molecular mechanism of the development of HCC in the transgenic mice has not been fully understood.

In the livers of HCV core gene transgenic mice, an age-dependent increase in oxidative stress and resultant DNA damage were found,[12] and these effects may contribute to or facilitate the development of HCC. Another possible mechanism of hepatocarcinogenesis is continuous enhancement of hepatocyte proliferation. Cell proliferation and apoptosis are highly regulated processes for maintaining homeostasis in many organs, and during the carcinogenic process, sustained imbalance generally precedes cancer.[13,14] For example, in patients with chronic HCV infection, high hepatocyte proliferative activity relative to apoptosis may reliably predict a new development of HCC.[15] However, there is no information about whether or not hepatocyte proliferation accelerates persistently in mice carrying the HCV core gene, and no information about how the core protein promotes hepatocyte proliferation *in vivo*.

In the current study, we began to examine changes in the parameters of hepatocyte proliferation and apoptosis in the transgenic mice.

Materials and Methods

Animals and treatments

HCV core gene transgenic mice on a C57BL/6N genetic background were produced as described previously.[10] Because HCC developed preferentially in male transgenic mice,[11] 9- and 22-month-old male mice (n = 8 for either age group) were adopted. Sex- and age-matched non-transgenic mice (n = 8 for either age group) were used as controls. These mice were fed an ordinary diet and were treated in a specific pathogen-free state according to the institutional guidelines. For additional experiment, male wild-type mice fed a control diet containing 0.5% clofibrate for 2 weeks (n = 8) were used. All mice were killed by cervical dislocation and the livers were excised. When a hepatic tumor was present, it was removed and the remaining liver tissue was used. All experiments were performed in accordance with animal study protocols approved by the Shinshu University School of Medicine.

Preparation of hepatocyte nuclear fraction

Approximately 200 mg of liver tissues was transferred to a chilled Dounce homogenizer (Wheaton, Milliville, NJ) and homogenized on ice by 30 strokes in 1.2 mL of nuclei buffer [300 mM sucrose in 10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 5 mM MgCl2, and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was filtered through gauze and centrifuged at 4,500 x g for 5 min at 4°C. The resulting pellet was resuspended, layered over 2 mL of nuclei buffer containing 2 M sucrose, and centrifuged at 23,000 x g for 1 hour at 4°C. The pellet obtained after ultracentrifugation was resuspended in 250 μL of nuclei buffer and used as the nuclear fraction. Preparation of nuclear fraction from isolated hepatocytes was performed as described elsewhere.[16]

Immunoblot analysis

Protein concentration was measured colorimetrically by a BCA[TM] Protein Assay kit (Pierce, Rockford, IL). For analysis of fatty acid-metabolizing enzymes and protein, whole liver lysate (10-20 μg protein) was subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.[17] For analysis of other proteins, hepatocyte nuclear fraction (100 μg protein) or whole liver lysate (200-300 μg protein) was

subjected to electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were incubated with the primary antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG. The origin of the primary rabbit polyclonal antibodies against fatty acid-metabolizing enzymes and protein was described previously.[17] For immunoblot analysis of peroxisome proliferator-activated receptor α (PPARα), a polyclonal anti-mouse antibody[18] or commercial antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The antibodies against cell-cycle regulators and oncogene products were purchased commercially (Santa Cruz Biotech.).[19] Equal loading of the protein obtained from whole liver lysate and nuclear fraction was confirmed by reprobing the membranes with an antibody against β-actin and histone H1, respectively. The band intensity of nuclear PPARα was quantified densitometrically, normalized to that of histone H1, and subsequently expressed as the fold changes relative to that of 9-month-old non-transgenic mice.

mRNA analysis

Total liver RNA was extracted with an RNeasy Mini Kit[TM] (Qiagen, Valencia, CA). Five μg of RNA was electrophoresed on 1.1 M formaldehyde-containing 1% agarose gels and transferred to nylon membranes by capillary blotting in 20 x SSC buffer (3 M NaCl and 300 mM sodium citrate, pH 7.0) overnight. The membranes were hybridized with ^{32}P-labeled cDNA probes. The blots were exposed to a phosphorimager screen cassette and were analyzed using a Molecular Dynamics Storm 860 Phosphorimager system (Sunnyvale, CA). The origin of the cDNA probes has been described elsewhere.[17-19] Northern blot of β-actin was used as the internal control. The blot intensity was quantified, normalized to that of β-actin, and subsequently expressed as the fold changes relative to that of 9-month-old non-transgenic mice.

Pulse-label and pulse-chase experiment

Parenchymal hepatocytes were isolated from transgenic and control mice by the modified *in situ* perfusion method.[20] After perfusion with 0.05% collagenase solution (Wako, Osaka, Japan), the isolated hepatocytes were washed three times by means of

differential centrifugation and the dead cells removed by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech., Buckinghamshire, UK). The live hepatocytes were washed and suspended in William's E medium containing 5% fetal bovine serum. 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 fetal bovine serum for 1 hour at 37°C. The medium was replaced with the same medium containing 300 μCi/ml of [^{35}S]methionine (Amersham Pharmacia Biotech.). After a 3-hour incubation, the labeled medium was changed to the standard medium and the preparation was chased for 4, 8, or 16 hours. The labeled cells were washed, homogenized and centrifuged for preparation of the nuclear fraction. The levels of radioactivity in the homogenates of the pulse-labeled preparations were similar between the transgenic and the non-transgenic mice, suggesting that the [^{35}S]methionine uptake capacity in the former hepatocytes is 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, 0.25 mM PMSF, 10 μg/mL aprotinin]. The lysate was incubated for 3 hours at 4°C with purified anti-PPARα antibody. The immune complexes were precipitated with *Staphylococcus aureus* protein A bound to agarose beads. After the precipitates had been washed in RIPA buffer, the labeled proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The nuclear fractions of the pulse-labeled preparations were also used for immunoblot analysis of PPARα.

Affinity chromatography for PPARα complex

All procedures were performed at 4°C. The nuclear fraction from the mouse liver was mixed with a four-fold volume of a solution containing 12.5 mM potassium phosphate, pH 7.5, 25 mM sodium chloride, 0.25% Tween 20 and 0.1 mM PMSF. The mixture was briefly sonicated with a microsonicator, the Powersonic Model 50 (Yamato, Tokyo, Japan), and then centrifuged at 100,000 x *g* for 20 minutes. The supernatant was applied to an immobilized anti-PPARα IgG column (1.0 x 4.0 cm), prepared with the Affigel HZ Immunoaffinity kit[R] (Bio-Rad, Hercules, CA) and equilibrated with 10 mM

potassium phosphate, pH 7.5, 20 mM sodium chloride and 0.2% Tween 20. The solution was again passed through the column and this was repeated at least three times. The column was washed and the elution performed with 150 mM sodium citrate, pH 3.0, and 200 mM sodium chloride, in a total volume of 2 mL. The eluate was resolved by 10% and 15% SDS-polyacrylamide gel electrophoresis for PPARα and the HCV core protein, respectively. The core protein expressed in COS cells was used as a positive marker.[21] The monoclonal antibody against the core protein was purchased commercially (ViroGen, Watertown, MA).

Cytochemical staining of peroxisomes

Liver peroxisome proliferation was evaluated by using 3,3'-diaminobenzidine (DAB) staining for catalase according to the method of Novikoff and Goldfischer with minor modifications.[22] Small pieces of liver were fixed with 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2, for 3 hours at 4°C, rinsed with sodium cacodylate buffer and cut into 100-μm sections with a Lancer[R] Vibratome 1000 (Lancer, Bridgeton, MO). These sections were then incubated for 1 hour at 37°C in the DAB reaction medium (0.2% DAB tetrahydrochloride in 50 mM propanediol, pH 9.7, 5 mM KCN, 0.05% H_2O_2) and postfixed with 1% OsO_4 in 100 mM sodium phosphate, pH 7.4 for 1 hour. The sections were dehydrated through a graded series of ethanol and acetone treatments and embedded in Epok 812 (Oken, Tokyo, Japan). One-μm sections were prepared, counterstained with 0.1% toluidine blue solution and examined by light microscopy. For electron microscopic examination, 0.1-μm sections were cut with a diamond knife, collected on grid meshes, stained with lead citrate and uranyl acetate, and visualized with a JEM 1200EX II electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 keV.

Morphometry of hepatic peroxisomes

Morphometric analysis of DAB-stained peroxisomes was carried out using electron photomicrographs. For each mouse, 10 independent fields in the pericentral area of liver lobuli were photomicrographed at an original magnification of 4,000x. At this magnification, peroxisomes smaller than 450 nm were clearly identified. Peroxisomes

were easily detected because of their high contrast due to the positive DAB reaction. In each frame, the number of peroxisomal profiles and the area of each individual profile were determined. The numerical density and volume density of peroxisomes were calculated using the following equations: numerical density (number/μm^2) = N_P / (A_T − A_{empty}), and volume density (%) = A_{TP} / (A_T − A_{empty}) x 100, where N_P is the peroxisome number in the test area, A_T is the test area, A_{empty} is the area of the vascular and biliary lumens and that of the hepatocyte nuclei and lipid droplets, and A_{TP} is the area of total peroxisomal profiles in the test area. The area was measured with a Luzex AP image analyzer (Nireco, Tokyo, Japan).

Immunofluorescence staining

Liver samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in Tissue-Tek O.C.T compound[TM] (Sakura Finetek Inc., Torrance, CA) and frozen. Frozen liver 5-μm sections were prepared, washed with PBS, blocked with bovine serum albumin for 1 hour, and incubated overnight with rabbit polyclonal antibodies against cyclin D1 (1:50 dilution)[19] and PPARα (1:100 dilution),[18] and with mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (1:100 dilution).[19] After five washes with PBS, these sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) or donkey anti-mouse IgG (Dako). The sections were mounted and viewed with an Olympus Fluoview confocal laser scanning microscope (Olympus, Tokyo, Japan). Two-thousand hepatocyte nuclei were examined for each mouse, and the number of hepatocyte nuclei stained with the antibodies against cyclin D1, PPARα and PCNA was counted and expressed as a percentage.

Assessment of apoptotic hepatocytes

Liver samples were cut into small pieces and then fixed in 4% paraformaldehyde in PBS. These samples were dehydrated, embedded in paraffin and cut into 4-μm sections. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed using a MEBSTAIN Apoptosis Kit II (Medical & Biological Laboratories, Nagoya, Japan). The number of apoptotic

hepatocytes in 2,000 hepatocytes was counted for each mouse, and expressed as a percentage.

Other methods

Hepatic caspase 3 activity was measured as described elsewhere.[23] For analysis of the nuclear contents of nonesterified fatty acids (NEFAs), approximately 150 μL of the hepatocyte nuclear fraction, containing 1-2 mg of protein, was treated with a microsonicator. Lipid extraction was performed according to a modification of the method developed by Folch et al,[24] and the nuclear content of NEFAs was measured with a NEFA C-test kit[TM] (Wako).

Statistical analysis

Statistical analysis was performed by means of Student's *t*-test. The results are expressed as the mean $\pm$ standard deviation. A probability value of less than 0.05 was considered to be statistically significant.

Results

Accelerated hepatocyte proliferation in HCV core gene transgenic mice

To evaluate hepatocyte proliferative activity, PCNA-positive hepatocytes were counted in male transgenic mice and non-transgenic mice. Although hepatic inflammation and hepatocyte necrosis were not detected in either group, the numbers of PCNA-positive hepatocytes were significantly increased in the 9-month-old transgenic mice compared with the 9-month-old non-transgenic mice (Figure 1A). The increase was more significant in the 22-month-old transgenic mice (Figure 1A). The numbers of PCNA-positive hepatocytes in the 22-month-old transgenic mice corresponded with those in HCV polyprotein-expressing transgenic mice with HCC.[25] On the other hand, the parameters of apoptosis, i.e., the numbers of TUNEL-positive hepatocytes and hepatic caspase 3 activity, remained unchanged between the two groups at the same ages (Figures 1B and C). These results suggest that spontaneous hepatocyte proliferation occurs as early as the age of 9 months and persists for a long time in HCV core gene transgenic mice.

Simultaneous induction of cell-cycle regulators and oncogene products in HCV core gene transgenic mouse livers

To examine the changes in the expression of proteins associated with hepatocyte division, the livers of the 9- and 22-month-old mice were subjected to immunoblot analysis. The levels of many proteins including cell-cycle regulators [cyclin-dependent kinase (CDK) 1, 2, and 4, cyclin D1 and E, and PCNA], and oncogene products (c-Myc, c-Fos, and c-Ha-Ras) were significantly higher in the 22-month-old transgenic mice than in the control mice (Figure 2). The levels of CDK inhibitors such as p16 and p21 were similar between the two groups. Similar results were obtained for the 9-month-old transgenic mice (data not shown). Time course changes in the expression of key G1-S checkpoint regulators, cyclin D1 and CDK4, are shown in Figure 3A. The simultaneous increase in the expression of cyclin D1 and CDK4 in the transgenic mice was continuous and more pronounced with age. Northern blot analysis revealed that the increase of these proteins occurred at the transcriptional level (Figure 3B and C). Thus, these results reveal that various proteins which accelerate cell-cycle progression were induced simultaneously, persistently, and age-dependently in the transgenic mice.

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Correlative induction of PPARα targets in HCV core gene transgenic mouse livers

As shown in Figure 2, the expression of many kinds of cell-cycle regulators and oncogene products is known to be induced by the functional activation of PPARα.[19,26-30] To investigate whether PPARα is activated in the livers of transgenic mice, the expression of representative PPARα target genes,[30] acyl-CoA oxidase (AOX), peroxisomal thiolase (PT), and liver-type fatty acid-binding protein (L-FABP), was examined. As demonstrated in Figure 3A, the levels of AOX, PT, and L-FABP were increased in the 9-month-old transgenic mice compared with the non-transgenic mice, and the increase was more pronounced in the 22-month-old transgenic mice. Northern blot analysis demonstrated that the increase in these PPARα targets was based on the increase in the transcriptional activity (Figure 3B and C). The increase in the mRNA expression of AOX, PT, and L-FABP corresponded exactly with that of cyclin D1 or CDK4 (Figure 3B and C). Therefore, these results demonstrate the strong correlation between continuous and age-dependent induction of cell-cycle regulators and functional activation of PPARα in these transgenic mice. Furthermore, the induction of these 5 proteins was also observed in wild-type mice treated with clofibrate, a potent PPARα activator; however, the degree of the induction of AOX and PT in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Figure 3), suggesting that the PPARα activation found in the transgenic mice was not as intense as that in the mice treated with clofibrate.

Histological evaluation of PPARα activation

An increase in the numbers of peroxisomes is associated with PPARα activation.[18] To determine whether peroxisome proliferation occurs in the HCV core gene transgenic mice, cytochemical staining for peroxisomal catalase was performed. A scattered distribution of hepatocytes with numerous peroxisomes was observed in the 9-month-old transgenic mice (Figure 4A). Such hepatocytes were also found in the 22-month-old transgenic mouse livers (Figure 4A). In contrast, almost all of the hepatocytes in the clofibrate-treated mice showed significant peroxisome proliferation (Figure 4A). To quantitatively evaluate the degree of peroxisome proliferation,

morphometric analysis of peroxisomes was conducted. The numerical density and volume density were significantly increased in the transgenic mice compared with those in the non-transgenic mice (Figure 4B). The volume density, the most reliable parameter of peroxisome proliferation, was increased age-dependently in the transgenic mice, but the degree of the increase was not as prominent as that observed in mice with clofibrate administration (Figure 4B). The finding that only some hepatocytes in the transgenic mice presented a marked peroxisome proliferation (Figure 4A) is noteworthy, since it seems to correlate with the finding that intense expression of the core protein was observed only in particular hepatocytes.[10] These histological analyses reveal that spontaneous, continuous, and age-dependent peroxisome proliferation and PPARα activation occur heterogeneously in the transgenic mouse livers, which is different from the response observed in the mice receiving clofibrate treatment.

Appearance of PPARα-, and cyclin D1-positive hepatocytes

We tried to detect abnormal hepatocytes to clarify the mechanism of hepatocarcinogenesis in the transgenic mice. On PPARα immunofluorescence staining, PPARα was primarily detected in the cytoplasm of the non-transgenic mice and the clofibrate-administered mice. Some hepatocytes having nuclei positively stained by anti-PPARα antibody were detected only in the transgenic mice (Figure 5A). Similar to the case of PPARα, the hepatocytes having nuclei stained intensively by anti-cyclin D1 antibody were found only in the transgenic mice (Figure 5A). A few hepatocytes stained by anti-CDK4 antibody were also observed only in the transgenic mice (data not shown). The frequency of appearance of PPARα-, or cyclin D1-positive hepatocytes was increased with age (Figure 5A and B). Thus, the appearance of these specific hepatocytes in the transgenic mice seemed to be, at least in part, associated with sustained, age-dependent, and heterogeneous PPARα activation in the transgenic mice.

Changes in PPARα levels

Since the expression of PPARα is known to be enhanced by its activation,[18,30] the quantitative change in PPARα was evaluated. The nuclear PPARα level in the transgenic mice was increased age-dependently, as expected (Figure 6A, upper panel

and B), but the PPARα level in the whole liver lysate remained unchanged (data not shown). The increase in nuclear PPARα in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Figure 6A, upper panel and B). Northern blot analysis revealed a higher PPARα mRNA level in the clofibrate-treated mice than in the controls, although this parameter in the transgenic mouse groups of each age was similar to that in the controls (Figure 6A, lower panel and B). These results indicate that the increase in nuclear PPARα in the transgenic mice occurs mainly at the post-transcriptional level, which is distinct from that observed in the clofibrate-treated wild-type mice.

Stabilization of PPARα through a possible interaction with HCV core protein in hepatocyte nuclei

The increased stability of PPARα in hepatocyte nuclei is thought to be one of the possible causes of a disproportional increase in the nuclear PPARα level. To examine this possibility, a pulse-chase experiment was performed using isolated hepatocytes. The half-life of nuclear PPARα was approximately 8 hours in the control mice and 13.5 hours in the transgenic mice (Figure 7A). In addition, the intensity of the labeled PPARα band (P in Figure 7A, upper panels) in the control mice was similar to that in the transgenic mice. The finding that the [^{35}S]methionine uptake in the hepatocytes from the control mice was similar to that from the transgenic mice suggests that the increase in nuclear PPARα in the hepatocytes from the transgenic mice (Figure 7A, lower right panel), as well as that *in vivo* (Figure 6A, upper panel), is not due to the increased PPARα transfer into the nucleus.

In the transgenic mice, HCV core protein accumulated in the nuclei, as evidenced by immunoelectron microscopy,[11] suggesting a possible interaction of the core protein with PPARα in the nuclei. We therefore examined this possibility by anti-PPARα IgG affinity chromatography. When proteins combining with PPARα in hepatocyte nuclei were subjected to immunoblot analysis, the core protein was clearly detected (Figure 7B). This result suggests the possibility of complex formation between the HCV core protein and PPARα, which is consistent with an interaction of the core protein with retinoid X receptor (RXR) α,[31] an essential heterodimeric partner of PPARα.[32] Thus,

HCV core protein may directly or indirectly affect the stability of PPARα in hepatocyte nuclei.

Increase in PPARα ligands

PPARα is a ligand-activated transcription factor. Since the transgenic mice were fed a standard laboratory chow, endogenous substances such as NEFAs would serve as ligands of PPARα[33]; therefore, the contents of NEFAs in hepatocyte nuclei were compared between the two groups. The levels of NEFAs in hepatocyte nuclei in the transgenic mice were approximately 5 times higher than those in the control mice at the same age (Figure 7C). This could account for the higher activation of PPARα in the transgenic mice than in the controls.

Discussion

A very large number of variables are involved in the induction of HCC by HCV core protein. While the precise mechanism underlying hepatocarcinogenesis in HCV core gene transgenic mice cannot be fully elucidated from this study, our results could provide some clues to explain this phenomenon. We found spontaneous, persistent, age-dependent, and heterogeneous PPARα activation in the transgenic mouse livers for the first time. This study thus advances our understanding of the association between HCV core protein-mediated hepatocarcinogenesis and persistent PPARα activation.

Hepatocyte proliferation is influenced by various factors, such as mitogenic chemicals, cytokines, growth factors, and transcription factors. It has been reported that various kinds of cell-cycle regulators and oncogene products are induced by PPARα activation.[19,26-30] In particular, cyclin D1, CDK4, PCNA, and c-Myc are potent and critical regulators of the G1-S checkpoint and cell-cycle progression,[13,14] and aberrant expression of these proteins is frequently detected in HCV-related HCC[34-37]. These key regulators are known to be induced in a PPARα-dependent manner in mice;[19,30] the continuous induction of these proteins and the resultant acceleration of hepatocyte proliferation found in the transgenic mice may be attributed to persistent PPARα activation. In the current study, we demonstrated that there was a great variety of the intensity of PPARα activation among different hepatocytes (Figure 4). This persistent and heterogeneous PPARα activation found especially in the transgenic mice may be linked with the age-dependent and multicentric hepatocarcinogenesis induced by the core protein.

It is well-known that the long-term administration of potent peroxisome proliferators such as fibrate drugs can induce hepatocarcinogenesis in rodents.[29] The findings observed in the transgenic mice markedly differ from those in mice with long-term treatment of peroxisome proliferators in several ways. Namely, the transgenic mice show no intense increase in AOX and PT (Figure 3), no increase in PPARα mRNA (Figure 6), heterogeneous peroxisome proliferation (Figure 4), and age-dependent emergence of hepatocytes having nuclei stained intensively by anti-PPARα or anti-cyclin D1 antibody (Figure 5). Therefore, the mode of PPARα activation and the mechanism of hepatocarcinogenesis caused by HCV core protein expression are indeed

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unique.

One of the mechanisms involved in the core protein-specific PPARα activation in mice is stabilization of PPARα in hepatocyte nuclei through a possible interaction with the core protein. In cultured cells expressing the core protein, it has been demonstrated that the core protein interacts with the PPARα-RXRα heterodimer and enhances the transcriptional activation mediated by PPARα regardless of the presence or absence of its ligands.[31] Since PPARα is ubiquitinated and degraded via the proteasome pathway,[38] it may be postulated that HCV core protein directly or indirectly influences the degradation pathway. It has been reported that the core protein binds to the proteasome activator PA28γ[39], which is known to combine with steroid receptor coactivator-3 and to accelerate its degradation.[40] Another possible mechanism is an increase in NEFAs in hepatocyte nuclei. The PPARα activation induced by the core protein enhances the expression of L-FABP,[30] which serves as a transporter of NEFAs into nuclei. Indeed, real-time confocal and multiphoton laser scanning microscopy has shown that L-FABP expression significantly increased the total uptake of medium- and long-chain fluorescent fatty acids into the nuclei of living cells.[41] Thus, increased L-FABP expression may facilitate the shuttling of NEFAs into hepatocyte nuclei for donating NEFAs to PPARα, leading to PPARα activation and further increase in L-FABP expression. Moreover, the binding of ligands causes conformational alternation of PPARα[42] and further stabilizes it in nuclei,[32] resulting in synergistic PPARα activation. Therefore, these findings concerning spontaneous and persistent PPARα activation induced by the core protein enable us to partially explain the precise molecular mechanism of hepatocarcinogenesis in HCV core gene transgenic mice.

The results obtained from the current study are consistent with the findings observed in chronically HCV-infected patients in several ways. That is, like the transgenic mice in the present study, chronically HCV-infected patients have been reported to show accelerated hepatocyte proliferation,[43] an increase in CDK4, cyclin D1 and E, PCNA, c-Myc and c-Fos,[34-37] and multicentric appearance of HCC.[44] Furthermore, it has been reported that a massive proliferation of peroxisomes was found in human non-tumorous liver tissue adjacent to HCC.[45] Thus the above findings, including the unique function of HCV core protein *in vivo* and the diverse and

significant roles of PPARα, may help to partially understand the onset and development of HCC in patients with chronic HCV infection. It has been demonstrated that the function of hepatic PPARα was impaired in patients with chronic HCV infection,[46] which is different from our results. Since HCC had not yet developed in the patients in the report, this discrepancy might derive from differences in the stage of the hepatocarcinogenic process.

The interpretation based on persistent activation of PPARα pertains to only one possible mechanism of hepatocarcinogenesis induced by the effects of HCV core protein. We cannot rule out the presence of other mechanisms. The exact relationship between PPARα activation and hepatocarcinogenesis may be elucidated by additional experiments in which PPARα activation is continuously inhibited in the same transgenic mice. Furthermore, the exact relationship may be confirmed when PPARα-null mice bearing the core protein gene do not represent development of HCC.

In conclusion, we demonstrated for the first time that spontaneous, persistent, age-dependent and heterogeneous activation of PPARα occurred in HCV core protein transgenic mice and caused continuous enhancement of hepatocyte proliferation, which may have contributed to the age-dependent and multicentric hepatocarcinogenesis observed in these mice. In addition, we observed nuclear stabilization of PPARα and an increase in NEFAs in the hepatocyte nuclei of the transgenic mice, which may have resulted in the HCV core protein-specific PPARα activation.

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Figure Legends

Figure 1. Increase in hepatocyte proliferative activity

(**A**) The number of PCNA-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number stained with anti-PCNA antibody was counted. Results are expressed as the mean $\pm$ standard deviation (n = 8). *, $P<0.05$ between the transgenic mice and the non-transgenic mice; NT, non-transgenic mice; T, transgenic mice; 9 mon, 9-month-old mice; 22 mon, 22-month-old mice.

(**B**) The number of apoptotic hepatocytes. The number of TUNEL-positive hepatocytes in 2,000 hepatocytes was determined for each mouse. Results are expressed as the mean $\pm$ standard deviation (n = 8).

(**C**) Caspase 3 activity. Results are expressed as the mean $\pm$ standard deviation (n = 8).

Figure 2. Immunoblot analysis of cell-cycle regulators and oncogene products

Whole liver lysate (200 µg) was loaded in each lane. The band of β-actin was used as the loading control. The apparent molecular weight is indicated in parentheses. 22 mon, 22-month-old mice; NT, non-transgenic mice; T, transgenic mice.

Figure 3. Analysis of PPARα-regulated proteins

(**A**) Immunoblot analysis of cell-cycle regulators and fatty acid-metabolizing enzymes and proteins. Since no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver pieces prepared from each mouse (n = 8 per group) was mixed and homogenized. Whole liver lysate (200 µg for cyclin D1 and CDK4, and 20 µg for others) was loaded in each lane. The band of β-actin was used as the loading control. Results are representative of four independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, non-transgenic mice; T, transgenic mice; NT*, non-transgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks; A and B, full-length and truncated AOX, respectively.

(**B**) Northern blot analysis concerning the proteins in Figure 3A. Ten mg of liver pieces from each mouse (n = 8 per group) was mixed and homogenized, and total liver RNA was extracted. Hepatic RNA (5 µg) was separated on a denaturing gel, transferred to

membranes and hybridized with the indicated ^{32}P-labeled cDNA probes. The blot of β-actin was used as the internal control. Results are representative of four independent experiments.

(**C**) Quantification of hepatic mRNA levels. The mRNA level was quantified using a phosphorimager, normalized to that of β-actin, and subsequently normalized to that of 9-month-old non-transgenic mice. Results were obtained from four independent experiments and expressed as the mean $\pm$ standard deviation. Abbreviations are identical with those in Figure 3B. *, $P<0.05$ between the transgenic mice and the non-transgenic mice.

Figure 4. Cytochemical staining for hepatic peroxisomes

(**A**) Light and electron photomicrographs of DAB-stained liver tissues. Peroxisomes are detected as darkly stained particles. The arrows in upper panels indicate hepatocytes showing profound peroxisome proliferation. The bars in the light and electron photomicrographs of 9-month-old non-transgenic mice indicate 50 μm and 2 μm, respectively. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, non-transgenic mice; T, transgenic mice; NT*, non-transgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks.

(**B**) Morphometric analysis of hepatic peroxisomes. The number of peroxisomes and the area of each individual peroxisome profile were measured in 10 photomicrographs for each mouse, and morphometric parameters such as numerical density and volume density were calculated. Results are expressed as the mean $\pm$ standard deviation (n = 8). Abbreviations are identical with those in Figure 4A. *, $P<0.05$ between the transgenic mice and the non-transgenic mice.

Figure 5. Immunofluorescence staining for PPARα and cyclin D1.

(**A**) Immunofluorescence staining using antibodies against PPARα and cyclin D1. The bars in the photomicrographs of 9-month-old non-transgenic mice indicate 50 μm. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, non-transgenic mice; T, transgenic mice; NT*, non-transgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks.

(**B**) The number of PPARα-, or cyclin D1-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number of nuclei intensively stained with anti-PPARα or anti-cyclin D1 antibody was counted. Results are expressed as the mean $\pm$ standard deviation (n = 8). Abbreviations are identical with those of Figure 5A. *, $P<0.05$ between the transgenic mice and the non-transgenic mice.

Figure 6. Analysis of PPARα

(**A**) (**Upper panels**) Immunoblot analysis of nuclear PPARα. Since few individual differences in the same mouse group were found in the preliminary experiments, 30 mg of liver pieces from each mouse (n = 8 per group) was mixed and homogenized to prepare the nuclear fraction. One-hundred μg of nuclear protein was separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and reacted with antibody against PPARα. The band of histone H1 was used as the loading control. Results are representative of four independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, non-transgenic mice; T, transgenic mice; NT*, non-transgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (**Lower panels**) Northern blot analysis of PPARα. A sample used in Figure 3B was adopted. Hepatic RNA (5 μg) was electrophoresed and hybridized with cDNAs for PPARα and β-actin, respectively. Results are representative of four independent experiments.

(**B**) Quantification of nuclear PPARα levels and PPARα mRNA levels. The nuclear PPARα level was quantified densitometrically and normalized to the histone H1 level. The mRNA level of PPARα was quantified using a phosphorimager and normalized to that of β-actin. Values were subsequently normalized to those of 9-month-old non-transgenic mice. Results were obtained from four independent experiments and expressed as the mean $\pm$ standard deviation. Abbreviations are identical with those in Figure 6A. *, $P<0.05$ between the transgenic mice and the non-transgenic mice.

Figure 7. Analyses of PPARα stability, interaction between PPARα with the core protein in hepatocyte nuclei, and nuclear contents of NEFAs

(**A**) Pulse-label and pulse-chase experiments for nuclear PPARα using isolated mouse

hepatocytes. (**Upper panels**) Labeled PPARα bands on X-ray film. Pulse-label and pulse-chase experiments were performed as described in the Materials and Methods. NT, non-transgenic mice; T, transgenic mice; P, pulse-label; 4hr, 8hr, 16hr, pulse-chase for 4, 8, 16 hours, respectively. (**Lower left panel**) Intensity plot of PPARα in five independent experiments. Values are normalized as a percentage of the values of the pulse-labeled band and expressed as the mean ± standard deviation. Open square, non-transgenic mice; black square, transgenic mice; *, $P<0.05$ between the transgenic mice and the non-transgenic mice. (**Lower right panel**) Immunoblot analysis of an isolated hepatocyte nuclear fraction. NT, non-transgenic mice; T, transgenic mice.

(**B**) Interaction between PPARα and HCV core protein in the nucleus. (**Left panel**) Immunoblot analysis (PPARα) of the eluate on anti-PPARα IgG affinity column chromatography. (**Right panel**) Immunoblot analysis (HCV core protein) of the same eluate. NT, non-transgenic mice; T, transgenic mice; COS, HCV core protein-overexpressing COS cell lysate.

(**C**) Nuclear contents of NEFAs. The levels of NEFAs were measured using a hepatocyte nuclear fraction. Results are expressed as the mean ± standard deviation (n = 8). *, $P<0.05$ between the transgenic mice and the non-transgenic mice; NT, non-transgenic mice; T, transgenic mice; 9 mon, 9-month-old mice; 22 mon, 22-month-old mice.