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Original Article

Effect of treatment with interferon α -2b and ribavirin in patients infected with genotype 2 hepatitis C virusYoshihiko Nagase,¹ Hiroshi Yotsuyanagi,^{1,2} Chiaki Okuse,¹ Kiyomi Yasuda,³ Tomohiro Kato,⁴ Kazuhiko Koike,² Michihiro Suzuki,¹ Kusuki Nishioka,⁴ Shiro Iino³ and Fumio Itoh¹

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Aim: Nearly 20% of chronic hepatitis C (CHC) patients with genotype 2 hepatitis C virus (HCV) infection are not curable, even by interferon (IFN)-ribavirin combination therapy. The aim of this study is to investigate the factors that determine the efficacy of combination therapy in patients with genotype 2 HCV infection.

Methods: Fifty patients with CHC who underwent a treatment of 6 MU IFN α -2b with ribavirin for 24 weeks were retrospectively analyzed.

Results: All the patients showed no serum HCV-RNA within 12 weeks after starting the therapy. Forty-one of the 50 patients (82%) achieved a sustained virological response (SVR). The age, sex, genotype (2a vs. 2b) and grade/stage of the liver by histopathology and pretreatment viral load were

not different between the sustained responders and relapsers. Univariate analysis showed that an earlier viral clearance from blood and a larger number of amino acid substitutions in the interferon sensitivity determining region (ISDR) were predictors of SVR. Multivariate analysis showed that a large number of amino acid substitutions in the ISDR was a predictor of SVR.

Conclusion: The characterization of the amino acid sequences of ISDR may be helpful for predicting a relapse after combination therapy in patients with genotype 2 HCV infection.

Key words: genotype, hepatitis C virus, interferon, ISDR, ribavirin

INTRODUCTION

CHRONIC HEPATITIS C (CHC) is an infection that affects more than 150 million people worldwide. Up to 50% of these people develop chronic liver disease leading to liver cirrhosis.^{1–3} Once liver cirrhosis has developed, up to 7% of these patients per year develop hepatocellular carcinoma.^{4–6} Antiviral treatment is crucial for the control of this disease.

Before the use of ribavirin, interferon (IFN) monotherapy was the only effective treatment for CHC.

Although many clinical trials and several meta-analyses have documented the efficacy of IFN monotherapy, the rate of sustained virological response (SVR) is low, particularly in patients with genotype 1 or 4 hepatitis C virus (HCV) infection.^{7–9}

The combination therapy of IFN and ribavirin has been shown to be more effective than IFN monotherapy for CHC.^{10–14} The baseline level of serum HCV-RNA before treatment and HCV genotype are predictors of a SVR to IFN therapy.¹⁵ With regard to HCV genotype, patients who are infected with genotype 2 or 3 HCV can achieve a higher SVR rate than those with genotype 1 HCV. However, even with genotype 2 HCV infection, combination therapy for 24 weeks failed to eradicate the virus in about 20% of patients,^{12–14} although the reason for this is still unclear.

Besides HCV genotype and viral load, mutations in the interferon sensitivity determining region (ISDR, aa 2209–2248) of the non-structural region 5A (NS5A) of

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HCV have also been reported to influence the efficacy of IFN. In genotype 1 HCV infection, the number of amino acid substitutions in ISDR is reported to be related to the efficacy of IFN therapy in Japan and Europe,¹⁶⁻²⁰ although this correlation is still controversial.²¹ In genotype 2 infection, the amino acid sequence of ISDR has been reported to also correlate with SVR to IFN monotherapy.²²⁻²⁴ Therefore, the efficacy of IFN-ribavirin combination therapy in genotype 2 HCV-infected patients may be determined by the amino acid sequence of ISDR, which has not yet been studied.

The aim of this study is to elucidate factors that determine the response to IFN-ribavirin combination therapy in patients with genotype 2 HCV infection.

METHODS

Patient selection

FROM 2001 TO 2003, 140 patients (84 men and 56 women; mean age, 53.8 ± 11.3 years) were treated with recombinant IFN α -2b (Intron A; Schering-Plough, Kenilworth, NJ) and ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ) combination therapy. Eighty-five patients had genotype 1 HCV infection (54 men and 31 women; mean age, 56.3 ± 10.5 years) and 55 patients had genotype 2 HCV infection (30 men and 25 women; mean age, 50.0 ± 11.6 years). All the patients with genotype 2 HCV infection were treated daily with IFN α -2b at 6 MU for two weeks, followed by treatment three times a week with IFN α -2b 6 MU for 22 weeks in combination with ribavirin. Ribavirin was given orally twice a day at a total daily dose of 600 mg for 24 weeks for patients who weighed 60 kg or less and 800 mg for patients who weighed more than 60 kg. Fifty of the 55 patients with genotype 2 HCV infection with available clinical data were retrospectively analyzed.

HCV markers

HCV genotype was determined by a direct sequencing of the amplified products generated during the Amplicor Monitor test (Roche Diagnostics, Branchburg, NJ)²⁵ with an ABI 3700 DNA sequencer (Perkin Elmer, Applied Biosystems, Foster City, CA).²⁶ HCV-RNA level was determined using Amplicor-M version 2 (Chugai-Roche Diagnostics, Tokyo, Japan).

Polymerase chain reaction (PCR) and determination of sequences of ISDR

Complementary DNA (cDNA) was prepared by reverse transcription using an RNA-PCR kit (Takara Bio, Shiga,

Japan). In brief, 1 μ L of RNA solution, extracted from 100 μ L of serum and dissolved in 25 μ L of RNase-free distilled water, was mixed with 4 μ L of 1.5 mM MgCl₂ solution, 2 μ L of 10 \times RNA-PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 8.5 μ L of RNase-free distilled H₂O, 2 μ L of a dNTP mixture (10 mM dATP, dCTP, dGTP, dTTP), 1 μ L of random 9-mers (5'-NNNNNNNNN-3'), 0.5 μ L of RNase inhibitor (Takara Bio, Shiga, Japan) and 1 μ L of reverse transcriptase (Takara Bio, Shiga, Japan), was reverse transcribed at 42 °C for 30 min.

The first round PCR was performed using the external primers (sense primer; nt 6824-6846; 5'-TCTCAG CTCCCTGCGATCCTGA-3' and antisense primer; nt 7155-7139; 5'-GATGGTATCGAAGGCTC-3') and 2.5 U of Ex Taq polymerase (Takara Bio, Shiga, Japan) with proofreading activity. The amplification conditions consisted of 94 °C for 16 min followed by 40 cycles of 94 °C for 1 min, 50 °C for one minute and 72 °C for one minute. One microliter of the first PCR product was used for the second PCR with internal primers (sense primer; nt 6950-6968; 5'-AGCTCCTCA GCGAGC CAGCT-3', and antisense primer; nt 7104-7085; 5'-GATGGTATCGAAGGCTC-3') and 0.5 μ L of amplitaq gold (Roche Diagnostics, Branchburg, NJ). The amplification conditions of the second PCR were the same as those of the first PCR. The second PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transillumination.

Amplification products were purified on Wizard PCR Preps DNA purification resin (Promega, Madison, WI) and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems, Foster City, CA) using the above PCR primers. Sequencing was performed using an automated DNA sequencer ABI 377 (Perkin Elmer, Applied Biosystems, Foster City, CA).

Histopathology

A liver biopsy was performed on each patient within six months before the start of therapy. The histopathological findings were assessed by grading inflammatory activity and the staging of fibrosis using the classification of Desmet *et al.*²⁷ by an experienced pathologist who had no knowledge of the clinical data of the patients.

Statistical analysis

The collected data were analyzed using the SPSS program, version 11.0J (SPSS, Chicago, IL). The distributions of continuous variables were analyzed using the

Table 1 Clinical background of patients

	Genotype of HCV			Difference
	2 (n = 50)	2a (n = 32)	2b (n = 18)	P (2a vs. 2b)
Age (years)	49.2 ± 11.8	50.6 ± 10.1	46.6 ± 12.2	0.25
Male	30 (60%)	20 (63%)	10 (56%)	0.63
Viral load (KIU/mL)	491.6 ± 286.2	420.3 ± 264.8	618.2 ± 279.0	0.02
Histopathology				
Grade (0/1/2/3)	0/29/17/2	0/16/13/1	0/13/4/1	0.34
Stage (0/1/2/3/4)	1/23/14/9/1	1/10/10/8/1	0/13/4/1/0	0.02
SVR	41 (82%)	27 (84%)	14 (78%)	0.15

SVR, sustained virological response.

Mann-Whitney *U*-test. Differences in proportions were tested using Fisher's exact test. Independent factors that may influence the response to combination therapy were identified using stepwise multiple logistic regression analysis. Variables with $P < 0.1$ at univariate analysis were retained for the multivariate logistic regression analysis. The significance of correlation was evaluated by Spearman's rank analysis. A two-tailed P -value of < 0.05 was considered to indicate statistical significance.

RESULTS

Baseline characteristics of treated patients

TABLE 1 SHOWS the clinical background of the treated patients with genotype 2 HCV infection. The patients comprised 30 men and 20 women with a mean age of 49.2 ± 11.8 years. The patients with genotype 2a have lower viral loads and more severe fibrosis than those with genotype 2b HCV infection. The rate of SVR was 84% (27 of 32) in the patients with genotype 2a and 78% (14 of 18) in those with genotype 2b.

Amino acid sequence of ISDR

The amino acid sequence of ISDR was determined in 29 of the 32 patients with genotype 2a and 17 of the 18

patients with genotype 2b. The number of amino acid substitutions in ISDR was positively correlated with viral load (Spearman's rank correlation coefficient $r = -0.53$, $P < 0.001$). Figure 1 shows the amino acid sequences of ISDR. The prototype sequences of genotype 2a (D10749)²⁸ and 2b (D10988)²⁹ were determined to be the reference sequence for genotype 2a and 2b, respectively. The rate of SVR in the patients with no amino acid substitutions (wild type) in their ISDR sequence was 57% (8/14). In the patients with one to three amino acid substitutions (intermediate) and four or more substitutions (mutant) in their ISDR sequences, the rates of SVR were 85% (22/26) and 100% (8/8), respectively. In the patients with genotype 2a HCV infection, the rates of SVR in the wild, intermediate and mutant type ISDR were 63% (5/8), 80% (12/15) and 100% (8/8), respectively. In genotype 2b HCV infection, the rate of SVR in wild and intermediate type ISDR was 50% (3/6) and 91% (10/11), respectively.

Predictors of response

The characteristics of patients with SVR and those without were compared (Table 2). By univariate analysis, time of viral clearance from blood ($P = 0.018$) and

Table 2 Univariate logistic regression analysis for factors responsible for sustained virological response

	SVR	non-SVR	Univariate analysis <i>P</i>	Odds ratio
Age	51 (22-68)	52 (28-63)	0.805	0.992
Gender	21:17	7:2	0.195	0.329
Genotype (2a vs. 2b)	25:13	5:4	0.561	1.636
Histology of liver				
Grading (0/1/2/3)	0/21/16/2	0/8/1/0	0.086	6.438
Staging (0/1/2/3/4)	1/18/12/7/1	0/5/2/2/0	0.897	1.058
Pretreatment viral load (KIU/mL)	430 (8.7->850)	710 (480->850)	0.323	0.999
Time of viral clearance from blood (days)	14 (7-70)	52 (28-63)	0.018	0.649
Number of substituted amino acids in ISDR	1 (0-1)	0 (0-2)	0.048	3.716

SVR, sustained virological response.

Case No.		Number of substituted amino acids	Category (type)	Outcome
D10749	2713 PSLRATCTTHGKAYDVMVDANLFMGGDVTRIESES 2748	0		
2a-1		0	wild	ETR
2a-2		0	wild	ETR
2a-3		0	wild	ETR
2a-4		0	wild	SVR
2a-5		0	wild	SVR
2a-6		0	wild	SVR
2a-7		0	wild	SVR
2a-8		0	wild	SVR
2a-9		1	intermediate	ETR
2a-10		1	intermediate	SVR
2a-11		1	intermediate	SVR
2a-12		1	intermediate	SVR
2a-13		1	intermediate	SVR
2a-14		1	intermediate	SVR
2a-15		1	intermediate	SVR
2a-16		1	intermediate	SVR
2a-17		1	intermediate	SVR
2a-18	A M R	2	intermediate	SVR
2a-19	N V S	2	intermediate	SVR
2a-20	T S	2	intermediate	SVR
2a-21	T E S	2	intermediate	SVR
2a-22	A N T S	3	intermediate	SVR
2a-23	S T S	3	intermediate	SVR
2a-24	S V L G I DY	4	mutant	SVR
2a-25	A L G I	4	mutant	SVR
2a-26	YCR S	4	mutant	SVR
2a-27	YCR S	4	mutant	SVR
2a-28	YCR S	4	mutant	SVR
2a-29	YCR S	4	mutant	SVR
2a-30	A F R E K	5	mutant	SVR
2a-31	A ER V LK SG I	9	mutant	SVR

Case No.		Number of substituted amino acids	Category (type)	Outcome
D10988	2713 PSLKATCTTHKMAYDCMVVDANLFMGGDVTRIEESDS 2748	0		
2b-1		0	wild	ETR
2b-2		0	wild	ETR
2b-3		0	wild	SVR
2b-4		0	wild	SVR
2b-5		0	wild	SVR
2b-6		0	wild	SVR
2b-7		1	intermediate	SVR
2b-8	L	1	intermediate	SVR
2b-9	N	1	intermediate	SVR
2b-10	N	1	intermediate	SVR
2b-11	S	1	intermediate	SVR
2b-12	S	1	intermediate	SVR
2b-13	R T	2	intermediate	ETR
2b-14	TT	2	intermediate	SVR
2b-15	T E	2	intermediate	SVR
2b-16	T I	2	intermediate	SVR
2b-17	G V N	3	intermediate	SVR

Figure 1 Figures 1a and 1b show patients with genotypes 2a and 2b, respectively. The rate of sustained virological response (SVR) in patients with no amino acid substitutions in interferon sensitivity determining region (ISDR) sequence (wild type) was 57% (8/14). In patients with one to three amino acid substitutions (intermediate) and four or more substitutions (mutant) in the ISDR sequences, the rates of SVR were 85% (22/26) and 100% (8/8), respectively. ETR, end of treatment for virological response.

amino acid mutations in the ISDR ($P=0.048$) were found to be significantly linked to SVR. Because these variables were mutually correlated, multivariate analysis including histological grading was performed. In the final step, amino acid mutations in the ISDR (odds ratio [OR], 4.280; 95% confidence interval [CI], 1.139-16.038; $P=0.031$) entered the model and could not be removed (Table 3). Therefore, amino acid mutations in ISDR are the only factor associated with SVR.

DISCUSSION

IN JAPAN, THE combination therapy of IFN and ribavirin for 24 weeks was approved in late 2001. It was shown that approximately 20% of patients infected with genotype 1b HCV with a high viral load attained SVR with this regimen.³⁰ Compared to those with genotype 1, patients with genotype 2 or 3 HCV infection are expected to achieve higher SVR rates.¹²⁻¹⁴ However,

Table 3 Multivariate logistic regression analysis for factors responsible for sustained virological response

	SVR	non-SVR	Multivariate analysis <i>P</i>	Odds ratio
Grading (0/1/2/3)	0/21/16/2	0/8/1/0	0.547	2.141 (0.180–25.463)
Time of viral clearance from blood (days)	14 (7–70)	52 (28–63)	0.091	0.552 (0.277–1.100)
Number of substituted amino acids in ISDR	1 (0–1)	0 (0–2)	0.031	4.280 (1.139–16.038)

ISDR, interferon sensitivity determining region; SVR, sustained virological response.

information on individual genotypes, in particular genotype 2, is quite limited,³¹ which prompted us to conduct this study.

In this study the SVR rate of patients with genotype 2 was 82%, which is lower than that found in a previous report by Zeuzem *et al.*³¹ According to the data of previous studies,^{32,33} a high SVR rate may be expected in genotype 2 or 3 even if the treatment period is 24 weeks. One possible reason for the low SVR rate in this study is the use of conventional IFN- α . Pegylated IFN- α is superior to conventional IFN- α for inducing sustained viral clearance.^{33,34} Another possible reason is ethnicity, because response to IFN-ribavirin combination therapy varies among races.^{35,36}

The number of mutations in the ISDR of NS5A is variable and influences the efficacy of IFN-ribavirin combination therapy. Studies from Japan and Europe showed that the number of amino acid substitutions in ISDR influences the efficacy of IFN monotherapy in genotype 1 infection.^{16–20} The efficacy of IFN-ribavirin combination therapy in genotype 1 infection is also influenced by the amino acid sequence of ISDR.³⁷ In genotype 2 infection, the amino acid sequence of ISDR has been reported to also correlate with the SVR to IFN monotherapy.^{22–24} Our results suggest that the amino acid sequence of ISDR may also influence the efficacy of combination therapy in genotype 2 infection.

It is interesting that mutations in ISDR confer susceptibility to IFN-ribavirin combination therapy. It was reported that NS5A suppresses PKR protein kinase, a mediator of IFN-induced antiviral resistance³⁸ in genotype 1 infection. Multiple ISDR mutations probably abrogate this action of NS5A to inhibit PKR.³⁹ However, whether the mechanisms are also applicable to genotype 2 infection is still unclear and needs clarification.

Our study showed that about 20% of the patients with genotype 2 HCV infection were not cured by the combination therapy for 24 weeks. However, all of the uncured patients were relapsers, whose viral loads were cleared from the serum at the end of treatment. Therefore, it can be expected that these patients may be cured by a longer treatment, which should be studied further.

Figure 1a showed that cases 26, 27, 28 and 29, with no common infectious source, had the same mutations. Most of previous reported cases with mutant-type strains of ISDR had different amino acid sequences, which seems contradictory to our results.^{22–24} However, one study showed that two of the four cases shared one mutant type sequence of ISDR.²³ These results imply that some viral strains with mutant type ISDR sequence are likely to be selected, which await further study.

To conclude, IFN-ribavirin combination therapy for 24 weeks cured 80% of the patients with genotype 2 HCV. Amino acid mutations in ISDR may determine the final outcome of the combination therapy.

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Short Communication

Prevalence of hepatitis B virus infection in Japanese patients with HIV

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Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretroviral therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998

patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

Key words: co-infection, hepatitis B, HIV, liver disease.

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.¹ The prevalence of HIV in the USA is also <1%, and the virus is estimated to have infected approximately 800 000 people.² Because of the common transmission routes, that is, parenteral transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the

prevalence of HBV co-infection is 6–14%.^{1,2} Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jirovecii* pneumonia and cytomegalovirus infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,^{3,4} in addition to HCV infection.⁵

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,^{1,6,7} which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.⁸ It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

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inhibitory effects on the replication of HBV.⁹⁻¹² A careless administration or discontinuation of NRTI on HIV-HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV-HBV co-infection may lead to the development of drug resistance.^{11,12} Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only.^{9,10}

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,¹³ where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.¹⁴ There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,¹⁴ and approximately 0.8 million chronic HBV carriers.¹⁵ However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

PATIENTS AND METHODS

IN THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv); (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men; (viii) the number of HBsAg-positive patients among (vii); (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use; (xi) the number of HBsAg-positive patients among (x); (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as "others"; (xiv) the number of HBsAg-positive patients among (xiii); and

(xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV-HCV co-infection study carried out in 2003.¹⁵ It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.¹⁴ However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study.

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).¹⁶ Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as "others", most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV-HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.¹⁶ Among these, 77 (3.4%)

Table 1 Prevalence rates of hepatitis B virus infection among HIV-positive patients

Routes of transmission	No. patients	HBsAg positive (% in HIV positive according to route)	ALT >100 IU/L (% in HBsAg positive according to route)
Blood products	508 (5.9%)	30 (40.0%)	12
Homosexual men	3213 (8.3%)	267 (32.2%)	86
Drug addicts	23 (13.0%)	3 (66.7%)	2
Others (heterosexual etc.)	2254 (3.4%)	77 (28.6%)	22
Total	5998	377 (6.3%)	122 (32.4%)

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV-HBV co-infected patients. This shows a contrast to the status of HIV-HCV co-infection, in which the majority of HIV-HCV co-infected Japanese patients contracted both viruses from blood products.¹⁵

There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20-49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV-HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV-HBV co-infected

patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV-HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

DISCUSSION

ALONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV-HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.¹⁴ In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if

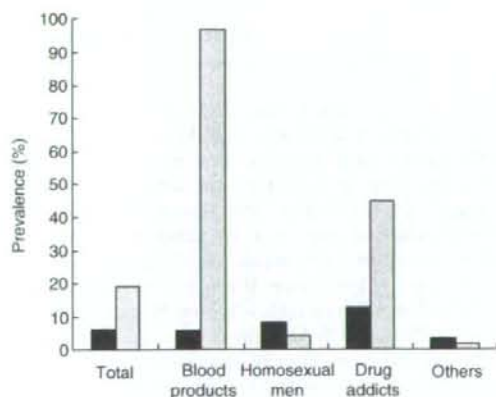


Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (□), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K et al.¹⁶

Table 2 Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

No. HIV (+)/ HBV (+)	No. HIV(+)				Total
	0	1-19	20-49	50+	
0	53	76	13	1	143
1-9	0	38	11	6	55
10+	0	0	0	9	9
Total	53	114	24	16	207

serum HBV-DNA levels were determined, but unfortunately, HBV-DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV-HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;¹⁶ the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.¹⁶ It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV¹⁷ may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.¹⁵

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV-HBV co-infected patients in the current study. Nonetheless, one-third of HIV-HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV-HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV-HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV-HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV-HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV-HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

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Hepatitis C virus 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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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 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 nontransgenic 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 (PPAR α). In these transgenic mice, spontaneous and persistent PPAR α activation occurred heterogeneously, which was different from that observed in mice treated with clofibrate, a potent peroxisome proliferator. We further demonstrated that stabilization of PPAR α through a possible interaction with HCV core protein and an increase in nonesterified fatty acids, which may serve as endogenous PPAR α ligands, in hepatocyte nuclei contributed to the core protein-specific PPAR α activation. In conclusion, these results offer the first suggestion that HCV core protein induces spontaneous, persistent, age-dependent and heterogeneous activation of PPAR α in transgenic mice, which may contribute to the age-dependent and multicentric hepatocarcinogenesis mediated by the core protein.

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Key words: cell-cycle regulator; peroxisome; nuclear stabilization; heterogeneous PPAR α activation

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 because of 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 apoptosis⁷ 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 de-

velopment 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.

Material and methods

Animals and treatments

HCV core gene transgenic mice on a C57BL/6N genetic background were produced as described earlier.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 nontransgenic 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.

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.

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Preparation of hepatocyte nuclear fraction

Approximately 200 mg of liver tissues was transferred to a chilled Dounce homogenizer (Wheaton, Millville, 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 4,500g 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 23,000g for 1 hr at 4°C. The pellet obtained after ultracentrifugation was resuspended in 250 μ L 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™ Protein Assay kit (Pierce, Rockford, IL). For analysis of fatty acid-metabolizing enzymes and protein, whole liver lysate (10–20 μ g protein) was subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.¹⁷ For analysis of other proteins, hepatocyte nuclear fraction (100 μ g protein) or whole liver lysate (200–300 μ g 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 earlier.¹⁷ For immunoblot analysis of peroxisome proliferator-activated receptor α (PPAR α), a polyclonal anti-mouse antibody¹⁸ 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 re-probing the membranes with an antibody against β -actin and histone H1, respectively. The band intensity of nuclear PPAR α was quantified densitometrically, normalized to that of histone H1, and subsequently expressed as the fold changes relative to that of 9-month-old nontransgenic mice.

mRNA analysis

Total liver RNA was extracted with an RNeasy Mini Kit™ (Qiagen, Valencia, CA). Five microgram of RNA was electrophoresed on 1.1 M formaldehyde-containing 1% agarose gels and transferred to nylon membranes by capillary blotting in 20 \times SSC buffer (3 M NaCl and 300 mM sodium citrate, pH 7.0) overnight. The membranes were hybridized with ³²P-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 nontransgenic 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 thrice 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 hr at 37°C. The medium

was replaced with the same medium containing 300 μ Ci/mL of [³⁵S]methionine (Amersham Pharmacia Biotech.). After 3-hr of incubation, the labeled medium was changed to the standard medium and the preparation was chased for 4, 8 or 16 hr. 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 nontransgenic mice, suggesting that the [³⁵S]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 μ g/mL aprotinin]. The lysate was incubated for 3 hr at 4°C with purified anti-PPAR α 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 PPAR α .

Affinity chromatography for PPAR α complex

All procedures were performed at 4°C. The nuclear fraction from the mouse liver was mixed with a 4-fold volume of a solution containing 12.5 mM potassium phosphate, pH 7.5, 25 mM NaCl, 0.25% Tween 20 and 0.1 mM PMSF. The mixture was briefly sonicated with a microsonicator, the Power Sonic Model 50 (Yamato, Tokyo, Japan), and then centrifuged at 100,000g for 20 min. The supernatant was applied to an immobilized anti-PPAR α IgG column (1.0 \times 4.0 cm²), prepared with the Affigel HZ Immunoaffinity kit⁸ (Bio-Rad, Hercules, CA) and equilibrated with 10 mM potassium phosphate, pH 7.5, 20 mM NaCl and 0.2% Tween 20. The solution was again passed through the column and this was repeated at least thrice. The column was washed and the elution performed with 150 mM sodium citrate, pH 3.0, and 200 mM NaCl, in a total volume of 2 mL. The eluate was resolved by 10 and 15% SDS-polyacrylamide gel electrophoresis for PPAR α 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 hr at 4°C, rinsed with sodium cacodylate buffer and cut into 100- μ m sections with a Lancer⁸ Vibratome 1000 (Lancer, Bridgeton, MO). These sections were then incubated for 1 hr at 37°C in the DAB reaction medium (0.2% DAB tetrahydrochloride in 50 mM propanediol, pH 9.7, 5 mM KCN, 0.05% H₂O₂) and postfixed with 1% OsO₄ in 100 mM sodium phosphate, pH 7.4 for 1 hr. The sections were dehydrated through a graded series of ethanol and acetone treatments and embedded in Epok 812 (Oken, Tokyo, Japan). One micrometer sections were prepared, counterstained with 0.1% toluidine blue solution and examined by light microscopy. For electron microscopic examination, 0.1- μ m 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 4,000 \times . At this magnification, peroxisomes smaller than 450 nm were clearly

identified. Peroxisomes were easily detected because of their high contrast because of 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) = $N_p/(A_T - A_{\text{empty}})$, and volume density (%) = $A_{TP}/(A_T - A_{\text{empty}}) \times 100$, 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 compoundTM (Sakura Finetek, Torrance, CA) and frozen. Frozen liver 5- μm sections were prepared, washed with PBS, blocked with bovine serum albumin for 1 hr and incubated overnight with rabbit polyclonal antibodies against cyclin D1 (1:50 dilution)¹⁹ and PPAR α (1:100 dilution),²⁸ and with mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (1:100 dilution).¹⁹ After 5 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, PPAR α 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 and 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), ~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 kitTM (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 nontransgenic 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 nontransgenic mice (Fig. 1a). The increase was more significant in the

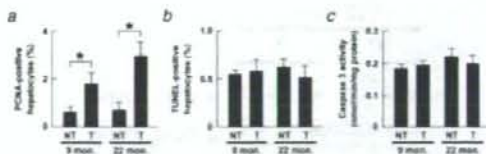


FIGURE 1 – Increase in hepatocyte proliferative activity. (a) The number of PCNA-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number stained with anti-PCNA antibody was counted. Results are expressed as the mean \pm standard deviation ($n = 8$). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice; NT, nontransgenic 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 ($n = 8$).

22-month-old transgenic mice (Fig. 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 2 groups at the same ages (Figs. 1b and 1c). 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 (Fig. 2). The levels of CDK inhibitors such as p16 and p21 were similar between the 2 groups. Similar results were obtained from 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 (Figs. 3b and 3c). Thus, these results reveal that various proteins which accelerate cell-cycle progression were induced simultaneously, persistently and age-dependently in the transgenic mice.

Correlative induction of PPAR α 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 PPAR α .^{19,26–30} To investigate whether PPAR α is activated in the livers of transgenic mice, the expression of representative PPAR α 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 nontransgenic mice, and the increase was more pronounced in the 22-month-old transgenic mice. Northern blot analysis demonstrated that the increase in these PPAR α targets was based on the increase in the transcriptional activity (Figs. 3b and 3c). The increase in the

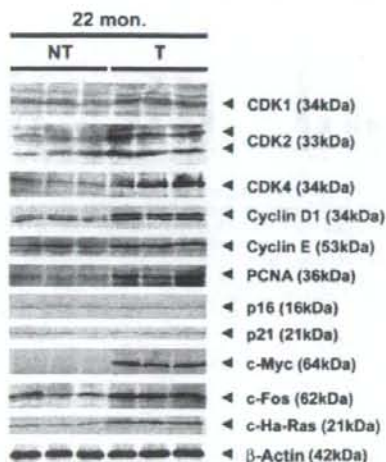


FIGURE 2 – Immunoblot analysis of cell-cycle regulators and oncogene products. Whole liver lysate (200 μ g) was loaded in each lane. The band of β -actin was used as the loading control. The apparent molecular weight is indicated in parentheses. 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice.

mRNA expression of AOX, PT and L-FABP corresponded exactly with that of cyclin D1 or CDK4 (Figs. 3b and 3c). Therefore, these results demonstrate the strong correlation between continuous and age-dependent induction of cell-cycle regulators and functional activation of PPAR α in these transgenic mice. Furthermore, the induction of these 5 proteins was also observed in wild-type mice treated with clofibrate, a potent PPAR α 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 (Fig. 3), suggesting that the PPAR α activation found in the transgenic mice was not as intense as that in the mice treated with clofibrate.

Histological evaluation of PPAR α activation

An increase in the numbers of peroxisomes is associated with PPAR α 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 (Fig. 4a). Such hepatocytes were also found in the 22-month-old transgenic mouse livers (Fig. 4a). In contrast, almost all of the hepatocytes in the clofibrate-treated mice showed significant peroxisome proliferation (Fig. 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 nontransgenic mice (Fig. 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 (Fig. 4b). The finding that only some hepatocytes in the transgenic mice presented a marked peroxisome proliferation (Fig. 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 PPAR α activation occur heterogeneously in the transgenic mouse

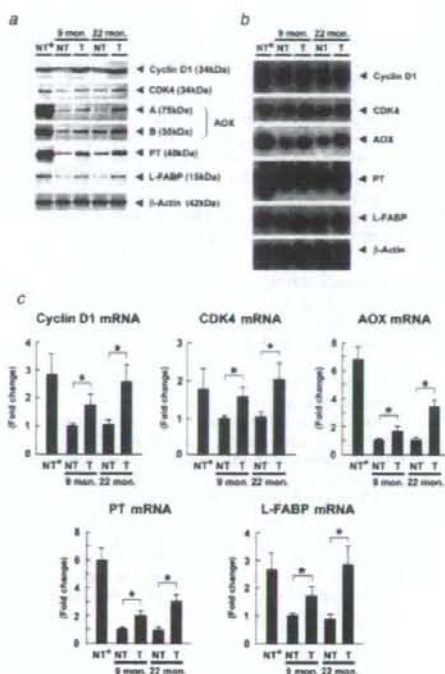


FIGURE 3 – Analysis of PPAR α -regulated proteins. (a) Immunoblot analysis of cell-cycle regulators and fatty acid-metabolizing enzymes and proteins. Since no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver pieces prepared from each mouse ($n = 8$ /group) was mixed and homogenized. Whole liver lysate (200 μ g for cyclin D1 and CDK4, and 20 μ g for others) was loaded in each lane. The band of β -actin was used as the loading control. Results are representative of 4 independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic 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 (a). Ten milligram of liver pieces from each mouse ($n = 8$ /group) was mixed and homogenized, and total liver RNA was extracted. Hepatic RNA (5 μ g) was separated on a denaturing gel, transferred to membranes and hybridized with the indicated ³²P-labeled cDNA probes. The blot of β -actin was used as the internal control. Results are representative of 4 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 nontransgenic mice. Results were obtained from 4 independent experiments and expressed as the mean \pm standard deviation. Abbreviations are identical with those in (b). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

livers, which is different from the response observed in the mice receiving clofibrate treatment.

Appearance of PPAR α - and cyclin D1-positive hepatocytes

We tried to detect abnormal hepatocytes to clarify the mechanism of hepatocarcinogenesis in the transgenic mice. On PPAR α immunofluorescence staining, PPAR α was primarily detected in the cytoplasm of the nontransgenic mice and the clofibrate-administered mice. Some hepatocytes having nuclei positively stained

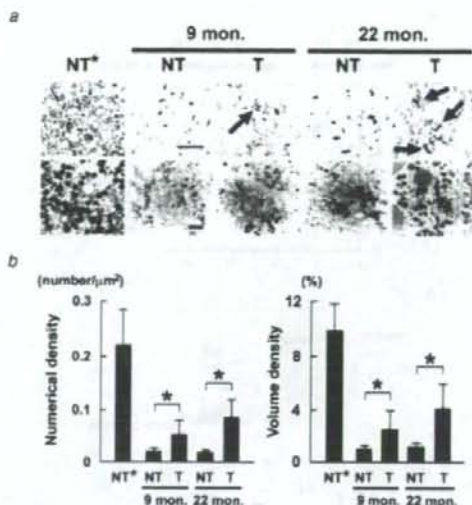


FIGURE 4—Cytochemical staining for hepatic peroxisomes. (a) Light and electron photomicrographs of DAB-stained liver tissues. Peroxisomes are detected as darkly stained particles. The arrows in upper panels indicate hepatocytes showing profound peroxisome proliferation. The bars in the light and electron photomicrographs of 9-month-old nontransgenic mice indicate 50 and 2 μm , respectively. (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 (a). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

by anti-PPAR α antibody were detected only in the transgenic mice (Fig. 5a). Similar to the case of PPAR α , the hepatocytes having nuclei stained intensively by anti-cyclin D1 antibody were found only in the transgenic mice (Fig. 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 PPAR α - or cyclin D1-positive hepatocytes was increased with age (Figs. 5a and 5b). 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 PPAR α activation in the transgenic mice.

Changes in PPAR α levels

Since the expression of PPAR α is known to be enhanced by its activation,^{18,30} the quantitative change in PPAR α was evaluated. The nuclear PPAR α level in the transgenic mice was increased age-dependently, as expected (Figs. 6a, upper panel and 6b), but the PPAR α level in the whole liver lysate remained unchanged (data not shown). The increase in nuclear PPAR α in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Figs. 6a, upper panel and 6b). Northern blot analysis revealed a higher PPAR α 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 (Figs. 6a, lower panel and 6b). These results indicate that the increase in

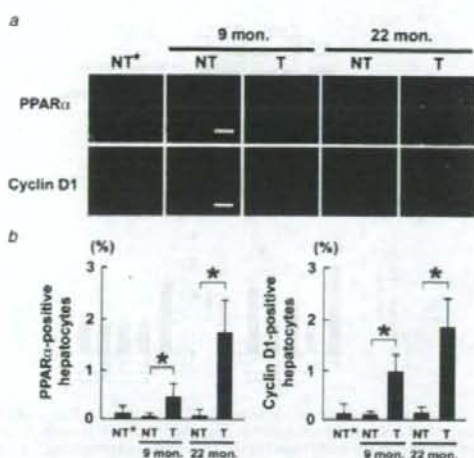


FIGURE 5—Immunofluorescence staining for PPAR α and cyclin D1. (a) Immunofluorescence staining using antibodies against PPAR α and cyclin D1. The bars in the photomicrographs of 9-month-old nontransgenic mice indicate 50 μm . 9 mon., 9-month-old mice; 22 mon., 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (b) The number of PPAR α - or cyclin D1-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number of nuclei intensively stained with anti-PPAR α or anti-cyclin D1 antibody was counted. Results are expressed as the mean \pm standard deviation ($n = 8$). Abbreviations are identical with those of (a). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

nuclear PPAR α 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 PPAR α through a possible interaction with HCV core protein in hepatocyte nuclei

The increased stability of PPAR α in hepatocyte nuclei is thought to be one of the possible causes of a disproportional increase in the nuclear PPAR α level. To examine this possibility, a pulse-chase experiment was performed using isolated hepatocytes. The half-life of nuclear PPAR α was ~ 7 hr in the control mice and 12.5 hr in the transgenic mice (Fig. 7a). In addition, the intensity of the labeled PPAR α band (P in Fig. 7a, upper panels) in the control mice was similar to that in the transgenic mice. The finding that the [³⁵S]methionine uptake in the hepatocytes from the control mice was similar to that from the transgenic mice suggests that the increase in nuclear PPAR α in the hepatocytes from the transgenic mice (Fig. 7a, lower right panel), as well as that *in vivo* (Fig. 6a, upper panel), is not because of the increased PPAR α 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 PPAR α in the nuclei. We therefore examined this possibility by anti-PPAR α IgG affinity chromatography. When proteins combining with PPAR α in hepatocyte nuclei were subjected to immunoblot analysis, the core protein was clearly detected (Fig. 7b). This result suggests the possibility of complex formation between the HCV core protein and PPAR α , which is consistent with an interaction of the core protein with retinoid X receptor (RXR) α ,³¹ an essential heterodimeric partner of PPAR α .³² Thus, HCV core protein may

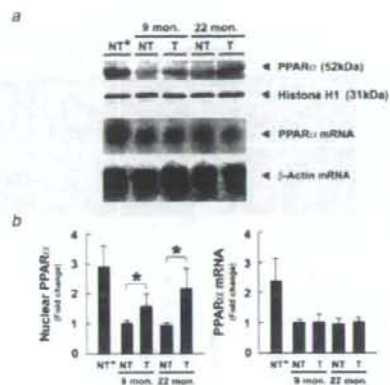


FIGURE 6 – Analysis of PPAR α . (a) (Upper panels) Immunoblot analysis of nuclear PPAR α . Since few individual differences in the same mouse group were found in the preliminary experiments, 30 mg of liver pieces from each mouse ($n = 8/\text{group}$) was mixed and homogenized to prepare the nuclear fraction. One-hundred microgram of nuclear protein was separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and reacted with antibody against PPAR α . The band of histone H1 was used as the loading control. Results are representative of 4 independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (Lower panels) Northern blot analysis of PPAR α . A sample used in Figure 3b was adopted. Hepatic RNA (5 μg) was electrophoresed and hybridized with cDNAs for PPAR α and β -actin, respectively. Results are representative of 4 independent experiments. (b) Quantification of nuclear PPAR α levels and PPAR α mRNA levels. The nuclear PPAR α level was quantified densitometrically and normalized to the histone H1 level. The mRNA level of PPAR α was quantified using a phosphorimager and normalized to that of β -actin. Values were subsequently normalized to those of 9-month-old nontransgenic mice. Results were obtained from 4 independent experiments and expressed as the mean \pm standard deviation. Abbreviations are identical with those in (a). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

directly or indirectly affect the stability of PPAR α in hepatocyte nuclei.

Increase in PPAR α ligands

PPAR α 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 PPAR α ³³; therefore, the contents of NEFAs in hepatocyte nuclei were compared between the 2 groups. The levels of NEFAs in hepatocyte nuclei in the transgenic mice were ~ 5 times higher than those in the control mice at the same age (Fig. 7c). This could account for the higher activation of PPAR α in the transgenic mice than in the controls.

Discussion

A 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 PPAR α activation in the transgenic mouse livers for the first time. This study thus advances our understanding of the association

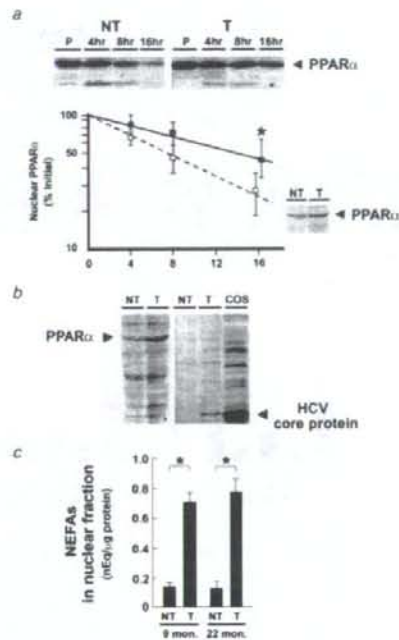


FIGURE 7 – Analyses of PPAR α stability, interaction between PPAR α with the core protein in hepatocyte nuclei, and nuclear contents of NEFAs. (a) Pulse-label and pulse-chase experiments for nuclear PPAR α using isolated mouse hepatocytes. (Upper panels) Labeled PPAR α bands on X-ray film. Pulse-label and pulse-chase experiments were performed as described in the Material and methods. NT, nontransgenic mice; T, transgenic mice; P, pulse-label; 4, 8, 16 hr, pulse-chase for 4, 8, 16 hr, respectively. (Lower left panel) Intensity plot of PPAR α in 5 independent experiments. Values are normalized as a percentage of the values of the pulse-labeled band and expressed as the mean \pm standard deviation. Open square, nontransgenic mice; black square, transgenic mice; *, $p < 0.05$ between the transgenic mice and the nontransgenic mice. (Lower right panel) Immunoblot analysis of an isolated hepatocyte nuclear fraction. NT, nontransgenic mice; T, transgenic mice. (b) Interaction between PPAR α and HCV core protein in the nucleus. (Left panel) Immunoblot analysis (PPAR α) of the eluate on anti-PPAR α IgG affinity column chromatography. (Right panel) Immunoblot analysis (HCV core protein) of the same eluate. NT, nontransgenic 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 \pm standard deviation ($n = 8$). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice; NT, nontransgenic mice; T, transgenic mice; 9 mon, 9-month-old mice; 22 mon, 22-month-old mice.

between HCV core protein-mediated hepatocarcinogenesis and persistent PPAR α 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 PPAR α 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 PPAR α -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 PPAR α activation. In the current study, we demonstrated that there was a great variety of the intensity of PPAR α activation among different hepatocytes (Fig. 4). This persistent and heterogeneous PPAR α 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 (Fig. 3), no increase in PPAR α mRNA (Fig. 6), heterogeneous peroxisome proliferation (Fig. 4) and age-dependent emergence of hepatocytes having nuclei stained intensively by anti-PPAR α or anti-cyclin D1 antibody (Fig. 5). Therefore, the mode of PPAR α activation and the mechanism of hepatocarcinogenesis caused by HCV core protein expression are indeed unique.

One of the mechanisms involved in the core protein-specific PPAR α activation in mice is stabilization of PPAR α 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 PPAR α -RXR α heterodimer and enhances the transcriptional activation mediated by PPAR α regardless of the presence or absence of its ligands.³¹ Since PPAR α 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 PA28 γ 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 PPAR α 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 PPAR α , leading to PPAR α activation and further increase in L-FABP expression. Moreover, the binding of ligands

causes conformational alternation of PPAR α ⁴² and further stabilizes it in nuclei,³² resulting in synergistic PPAR α activation. Therefore, these findings concerning spontaneous and persistent PPAR α 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 earlier findings, including the unique function of HCV core protein *in vivo* and the diverse and significant roles of PPAR α , 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 PPAR α 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 PPAR α 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 PPAR α activation and hepatocarcinogenesis may be elucidated by additional experiments in which PPAR α activation is continuously inhibited in the same transgenic mice. Furthermore, the exact relationship may be confirmed when PPAR α -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 PPAR α 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 PPAR α and an increase in NEFAs in the hepatocyte nuclei of the transgenic mice, which may have resulted in the HCV core protein-specific PPAR α activation.

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