be statistically significant. Statistical analyses were performed using SPSS, version 15.0J (SPSS).

Results. We first compared the demographic, clinical, and virologic features of the 23 patients who experienced HBV reactivation with those of the 529 patients with acute hepatitis B (table 1). The reactivation group had a significantly higher median age and median serum HBV DNA level ($P < .001$) and significantly lower peak ALT and albumin levels ($P < .001$). Although HBV genotype was not determined for one-half of the patients with acute hepatitis B, marked differences in the distribution of genotypes were seen; HBV type A occurred less frequently ($P = .003$) among patients with HBV reactivation than among those with acute hepatitis. However, HBV type B occurred more frequently among patients with HBV reactivation ($P < .001$).

FHF was more common among patients with HBV reactivation than among those with acute hepatitis ($P = .048$). Of the 23 cases of HBV reactivation, 6 (26%) resulted in liver-related death, 11 (48%) resolved, and 6 (26%) led to chronic hepatitis B. In contrast, of the 529 cases of acute hepatitis B, 490 (93%) were self-limited, 16 (3%) became chronic, and 21 (4%) resulted in death. These results revealed that liver-related mortality was significantly higher in the group with HBV reactivation than in the group with acute hepatitis ($P < .001$).

We then compared the clinical features of FHF between the groups (table 2). Patients with HBV reactivation had a higher median age, significantly lower peak ALT levels ($P = .006$), higher HBV DNA levels ($P = .035$), and higher mortality ($P = .031$) than did patients with acute hepatitis B.

Malignant lymphoma–associated morbidity was significantly higher among patients with HBV reactivation who developed FHF than among those who did not develop FHF (table 3). A rituximab-containing treatment regimen was administered to all patients who experienced FHF, compared with only 4 (22%) of 18 patients who did not experience FHF ($P = .004$). Lamivudine was administered to 16 (89%) of 18 patients who did not experience FHF and to all patients who experienced FHF at 7 and 20 days after hospital admission, respectively; this suggests that lamivudine treatment could not prevent FHF after HBV reactivation. Eventually, liver-related mortality occurred exclusively in patients who experienced FHF. There were no statistically significant differences between the 2 subgroups regarding HBV markers.

Discussion. Although a prospective study by Hui et al. [23] revealed that the incidence of HBV reactivation among HBsAg-negative patients after chemotherapy was 3.3%, there are no data available on HBV reactivation in Japan. In our nationwide cross-sectional study, a total of 552 newly HBsAg-positive patients were registered from 63 tertiary care hospitals. Overall, HBV reactivation was found in 4% of patients with resolved infection after chemotherapy. Serum and liver samples were not available before chemotherapy for most of these patients; therefore, we were unable to prove specifically whether reactivation was a result of occult or acute HBV infection. However,

Table 1. Demographic and clinical characteristics of patients with hepatitis B virus (HBV) reactivation, compared with those of patients with acute hepatitis B.

Characteristic	Patients with HBV reactivation	Patients with acute hepatitis B	*P*
Age, median years (95% CI)	63 (39–83)	33 (19–64)	<.001
Male sex	14/23 (61)	374/529 (71)	NS
Peak ALT level, median IU/L (95% CI)	929 (137–2441)	2300 (299–6626)	<.001
Peak bilirubin level, median mg/dL (95% CI)	10.3 (0.3–58.6)	6.4 (1.0–23.7)	NS
Lowest albumin level, median g/dL (95% CI)	3.2 (2.1–3.7)	3.6 (2.7–4.2)	<.001
Most prolonged PT%, median % (95% CI)	65.0 (10.2–121.4)	75.0 (11.0–103.1)	NS
HBV DNA level, median log copies/mL (95% CI)	7.5 (4.0 to >7.6)	5.5 (2.6 to >7.6)	<.001
Genotype			
A	0/19 (0)	57/232 (25)	.003
B	8/19 (42)	27/232 (12)	<.001
C	11/19 (58)	141/232 (61)	NS
Other	0/19 (0)	7/232 (3)	
Treatment			
Lamivudine	20/23 (87)	118/529 (22)	<.001
IFN	5/23 (22)	12/529 (2)	<.001
Fulminant hepatic failure	5/23 (22)	45/529 (9)	.048
Liver-related death	6/23 (26)	21/529 (4)	<.001

NOTE. Data no. (%) of patients, unless otherwise indicated. ALT, alanine aminotransferase; NS, not statistically significant; PT, prothrombin time.

Table 2. Demographic and clinical characteristics of patients with hepatitis B virus (HBV) reactivation who experienced fulminant hepatic failure (FHF), compared with those of patients with acute hepatitis B who experienced FHF.

Characteristic	Patients with FHF		*P*
	With HBV reactivation	With acute hepatitis B	
Age, median years (95% CI)	63 (47–64)	48 (18–72)	.029
Male sex	3/5 (60)	26/45 (58)	NS
Peak ALT level, median IU/L (95% CI)	907 (359–1823)	5995 (589–11,858)	.006
Peak bilirubin level, median mg/dL (95% CI)	20.8 (10.2–45.7)	9.9 (4.9–30.5)	.099
Lowest albumin level, median g/dL (95% CI)	2.6 (2.1–3.0)	2.9 (1.9–3.9)	NS
Most prolonged PT%, median % (95% CI)	22.0 (8.7–32.3)	16.0 (0.2–37.0)	NS
HBV DNA level, median log copies/mL (95% CI)	7.6 (5.6 to >7.6)	5.7 (2.6 to >7.6)	.035
Genotype			
A	0/5 (0)	2/16 (13)	NS
B	1/5 (20)	3/16 (19)	NS
C	4/5 (80)	11/16 (69)	NS
Received lamivudine treatment	5/5 (100)	29/45 (81)	NS
Liver-related death	5/5 (100)	21/45 (47)	.031

NOTE. Data are no. (%) of patients, unless otherwise indicated. ALT, alanine aminotransferase; NS, not statistically significant; PT, prothrombin time.

because all patients were negative for HBsAg and positive for antibody to hepatitis B core antigen before treatment, we presumed that reactivation was occult in nature.

In our study, patients who experienced HBV reactivation were significantly older and had lower serum albumin levels, compared with patients with acute hepatitis B. The immune status of many patients may have been further decreased by cytotoxic chemotherapy. Approximately 20% of the patients who experienced HBV reactivation developed FHF. Surprisingly, mortality was 100%, implying that FHF in these cases is severe. Both the prevalence of and mortality associated with FHF were significantly higher among patients who experienced HBV reactivation than among those with acute HBV infection. Although the group with HBV reactivation also had lower albumin levels at the onset of lamivudine therapy, the development of FHF could not be predicted from this study. Thus, it is crucial to prevent FHF in patients with HBV reactivation with use of agents other than—or complimentary to—lamivudine. Unfortunately, preemptive therapy is not recommended because of the difficulties in detecting reactivation. Hui et al. [23] recommended monthly testing of HBV DNA levels and immediate antiviral therapy when levels are 100-fold the levels before chemotherapy. However, this strategy is still controversial [28, 29] and needs testing in a randomized study.

A recent study revealed that HBV type Bj and G1896A mutations were independently associated with a fulminant outcome in patients with acute HBV infection [30]. However, HBV genotype, serum HBV DNA level, or mutations in G1896A or A1762T/G1764A did not influence the development of FHF in patients who experienced HBV reactivation in this study. HBV reactivation in patients infected with HBV genotype A was also not found in this study, which may be explained by the fact that this genotype occurs in only 1.7% of patients with chronic hepatitis B in Japan [31].

Because our study and other studies [23] have confirmed that HBV reactivation can be fatal, we need to emphasize greater testing of HBV markers, including antibody to hepatitis B core antigen, antibody to HBsAg, and HBV DNA levels before administration of chemotherapy, especially therapy containing rituximab. Patients with resolved HBV infection should be routinely monitored for liver function and HBV DNA levels, and antiviral therapy should be administered immediately when evidence of HBV reactivation is found.

In conclusion, HBV reactivation is found in 4% of newly HBsAg-positive patients with resolved HBV infection in Japan. One-fourth of cases of HBV reactivation develop into FHF, and mortality is extremely high. Because our study was unable to distinguish HBV reactivation from occult HBV infection and could not clarify whether antiviral therapy was effective, a prospective study is being planned to clarify the mechanism of HBV reactivation and the benefits of antiviral therapy.

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Table 3. Demographic and clinical characteristics of patients with hepatitis B virus (HBV) reactivation who did and did not experience fulminant hepatic failure (FHF).

Characteristic	Patients with HBV reactivation		
	Experienced FHF (n = 5)	Did not experience FHF (n = 18)	P
Age, median years (95% CI)	63 (47–64)	63 (39–78)	NS
Male sex	3 (60)	11 (61)	NS
Peak ALT level, median IU/L (95% CI)	907 (359–1823)	1016 (124–2524)	NS
Peak bilirubin level, median mg/dL (95% CI)	20.8 (10.2–45.7)	7.6 (0.3–24.9)	.094
Lowest albumin level, median g/dL (95% CI)	2.6 (2.1–3.0)	3.3 (2.2–3.6)	.015
Most prolonged PT%, median % (95% CI)	22.0 (8.7–32.3)	77.5 (18.0–101.8)	<.001
ALT level,[a] median IU/L (95% CI)	176 (83–1035)	266 (58–1690)	NS
Bilirubin level,[a] median mg/dL (95% CI)	0.7 (0.4–7.2)	0.7 (0.3–13.6)	NS
Albumin level,[a] median g/dL (95% CI)	3.4 (2.5–3.5)	3.9 (2.8–4.5)	.035
PT%,[a] median % (95% CI)	42.2 (16.4–46.4)	83.7 (38.7–123.5)	NS
HBV DNA level, median log copies/mL (95% CI)	7.6 (5.6 to >7.6)	7.5 (4.0 to >7.6)	NS
Genotype			
Bj	1 (20)	7/14 (50)	NS
C	4 (80)	7/14 (50)	NS
Mutation			
G1896A	4 (80)	5/12 (42)	NS
A1762T/G1764A	2 (40)	2/12 (17)	NS
Non-Hodgkin lymphoma	5 (100)	8 (44)	.046
Received a rituximab-containing treatment regimen	5 (100)	4 (22)	.004
Treatment			
Lamivudine	5 (100)	16 (89)	NS
IFN	1 (20)	4 (22)	NS
Liver-related death	5 (100)	1 (6)	<.001

NOTE. Data are no. (%) of patients, unless otherwise indicated. ALT, alanine aminotransferase; NS, not statistically significant; PT, prothrombin time.

[a] Laboratory data are from the start of lamivudine therapy.

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Techniques for the Early Detection of Drug Resistance

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Hepatitis B Core-Related Antigen Assay Is Useful for Monitoring the Antiviral Effects of Nucleoside Analogue Therapy

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Key Words
Drug resistance · HBV DNA · Hepatitis B core-related antigen · Lamivudine · Nucleoside analogue · Recurrence, hepatitis

Abstract
Objective: The clinical significance of the hepatitis B virus core-related antigen (HBcrAg) assay in monitoring the antiviral effects of lamivudine is reviewed. ***Methods:*** The HBcrAg assay simultaneously measured serum levels of hepatitis B core (HBc) and e (HBe) antigens using monoclonal antibodies which recognize common epitopes of these two denatured antigens. ***Results:*** Although serum HBcrAg levels correlated linearly with those of hepatitis B virus (HBV) DNA in natural course, the decrease in HBcrAg was significantly slower than in HBV DNA after initiation of lamivudine administration. We analyzed the clinical significance of HBV DNA and HBcrAg levels to predict the occurrence of lamivudine resistance. HBV DNA measurement may be useful to identify patients who are at high risk of developing lamivudine resistance, and HBcrAg measurement may help to detect patients who are at low risk of drug resistance. The measurement of HBcrAg was also found to be a useful prognosticator for reactivation of hepatitis after cessation of lamivudine administration. ***Conclusion:*** The HBcrAg assay is indeed useful for monitoring the antiviral effects of lamivudine, and we propose that it be adopted as a serum marker which reflects the amount of HBV covalently closed circular DNA in hepatocytes.

Introduction

Measurement of hepatitis B virus (HBV) DNA in serum is useful for monitoring the antiviral effects of nucleoside or nucleotide analogue therapy. However, a negative result for HBV DNA does not necessarily indicate a good therapeutic outcome since drug resistance may occur even if HBV DNA levels remain undetectable during therapy and reactivation of HBV replication may occur afterwards [1, 2]. Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B core-related antigen (HBcrAg) [3, 4]. HBcrAg consists of HBV core and e antigens: both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical [5–7]. HBcrAg CLEIA simultaneously measures serum levels of hepatitis B core and e antigens using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present report, we reviewed the clinical significance of the HBcrAg assay in monitoring the antiviral effects of lamivudine treatment.

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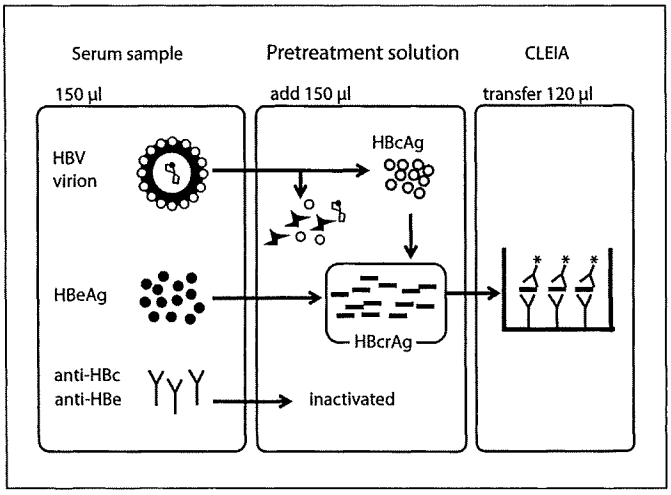

Fig. 1. Principles of the HBcrAg assay.

HBcrAg CLEIA

The principles of the HBcrAg assay are shown in figure 1. Briefly, 150 µl of serum sample are incubated with the same volume of pretreatment solution containing several detergents. Under these conditions, HBV virions are destroyed into denatured core proteins, e antigens are denatured and circulating antibodies to core and e antigens, which may interfere with the enzyme immunoassay (EIA), are inactivated. After incubation, 120 µl of pretreated specimen are subjected to the chemiluminescence EIA, and HBcrAg concentration is expressed as units per milliliter [3].

In serum samples not subjected to antiviral therapy, concentrations of HBcrAg and HBV DNA correlate linearly. The sensitivity of the HBcrAg assay for HBV viremia is better than that of the transcription-mediated amplification assay, which has a lower detection limit of 3.7 log copies/ml [8]. These findings indicate that the HBcrAg assay can measure viral load with high sensitivity [4].

Prediction of Lamivudine Resistance

Serum HBcrAg levels reflect viral load in the natural course because they correlate linearly with those of HBV DNA [4]. On the other hand, the characteristics of HBcrAg are somewhat different from HBV DNA in patients undergoing antiviral therapies, e.g. with lamivudine; specifically, the decrease in HBcrAg levels is significantly slower than that of HBV DNA after the initiation of lamivudine administration [9, 10].

We analyzed the clinical significance of HBV DNA and HBcrAg levels as a prognosticator of the occurrence of lamivudine resistance in a previous study [10]. Of the 81 patients enrolled, 27 (33%) showed lamivudine resistance during a median follow-up of 12 months. In patients without lamivudine resistance, HBV DNA decreased rapidly and remained below the detection limits in almost all patients. However, HBV DNA levels did not reach undetectable levels in the majority of patients with lamivudine resistance. Whereas HBcrAg levels decreased slowly to levels <4.7 log U/ml in most patients without lamivudine resistance, they did not decrease in cases with lamivudine resistance; HBcrAg levels remained >4.7 log U/ml until at least 6 months of treatment.

The cumulative occurrence of lamivudine resistance was then compared between both patient groups classified according to HBV DNA and HBcrAg levels after 6 months of therapy, with cutoff values for HBV DNA and HBcrAg set at 2.6 log copies/ml and 4.7 log U/ml, respectively. Lamivudine resistance at 2 years was as high as 80% in patients with HBV DNA ≥2.6 log copies/ml, but was about 30% in patients with levels <2.6 log copies/ml ($p < 0.001$). For HBcrAg, lamivudine resistance at 2 years was 50–60% in patients with HBcrAg levels ≥4.7 log U/ml, while no patients with levels below that developed lamivudine resistance ($p = 0.005$).

Taken together, our results suggest that measurement not only of HBV DNA, but also of HBcrAg, is useful to predict the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing lamivudine resistance, and measurement of HBcrAg is valuable for identifying those who are at low risk.

Reactivation of Hepatitis after Halting Lamivudine Administration

During lamivudine administration, the concentration of serum HBV DNA decreases and usually becomes undetectable even to high-sensitivity HBV DNA assays. However, this threshold may be an inadequate indicator for safely discontinuing lamivudine administration since active hepatitis often recurs in patients after treatment [11]. Therefore, we evaluated the clinical significance of the HBcrAg assay in predicting the likelihood of non-reactivation of hepatitis after discontinuing lamivudine administration for HBV treatment [9].

A total of 34 patients with chronic hepatitis B were enrolled, and lamivudine was administered for at least 6 months. Reactivation of hepatitis was defined as an elevation in ALT levels to >80 IU/l within 12 months of cessation.

Several factors just prior to lamivudine therapy were compared between patients with and without hepatitis reactivation after halting lamivudine administration, but did not significantly differ (e.g. age, gender, genotype, ALT, HBV DNA, HBcrAg or prevalence of HBeAg). Similarly, at the cessation of lamivudine therapy, several factors were compared, but the duration of lamivudine administration and the level of ALT did not differ between both groups and HBV DNA was below the detection limits in almost all patients. Thus, we can presume that HBV DNA did not differ between both groups. Interestingly, HBcrAg levels were significantly higher in patients with hepatitis reactivation than in those without (range 25–75%, 4.9; 4.7–5.9 vs. 3.2; <3.0–4.5 log U/ml, p = 0.009), indicating that measurement of HBcrAg is useful for predicting reactivation of hepatitis following lamivudine administration.

The ability of HBcrAg concentration to predict non-recurrence of hepatitis was analyzed using receiver-operating characteristic analysis, which showed a wide area under the curve of 0.764 in predicting non-reactivation of HBV. With the cutoff value set at 4.5 log U/ml, both specificity and sensitivity almost reached 80%. An even higher specificity of 0.9 can be obtained with an HBcrAg cutoff value of 4.0 log U/ml. In this case, the sensitivity would still be nearly 0.6.

Hypothesis

The replication process of HBV in hepatocytes is shown in figure 2. HBV is an enveloped DNA virus containing a relaxed circular DNA genome converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells [7, 12–14]. These cccDNA molecules serve as the transcriptional templates for the production of viral RNAs that encode both viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of pregenomic RNA, it directly suppresses the production of HBV virions [15, 16]. This explains why serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because this process does not require reverse transcription. It has also been reported that the amount of HBV cccDNA, which serves as a template for mRNA, decreases quite slowly following commencement of nucleoside analogues [17–19]. Thus, it is reasonable that serum HBcrAg levels decrease much slower than HBV DNA levels after the initiation of lamivudine therapy.

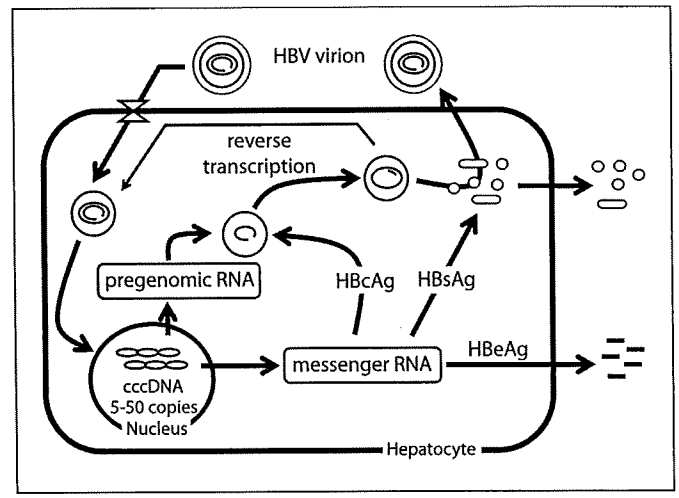

Fig. 2. Replication of HBV in hepatocytes.

Elevated levels of HBV cccDNA in hepatocytes have been associated with both more frequent occurrence of lamivudine resistance during lamivudine administration and more severe reactivation of hepatitis after discontinuation of lamivudine. Direct measurement of HBV cccDNA in hepatocytes is ideal for monitoring the antiviral effect of nucleotide analogues, but is not practical for clinical use because it requires a liver biopsy. Thus, a serum marker that reflects HBV cccDNA levels is needed. Our results suggest that serum HBcrAg levels reflect HBV cccDNA in hepatocytes more accurately than serum HBV DNA, which is also supported by the theoretical evidence described in the previous paragraph. We thus hypothesize that the HBcrAg assay can indeed be a representative serum marker which reflects the amount of HBV cccDNA in hepatocytes. Further studies are required to clarify the exact significance of the HBcrAg assay since a direct association between HBV cccDNA levels in hepatocytes and HBcrAg levels in serum remains to be shown.

Lamivudine has already been eliminated from first-line therapy in naïve chronic hepatitis B patients due to

the increased incidence of resistant mutations compared with newer antiviral agents, e.g. adefovir dipivoxil and entecavir [20]. The distinct characteristic of the HBcrAg assay under lamivudine therapy that differentiates it from other HBV DNA assays is that lamivudine suppresses the production of HBV virions by inhibiting reverse transcription of pregenomic RNA, but does not suppress the production of viral proteins, in which reverse transcription is unnecessary. Thus, it is possible that the HBcrAg assay may also be useful for patients undergoing entecavir or adefovir dipivoxil administration since the main mechanism of HBV replication suppression is similar. As a considerable number of patients who began lamivudine administration in the past are still taking this treatment now, the present study may be valuable for patients who consider changing therapy in the future, though further studies are required to determine whether the HBcrAg assay is indeed applicable to antiviral agents other than lamivudine.

Disclosure Statement

The authors declare that they have no financial conflict of interest.

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CLINICAL STUDIES

Insulin resistance and hepatitis C virus: a case–control study of non-obese, non-alcoholic and non-steatotic hepatitis virus carriers with persistently normal serum aminotransferase

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Keywords
adiponectin – γ-glutamyltransferase – HCV core protein – insulin resistance – waist circumference

Abbreviations
ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet count ratio index; AST, aspartate aminotransferase; BMI, body mass index; FPG, fasting plasma glucose; γ-GT, γ-glutamyltransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA, homoeostasis model assessment; hsCRP, high-sensitivity C-reactive protein; IR, insulin resistance; IRI, immunoreactive insulin; NASH, non-alcoholic steatohepatitis; PNALT, persistently normal serum alanine aminotransferase; TG, triglyceride; TNF, tumour necrosis factor; US, ultrasonography.

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Abstract

Background/Aims: Recent studies using transgenic mouse models have demonstrated that the presence of hepatitis C virus (HCV) singularly induces insulin resistance (IR). When evaluated in humans, the exclusion of other factors influencing IR, such as obesity, alcohol intake, hepatic inflammation and steatosis is needed, but only few studies have been performed to these ends. Therefore, we aimed at exploring the singular effects of HCV on glucose metabolism through analysis of HCV carriers with persistently normal serum aminotransferase. *Methods:* Non-obese, non-diabetic and non-alcoholic HCV carriers ($n = 30$) were enrolled with 30 hepatitis B virus carriers matched by age, gender, body mass index and waist-to-hip ratio. All patients maintained normal serum aminotransferase (<30 U/L), hyaluronic acid (<50 ng/ml) and platelet count ($>150 \times 10^3/\mu l$) for more than 5 years without additional treatments, and had no signs of steatosis. We then compared fasting plasma glucose, serum insulin and adiponectin, and homoeostasis model assessment of IR (HOMA-IR) and HOMA-β indices between the groups. *Results:* There were no significant differences in IR/secretion-associated markers or serum adiponectin. Multivariate analysis demonstrated that the presence of HCV was not an independent predictor of IR. HOMA-IR was strongly correlated with waist circumferences and serum γ-glutamyltransferase in HCV carriers, but not with serum aminotransferase, high-sensitivity C-reactive protein, hyaluronic acid or HCV core antigen. *Conclusions:* These results suggest that the presence of HCV alone does not affect IR. Coexistence of hepatitis, steatosis and/or fibrosis may be important to the pathogenesis of IR induced by chronic HCV infection.

There have been several epidemiological studies that have demonstrated a close association between chronic hepatitis C virus (HCV) infection and diabetes mellitus (1–3), and insulin resistance (IR) is the main feature of impaired glucose metabolism caused by HCV infection (4). Although the mechanism of IR has not been fully elucidated, increased triglyceride (TG) (5, 6) and/or iron accumulation (7–9) in the liver and advanced hepatic fibrosis (10–12) are thought to contribute to the phenomenon of IR. The

homoeostasis model assessment of IR (HOMA-IR) in the early stages of chronic hepatitis C patients is reported to be greater than that in healthy volunteers matched by age, gender, body mass index (BMI) and waist-to-hip ratio (13), suggesting the presence of a HCV-specific mechanism of IR independent of progression of hepatic fibrosis.

The possibility that HCV and/or the core protein itself can induce HCV-specific IR is based on several findings. Firstly, insulin receptor substrates-1 and -2, which are central molecules in the insulin signalling cascade, are downregulated in the livers of HCV core protein transgenic mice and in the core protein-transfected human hepatoma cell lines (14). Secondly, transgenic mice constitutively expressing core protein in livers exhibited marked hyperinsulinaemia and IR, which were ameliorated by inhibition of the tumour necrosis factor (TNF)-α pathway (15). Finally, HCV eradication by interferon therapy improved IR in patients with chronic hepatitis C (16). However, because factors such as obesity, hepatocyte injury, hepatic inflammation and steatosis have been shown to affect the onset of IR (17–19), they need to be excluded when the sole effect of HCV-specific IR in humans is discussed. As far as we know, there are very few studies that assess IR in chronically HCV-infected patients after careful adjustment for these factors. Hence, we sought to determine the contribution of HCV alone to the pathogenesis of IR by comparing the IR/secretion-related parameters between HCV and hepatitis B virus (HBV) carriers with persistently normal serum alanine aminotransferase (<30 U/L) (PNALT) (20). Before comparison, several other known factors that influence IR, such as obesity, alcohol intake (21), hepatic steatosis and fibrosis, were excluded, and both groups were carefully matched by age, gender, BMI and waist-to-hip ratio. IR was not found in non-obese, non-diabetic, non-alcoholic and non-steatotic HCV carriers with PNALT, implying that the presence of HCV *per se* cannot induce IR. In other words, our results suggest that various other hepatic abnormalities caused by HCV infection, including hepatitis, steatosis or fibrosis, are probably necessary for the pathogenesis of IR in chronically HCV-infected patients.

Patients and methods

Patients

Patient selection was carried out as shown in Figure 1. HCV and HBV carriers with PNALT were defined as patients who were positive for HCV-RNA and HBV surface antigen in sera, respectively, but who had normal serum alanine aminotransferase (ALT)

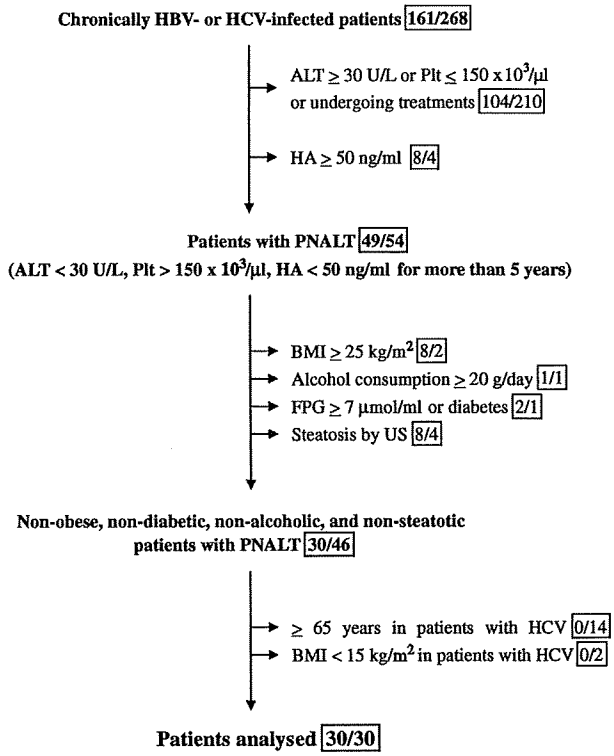

Fig. 1. Patient selection criteria. The left and right numbers in each box show the number of patients with chronic infection of HBV and HCV respectively. ALT, alanine aminotransferase; BMI, body mass index; FPG, fasting plasma glucose; HA, hyaluronic acid; HBV, hepatitis B virus; HCV, hepatitis C virus; Plt, platelet count; PNALT, persistently normal serum ALT; US, ultrasonography.

(<30 U/L), hyaluronic acid (<50 ng/ml) and platelet count (>150 × 10^3/μl) for more than 5 years without any treatments. Serum ALT and platelet count had been measured at least every 3 months, and serum hyaluronic acid had been measured every 6 months before the study. Patients who had previously been administered interferon injections and/or antiviral or hepatoprotective drugs were excluded. All HBV carriers were negative for hepatitis B e antigen and had viral loads <1000 copies/ml. To minimize any other factors affecting IR, additional criteria that have been strictly upheld for more than 5 years include : (i) BMI <25 kg/m^2, as calculated every 3 months; (ii) alcohol consumption <20 g/day; (iii) fasting plasma glucose (FPG) <7 μmol/ml, or taking no insulin or oral hypoglycaemic drugs; (iv) absence of steatosis, advanced fibrosis and cirrhosis, as detected by repeated abdominal ultrasonography (US) every 6 months; (v) absence of ongoing treatment with corticosteroids or any other medication known to affect glucose tolerance or insulin secretion and (vi) absence

of other concomitant diseases such as human immunodeficiency virus infection, hereditary haemochromatosis, pancreatitis, renal failure or neoplasia. Subsequently, both groups were carefully matched by age, gender, BMI and waist-to-hip ratio, and non-obese, non-diabetic, non-alcoholic and non-steatotic HCV ($n = 30$) and HBV ($n = 30$) carriers with PNALT were enrolled in this study (Fig. 1).

Informed consent, in writing, was obtained from all patients. Body height, weight and waist and hip circumferences were measured in the fasting state by hospital staff unaware of the patients' medical information. Any underlying diseases, medical interventions, past medical history and family history of diabetes were also recorded. Patients were considered hypertensive if their systolic/diastolic pressure was $> 140/90$ mmHg or if they were taking antihypertensive drugs. Patients were considered to have hyperlipidaemia if their fasting serum cholesterol and TG were ≥ 220 and 150 mg/dl, respectively, or if they were taking lipid-lowering drugs (5, 22). The presence of metabolic syndrome was judged according to the new definition released by the Japanese Committee for the Diagnostic Criteria of Metabolic Syndrome (23).

Laboratory examination

Venous blood samples were drawn from patients after an overnight fast. Serum insulin was determined by the radioimmunoassay method, and other data were measured by standard methods using a conventional automated analyser. HOMA-IR was calculated using the following equation: [FPG (μmol/ml) × immunoreactive insulin (IRI) (μU/ml)]/22.5. A HOMA-IR > 1.73 was considered indicative of the presence of IR, which was estimated using the *M*-value from the euglycaemic–hyperinsulinaemic clamp method in the Japanese population (24). HOMA-β, a parameter reflecting the insulin secretion ability of pancreatic β-cells, was calculated as follows: [360 × IRI (μU/ml)]/[FPG (μmol/ml)/0.0555–63]. Serum adiponectin was measured by means of an enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical Co. Ltd, Tokyo, Japan). Serum hyaluronic acid and high-sensitivity C-reactive protein (hsCRP) were examined by latex agglutination-turbidimetric immunoassay (Fujirebio Inc., Tokyo, Japan) and latex nephelometry (Dede Behring, Deerfield, IL, USA) respectively. The amount of HCV core antigen in serum was determined by a chemiluminescence enzyme immunoassay method (Eiken Chemical Co. Ltd, Tokyo, Japan). Serum aspartate aminotransferase-to-platelet count ratio index (APRI), a well-known indicator of hepatic fibrosis (25, 26), was calculated as follows: aspartate aminotransferase AST (U/L)/33 (upper limit of normal range of AST)/platelet count ($\times 10^3$/μl) × 100.

Imaging examination

Each patient underwent abdominal US (Hitachi model EUB-525 equipped with a 3.5 MHz convex-type transducer; Hitachi, Tokyo, Japan) in a fasting state. The presence of hepatic steatosis was assessed according to findings such as hepatorenal contrast, blurring of vascular walls and profound attenuation of the diaphragm (5, 27). The presence of advanced fibrosis or cirrhosis was evaluated by the presence of splenomegaly, hypertrophy of left or caudal lobes and surface irregularity (28). Images were evaluated and judged by an independent ultrasonographer uninformed about the clinical data of the patients.

Ethics

This study was carried out in accordance with the World Medical Association Helsinki Declaration and was approved by the hospital's human ethics committee.

Statistical analysis

Results are expressed as mean ± SD or median and range (in parenthesis). Statistical analyses were performed using SPSS software 11.5J for Windows (SPSS Inc., Chicago, IL, USA). Comparisons between the two groups were made using Fisher's exact probability test for categorical variables and the Student's *t* test for continuous variables. All *P*-values were based on a two-sided test of statistical significance. Correlation coefficients were calculated using Spearman's rank correlation analysis. A *P*-value of < 0.05 was considered statistically significant.

Results

Clinical features and biochemical parameters of hepatitis virus carriers with persistently normal serum alanine aminotransferase

The clinical features of both groups are shown in Table 1. The prevalence of hypertension and hyperlipidaemia was similar between the groups, and no participant had a family history of diabetes or fulfilled the Japanese criteria for metabolic syndrome. There were no significant differences in age, gender, BMI, waist-to-hip ratio, platelet count, hsCRP, serum AST or ALT, γ-glutamyltransferase (γ-GT), hyaluronic acid or APRI. Although it has been reported that serum cholesterol and ferritin are elevated in patients with chronic hepatitis C compared with those with chronic hepatitis B (29, 30), these factors were similar between

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Table 1. Clinical features and biochemical parameters of the hepatitis virus carriers with persistently normal serum alanine aminotransferase

	HBV+	HCV+	*P*
n	30	30	
Age (years)	50 ± 9	53 ± 12	0.42
Gender (male/female)	7/23	8/22	1.00
Hypertension (*n*)	1 (3%)	1 (3%)	1.00
Hyperlipidaemia (*n*)	0 (0%)	1 (3%)	0.50
Metabolic syndrome (*n*)	0 (0%)	0 (0%)	–
BMI (kg/m^2)	21.2 ± 2.2	20.9 ± 2.6	0.71
Waist circumference (cm)	71.7 ± 8.8	71.8 ± 9.0	0.97
Hip circumference (cm)	90.0 ± 5.0	90.0 ± 6.3	0.99
Waist-to-hip ratio	0.80 ± 0.09	0.80 ± 0.08	0.99
Platelet (× 10^3/μl)	210 ± 34	223 ± 50	0.33
hsCRP (mg/dl)	0.036 ± 0.027	0.027 ± 0.023	0.26
Albumin (g/dl)	4.5 ± 0.2	4.4 ± 0.3	0.68
AST (U/L)	20 ± 3	22 ± 6	0.17
ALT (U/L)	17 ± 4	19 ± 6	0.14
γ-GT (U/L)	19 ± 11	24 ± 22	0.37
Choline esterase (U/L)	315 ± 106	282 ± 55	0.25
Total cholesterol (mg/dl)	202 ± 27	194 ± 40	0.46
TG (mg/dl)	71 ± 17	74 ± 36	0.77
HDL cholesterol (mg/dl)	69 ± 16	67 ± 15	0.64
Iron (μg/dl)	100 ± 38	103 ± 43	0.83
Transferrin saturation (%)	32 ± 13	31 ± 14	0.79
Ferritin (ng/ml)	84 ± 74	55 ± 50	0.13
Hyaluronic acid (ng/ml)	24 ± 14	25 ± 14	0.77
APRI	0.31 ± 0.07	0.33 ± 0.13	0.48
HCV core antigen (fmol/L)		10550 (95–25800)	

Qualitative data are expressed as a percentage and quantitative data are expressed as mean ± SD or median (range). The *P*-value for qualitative and quantitative data was calculated using Fisher's exact probability test and Student's *t* test respectively.

ALT, alanine aminotransferase; APRI, AST-to-platelet ratio index; AST, aspartate aminotransferase; BMI, body mass index; γ-GT, γ-glutamyltransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; hsCRP, high-sensitivity C-reactive protein; HDL, high density lipoprotein; SD, standard deviation; TG, triglyceride.

the groups. HCV genotype was 1b in all patients, and overall HCV core antigen concentrations were variable.

Comparison of insulin resistance/secretion-related parameters between hepatitis C virus and hepatitis B virus carriers with persistently normal serum alanine aminotransferase

Next, serum insulin and adiponectin, as well as the parameters related to IR (HOMA-IR) and secretion (HOMA-β), were compared between HCV and HBV carriers. We found no significant differences in FPG (5.27 ± 0.72 vs. 5.33 ± 0.78 μmol/ml, $P = 0.83$), serum IRI (5.1 ± 2.8 vs. 7.9 ± 6.6 μU/ml, $P = 0.11$) or adiponectin (16.5 ± 6.3 vs. 13.9 ± 5.9 μg/ml, $P = 0.21$) (Table 2). HOMA-IR in HCV carriers was

Table 2. Comparison of parameters associated with insulin resistance/secretion between hepatitis B virus and hepatitis C virus carriers with persistently normal serum alanine aminotransferase

	HBV+	HCV+	*P*
n	30	30	
FPG (μmol/ml)	5.33 ± 0.78	5.27 ± 0.72	0.83
Glycohaemoglobin (%)	5.3 ± 0.3	5.2 ± 0.4	0.21
IRI (μU/ml)	7.9 ± 6.6	5.1 ± 2.8	0.11
HOMA-IR >1.73 (*n*)	7 (23%)	4 (13%)	0.51
HOMA-IR	1.8 ± 1.3	1.2 ± 0.8	0.12
HOMA-β	123 ± 84	59 ± 31	0.18
Adiponectin (μg/ml)	13.9 ± 5.9	16.5 ± 6.3	0.21

Qualitative data are expressed as a percentage and quantitative data are expressed as mean ± SD. The *P*-value for qualitative and quantitative data was calculated using Fisher's exact probability test and Student's *t* test respectively. HOMA-IR and HOMA-β were calculated according to the formulas described in 'Patients and methods'. A HOMA-IR of > 1.73 was judged positive for the presence of insulin resistance.

FPG, fasting plasma glucose; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA, homoeostasis model assessment; HOMA-IR, homoeostasis model assessment of insulin resistance; IRI, immunoreactive insulin; SD, standard deviation.

similar to that in HBV carriers (1.2 ± 0.8 vs. 1.8 ± 1.3, $P = 0.12$), and HOMA-β did not differ between the two groups (59 ± 31 vs. 123 ± 84, $P = 0.18$) as well. These results demonstrate that HCV-specific IR does not occur in non-obese, non-steatotic HCV carriers with PNALT.

Multivariate analysis

Multivariate analysis was performed to investigate the contribution of HCV infection to IR. There were no independent predictors of IR, including the presence of HCV.

Correlation between clinical parameters and insulin resistance/secretion markers in hepatitis C virus carriers with persistently normal serum alanine aminotransferase

To explore the clinical indicators associated with IR/secretion in the HCV carriers with PNALT, correlations between several clinical parameters with HOMA-IR, serum IRI/adiponectin and HOMA-β were analysed. HOMA-IR was strongly correlated with waist circumference ($r = 0.580$, $P = 0.006$), serum γ-GT ($r = 0.554$, $P = 0.004$) and TG ($r = 0.529$, $P = 0.007$) (Table 3). HOMA-IR was also associated with hip circumference ($r = 0.496$, $P = 0.022$), but this was weaker than that of the aforementioned indicators. Interestingly, HOMA-IR did not correlate with serum adiponectin ($r = -0.303$, $P = 0.170$), which was inversely correlated with waist

Table 3. Correlations between immunoreactive insulin/homoeostasis model assessment of insulin resistance/adiponectin/homoeostasis model assessment-β and clinical parameters in hepatitis C virus carriers with persistently normal serum alanine aminotransferase

	IRI	HOMA-IR	Adiponectin	HOMA-β
Waist circumference	0.600**	0.580**	-0.567**	NS
Hip circumference	0.525*	0.496*	NS	0.452*
Waist-to-hip ratio	NS	NS	-0.700**	NS
γ-GT	0.546**	0.554**	-0.631**	NS
TG	0.508**	0.529**	NS	0.410*
HDL-cholesterol	NS	NS	0.473*	NS

Correlation coefficients were calculated by Spearman's rank correlation analysis.

*$P < 0.05$.

**$P < 0.01$.

γ-GT, γ-glutamyltransferase; HDL, high density lipoprotein; HOMA, homoeostasis model assessment; HOMA-IR, homoeostasis model assessment of insulin resistance; IRI, immunoreactive insulin; NS, not significant; TG, triglyceride.

circumference ($r = -0.567$, $P = 0.007$), waist-to-hip ratio ($r = -0.700$, $P < 0.001$) and γ-GT ($r = -0.631$, $P = 0.002$), and positively correlated with high-density lipoprotein–cholesterol ($r = 0.473$, $P = 0.026$). On the other hand, there were no significant correlations between HOMA-IR or serum adiponectin with serum AST, ALT, hsCRP, platelet count, hyaluronic acid, APRI, HCV core antigen, ferritin or transferrin saturation ratio. The HOMA-β was weakly associated with hip circumference ($r = 0.452$, $P = 0.040$) and serum TG ($r = 0.410$, $P = 0.042$). No similar correlations were found in asymptomatic HBV carriers, demonstrating the existence of a positive and strong association between HOMA-IR/serum adiponectin, waist circumference and serum γ-GT and TG in chronically HCV-infected patients.

Discussion

In the current study, there were no significant differences in HOMA-IR and serum adiponectin between non-obese, non-diabetic, non-alcoholic and non-steatotic HCV and HBV carriers with PNALT. According to multivariate analysis, the presence of HCV was not an independent predictor of IR. HOMA-IR in the HCV carriers was strongly associated with waist circumference and serum γ-GT and TG, but not with the indicators of obesity (BMI), hepatocyte injury (serum AST or ALT), hepatic fibrosis (platelet count, serum hyaluronic acid or APRI), iron accumulation (serum ferritin or transferrin saturation ratio), systemic inflammation (serum hsCRP) or amount of HCV core protein. These results support the premise that the presence of HCV alone cannot induce IR. To our knowledge, this is the first study to evaluate HOMA-IR and serum adiponectin in non-obese, non-diabetic HCV carriers with PNALT and compare them with HBV carriers.

We defined the presence of IR as a HOMA-IR of >1.73. Although considerably lower than that in Hispanic and Caucasian populations, this cutoff value is consistent with the results of a previous study using young, lean, healthy individuals (31) that showed a significantly lower insulin sensitivity index in Asian groups compared with other ethnic groups.

Several lines of evidence have shown that the presence of obesity, alcohol consumption and hepatic steatosis all contribute to the onset of IR (5, 6, 17, 18, 21). To exclude these factors as much as possible, we first selected non-obese hepatitis virus carriers devoid of a history of habitual alcohol intake and hepatic steatosis and closely matched by BMI and waist-to-hip ratio. Our results revealed that waist circumference, an important anthropometric predictor of visceral fat accumulation (32), was significantly correlated with HOMA-IR and inversely associated with serum adiponectin in HCV carriers. Surprisingly, BMI was not found to strongly affect HOMA-IR in our cohort. Several studies have documented a close relationship between visceral fat accumulation and IR in healthy volunteers (33, 34), and the results of this study support such a strong contribution to IR development in chronically HCV-infected patients as well.

We judged the presence of hepatic steatosis using abdominal US. Although US is a safe, non-invasive and accurate method of detecting moderate-to-severe steatosis, its diagnostic accuracy declines sharply in cases of mild steatosis (liver fat <25%). Indeed, clear differentiation between non-alcoholic steatohepatitis (NASH) with mild steatosis or cryptogenic chronic hepatitis is sometimes difficult by such imaging modalities. We previously reported that in Japanese patients with persistent ALT elevation, despite no detection of steatosis by US, obesity, hyperferritinaemia and high HOMA-IR are predictors of NASH with mild steatosis (5). The close relationship between IR and hepatic steatosis has also been documented elsewhere in the Asian population (31). In this study, patients with a BMI of more than 25 kg/m^2 were excluded, and most patients demonstrated normal serum ferritin and HOMA-IR. Thus, the possibility that patients with mild steatosis were included is presumably low.

Because advanced fibrosis may also lead to hyperinsulinaemia probably because of decreased insulin clearance capacity (11, 12), we also limited our patients to those having no or mild fibrosis, as estimated

by platelet count, serum hyaluronic acid and ultrasonographical findings; it is known that chronic hepatitis C patients presenting with serum hyaluronic acid < 50 ng/ml correspond to the absence of severe fibrosis (35). Moreover, more than 90% of HCV carriers with PNALT presenting with platelet count more than $150 \times 10^3/\mu l$ are reported to have normal or mild liver histologies (20). Although percutaneous liver biopsies could not be performed in our patients, selection according to the above strict criteria enabled us to confidently exclude the possibility of IR caused by advanced fibrosis. Low APRIs in both groups also confirm the relevance of our selection criteria.

Under these conditions, we were able to discover that serum γ-GT was closely associated with HOMA-IR in non-obese, non-diabetic, non-alcoholic and non-steatotic HCV carriers with PNALT. These results are consistent with those of previous studies in that serum γ-GT is an important risk indicator for developing metabolic syndrome and type-2 diabetes (36, 37). A positive association between serum γ-GT and hepatic TNF-α expression has been documented in patients with chronic HCV infection (38). Because an activated TNF-α system is one of the major causes of IR development (15), the close relationship seen between serum γ-GT and HOMA-IR may partially reflect the local enhancement of TNF-α expression in HCV-infected livers. Although serum γ-GT is a non-specific marker of liver injury, it may also become a useful predictor of IR in HCV-infected patients.

Interestingly, serum HCV core protein concentration was not associated with HOMA-IR, which was inconsistent with a previous report demonstrating an association between HOMA-IR and the amount of serum HCV core antigen (14). Although the direct contribution of core protein to the pathogenesis of HCV-induced IR has been shown in transgenic mouse lines (14, 15), recent studies have uncovered that the occurrence of core protein-induced IR is not derived from the protein's intrinsic effect. For example, in mice constitutively expressing HCV core protein, deletion of the proteasome activator PA28γ gene did not induce hyperinsulinaemia or IR, despite the presence of core protein (39). Moreover, we obtained similar results from peroxisome proliferator-activated receptor α-null mice bearing the core protein gene (40). Thus, the results in this study support the notion that the core protein itself does not have the potential to induce IR.

In humans, the relationship between serum adiponectin and presence of HCV is a matter of controversy. Several studies have reported that low adiponectin is significantly associated with high HOMA-IR in patients with chronic hepatitis C (41, 42). On the other hand, a recent large-scale study has clearly shown that chronic HCV infection has little influence on serum adiponectin (43). Here, we also demonstrated no correlation between HOMA-IR and serum adiponectin in non-steatotic HCV-infected patients with PNALT, as well as no differences in adiponectin levels compared with matched HBV carriers. Our results lead us to conclude that the probability of HCV itself modulating adiponectin expression is low.

Also, in this study, serum hsCRP, a surrogate marker of subclinical systemic inflammation, did not differ between HCV and HBV carriers. It has been documented by a case–control study that serum levels of pro-inflammatory cytokines inducing IR, such as TNF-α and interleukin-6, were higher in patients with chronic hepatitis C than in those with other causes of hepatitis, despite similar levels of hepatitis activity (19). Thus, activation of TNF-α-mediated pathway may contribute to HCV-specific IR.

The present study suggests the contribution of the hepatic inflammatory component to the development of IR in chronic hepatitis C. However, our results do not necessarily mean that IR frequently found in chronic hepatitis C patients is mediated by hepatitis alone. HOMA-IR was reported to be higher in patients with chronic hepatitis C than in those with other causes of hepatitis, independent of severity of hepatic inflammation and fibrosis (2, 19), indicating the diabetogenic potential of HCV. HCV might lead to a latent disturbance of the insulin signalling cascade, which cannot be detected by a simple indicator (i.e. HOMA-IR), and trigger IR in cooperation with other factors such as hepatitis.

Clinically, it is well known that elevated HOMA-IR is one of the primary predictors of hyporesponsiveness or failure of interferon therapy in persistently HCV-infected patients (44, 45). The demonstration of a strong relationship between HOMA-IR and waist circumference, serum γ-GT and TG in HCV-infected humans without obesity, diabetes, hepatocyte damage, hepatitis or obvious steatosis indicates that simple nutritional intervention and exercise to reduce visceral fat mass can further ameliorate the outcome of antiviral therapy. In addition, the combination therapy of insulin-sensitizing TG-lowering agents and interferon injections might prove beneficial. In fact, additional treatment with bezafibrate, a typical TG-lowering agent (46), was reported to achieve a higher complete response rate with interferon and ribavirin combination therapy (47). Therefore, accurate evaluation of metabolic disturbances, such as visceral fat accumulation and high levels of serum TG and HOMA-IR, and the ensuing steps taken to regulate

them, round out a list of therapeutic strategies for HCV-infected patients.

There are some limitations in the present study. Firstly, the sample size is limited. Large-scale case–control studies using the same selection criteria will be able to further ascertain the association between HCV infection and development of IR. Secondly, the patients were selected from a homogenous race (i.e. Japanese), and the pathogenesis of HCV-specific IR might differ between races. Finally, we were not able to access the changes in IR with ageing, necessitating further long-term follow-up of our patients to address the issue.

In conclusion, the results of this study demonstrate that the presence of HCV *per se* cannot induce IR; rather, it may be other factors, such as the presence of active hepatitis, hepatic steatosis or fibrosis, that are important to HCV-specific IR. In addition, waist circumference and serum γ-GT and TG were strongly associated with HOMA-IR in non-obese, non-alcoholic and non-steatotic HCV carriers with PNALT, suggesting the likelihood that these parameters are useful and reliable indicators of IR in HCV-infected patients. Although our data offer novel information about the pathogenesis of HCV-specific IR, further large-scale studies are needed to confirm our results.

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Conflicts of interests: none.

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Susceptibility of Chimeric Mice with Livers Repopulated by Serially Subcultured Human Hepatocytes to Hepatitis B Virus

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We previously identified a small population of replicative hepatocytes in long-term cultures of human adult parenchymal hepatocytes (PHs) at a frequency of 0.01%-0.09%. These hepatocytes were able to grow continuously through serial subcultures as colony-forming parenchymal hepatocytes (CFPHs). In the present study, we generated gene expression profiles for cultured CFPHs and found that they expressed cytokeratin 19, CD90 (Thy-1), and CD44, but not mature hepatocyte markers such as tryptophan-2,3-dioxygenase (TO) and glucose-6-phosphatase (G6P), confirming that these cells are hepatic progenitor-like cells. The cultured CFPHs were resistant to infection with human hepatitis B virus (HBV). To examine the growth and differentiation capacity of the cells *in vivo*, serially subcultured CFPHs were transplanted into the progeny of a cross between albumin promoter/enhancer-driven urokinase plasminogen activator-transgenic mice and severe combined immunodeficient (SCID) mice. The cells were engrafted into the liver and were able to grow for at least 10 weeks, ultimately reaching a maximum occupancy rate of 27%. The CFPHs in the host liver expressed differentiation markers such as TO, G6P, and cytochrome P450 subtypes and could be infected with HBV. CFPH-chimeric mice with a relatively high replacement rate exhibited viremia and had high serum levels of hepatitis B surface antigen. *Conclusion:* Serially subcultured human hepatic progenitor-like cells from postnatal livers successfully repopulated injured livers and exhibited several phenotypes of mature hepatocytes, including susceptibility to HBV. *In vitro*–expanded CFPHs can be used to characterize the differentiation state of human hepatic progenitor-like cells. (HEPATOLOGY 2008;47:435-446.)

Abbreviations: 9MM, 9-month-old Caucasian male; 10YF, 10-year-old Caucasian female; 12YM, 12-year-old Asian male; 16YF, 16-year-old Asian female; AAT, α1-antitrypsin; AFP, α-fetoprotein; ALB, albumin; BGP, biliary glycoprotein; BrdU, 5-bromo-2'-deoxyuridine; CFPH, colony-forming parenchymal hepatocyte; CK, cytokeratin; G6P, glucose-6-phosphatase; h, human; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; CYP, cytochrome P450; m, mouse; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; PH, parenchymal hepatocyte; RI, replacement index; RT-PCR, reverse-transcription polymerase chain reaction; SH, small hepatocyte; TO, tryptophan-2,3-dioxygenase; uPA, urokinase plasminogen activator.

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Studies using rodents with damaged livers have shown that parenchymal hepatocytes (PHs) have great growth potential. When mouse (*m*) hepatocytes were transplanted into the livers of albumin promoter/enhancer-driven urokinase plasminogen activator (uPA)-transgenic mice,[1] they engrafted and repopulated the host liver. Serial transplantation experiments using *m*-hepatocytes in mice with tyrosinemia showed their enormous growth capacity.[2] The replicative potential of rat hepatocytes has also been demonstrated by transplanting them into the partially hepatectomized liver of a retrorsine-treated rat,[3] and uPA-transgenic mice crossed with severely immunodeficient mice, such as severe combined immunodeficient (SCID)/beige mice,[4] SCID mice,[5,6] or recombination activation gene 2 knockout mice[7] have been used to show the growth potential of human (*h*)-hepatocytes. When transplanted into uPA/SCID mice, PHs from a human juvenile male grew in the host liver to a level at which the proportion (replacement index) of the area of repopulated *h*-hepatocytes to the total number (host and donor) of hepatocytes reached 96% at 64 days posttransplantation.[5] Such *h*-hepatocyte–chimeric mice have been used to study the pharmacological responses of *h*-hepatocytes[5] and to investigate *h*-hepatitis viral infections.[4,6-8]

In contrast, normal hepatocytes have limited replicative capacity *in vitro* and acquire an abnormal phenotype if they are cultured for extended periods.[9,10] Studies on hepatocytes cultured in a newly devised medium (hepatocyte clonal growth medium[11,12]) revealed a subpopulation of highly replicative PHs, known as small hepatocytes (SHs), in both rats[12] and humans.[13] Their occupancy rate in *h*-liver ranged from 0.01% to 0.09% and was dependent on donor age.[13] The *h*-SHs formed colonies and grew continuously through several subcultures, which led us to name them colony-forming PHs (CFPHs).[13] Replication of the CFPHs was donor age–dependent up to passage 7 ($p = 7$),[13] and the cells did not exhibit a normal hepatocytic phenotype. Instead, they exhibited the traits of hepatocytes or biliary cells depending on the culture conditions. In addition, the CFPHs were not susceptible to infection with hepatitis B virus (HBV) (unpublished data).

In this study, we generated gene expression profiles of CFPHs and transplanted serially subcultured CFPHs into homozygous uPA/SCID mice to examine their growth and differentiation capacity. Our results indicate that the cells were engrafted onto the liver parenchyma and repopulated the tissue, ultimately differentiating into mature hepatocytes. Importantly, the *in vitro*–propagated CFPHs became susceptible to infection with HBV. This study supports our previous suggestion that CFPHs from *h*-postnatal liver are hepatic progenitor-like cells with the potential to assume a normal hepatocytic phenotype.[13]

Materials and Methods

h-*Hepatocytes.* This study was performed with the approval of the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board. PHs were isolated as described[13,14] from livers donated by a 12-year-old Asian male (12YM) and a 16-year-old Asian female (16YF) according to the guidelines of the 1975 Declaration of Helsinki. Cryopreserved PHs from a 9-month-old Caucasian male (9MM) and a 10-year-old Caucasian female (10YF) were obtained from In Vitro Technologies (Baltimore, MD) and BD Biosciences (San Jose, CA), respectively.

Culture of CFPHs. Cryopreserved PHs from the 9MM, 12YM, and 16YF were thawed[5] and serially subcultured to obtain *in vitro*–expanded CFPHs.[13] Commercial 9MM PHs and freshly isolated 12YM and 16YF PHs were each subcultured to $p = 3$. The expanded cells were then cryopreserved, thawed upon use, and cultured on collagen-coated plates for 14-20 days as described.[13]

Flow Cytometry. We detached 12YM CFPHs ($p = 4$ or 5) from culture plates by treatment with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA), suspended, incubated on ice for 30 minutes with *m*-monoclonal antibodies against *h*Thy-1 (clone F15-42-1; Chemicon, Temecula, CA), and incubated with antibodies against *m*-immunoglobulin G Alexa-488 (Molecular Probes, Eugene, OR). We used *m*-immunoglobulin G_1 as a negative control. The cells were then analyzed and separated using a fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, NJ) as reported.[12]

Transplantation of PHs and CFPHs. We detached 9MM and 12YM CFPHs ($p = 4$) from their culture plates and treated for 1 hour with DMEM containing 10% fetal bovine serum and 3 μg/mL anti-*h*-integrin $\alpha 1$ monoclonal antibodies (clone FB12, Chemicon).[15] This procedure improved engraftment of the CFPHs in uPA/SCID *m*-liver and reduced host mortality.

Transplantation of PHs and CFPHs was performed as described previously.[5] Homozygous uPA/SCID mice were injected with 0.75×10^6 9MM and 12YM PHs or $0.75\text{-}1.0 \times 10^6$ *in vitro*–expanded 9MM and 12YM CFPHs into the inferior splenic pole. When necessary, 10 mM 5-bromo-2′-deoxyuridine (BrdU) (Sigma, St. Louis, MO) and 1.2 mM 5-fluoro-2′-deoxyuridine (Wako, Osaka, Japan) in saline were injected intraperitoneally into the mice at 10 μL/g body weight 1 hour prior to death. The animals were treated according to the guidelines of our local committee on animal experiments.

Table 1. Summary of CFPH and PH Transplantation Experiments in uPA/SCID Mice

Group	Donor Cells	Time of Sacrifice (Weeks After Transplantation)	No. of Transplanted Mice	No. of Mice with Engraftment* [RE (%)]	RI† [Mean ± SD (n)]
A	12YM CFPHs (p = 4)	3	9	3 (33)	0.06-0.19% [0.14 ± 0.07% (n = 3)]
B	9MM CFPHs (p = 4)	3	6	4 (67)	0.03-0.05% [0.04 ± 0.01% (n = 4)]
C	9MM PHs	3	3	3 (100)	5.1-19.4% [6.4 ± 2.9% (n = 3)]
D	12YM CFPHs (p = 4)	9-10	27	14 (52)	0.2-27.0% [6.6 ± 8.3% (n = 14)]
E	9MM PHs	10-11	23‡	23 (100)‡	32.6-82.2% [57.4% (n = 2)]
F	12YM PHs	10	6	4 (67)	31.0-77.0% [62.3 ± 23.8% (n = 4)]
G§	12YM CFPHs (p = 4)	17-20	4	ND	ND

Abbreviation: ND, not determined.

*Number of mice whose livers were engrafted with transplanted PHs or CFPHs. The RE was determined via *h*ALB immunohistochemistry on sections prepared from 5 lobes of a liver.

†Ranges of RI of chimeric mice used in each group.

‡Data from Tateno et al.[5]

§Mice from group G were used for HBV infection studies.

We transplanted 9MM and 12YM CFPHs into 6 and 40 uPA/SCID mice, respectively. The mice were then killed 3, 9, or 10 weeks later, depending on the experimental purpose. In a previous report, we used 9MM and 12YM PHs as donor cells.[5] In this study, we used some of the preserved livers from these mice for histological examinations and as sources of RNA for reverse-transcription polymerase chain reaction (RT-PCR) analysis. The mice used in our transplantation experiments were separated into 7 groups (A-G) as shown in Table 1, which includes the rates of engraftment and replacement indices (RIs) of the chimeric mice.

Blood samples (5 μL) were collected periodically after transplantation from the tail veins of the hosts, and the level of *h*-albumin (ALB) in each was determined using a Human Albumin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX) to monitor the growth of the transplanted CFPHs.

RT-PCR. An RNeasy Tissue Kit (Qiagen, Valencia, CA) was used to isolate total RNA from freeze-thawed 9MM and 10YF PHs, cells of the *h*-hepatoma cell line HepG2, and 12YM and 16YF CFPHs (p = 4). RNA was also isolated with Isogen (Nippon Gene, Tokyo, Japan) from the livers of homozygous uPA/SCID mice and mice chimeric for 12YM PHs or 12YM CFPHs. Each RNA sample was treated with deoxyribonuclease (Takara Bio, Kyoto, Japan) and used as the template for RT-PCR. The RNA (1 μg) was reverse-transcribed with random hexamers using PowerScript Reverse Transcriptase (Clontech, Kyoto, Japan). All reactions were performed with Ex Taq (Takara Bio). Semiquantitative PCR was performed to allow linear amplification of the targets. The following *h*-specific or *m* and *h* cross-reactive genes were subjected to RT-PCR under the conditions shown in Supplementary Table 1: ALB, α1-antitrypsin (AAT), tryptophan-2,3-dioxygenase (TO), glucose-6-phosphatase (G6P), α-fetoprotein (AFP), cytokeratin 19 (CK19), biliary glycoprotein (BGP), Thy-1, CD44, multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), MRP2, and glyceraldehyde-3-phosphate dehydrogenase.

In Situ *Hybridization.* Cryosections (7 μm thick) were fixed with 4% paraformaldehyde, then incubated with 100 ng/mL proteinase K for 10 minutes at 37°C. The sections were then treated at 90°C for 6 minutes and hybridized for 2 hours at 37°C with biotinylated h-DNA probes (Dako, Glostrup, Denmark). The sections were also used to detect whole *h*-genomic DNA using the GenPoint System (Dako) according to the manufacturer's instructions. Finally, they were stained with hematoxylin-eosin.

Immunohistochemistry and Histochemistry. Formalin-fixed livers were embedded in paraffin and sectioned 5 μm thick. The sections were heated in a microwave oven for 5 minutes in Target Retrieval Solution (Dako), then placed at room temperature for 20 minutes. The livers used to generate frozen sections were embedded in OCT compound (Sakura Finechemicals, Tokyo, Japan), frozen in liquid nitrogen, and sectioned 5 μm thick. The cultured cells were fixed in cold ethanol for 10 minutes. The primary antibodies and conditions used for immunohistochemistry are listed in Supplementary Table 2. For bright-field immunohistochemistry, the antibodies were visualized using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) using DAB substrates. Fluorescense immunohistochemistry was performed using Alexa 488–conjugated or Alexa 594–conjugated secondary antibodies (Molecular Probes). The nuclei were stained with Hoechst 33258. Glycogens were visualized using a periodic acid-Schiff (PAS) staining kit (Muto Pure Chemicals, Tokyo, Japan). RIs were determined using

*h*ALB-immunostained sections of chimeric *m*-livers as reported previously.[5]

HBV Infection. We obtained *h*-serum containing high-titer HBV DNA (8.1 $\log_{10}$ genome equivalents/mL serum) from an HBV genotype C carrier after obtaining informed consent. The serum was kept at −80°C until use. Four CFPH-chimeric mice were intravenously injected with 100 μL of the HBV-positive serum 9-12 weeks after transplantation.

HBV Marker Analysis. Hepatitis B surface antigen (HBsAg) was measured using an Architect Analyzer (Abbott, Osaka, Japan). Serum DNA was extracted using a SMITEST EX-R&D Nucleic Acid Extraction Kit (Genome Science Laboratories, Fukushima, Japan). Small amounts of HBV DNA (<300 copies/mL) were detected via nested PCR.[8] If HBV DNA was detected during the initial round of PCR, the copy number was determined via real-time PCR as reported.[8]

Results

***Phenotypes of CFPHs* In Vitro.** We seeded 9MM and 12YM PHs on culture dishes and confirmed that the CFPHs from the 2 donors were similar in morphology and replicative capacity. A small number of the CFPHs (0.01%-0.09% of the seeded PHs) began to replicate after 5 days, and the number of replicating cells gradually increased until colonies appeared at 17 days (Fig. 1A); after 21 days, the cells covered the surface of the dish (Fig. 1B). Most of the seeded PHs were not replicative, and they gradually flattened, acquiring a senescent morphology within 20 days of seeding (Fig. 1A). The CFPHs showed an epithelial cell–like morphology with scant cytoplasm (Fig. 1B), and they retained this appearance during subculture (Fig. 1C). The population doubling time (PDT) of the CFPHs gradually increased as the passage number increased. Up to p = 4, the CFPHs from the young donors replicated with a population doubling time of 170-220 hours; subsequently, the population doubling time increased until the cells finally became senescent.[13]

The expression of several marker genes was compared among PHs, HepG2 cells, and CFPHs (Fig. 1D). In our experience, no significant differences exist in the marker gene expression profiles of PHs among different donors, and the same trend applies to subcultured CFPHs.[13] At p = 4, the CFPHs expressed less ALB and AAT messenger RNA compared with the PHs. The PHs expressed TO and G6P, both of which are markers of mature hepatocytes, whereas the CFPHs did not. CK19, a hepatic progenitor/biliary cell marker, was expressed in both the CFPHs and HepG2 cells, but not in the PHs. BGP, a cell–cell adhesion molecule in epithelium, endothelium, and myeloid cells,[16] was expressed in the PHs and HepG2 cells, but only faintly in the CFPHs. The CFPHs, but not the PHs or HepG2 cells, expressed Thy-1, a hematopoietic/hepatic progenitor cell marker. AFP, a hepatic progenitor/carcinoma cell marker, was only detectable in HepG2 cells. CD44, an SH[17] or oval cell marker,[18] was strongly expressed in CFPHs, but only faintly in PHs and HepG2 cells. PHs and CFPHs faintly expressed MDR1. PHs expressed MRP2, but not MRP1. In contrast, CFPHs expressed MRP1, but not MRP2. A change from MRP2 to MRP1 expression during culture has been reported in rat hepatocytes.[19]

Thy-1 and CD44 expression in CFPHs was assessed via immunocytochemistry (Fig. 1E-F). A few CFPHs were positive for Thy-1 (Fig. 1E), whereas the majority was strongly positive for CD44 (Fig. 1F). Fluorescence-activated cell sorting indicated that a minor population of the CFPHs expressed Thy-1 (Fig. 1G-H), with an occupancy rate of 1%-3% (Fig. 1H). The CFPHs expressed CK7, CK8, CK18, and CK19 in the preconfluent state and became CK7- and CK19-negative in condensed regions postconfluence (data not shown), which is in agreement with our previous findings.[13] Other hepatic stem cell markers such as CD34 and c-kit were undetectable in our CFPHs (data not shown).

Repopulation of CFPHs in uPA/SCID Mouse Liver. We transplanted 12YM CFPHs (p = 4) into 27 homozygous uPA/SCID mice. The serum concentration of *h*ALB was monitored posttransplantation as a measure of the RI of CFPHs (Fig. 2A). Approximately half of the hosts had no or only a small increase in the level of *h*ALB throughout the experimental period. The remaining mice showed a continuous increase in the concentration of *h*ALB, which reached >10 μg/mL after 9 to 10 weeks. Animal 27 showed the greatest increase, reaching 0.7 mg/mL after 10 weeks. The RI of each of the 14 mice in which blood *h*ALB concentration was >8 μg/mL after 9 to 10 weeks was determined by dividing the *h*ALB-positive areas by the entire area measured,[5] and the data were plotted against the corresponding blood *h*ALB concentrations (Fig. 2B). RIs between 0.2% and 27.0% were well correlated with blood *h*ALB concentrations in the 9-728 μg/mL range.

Livers of mice engrafted with the CFPHs were subjected to immunohistochemical staining for *h*ALB (Fig. 3A-D,H) and *in situ* hybridization using *h*-genomic DNA probes (Fig. 3I). *h*ALB-positive cells were visible within 3 weeks posttransplantation as single cells or small clusters consisting of up to 25 cells (Fig. 3A-B). Larger clusters containing 20-450 *h*ALB-positive cells appeared after 9 to 10 weeks (Fig. 3C for animal 2 and Fig. 3D for animals 17 and 27). To detect replicating CFPHs, the mice were