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CLINICAL STUDIES

Diabetes pattern on the 75 g oral glucose tolerance test is a risk factor for hepatocellular carcinoma in patients with hepatitis C virus

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Keywords
diabetes mellitus – glucose tolerance test – hepatitis C virus – hepatocellular carcinoma – oxidative stress – tumor necrosis factor-alpha

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Abstract

Background: Patients with hepatitis C virus (HCV) frequently show glucose intolerance. Diabetes mellitus (DM) has been proposed to be a risk factor for hepatocellular carcinoma (HCC). *Aims:* The aim of this study is to clarify the influence of glucose intolerance as evaluated by the 75 g oral glucose tolerance test (OGTT) on hepatocarcinogenesis in patients with HCV. *Methods:* This study was carried out in a cohort of 197 patients with HCV who had not been previously diagnosed as having DM. All patients underwent the 75 g OGTT at entry. They were also screened for HCC and, thereafter, the rate of hepatocarcinogenesis was compared between the patients with and without glucose intolerance. *Results:* Based on the results of the 75 g OGTT, 125 (63%) had normal glucose tolerance (NGT), 49 (25%) had impaired glucose tolerance (IGT) and 23 (12%) had the DM pattern. HCC occurred more frequently in patients with the DM pattern than in patients with either NGT or IGT. Even in patients without advanced liver fibrosis, HCC was more frequently observed in patients with DM than in patients with NGT. A multiple logistic regression analysis showed advanced liver fibrosis, the DM pattern on the 75 g OGTT, an older age and γ-glutamyltransferase to all be independent risk factors related to hepatocarcinogenesis. *Conclusions:* A DM pattern on the 75 g OGTT was thus found to be associated with hepatocarcinogenesis and the 75 g OGTT is considered to be useful for identifying this risk factor for HCC in patients with HCV.

There is a high frequency of chronic hepatitis C (CH-C) worldwide and this condition progresses to cirrhosis and hepatocellular carcinoma (HCC) over a period of 20–30 years (1–3). Interferon (IFN) is often administered to treat CH-C and it can induce viral clearance and yield biochemical and histological improvement. Diabetes mellitus (DM) reduces the therapeutic effectiveness of IFN-α-2b plus ribavirin in patients with hepatitis C virus (HCV) (4). DM is often found in patients with HCV, especially in those with liver cirrhosis (5, 6). HCV infection itself may be associated with glucose intolerance. HCV core transgenic mouse demonstrates that glucose intolerance is usually due to insulin resistance induced by the HCV core protein (7). Tumour necrosis factor (TNF)-α has a crucial role in insulin resistance in this mouse model.

There are several risk factors for hepatocarcinogenesis associated with HCV, such as age, gender, total alcohol intake, cirrhosis, hepatic steatosis, HCV genotype and oxidative stress (8–12). DM is also a risk factor of HCC (13, 14). DM has a negative impact on patients with HCV because it reduces the efficacy of anti-HCV treatment and contributes to the development of HCC.

An accurate diagnosis of DM is sometimes difficult in patients with CH-C. A 75 g oral glucose tolerance test (OGTT) is frequently used to assess glucose tolerance. It identifies patients at the early stage of glucose intolerance, such as impaired glucose tolerance (IGT) and impaired fasting glucose (IFG). Previous reports of hepatocarcinogenesis in patients with HCV have not assessed the early stage of glucose intolerance by OGTT. Therefore, to clarify the association of DM with hepatocarcinogenesis, hepatocarcinogenesis was investigated in the patients with HCV evaluated with the 75 g OGTT. The results showed that DM or the early stage of glucose intolerance contributes to HCC in patients with HCV.

Patients and methods

Patients

This study retrospectively enrolled 197 patients with HCV who were admitted to the Ehime University Hospital from April 1992 to June 2006. Their glucose intolerance was evaluated with the 75 g OGTT and they underwent a liver biopsy. In this study, we excluded any patients with IFG because there were only two patients

with IFG during the study period. All patients were administered antiviral therapy by IFN during observations. They were positive for both anti-HCV antibody and serum HCV-RNA detected by the polymerase chain reaction (PCR). Hepatitis B virus (HBV) infection or autoimmune liver diseases were excluded. This study protocol was approved by the Institutional Review Board of Ehime University Hospital.

Evaluation of glucose intolerance

No patient had been treated by medical agents for diabetes before enrollment in this study. Their glucose intolerance was evaluated based on a 75 g OGTT according to the World Health Organization criteria: normal glucose tolerance (NGT), fasting plasma glucose (FPG) < 110 mg/dl and 2-h plasma glucose (PG) < 140 mg/dl; IGT, FPG < 110 mg/dl and 2-h PG between 140 and 200 mg/dl and DM, FPG ≥ 126 mg/dl or a 2-h PG level ≥ 200 mg/dl (15). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as reported previously (16).

Liver histopathological examination

Among the liver biopsy specimens obtained from all patients for histological examination, 72, 55, 53 and 17 were from patients with mild, moderate and severe fibrosis and liver cirrhosis respectively. In addition, 99, 95 and three were from patients with mild, moderate and severe histological activity respectively. The specimens were histologically classified according to the criteria of the International Hepatitis Group (17).

The degree of steatosis was determined based on the Brunt grading system (18) by the pathologist who reviewed the biopsy specimens without any information concerning the clinical characteristics. The steatosis grades were as follows: grade 0, no steatosis; grade 1, up to 33%; grade 2, up to 65% and grade 3, more than 66%.

Estimation of hepatitis B virus and hepatitis C virus markers and laboratory investigations

The presence of hepatitis B surface antigen and anti-HCV antibody was determined using an enzyme immunoassay kit (Dainabot, Tokyo, Japan; Kokusai-Shiyaku, Kobe, Japan). The HCV-RNA titres immediately before IFN therapy were determined using either an Amplicor-Monitor (Roche Diagnostics, Branchburg, NJ, USA) and expressed as kcopies/ml or kilo-international units/ml (KIU/ml) or the branched DNA probe assay (Chiron, Emeryville, CA, USA) and expressed as 10^6 genomic equivalents/ml (mEq/ml). The patients were separated according to a viral load of more (high viral load) or less (low viral load) than 8×10^6 mEq/ml by a branched DNA probe assay or 800 kcopies/ml (800 KIU/ml) by the Amplicor-Monitor assay. The serotype of HCV was determined using an enzyme immunoassay (Ohtsuka Laboratories Co. Ltd, Tokushima, Japan).

Treatment schedule of interferon therapy

All the 197 patients were treated with antiviral therapy at the Ehime University Hospital. Twenty-eight patients were treated with a natural IFN-β injection intravenously every day for 6–12 weeks at an average dose of 414 million international units (MIU). One hundred and fourteen patients were treated with IFN-α alone. IFN-α was administered intramuscularly every day for the first 14 days and then three times each week for 12–22 weeks, at an average dose of 653 MIU. Seven patients were treated with intravenous injections of IFN-β and IFN-α daily for 2–4 weeks and intramuscular injections of IFN-α three times each week for 16–22 weeks, at an average dose of 505 MIU. Twenty-six patients were treated with IFN-α-2b intramuscularly every day for the first 2 weeks and then three times a week for the following 22 weeks, at an average dose of 672 MIU. Ribavirin was combined with IFN-α-2b at a daily dose of 600 or 800 mg, depending on the body weight (< 60 or ≥ 60 kg respectively). Five patients were treated with pegylated IFN-α-2a once a week for 24–48 weeks. Seventeen patients were treated with pegylated IFN-α-2b once a week for 48 weeks in combination with ribavirin at a daily dose of 600 or 800 mg, depending on body weight (< 60 or 60–80 respectively).

Criteria for interferon effectiveness

All patients were followed-up for 24 weeks after antiviral therapy. HCV-RNA was assayed periodically during this period. Ninety-one patients (46%) were virologically sustained responders, who had no detectable HCV-RNA according to PCR assays during the follow-up period, and 106 (54%) were non-responders, who remained positive for HCV-RNA after antiviral therapy, irrespective of the HCV-RNA levels or a relapse during the follow-up period.

Diagnosis of hepatocellular carcinoma

All patients were examined for HCC by either abdominal ultrasonography or contrast-enhanced computed tomography every 3–6 months. If a mass lesion was detected, magnetic resonance imaging, abdominal angiography and a tumour biopsy guided by ultrasonography were performed to confirm the diagnosis of HCC. The mean follow-up period was 78 ± 45 months.

Measurement of protein carbonyls and tumour necrosis factor-α

In order to evaluate the levels of oxidative stress and TNF-α of the patients, separate serum aliquots were frozen at -20 °C and stored until the measurement. A patient was selected from each group according to the 75 g OGTT, whose sex and liver fibrosis stage were matched. Twenty-four, 23 and 24 patients from NGT, IGT and DM were eligible. The serum protein carbonyl levels were measured using a commercially available ELISA kit (BioCell

Corporation Limited, Auckland, New Zealand) involving a reaction of the protein with dinitrophenylhydrazine and detection with an antidinitrophenylhydrazine antibody according to the manufacturer's instructions. The total protein concentrations were measured for reference using the DC protein assay (Bio-Rad Laboratories, Richmond, CA, USA). The level of TNF-α was measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The detection limit was 0.5 pg/ml.

Statistical analysis

All data are expressed as the mean ± standard deviation. Student's *t*-test was used to evaluate the continuous variables. Multiple comparisons were analysed using ANOVA and Tukey's HSD test. The difference in proportions was evaluated using the χ^2 test or Fisher's exact test. The cumulative hepatocarcinogenesis was calculated using the Kaplan–Meier method and the differences between the survival curves were tested using the log-rank test. Variables were assessed to determine independent predictive factors that relate to hepatocarcinogenesis using the Cox regression analysis. At first, we selected variables with a *P*-value of < 0.15, which is known to be related with hepatocarcinogenesis. Next, we used those variables in a Cox regression analysis.

The model was simplified in a stepwise fashion by removing variables with $P > 0.05$. A value of $P < 0.05$ was considered to be significant. Calculations were performed using SPSS for Windows, Release 15.0J (SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics according to the results of the 75 g oral glucose tolerance test

The characteristics of patients classified as glucose intolerance are compared in Table 1. Based on the results of the 75 g OGTT, 125 (63%) were NGT, 49 (25%) were IGT and 23 (12%) were DM pattern. The DM pattern was seen in 4% of the patients with mild fibrosis (F1), 15% of the patients with moderate fibrosis (F2), 11% of the patients with severe fibrosis (F3) and 35% of the patients with liver cirrhosis (F4; Fig. 1). The rate of males was significantly higher in IGT than in NGT ($P < 0.05$). The patients with the DM pattern seemed to have more progressive liver fibrosis but there was no difference in the state of liver fibrosis (F1, F2 vs. F3, F4) between the patients with DM and NGT. The steatosis grade of liver biopsy specimens was not significantly different according to glucose intolerance. The patients with the DM pattern tended to consume more alcohol than the patients with NGT ($P < 0.05$). The body mass index (BMI), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly higher in the patients with DM than in those with NGT ($P < 0.05$, $P < 0.01$, $P < 0.01$ respectively). HOMA-IR was significantly higher in patients with DM than in those with NGT and IGT ($P < 0.05$ and $P < 0.01$ respectively). Furthermore, the average of HOMA-IR even in the patients with NGT was over 2, which is considered as indicative of insulin resistance. The platelet count and insulinogenic index (ΔIRI/ΔBS) were significantly lower in patients with DM than in those with NGT ($P < 0.05$ and $P < 0.01$ respectively) and ΔIRI/ΔBS were also significantly lower in the patients with IGT than in those with NGT ($P < 0.01$). Triglyceride was significantly higher in patients with DM and IGT than in those with NGT ($P < 0.05$ and $P < 0.05$ respectively). No difference was found in either the virological state or the response to IFN therapy among these groups.

Evaluation of protein carbonyl and tumour necrosis factor-α levels among the patients with or without glucose intolerance

Serum protein carbonyl levels were compared among the patients with NGT, IGT and DM (Fig. 2A). The levels of protein carbonyl in the patients with NGT, IGT and DM were 5.9 ± 2.1, 6.3 ± 1.9 and 6.3 ± 1.7 respectively. No significant differences were observed in the protein carbonyl levels. Furthermore, the protein carbonyl levels were evaluated between the patients with and without HCC (Fig. 2B). No significant difference was found between these two groups. The levels of TNF-α in the patients with NGT, IGT and DM were 36.0 ± 56.1, 42.1 ± 82.4 and 14.8 ± 22.3 respectively. The range of each value was wide and no significant differences of TNF-α were identified between the patients with and without HCC as well as in the patients with and without glucose intolerance.

Cumulative hepatocarcinogenesis rates according to the patterns of 75 g oral glucose tolerance test

During the follow-up period, 18 patients (9.1%) developed HCC. In the patients with NGT, IGT and DM pattern, the cumulative hepatocarcinogenesis rates were 7.1%, 0% and 10.3% at the end of 5 years and 9.2%, 8.4% and 60.7% at the end of 10 years respectively. Hepatocarcinogenesis was significantly higher in patients with the DM pattern than in patients with NGT and IGT ($P < 0.01$ and < 0.01 respectively; Fig. 3). Next, the cumulative hepatocarcinogenesis rates in the patients with mild liver fibrosis (F1, F2) were evaluated to determine the impact of glucose intolerance, especially in patients with an early stage of fibrosis. The cumulative hepatocarcinogenesis rates were significantly higher in patients with the DM pattern than in patients with NGT ($P < 0.01$; Fig. 4).

Multivariate analysis of factors associated with hepatocarcinogenesis

The data were analysed to determine the variables that independently influence hepatocarcinogenesis in patients with HCV. A univariate analysis showed eight

Table 1. Clinical and virological characteristics of the 197 patients with hepatitis C virus according to the 75 g oral glucose tolerance test

	NGT ($n=125$)	IGT ($n=49$)	DM ($n=23$)
Sex (M/F)†	70/55	38/11	18/5
Age (years)	51 ± 12	52 ± 11	55 ± 9
Body mass index (kg/m^2)*	23.5 ± 3.4	23.8 ± 2.7	25.6 ± 3.6
Alcohol consumption* (< 500 kg/≥ 500 kg)	111/14	37/12	15/8
Total protein (g/dl)	7.4 ± 0.6	7.5 ± 0.7	7.7 ± 0.6
Serum albumin (g/dl)	4.2 ± 0.4	4.2 ± 0.5	4.2 ± 0.4
AST (IU/L)**	64 ± 39	72 ± 45	94 ± 60
ALT (IU/L)**	96 ± 70	112 ± 76	149 ± 120
γ-GTP (IU/L)	66 ± 81	81 ± 62	95 ± 73
α-fetoprotein (ng/ml)	12.6 ± 23.6	14.0 ± 19.5	18.7 ± 18.9
Platelet count (× 10^4/μl)*	17.8 ± 6.5	16.8 ± 5.4	14.1 ± 6.4
Total cholesterol (mg/dl)	174 ± 32	173 ± 22	173 ± 39
Triglyceride (mg/dl)*,†	102 ± 47	140 ± 123	147 ± 89
IRI-AUC180	280.6 ± 138.4	309.1 ± 164.9	316.9 ± 151.5
HOMA-IR**,§	2.6 ± 2.2	2.7 ± 1.3	6.8 ± 12.3
ΔIRI/ΔBS**,‡	1.58 ± 1.09	1.00 ± 0.81	0.49 ± 0.40
HCV serotype			
1/2/1+2	79/42/4	32/15/2	13/10/0
HCV-RNA titre			
High/low	17/108	37/12	7/16
Histological fibrosis			
Mild	57	12	3
Moderate	31	16	8
Severe	30	17	6
Cirrhosis	7	4	6
Histological activity*			
Mild	66	25	8
Moderate	58	23	14
Severe	1	1	1
Steatosis grade			
Grade 1	82	30	13
Grade 2	31	13	8
Grade 3	10	5	2
Grade 4	2	1	0
Response to interferon (SVR/non-SVR)	61/64	18/31	12/11

Data are expressed as mean ± SD. The homeostasis model for assessment of insulin resistance (HOMA-IR) was evaluated only in patients with fasting plasma glucose < 140 mg/dl. The steatosis grade was determined by the Brunt grading system.

*$P < 0.05$ compared between DM and NGT.

**$P < 0.01$ compared between DM and NGT.

†$P < 0.05$ compared between NGT and IGT.

‡$P < 0.01$ compared between NGT and IGT.

§$P < 0.01$ compared between DM and IGT.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; ΔIRI/ΔBS, insulinogenic index; DM, diabetes mellitus; F, female; γ-GTP, γ-glutamyltransferase; HCV, hepatitis C virus; IGT, impaired glucose tolerance; IRI, immunoreactive insulin; M, male; NGT, normal glucose tolerance; SD, standard deviation; SVR, sustained viral response.

parameters that significantly influenced hepatocarcinogenesis: advanced liver fibrosis (F3, F4) ($P < 0.01$), DM pattern on 75 g OGTT ($P < 0.01$), an older age ($P < 0.01$), platelet counts ($P < 0.01$), AST ($P < 0.01$), ALT ($P = 0.013$), prothrombin time ($P = 0.010$) and the cumulative alcohol intake ($P = 0.043$) and a marginally significant association was observed in the patients without a sustained viral response by antiviral therapy ($P = 0.070$), serum albumin ($P = 0.091$), α-foetoprotein ($P = 0.109$) and γ-glutamyltransferase (GTP) ($P = 0.144$). Next, these results were analysed to identify the independent risk factors of hepatocarcinogenesis. A multiple logistic regression analysis showed advanced liver fibrosis (F3, F4), a DM pattern on the 75 g OGTT, an older age and γ-GTP to all be independent risk factors associated with hepatocarcinogenesis [odds ratio 8.135 (1.677–12.766), $P = 0.005$; 4.627 (1.677–12.766), $P = 0.003$; 1.094 (1.021–1.172), $P = 0.011$; 1.007 (1.002–1.011), $P = 0.002$ respectively; Table 2].

Discussion

The present study shows evidence that DM evaluated by the 75 g OGTT is a risk factor of hepatocarcinogenesis in

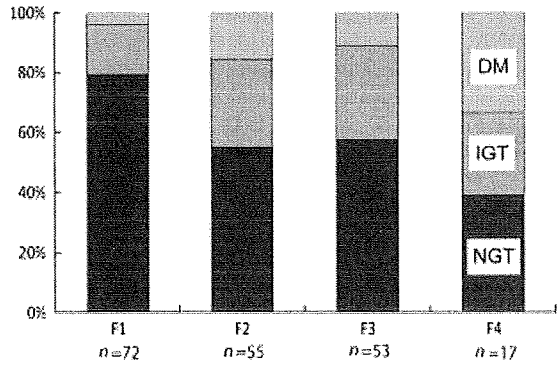

Fig. 1. Results of the 75 g oral glucose tolerance test according to the degree of liver fibrosis. DM, diabetes mellitus; IGT, impaired glucose tolerance; NGT, normal glucose tolerance.

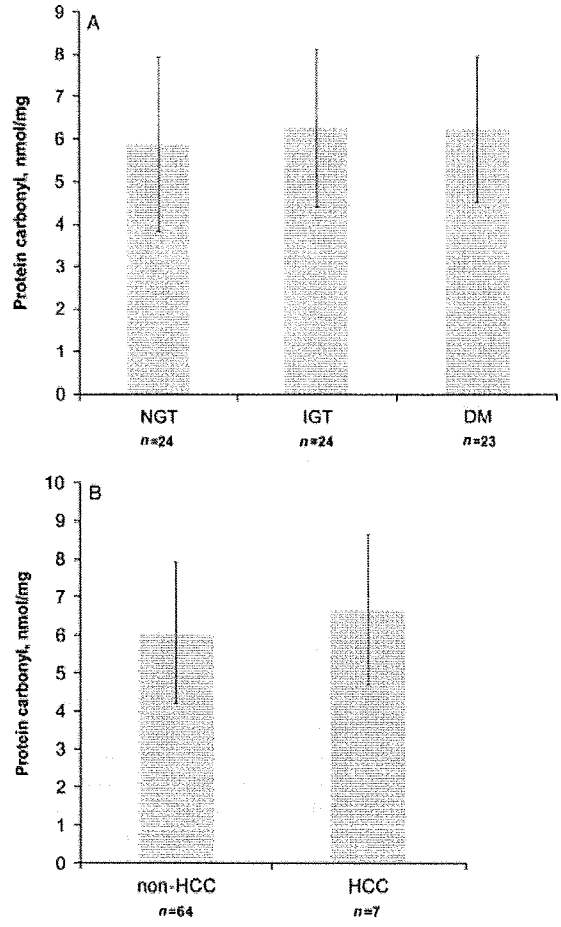

Fig. 2. The serum carbonyl levels were compared between patients (A) with or without glucose intolerance and (B) between patients with and without HCC. DM, diabetes mellitus; HCC, hepatocellular carcinoma; IGT, impaired glucose tolerance; NGT, normal glucose tolerance.

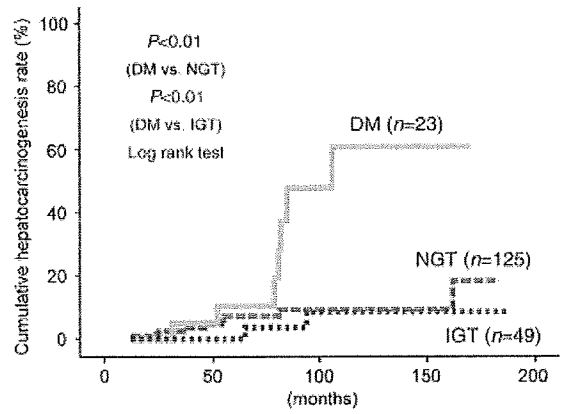

Fig. 3. Cumulative hepatocarcinogenesis rates based on the 75 g oral glucose tolerance test. The rate is significantly higher in patients with a DM pattern than in those with NGT and IGT. DM, diabetes mellitus; IGT, impaired glucose tolerance; NGT, normal glucose tolerance.

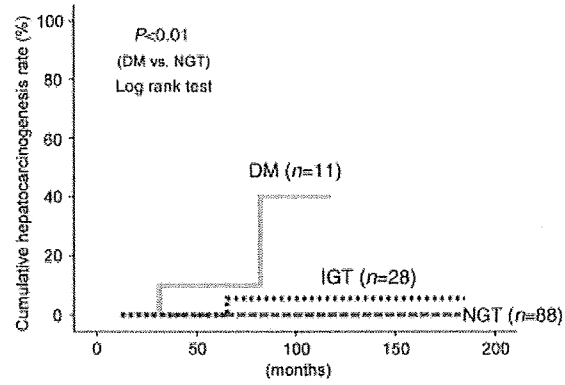

Fig. 4. The cumulative hepatocarcinogenesis rate based on the 75 g oral glucose tolerance test was significantly higher in patients with DM in comparison with those with NGT, even in those patients without advanced liver fibrosis. DM, diabetes mellitus; IGT, impaired glucose tolerance; NGT, normal glucose tolerance.

Table 2. Multiple logistic regression analysis of the variables on hepatocarcinogenesis in the patients with hepatitis C virus

Variable	Category	*P*-value	Hazard ratio (95% CI)*
Hepatic fibrosis stage	1: F1, F2 2: F3, F4	0.005	8.135 (1.870–35.394)
Result of 75 g OGTT	1: NGT, IGT 2: DM pattern	0.003	4.627 (1.677–12.766)
Age (years)		0.011	1.094 (1.021–1.172)
γ-GTP		0.002	1.007 (1.002–1.011)

*Values are the hazard ratio of hepatocarcinogenesis.
CI, confidence interval; DM, diabetes mellitus; γ-GTP, γ-glutamyltransferase; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test.

patients with HCV. Several reports have been published regarding the relationship between DM and hepatocarcinogenesis in patients with HCV. One described DM to be a risk factor of hepatocarcinogenesis in patients with HCV (19). Another showed DM to be a risk factor in patients with HCV who have advanced liver fibrosis (20). A study in Taiwan investigated the risk of HCC associated with HBV/HCV infections and metabolic status, such as obesity and DM (21). It showed that diabetes and obesity increased the risk of HCC, especially in patients with HCV. These reports addressed hepatocarcinogenesis in patients with HCV complicated with previously diagnosed and/or treated DM. The current study showed that patients with HCV with glucose intolerance based on the 75 g OGTT had a malignant potential even if they had not been diagnosed with DM.

The patients with IGT progressed to DM if their hepatitis continued and progressed to advanced liver fibrosis over several years and, therefore, IGT is considered to be a possible risk factor for carcinogenesis by identifying a condition that could progress to DM.

In patients without advanced liver fibrosis, hepatocarcinogenesis was found more frequently in patients with DM. Because there were few occurrences of HCC, the malignant potential of DM could not be defined in the patients without advanced liver fibrosis. However, these results indicate that a strict evaluation of glucose intolerance is needed in patients with HCV even if they do not progress to eventually develop advanced liver fibrosis.

Hepatocellular carcinoma can be prevented in many ways for patients with HCV. Especially, the eradication of HCV by anti-HCV therapy with IFN has a high impact in preventing the occurrence of HCC (22). Even in patients with HCV who failed to clear HCV-RNA, HCC was observed to be prevented (23). In our subjects, all patients were treated with IFN. However, the effect of antiviral therapy in regard to the occurrence of HCC was not marginally significant. In a multivariate analysis, the eradication of HCV did not significantly correlate with the occurrence of HCC in our current study.

Steatosis has been reported to be a risk factor of HCC for patients with HCV (24). We evaluated the degree of the hepatic steatosis and examined the association with the hepatocarcinogenesis. However, no relationship between steatosis and hepatocarcinogenesis was observed. Our patients mainly had the HCV genotype 1 or 2, but not genotype 3, and their BMI was generally not too high. Therefore, the steatosis grade was relatively low and it is therefore not considered to have affected hepatocarcinogenesis.

It is unclear how glucose intolerance influences hepatocarcinogenesis. Oxidative stress could be an important factor in hepatocarcinogenesis (25). Recently, protein carbonyl was proposed as a good marker of oxidative protein in blood samples (26). The serum protein carbonyl levels were measured in some patients at the time of enrolment. However, no differences were observed in the protein carbonyl levels among the patients with or without glucose intolerance. The accumulation of DNA damage due to oxidative stress should be important for hepatocarcinogenesis. However, no evidence of the mechanism by which glucose could contribute to hepatocarcinogenesis through oxidative stress was seen.

Hyperglycaemia increases the cancer risk (27) and postprandial hyperinsulinaemia is associated with accelerated HCC growth (28). Insulin may therefore be a key molecule associated with hepatocarcinogenesis. Insulin acts as a growth factor via the activation of mitogen-activated protein kinases (29, 30). Therefore, hyperinsulinaemia may be one reason that hepatocarcinogenesis is associated with hyperglycaemia. Furthermore, HOMA-IR was significantly higher in patients with the DM pattern than in those with NGT and IGT. These findings indicate that insulin resistance is important for a progression to DM in the patients with HCV. Insulin resistance, which leads to hyperinsulinaemia, is partially caused by increased production levels of TNF-α. In fact, the production of TNF-α has been reported to increase in chronic liver injury (31) and TNF-α is one of the causes of DM in patients with CH-C (32, 33). The study using a mouse model transgenic for the HCV core gene revealed that HCV causes insulin resistance and that a high level of TNF-α contributes to insulin resistance in these transgenic mice (7). Therefore, hyperinsulinaemia might be induced by TNF-α and other inflammatory cytokines. In the current cohort, TNF-α did not significantly increase in the patients with glucose intolerance. Other mechanisms may therefore be associated with hyperinsulinaemia and insulin resistance. HOMA-IR was significantly higher in patients with the DM pattern than in those with NGT and IGT. In addition, obesity is a risk factor for HCC in patients with decompensated cirrhosis (34). The BMI of the patients with the DM pattern was found to be higher than that of NGT. These results support the higher incidence of HCC in patients with a DM pattern.

It is unknown whether the good control of blood glucose levels prevents hepatocarcinogenesis. There is no evidence that successful treatment for glucose intolerance can reduce hepatocarcinogenesis. In an animal model, blood glucose control suppressed the incidence of squamous cell carcinoma in alloxan-induced diabetic rats through suppressing inflammation (35). However, the direct relationship between the treated DM and carcinogenesis still remains controversial. In our subjects, we did not have any detailed follow-up data and, therefore, it is difficult to evaluate whether the good control of blood glucose levels can reduce the occurrence of HCC.

In conclusion, this study revealed that DM pattern identified by the 75 g OGTT could be an independent risk factor of hepatocarcinogenesis in patients with HCV. These results indicate that an accurate evaluation of glucose intolerance would therefore be useful for estimating the risk of hepatocarcinogenesis in HCV patients. It is necessary to determine how the control of

hyperglycaemia reduces the potential of hepatocarcinogenesis and to determine how the incidence of HCC can be reduced in HCV patients with DM.

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Clinical trial: extended treatment duration of peginterferon-alpha2b plus ribavirin for 72 and 96 weeks in hepatitis C genotype 1-infected late responders

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SUMMARY

Background
The benefits of prolonging peginterferon and ribavirin after 48 weeks of treatment to maximize sustained virological responses (SVR) in hepatitis C virus (HCV) genotype 1-infected patients remain to be understood.

Aim
To investigate whether extended treatment longer than 72 weeks may be superior to 72-week treatment.

Methods
A total of 120 treatment-naïve or retreated patients with HCV genotype 1 were treated with peginterferon-alpha-2b (1.5 μg/kg/week) plus weight-based ribavirin. We had 34 late responders, in whom HCV RNA first became undetectable at week 12–48, and randomized them into three groups receiving standard-dose peginterferon-alpha-2b plus low-dose ribavirin (200 mg/day) for extended 24 weeks (group A), receiving low-dose peginterferon-alpha-2b (0.75 μg/kg/week) plus low-dose ribavirin for extended 48 weeks (group B) or no extended treatment (group C), and evaluated the outcome according to their virological response.

Results
Multivariate analysis showed that the treatment for 96 weeks was identified as a significant, independent factor associated with SVR in HCV genotype 1-infected late responders in comparison with group A [odds ratio (OR), 10.002; $P = 0.080$] and group C (OR, 17.748; $P = 0.025$).

Conclusion
Extending the treatment duration from 48 weeks to 96 weeks improves SVR rates in genotype 1-infected patients with late virological response to peginterferon-alpha-2b and ribavirin.

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INTRODUCTION

The hepatitis C virus (HCV) is a common cause of cirrhosis and hepatocellular carcinoma. For the management of HCV genotype 1 infection, 48 weeks of therapy with pegylated interferon plus ribavirin is recommended.[1] Although the introduction of pegylated interferon in combination with ribavirin in recent years greatly improved the treatment outcome of HCV infection, the treatment outcome of HCV type 1-infected patients remains unsatisfactory and sustained virological responses (SVR) can be obtained in only approximately 45%.[2–4]

Hepatitis C virus kinetics during the early phase of treatment is recognized as a predictor of the final therapeutic outcome. Assessment of early virological response (EVR) correlates closely with the likelihood of the ultimate eradication of HCV in patients treated with ribavirin in combination with interferon[5] or pegylated interferon.[6–8] After 48 weeks of treatment, the likelihood of SVR was approximately 90% in patients who achieved undetectable serum HCV RNA at week 4 of treatment in subjects infected with HCV genotype 1, whereas patients with less than 2-log decrease in HCV RNA levels by week 12 of treatment had virtually no chance of developing SVR.[6, 7] On the basis of these findings, discontinuation of treatment in nonresponders at this time was recommended to avoid unnecessary therapy.[1, 9] However, high relapse rates in slow responders may indicate that treatment was not administered for a sufficient duration in patients with slow virological response.

An analysis based on a mathematic model from a phase III randomized trial of peginterferon-alpha-2a and ribavirin, Drusano and Preston suggested that the rate of SVR in patients infected with HCV genotype 1 directly correlates with the duration of treatment once HCV RNA has been cleared from serum.[10] As the average time to clear serum HCV RNA was over 30 weeks, the authors concluded that 48-week duration of therapy was inadequate for most patients with genotype 1. Indiscriminate extension of treatment in patients with HCV genotype 1 is not beneficial. It has been currently reported that there is a subgroup of genotype 1-infected patients, the so-called 'slow responders', who benefit from extending the treatment duration from 48 weeks to 72 weeks that significantly improves SVR rates.[11–14] Therefore, prolonged treatment has the potential to improve cure rates, although it will increase the cost of treatment and may increase the probability that a patient will experience adverse events. However, prolonged duration and optimal doses of pegylated interferon or ribavirin after 48 weeks of treatment to maximize SVR still remain to be understood. We aimed to investigate whether extended treatment longer than 72 weeks using the dose reduction of pegylated interferon after 48 weeks of treatment may be superior to the 72-week treatment using the standard dose of pegylated interferon. To tolerate such a long treatment, we tapered doses of pegylated interferon and/or ribavirin substantially after 48 weeks of treatment.

In hepatitis C genotype 1 patients, a slow virological responder was commonly defined as a patient with at least a 2-log decrement in baseline serum HCV RNA, albeit detectable viraemia at 12 weeks and undetectable serum HCV RNA at 24 weeks.[13] However, Mangia *et al.* reported that SVR rates of HCV genotype 1 patients who first achieved undetectable HCV RNA at week 12 were 38.1% and 63.4% in 48 weeks and 72 weeks treatment respectively.[14] In a multicentre study in Japan, SVR rate of HCV genotype 1b patients in whom HCV RNA became negative for the first time at week 12 was 41.2% in 48 weeks treatment, although SVR rate of patients in whom HCV RNA became negative within 8 weeks was over 80% (personal communication to Dr Kuboki).[15] These studies indicate that extended treatment duration is recommended in patients with undetectable HCV RNA at week 12 to improve cure rates.

Following these concepts, we randomized HCV genotype 1-infected late responders, in whom HCV RNA was positive at 8 weeks of treatment and negative for the first time during 12–48 weeks of treatment, into groups receiving standard-dose peginterferon-alpha-2b (1.5 μg/kg/week) plus low-dose ribavirin (200 mg/day) for additional 24 weeks (total 72 weeks) or receiving low-dose peginterferon-alpha-2b (0.75 μg/kg/week) plus low-dose ribavirin (200 mg/day) for additional 48 weeks (total 96 weeks) and evaluated the outcome according to their virological response.

METHODS

Patients

The purpose of this study was to assess prospectively the efficacy of extended treatment duration of peginterferon-alpha-2b plus ribavirin in HCV genotype

1-infected late responders. Adult patients of both genders aged more than 18 years testing positive for anti-HCV, with consistent detection of HCV RNA above 100 000 IU/mL by reverse-transcription polymerase chain reaction [RT-PCR; Amplicor HCV (version 2), Roche Diagnostics, Branchburg, NJ, USA] and elevated serum alanine aminotransferase (ALT) levels were eligible for enrollment. Patients were excluded if they had decompensated liver diseases, other causes of liver disease, hepatitis B infection, haemoglobin values <13 g/dL, white blood cell count <4000/μL, thrombocytopenia <100 000 /μL, neoplastic, severe cardiac, neurological, autoimmune or thyroid disease. Also excluded were patients with alcohol or drug abuse, women who were pregnant or considering pregnancy in the next 18 months or men whose partners were considering pregnancy in the next 18 months. A late responder was defined as a patient with HCV RNA positive at 8 weeks of treatment and negative for the first time during 12–48 weeks of treatment. Written informed consent was obtained from all patients, and an institutional review board at each participating centre approved the study protocol.

Study design

This study was conducted between December 2004 and December 2005 at eight centres (two university hospitals and six general hospitals) in Japan. In this partially randomized, open-label, parallel-group, multicentre study, one hundred twenty treatment-naïve or retreated patients who met the criteria for entry were enrolled and received treatment with subcutaneous peginterferon-alpha-2b (1.5 μg/kg/week) (Peg-Intron; Schering Corp., Kenilworth, NJ, USA) and oral ribavirin (600–1000 mg/day based on weight: ≤60 kg, 600 mg; 61–80 kg, 800 mg; and >80 kg, 1000 mg) (Schering Corp.) for 48 weeks. Thirty-seven of 120 patients had been treated previously with conventional interferon or conventional interferon plus ribavirin for 24 weeks ('relapsers' and 'nonresponders' were included), but had not been treated previously with pegylated interferon and ribavirin. Pegylated interferon and ribavirin dose modifications followed standard criteria and procedures. The late responders whose serum HCV RNA became undetectable during 12–48 weeks after treatment were randomized to 1 of the 3 treatment groups: extended therapy for an additional 24 weeks with standard-dose peginterferon-alpha2b (1.5 μg/kg/week) plus low-dose ribavirin (200 mg/day) (total treatment duration of 72 weeks; group A); extended therapy for an additional 48 weeks with low-dose peginterferon-alpha-2b (0.75 μg/kg/week) plus low-dose ribavirin (200 mg/day) (total treatment duration of 96 weeks; group B); and not-extended therapy (total treatment duration of 48 weeks; group C).

Liver biopsies, which were not mandatory for the patients to be enrolled, were performed in 100 patients within 6 months before study entry, and histological changes were recorded according to the criteria of Desmet *et al.*,[16] with the grading of activity and the staging of fibrosis being defined as A0 (no histological activity), A1 (mild activity), A2 (moderate activity), A3 (severe activity), and as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis) and F4 (cirrhosis) respectively.

On the basis of SVR rate of 35% in the 48 weeks treatment (Group C in our study)[14, 15] and predicted improvement of the rate of 50% or higher in Group A or B (SVR rate of 85%), we calculated the required sample size of 14 for each group with α-error of 0.05 and ß-error of 0.80.

Measurement of HCV RNA

Serum samples were collected in each institution and centrally stored at −80 °C. Anti-HCV was tested by third-generation enzyme-linked immunoassay (Abbott Laboratories, North Chicago, IL, USA). Quantification of serum HCV RNA was performed by a single central laboratory (SRL Laboratory Co., Tokyo, Japan) to avoid variability between available assays using RT-PCR (Amplicor HCV Monitor test [version 2], Roche Diagnostics, Branchburg, NJ, USA) with a lower limit of detection of 600 IU/mL. Serum HCV qualitative test (detection limit 50 IU/mL; Amplicor HCV kit [version 2], Roche Diagnostics) was assessed at every 4 weeks after treatment. HCV genotyping was performed by RT-PCR using genotype-specific primers[17] in a single central laboratory (SRL Laboratory Co.) using a modification of a method described by Ohno *et al.*[18]

Determination of nucleotide and deduced amino-acid sequences of the IFN-sensitivity-determining-region (ISDR) and core region

RNA coding for ISDR in the NS5A region was amplified by nested RT-PCR. For direct sequencing of the NS5A (2209–2248) region, after the first-round PCR,

the second round of nested RT-PCR was performed using an external sense primer and internal antisense primer.[19] The second-round PCR products were purified and directly sequenced using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystem, Warrington, UK) in a 310 DNA sequencer (ABI 3100 Genetic Analyzer, Perkin Elmer Applied Biosystems). Electropherograms were analysed using Sequence Navigator software (Perkin Elmer Applied Biosystems). The deduced amino acid sequences of ISDR were compared with the sequence of the prototype isolate of HCV-J. Detection of amino acid substitutions of aa 70 and aa 91 in core region of HCV genotype 1b was performed using mutation-specific primer as an alternative to the direct sequencing method.[20] The major protein type was determined based on the relative intensity of the bands for wild (aa 70, arginine; aa 91, leucine) and mutant (aa 70, glutamine/histidine; aa 91, methionine) in agarose gel electrophoresis. All of the above procedures were performed centrally by SRL Laboratory.

Efficacy end points

The primary aim of the study was to assess the effect of extended treatment duration of peginterferon-alpha-2b plus ribavirin on sustained virological response (SVR) for patients with late virological response defined as HCV-RNA positive at week 8, but negative at weeks 12–48. SVR was defined as the sustained disappearance of serum HCV RNA for 24 weeks after the end of treatment. Treatment failure was categorized as relapse (reappearance of HCV RNA during the follow-up period after an end of treatment response), nonresponse (HCV RNA positive at week 48) or discontinuation (treatment withdrawn for any reason).

The secondary endpoint was the evaluation of discontinuation. It was thought important to decrease the numbers of patients with discontinuation to achieve higher SVR.

Statistical analysis

The efficacy analysis was conducted on an intention-to-treat basis. All patients who received at least one dose of study medication were included in the intention-to-treat population. The baseline characteristics of patients randomized to groups A, B and C were compared using Fisher's exact test for categorical data and Kruskal–Wallis test for continuous variables. Univariate and stepwise multivariate logistic regression analyses were used to determine independent predictive factors that were associated with SVR. Correlations were tested using Pearson's rank correlation coefficient.

RESULTS

Patient profiles

The median age of the enrolled population of 120 patients from eight centres was 60 years, 61% were men, and 98% were infected with genotype 1b. The trial participant flow is shown in Figure 1. Of 120 patients with genotype 1 infection treated with peginterferon-alpha-2b and ribavirin during that study period, 39 patients (33%) were late responders to therapy and met inclusion criteria. However, only 35 patients participated and were randomized, because four late responders declined to participate in the study. Of 120 enrolled patients, 25 patients (21%) stopped treatment within 48 weeks.

Thirty-five late responders, all of whom were genotype 1b, were assigned to group A (n = 12), group B (n = 10) or group C (n = 13). However, one patient of group B who was found to be HCV RNA negative at week 8 and did not meet inclusion criteria was excluded for this analysis. Baseline demographic, biochemical and virological characteristics of patients did not differ among three groups (Table 1). Time when patients first achieved undetectable HCV RNA did not differ among three groups.

Outcomes of patients

At week 48 of treatment, HCV RNA was undetectable in all of 34 patients in groups A, B and C. At the end of therapy, HCV RNA was undetectable in 92%, 100% or 100% of patients from each group A, B or C respectively (Figure 2). At the end of the follow-up period, virological response was sustained in 58% (7/12) of patients in group A, 89% (8/9) of patients in group B and 38% (5/13) of patients in group C (Figure 2). Surprisingly, one patient in group B who first became HCV negative at week 28 of treatment achieved SVR. As shown in Figure 2, relapse rate was lesser in patients treated for 96 weeks (11%) than in those treated for 72 weeks (42%, P = 0.178) or 48 weeks (62%, P = 0.031). Moreover, we have assessed the treatment outcome of patients who had detectable HCV RNA at

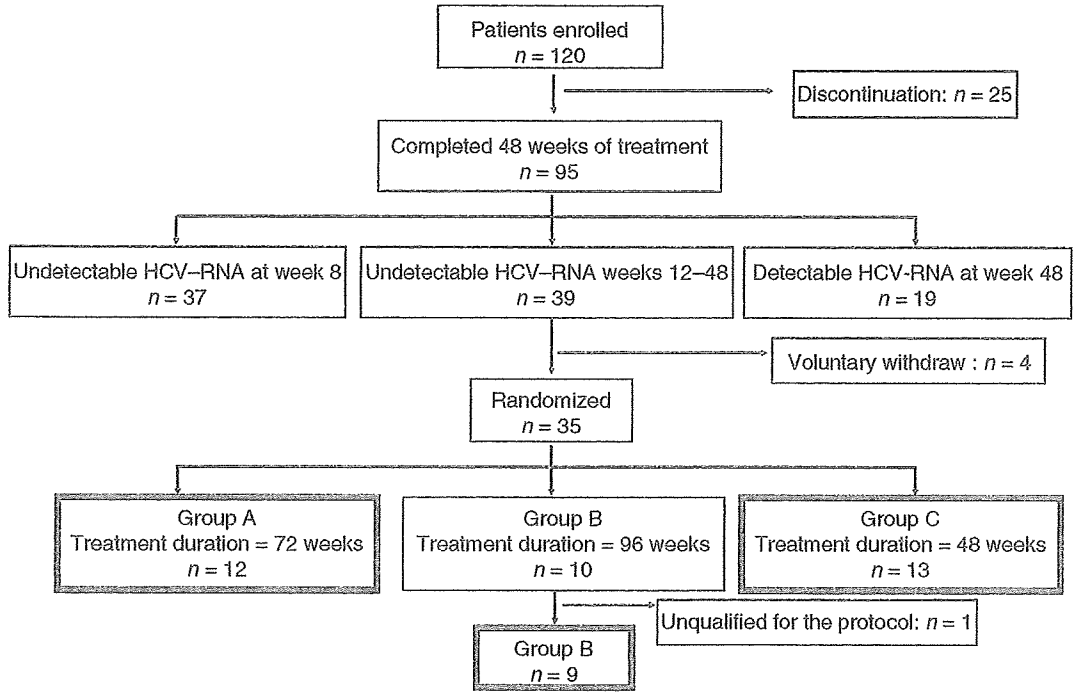

Figure 1. Flow of participants throughout the study.

week 12, but undetectable HCV RNA at week 48. Although patient numbers among the treatment subgroup were limited, virological response was sustained in 33% (2/6) of patients in group A, 67% (2/3) of patients in group B and 0% (0/5) of patients in group C.

During the extended treatment for patients in groups A and B, only one patient in group A discontinued ribavirin intake, but none except the patient in both groups needed dose reductions of peginterferon-alpha-2b or ribavirin. In addition, haemoglobin levels increased again in groups A and B after 48-week treatment probably because of the dose reduction of ribavirin during the extended treatment (Figure 3). The rate of SVR among HCV genotype 1-infected patients was significantly higher in patients treated for 96 weeks than in those treated for 48 weeks ($P = 0.034$, Table 2 and Figure 2), although the difference between the rates of SVR in group A and group C was not significant (Table 2). The rate of SVR of patients in group B (89%) was comparable to that of patients achieving early virological response whose HCV RNA was negative at week 8 [78% (29/37)].

Several baseline and on-treatment predictors of SVR (group, age, activity grade, total cholesterol), which P values were lower than 0.2 using Fisher's exact test, were examined by logistic regression analysis. The stepwise multivariate logistic regression analyses for four variables showed that group and age were independent predictive factors of SVR. The treatment for 96 weeks was identified as a significant, independent factor associated with SVR in HCV genotype 1-infected late responders [group B vs. group A; odds ratio (OR), 10.002; confidence interval (CI), 0.757–132.148; $P = 0.080$, group B vs. group C; OR, 17.748; 95% CI, 1.427–220.746; $P = 0.025$].

Sustained virological responses in the total study population

Sustained virological responses was obtained in 52 of 120 (43%) of the total intention-to-treat population and 52 of 117 (44%) of those with 24-week follow-up data. SVR was obtained in 2 of 25 (8%) of patients with treatment discontinuation. For the improvement of SVR in the total population, it must be important to decrease the number of patients with treatment discontinuation. Interestingly, we found that the number of patients enrolled per hospital was significantly associated with the reduced ratio of patients with treatment discontinuation (Figure 4).

Table 1. Characteristics of patients at baseline

Treatment duration	Group A (N = 12) 72 weeks	Group B (N = 9) 96 weeks	Group C (N = 13) 48 weeks	P-value
Gender				
Male	8 (67%)	5 (56%)	8 (62%)	0.908
Female	4 (33%)	4 (44%)	5 (38%)	
Age (year)	54 (35–73)	60 (48–70)	62 (35–71)	0.657
Serum ALT (IU/L)*	52 (26–255)	61 (40–108)	64 (17–171)	0.437
HCV RNA (KIU/mL)				
<1500	4 (33%)	5 (56%)	3 (23%)	0.317
≥1500	8 (67%)	4 (44%)	10 (77%)	
Number of mutations in ISDR				
0	5 (45%)	6 (67%)	9 (75%)	0.282
1–3	5 (45%)	3 (33%)	3 (25%)	
4-	1 (9%)	0 (0%)	0 (0%)	
Core 70 mutation				
W	9 (75%)	8 (89%)	9 (75%)	0.708
M	3 (25%)	1 (11%)	3 (25%)	
Core 91 mutation				
W	9 (75%)	6 (67%)	10 (83%)	0.595
M	3 (25%)	3 (33%)	2 (17%)	
Fibrotic stage				
0–1	2 (22%)	3 (33%)	5 (38%)	0.559
2–4	7 (77%)	6 (67%)	8 (62%)	
Activity grade				
0–1	3 (33%)	4 (44%)	4 (31%)	0.893
2–3	6 (67%)	5 (56%)	9 (69%)	
Loss of HCV RNA (week)				
12	6	6	8	0.653
16	2	0	4	
20	0	0	0	
24	3	1	1	
28	1	1	0	
32	0	1	0	

HCV, hepatitis C virus; ISDR, interferon-sensitivity-determining-region.
*Normal range of ALT: 7–40 IU/L.
Fisher's exact test was used for categorical data to compare differences, and continuous variables were compared by Kruskal-Wallis test.
Hepatic histology was not evaluated in three patients in group A, because liver biopsy was not performed.
Lack of completeness was due to incomplete sampling.

DISCUSSION

A 48-week treatment with pegylated interferon plus ribavirin has now become the standard of care for patients with HCV genotype 1. The duration of antiviral therapy is one of the most important factors influencing treatment outcome, especially in HCV genotype 1-infected patients.[11–14] Berg *et al.* investigated the efficacy of 48 weeks vs. 72 weeks of treatment with peginterferon-alfa-2a plus ribavirin in treatment-naïve patients with HCV type 1 infection. In this study, prolongation of the therapeutic regimen for up to 72 weeks does not lead to higher SVR rates in the intention-to treat population, but patients who still are HCV-RNA positive at week 12 show significantly higher SVR rates when treated for 72 weeks instead of 48 weeks.[11] Sánchez-Tapias *et al.* have recently demonstrated that extension of treatment with peginterferon-alfa-2a plus ribavirin from 48 to 72 weeks significantly increases the rate of SVR in patients with detectable viraemia at week 4 of treatment.[12] Pearlman *et al.* have demonstrated that extending the treatment

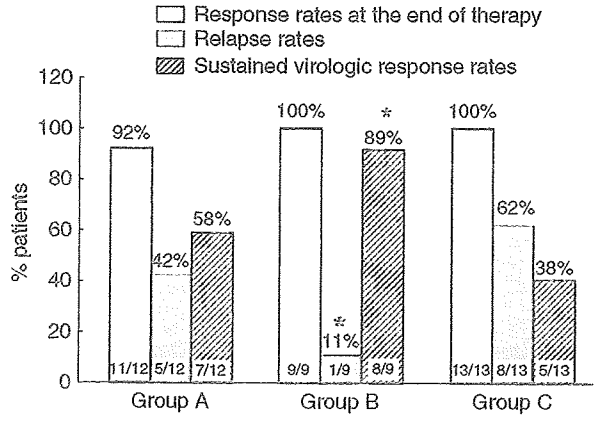

Figure 2. Frequency of virological response rates at the end of therapy and virological relapse rates in groups A, B and C. These rates are shown as a percentage and the number of patients with virological response or virological relapse in relation to the total number of patients examined is shown at the bottom of each column. *$P < 0.05$ compared with group C.

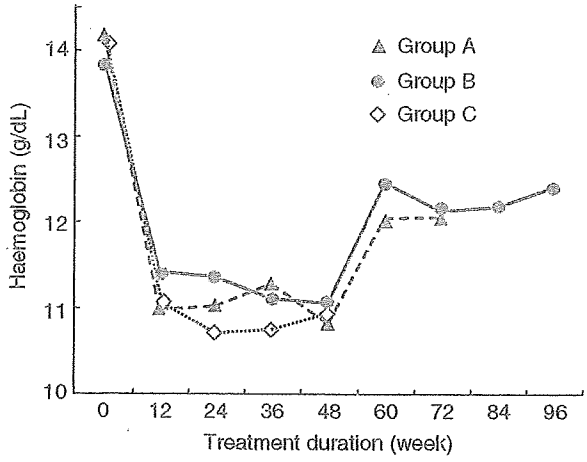

Figure 3. Time course of haemoglobin levels in groups A, B and C during therapy. The patients in groups A and B were given low-dose ribavirin (200 mg/day) beyond 48 weeks.

duration from 48 weeks to 72 weeks in genotype 1-infected patients with slow virological response to peginterferon- alfa2b plus ribavirin, which was defined by achieving at least a 2-log decrement in HCV RNA from baseline, yet having detectable HCV RNA at 12 weeks and undetectable HCV RNA at 24 weeks, significantly improves SVR rates.[13]

However, all of the aforementioned studies extended the treatment duration to 72 weeks to improve SVR rates in slow responders with HCV genotype 1. Furthermore, it is unclear if the standard doses of peginterferons and ribavirin continued to be used after week 48, although these patients achieved undetectable HCV RNA before 24 weeks. It was reported that treatment discontinuation was more frequent in patients treated for 72 weeks than those for 48 weeks.[13] In the prediction model developed by Drusano and Preston, it was concluded that type 1-infected patients required the continuous absence of detectable HCV RNA in serum for 36 weeks to attain 90% probabilities of SVR,[10] suggesting the importance of treatment duration when serum HCV RNA is continuously negative. In the present study, we used the minimum dose of ribavirin (200 mg/day) beyond 48 weeks in late responders who first became HCV RNA undetectable after 12 weeks and compared the efficacy and safety of additional 24 weeks of treatment (total 72 weeks) with the standard dose of peginterferon- alfa2b with those of additional 48 weeks of treatment (total 96 weeks) with the half dose of peginterferon- alfa2b.

Our data showed that SVR rates were higher in the 96-week group as compared with the 72-week group as well as the 48-week group (89% vs. 58% or 38%, respectively). The SVR rates seem to be higher than those previously reported.[11–13] The differences in the SVR rates could be because of our criteria of late responders that include patients with a first virological response at week 12. Only one of 21 patients in group A and B became HCV RNA positive during the extended treatment after 48 weeks of treatment, suggesting that the intentional dose reductions of peginterferon-alpha-2b and ribavirin between weeks 48 and 96 did not cause adverse effects on viral load. Only one of 21 patients discontinued ribavirin intake, but the others did not need dose reductions of peginterferon-alpha-2b or ribavirin and therapy discontinuation during the extended treatment, indicating that the intentional dose reductions of peginterferon-alpha-2b and ribavirin between weeks 48 and 96 were safe for patients with chronic hepatitis C genotype 1. Moreover, the intentional dose reductions during the last part of the treatment improved haemoglobin levels (Figure 3), which might result in tolerating a long treatment.

Among patients who discontinued treatment up to week 48, the rate of SVR was 8% (intention-to-treat analysis), which is much lower than that with patients treated for at least 48-weeks [53% (50/95)]. These data highlight the relevance of encouraging adherence to therapy.[21] Interestingly, the number of patients

Table 2. Predictors of sustained virological response to combination therapy

Factor	Definition	OR (95% CI)	*P*-value
Univariate logistic regression analysis			
Group	B vs. C	12.800 (1.208–135.579)	0.034
	B vs. A	5.714 (0.532–61.409)	0.150
	A vs. C	2.240 (0.451–11.114)	0.324
Gender	Female	2.045 (0.477–8.773)	0.336
Age	<60 years	3.344 (0.802–13.941)	0.098
Previous IFN course	Naïve	1.750 (0.420–7.288)	0.442
Serum ALT*	≥63 (IU/L)	2.500 (0.584–10.696)	0.217
Total cholesterol	≥170 (mg/dL)	4.480 (0.986–20.354)	0.052
HCV RNA	<1500 KIU/mL	3.000 (0.636–14.150)	0.165
ISDR mutation	W	1.072 (0.250–4.591)	0.926
Core 70 mutation	W	1.200 (0.221–6.520)	0.833
Core 91 mutation	W	0.900 (0.175–4.630)	0.900
Fibrotic stage	2–4	1.625 (0.355–7.434)	0.532
Activity grade	2–3	2.229 (0.497–9.997)	0.295
Stepwise multivariate logistic regression analysis			
Group	B vs. C	17.748 (1.427–220.746)	0.025
	B vs. A	10.002 (0.757–132.148)	0.080
	A vs. C	1.774 (0.315–10.010)	0.516
Age	<60 years	4.963 (0.922–26.710)	0.062

OR, odds ratio; 95% CI, 95% confidence interval; HCV, hepatitis C virus; ISDR, interferon-sensitivity-determining-region.
Normal range of ALT: 7–40 IU/L.

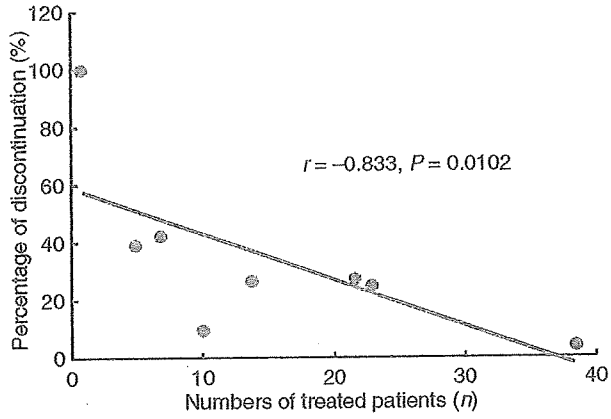

Figure 4. Correlation between the number of treated patients and percentage of discontinuation at each hospital. All 25 patients who stopped treatment within 48 weeks of treatment were analysed. Pearson's correlation coefficient is indicated on the graph.

enrolled per hospital was negatively associated with the numbers of patients with treatment discontinuation. These findings imply that the differences in improved adherence could be the result of physician-driven care and continuity based on the experience of each physician, because almost all of our patients were seen by the same treating physician on a monthly basis throughout the trial. Moreover, low attrition rates in hospitals where a greater numbers of patients were cared could be explained by the patients' knowledge concerning the adverse and beneficial effects of this combination therapy informed by physicians before and during treatment.

Limitation of this study is the small number of patients as compared to the predicted sample size. Thus, the statistical power is weaker than that of the initial design, and the possibility of ß-error remains.

In conclusion, extension of the treatment duration with peginterferon-alfa-2b plus ribavirin up to 96 weeks significantly increased the likelihood of achieving SVR in HCV genotype 1-infected late responders whose serum HCV RNA became undetectable for the first time during 12–48 weeks after treatment. Treatment extension did not increase the rate of dose reduction or treatment discontinuation.

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Role of CD44 in CTL-induced acute liver injury in hepatitis B virus transgenic mice

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Background. There are many uncertain points regarding leukocyte movement in the liver, especially interaction between liver sinus endothelial cells (LSECs) and cytotoxic T lymphocytes (CTLs). We examined the role of CD44 in these interactions using the hepatitis model. ***Methods.*** CTLs were administered to hepatitis B virus transgenic mice (HBVTg) mice and HBVTg × CD44 knockout (KO) mice, and alanine aminotransferase activity (ALT), number of intrahepatic leukocytes, cytokines, and chemokine mRNA level were examined. To determine the number and distribution of CTLs in the liver, 5,6-carboxyfluoroscein succinimidyl ester (CFSE)-labeled CTLs was administered to HBVTg mice with or without CD44. ***Results.*** Serum ALT activity increased after 12 h, although it had declined to 4 h in the CD44KO × HBVTg mice after CTL injection. Similarly, the levels of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and macrophage inflammatory protein (MIP)-2 mRNAs were reduced in 4 h, although the levels were increased after 12 h in the CD44KO × HBVTg mice. The number of apoptotic hepatocytes increased intentionally at 24 h in the CD44KO × HBVTg livers, and this was thought to result from the lower activity of initial nuclear factor kappa B (NF-κB). Although the number of CTLs was lower at 4 h in the CD44KO × HBVTg livers, the difference of intercellular adhesion molecule (ICAM)-1 and CD86 expression on LSECs was not detected. ***Conclusions.*** CD44 exerts and important effect on LSECs for CTL migration into the hepatocytes. However, because the CD44-deficient state exacerbated hepatic injury, attention is necessary for hepatitis treatment as CD44 target therapy.

Key words: HBV, CD44, CTL, acute hepatitis

Introduction

We previously demonstrated that inflammatory cell infiltration plays a key role in hepatitis B virus (HBV)-specific cytotoxic T lymphocyte (CTL)-induced hepatitis, because neutrophil elastase inhibitor and antimacrophage migration inhibitory factor (MIF) antibody treatments, which suppress inflammatory cell recruitment, reduced liver injury.[1,2] Furthermore, other reports have also demonstrated that depletion of neutrophils and macrophages reduced liver injury in the HBV transgenic (Tg) mice model, indicating that recruited inflammatory cells are involved in exacerbation of hepatitis.[3] As these results suggested that inhibition of inflammatory cells represents a possible new therapy for acute hepatitis, we investigated the usefulness of adhesion molecule blockade and focused on CD44.

Protection and inhibition of adhesion molecules have been proposed as promising therapeutic strategies for inflammatory diseases. CD44 has been generally identified as one of these adhesion molecules because binding of CD44 on activated T lymphocytes to endothelial hyaluronan mediates a primary adhesive interaction and permits extravasations at sites of inflammation.[4,5] Recently, CD44 was reported to have a variety of functions in immune regulation, signal transduction, and tumor migration or growth.[6] Previous reports have indicated that anti-CD44 antibody treatment effectively suppresses the disease activities of pulmonary eosinophilia, experimental colitis, and encephalomyelitis.[7–10] Furthermore, CD44-deficient (CD44KO) mice exhibit suppression of lipopolysaccharide (LPS)-induced lung injury or granuloma formation.[11,12] Based on these findings, we focused on the roles of CD44 in HBV-specific CTL-mediated acute hepatitis model mice.[13,14]

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Methods

Mice

The HBVTg lineage 107-5D used[13] in this study has previously been described and was generously provided by Dr. Francis V. Chisari (Scripps Research Institute, La Jolla, CA, USA). CD44-knockout (CD44KO) mice were kindly provided by Dr. Tak W. Mak (University of Toronto, Toronto, Canada).[11] In all experiments, the mice were matched for age (8 weeks), sex (female), and serum hepatitis B surface antigen (HBsAg) lev el before experimental manipulation. Serum HBsAg was examined by Espline HBsAg kit (Fuji Rebio, Tokyo, Japan). All animals were housed in pathogen-free rooms under strict barrier conditions and received humane care according to the guidelines of the Animal Care Committee of the University of Tokyo School of Medicine.

CTL clones

HBVTg mice were injected with an HBsAg-specific, H-2d-restricted, $CD8^+$ CTL clone (designated 6C2) that recognizes an epitope (IPQSLDSWWTSL) located between residues 28 and 39 of HBsAg.[2] At 5 days after the last stimulation, the cells were washed, counted, and injected intravenously into HBVTg mice.

RNA analyses

Total RNA was isolated from frozen livers (left lobe) and isolated cells and analyzed for inflammatory cytokine or chemokine messenger RNAs (mRNAs) using an RNase protection assay (RPA) as described previously.[15]

Biochemical and histological analyses

The extent of hepatocellular injury was monitored both histologically and biochemically at multiple time points after the CTL injection. For biochemical analysis, the serum alanine aminotransferase (sALT) activity was measured using a standard clinical automatic analyzer. For histological analysis, liver tissue was fixed in 10% zinc-buffered formalin, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin.

5,6-Carboxyfluoroscein succinimidyl ester (CFSE) staining

For labeling CTLs, the culture CTLs were resuspended in phosphate-buffered saline (PBS; 1×10^5 cells/ml) containing 5- (and 6-)CFSE (Molecular Probes, Eugene, OR, USA) at a final concentration of 1 μM, incubated at 37°C for 10 min, and washed three times. After this procedure, CFSE-labeled CTLs were injected into mice.

Immunohistochemistry

For immunofluorescent microscopic analyses, liver sections were fixed with acetone at 4°C for 10 min and preincubated with 10 mg/ml anti-CD16/32 antibody (clone 2.4G2; BD Pharmingen, La Jolla, CA, USA) for 30 min. A antimouse antibody to rabbit type IV collagen (LSL, Tokyo, Japan) was used to outline hepatic sinusoids. As a secondary step, Alexa Fluor 594 goat antirabbit IgG antibody (Invitrogen, Carlsbad, CA, USA) was used. After each step of the staining, the sections were washed three times with PBS for 10 minutes each. Finally, the sections were observed using a DMRA fluorescence microscope and the QFISH software (Leica Microsystems Imaging Solutions, Cambridge, UK).

Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay

Apoptotic cells were estimated by the TUNEL assay, which relies on the incorporation of labeled dUTP at DNA break sites. All the reagents, including the buffers, were part of a TUNEL assay kit (Apop Tag; Oncor, Gaithersburg, MD, USA), and the procedure was performed according to the manufacturer's instructions.[15]

Isolation of intrahepatic leukocytes (IHLs) and peripheral blood mononuclear cells (PBMCs)

To isolate IHLs, single-cell suspensions were prepared from liver perfused with phosphate-buffered saline (PBS) via the inferior vena cava and digested in 10 ml RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) containing 0.02% (wt/vol) collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) and 0.002% (wt/vol) DNase I (Sigma-Aldrich) for 40 min at 37°C. Cells were overlaid on Lymphocyte M (Cedarlane Laboratories, Ontario, Canada) in PBS.[16] To isolate PBMCs, peripheral blood (0.4 ml) was obtained by cardiac puncture under ether anesthesia. After density separation (Lympholyte-Mouse; Cedarlane, Westbury, NY, USA), cell counts and immunofluorescence analyses were performed.

Fluorescence-activated cell sorting (FACS) analysis

IHLs were harvested from mice at the indicated times after the CTL injection. Cells were then surface stained with anti-CD3-FITC monoclonal antibody (mAb), anti-NK1.1-PE mAb, anti-CD11b-allophycocyanin (APC), anti-Gr-1-FITC mAb, anti-ICAM-1-PE, anti-CD44-PE,