cirrhosis. This lack of specificity for HCC means that AFP has a comparatively high false-positive rate [37]. The C3a fragment may also be elevated during hepatocyte regeneration following liver damage [38], and early diagnosis of small HCC tumors may be difficult with one marker alone. Therefore, the false-positive rates for HCC must be carefully considered [39–41]. Also, a combination of markers, including AFP, DCP, and the C3a fragment, in the serum should be verified to improve the diagnostic rate.

The ProteinChip SELDI system can separate and partially characterize multiple proteins in tissue and serum samples. Our previous report used a panel of proteins to diagnose early HCC with the ProteinChip SELDI system [15]. This panel diagnosis of seven protein peaks included a discriminant peak of 4060 m/z. This 4060 m/z peak may be a double-charged 8130 m/z peak, although the C3a fragment (8130 m/z) was not used to develop this diagnostic method. These results suggest that the C3a fragment is a useful HCC biomarker, regardless of whether this fragment carries a single or double charge. In addition, the panel diagnosis method is more useful than measuring the C3a fragment alone to diagnose and predict the occurrence of HCC. However, this method must be performed using the ProteinChip SELDI system, which is expensive and does not detect putative interactions between various proteins. Identifying a specific HCC protein such as the C3a fragment will also further our understanding of the molecular mechanisms of hepatocarcinogenesis. Therefore, the C3a fragment should not only be considered a simple HCC tumor marker, but should also be evaluated for its contribution to HCC carcinogenesis.

In conclusions, serum profiling with the ProteinChip SELDI system may be used to distinguish HCC from chronic liver disease without HCC and to detect early HCC in HCV-infected patients. Because we identified the C3a fragment (8.1 k m/z) in serum samples from HCC patients, the C3a fragment is a promising marker that can be used to screen for HCV-HCC and to develop new therapeutic targets.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinical significance of alanine aminotransferase levels and the effect of ursodeoxycholic acid in hemodialysis patients with chronic hepatitis C

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Abstract

Background The natural history of hepatitis C virus (HCV) carriers and the effect of ursodeoxycholic acid (UDCA) have not been fully elucidated among hemodialysis (HD) patients.

Methods Eighty-four anti-HCV antibody- and HCV RNA-positive and 154 anti-HCV antibody-negative HD patients who were retrospectively observed for at least 3 years were analyzed. We investigated the factors associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) and decreased platelet count (PLT) (more than 20% decrease during the follow-up period), which were considered to be indicators of hepatic fibrosis. In addition, another 16 HD patients with HCV who received 300 mg/day UDCA orally for at least 6 months were investigated. Changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT) and PLT were assessed.

Results After the 60.3-months mean follow-up period, HCV infection was independently associated with both thrombocytopenia [odds ratio (OR) 2.589] and decreased PLT (OR 2.339) in 238 HD patients. In 84 HD patients with HCV, the average ALT levels ($\geq$ 15 IU/L) during the follow-up period was associated with thrombocytopenia (OR 3.882) and decreased PLT (OR 4.470). In addition, ALT, AST and GGT significantly decreased at 6 months after starting UDCA, but PLT did not change in 16 HD patients with HCV.

Conclusions These results indicate that HCV infection is a risk for thrombocytopenia which should be associated with hepatic fibrosis in HD patients. In addition, the clinical course of ALT levels predicts the progression of thrombocytopenia, and UDCA may effectively lower ALT levels in HD patients with HCV.

Keywords Hemodialysis · HCV · Thrombocytopenia · ALT · Ursodeoxycholic acid

Introduction

Chronic kidney disease (CKD) patients who are on hemodialysis (HD) continue to have a higher prevalence of hepatitis C virus (HCV) infection than the general population [1–4]. The prevalence of anti-HCV seropositivity among patients undergoing regular dialysis in developed countries ranges between 7 and 40% [5–8].

HCV infection in HD patients is usually recognized as asymptomatic and cirrhosis is infrequent in this population [9]. One of the reasons for these findings is that the clinical course of chronic hepatitis C extends over decades and dialysis patients generally have higher morbidity and mortality rates than the general population, making the long-term consequences of HCV infection with HD difficult to establish [6]. However, more recently, the prognosis of HD patients has been improving, so addressing HCV infection in these patients is becoming more important [10].

The strong association between serum alanine aminotransferase (ALT) levels and the fibrosis progression rate or occurrence of hepatocellular carcinoma has been well documented in HCV carriers without HD [11–13]. HD

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patients with persistent HCV infection also have higher ALT levels than those patients without HCV, and ALT values may predict the outcome of HCV infection in patients with HD [14]. In contrast, ALT values are still typically within the normal range in HCV carriers with HD and ALT values are lower in HCV carriers with HD than those without HD. Recently, the risk of liver disease-related deaths is higher in chronic hepatitis C patients with ALT levels closer to the upper limit of the normal range (ULN) (20–29 IU/L) compared to patients with lower ALT levels (< 20 IU/L) [15, 16]. In addition, it has been proposed that the cut-off for serum ALT levels should be reduced by half to screen for hepatic damage in HCV carriers with HD [17]. However, the association between serum ALT levels in those patients with HCV and fibrosis progression has not been fully elucidated.

Platelet count (PLT) is a simple biomarker of hepatic fibrosis in HCV carriers [18]. PLT is also lower in HCV RNA-positive HD patients than in HD patients with HCV RNA-negative serum [16]. In addition, severe hepatic fibrosis is independently associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) in HCV carriers with end-stage renal disease [19]. This study evaluated the association of ALT status over a long period and changes in PLT, which was considered an indicator of hepatic fibrosis, in HD patients.

Several trials have examined the efficacy of interferon monotherapy or interferon plus ribavirin combination therapy in HD patients with HCV, and some of these patients obtained a sustained virological response [20]. However, the virological response was limited and side effects may occur more frequently in patients with HD than in those without HD [21, 22]. Therefore, other therapies should be considered for these patients. For chronic hepatitis C patients with or without HD, ursodeoxycholic acid (UDCA) has already been used up to 150 mg/day as routine care in Japan. In addition, the effect of UDCA up to 900 mg/day in HCV carriers who are not undergoing HD was investigated [23], and the use of UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients after April 2007 in Japan. However, the effect of UDCA was not fully elucidated in HCV carriers with HD. Therefore, in this retrospective study we investigated the clinical significance of biochemical markers in the natural course of disease with particular emphasis on PLT and assessed the effect of oral UDCA on serum biomarkers in those patients with HCV.

Materials and methods

Study population

The patients in this study were retrospectively recruited. This study was approved by the Kagoshima University Graduate School of Medical and Dental Sciences. The study population consisted of patients who were on HD in August 2008 and whose data were obtained at least 3 years before August 2008 at 17 HD facilities in Kagoshima, Japan. Their alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total cholesterol (TC) and PLT were monitored once or twice each month. In 2539 patients, 243 patients were positive for anti-HCV, 143 patients were excluded because they were positive for hepatitis B virus surface (HBs) antigen, they were positive for anti-HCV antibody (anti-HCV) but were not examined for HCV RNA, they had received antiviral treatment or they had hepatocellular carcinoma (HCC). The final population enrolled in this study consisted of 100 patients. Among this cohort of 100 HD patients who were both anti-HCV- and HCV RNA-positive, 84 subjects had not received UDCA and were enrolled in study 1 (HD + HCV Group) and 16 subjects had already received 300 mg/day UDCA for at least 3 months after April 2007 when UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients in Japan and were enrolled in study 2 (UDCA Group). The control subjects in study 1 were 154 HD patients who were anti-HCV-negative (HD Group), and the controls in study 2 were the 84 HD patients among the study 1 population who were both anti-HCV- and HCV RNA-positive but had not received previous treatments including UDCA and were observed until August 2008 (non-UDCA Group). Of the 84 HD patients who were controls in study 2, 2 patients died before November 2008. Blood samples were obtained before routine HD procedures and then were used to assay for ALT, AST, GGT, TC and PLT. The relationship of these markers to PLT and the percent change in PLT were examined, and the percent change in PLT was calculated according to the formula: $\Delta\%PLT = [PLT\ (at\ the\ end\ of\ study) - PLT\ (at\ enrollment)]/PLT\ (at\ enrollment) \times 100$.

Serum HCV markers

Serum anti-HCV and HBsAg were determined using a commercially available third-generation enzyme-linked immunosorbent assay and anti-HBs assay, respectively. For anti-HCV antibody-positive patients, HCV RNA was quantified using the COBAS TaqMan HCV kit (COBAS AmpliPrep/COBAS TaqMan HCV assay, Roche Diagnostics, Tokyo, Japan) during the follow-up period. The serologically defined HCV genotype (HCV serotype) was also determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping, International Reagents Co., Tokyo, Japan). In some patients, the HCV genotype was examined (HCV Core Genotype, SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b with serotype II. No other HCV genotype was detected in this study population.

Study 1

The HD + HCV Group, which contained 84 HD patients with HCV, was compared to the HD Group, which contained 154 HD anti-HCV-negative patients. We compared the basal characteristics at enrollment and the changes in PLT during the follow-up period between the two groups. In addition, we divided the HD + HCV patients into the following four groups according to the average ALT level of all available ALT levels during the follow-up period: Group A, ALT < 15; Group B, 15 ≤ ALT < 20; Group C, 20 ≤ ALT < 30; and Group D, 30 ≤ ALT. Clinical characteristics at baseline or average ALT levels and change in PLT during the follow-up period were compared between these four groups.

Study 2

Sixteen patients with HD and HCV had been treated with 300 mg/day UDCA orally for at least 3 months after April 2007, when UDCA up to 900 mg/day was approved for chronic hepatitis C patients, until August 2008 (UDCA Group). These patients were observed every month for at least 6 months before the administration of UDCA and then monitored for the efficacy of UDCA for more than 3 months until August 2008. Then, these patients were observed for a total of at least 6 months until November 2008. We compared the basal characteristics between the UDCA Group just before UDCA treatment and the non-UDCA Group in May 2008. In addition, the changes in ALT, AST, GGT and PLT during the follow-up period were compared between the two groups. For example, the percent of ALT was calculated according to the formula: %ALT = [ALT (−6, 0, 1, 2, 3 or 6 M]/ALT[0 M] × 100).

Statistical analysis

When appropriate, χ^2 test, Fisher's exact test, Student's *t* test and Mann–Whitney *U* test were used to compare the frequencies or means. Logistic regression models were used for calculating the odds ratios (ORs), 95% confidential intervals (CIs) and *P* values. Statistical analyses were performed using STATVIEW (version 5.0; Abacus Concepts, Berkeley, CA), or SPSS (SPSS Inc., Chicago, IL) software programs. A *P* value less than 0.05 was considered statistically significant.

Results

Demographic characteristics of study 1 subjects

As shown in Table 1, 84 HD patients among the anti-HCV-positive patients were HCV carriers (positive for HCV RNA). One hundred fifty-four HD patients were anti-HCV-negative. On average, the frequency of males, levels of AST, ALT and GGT were higher and TC and PLT were lower at baseline in patients with HCV than those in patients without HCV. The follow-up period was also shorter in patients with HCV than those in patients without HCV. In contrast, there were no significant differences between the two groups with respect to age and duration of dialysis.

Table 1 Baseline characteristics of hemodialysis patients

	HCV (+)[a]	HCV (−)[b]	*P* value
Number	84	154	
Age (year)	64.4 ± 10.3	62.2 ± 12.5	0.165
Sex (male/female)	54/30	77/77	0.034
Duration of HD (years)	13.5 ± 9.6	11.8 ± 7.4	0.669
Follow-up period (months)	56.8 ± 15.8	62.2 ± 7.9	0.039
HCV RNA (Log IU/mL)[c]	4.9 ± 1.4	–	
Serotype (I/II/undetermined)[c]	59/21/4	–	
AST (IU/L)	19.7 ± 8.5	14.9 ± 6.7	<0.001
ALT (IU/L)	18.5 ± 9.3	13.2 ± 7.1	<0.001
GGT (IU/L)	41.5 ± 43.0	30.1 ± 42.1	0.002
TC (mg/dl)	153.7 ± 41.0	167.1 ± 35.0	0.003
PLT (× 10^5/μl)	1.59 ± 0.53	1.93 ± 0.73	<0.001

Unless otherwise indicated, data are given as the mean ± SD or number of patients

HD hemodialysis, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *TC* total cholesterol, *PLT* platelet count

[a] HCV (+), both anti-HCV antibody and HCV RNA positive

[b] HCV (−); anti-HCV antibody negative

[c] HCV RNA and serotype were examined during follow-up period

Predictors of thrombocytopenia in HD patients

Table 2 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT < 1.3 × 10^5/μl) at the end of study 1 (August 2008) using 9 baseline characteristics in all HD patients with or without HCV. Older age, HCV viremia, elevated AST, ALT, and GGT levels were significantly associated with thrombocytopenia. In addition, a multivariate analysis revealed that HCV viremia was independently associated with thrombocytopenia (Table 2). Furthermore, after the 60.3-month mean follow-up period (mean of HD + HCV Group, 56.7 months; HD Group, 62.2 months), PLT in the HD + HCV Group had decreased (from 1.59 × 10^5/μL to 1.22 × 10^5/μL) significantly compared to that in the HD Group (from 1.93 × 10^5/μL to 1.77 × 10^5/μL) (average Δ%PLT in each patient: −22.4 vs. −5.3%, $P < 0.001$). Variables that were statistically significant by a univariate analysis were further analyzed to identify variables that

Table 2 Univariate and multivariate analyses of variables associated with thrombocytopenia (< 1.3 × 10^5/μl) in HD patients

Variables	Odds ratio	95% CI	*P* value
Univariate analysis			
Age (years)			
<60	1.0		
≥60	1.994	1.141–3.484	0.015
Sex			
Female	1.0		
Male	1.494	0.868–2.571	0.147
Duration of dialysis (years)			
<10	1.0		
≥10	1.065	0.624–1.818	0.816
Follow-up period (months)			
<55	1.0		
≥55	0.727	0.4–1.321	0.296
HCV			
(−)	1.0		
(+)	4.533	2.555–8.043	<0.0001
AST (IU/L)			
<30	1.0		
≥30	7.741	2.095–28.603	0.002
ALT (IU/L)			
<20	1.0		
≥20	3.793	2.017–7.133	<0.0001
GGT (IU/L)			
<50	1.0		
≥50	2.836	1.396–5.758	0.004
TC (mg/dl)			
<150	1.0		
≥150	0.58	0.296–1.135	0.112
Multivariate analysis			
Age (years)			
<60	1.0		
≥60	1.783	0.937–3.394	0.078
HCV			
(−)	1.0		
(+)	2.589	1.317–5.091	0.006
AST (IU/L)			
<30	1.0		
≥30	5.123	0.996–26.339	0.050
ALT (IU/L)			
<20	1.0		
≥20	1.75	0.786–3.896	0.171
GGT (IU/L)			
<50	1.0		
≥50	1.743	0.783–3.88	0.174

Abbreviations as in Table 1

were independently associated with a more than 20% decrease in PLT. As a result, male sex (OR 2.375; 95% CI, 1.319–4.278; $P = 0.004$) and HCV viremia (OR 2.339; 95% CI, 1.295–4.224; $P = 0.005$) were factors that were independently associated with more than a 20% decrease in PLT.

Predictors of thrombocytopenia in HD patients with HCV

Table 3 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT < 1.3 × 10^5/μL) at the end of study 1 (August 2008) using 10 baseline characteristics in HD patients with HCV. The patients with HCV and thrombocytopenia had significantly higher frequencies of elevated ALT and GGT levels at baseline. However, age, sex, duration of HD, follow-up period, history of diabetes mellitus (DM), and elevated AST and TC levels were not significantly different between patients with and without thrombocytopenia. In addition, elevated ALT and GGT levels at baseline were not significantly associated with thrombocytopenia in patients with HCV by a multivariate analysis.

On the other hand, a univariate analysis that compared a decrease in PLT of more than 20% with a decrease less than 20% revealed that male sex and elevated ALT levels at baseline were associated with decreased PLT in patients with HCV. A multivariate analysis of two variables that were statistically significant by a univariate analysis also revealed that high ALT levels (ALT ≥ 20 IU/L) at baseline were independently associated with decreased PLT in patients with HCV (OR 3.318; 95% CI, 1.256-8.764; $P = 0.016$).

Furthermore, we divided patients with HCV into four groups according to average ALT levels during the follow-up period. As Table 4 shows, 30, 19, 18 and 17 patients were in Groups A, B, C and D, respectively. Age, duration of dialysis, follow-up period, HCV RNA levels, distribution of HCV serotype, frequency of diabetes mellitus, TC levels and PLT were not significantly different between the four groups. However, serum AST levels and ALT levels at baseline were significantly different, and these levels gradually increased from Group A to D. The distribution of sex was also significantly different and the frequency of males was higher in Groups B, C and D than in Group A. The decreasing rate of change in PLT was significantly higher in Groups B, C, and D compared to Group A (Fig. 1). In addition, the average ALT levels (≥ 15 IU/L) during the follow-up period were independently associated with thrombocytopenia (OR 3.882; 95% CI, 1.257–11.987;

Table 3 Univariate and multivariate analyses of variables associated with thrombocytopenia (PLT < 1.3 × 10^5/ul) in HD + HCV patients

Variables	Odds ratio	95% CI	*P* value
Univariate analysis			
Age (years)			
<60	1.0		
≥60	0.616	0.247–1.534	0.298
Sex			
Female	1.0		
Male	1.273	0.518–3.129	0.599
Duration of HD (years)			
<10	1.0		
≥10	1.321	0.555–3.141	0.529
Follow-up period (months)			
<55	1.0		
≥55	1.057	0.445–2.515	0.899
History of diabetes mellitus			
−	1.0		
+	1.426	0.557–3.646	0.459
Serotype			
I	1.0		
II	1.051	0.384–2.871	0.923
AST (IU/L)			
<30	1.0		
≥30	3.4	0.676–17.103	0.138
ALT (IU/L)			
<20	1.0		
≥20	2.686	1.083–6.662	0.033
GGT (IU/L)			
<50	1.0		
≥50	4.333	1.235–15.206	0.022
TC (mg/dl)			
<150	1.0		
≥150	0.727	0.27–1.958	0.528
Multivariate analysis			
ALT (IU/L)			
<20	1.0		
≥20	1.972	0.665–5.847	0.221
GGT (IU/L)			
<50	1.0		
≥50	3.305	0.876–12.467	0.078

Abbreviations as in Table 1

$P = 0.018$) by multivariate analysis using two variables including average ALT levels and GGT at baseline. The average ALT levels were also associated with decreased PLT (OR 4.470; 95% CI, 1.571–12.719; $P = 0.005$) by multivariate analysis using average ALT levels and sex. These results indicate that the clinical course of ALT levels is associated with thrombocytopenia and a decrease in PLT in patients with HCV.

Demographics of HD patients with HCV who were treated with UDCA

We enrolled 16 HD patients with HCV who were treated with 300 mg/day UDCA orally for more than 3 months in August 2008, and compared these patients (UDCA group) to 84 HD patients with HCV who were not treated with UDCA (non-UDCA group). The UDCA group and non-UDCA group showed similar demographics in regard to age, sex, HCV RNA levels, distribution of HCV serotype, GGT and PLT. The UDCA group, however, had a shorter duration of dialysis and higher AST and ALT levels just before UDCA administration compared to those in the non-UDCA group in May 2008 (Table 5).

Efficacy of UDCA in HD patients with HCV

After administering UDCA, percent of ALT and AST significantly decreased after one month and remained constant up to 6 months compared to the non-UDCA group (Fig. 2). Percent of GGT also significantly decreased after 2 months of UDCA treatment compared to the non-UDCA group. In addition, ALT, AST and GGT levels significantly decreased after UDCA treatment compared to levels before treatment, but PLT did not change during the 6 months of UDCA treatment (Fig. 2). In contrast, serum AST, ALT, GGT and PLT in the non-UDCA group did not change during the 6-month period from May 2008 to November 2008.

Discussion

Our study indicated that HD patients persistently infected with HCV are at risk for thrombocytopenia (less than 1.3 × 10^5/μL) and a decrease in PLT (more than 20%), although the exact dates of HCV infection were not clear in our study population. In addition, the basal or clinical course of ALT levels appears to predict decreased PLT or thrombocytopenia in patients with HCV. In this study population, the prevalence [243 anti-HCV positive among 2539 HD patients (9.6%)] and age distribution (average age was 63 years old) of anti-HCV antibody-positive subjects and the frequency of the HCV serotype I (74%) were similar to previous reports on HD patients with HCV in Japan [24–26], suggesting that the clinical course of anti-HCV-positive subjects in this study reflects those in Japan as a whole.

It is known that patients on HD often have thrombocytopenia [27], and there is a negative correlation between the dialysis period and PLTs [27, 28]. It was also reported that megakaryocytes are produced at lower levels in the bone marrow [28], platelets are destroyed due to the

Table 4 Baseline characteristics of four groups of HD patients with HCV according to the clinical course of average ALT levels

Average ALT	A; ALT < 15	B; 15 ≤ ALT < 20	C; 20 ≤ ALT < 30	D; 30 < ALT	*P* value
Number	30	19	18	17	
Age (years)	67.8 ± 10.8	60.8 ± 10.6	64.0 ± 9.7	63.1 ± 8.7	0.105
Sex male/female	11/19	15/4	14/4	14/3	0.001
Duration of dialysis (years)	14.4 ± 10.7	14.2 ± 9.2	12.8 ± 8.8	11.7 ± 9.1	0.945
Follow-up period (months)	53.2 ± 14.3	55.4 ± 16.4	64.2 ± 16.0	57.5 ± 16.3	0.290
HCV-RNA (Log IU/mL)	4.9 ± 1.6	4.8 ± 1.3	5.2 ± 1.2	4.8 ± 1.4	0.774
HCV Serotype (I/II/undetermined)	21/7/2	13/6/0	13/4/1	12/4/1	0.949
History of diabetes mellitus (-)/(+)	23/7	12/7	12/6	10/7	0.592
AST (IU/L)	15.0 ± 4.7	19.8 ± 8.6	22.8 ± 9.8	24.9 ± 8.0	<0.001
ALT (IU/L)	10.4 ± 4.1	19.3 ± 6.8	22.3 ± 8.0	27.8 ± 7.9	<0.001
GGT (IU/L)	21.3 ± 15.2	34.8 ± 22.1	81.2 ± 71.2	48.5 ± 35.2	<0.001
TC (mg/dl)	149.7 ± 31.4	152.3 ± 46.1	154.9 ± 37.0	161.2 ± 57.2	0.970
PLT (× 10^5/μl)	1.62 ± 0.55	1.62 ± 0.61	1.46 ± 0.42	1.64 ± 0.51	0.764

Abbreviations as in Table 1

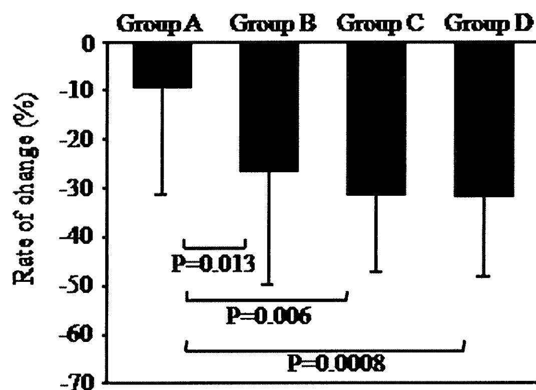

Fig. 1 Comparison of the rate of change in platelet counts by average alanine aminotransferase (ALT) levels during the follow-up period. Group A, average ALT < 15; Group B, 15 ≤ average ALT < 20; Group C, 20 ≤ average ALT < 30; Group D, 30 ≤ average ALT

Table 5 Demographics of HD patients with HCV who were treated with UDCA

	UDCA[a]	Non-UDCA[b]	*P* value
Number	16	84	
Age (years)	66.4 ± 8.6	69.2 ± 10.2	0.261
Sex male/female	9/7	54/30	0.743
Duration of dialysis (years)	6.5 ± 6.6	18.2 ± 9.9	<0.001
HCV-RNA	4.1 ± 2.6	4.9 ± 1.4	0.918
Serotype (I/II/undetermined)	12/4/0	59/21/4	0.669
AST (IU/L)	30.2 ± 24.2	19.2 ± 10.2	0.008
ALT (IU/L)	25.3 ± 16.9	17.1 ± 9.9	0.004
GGT (IU/L)	32.3 ± 23.4	41.4 ± 39.1	0.793
PLT (× 10^5/μl)	1.55 ± 0.56	1.39 ± 0.56	0.577

Abbreviations as in Table 1

[a] Data was obtained at just before the treatment period

[b] Data was obtained in May 2008

appearance of the anti-platelet antibodies [28, 29] and uremic materials reduce the effects of hemopoietic cells [30]. In our study, PLT in HD patients without HCV was significantly decreased after 62.2 months (−5.3%). However, PLT decreased even more dramatically in HD patients with HCV after 56.7 months (−22.4%) compared to patients without HCV. In addition, persistent HCV infection was independently associated with thrombocytopenia and a decrease in PLT in HD patients by a multivariate analysis, but dialysis period was not associated with those. Although the data regarding liver histology and serum markers of hepatic fibrosis were lacking in our study, it has also been reported that severe hepatic fibrosis is associated with thrombocytopenia in HCV carriers with end-stage renal disease [19]. These results suggest that thrombocytopenia is more associated with HCV viremia than with the HD procedure or dialysis period in HD patients.

Hepatocellular carcinoma (HCC) and hepatic failure are critical complications in HCV patients, even in those undergoing HD [10, 31]. These complications occur more frequently in patients with advanced hepatic fibrosis [32, 33]. It has been reported that hepatic fibrosis can be predicted by thrombocytopenia in chronic hepatitis C patients with or without HD [19, 34]. In addition, hepatitis is usually assessed by ALT levels, and changes in ALT levels have been shown to be the most important factor that affects hepatic fibrosis in chronic hepatitis C patients without HD [11, 12]. In this study, we showed that basal ALT levels are associated with thrombocytopenia by a univariate analysis and with decreased PLT by a multivariate analysis. The clinical

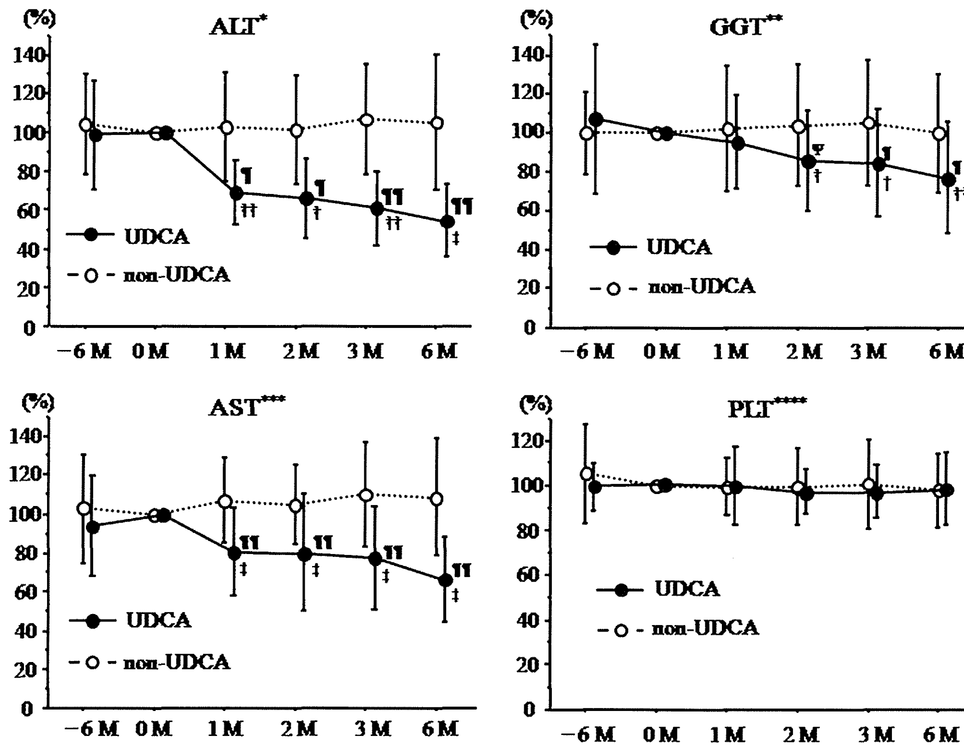

Fig. 2 Efficacy of ursodeoxycholic acid (UDCA) in hemodialysis (HD) patients with hepatitis C virus. Percent of ALT, AST, GGT and PLT in the UDCA group ($n = 16$) 6 months (−6 M) or just (0 M) before and during the treatment period [1, 2, 3 or 6 months (M)] compared to patients in the non-UDCA group ($n = 84$ excluding 6 M) in December 2007 (−6 M), May 2008 (0 M), June (1 M), July (2 M), August (3 M) or November 2008 (6 M, $n = 82$; two patients died before November 2008). Data are expressed as mean ± standard deviation. Closed (*black*) and open circles indicate the UDCA group and non-UDCA group, respectively. The percent of ALT was calculated according to the formula: %ALT = (ALT[−6 M, 0 M, 1 M, 2 M, 3 M or 6 M]/ALT[0 M]) × 100. *ALT* alanine aminotransferase, *AST* asparate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *PLT* platelet count. $^{\Psi}P < 0.05$ (UDCA vs. non-UDCA). $^{¶}P < 0.01$ (UDCA vs. non-UDCA), $^{¶¶}P < 0.001$ (UDCA vs. non-UDCA), $^{\dagger}P < 0.05$ (vs. 0 M), $^{\dagger\dagger}P < 0.01$ (vs. 0 M), $^{\ddagger}P < 0.001$ (vs. 0 M)

course of ALT is also associated with these clinical changes. These results indicate that ALT is an important predictor of thrombocytopenia which should be associated with hepatic fibrosis in HD patients with HCV. In contrast, serum ALT levels are significantly lower in chronic hepatitis C patients on HD than in chronic hepatitis C patients with normal renal function [19]. It was reported that a vitamin B6 deficiency [35], uremic toxins [36], or ultraviolet-absorbing materials [37] are associated with low ALT levels in HD patients. Furthermore, ALT levels have been reported to predict liver disease-related deaths in HD patients, even when ALT levels are in the normal range [38, 39]. Our study also revealed that both patients with abnormal ALT levels (Group D) and normal ALT levels close to the ULN (Groups B and C) had a significant decrease in PLT compared to patients with low ALT levels (Group A). These findings suggest that ALT levels can be used to assess liver damage in HD patients with HCV, although the normal range of ALT should be determined in those patients with HCV in a large cohort study or by liver biopsy.

HCV carriers with persistently normal ALT (PNALT) are more often females than chronic hepatitis C patients with abnormal ALT [40]. This distinction is likely due to lifestyle differences such as alcohol consumption [40], hormonal factors [41] or lower serum iron levels [42]. Although the normal range of ALT in HD patients with HCV may be different compared to the range in HCV carriers with normal renal function, our study demonstrated that females are more likely to have lower ALT levels, even in HD patients (Table 4). This difference in sex may also affect the decrease in PLT. In contrast, the frequency of serotype II, which is reportedly higher in PNALT patients than in chronic hepatitis C patients with abnormal ALT [43], was not different between the four groups in this study (Table 4). A further analysis of the factors associated with elevated ALT levels in those patients with HCV is required.

Interferon therapy has been shown to improve hepatic fibrosis [44] and to reduce the occurrence of HCC in chronic hepatitis C patients with normal renal function. Compared to untreated patients, the risk of HCC after interferon treatment in patients who did not achieve a virological response was shown to be 0.20, 0.36 and 0.91 in chronic hepatitis C patients whose ALT levels were

normal, moderately elevated (less than twice the upper normal limit) and highly elevated, respectively [45]. These results indicate that ALT might predict the mortality of patients with liver-related diseases who have or have not received interferon treatment. Although lower serum ALT levels decreased the risk of HCC, biochemical and virological responses were limited [20, 46] and HD was one of the factors associated with patients who did not respond to interferon and ribavirin treatment [21, 22]. Other therapies that lower serum ALT levels but do not involve interferon-based treatment need to be investigated. Recently, it has been established that UDCA up to 900 mg/day dose-dependently improves biochemical indices such as serum ALT, GGT and bilirubin [23]. Although UDCA seems to lower serum ALT levels, the risk of liver fibrosis, and possibly the incidence of hepatocellular carcinoma, liver histology, serum hepatic fibrosis markers and prognosis (including the incidence of HCC) should also be evaluated over a long time period in HCV carriers with or without HD.

Our study had several limitations; a small number of patients was simply treated with UDCA as routine care, selection of patients depended on each physician and then the data collected retrospectively after a specified duration of therapy. However, this study showed that UDCA effectively had reduced serum ALT, AST and GGT levels in HD patients with HCV. Interestingly, UDCA decreased ALT levels even in patients with normal ALT levels less than 30 IU/L (data not shown). Therefore, HCV patients with normal ALT levels should also be considered for the indication of treatment.

Although the patients in this study were treated with 300 mg/day UDCA, it has also been reported that a 600 mg/day dose of UDCA more effectively decreases ALT and AST levels than a 150 mg/day dose in chronic hepatitis C patients with normal renal function [23]. In addition, PLT did not change during UDCA treatment. Future studies need to investigate the dose-dependent effects of UDCA on ALT levels and prospective double-blind UDCA treatment over a long period in HD patients with HCV.

In conclusion, HCV viremia and ALT levels at basal conditions and during the clinical course of disease were associated with thrombocytopenia and decreased PLT in HD patients. We recommend that HCV carriers on HD who have ALT levels greater than 15 IU/mL be considered for treatment. In addition, UDCA should be considered for HD patients who have chronic hepatitis due to HCV infection but cannot receive interferon-based therapy.

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MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Animal model for study of human hepatitis viruses

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Key words
hepatitis B virus, hepatitis C virus, uPA/scid mouse model.

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Abstract

Human hepatitis B virus (HBV) and hepatitis C virus (HCV) infect only chimpanzees and humans. Analysis of both viruses has long been hampered by the absence of a small animal model. The recent development of human hepatocyte chimeric mice has enabled us to carry out studies on viral replication and cellular changes induced by replication of human hepatitis viruses. Various therapeutic agents have also been tested using this model. In the present review, we summarize published studies using chimeric mice and discuss the merits and shortcomings of this model.

Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are pathogens that cause chronic infection in humans. There are 360 million and 170 million people infected worldwide with HBV or HCV, respectively.[1,2] Infected individuals develop acute hepatitis, chronic hepatitis and liver cirrhosis. The viruses are also important causative agents of hepatocellular carcinoma, especially in the Asia–Pacific region.[3] Study of the biology and development of therapies for each virus has long been hampered by the lack of a small animal model that supports hepatitis virus infection. This is probably as a result of the lack of receptor molecules necessary for viral infection in animal liver cells.

Transgenic mice that express over-length HBV-DNA export viral particles into the serum,[4] and such animals can be used to evaluate antiviral agents,[5–7] as well as HBV-targeted siRNA[8]. However, the virus life cycle is not established in this model, and it is inappropriate for studying drug-resistant HBV strains. Accordingly, researchers attempted to transplant human hepatocytes into mice. The development of the trimera mouse was one such attempt, in which human hepatocytes were transplanted under the kidney capsule of immune-deficient mice after lethal irradiation.[9,10] However, the number of hepatocytes that could survive on the kidney capsule was small, and normal liver architecture was not present. Although 85% of HBV-inoculated animals developed HBV viremia, the titer was less than 10^5 virus particles or IU/mL.[9] Similarly, 85% of HCV-inoculated animals also developed viremia,[10] but the level of the viremia only reached 10^5/mL. Thus, the advent of human hepatocyte transplanted uPA/scid mice has provided the first really useful model for acute and chronic infections of human hepatitis virus.

Human liver cell transplanted uPA/scid mice

Transgenic mice in which the urokinase gene is driven by the human albumin promoter/enhancer were developed and shown to have accelerated hepatocyte death and consequent chronic stimulation of hepatocyte growth.[11] Transplanted rat hepatocytes proliferated and repopulated injured livers in immunodeficient uPA mice, which were produced by mating uPA transgenic mice with scid mice.[12] Human hepatocytes were then transplanted into uPA/scid mice; these cells proliferated and replaced the apoptotic mice liver cells (Fig. 1).

Such human hepatocyte chimeric mice have been shown to be susceptible to both HBV[16] and HCV[17] infections. Repopulation levels by human hepatocytes have been estimated by measuring human albumin levels in mouse serum. Replication levels of both HBV[13] and HCV[17] were higher in mice in which the repopulation index was higher. A unique attempt to remove mouse residual liver cells with the herpes simplex virus type-1 thymidine kinase (HSVtk)/ganciclovir (GCV) system failed to result in a higher repopulation rate as a result of damage to the transplanted human hepatocyte caused by bystander effects.[18] Despite this, mice with livers that have been highly repopulated with human hepatocytes

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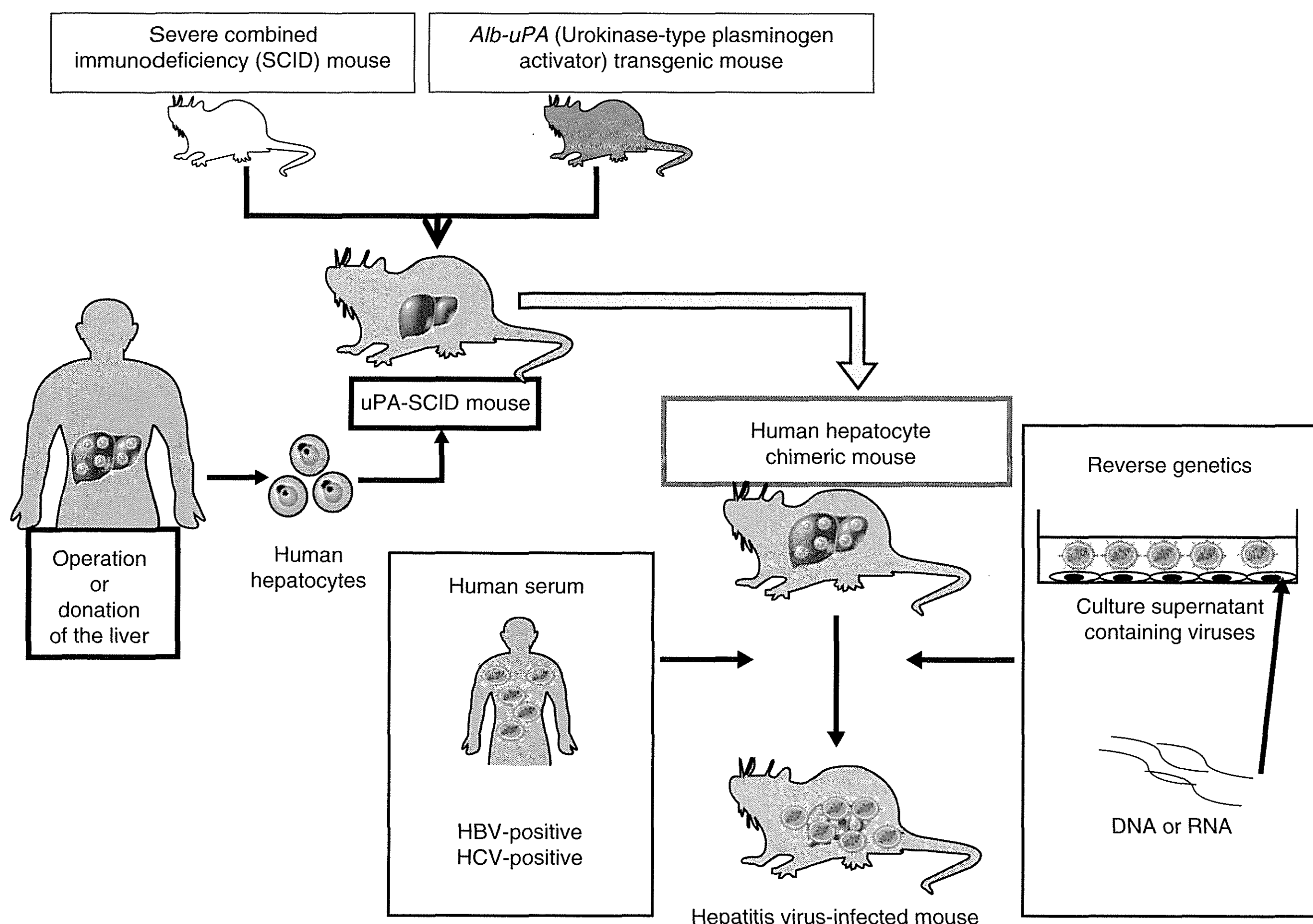

Figure 1 Generation of human hepatocyte chimeric mice and hepatitis virus infection model. A uPA/scid mouse was created by mating uPA transgenic mouse and scid mouse. Human hepatocytes obtained by surgical resection or donation were transplanted to newborn mice. The chimeric mice can be infected with hepatitis B virus (HBV) or hepatitis C (HBC) virus by injecting human serum containing these viruses. Alternatively, the mice can be infected by HBV[13] or HCV[14] created in cell culture or by injecting HCV RNA into the mouse liver.[15]

are susceptible to infection with both HBV and HCV, and as such comprised the most effective small animal model for chronic hepatitis so far developed.[19,20] An example of a highly repopulated mouse liver that we are using in experiments is shown in Figure 2.

Highly repopulated mice have been shown to be a valuable model for the study of drug metabolism.[21–29] Advances in technology for human hepatocyte transplantation have enabled serial passage of human hepatocytes in uPA/scid mice and have been shown to retain infectivity for HBV.[30]

This mouse model and other animal models for the study of hepatitis viruses have been summarized in reviews by Meuleman and Leroux-Roels,[31] Dandri *et al.*,[32,33] Barth *et al.*,[34] and Kneteman and Toso.[35] The present review will focus on key issues and updated information.

Study of hepatitis B virus infection using human hepatocyte chimeric mice

Since the initial reports of successful transmission of HBV to human hepatocyte chimeric mice in 2001 and 2004,[16,27] several researchers have reported transmission of HBV into similar mice.[13,36,37] In these studies, passage experiments studies show that HBV replicating in mice retain infectivity.[13,36] Further, the presence of viral proteins has been shown immunohistochemically in human hepatocytes transplanted into mouse livers, but these are not present in mouse hepatocytes.[13,36,37] Formation of viral particles in infected mouse livers can be shown by electron microscopy.[36,37] Genetically engineered viruses lacking HBe-antigen have also been shown to infect chimeric mice, proving that e antigen is dispensable for viral infection and replication.[13] In contrast, HBx protein has been shown to be indispensable for viral replication.[38] Transcomplementation of HBx protein with hydrodynamic injection restored HBV infectivity in mice. Interestingly, all revertant viruses show a restored ability to express HBx.[38]

By infecting chimeric mice with genotype A, B and C, differing proliferative capacity has been shown between HBV genotypes.[37] In mice infected for a relatively short time, there are no morphological changes in HBV infected mice livers in studies.[13,36] In contrast, the occurrence of liver cell damage has been reported after long-term infection of chimeric mice with HBV[39] or with specific strains of HBV;[40] these findings are consistent with direct cytopathic effects of HBV under certain conditions.

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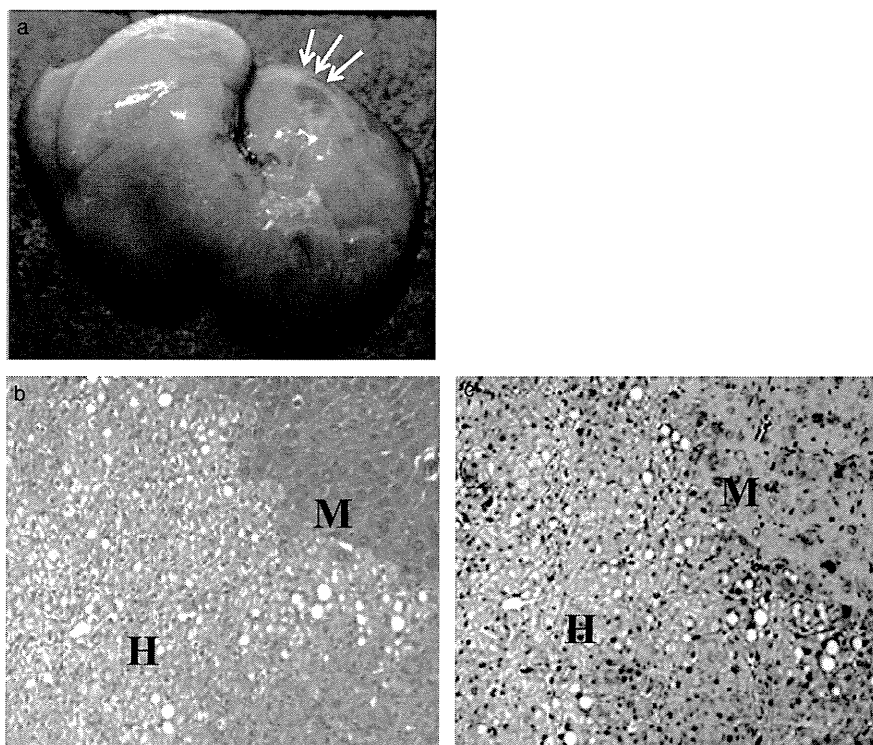

Figure 2 Representative uPA/scid mouse livers repopulated by human hepatocytes. (a) Mouse liver almost completely repopulated by human hepatocytes. Only a small portion of mouse hepatocytes are shown by arrows. (b) Microscopic figure of the mouse liver. M and H indicate regions consisting of mouse and human hepatocytes, respectively (Hematoxylin–eosin staining, magnification: ×100). (c) Microscopic figure of the mouse liver stained with antibody directed against human serum albumin.

The biological properties of a newly identified unique strain of HBV, genotype G, which replicates only in the presence of another genotype, were confirmed using the chimeric mouse.[41] Infectivity of another novel HBV strain, identified from a Japanese patient, that is divergent from known human and ape HBV has also been confirmed.[42] Titration of HBV infectivity, which previously could only be carried out using chimpanzees, can be carried out effectively using chimeric mice.[43]

Taking advantage of the absence of human immune cells in the chimeric mice, Noguchi *et al.*[44] showed that hypermutation of HBV increases in human hepatocytes under interferon treatment. Dandri *et al.* measured viral half-life in human and chimeric mice repopulated with wooly monkey hepatocytes.[45] The results clearly showed that viral half-life is shortened by immunological mechanisms in humans with low viral levels, but not in chimeric mice where functional immunity is absent. Hiraga *et al.*[46] showed an absence of interference between HBV and HCV.

Evaluation of therapeutic agents is the most important role for this mouse model. Tsuge *et al.*[13] assessed the effect of interferon and lamivudine using chimeric mice. Similarly, Dandri *et al.*[47] showed the effects of adefovir using uPA/scid mice repopulated with tupaia hepatocytes, which also support replication of human HBV. Oga *et al.*[48] identified a novel lamivudine-resistant variant that has an amino acid substitution outside of the YMDD motif. They showed that lamivudine was ineffective against the novel mutant strain. It is thus apparent that this mouse/human liver chimeric model is ideal to study the susceptibility of mutant strains to various drugs, because mutant viruses can easily be made and infected into chimeric mice.[13] The model has also been utilized to evaluate viral entry inhibitors derived from the large envelope protein.[49]

Study of hepatitis C virus using human hepatocyte chimeric mice

As observed in studies on HBV, HCV infection efficiency was poor and levels of viremia were low in mice where the repopulation rate of the mouse liver with human hepatocyte was low.[17,50] As shown in Figure 3, human albumin levels in mouse serum were significantly higher in mice in which measurable viremia developed (Hiraga *et al.* unpublished data). Recent studies have therefore been carried out using highly repopulated mice. The usefulness of a newly developed HCV assay,[51] and infectivity of a newly identified intergenotypic recombinant strain,[52] have been reported using the chimeric mice.

Using the remarkable replication ability of the JFH1 genotype 2a strain,[53] infectivity of JFH1 or intergenotypic chimeric viral particles, previously shown in cell culture, has now been shown to be infectious in chimeric mice.[54–56] Infectivity of viruses that were replicated in chimeric mice in cell culture has also been shown, and virus fitness has been studied.[55,56] The role of the HCV core+1 open reading frame and core *cis*-acting RNA elements has also been examined using the chimeric virus.[57] These elegant studies have the limitation that the non-structural part of the virus is limited to that of JFH1. Hiraga *et al.*[14] have shown that infectious clones of genotype 1a and JFH1 can be infected with direct injection of *in vitro* transcribed RNA into the mouse liver.[14] Similarly, Kimura *et al.*[15] reported the establishment of infectious clones of genotype 1b and ablation of RNA polymerase by site-directed mutagenesis abolish infectivity. These infectious clones will be useful for the study of drug-resistant strains.

The model of HCV infection has also been used to show that infection of the virus can be prevented by antibodies against

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Table 1 New therapeutic strategies tested by human hepatocyte chimeric mice

n	Drug or cell	Strategy	Reference
1	Interferon alpha 2b	Activation of antiviral genes	Kneteman *et al.*[65]
	BILN-2061	NS3-4A protease inhibition	
	HCV371	NS5B polymerase inhibition	
2	Modified BID	Induction of apoptosis	Hsu *et al.*[66]
3	Serine palmitoyltransferase inhibitor	Disruption of lipid raft	Umehara *et al.*[67]
4	Lymphoblastoid interferon alpha	Activation of antiviral genes	Hiraga *et al.*[14]
5	Amphipathic DNA polymers	Blocking viral entry	Matsumura *et al.*[60]
6	Sec-butyl-analogue of HCV-371	NS5B polymerase inhibition	LaPorte *et al.*[68]
7	HCV796	NS5B polymerase inhibition	Kneteman *et al.*[69]
8	Liver allograft-derived lymphocyte	Adoptive immunotherapy	Ohira *et al.*[70]
9	Telaprevir	NS3-4A protease inhibition	Kamiya *et al.*[71]

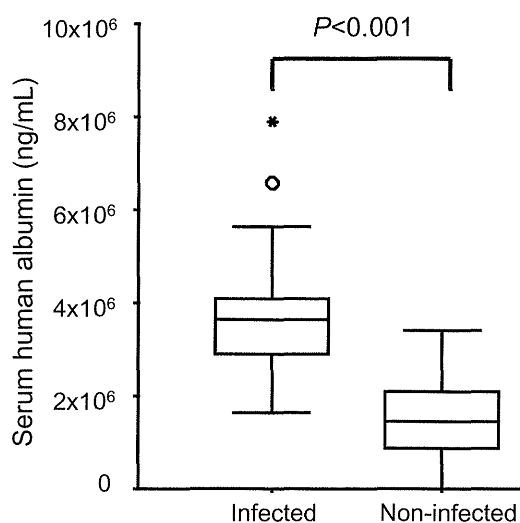

Figure 3 Human albumin levels in mice used in the hepatitis C virus (HCV) infection experiments. A total of 54 mice were injected with HCV positive serum samples containing 5×10^5 virus particles. A total of 24 mice became persistently positive for HCV-RNA, but 30 mice did not. Serum human albumin levels 2 weeks after human hepatocyte transplantation were compared between infected and non-infected mice.

CD81,[58] polyclonal human immunoglobulin directed to a similar strain,[59] and amphipathic DNA polymers.[60] Notably, the presence of broadly neutralizing antibodies to HCV that protect against heterologous viral infection has been reported, suggesting the possibility of a prophylactic vaccine against HCV.[61]

With respect to evasion of the virus against the innate immune response, altered intrahepatic expression profiles in the early phase of infection is of particular interest. The chimeric mice model is ideal for such studies; cross-hybridization of mouse and human can be avoided by careful experimental procedures.[62] Microarray analysis of livers of HCV infected and non-infected mice showed transcriptional activation of genes related to innate immune response, lipid metabolism, endoplasmic reticulum (ER) stress and apoptosis in HCV-infected mice.[63,64] The HCV infected mouse model is particularly useful for the study of newly developed HCV agents. The effect of recently developed chemicals and a unique therapy using intrahepatic lymphocytes have been shown using this model (Table 1). However, none of these therapies have yet been able to completely eradicate HCV from mice. It is noteworthy that ultra-rapid cardiotoxicity has been reported with the protease inhibitor BILN 2061 in the uPA/scid mice, but not in scid mice, implicating involvement of the uPA transgene.[72] Care should therefore be taken in interpreting the results obtained by this model.

Conclusion

Development of a small animal model using human hepatocyte chimeric mice has enabled us to study key aspects of HBV and HCV biology. The characteristic feature of the absence of human immune cells is suitable for studying viral replication and observing changes occurring in liver cells during viral infection, such as the innate immune response and cellular stress and metabolic responses. The model is also useful for studying the effect of drugs without the influence of cytokines and cytotoxic T lymphocytes. Nonetheless, the model is insufficient to study carcinogenesis of hepatitis viruses, because non-parenchymal cells in mouse liver are of mouse origin and do not support inflammation and fibrosis, which are probably closely related to carcinogenesis. The lack of human immune cells also limits the study of inflammation and immunity. Furthermore, the availability of human hepatocytes is limited. Despite these limitations, the current model shows great potential as a mouse model for the study of hepatitis viruses. Development of a small animal model with or without human immunity using stem cells or iPS cells would be an ideal model in the future.

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Impact of Radiation and Hepatitis Virus Infection on Risk of Hepatocellular Carcinoma

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In cohort studies of atomic bomb survivors and Mayak nuclear facility workers, radiation-associated increases in liver cancer risk were observed, but hepatitis B virus (HBV) and hepatitis C virus (HCV) infections were not taken strictly into account. We identified 359 hepatocellular carcinoma (HCC) cases between 1970 and 2002 in the cohort of atomic bomb survivors and estimated cumulative incidence of HCC by radiation dose. To investigate contributions of radiation exposure and hepatitis virus infection to HCC risk, we conducted a nested case-control study using sera stored before HCC diagnosis in the longitudinal cohort of atomic bomb survivors. The study included 224 HCC cases and 644 controls that were matched to the cases on gender, age, city, and time and method of serum storage, and countermatched on radiation dose. The cumulative incidence of HCC by follow-up time and age increased significantly with radiation dose. The relative risk (RR) of HCC for radiation at 1 Gy was 1.67 (95% confidence interval: 1.22-2.35) with adjustment for alcohol consumption, body mass index (BMI), and smoking habit, whereas the RRs for HBV or HCV infection alone were 63 (20-241) and 83 (36-231) with such adjustment, respectively. Those estimates changed little when radiation and hepatitis virus infection were fit simultaneously. The RR of non-B, non-C HCC at 1 Gy was 1.90 (1.02-3.92) without adjustment for alcohol consumption, BMI, or smoking habit and 2.74 (1.26-7.04) with such adjustment. *Conclusion:* These results indicate that radiation exposure and HBV and HCV infection are associated independently with increased HCC risk. In particular, radiation exposure was a significant risk factor for non-B, non-C HCC with no apparent confounding by alcohol consumption, BMI, or smoking habit. (Hepatology 2011;53:1237-1245)

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) are recognized as critically important risk factors for HCC. Our previous study actually showed that about 63% of HCC in atomic bomb survivors is related to HCV infection, 14% to HBV infection, and 2% to both HBV and HCV infections.[1] However, an increase of non-B, non-C HCC without HBV and HCV infection has been noted recently in Japan.[2,3] The etiology of non-B, non-C HCC has been poorly understood, although alcoholic hepatitis, nonalcoholic fatty liver disease (NAFLD) including nonalcoholic steatohepatitis (NASH), and hemochromatosis[4,5] are known as risk factors. In Japan, NAFLD has increased along with Westernization of lifestyle, and most NASH cases have developed due to such lifestyle-related diseases such as obesity, diabetes mellitus, and hyperlipidemia.[6] Obesity and diabetes mellitus, as well as NAFLD, have also recently received increased attention as risk factors for HCC.[1,7-12]

Abbreviations: AHS, Adult Health Study; BMI, body mass index; CI, confidence interval; ERR: excess relative risk; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; RERF, Radiation Effects Research Foundation; RR, relative risk.

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An increased risk of liver cancer with radiation dose among atomic bomb survivors has been reported based on tumor registries, mortality studies, and pathology review,[13-16] but hepatitis virus infection status was not taken into account. In three previous HBV studies at the Radiation Effects Research Foundation (RERF), the HBV surface antigen (HBsAg)-positive proportion increased with radiation dose.[17-19] Previous research at RERF demonstrated no increase in the prevalence of anti-HCV antibody (anti-HCV Ab) with radiation dose,[20] but reported supermultiplicative effects between radiation exposure and chronic HCV infection in the etiology of HCC without cirrhosis.[21]

On the other hand, the cohort study in workers at the Mayak nuclear facility demonstrated that the risk of liver cancer mortality was significantly associated with plutonium exposure,[22] and that the incidence of HCC was marginally significantly associated with plutonium exposure.[23] In the latest analysis, a significant plutonium dose-response relationship was observed for liver cancer mortality, with risk reasonably described by a linear function.[24] However, liver cancer in those analyses included hepatoblastoma and intrahepatic cholangiocarcinoma as well as HCC. In addition, hepatitis virus infection status was not taken into account in a strict and in-depth manner, although HCC accounted for most of the liver cancer.

A lifespan study using B6C3F1 mice exposed to continuous low-dose-rate γ rays demonstrated that the incidence of HCC was significantly increased in male mice exposed to total doses equivalent to 8,000, 400, and 20 mGy and in females exposed to 8,000 mGy. However, the incidence of other liver tumors did not significantly increase except for that of hepatoblastoma in males exposed to 400 mGy.[25]

With the aim of determining whether radiation exposure is an independent risk factor for HCC, even after adjusting for hepatitis virus infection, alcohol consumption, body mass index (BMI), and smoking habit, we conducted a nested case-control study among atomic bomb survivors using stored sera. We also evaluated whether radiation, alcohol consumption, increase of BMI, and smoking habit contribute to increased risk for non-B, non-C HCC.

Patients and Methods

Cohorts. The Atomic Bomb Casualty Commission (ABCC) and its successor, the RERF, established the Adult Health Study (AHS) longitudinal cohort in 1958, in which more than 20,000 gender-, age-, and city-matched proximal and distal atomic bomb survivors and persons not present in the cities at the time of bombings are examined biennially in outpatient clinics in Hiroshima and Nagasaki.

Cases and Controls. Incident cancer cases were identified through the Hiroshima Tumor and Tissue Registry and Nagasaki Cancer Registry, supplemented by additional cases detected by way of pathological review of related diseases.[26] As described in our previous study,[1] 359 primary HCC cases were diagnosed among 18,660 AHS participants between 1970 and 2002 who visited our outpatient clinics before their diagnosis. Of these, 229 cases had serum samples obtained within 6 years before HCC diagnosis. After excluding five cases with inadequate stored serum, 224 cases remained for our study. There were no important differences in characteristics such as gender, age at HCC diagnosis, city, alcohol consumption, BMI, or radiation dose to the liver (among exposed persons) between HCC cases excluded due to nonavailability of stored serum and those included in the present study.

Three control sera per case were selected from the at-risk cohort members matched on gender, age, city, and time and method of serum storage, and countermatched on radiation dose in nested case-control fashion.[27] Countermatching (to increase statistical efficiency for studying joint effects of radiation and other factors) was performed using four strata based on whole-body (skin) dose: zero dose (<0.0005 Gy), <0.05 Gy, <0.75 Gy, and ≥ 0.75 Gy (nonzero categories correspond roughly to tertiles of skin dose among all eligible exposed cases). At the time of each case diagnosis, one control serum was selected for each of the three dose strata not occupied by the case. Although the total number of potential matched control serum samples is 672, due to occasional lack of subjects with stored sera who met the matching and countermatching criteria, the total number of control serum samples actually selected was 644, which comprised 488 sera from unique noncase subjects and 156 sera from subjects sampled on repeated occasions.

Laboratory Tests. Virological assays were performed on 211 case and 640 control sera, because 13 case samples and four control samples had insufficient stored sera for these assays. HBsAg and antibody to hepatitis B core antigen (anti-HBc Ab) were measured by enzyme immunoassay (EIA), and anti-HCV Ab was measured by second-generation EIA as described.[28,29] Qualitative detection of HCV RNA among anti-HCV-positive samples was performed using a thermocycler (Whatman Biometra, Goettingen, Germany) based on the nested polymerase chain reaction (PCR) method, as described.[29] HBV infection (HBV+) status was

defined as positive for HBsAg or having a high titer of anti-HBc Ab. HCV infection (HCV+) status was defined as positive for HCV RNA. Non-B, non-C status was defined as negative for HBsAg and not having a high titer of anti-HBc Ab (HBV−) as well as negative for HCV RNA (HCV−).

Radiation Dose. Radiation dose to the liver was estimated for each subject according to Dosimetry System DS02.[30] A weighted sum of the gamma dose in gray plus 10 times the neutron dose in gray was used. Because of the countermatched selection of cases, direct comparison of doses between cases and controls in the study requires that control doses be weighted by the inverses of their selection probabilities.

Information on Alcohol Consumption, BMI, and Smoking Habit. Information on alcohol consumption was obtained from the 1965 AHS questionnaire when available, with missing data complemented using the 1978 mail survey. Alcohol consumption was quantified as volume of each type of alcoholic beverage; mean ethanol amounts were calculated as grams per day as described.[31] BMI (kg/m^2) was calculated from height and weight measured at the AHS examination. Subjects were classified based on BMI quintiles with cutpoints of 19.5, 21.2, 22.9, and 25.0. Following the recommendations for Asian people by the World Health Organization (WHO), the International Association for the Study of Obesity, and the International obesity Task Force,[32] 21.3 to 22.9 kg/m^2 was considered normal, 23.0 to 25.0 kg/m^2 as overweight, and >25.0 kg/m^2 as obese. We used information on BMI obtained 10 years before the time of HCC diagnosis or control matching because this condition is subject to change due to disease progression in the later stages before development of HCC. Information on smoking habit was obtained from the 1965 questionnaire; subjects were categorized as never, current (at time of survey), or former smoker.

Ethical Considerations. This study (RERF Research Protocol 1-04) was reviewed and approved by the Research Protocol Review Committee and the Human Investigation Committee of RERF.

Statistical Analyses. The nested case-control design was analyzed using a partial likelihood method analogous to that used for cohort follow-up studies,[33] which is in practice the same as the conditional binary data likelihood for matched case-control studies[34] except that the subjects (cases and "controls") in the study are not completely independent due to repeated selection. Cumulative incidence of HCC by follow-up time (year) and age was derived according to the method of Nelson and Aalen, using Cox regression to adjust for age at start of follow-up. Cumulative incidence by radiation dose groups (0-0.0009, 0.001-0.999, and 1.0+ Gray) was compared using the Gehan/Breslow generalized Wilcoxon test. All factors other than radiation were analyzed using relative risks (RRs) estimated by a log-linear model. Although radiation exposure could have been adjusted by matching on radiation dose as an additional matching factor in the control selection,[35] in addition to assessing effects of lifestyle factors and viral hepatitis, another purpose of the present study was to examine the effects of radiation exposure after adjustment for possible confounding and interaction by these factors, so matching on radiation—which precludes analysis of radiation risk—was not desirable; rather, we countermatched on radiation.[27,33,36] Radiation risk was analyzed using an excess relative risk (ERR) model (ERR = RR-1) as done previously.[37] The cumulative hazard estimator and comparisons by radiation dose groups were computed using Stata (StataCorp, College Station, TX; v. 11.1); all other analyses were conducted using Epicure (HiroSoft International, Seattle, WA; v. 1.81).

Results

Characteristics of Cases and Controls. Characteristics of the 224 HCC cases and 644 matched controls are shown in Table 1. HCC cases and controls were comparable with respect to gender, age, city, and time and method of serum storage by design. Prevalence of HBV and/or HCV infection status in HCC cases is higher than those in controls. Higher proportions of HCC cases had a history of alcohol consumption of more than 40 g of ethanol per day, were obese (BMI >25.0 kg/m^2), and were current smokers, compared with the controls. HCC cases also received on average higher radiation doses to the liver, compared with the controls.

Cumulative Incidence of HCC by Radiation Dose. Figure 1A,B shows the cumulative incidence of HCC by radiation dose using either follow-up time (adjusted for age at start of follow-up) or age. Of 359 HCC cases diagnosed among 18,660 AHS subjects between 1970 and 2002, the analysis was performed using 322 HCC cases, based on 16,766 subjects with known radiation dose. A significant increase with radiation dose was seen with cumulative incidence both by follow-up time (P = 0.028) (Fig. 1A) and by age (P = 0.0003) (Fig. 1B). The effect of radiation was especially evident at age 60 years or later.

Risk of HCC for Radiation and Hepatitis Virus Infection. Table 2 shows risk of HCC with and without adjustment for categorical alcohol consumption,