with 17% with 800 mg/day). These studies showed that either drug dose can be reduced for genotype 2 and 3 patients without compromising antiviral efficacy. In the present study, neither Peg-IFN nor ribavirin drug exposure participated in reaching RVR and SVR. In particular, more than 90% of patients having RVR achieved SVR regardless of the drug exposure level, as long as the mean Peg-IFN dose was over 0.5 μg/kg/week and ribavirin was over 5.0 mg/kg/day. The results of our study suggested that genotype 2 patients may receive reduced levels of both drug doses on the condition that they can complete the full 24-week course of combination therapy. Randomized, prospective trials that reduced both Peg-IFN and ribavirin should be conducted for CHC patients to clarify this.

In the present study, while the treatment outcome was independent of the individual ribavirin exposure in patients who had completed the 24-week treatment, the most common reason to withdraw the treatment was decreased haemoglobin because of ribavirin medication. Based on the results of randomized controlled trials [6], using a ribavirin dose of 800 mg/day is recommended for genotype 2/3 patients [1–3]. However, several studies have shown that some patients cannot tolerate even this suboptimal ribavirin dose. This is a serious problem for patients with the risk of anaemia, especially elderly patients. The ageing of patients is progressing around the world, requiring improvement in treatment tolerability. Recently, Andriulli *et al.* [25] examined the effect of ribavirin in a 12-week course of therapy on CHC genotype 2 patients with RVR in two groups, one continuing with ribavirin and the other receiving Peg-IFN alpha-2a alone after week 6. The relapse rates were higher (46% *vs* 17%; $P < 0.001$) and overall SVR rates were lower (54 *vs* 82%; $P < 0.001$) in patients who stopped receiving ribavirin at week 6. Thus, ribavirin medication throughout the treatment period is necessary to raise the SVR rate even in genotype 2 or 3 patients with RVR. In the present study, the ribavirin dose could be reduced without loss of efficacy for genotype 2 patients, as long as the patients were treated for 24 weeks. Therefore, in the patients with the risk of anaemia, it would be better to reduce the dose of ribavirin before anaemia arises rather than being forced to discontinue the combination therapy because of anaemia caused by ribavirin medication. We previously reported that in CHC patients treated by IFN or Peg-IFN in ribavirin combination therapy, a decline of haemoglobin concentration by 2 g/dL at the end of 2 weeks from the start of the treatment can be used to identify patients likely to develop severe anaemia [26,27]. This kind of predictive factor for the progression to severe anaemia can be of much help in reducing ribavirin with appropriate timing.

Our study has some limitations. First, it is a retrospective study, and we could not obtain complete information for all patients. However, this is the first study of Peg-IFN and ribavirin combination therapy in which the drug dose of Peg-IFN and ribavirin taken by each patient was assessed independently for HCV genotype 2 patients. Our results can be taken as an evidence offering suggestions for the treatment of CHC genotype 2 patients. Second, this cohort included patients with different histories of past IFN treatment. Patients who had failed to recover with previous IFN-based treatment were likely to experience treatment failure again [28]. Therefore, we examined the predictors of treatment response separately according to treatment history, and confirmed that in both naïve and treatment-experienced patients, the mean dose of Peg-IFN and ribavirin showed no correlation with SVR or RVR in both groups.

In conclusion, our study demonstrates that RVR is an important treatment predictor and more than 90% of patients having RVR achieve SVR with combination therapy of Peg-IFN and ribavirin for genotype 2 infected CHC patients regardless of the drug exposure. Further prospective, randomized studies are necessary to assess whether the standard or a reduced dose of each drug can produce equivalent outcomes.

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Reduced risk of hepatocellular carcinoma after interferon therapy in aged patients with chronic hepatitis C is limited to sustained virological responders

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SUMMARY. This study was undertaken to investigate the effect of interferon (IFN) monotherapy on the risk of hepatocellular carcinoma (HCC) in aged-patients with chronic hepatitis C. Seven hundred and twenty-five patients with histologically proven chronic hepatitis C were enrolled in this retrospective cohort study; 531 received IFN monotherapy for 6 months between 1992 and 1995, and 157 were collected as a historical control. The effect of IFN therapy on the development of HCC was compared between the patients with chronic hepatitis C under 60 years old (non-aged group, $n = 531$) and those 60 and over (aged group, $n = 194$). A stepwise Cox proportional-hazards regression analysis in the non-aged group revealed that IFN therapy (risk ratio 0.52, 95% CI 0.33–0.81, $P = 0.004$), older age ($P = 0.001$), and higher histological stage ($P < 0.001$) were independent factors associated with the development of HCC. In the aged-group, only higher histological stage ($P = 0.002$) and male gender ($P = 0.011$), but not IFN therapy (risk ratio 0.77, 95% CI 0.42–1.40, $P = 0.386$), were identified as independent risk factors for HCC, although HCC was significantly reduced when sustained virological response (SVR) was obtained (risk ratio 0.23, 95% CI 0.08–0.64, $P = 0.005$). In conclusion, inhibitory effect of IFN on development of HCC in the patients with chronic hepatitis C aged 60 and over was limited to the patients achieving SVR when treated with 6 months-IFN monotherapy.

Keywords: aged patients, chronic hepatitis C, hepatocellular carcinoma, interferon, sustained virological response.

INTRODUCTION

In Japan, based on the epidemiological surveillance as well as the study on molecular tracing of hepatitis C virus (HCV), HCV infection is considered to spread from the 1920s and to expand more after World War II [1–5], The data of first-time blood donor candidates in Osaka demonstrated that the prevalence of anti-HCV antibodies among the candidates born in 1925–1935 was 7–10%, which was much higher than the prevalence of anti-HCV antibodies among the younger population [6]. Accordingly, chronic hepatitis C patients have become aged in Japan and HCV-related hepatocellular carcinoma (HCC) patients have also been shown to be old with a peak around age 70 and tended to decrease [1,3,5]. More importantly, the main cause of death in the patients with chronic hepatitis C has been reported to be HCC [7–10].

In the 1990s, interferon (IFN) therapy was used for the treatment of the patients with chronic hepatitis C worldwide and it has been shown by many studies including our reports that IFN therapy reduced the risk of HCC in patients with chronic hepatitis C [7,11–17]. This inhibitory effect of IFN therapy on hepatocarcinogenesis is notable when sustained virological response (SVR) was obtained, although SVR rate of IFN monotherapy was not very high. It has been also

Abbreviations: IFN, interferon; HCC, hepatocellular carcinoma; SVR, sustained virological response; HCV, hepatitis C virus; non-SVR, nonsustained virological response.

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reported that HCC development was significantly reduced in the patient achieving SVR as compared with those without SVR in chronic hepatitis C patients treated with IFN and ribavirin [18].

For the treatment of the patients with chronic hepatitis C, a combination of peginterferon and ribavirin has become a standard therapy, which has a high SVR rate [19–21]. However, the combination treatment has several adverse effects such as haemolytic anaemia which may not be tolerable for aged patients with chronic hepatitis C. On the other hand, aging is a significant risk factor for HCC in chronic hepatitis C patients. Accordingly, it is an important issue whether IFN monotherapy could reduce incidence of HCC in aged patients with chronic hepatitis C. Recently, Arase *et al.* [22] reported that long-term IFN monotherapy using low-dose of natural IFN-α was effective in preventing hepatocarcinogenesis in aged patients with chronic hepatitis C. In contrast, the hepatitis C antiviral long-term treatment against cirrhosis (HALT-C) Trial has shown that maintenance peginterferon therapy for 3.5 years did not reduce the incidence of HCC and the rate of disease progression in chronic hepatitis C patients with bridging fibrosis or cirrhosis who failed to respond to the combination therapy of peginterfeon-α2a and ribavirin [23,24].

We conducted a long-term multicenter retrospective cohort study to clarify the effect of 6-month IFN monotherapy on the incidence of HCC in aged patients with chronic hepatitis C.

MATERIAL AND METHODS

Patients

This study was conducted at Osaka University Hospital and six university-affiliated hospitals. IFN-treated patients consisted of 568 consecutive patients with chronic hepatitis C who had undergone liver biopsy 1 week to 2 months before IFN therapy and received either human lymphoblastoid IFN, recombinant IFN-α2a or recombinant IFN-α2b for 6 months between 1992 and 1995. The control group consisted of 158 consecutive patients with chronic hepatitis or cirrhosis who had undergone liver biopsy between January 1986 and December 1989, when IFN therapy had not been available in Japan. All the patients were positive for anti-HCV. The inclusion criteria in this study were as follows: (1) histological diagnosis of chronic hepatitis or cirrhosis; (2) no history of clinical signs at entry into the study of complications of cirrhosis, i.e. ascites, jaundice, encephalopathy, or variceal bleeding; (3) no previous IFN therapy; (4) no evidence of HCC at entry into the study as assessed by ultrasonography and/or computed tomography; (5) absence of serum hepatitis B surface antigen; (6) absence of co-existing liver diseases such as autoimmune hepatitis or primary biliary cirrhosis and (7) absence of excessive alcohol consumption (>80 g/day).

Sustained virological response was defined as persistent HCV RNA negativity during IFN therapy and follow-up. Patients showing positive HCV RNA after IFN therapy were classified as nonsustained virological response (non-SVR). In the patients with non-SVR, patients whose ALT levels decreased to the normal range and remained normal during IFN therapy were classified as transient biochemical response and patients without a decrease of ALT levels of the normal range during the therapy were classified as zbiochemical nonresponse.

Hepatitis C virus antibody was measured by first-, second-, or third-generation enzyme-linked immunosorbent assays (Ortho Diagnostics, Tokyo, Japan). Serum HCV RNA was measured by reverse transcription polymerase chain reaction or complementary DNA assay [25].

Follow-up

The starting date of follow-up of the patients was defined as the date of liver biopsy. Abdominal ultrasonography or computed tomography and biochemical examinations including α-fetoprotein were performed every 3–6 months during follow-up equally in the IFN-treated and control patients. The diagnosis of HCC was confirmed by needle biopsy, by surgically resected tumour specimens, or by typical radiological findings on hepatic angiography or dynamic computed tomography. In the patients residing in Osaka whose follow-up data were not obtained, the Osaka Cancer Registry was used to determine whether HCC had occurred and the data were available until the end of 2002 in this study [13,26]. Accordingly, we decided to use the date of the development of HCC or the end of 2002 as the end of follow-up. As the longest observation period of the patients in the IFN group was 11 years, only the follow-up data for the first 11 years were considered in the control group. The study protocol was in accordance with the Helsinki Declaration of 1975 (revised in 1983) and approved by the Ethical Committee of the Ikeda Municipal Hospital.

Histological evaluation

The sections were stained with haematoxylin–eosin and Azan-Mallory and histology of liver biopsy specimens was scored by two authors in a blinded manner using two scoring methods as described before [13]. Briefly, fibrosis score of Desmet *et al.* was used for the assessment of histological staging and a total score of histological activity (components 1–3) using the Knodell histological activity index was used for the assessment of histological grading [13,27,28].

Statistical analysis

Patients who did not complete the treatment protocol were included for the analysis on an intention-to-treat basis. The chi-square test and Student's *t*-test were used to compare the

baseline characteristics. The Kaplan–Meier method was used to calculate the cumulative incidence of HCC, and the log-rank test was used to compare the cumulative incidence of HCC between the groups. To estimate independent risk factors for the development of HCC, a stepwise Cox proportional-hazards regression analysis was used. For the analysis, IFN therapy, age, gender, and histological staging and activity scores were used as variables. A P value <0.05 was considered statistically significant. Data are presented as the mean ± SD and were analysed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Table 1 shows the baseline characteristics of the aged (60 years old and over) and non-aged (under 60 years old) groups. Both the histological stage and activity were significantly higher in the aged group than in the non-aged group. The proportion of male patients of the non-aged group was significantly higher than that of the aged group. In Table 2, baseline characteristics of controls and IFN-treated patients in the aged and non-age groups were compared. In the non-aged group, age at entry, proportion of male gender, histological activity score, serum ALT level and platelet count did not differ between the control and IFN-treated patients. However, histological stage of IFN-treated patients was less advanced as compared with that of the control patients. In the age-group, age at entry, proportion of male gender, histological stage and activity, serum ALT level and platelet count did not differ between the control and IFN-treated patients.

During the follow-up period, HCC was found in 35 controls and 44 IFN-treated patients among the non-aged group and in 14 controls and 48 IFN-treated patients among the aged group. The median tumour sizes of HCC in controls and IFN-treated patients at the time of discovery on ultrasonography or computed tomography were 22 mm (range, 10–55 mm) and 19 mm (range, 8–52 mm) respectively ($P \geq 0.2$). In the non-aged group, the cumulative incidence of HCC estimated by the Kaplan–Meier Method of IFN-treated patients was significantly lower than that of control patients (log-rank test, $P < 0.001$, Fig. 1a), whereas there was no difference in the cumulative incidence of HCC between controls and IFN-treated patients in the aged group (log-rank test, $P = 0.498$, Fig. 1b). The cumulative incidence of HCC of SVR and non-SVR patients and controls of the aged and non-aged groups are shown in Fig. 2. The 10-year incidences of HCC for controls, non-SVR and SVR patients in the non-aged group were 30.1%, 15.8%, 4.5% respectively (log-rank test, $P < 0.001$, Fig. 2a). Also, the 10-year incidences of HCC for controls, non-SVR and SVR patients in the aged group were 39.1%, 38.9%, 12.7% respectively (log-rank test, $P = 0.015$, Fig. 2b).

In Table 3, risk ratios for the development of HCC calculated by a stepwise Cox regression analysis in the aged and non-aged patients with chronic hepatitis C according to virological and biochemical responses to IFN are summarized. In the 410 IFN-treated patients of non-aged group, 134 patients (32.7%) achieved SVR and the remaining 276 showed non-SVR (Table 3). Of this 276 patients showing non-SVR, 163 showed transient biochemical response and 113 showed biochemical nonresponse during the IFN treatment. On the other hand, 41 (25.9%) of 158 IFN-treated patients of the aged group obtained SVR and the other 117 did not obtain SVR (Table 3). Of the 117 non-SVR patients, 57 showed transient biochemical response and 60

Table 1 Baseline characteristics of aged and non-aged patients with chronic hepatitis C

	Non-aged group (n = 531)	Aged group (n = 194)	P value
Control group (n)/IFN group (n)	121/410	36/158	0.262
Age	48.1 ± 9.7	63.7 ± 3.3	<0.001
Gender			
Male	353	108	0.009
Female	178	86	
Histological stage*			
F0, 1	186	37	0.001
F2	157	69	
F3	141	69	
F4	47	19	
Histological activity†			
<10	329	104	0.049
≧ 10	202	90	
ALT (IU/L)	117 ± 86	104 ± 60	0.053
Platelete count ($10^4/\mu$L)	15.4 ± 5.6	14.4 ± 5.6	0.040

*According to Desmet *et al.*[27] †Based on components 1–3 of the Knodell histological activity.

Table 2 Baseline characteristics of controls and IFN-treated patients in aged and non-aged groups

	Non-aged group			Aged group		
	Controls	IFN-treated	*P* value	Controls	IFN-treated	*P* value
n	121	410		36	158	
Age	48.4 ± 10.5	48.0 ± 9.4	0.736	64.6 ± 3.6	63.5 ± 3.2	0.059
Gender						
Male	75	278	0.273	22	86	0.579
Female	46	86		14	72	
Histologic stage*						
F0,1	27	159	<0.001	8	29	0.933
F2	28	129		12	57	
F3	47	94		12	57	
F4	19	28		4	15	
Histologic activity†						
<10	72	257	0.525	20	84	0.854
≧ 10	49	153		16	74	
ALT (IU/L)	127 ± 80	114 ± 88	0.132	110 ± 85	103 ± 53	0.523
Platelete count ($10^4/\mu L$)	15.2 ± 6.1	15.4 ± 5.4	0.766	15.0 ± 5.4	14.3 ± 5.7	0.486
HCV RNA load						
High	ND‡	166		ND‡	54	
Low	ND‡	116		ND‡	30	
HCV RNA serotype						
1	ND‡	231		ND‡	90	
2	ND‡	102		ND‡	32	

*According to Desmet *et al.* [27] †Based on components 1–3 of the Knodell histologic activity. ‡Not done.

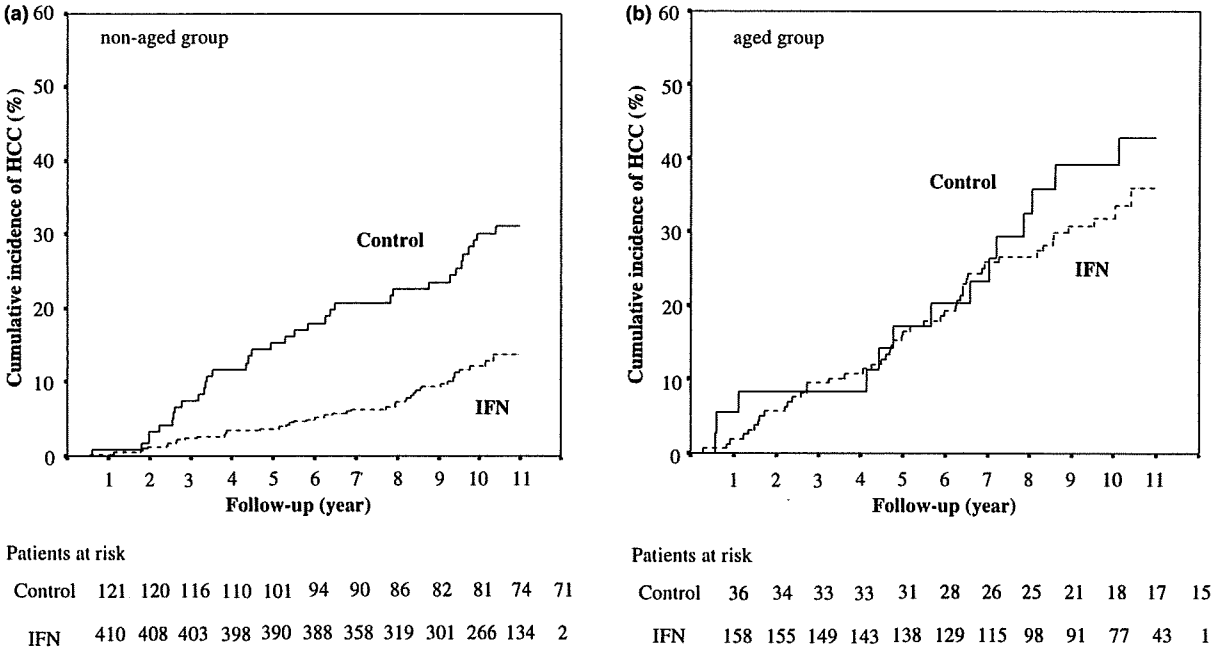
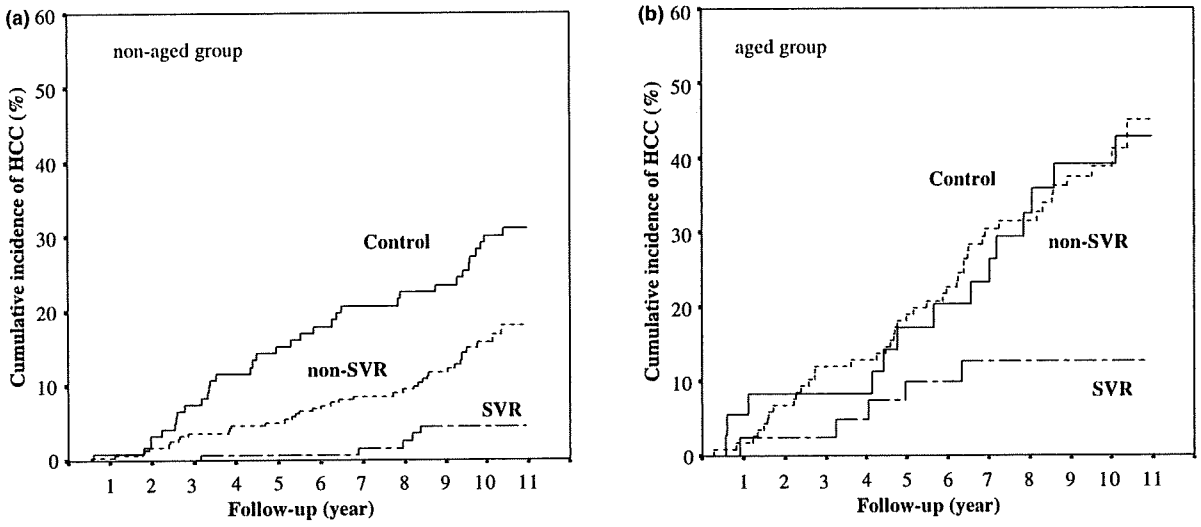


Fig. 1 Cumulative incidence of hepatocellular carcinoma in IFN-treated (dotted line) and control (solid line) patients of the non-aged group (a) and the aged group (b). A log-rank test of the two curves showed a significant difference in the non-aged group ($P < 0.001$), whereas no significant difference was observed in the aged group ($P = 0.498$).



Patients at risk

Control	121	120	116	110	101	94	90	86	82	81	74	71
Non-SVR	276	274	269	264	259	257	239	212	199	179	85	2
SVR	134	134	134	134	131	131	119	107	102	87	49	0

Patients at risk

Control	36	34	33	33	31	28	26	25	21	18	17	15
Non-SVR	117	115	115	103	100	93	82	67	60	50	26	1
SVR	41	40	40	40	38	36	33	31	31	27	17	0

Fig. 2 (a) Cumulative incidence of hepatocellular carcinoma categorized by sustained virological response (dashed line), nonsustained virological response (dotted line), and controls (solid line) of the non-aged group (a) and the aged group (b). A log-rank test of the three curves showed a significant difference between these groups (non-aged group, $P < 0.001$; aged group, $P = 0.015$).

showed biochemical nonresponse. In the non-aged group, stepwise Cox regression analysis identified IFN therapy (risk ratio 0.52, 95% CI 0.33–0.81, $P = 0.004$), older age (risk ratio 1.07, 95% CI 1.03–1.10, $P = 0.001$), and higher histological stage (score 3 or 4) (risk ratio 4.03, 95% CI 2.41–6.76, $P < 0.001$) as independent risk factors associated with the development of HCC. In the non-aged group, the development of HCC was strongly suppressed when SVR was achieved (risk ratio 0.20, 95% CI 0.08–0.50, $P < 0.001$) (Table 3). In the patients with transient biochemical response of the non-SVR group among the non-aged group, HCC development was also significantly reduced (risk ratio 0.47, 95% CI 0.26–0.86, $P = 0.015$). In the aged group, stepwise Cox regression analysis revealed that only higher histological stage (score 3 or 4) (risk ratio 2.27, 95% CI 1.36–3.78, $P = 0.002$) and male gender (risk ratio 2.00, 95% CI 1.17–3.41, $P = 0.011$) were independent factors responsible for the development of HCC (Table 3). Although IFN therapy was not identified as an independent variable for HCC, the risk of HCC was significantly decreased in the patients with SVR in the aged group as shown in the Table 3 (risk ratio 0.23, 95% CI 0.08–0.64, $P = 0.005$). In the

Table 3 Risk ratios for hepatocellular carcinoma in aged and non-aged patients with chronic hepatitis C according to virological and biochemical responses to interferon*

	Non-aged group (n = 531)				Aged group (n = 194)			
	n	Risk ratio	95% CI	P value	n	Risk ratio	95% CI	P value
Control group	121	1.00			36	1.00		
IFN group	410	0.52	0.33–0.81	0.004	158	0.77	0.42–1.40	0.388
Sustained virological response	134	0.20	0.08–0.50	0.001	41	0.23	0.08–0.64	0.005
Nonsustained virological response	276	0.65	0.41–1.03	0.068	117	1.07	0.58–1.97	0.821
Transient biochemical response†	163	0.47	0.26–0.86	0.015	57	0.67	0.32–1.43	0.303
Biochemical nonresponse†	113	0.86	0.51–1.47	0.584	60	1.46	0.77–2.78	0.245

*A stepwise Cox regression analysis was carried out by using interferon therapy, age, gender, and histologic stage and histologic activity scores as variables. †Nonsustained virological response was classified into transient biochemical response and biochemical nonresponse according to the ALT response during the interferon treatment.

patients with transient biochemical response of the non-SVR group of aged patients, HCC development was not reduced (risk ratio 0.67, 95% CI 0.32–1.43, $P = 0.303$, Table 3) in contrast to the patients showing transient biochemical response in the non-aged group.

As the cumulative incidence of HCC calculated by the Kaplan–Meier Method of the patients with SVR in the aged group was much higher than that in the non-aged group, we also carried out Cox proportional-hazards regression analysis to estimate risk factors responsible for HCC development in the 175 patients achieving SVR. As a result, older age (risk ratio 1.09, 95% CI 1.01–1.18, $P = 0.025$) and higher histological activity before IFN therapy started (10 or more of the total score of components 1–3 in Knodell's histological activity index) (risk ratio 4.16, 95% CI 1.07–16.25, $P = 0.040$) were identified as risk factors associated with HCC among the patients with SVR.

DISCUSSION

In this long-term retrospective cohort study, an inhibitory effect of 6 months-IFN monotherapy in early 1990s on the cumulative incidence of HCC were compared between the patients with histologically proven chronic hepatitis C under 60 years old (non-aged group) and those 60 years old and over (aged group). Because of retrospective analysis, there were some differences in baseline characteristics between the two groups. In the aged group, the histological stage and activity as well as the proportion of male patients were significantly higher than in the non-aged group. Also, SVR rate in the aged group was lower than that in the non-aged group. To avoid the influence of these biases, we performed Cox proportional-hazards regression analysis to see whether IFN monotherapy reduced the risk of HCC in the aged and non-aged groups. Then, we found that IFN therapy for 6 months significantly reduced the risk of HCC (risk ratio 0.52) in the non-aged group, whereas this inhibitory effect of IFN monotherapy on HCC development was recognized only in the patients achieving SVR among the aged-patients.

It is difficult to explain why IFN had no inhibitory effect on HCC development in the aged patients, whereas IFN had significant inhibitory effect in the non-aged patients of this study. Many clinical studies have demonstrated that aging was an independent risk factor associated with HCV-related HCC other than advanced histological staging and male gender [7,11–17,29]. However, molecular mechanism of the impact of aging on haepetocarcinogenesis has not been elucidated. Moriya *et al.* reported that lipid hydroperoxide products accumulated in the liver without inflammation and may play a role in the development of HCC in HCV core gene transgenic mice [30,31]. A long-term infection of HCV may lead to HCC through some molecular alterations.

Recently, there have been two controversial reports from the United States and Japan as to the long-term effect of low-dose IFN therapy on the incidence of HCC in chronic hepatitis C [22,24]. The report from Japan was a non-randomized retrospective study and observed beneficial effect of long-term natural IFN-α therapy on hepatocarcinogenesis in aged chronic hepatitis C patients [22]. The HALT-C Trial from the United States, a large prospective randomized study, reported that treatment with peginterfeon-α2a at a dose of 90 μg weekly for 3.5 years did not prevent HCC development in the patients with bridging fibrosis or cirrhosis who did not obtain SVR by combination therapy of peginterferon and ribavirin [24]. The result was consistent with our data in the aged patients. However, the annual incidence of HCC of the HALT-C Trial, about 1%, was much lower than that in the aged group in this study, about 4%. Accordingly, a randomized prospective study to determine the effect of long-term IFN or peginterferon therapy on the incidence of HCC in chronic hepatitis C, especially in the aged patients, may be needed in Japan.

This study has a limitation, because we used historical controls as control patients. A lead-time bias may have occurred. Detection of HCC by the screening program could be less effective in controls than IFN-treated patients. In that case, we might underestimate the effect of IFN on the cumulative incidence of HCC. However, such underestimation may be unlikely as the tumour sizes at the time of detection were not different between the control and IFN-treated patients.

The 10-year incidence of HCC for SVR patients of the aged group (12.7%) was much higher than that of non-aged group (4.5%) in our study. Makiyama *et al.* [32] studied the risk factors for developing HCC after obtaining sustained biochemical response to IFN therapy in chronic hepatitis C and reported that older age, male gender and advanced fibrosis were associated with HCC. Consistent with their results, we found that older age was an independent risk factor for HCC in the patients with SVR, suggesting a high potential of developing HCC even after eradication of HCV RNA in the aged patients. Another possibility is that malignant foci, which could not be detected by imaging modalities, had already existed before IFN therapy. Our finding indicates that even in the patients showing SVR, a follow-up examination to investigate HCC should be carried out for at least 10 years, particularly in the aged patients.

In conclusion, IFN monotherapy reduced the risk of HCC in the patients with chronic hepatitis C under 60 years old. In contrast, this inhibitory effect of IFN on hepatocacinogenesis was limited to patients showing SVR in the aged-patients when treated with 6 months-IFN monotherapy. These results suggest that combination therapy of peginterferon and ribavirin is recommended even in the aged patients with chronic hepatitis C to obtain better preventive effect of IFN on HCC development. For reasons of relatively high cumulative incidence of HCC in the aged chronic hepatitis C patients with SVR to IFN therapy, they should be followed carefully even after eradication of HCV by IFN therapy.

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Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribavirin

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SUMMARY. The impact of ribavirin exposure on virologic relapse remains controversial in combination therapy with pegylated interferon (Peg-IFN) and ribavirin for patients with chronic hepatitis C (CH-C) genotype 1. The present study was conducted to investigate this. Nine hundred and eighty-four patients with CH-C genotype 1 were enrolled. The drug exposure of each medication was calculated by averaging the dose actually taken. For the 472 patients who were HCV RNA negative at week 24 and week 48, multivariate logistic regression analysis showed that the degree of fibrosis ($P = 0.002$), the timing of HCV RNA negativiation ($P < 0.001$) and the mean doses of ribavirin ($P < 0.001$) were significantly associated with relapse, but those of Peg-IFN were not. Stepwise reduction of the ribavirin dose was associated with a stepwise increase in relapse rate from 11% to 60%. For patients with complete early virologic response (c-EVR) defined as HCV RNA negativity at week 12, only 4% relapse was found in patients given ≥12 mg/kg/day of ribavirin and ribavirin exposure affected the relapse even after treatment week 12, while Peg-IFN could be reduced to 0.6 μg/kg/week after week 12 without the increase of relapse rate. Ribavirin showed dose-dependent correlation with the relapse. Maintaining as high a ribavirin dose as possible (≥12 mg/kg/day) during the full treatment period can lead to suppression of the relapse in HCV genotype 1 patients responding to Peg-IFN alpha-2b plus ribavirin, especially in c-EVR patients.

Keywords: chronic hepatitis C, drug exposure, pegylated interferon plus ribavirin, virologic relapse.

INTRODUCTION

Combination therapy of pegylated interferon (Peg-IFN) plus ribavirin is very effective for patients with chronic hepatitis C (CH-C). However, sustained virologic response (SVR) in current therapy occurs in only 40–50% of patients with hepatitis C virus (HCV) genotype 1 [1–4]. Also, SVR is reduced in patients with genotype 1 who require reduction of either Peg-IFN or ribavirin, although dose reduction has little influence on SVR in those with genotype 2 or 3 [1–3,5,6]. Therefore, it is important to clarify the degree to which these medications can be reduced without adversely affecting SVR in patients with CH-C genotype 1.

In an early report on the relationship between drug exposure and antiviral effect in patients with CH-C genotype 1, patients who received ≥80% of their total planned cumulative doses of Peg-IFN and ribavirin for ≥80% of the scheduled duration of therapy had an SVR of 51% compared with only 34% for patients who received lesser amounts of one or both

Abbreviations: CH-C, chronic hepatitis C; c-EVR, complete early virologic response; ETR, end-of-treatment virologic response; Hb, haemoglobin; HCV, hepatitis C virus; IFN, interferon; LVR, late virologic response; Peg-IFN, pegylated interferon; PP, per protocol; Plt, platelet; RVR, rapid virologic response; SVR, sustained virologic response; VR, virologic response; WBC, white blood cell.

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medications [7]. On the other hand, Shiffman *et al.* [8] recently reported that reducing ribavirin did not affect SVR as long as the dose of Peg-IFN was maintained, while reducing the Peg-IFN dose significantly reduced SVR. The results of these observations are consistent with respect to the effect of Peg-IFN on SVR. However, what is controversial is whether or not reducing the ribavirin dose affects the antiviral effect.

Adding ribavirin to either interferon (IFN) or Peg-IFN monotherapy for patients with CH-C genotype 1 has been shown to reduce the relapse rate in large randomized trials [1,2,9–11]. In detail, adding ribavirin to the usual IFN monotherapy (3MIU, three-times-weekly) in 48-week treatment raised the end-of-treatment virologic response (ETR) rate from approximately 30% to 50% and also lowered the relapse rate from mid-40% to approximately 20% [9–11]. Lindsay *et al.* [12] reported that Peg-IFN alpha-2b (Peg-IFN α-2b) monotherapy (1.5 μg/kg, once-weekly), as compared with IFN alpha-2b (IFNα-2b) monotherapy (3MIU, three-times-weekly), improved ETR (49% vs. 24%), but not the relapse rate (53% vs. 50%). In the trial of Peg-IFN alpha-2a (Peg-IFN α-2a) plus ribavirin *vs* IFN α-2b plus ribavirin or Peg-IFN α-2a alone, the ETR rates were 69%, 52% and 59%, and the relapse rates were 19%, 15% and 52%, respectively [2]. These findings from large-scale trials indicate that the main role of ribavirin is to reduce relapse in the combination therapy with Peg-IFN, although ribavirin affects both ETR and relapse in combination therapy with the usual IFN.

In the present study, we tried to determine whether or not dose reduction of ribavirin (or Peg-IFN) has an effect on virologic relapse in Peg-IFN plus ribavirin treatment for patients with CH-C genotype 1.

PATIENTS AND METHODS

Patients

This study was a multicentre trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 984 patients with CH-C were enrolled in this study between December 2004 and September 2006, and treated with a combination of Peg-IFN α-2b plus ribavirin. The baseline characteristics of the patients are shown in Table 1. All patients were Japanese infected with HCV genotype 1 and a viral load of more than 10^5 IU/mL. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis), coinfection with hepatitis B or anti-human immunodeficiency virus. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

Treatment

All patients received Peg-IFN α-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough) for the duration of the study of 48 weeks. As a starting dose, Peg-IFN α-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg) according to the manufacturer's drug information available in Japan.

Table 1 Baseline characteristics of patients and drug doses at start of treatment

Factor	Mean ± SD or *n*
n	984
Age (years)	56.3 ± 10.1
Sex (male/female)	555/429
Body weight (kg)	61.8 ± 11.5
History of IFN treatment Naïve/experienced (relapser/nonresponder)*	575/409 (160/182)
White blood cells (/mm^3)	5052 ± 1550
Neutrophils (/mm^3)	2577 ± 1092
Red blood cells (×10^4/mm^3)	442 ± 47
Haemoglobin (g/dL)	14.1 ± 1.4
Platelets (×10^4/mm^3)	15.9 ± 5.5
AST (IU/L)	66 ± 45
ALT (IU/L)	79 ± 61
Serum HCV RNA (kIU/mL)†	1600
Histology (METAVIR)‡	
Fibrosis; 0/1/2/3/4	49/314/197/105/18
Activity; 0/1/2/3	23/329/304/27
Peg-IFN dose (μg/kg/week)	1.45 ± 0.17
Ribavirin dose (mg/kg/day)	11.4 ± 1.6

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus. *Viral response to previous treatment was unknown in 57 patients, and 10 patients had discontinued treatment. †Data shown are median values. ‡301 missing.

Dose reduction and discontinuance

Dose modification also followed, as a rule, the manufacturer's drug information according to the intensity of the haematologic adverse effects. The dose of Peg-IFN α-2b was reduced to 50% of the assigned dose when the white blood cell (WBC) count was below 1500/mm^3, the neutrophil count below 750/mm^3 or the platelet (Plt) count below 8×10^4/mm^3, and was discontinued when the WBC count was below 1000/mm^3, the neutrophil count below 500/mm^3 or the Plt count below 5×10^4/mm^3. Ribavirin was also reduced from 1000 mg to 600 mg, 800 mg to 600 mg, or 600 mg to 400 mg when the haemoglobin (Hb)

concentration decreased to less than 10 g/dL, and was discontinued when the Hb concentration decreased to less than 8.5 g/dL. Both Peg-IFN α-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. No ferric medicine or haematopoietic growth factors, such as epoetin alpha, or granulocyte-macrophage colony stimulating factor, were administered.

Virologic assessment and definition of virologic response

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 kIU/mL; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analysed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL; Roche Diagnostics). Complete early virologic response (c-EVR) was defined as the absence of detectable serum HCV RNA at treatment week 12, the late virologic response (LVR) was defined as undetectable serum HCV RNA for the first time at 13–24 weeks of treatment, and the virologic response (VR) was defined as HCV RNA negativity at week 24 and week 48. SVR was defined as the absence of detectable serum HCV RNA at week 72. Patients with less than a 2-log decrease in HCV RNA level at treatment week 12 compared with the baseline had to stop treatment according to the protocol and were regarded as nonresponders. All patients with detectable serum HCV RNA at treatment week 24 were also considered to be nonresponders and were excluded from further treatment.

Assessment of drug exposure

The amounts of Peg-IFN α-2b and ribavirin actually taken by each patient during the full treatment period were evaluated by reviewing the medical records. The mean doses of Peg-IFN α-2b and ribavirin were calculated individually as averages on the basis of body weight at baseline: Peg-IFN α-2b expressed as μg/kg/week, ribavirin expressed as mg/kg/day.

Evaluation of impact of drug exposure on virologic relapse

We evaluated the relationship between the drug exposure of both drugs and relapse by two different methods, univariate and multivariate analysis for relapse and independent evaluation of both drugs for relapse according to the degree of drug exposure. The former was performed with the factors of mean administration doses of both drugs, including the factors at baseline and the timing of HCV RNA negativiation. The latter was examined by classifying Peg-IFN α-2b exposure into five categories (up to 0.6 μg/kg; from 0.6 to less than 0.9 μg/kg; from 0.9 to less than 1.2 μg/kg; from 1.2 to less than 1.5 μg/kg; from 1.5 μg/kg) and ribavirin exposure into five categories (up to 6 mg/kg; from 6 to less than 8 mg/kg; from 8 to less than 10 mg/kg; from 10 to less than 12 mg/kg; from 12 mg/kg).

Statistical analysis

Baseline data are expressed as means ± SD or median values. Virologic response was evaluated using per protocol (PP) analysis. To analyse the difference between baseline data including drug exposure and virologic response, univariate analysis using the Mann–Whitney *U*-test or chi-square test and multivariate analysis using logistic regression analysis were performed. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. A two-tailed *P* value <0.05 was considered significant. The analysis was conducted with SPSS version 15.0J (SPSS Inc., Chicago, IL, USA).

RESULTS

Progress of patients and dose reduction of Peg-IFN α-2b and ribavirin

The progress of patients in this study is shown in Fig. 1. Of the 984 patients, 903 completed 12 weeks of treatment and the c-EVR rate was 49% (445/903), based on PP study. To analyse for relapse, 472 patients with VR were assessed, with 178 (38%) showing Peg-IFN dose reduction without discontinuation and 246 (52%) with ribavirin dose reduction without discontinuation during the full (48 weeks) treatment period. The relapse rate was 26% (125/472) in the patients with undetectable HCV RNA level at the end of treatment. No difference was found in relapse rates between the IFN naïve patients and IFN experienced patients (IFN naïve; 25%, 72/287 *vs* IFN experienced; 29%, 53/185, $P = 0.40$). The SVR rate was 43% (347/812) in the PP study.

Impact of drug exposure during 0–48 weeks on relapse among patients with VR

The mean dose of Peg-IFN α-2b actually taken during the full treatment period by each patient was 1.32 μg/kg/week (range, 0.49–2.16 μg/kg/week; median, 1.38 μg/kg/week) and that of ribavirin was 9.8 mg/kg/day (range, 3.3–16.2 mg/kg/day; median, 10.1 mg/kg/day) in patients with VR.

The result of univariate analysis for relapse among the patients with VR is shown in Table 2a. The degree of fibrosis, the timing of HCV RNA negativiation, Plt value and the mean doses of ribavirin were factors significantly associated with relapse, but those of Peg-IFN α-2b were not. The mean dose of ribavirin as well as the degree of fibrosis and the timing of HCV RNA negativiation was selected as a significant independent factor by multivariate logistic regression analysis (Table 2b).

Next, we analysed the relationship of the relapse rate and the mean ribavirin dose. The overall relapse rate among patients with VR was 26% (125/472). The

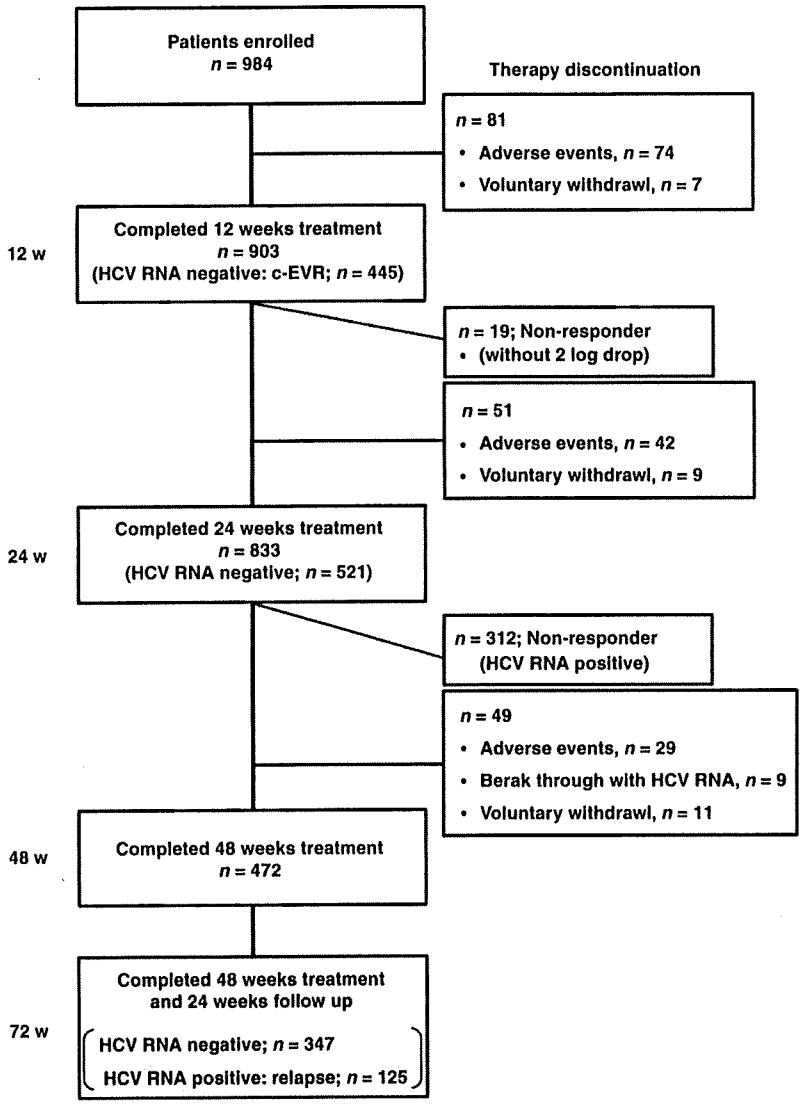

Fig. 1 Flow of patients throughout the study.

relapse rate was 60% (9/15) in patients receiving less than 6 mg/kg/day of ribavirin, and declined to 41% (32/79) at 6–8 mg/kg/day, 27% (34/124) at 8–10 mg/kg/day, 22% (43/193) at 10–12 mg/kg/day and 11% (7/61) in patients given ≥12 mg/kg/day ($P < 0.0001$). Figure 2 shows the relationship of the relapse rate and the mean ribavirin dose for two dosage groups of Peg-IFN α-2b: the group given ≥1.4 μg/kg/week of Peg-IFN and that given <1.4 μg/kg/week (1.4 μg/kg/week was the median value). In both groups, ribavirin was dose-dependently correlated with relapse. More than 12 mg/kg/day of the mean ribavirin exposure could suppress the relapse rate to 20% (4/20) in the group given <1.4 μg/kg/week and strongly suppress it to 7% (3/41) in the group given ≥1.4 μg/kg/week of Peg-IFN.

Impact of drug exposure during 0–48 weeks on relapse according to the timing of HCV RNA negativiation

Relapse rates among patients with c-EVR

The overall relapse rate among patients with c-EVR was 19% (75/391). We separately analysed the relapse rate among the patients with c-EVR according to the degree of exposure to both drugs. Table 3a shows the relapse rates among the patients with c-EVR according to the categories of Peg-IFN α-2b and ribavirin doses during the full treatment period. The relapse rate showed a decline according to the increase in the dose of ribavirin ($P = 0.0002$). The relapse rate was suppressed at an average of 15% (13–16%) in the patients who received 10–12 mg/kg/day of ribavirin, and the average was only 4% for those who received more than 12 mg/kg/day

Table 2 Factors associated with relapse among the patients with virologic response

(a) Univariate analysis

Factor	Nonrelapser	Relapser	*P* value
n	347	125	
Age (years)	53.9 ± 10.7	56.2 ± 9.2	0.07
Sex (male/female)	213/134	66/59	0.09
Serum HCV RNA (kIU/mL)*	1600	1800	0.34
White blood cells (/mm^3)	5335 ± 1517	5075 ± 1428	0.08
Neutrophils (/mm^3)	2797 ± 1143	2625 ± 1021	0.17
Red blood cells (×10^4/mm^3)	450 ± 45	446 ± 50	0.25
Haemoglobin (g/dL)	14.3 ± 1.4	14.2 ± 1.5	0.45
Platelets (×10^4/mm^3)	17.6 ± 5.3	16.4 ± 5.1	0.03
AST (IU/L)	60 ± 42	58 ± 33	0.75
ALT (IU/L)	75 ± 60	71 ± 50	0.98
Histology (METAVIR)†			
Fibrosis: 0–2/3–4	222/20	74/19	0.002
Activity: 0–1/2–3	140/102	52/41	0.75
Peg-IFN dose (μg/kg/week)‡	1.33 ± 0.26	1.27 ± 0.29	0.07
Ribavirin dose (mg/kg/day)‡	10.1 ± 1.9	9.1 ± 2.1	<0.001
Virologic response§: c-EVR/LVR	316/31	75/50	<0.001

(b) Multivariate analysis

Factor	Category	Odds ratio	95% CI	*P* value
Platelets	By 1 × 10^4/mm^3	–	–	NS
Fibrosis¶	0–2/3–4	1/3.192	1.515–6.725	0.002
Ribavirin dose‡	By 1 mg/kg/day	0.790	0.696–0.896	<0.001
Virologic response§	c-EVR/LVR	1/6.290	3.385–11.690	< 0.001

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus; c-EVR, complete early virologic response; LVR, late virologic response; NS, not significant difference Peg-IFN, pegylated interferon.
*Data shown are median values. †137 missing. ‡Mean doses during 0–48 weeks. §The timing of HCV RNA negativiation. ¶METAVIR fibrosis score.

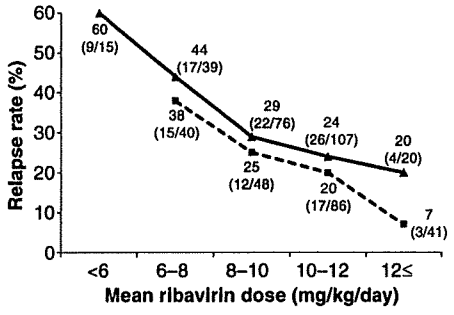

Fig. 2 Relapse rate according to Peg-IFN α-2b and ribavirin doses during treatment of patients who completed treatment, which was stratified with the mean ribavirin doses. (-▲-) Group with the mean Peg-IFN dose <1.4 μg/kg/week; (··■··) Group with the mean Peg-IFN dose ≥1.4 μg/kg/week. The ribavirin dose was dose-dependently correlated with the virologic relapse in both groups ($P < 0.0001$). There was no significant difference between the two Peg-IFN α-2b-dose groups ($P = 0.17$).

of ribavirin. In contrast, the relapse rate was not affected by the dose of Peg-IFN α-2b when the patients were given more than 0.9 μg/kg/week of Peg-IFN α-2b. On the other hand, with respect to patients with rapid virologic response (RVR) defined as the absence of detectable serum HCV RNA at treatment week 4 ($n = 41$), none showed relapse and all attained SVR irrespective of the dose of Peg-IFN α-2b or ribavirin (prevalence of patients: the mean dose of Peg-IFN α-2b; <0.9 : 0.9–1.2 : 1.2–1.5 : 1.5 μg/kg/week ≤ = 7 : 17 : 34 : 42%, the mean dose of ribavirin; <8 : 8–10 : 10–12 : 12 mg/kg/day ≤ = 15 : 24 : 41 : 20%).

Relapse rates among patients with LVR

Among the patients with LVR, the ribavirin exposure during treatment was also the factor correlated adversely with the relapse rate ($P = 0.03$). However, the overall relapse rate was 62% (50/81), which was much higher than that of the c-EVR patients ($P < 0.0001$) and 45% (5/11) of patients with LVR relapsed even in the group given more than 12 mg/kg/day of the average ribavirin dose (Table 3b).

Table 3 Relapse rate according to Peg-IFN and ribavirin doses during week 0–48 for patients with c-EVR and LVR who completed 48 weeks of treatment

(a) C-EVR

Peg-IFN dose (μg/kg/week)†	Ribavirin dose (mg/kg/day)*								Total	
	12≤		10–12		8–10		<8			
≥1.5	0%	(0/28)	13%	(4/31)	14%	(3/21)	29%	(5/17)	12%	(12/97)
1.2–1.5	20%	(2/10)	16%	(16/100)	25%	(16/65)	23%	(7/30)	20%	(41/205)
0.9–1.2	0%	(0/7)	13%	(2/15)	15%	(2/13)	38%	(6/16)	20%	(10/51)
<0.9	0%	(0/5)	15%	(2/13)	55%	(6/11)	44%	(4/9)	32%	(12/38)
Total	4%	(2/50)	15%	(24/159)	25%	(27/110)	31%	(22/72)	19%	(75/391)

(b) LVR

Peg-IFN dose (μg/kg/week)§	Ribavirin dose (mg/kg/day)‡								Total	
	12≤		10–12		8–10		<8			
≥1.5	43%	(3/7)	50%	(1/2)	100%	(2/2)	100%	(4/4)	67%	(10/15)
1.2–1.5		(1/1)	60%	(12/20)	29%	(2/7)	82%	(9/11)	62%	(24/39)
<1.2	33%	(1/3)	50%	(6/12)	60%	(3/5)	86%	(6/7)	59%	(16/27)
Total	45%	(5/11)	56%	(19/34)	50%	(7/14)	86%	(19/22)	62%	(50/81)

Peg-IFN, pegylated interferon; c-EVR, complete early virologic response; LVR, late virologic response.
*P = 0.0002 for comparison of the four ribavirin groups. †P = 0.08 for comparison of the four Peg-IFN groups. ‡P = 0.03 for comparison of the four ribavirin groups. §P = 0.57 for comparison of the three Peg-IFN groups.

Impact of dose reduction after week 12 on relapse among patients with c-EVR

Among c-EVR patients with no or little reduction of Peg-IFN α-2b (the average dose ≥1.2 μg/kg/week) during the first 12 weeks, no significant difference was found in the relapse rate between those whose average dose of Peg-IFN α-2b was reduced to 0.6–1.2 μg/kg/week during 12–48 weeks (17%, 7/41) and those without reduction of Peg-IFN α-2b (average dose ≥1.2 μg/kg/week) (18%, 53/295) (P = 0.86) (Table 4a). Reducing the dose of Peg-IFN α-2b after week 12 in patients in whom HCV RNA had already become undetectable before week 12 did not appear to adversely influence virologic relapse when the average dose of Peg-IFN α-2b was more than 0.6 μg/kg/week during 12–48 weeks, irrespective of the mean dose of Peg-IFN α-2b during the first 12 weeks. On the other hand, the ribavirin dose reduction after week 12 tended to affect the relapse rate in patients given ≥10 mg/kg/day of the ribavirin dose during the first 12 weeks (Table 4b).

Impact of drug exposure during 0–48 weeks on relapse among VR patients with advanced fibrosis

In the evaluation of the 39 patients with VR with progression of fibrosis or cirrhosis (METAVIR fibrosis score 3 or 4) enrolled in this study, ribavirin exposure during treatment significantly correlated with relapse (nonrelapser, 10.5 ± 2.1 mg/kg/day *vs* relapser, 8.8 ± 2.3 mg/kg/day; P = 0.007). Among patients with advanced fibrosis (score 3–4), the relapse rate in patients given ≥10 mg/kg/day of the average ribavirin dose was significantly low (36%, 9/25) in comparison with that in patients given <10 mg/kg/day of ribavirin (71%, 10/14) (P = 0.048).

DISCUSSION

Previous studies have suggested that reducing the ribavirin dose within the first 12–20 weeks of treatment in patients with HCV genotype 1 was associated with a decline of SVR [7,13,14]. However, Shiffman *et al.* [8] recently reported that reducing the mean dose of ribavirin during the first 20 weeks of treatment had little impact on relapse for patients with CH-C genotype 1 and that SVR may not be adversely affected as long as the total cumulative ribavirin dose remains above 60%. As the reason for the inconsistency in the impact of reducing ribavirin on the antiviral effect, it was suggested that sample sizes of the previous studies were insufficient to assess the impact of reducing the dose of ribavirin independent of Peg-IFN. However, in Shiffman's study, while the impact of reducing the dose of Peg-IFN or ribavirin on SVR was indeed closely examined independently of each other with a large sample size, the subjects were limited to patients with advanced fibrosis or cirrhosis and prior nonresponse to Peg-IFN ± ribavirin who were enrolled in the Hepatitis Antiviral Long-term Treatment Against Cirrhosis (HALT-C) trial. Reddy *et al.* [15] analysed the drug exposure retrospectively for 569 CH-C patients with genotype 1 enrolled in clinical trials of Peg-IFN α-2a plus

Table 4 Relapse rate according to drug doses during week 0–12 and 12–48 for patients with c-EVR who completed 48 weeks of treatment

(a) Peg-IFN

Peg-IFN dose (mean, μg/kg/week)		12–48 weeks			
		≥1.2	0.9–1.2	0.6–0.9	<0.6
0–12 weeks	≥1.2	18% (53/295)	17% (5/30)	18% (2/11)	(1/1)
	0.9–1.2	–	22% (4/18)	33% (4/12)	60% (3/5)
	<0.9	(0/1)	(0/1)	17% (2/12)	20% (1/5)
Total*		18% (53/296)	18% (9/49)	23% (8/35)	45% (5/11)

(b) Ribavirin

Ribavirin dose (mean, mg/kg/day)		12–48 weeks			
		≥12	10–12	8–10	<8
0–12 weeks	≥12	4% (2/47)	13% (3/23)	13% (1/8)	33% (1/3)
	10–12	–	15% (18/123)	22% (12/54)	20% (5/25)
	8–10	–	(1/1)	26% (10/38)	26% (10/39)
	<8	–	–	–	40% (12/30)
Total†		4% (2/47)	15% (22/147)	23% (23/100)	29% (28/97)

c-EVR, complete early virologic response; Peg-IFN, pegylated interferon.
*$P = 0.18$ for comparison of the four Peg-IFN groups. †$P < 0.0001$ for comparison of the four ribavirin groups.

ribavirin, and concluded that SVR was not affected adversely by ribavirin reduction unless the cumulative ribavirin exposure was less than 60%. This supported Shiffman's data, but in Reddy's study, the stepwise reduction in ribavirin dose was shown to be associated with a stepwise increase in relapse rate from 19% to 54%. Thus, the impact of ribavirin drug exposure on the antiviral effect (relapse) in patients with CH-C genotype 1 remains unclear. Further examination is needed to determine whether or not ribavirin can be reduced to a certain degree without adversely affecting virologic relapse or SVR in Peg-IFN and ribavirin combination therapy for CH-C genotype 1.

In order to raise the SVR rate in patients with genotype 1, two strategies are possible: one is enhancing the virologic response of HCV RNA negativity and another is reducing relapse. In Peg-IFN plus ribavirin treatment, raising the doses of either or both drugs (dose-up strategy) is the only way to enhance the virologic response of HCV RNA negativity, but this is always accompanied by a high risk and the discontinuation rate can increase with the dose-up of drug, although the virologic response among patients completing the therapy can be improved [16,17]. Therefore, in this study, we tried to manage the drug dose to reduce relapse in virologic responders with HCV RNA negativity. Large-scale clinical trials [1,2,9–12] have revealed that adding ribavirin to IFN or Peg-IFN monotherapy for patients with CH-C reduced the relapse rate from approximately 50% to under 20%. Bronowicki *et al.* [18] examined the effect of ribavirin on CH-C genotype 1 in Peg-IFN α-2a plus ribavirin treatment by randomizing patients with HCV RNA negativity by week 24 into two groups, one continuing with ribavirin and the other receiving Peg-IFN α-2a alone after week 24. As a result, the virologic responders who stopped ribavirin treatment at week 24 were found to have a significantly higher rate of breakthroughs during therapy and higher relapse rates after therapy in comparison with those who received Peg-IFN plus ribavirin for the full treatment period (relapse rate; 42% vs. 29%, $P = 0.02$). These findings indicate that ribavirin plays a very important role in reducing relapse. However, the relationship between ribavirin dose and relapse rate has not been examined in detail. Considering that ribavirin has little influence on HCV RNA negativiation [1,2,9–12], its dose impact on the antiviral effect should be carefully examined, not for the SVR rate of all patients, but for the relapse rate of patients responding to Peg-IFN plus ribavirin, as evaluating of ribavirin by SVR including HCV RNA negativiation cannot differentiate it from the strong influence of the Peg-IFN effect, which affects HCV RNA negativiation dose-dependently [19]. Here, we examined the correlation between the average dose of drugs and the virologic relapse for patients responding to the treatment.

We performed univariate and multivariate analysis for relapse among the factors of mean administration doses of both drugs, including baseline factors and the timing of HCV RNA negativiation. We found exposure to ribavirin dose, timing of HCV RNA negativiation and the degree of liver fibrosis to be the independent factors affecting the virologic relapse in patients with VR. This indicates that management

of the ribavirin dose, which is the variable factor, unlike baseline factors, plays an important role in suppressing the virologic relapse in patients with CH-C genotype 1 treated by Peg-IFN plus ribavirin treatment. This suggests that maintaining the ribavirin dose should lower the relapse rate even in patients with advanced fibrosis who are liable to relapse. In fact, among patients with advanced fibrosis (METAVIR score 3–4), the relapse rate in those given ≥10 mg/kg/day of the average ribavirin dose was significantly lower than that in patients given <10 mg/kg/day of ribavirin (36% vs. 71%). However, the sample size was too small for subsequent analysis with stratification. Further study is needed to clarify the impact of ribavirin dose on viral relapse in patients with progression of fibrosis.

The relapse rate among patients with c-EVR showed a decline according to the increase in ribavirin dose during treatment week 0–48 and was not affected by the Peg-IFN α-2b dose when the patients were given more than 0.9 μg/kg/week of Peg-IFN α-2b. Among the patients with c-EVR, none with RVR had a relapse and all attained SVR irrespective of the dose of Peg-IFN α-2b or ribavirin. Examination of the impact of dose reduction after week 12 on relapse among patients with c-EVR showed that the ribavirin dose reduction after week 12 tended to affect the relapse rate in patients given ≥10 mg/kg/day of the ribavirin dose during the first 12 weeks, while the Peg-IFN α-2b dose after week 12 could be reduced without any increase in relapse rate in patients given more than 0.6 μg/kg/week of the average dose of Peg-IFN α-2b. On the other hand, maintaining the ribavirin did not lead to reduce the relapse rate in patients with LVR. About half relapsed even when given ≥12 mg/kg/day of the average ribavirin dose. This suggested that the relapse rate could not be reduced by management of the ribavirin dose in patients with LVR. Extended therapy should be chosen in LVR patients as shown in the previous studies [20–23].

Shiffman *et al.* [24] recently reported that maintaining the Hb level with epoetin alpha did not enhance SVR if ribavirin was started at the standard dose (800–1400 mg/day, mean dose 13.3 mg/kg/day), although discontinuance and the reduction rates of ribavirin were decreased and a higher mean dose of ribavirin was administered in comparison with those treated with Peg-IFN plus ribavirin without epoetin. If these findings apply to patients with CH-C genotype 1, this would suggest that the ribavirin dose does not need to be maintained during treatment with Peg-IFN plus ribavirin, which would not agree with our findings. However, closer examination of the Shiffman *et al.* study shows that Peg-IFN plus a higher dose of ribavirin (1000–1600 mg/day, mean dose 15.2 mg/kg/day) with epoetin was found to suppress the relapse rate and enhance SVR. These data agree with ours with respect to the point that higher doses of ribavirin are associated with a lower relapse rate. What differs is the ribavirin dose needed to suppress the relapse. This is likely to be due to ethnic differences between the subjects. In Shiffman's study, approximately 40% were African-Americans in whom the virologic response is well established as being significantly lower than those of other ethnic groups [25,26], while in our study, all subjects were Japanese. In the African-Americans treated with Peg-IFN plus standard-dose ribavirin, the relapse rate (calculated from 48% of ETR and 19% of SVR) was 60%, while 18% relapse (from 38% of ETR and 31% of SVR) occurred in those given Peg-IFN plus high-dose ribavirin. The relapse rate of patients with c-EVR in our study was 19%, which was very close to that for those with Peg-IFN plus high-dose ribavirin in Shiffman's study. Ribavirin does not have a direct antiviral action against HCV [27,28], and is considered to play an important role in accelerating HCV-infected cell clearance [29] and eradicating them completely when an immune response against infected cells is induced by IFN or Peg-IFN [30,31]. Therefore, the difference between patients who are easy or difficult to treat due to ethnic differences or differences in response to Peg-IFN can result in the need for different doses of ribavirin to suppress the relapse rate in patients with CH-C genotype 1.

In conclusion, our results have demonstrated that ribavirin is dose-dependently correlated with a relapse in patients with CH-C genotype 1 responding to Peg-IFN plus ribavirin. Maintaining a high dose (≥12 mg/kg/day) of ribavirin during the full treatment period could strongly suppress the relapse in such patients, while Peg-IFN α-2b could be reduced without affecting relapse in patients with c-EVR. This possibility should be explored in a prospective study.

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Mcl-1 and Bcl-xL Cooperatively Maintain Integrity of Hepatocytes in Developing and Adult Murine Liver

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Anti-apoptotic members of the Bcl-2 family, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1, inhibit the mitochondrial pathway of apoptosis. Bcl-xL and Mcl-1 are constitutively expressed in the liver. Although previous research established Bcl-xL as a critical apoptosis antagonist in differentiated hepatocytes, the significance of Mcl-1 in the liver, especially in conjunction with Bcl-xL, has not been clear. To examine this question, we generated hepatocyte-specific Mcl-1–deficient mice by crossing *mcl-1*$^{flox/flox}$ mice and *AlbCre* mice and further crossed them with *bcl-x*$^{flox/flox}$ mice, giving Mcl-1/Bcl-xL–deficient mice. The *mcl-1*$^{flox/flox}$ *AlbCre* mice showed spontaneous apoptosis of hepatocytes after birth, as evidenced by elevated levels of serum alanine aminotransferase (ALT) and caspase-3/7 activity and an increased number of terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL)-positive cells in the liver; these phenotypes were very close to those previously found in hepatocyte-specific Bcl-xL–deficient mice. Although *mcl-1*$^{flox/+}$ *AlbCre* mice did not display apoptosis, their susceptibility to Fas-mediated liver injury significantly increased. Further crossing of Mcl-1 mice with Bcl-xL mice showed that *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice also showed spontaneous hepatocyte apoptosis similar to Bcl-xL–deficient or Mcl-1–deficient mice. In contrast, *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice displayed a decreased number of hepatocytes and a reduced volume of the liver on day 18.5 of embryogenesis and rapidly died within 1 day after birth, developing hepatic failure evidenced by increased levels of blood ammonia and bilirubin. *Conclusion:* Mcl-1 is critical for blocking apoptosis in adult liver and, in the absence of Bcl-xL, is essential for normal liver development. Mcl-1 and Bcl-xL are two major anti-apoptotic Bcl-2 family proteins expressed in the liver and cooperatively control hepatic integrity during liver development and in adult liver homeostasis in a gene dose-dependent manner. (HEPATOLOGY 2009;50:1217-1226.)

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Abbreviations: ALT, alanine aminotransferase; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling.

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The mitochondrial pathway of apoptosis is regulated by the Bcl-2 family proteins.[1,2] They are functionally divided into two basic groups: pro-apoptotic and anti-apoptotic members. Pro-apoptotic members are further divided into multi-domain members, such as Bax and Bak, and BH3-only proteins. Bax/Bak triggers release from mitochondria of cytochrome c, presumably by forming pores at the mitochondrial outer membrane. Cytochrome c released into the cytosol activates multiple caspases, which cut a variety of cellular substrates and dismantle the cell.[3] The release of Bax/Bak–mediated cytochrome c is considered to be a point of no return and a commitment to cell death.[4] Killing by BH3-only proteins, such as Bid, Bim, or Puma, requires Bax or Bak, placing them upstream of Bak/Bax activation. BH3-only proteins are transcriptionally or posttranslationally activated by a variety of cellular stresses. They are considered to be sensors that transmit apoptotic stimuli to mitochondria. Anti-apoptotic members, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1, inhibit the mitochon-

drial pathway of apoptosis either by directly blocking Bak/Bax activation or by sequestering BH3-only proteins from Bak or Bax.

Mcl-1 has increasingly attracted attention because of its role in liver disease. Several reports have shown that Mcl-1 is overexpressed in a subset of human hepatocellular carcinomas and provides apoptosis resistance.[5-7] The multi-kinase inhibitor sorafenib, which was recently approved by the Food and Drug Administration as a chemotherapeutic agent for hepatocellular carcinoma,[8] is capable of down-regulating Mcl-1 expression and producing apoptosis in hepatoma cells.[9] Cycloxygenase 2 or hepatocyte growth factor up-regulates Mcl-1 expression in hepatocytes and improves Fas-mediated liver injury.[10,11] Recently, enforced expression of Mcl-1 was reported to reduce liver injury induced by anti-Fas injection in mice.[12] However, little is known about the physiologic significance of Mcl-1 in hepatocytes.

We previously reported that hepatocyte-specific Bcl-xL knockout mice were born and grew up but developed spontaneous hepatocyte apoptosis, identifying Bcl-xL as a critical apoptosis antagonist in hepatocytes.[13] This raises a question of whether other anti-apoptotic Bcl-2 family members, such as Mcl-1, have a significant role in regulating hepatocyte apoptosis and what the relationship is among those molecules. To this end, in the current study, we generated hepatocyte-specific Mcl-1 knockout as well as Bcl-xL/Mcl-1 double knockout mice and found that, like Bcl-xL, Mcl-1 is critical for maintaining hepatocyte integrity in adult liver, but not essential for liver development. However, both deficiencies cause a severe defect in liver development and lethality during the early neonatal period because of severe hepatic failure. The current study identifies Bcl-xL and Mcl-1 as two major anti-apoptotic Bcl-2 family proteins in the liver and demonstrates their gene dose–dependent effects for controlling hepatic integrity.

Materials and Methods

Mice. Mice carrying the *mcl-1* gene encoding amino acids 1 through 179 flanked by 2 *lox*P (*mcl-1*$^{flox/flox}$) were provided by Dr. You-Wen He of Duke University.[14] Mice carrying a *bcl-x* gene with two *lox*P sequencers at the promoter region and a second intron (*bcl-x*$^{flox/flox}$) were described previously.[15] Heterozygous AlbCre transgenic mice expressing Cre recombinase gene under the promoter of the albumin gene were described previously.[13] We generated hepatocyte-specific Mcl-1 knockout mice (*mcl-1*$^{flox/flox}$ *AlbCre*) by mating *mcl-1*$^{flox/flox}$ and *AlbCre* mice. We then used these knockout mice to generate hepatocyte-specific Bcl-xL/Mcl-1 knockout mice (*bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre*) by mating them with *bcl-x*$^{flox/flox}$ mice. Traditional Bid knockout mice were described previously.[16] They were maintained in a specific pathogen–free facility and treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Genotyping. Genomic DNA was extracted from the tail and subjected for polymerase chain reaction (PCR) for genotyping mice. The primers used were as follows: 5′-GCCACCTCATCAGTCGGG-3′ and 5′-TCAGAAGCCGCAATATCCCC-3′ for the *bcl-x* allele; 5′-GGTTCCCTGTCTCCTTACTTACTGTAG-3′ and 5′-CTCCTAACCACTGTTCCTGACATCC-3′ for the *mcl-1* allele; 5′-GCGGTCTGGCAGTAAAAACTATC-3′, 5′-GTGAAACAGCATTGCTGTCACTT-3′, 5′CTAGGCCACAGAATTGAAAGATCT-3′ 5′-GTAGGTGGAAATTCTAGCATCATCC-3′ for the *AlbCre* allele; 5′-CCGAAA TGTCCCATAAGAG-3′, 5′-GAGATGGACCACAACATC-3′, and 5′ TGCTACTTCCATTTGTCACGTCCT-3′ for the *bid* allele. PCR products were electrophoretically separated using 2% agarose gels. The expected sizes of the PCR products were as follows: 165 bp for the wild-type *bcl-x* allele, 195 bp for the floxed *bcl-x* allele, 200 bp for the wild-type *mcl-1* allele, 300 bp for the floxed *mcl-1* allele, 130 bp for the wild-type *bid* allele, and 350 bp for the *bid* knockout allele. *AlbCre*-negative mice showed a 350-bp band, and heterozygous *AlbCre* mice showed 100-bp and 350-bp double bands.

Apoptosis Assay. To measure serum ALT level and caspase-3/7 activity, blood was collected from the inferior vena cava of mice and centrifuged. Serum was stored at −20°C until use. Serum ALT levels were measured by a standard method at Oriental Kobo Life Science Laboratory (Nagahama, Japan), and serum caspase-3/7 activity was measured by a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Tokyo, Japan). For histological analysis, livers were formalin-fixed, embedded in paraffin, and thin sliced. The liver sections were stained with hematoxylin-eosin. To detect cells with oligonucleosomal DNA breaks, the sections were also subjected to terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL) staining, according to a previously reported procedure.[17] For Fas-stimulating study, anti-Fas antibody (Jo2 clone) (PharMingen, San Diego, CA) was intraperitoneally injected into mice 3 hours before sacrifice.

Western Blot Analysis. Approximately 25 mg liver tissues was lysed with a lysis buffer (1% NP-40, 0.5%