INTRODUCTION

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

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

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

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

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

METHODS

Patients

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

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

Study design

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

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

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

Measurement of HCV RNA

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

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

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

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

Efficacy end points

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

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

Statistical analysis

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

RESULTS

Patient profiles

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

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

Outcomes of patients

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

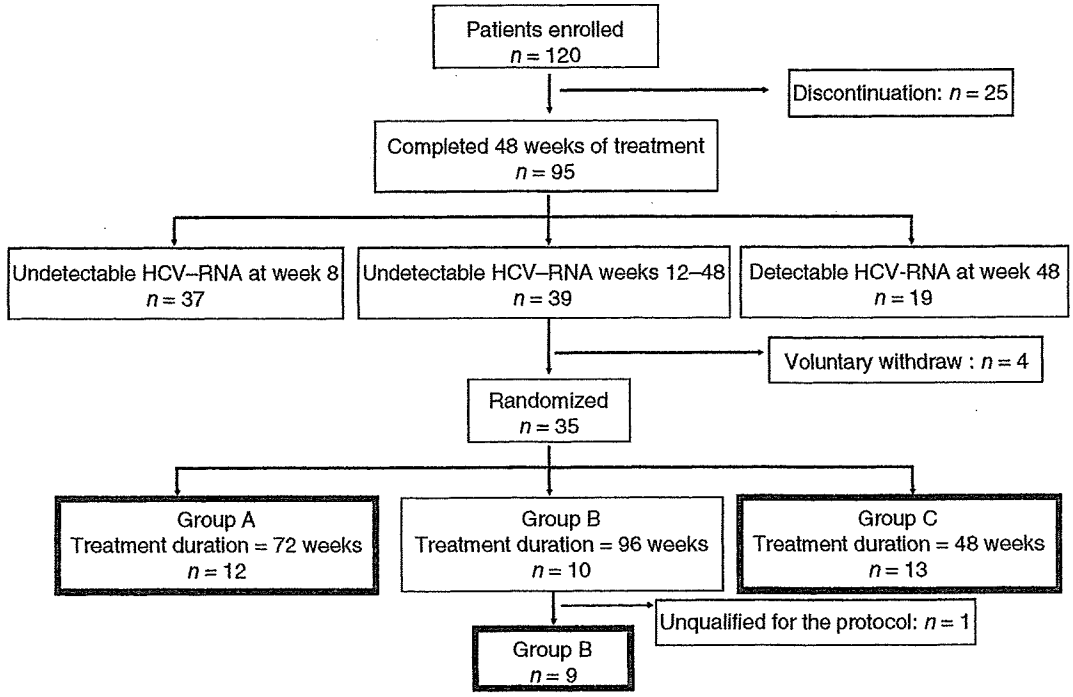


Figure 1. Flow of participants throughout the study.

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

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

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

Sustained virological responses in the total study population

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

Table 1. Characteristics of patients at baseline

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

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

DISCUSSION

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

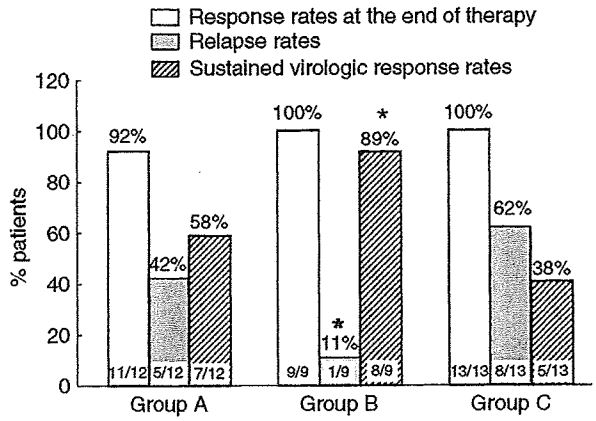


Figure 2. Frequency of virological response rates at the end of therapy and virological relapse rates in groups A, B and C. These rates are shown as a percentage and the number of patients with virological response or virological relapse in relation to the total number of patients examined is shown at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

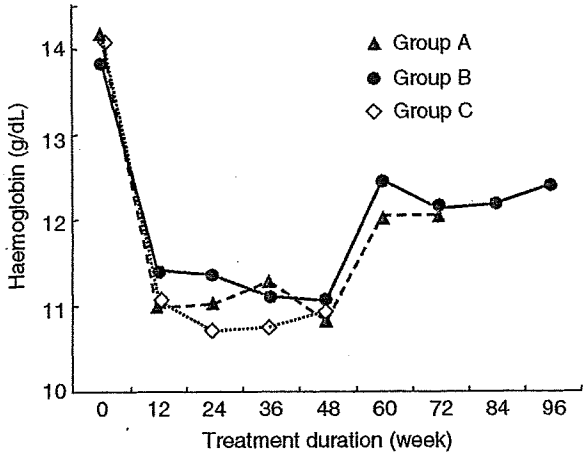


Figure 3. Time course of haemoglobin levels in groups A, B and C during therapy. The patients in groups A and B were given low-dose ribavirin (200 mg/day) beyond 48 weeks.

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

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

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

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

Table 2. Predictors of sustained virological response to combination therapy

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

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

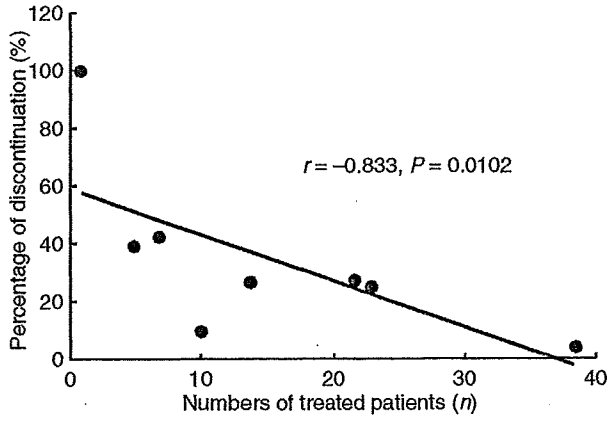


Figure 4. Correlation between the number of treated patients and percentage of discontinuation at each hospital. All 25 patients who stopped treatment within 48 weeks of treatment were analysed. Pearson's correlation coefficient is indicated on the graph.

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

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

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

ACKNOWLEDGEMENTS

Declaration of personal and funding interests: None.

REFERENCES

1 Seeff LB, Hoofnagle JH. National Institutes of Health consensus development conference: management of hepatitis C: 2002. *Hepatology* 2002; 36(Suppl): S1–20.

2 Manns MP, McHutchinson JG, Gordon SC, *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet* 2001; 358: 958–65.

3 Fried MW, Shiffman ML, Reddy KR, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–82.

4 Hadziyannis S, Sette H Jr, Morgan TR, *et al.* Peginterferon-alfa-2a plus ribavirin combination therapy in chronic hepatitis C. A randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; 40: 346–55.

5 Nagaki M, Imose M, Naiki T, *et al.* Prospective study on early virologic response to treatment with interferon alpha-2b plus rebavirin in patients with chronic hepatitis C genotype 1b. *Hepatol Res* 2005; 33: 285–91.

6 Davis GL, Wong JB, McHutchison JG, *et al.* Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; 38: 645–52.

7 Ferenci P, Fried MW, Shifmann ML, *et al.* Predicting sustained virological response in chronic hepatitis C patients treated with peginterferon alfa-2a (40KD)/ribavirin. *J Hepatol* 2005; 43: 425–33.

8 Zeuzem S, Buti M, Ferenci P, *et al.* Efficacy of 24 weeks treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C infected with genotype 1 and low pretreatment viremia. *J Hepatol* 2006; 44: 97–103.

9 Strader DB, Wright T, Thomas DL, Seeff LB, American Association for the Study of Liver Diseases. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; 39: 1147–71.

10 Drusano GL, Preston SL. A 48-week duration of therapy with pegylated interferon alpha 2b plus ribavirin may be too short to maximize long-term response among patients infected with genotype-1 hepatitis C virus. *J Infect Dis* 2004; 189: 964–70.

11 Berg T, von Wagner M, Nasser S, *et al.* Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006; 130: 1086–97.

12 Sánchez-Tapias JM, Diago M, Escartín P, *et al.* Peginterferon-alfa2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. *Gastroenterology* 2006; 131: 451–60.

13 Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis c genotype 1-infected slow responders. *Hepatology* 2007; 46: 1688–94.

14 Mangia A, Minerva N, Bacca D, *et al.* Individualized treatment duration for hepatitis C genotype 1 patients: A randomized controlled trial. *Hepatology* 2008; 47: 43–50.

15 Kuboki M, Iino S, Okuno T, *et al.* Peginterferon α-2a (40KD) plus ribavirin for the treatment of chronic hepatitis C in Japanese patients. *J Gastroen Hepatol* 2007; 22: 645–52.

16 Desmet VJ, Gerber M, Hoofnagle JH, *et al.* Classification of chronic hepatitis: diagnosis, grading, and staging. *Hepatology* 1994; 19: 1513–20.

17 Okamoto H, Sugiyama Y, Okada S, *et al.* Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J Gen Viol* 1992; 73: 673–9.

18 Ohno T, Mizokami M, Wu R-R, *et al.* New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* 1997; 35: 201–7.

19 Enomoto N, Sakuma I, Asahina Y, *et al.* Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b: sensitivity to interferon is conferred by amino acid substitutions in NS5A region. *J Clin Invest* 1995; 96: 224–30.

20 Akuta N, Suzuki F, Kawamura Y, *et al.* Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007; 46: 403–10.

21 McHutchison JG, Manns M, Patel K, *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; 123: 1061–9.

J Gastroenterol 2009; 44:218–227
DOI 10.1007/s00535-008-2300-8

Journal of Gastroenterology

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

Kiminori Kimura[1,2], Masahito Nagaki[3], Masanao Saio[4], Hisataka Moriwaki[3], and Kazuhiro Kakimi[2]

[1]Division of Hepatology, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan
[2]Department of Immunotherapeutics (Medinet), Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[3]First Department of Internal Medicine, Gifu University School of Medicine, Gifu, Japan
[4]Department of Immunopathology, Gifu University School of Medicine, Gifu, Japan

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

Key words: HBV, CD44, CTL, acute hepatitis

Introduction

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

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

Received: June 9, 2008 / Accepted: September 10, 2008
Reprint requests to: K. Kimura

Methods

Mice

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

CTL clones

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

RNA analyses

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

Biochemical and histological analyses

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

5,6-Carboxyfluoroscein succinimidyl ester (CFSE) staining

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

Immunohistochemistry

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

Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay

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

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

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

Fluorescence-activated cell sorting (FACS) analysis

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

anti-CD86-PE mAb (BD Bioscience, La Jolla, CA, USA), and anti-LSECs-FITC mAb (Miltenyi Biotec, Bergisch Gladbach, Germany), washed in FACS buffer [PBS containing 1% fetal calf serum (FCS)], and fixed in 2% paraformaldehyde for 30 min at room temperature. Samples were acquired using a FACScalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA), and the data were analyzed using the CELLQuest software (BD Immunocytometry Systems).

Isolation of CD11b-positive cells

CD11b-positive cells were purified by positive selection using antimouse CD11b beads and a magnetic cell separation system column (Miltenyi Biotec) according to the manufacturer's directions. After incubating with isolated $CD11b^+$ cells and FITC-dextran (Sigma), phagocyte activity was analyzed by FACS.

Data analysis

All data are expressed as the mean ± SD. The significance of differences among mean values was evaluated according to the Mann–Whitney *U* test.

Results

To examine the effects of CD44 on liver inflammation, we first generated HBV transgenic (HBVTg) mice backcrossed with CD44KO mice[11] (CD44KO × HBVTg mice). HBV Tg mice named lineage 107-5D (inbred B10.D2, H-2d) in which, constitutively, the HBV envelope coding region is under the control of the mouse albumin promoter.[13] In addition, we confirmed no significant difference with cell number and phenotype in CD44 present or absent intrahepatic leukocytes (IHLs).

We injected 5×10^6 HBV-specific CTL clones (epitope 28–39) into HBVTg or CD44KO × HBVTg mice, and subsequently monitored their sALT activities and intrahepatic leukocyte (IHL) numbers at various time points. As shown in Fig. 1A, the sALT activity in HBVTg mice began to increase at 4 h (287 ± 36 IU/l) and reached a peak at 24 h (8677 ± 1224 IU/l) after CTL injection, as previously reported.[1] On the other hand, the sALT activity in CD44KO × HBVTg mice was significantly reduced at 4 h (168 ± 21 IU/l) compared with HBVTg mice, but exacerbated at 36 h (13650 ± 1490 IU/l) after CTL injection. Consistent with the sALT activity, the total number of IHLs was significantly reduced in CD44KO × HBVTg mice at 4 h, with particular reductions in the neutrophil ($Gr\text{-}1^{++}/CD11b^+$) and macrophage ($Gr\text{-}1^+/CD11b^+$) populations. In contrast, the total number of IHLs, including neutrophils and macrophages, was increased at 24 h in these mice. Furthermore, we analyzed the messenger RNA (mRNA) expression levels for inflammatory cytokines, chemokines, and cell markers in the liver using RNase protection assays (RPAs). The results exhibited similar expression patterns to the elevation of sALT activity, because the mRNA levels of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin (IL)-1α/β in the liver of CD44KO × HBVTg mice were reduced at 4 and 8 h but recovered to almost their normal levels at 24 and 36 h (see also Supplementary Fig. 1). In addition, we found that the mRNA levels of various chemokines in the liver of CD44KO × HBVTg mice were reduced at 4 h, in association with the reduced numbers of IHLs.

Regarding the suppressive effects on liver injury at 4 h, we hypothesized that the number of transferred CTLs would be reduced in the liver by the CD44KO conditions. To clarify this hypothesis, we injected 5,6-carboxyfluoroscein succinimidyl ester (CFSE)-labeled CTLs into HBVTg mice with or without CD44 and then counted the numbers of transferred CTLs among the IHLs and PBMCs by fluorescence-activated cell sorting (FACS) and in the liver by immunohistochemistry. The number of CFSE-labeled CTLs was seen to be lower (Fig. 2A) at 4 h, but not at 24 h, in the liver of CD44KO × HBVTg mice compared with the liver of HBVTg mice. In contrast, transferred CTLs were not detected in the PBMCs of both types of mice. Consistent with the FACS data, immunohistochemical analysis revealed that the number of CFSE-labeled CTLs was significantly lower in the liver of CD44KO × HBVTg mice. Collectively, these results suggest that HBV-specific CTLs migrate into the liver in a CD44-dependent manner.

It is well known that CTLs have to pass through liver sinus endothelial cells (LSECs) to enter the hepatocytes.[17–19] Based on this knowledge, we examined the role of LSECs in this model. First, we analyzed CD44 expression on LSECs in the liver of HBVTg mice. LSECs expressed CD44 under steady-state conditions but upregulated its expression at 4 and 24 h after CTL injection (Fig. 3). We also analyzed the levels of a costimulatory molecule (CD86) and an adhesion molecule (ICAM-1) on LSECs in the presence or absence of CD44. We found that these levels were similar in both types of mice, indicating that CD44 on LSECs plays a key role in CTL migration.

As already mentioned, the sALT activity was suppressed in CD44KO × HBVTg mice in association with the reduced number of IHLs and levels of cytokine or chemokine mRNA at 4 h. However, liver inflammation was reinforced at the late phase. To evaluate the mechanism of the latter observation, we analyzed the induction of hepatocyte apoptosis and NF-κB activity in the liver, as we previously demonstrated that low intrahe-

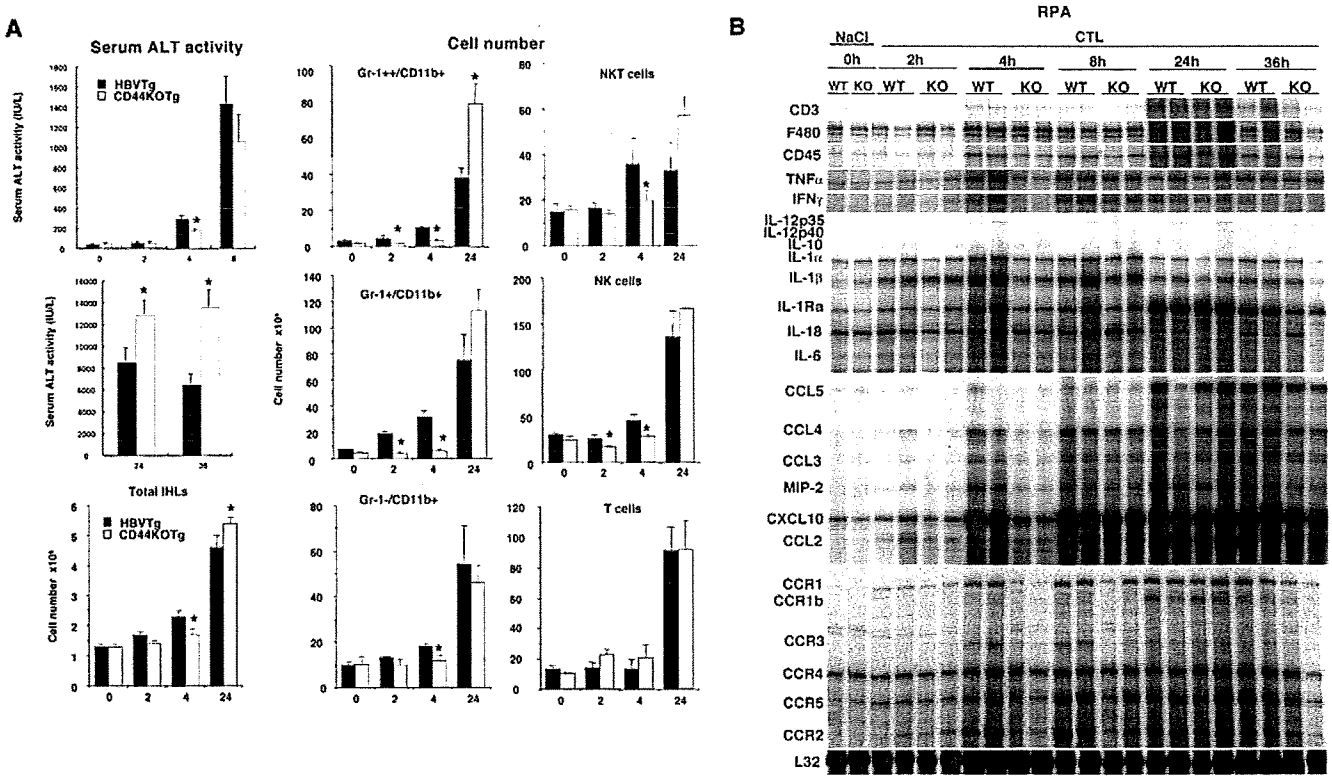


Fig. 1. **A** CD44KO mice exhibit suppressed cytotoxic T lymphocyte (CTL)-induced liver injury and cell recruitment at the early phase but not the late phase. Hepatitis B virus transgenic (HBVTg) or CD44KO × HBVTg mice were injected intravenously with 5×10^6 6C2 cells or NaCl. The mean serum alanine transferase (sALT) activity measured at the time of autopsy is indicated for each group and expressed in IU/l (mean ± SD). $^*P < 0.05$. Intrahepatic leukocytes (IHLs) were isolated from the same animals, and the effects of CD44 blockade were analyzed. The numbers in each subset of cells in the liver were calculated by multiplying the total number of IHLs by the frequency of each subset in the IHL population by fluorescence-activated cell sorting (FACS) analysis. The data represent means ± SD for three mice. $^*P < 0.05$. **B** Inflammatory cytokine and chemokine expression levels in the liver. The mRNA expression levels of the indicated inflammatory cytokines, chemokines, and cell-surface markers in total hepatic RNA samples (20 μg) from the same mice as in **A** were analyzed by RNase protection assays (*RPA*). The mRNA level of the housekeeping gene encoding ribosomal protein L32 was used to normalize the quantity of RNA loaded in each lane

patic NF-κB activity easily induces hepatocyte apoptosis.[15,20] To determine the histological changes in the liver of HBVTg mice with or without CD44 at 24 h after CTL injection, we stained liver tissues with hematoxylin and eosin and in situ TUNEL. The histological analysis revealed widely scattered inflammatory foci in the liver parenchyma and around the portal tract, containing mostly lymph mononuclear cells and a few apoptotic hepatocytes, in HBVTg mice (Fig. 4A). On the other hand, there was a marked increase in apoptotic hepatocytes in the parenchyma (Fig. 4A) in CD44KO × HBVTg mice, and lymph mononuclear cells had infiltrated around the central vein. In addition, the liver of CD44KO × HBVTg mice showed a marked increase in the number of TUNEL-positive cells after CTL injection (Fig. 4A), as quantified in Fig. 4B. These results suggest that the liver of CD44KO × HBVTg mice underwent massive apoptosis of hepatocytes, in association with the marked elevation of sALT activity. Furthermore, we analyzed the difference in CTL-induced NF-kB activation in the liver by an electrophoretic mobility shift assay (EMSA). We found that induction of NF-kB activity revealed the same level in the liver with CD44KO × HBVTg or HBVTg mice (Fig. 4C). We observed induction of NF-kB activity of HBVTg mice at 4 h after the injection, as previously reported.[21] In contrast, and consistent with the cytokine profile, NF-κB activity was reduced with CD44KO × HBVTg mice at 4 h after the injection, suggesting that this reduction represents one of the reasons for the increase in apoptotic hepatocytes. Next, we investigated the phagocytic activity of CD44KO macrophages, because recent reports have suggested that CD44 is a competent phagocyte receptor and CD44KO mice exhibited poor clear-

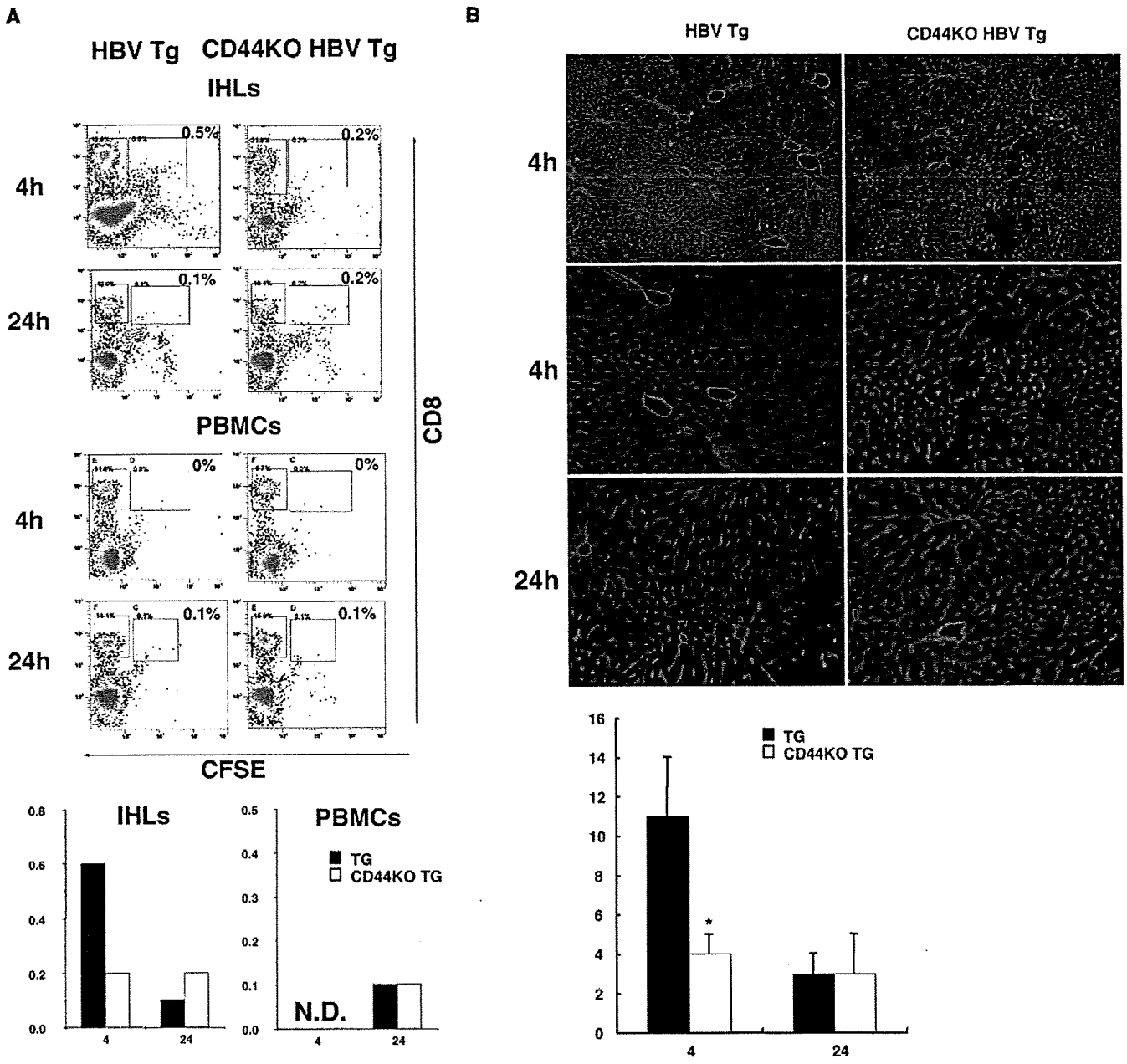


Fig. 2. Numbers of transferred CTLs in the liver labeled by 5,6-carboxyfluoroscein succinimidyl ester (CFSE). **A** To detect CFSE-labeled CTLs in the liver and peripheral blood mononuclear cells (PBMCs), we isolated IHLs and PBMCs from CD44KO × HBVTg and HBVTg mice at 4 and 24 h after injection. The samples were stained with an antimouse APC-CD8 antibody and analyzed by FACS. *N.D.*, not detected. **B** Liver sections were obtained from the same mice after injection of CFSE-labeled CTLs and stained with an antimouse type 4 collagen antibody and Alexa Fluor 594-conjugated goat antirabbit IgG as a secondary antibody to distinguish liver sinus endothelial cells (LSECs). The percentages of CFSE-positive cells in the liver were calculated. Data are expressed as means ± SD for three mice. $*P < 0.05$

ance of apoptotic cells by macrophages.[22,23] We found that dextran uptake by isolated $CD11b^+$ cells (mostly macrophages) from CD44KOxHBVTg mice was decreased compared with the uptake by $CD11b^+$ cells from HBVTg mice (Fig. 4D), indicating that the increase in apoptotic hepatocytes may be partially the result of dysfunction of clearance by macrophages.

Discussion

In the present study, we have demonstrated that, although CD44 deficiency has no suppressive effect on CTL-induced acute hepatitis, it contributes to CTL migration into the liver. It is already established that adhesion on lymphocytes, including CTLs and endothe-

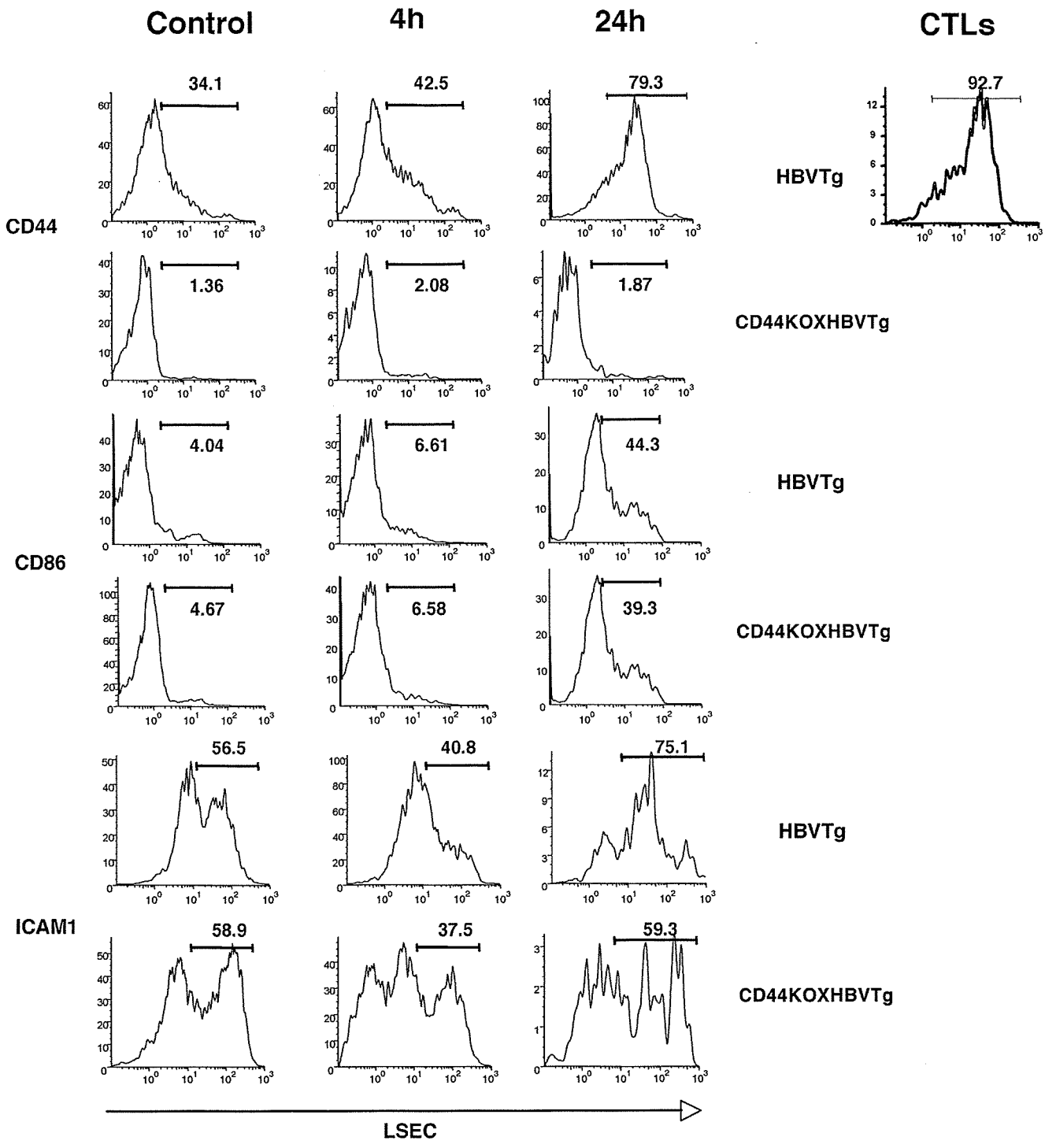


Fig. 3. FACS analysis of LSECs in the liver. The expression levels of CD44, CD86, and ICAM-1 on LSECs were examined at 4 and 24 h after CTL injection in CD44KO × HBVTg or HBVTg mice. IHLs were stained with anti-LSEC fluorescein isothiocyanate (FITC) and anti-CD44-PE, anti-CD86-PE, or anti-ICAM-1-PE antibodies. Representative results of three independent experiments are shown

lial cells, involves multiple steps at inflamed sites.[24–26] Although CD44 on lymphocytes is thought to be essential for adherence to endothelial cells, we have shown that CD44 on LSECs is also important for inflammatory cell migration into the liver. However, the precise mechanism of the interaction between CTLs and CD44 on LSECs remains unknown. As previously reported,[27,28] CD44 is a major receptor for hyaluronan, a nonsulfated

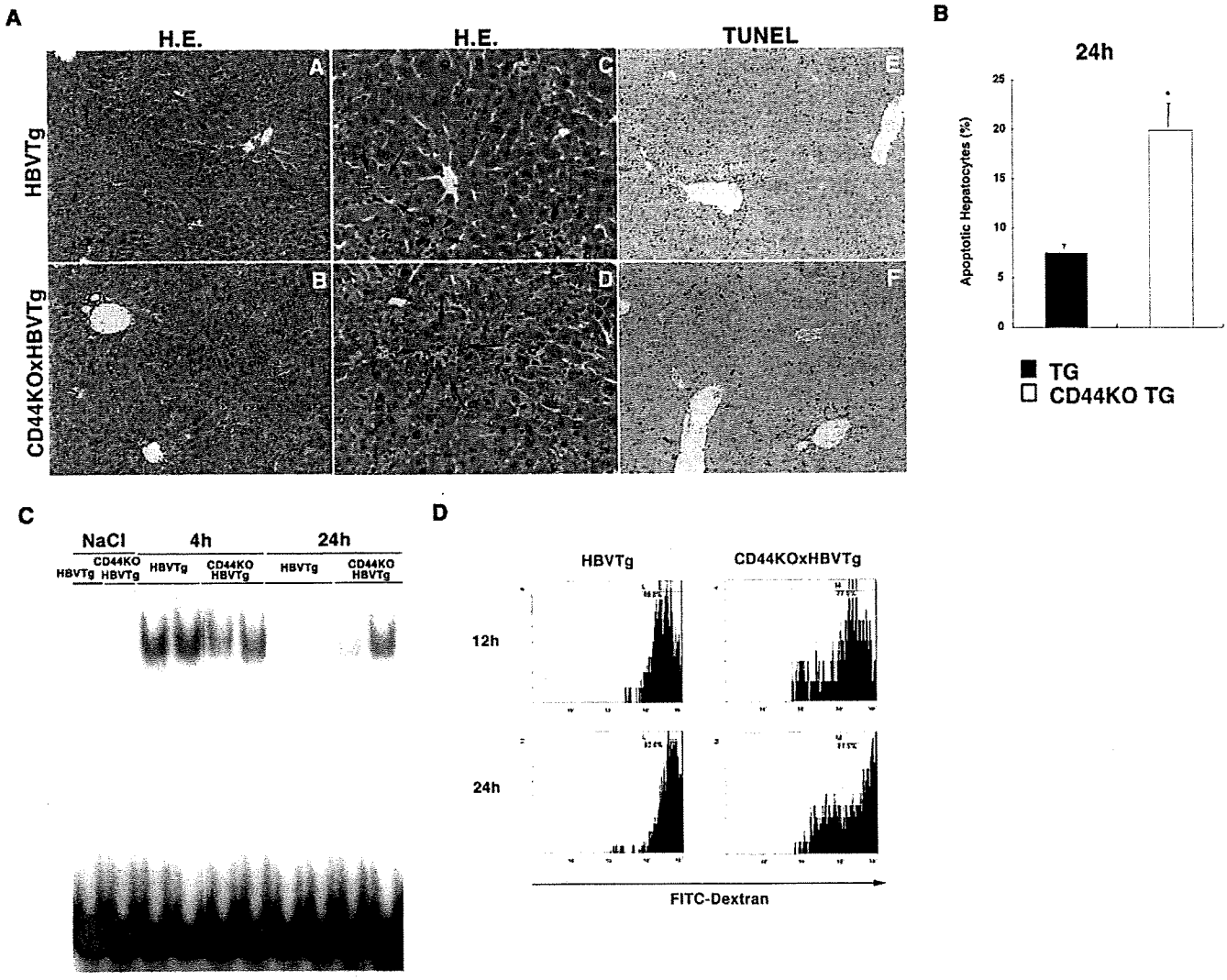
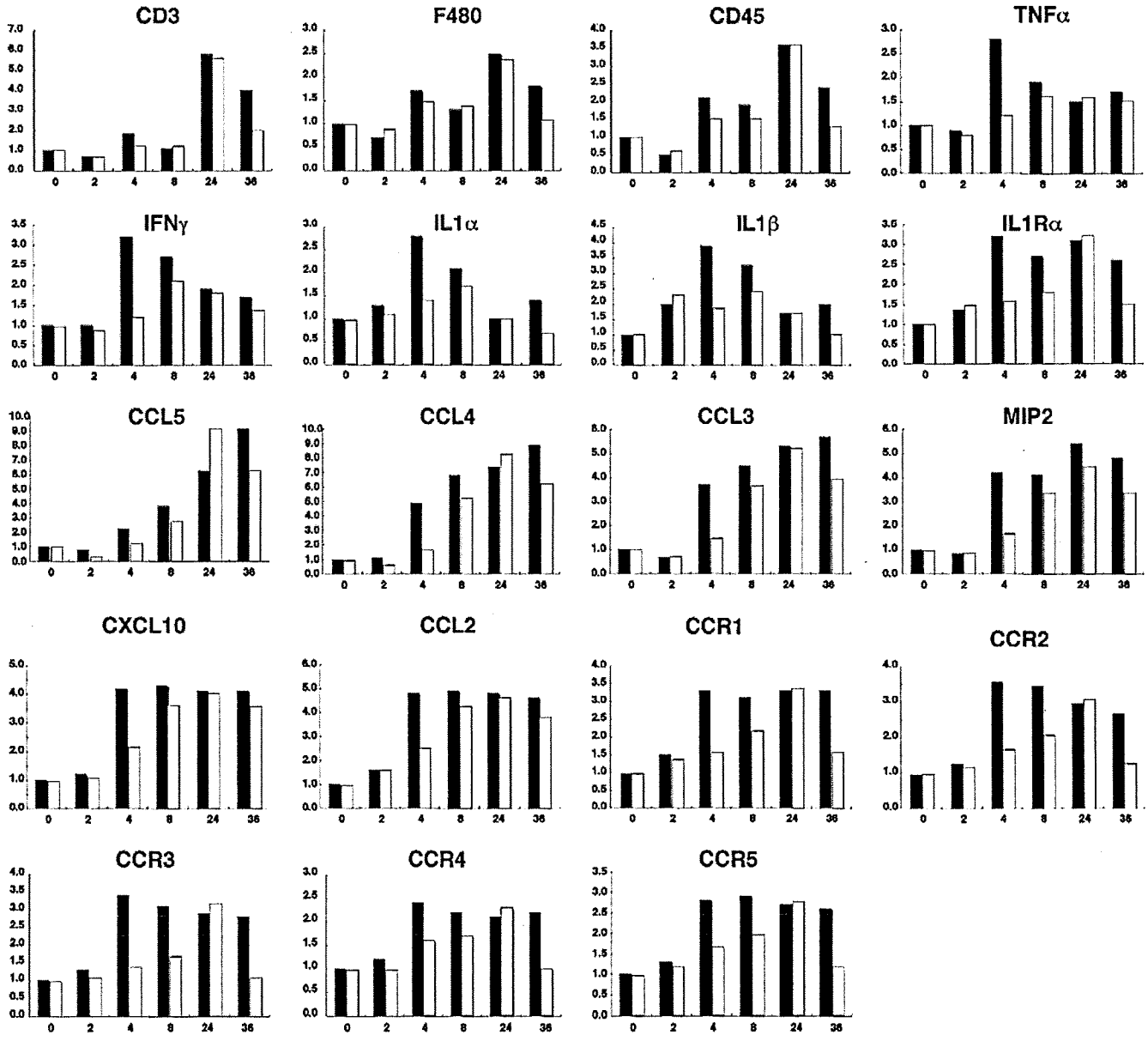


Fig. 4. Histological analysis of the livers of CD44KO × HBVTg or HBVTg mice after CTL injection and NF-κB activity. **A** Liver sections were obtained from mice killed at 24 h after CTL injection and stained with hematoxylin and eosin (*H.E.; left panels*). Note, in HBVTg mice at 24 h, that small inflammatory foci containing mostly lymph mononuclear cells (*arrows*) are observed in the liver. In CD44KO × HBVTg mice, larger foci containing more lymph mononuclear cells and liver necrosis are detected in the parenchyma (*arrows*). To evaluate the induction of apoptosis, liver sections were stained by the in situ TUNEL assay (*right panels*). In CD44KO × HBVTg mice at 24 h, TUNEL-positive hepatocytes are detected in the parenchyma (**B**). The percentages of apoptotic hepatocytes among the total hepatocytes were determined by TUNEL staining. Data are expressed as means ± SD for three mice. $^*P < 0.05$. **C** Electrophoretic mobility shift assays (EMSAs) were carried out on nuclear extracts from the livers of mice at 0, 4, or 24 h after CTL administration. **D** Phagocytic activities. To clarify whether CD44KO macrophages exhibit dysfunction of phagocytes, we isolated $CD11b^+$ cells from IHLs from CD44KO × HBVTg and HBVTg mice using MACS beads. After incubating both types of isolated $CD11b^+$ cells with FITC-dextran for 12 and 24 h, FACS analyses were performed

glycosaminoglycan ubiquitously distributed in extracellular spaces.[6] In addition, proteoglycan forms of CD44 exhibit affinities for other matrix components, including fibronectin, collagens, and osteopontin, as well as growth factors and cytokines.[29] Although we found that osteopontin was present on CTLs (data not shown), we cannot currently confirm the counterpart molecule for CD44 on CTLs. It is also curious why CD44KO inflammatory cells were able to infiltrate the liver at the late phase, when their migration was suppressed in the early phase. As one possible reason, we consider that, once the inflammatory cascade has started and many inflammatory cells migrate to the inflamed tissue, blockade of a single molecule, in this case CD44, is insufficient to protect against inflammation. These facts remind us that antiadhesion molecule treatment requires optimal timing and that its clinical application requires further experiments.

On the other hand, controversial findings have indicated that CD44 ligation has anti- or proapoptotic effects in cell lines.[30,31] To rule out the possibility that the transferred CTLs were susceptible to apoptosis in CD44KO mice, we analyzed annexin-V staining of CTLs after coculture with isolated LSECs from CD44KO × HBVTg or HBVTg mice, as previously reported.[15] However, there was no difference in the numbers of annexin V-positive CTLs in the mice (data not shown).

Thus, it is of note to mention that antiadhesion molecule-targeted therapy should be viewed with caution for the liver immune system. Although our findings require confirmation, blockade of CD44 alone in the present study led to inadequate cytokine production as a result of suppressed inflammation brought about by low NF-κB induction and subsequently led to increased numbers of apoptotic hepatocytes and severe liver injury. These dual effects (suppression and exacerbation) on the regulation of liver inflammation have been reported with regard to NF-κB regulation.[15,16] These results suggested that insufficient antiinflammatory effects rather exacerbate hepatic injury, and it seems that it is necessary to suppress the inflammation sufficiently during acute hepatitis in the environment of the liver.



Supplementary Fig. 1. Supplementary data. Densitometric analysis: mRNA expression levels were calculated as relative percentages to the mRNA expression level of the *L32* housekeeping gene

Furthermore, we showed here that HBV-specific CTLs play an important role as the trigger for severe liver injury. As was shown in Fig. 2, the number of CFSE-labeled CTLs was reduced at 24 h compared with 4 h in HBVTg mice when CD44 was either present or absent; however, sALT activity exhibited a peak at 24 h, suggesting that severe liver injury was mainly caused by antigen-nonspecific inflammatory cells, NK cells, macrophages, and neutrophils, as previously reported.[2,3,13]

Although we showed the number of CFSE-labeled CTLs in the liver and PBMCs (see Fig. 2), we also investigated the number in another tissue, the spleen. However, we could not detect CTLs in the CD44 × HBVTg or HBVTg spleen at 2, 4, and 24 h after injection (data not shown). These results suggested to us the importance of the affinity and avidity of HBV-specific CTLs in HBV-expressing hepatocytes.

In conclusion, these findings mean that although CD44 blockade is useful for the migration of virus-specific CTLs to the liver, it may require further improvement for use as a clinical therapy.

Acknowledgments. We thank Dr. Francis V. Chisari (Scripps Research Institute) for providing us with HBV transgenic mice and Dr. Tak W. Mak (University of Toronto) for providing us with CD44KO mice. We also thank Tomomi Saeki and Yumiko Okuda for technical support. This study was supported by Grants-in-Aid (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant No. 87100000183) and a Grant from the Tokai Science Academy to Kimura K.

References

1. Kimura K, Nagaki M, Nishihira J, Satake S, Kuwata K, Moriwaki H. Role of macrophage migration inhibitory factor in hepatitis B virus-specific cytotoxic-T-lymphocyte-induced liver injury. Clin Vaccine Immunol 2006;13:415–9.
2. Takai S, Kimura K, Nagaki M, Satake S, Kakimi K, Moriwaki H. Blockade of neutrophil elastase attenuates severe liver injury in hepatitis B transgenic mice. J Virol 2005;79:15142–50.
3. Sitia G, Isogawa M, Kakimi K, Wieland SF, Chisari FV, Guidotti LG. Depletion of neutrophils blocks the recruitment of antigen-nonspecific cells into the liver without affecting the antiviral activity of hepatitis B virus-specific cytotoxic T lymphocytes. Proc Natl Acad Sci U S A 2002;99:13717–22.
4. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301–14.
5. Springer TA. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. Annu Rev Physiol 1995;57: 827–72.
6. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003;4: 33–45.
7. Brocke S, Piercy C, Steinman L, Weissman IL, Veromaa T. Antibodies to CD44 and integrin alpha4, but not L-selectin, prevent central nervous system inflammation and experimental encephalomyelitis by blocking secondary leukocyte recruitment. Proc Natl Acad Sci U S A 1999;96:6896–901.
8. Katoh S, Matsumoto N, Kawakita K, Tominaga A, Kincade PW, Matsukura S. A role for CD44 in an antigen-induced murine model of pulmonary eosinophilia. J Clin Invest 2003;111: 1563–70.
9. McKallip RJ, Fisher M, Gunthert U, Szakal AK, Nagarkatti PS, Nagarkatti M. Role of CD44 and its v7 isoform in staphylococcal enterotoxin B-induced toxic shock: CD44 deficiency on hepatic mononuclear cells leads to reduced activation-induced apoptosis that results in increased liver damage. Infect Immun 2005;73: 50–61.
10. Mikecz K, Brennan FR, Kim JH, Glant TT. Anti-CD44 treatment abrogates tissue oedema and leukocyte infiltration in murine arthritis. Nat Med 1995;1:558–63.
11. Schmits R, Filmus J, Gerwin N, Senaldi G, Kiefer F, Kundig T, et al. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. Blood 1997;90:2217–33.
12. Wang Q, Teder P, Judd NP, Noble PW, Doerschuk CM. CD44 deficiency leads to enhanced neutrophil migration and lung injury in *Escherichia coli* pneumonia in mice. Am J Pathol 2002;161: 2219–28.
13. Ando K, Moriyama T, Guidotti LG, Wirth S, Schreiber RD, Schlicht HJ, et al. Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis. J Exp Med 1993;178:1541–54.
14. Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity 1996;4:25–36.
15. Kimura K, Nagaki M, Takai S, Satake S, Moriwaki H. Pivotal role of nuclear factor kappa B signaling in anti-CD40-induced liver injury in mice. Hepatology 2004;40:1180–9.
16. Kimura K, Nagaki M, Kakimi K, Saio M, Saeki T, Okuda Y, et al. Critical role of CD44 in hepatotoxin-mediated liver injury. J Hepatol 2008;48:952–61.
17. Crispe IN. Hepatic T cells and liver tolerance. Nat Rev Immunol 2003;3:51–62.
18. Knolle P, Lohr H, Treichel U, Dienes HP, Lohse A, Schlaack J, et al. Parenchymal and nonparenchymal liver cells and their interaction in the local immune response. Z Gastroenterol 1995;33: 613–20.
19. Knolle PA, Gerken G, Loser E, Dienes HP, Gantner F, Tiegs G, et al. Role of sinusoidal endothelial cells of the liver in concanavalin A-induced hepatic injury in mice. Hepatology 1996;24: 824–9.
20. Nagaki M, Naiki T, Brenner DA, Osawa Y, Imose M, Hayashi H, et al. Tumor necrosis factor alpha prevents tumor necrosis factor receptor-mediated mouse hepatocyte apoptosis, but not fas-mediated apoptosis: role of nuclear factor-kappa B. Hepatology 2000;32:1272–9.
21. Satake S, Nagaki M, Kimura K, Moriwaki H. Inhibition of nuclear factor-kappa B induces inflammatory cell migration and exacerbates severe liver injury in hepatitis B virus transgenic mice. Hepatol Res 2007;37:524–30.
22. Teder P, Vandivier RW, Jiang D, Liang J, Cohn L, Pure E, et al. Resolution of lung inflammation by CD44. Science 2002;296: 155–8.
23. Vachon E, Martin R, Plumb J, Kwok V, Vandivier RW, Glogauer M, et al. CD44 is a phagocytic receptor. Blood 2006;107: 4149–58.
24. Kraal G, Mebius RE. High endothelial venules: lymphocyte traffic control and controlled traffic. Adv Immunol 1997;65: 347–95.
25. Siegelman MH, DeGrendele HC, Estess P. Activation and interaction of CD44 and hyaluronan in immunological systems. J Leukoc Biol 1999;66:315–21.
26. Stoolman LM. Adhesion molecules controlling lymphocyte migration. Cell 1989;56:907–10.
27. Cichy J, Pure E. The liberation of CD44. J Cell Biol 2003;161: 839–43.
28. Goodison S, Urquidi V, Tarin D. CD44 cell adhesion molecules. Mol Pathol 1999;52:189–96.

29. Weber GF, Ashkar S, Glimcher MJ, Cantor H. Receptor–ligand interaction between CD44 and osteopontin (Eta-1). Science 1996; 271:509–12.
30. Allouche M, Charrad RS, Bettaieb A, Greenland C, Grignon C, Smadja-Joffe F. Ligation of the CD44 adhesion molecule inhibits drug-induced apoptosis in human myeloid leukemia cells. Blood 2000;96:1187–90.
31. Lakshman M, Subramaniam V, Jothy S. CD44 negatively regulates apoptosis in murine colonic epithelium via the mitochondrial pathway. Exp Mol Pathol 2004;76:196–204.

Hepatology Research 2009; 39: 93–105 doi: 10.1111/j.1872-034X.2008.00409.x

Original Article

Pathological role of CD44 on NKT cells in carbon tetrachloride-mediated liver injury

Kiminori Kimura,[1,2] Masahito Nagaki,[3] Tomokazu Matsuura,[4] Hisataka Moriwaki[3] and Kazuhiro Kakimi[2]

[1]*Division of Hepatology, Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital, 18-22-3, Honkomagome, Bunkyoku, Tokyo 113-8677, Japan*
[2]*Department of Immunotherapeutics (Medinet), Graduate School of Medicine, The University of Tokyo,*
[4]*Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo, and* [3]*First Department of Internal Medicine, Gifu University Graduate School of Medicine, Gifu, Japan*

Aim: CD44 has a variety of functions in immune regulation and signal transduction. Although CD44 is involved in the induction of several inflammatory diseases, it remains unknown whether CD44-targeting therapies are useful for liver diseases. Here, we examined whether CD44 blockade is effective in a chemical-induced liver injury model.

Methods: We injected CD44 knock out (KO) or wild type mice with carbon tetrachloride (CCl_4) and examined the difference of liver injury by immunological or histological analysis.

Results: Although CD44KO mice exhibited suppressed liver injury at 6 h after CCl_4 injection with decreased inflammatory cell numbers and cytokine production, these mice showed severe liver injury at 24 h. We found that NKT cells played an important role in liver injury with increased infiltration of the liver after migration, which was independent of the CD44 pathway. In CD44NKT double-KO mice, liver injury was suppressed with reduced cytokine production and macrophage infiltration compared with CD44KO mice. Furthermore, MIP-2 derived from NKT cells or tumor necrosis factor alpha from macrophages contributed to exacerbation of the liver injury, since neutralization of MIP-2 provided significant protection against liver injury in CD44KO mice. Finally, we found that CD44KO mice exhibited excessive liver fibrosis compared with wild-type mice after repeated CCl_4 injections.

Conclusion: We found that CD44 has unique characteristics for inflammatory liver diseases associated with NKT cell infiltration and activation. Furthermore, CD44-targeting therapies may need to be viewed with caution for liver diseases due to the actions of the liver immune system.

Key words: CD44, cytokine, inflammation, liver fibrosis, NKT cell

INTRODUCTION

THE LIVER HAS a variety of characteristic features in its immune responses to foreign pathogens.[1,2] The liver contains a large population of lymphocytes, including $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells and natural killer T (NKT) cells.[3–5] These populations can be rapidly expanded during inflammatory liver disease or in response to viral infection. It has been suggested that most lymphocytes in the normal liver are activated terminally-differentiated T cells that are removed from circulation by the liver, where they are destined to die by apoptosis.[1,6] Furthermore, it is well established that antigen-specific $CD8^+$ cells play an important role in the clearance of hepatitis B and C viruses.[3,7] In addition, recent reports have shown that antigen-nonspecific cells, NKT cells, NK cells and B cells could also act as effector cells in the activated state.[8–10] In view of these findings, blockade of inflammatory cell infiltration into the liver is thought to be an ideal therapy for various diseases of the liver.

For a lymphocyte to be recruited from the circulating blood stream, it must first recognize and then bind to adhesion molecules expressed on endothelial cells.[11] A multistep model of leukocyte adhesion to the vascular

Correspondence: Dr Kiminori Kimura, M.D. Ph.D. Division of Hepatology, Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital, 18-22-3, Honkomagome, Bunkyoku, Tokyo 113-8677, Japan. Email: kkimura@cick.jp
Received 10 June 2008; revision 26 June 2008; accepted 30 June 2008.

endothelium has been described elsewhere and is broadly applicable in different tissues.[12,13] It has also been demonstrated that tethering or rolling receptors expressed on endothelial cells capture free-floating leukocytes. Recruitment of leukocytes in the post-sinusoidal venules of the liver follow a similar paradigm. However, distinct from most other organs, many leukocytes can also be seen adhering in the sinusoids, which are specialized hepatic capillaries.[11]

CD44-hyaluronan interactions play an important role in regulating leukocyte extravasation into inflammatory sites and mediate efficient phagocytosis.[14–17] In addition, a growing body of evidence suggests that CD44 serves as a key factor in the resolution of inflammation through removal of matrix breakdown products and clearance of apoptotic neutrophils, and mediates fibroblast migration and invasion in the provisional matrix of the wound.[16]

NKT cells express an invariant T cell receptor chain (V14-J281 in mice) and recognize glycolipid antigens, such as α-galactosylceramide (αGalCer), in association with the major histocompatibility complex (MHC) class I-like molecule CD1d.[18,19] NKT cells are unique for cytokine production, since they are autoreactive and produce both Th1 and Th2 cytokines, including interleukin (IL)-4, IL-5 and IFN-γ, upon stimulation with αGalCer.[20–22] A recent report demonstrates that the adhesion molecule lymphocyte function-associated antigen (LFA)-1 plays a role in NKT cell activation (especially, Th2 responses) in response to αGalCer treatment.[23–25] However, the relationship between NKT cells and adhesion molecules during inflammatory events remains unknown.

In the present study, we examine the suppressive effects of CD44 on inflammation in mouse models of hepatotoxin (CCl_4)-induced acute and chronic liver diseases.

EXPERIMENTAL PROCEDURES

Animals

CD44KO MICE (BACKGROUND, C57BL/6) were generously provided by Dr. Tak W. Mak (University of Toronto, Canada).[26] Vα14 NKT-deficient (NKT-KO) mice were generated as described[20] and C57BL/6 (B6) mice were purchased from SLC (Japan). All animals were housed in pathogen-free rooms under strict barrier conditions, and received humane care according to the guidelines of the Animal Care Committee of the University of Tokyo, School of Medicine.

Administration of CCl_4 and αGalCer

For acute CCl_4-induced liver damage studies, a single dose of CCl_4 (2.0 mL/kg body weight; 1 : 4 v/v in mineral oil) was administered by i.p. injection. For chronic CCl_4-induced liver damage studies, a dose of CCl_4 (2.0 mL/kg body weight) was administered twice per week. For activation of Vα14 NKT cells with αGalCer, mice were injected i.p. with αGalCer (100 μg/kg) or vehicle as previously described.[21] For *in vitro* experiments, we added CD44KO and wild type (WT) mice derived intrahepatic leukocytes (IHLs) with αGalCer (10 ng/mL). αGalCer (KRN7000) was kindly provided by Kirin Brewery (Japan).

Tissue RNA analyses

Frozen liver was mechanically pulverized under liquid nitrogen and total RNA was isolated for ribonuclease protection assay (RPAs), as previously described.[10] All RPA reagents were purchased from BD PharMingen (CA, USA).

Biochemical and histological analyses

The extent of hepatocellular injury was monitored biochemically by measuring the activity of serum alanine aminotransferase (sALT) at multiple time points using a standard automatic clinical analyzer. For histological analysis, liver tissue was fixed in 10% zinc-buffered formalin, embedded in paraffin, sectioned (3 μm) and stained with hematoxylin and eosin or AZAN for light-microscopic evaluation.

Immunohistochemistry

Immunohistochemical staining with anti-mouse α-smooth muscle actin (αSMA) and anti-mouse proliferating cell nuclear antigen (PCNA) monoclonal antibodies (mAbs) was performed using an avidin-biotin-peroxidase complex technique as described previously.[10] The numbers of positive cells were counted in at least 50 high-power (× 400) fields and the results were expressed as percentages (positive cells/total cells). Terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) assays were performed according to the manufacturer's instructions, as described previously.[27]

Preparation of intrahepatic leukocytes (IHLs)

To isolate IHLs, single-cell suspensions were prepared from liver perfused with phosphate-buffered saline (PBS) via the inferior vena cava and digested with 10 mL of RPMI 1640 (Life Technologies, MD, USA) containing 0.02% (w/v) collagenase IV (Sigma–Aldrich, MO, USA)