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Original Article

Hepatitis C virus kinetics during the first phase of pegylated interferon-α-2b with ribavirin therapy in patients with living donor liver transplantation

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Aim: To identify the problems of pegylated interferon (PEG IFN) with ribavirin therapy against hepatitis C virus (HCV) reinfection in living donor liver transplantation (LDLT) patients. HCV kinetics during the PEG IFN with ribavirin therapy were analyzed in LDLT patients, as well as in chronic hepatitis C (CHC) patients.

Methods: The study included 80 consecutive HCV infected patients undergoing PEG IFN with ribavirin therapy (64 CHC and 16 LDLT patients) who attended the Nagasaki University Hospital for an initial visit between January 2005 and December 2007.

Results: The sustained viral response (VR) rate of the CHC group (80%) was superior to the LDLT group (22%). The viral disappearance rate of the CHC group was also superior to the LDLT group, regardless of the HCV serotype. The HCV core antigen (cAg) titer under treatment in the LDLT group was more than that of the CHC group from day 0 to week 12. The HCV cAg decrease rate of the LDLT group on the first day of treatment was less than that of the CHC group.

Conclusion: The HCV infection of a transplanted liver is more refractory to treatment than a non-transplanted liver. The low reduction HCV cAg rate on day 1 is one of the problems of the combination therapy.

Key words: chronic hepatitis C, first phase, hepatitis C virus, interferon, living donor liver transplantation

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is widespread throughout the world. Chronic HCV infection leads to cirrhosis and hepatocellular carcinoma. Liver transplantation for HCV-related liver disease has been an option worldwide.[1] Recently, it has been shown that the prognosis for liver transplanted (LT) patients with HCV-related disease deteriorates over time,[2] thus resulting in a poorer outcome than in the non-HCV course.[3] The transplanted liver for HCV-related disease undergoes a rapidly progressive fibrosis and acute graft failure.[3,4] Consequently, anti-HCV treatment after LT is important for the prognosis. Interferon (IFN) has been recognized as the only treatment method for HCV infection. For the transplanted liver, it is known that IFN treatment improves liver fibrosis or halts the progression.[5] Recently, the combination of pegylated IFN (PEG IFN) with ribavirin was used and produced an excellent result for non-transplanted patients with HCV.[6] However, that was not the case for the HCV re-infected transplanted liver.[7] It is important that the cause of refractory HCV infection in the transplanted liver be more fully clarified. Immunosuppressant therapy, especially with glucocorticoid, has been speculated to be the cause of the refractory nature of the transplanted liver to IFN.[8,9] The cause of this is considered to be that glucocorticoid downregulated the IFN signal transduction in the hepatocytes.[8] The authors recently found that calcineurin inhibitors also inhibited IFN induced STAT-1 phosphorylation and antiviral activity in the HCV

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replicon system.[10] Therefore, the problem of IFN signaling in the hepatocyte induced an IFN refractory condition[11] and decreased the first phase of HCV decline, which was IFN induced HCV decay during the first day of IFN treatment.[12]

In the present study, we attempted to better understand PEG IFN and ribavirin therapy by comparing patients with chronic hepatitis from HCV infection (CHC) with living donor LT (LDLT) patients. When the non-transplanted CHC patients were used as a reference against the HCV reinfected LDLT patients, we expected that the differences in the clinical data in the two groups would help to clarify the problem of IFN refractory HCV infection, and shed light on the analysis of HCV kinetics under IFN and ribavirin treatment, and to elucidate the damaged segment of the IFN induced antiviral mechanism in the LDLT condition.

PATIENTS AND METHODS

Patients

THE PRESENT RESEARCH is a prospective study. The study included 80 consecutive HCV-infected patients undergoing PEG IFN with ribavirin combination therapy (64 CHC and 16 LDLT patients) who attended the Nagasaki University Hospital for an initial visit between January 2005 and December 2007. All patients received the targeted dose of 1.5 μg/kg PEG IFN-α-2b (Pegintron; Schering-Prough K.K., Osaka, Japan) once weekly with daily ribavirin (Rebetol; Schering-Prough K.K., Osaka, Japan) for a total dose of 600 mg (bodyweight < 60 kg), 800 mg (60 kg < bodyweight < 80 kg) or 1000 mg (bodyweight > 80 kg) according to bodyweight (BW). The number of patients who were judged to have obtained a curative effect from IFN therapy was 42 in total, and 12 were LDLT patients. If the HCV-RNA had been negative in the patient serum until 12 weeks after the initiation of treatment or positive at 24 weeks, PEG IFN with ribavirin therapy was stopped at week 48. If the HCV-RNA had been negative from weeks 12 to 24, PEG IFN with ribavirin therapy was continued for 24 weeks to a predetermined 48 weeks. CHC patients were diagnosed on the basis of a persistently raised alanine aminotransferase (ALT) level and biopsy proven disease. All LDLT patients, who had undergone liver transplantation for HCV related cirrhosis at Nagasaki University Hospital from June 2002 to May 2007, had the HCV-RNA in their serum at the commencement of PEG IFN with ribavirin treatment. To prevent HCV related hepatitis after liver transplantation, pre-emptive therapy using IFN is the strategy used at the Nagasaki University Hospital. After the recovery of the general condition without ascites and icterus after transplantation, and establishment of the diagnosis using the liver biopsy, PEG IFN with ribavirin therapy was started. The interval between LDLT and IFN treatment was a mean of 281 days (range 16–989 days). Tacrolimus (Astellas, Tokyo, Japan), an immunosuppressive agent, was used together with steroids for all LDLT patients as the induction therapy. When IFN therapy was commenced, tacrolimus was switched to cyclosporin (Novartis, Tokyo, Japan) in 12/16 cases. A percutaneous liver biopsy assisted by ultrasonography was carried out in all cases. Liver histology was evaluated according to the degree of fibrosis and necroinflammatory activity.[13] The extent of fibrosis (staging) was classified as follows: F1 (periportal expansion), F2 (portoportal septa), F3 (portocentral linkage or bridging fibrosis) and F4 (cirrhosis). The necroinflammatory activity (grading) was classified as follows: A1 (mild), A2 (moderate) and A3 (severe). Liver biopsy specimens were fixed in 10% formalin, embedded in paraffin, cut to a thickness of 4 μm, and subjected to hematoxylin–eosin and Azan–Mallory staining.

Hepatitis C virus kinetics assessment

We compared the HCV viral load in both groups, determined by the HCV core antigen (cAg), at baseline (D0), day 1 (D1), week 1 (W1), week 2 (W2), week 4 (W4), week 8 (W8), week 12 (W12), week 24 (W24) and week 48 (W48). The HCV viral serotype (ST) and HCV cAg were determined using available kits. In this assay, HCV serotypes 1 and 2 correspond to genotypes 1 and 2 of Simmonds' classification,[14] respectively. The HCV cAg correlates with HCV-RNA by quantitative PCR.[15] HCV cAg was measured at the indicated times and HCV-RNA qualitative PCR, the amplicor monitor method, was used after the level was under the detection range of HCV cAg in every month. In the present study, we proposed the calculation of the decreased HCV viral load during PEG IFN with ribavirin treatment and set as follows: a negative HCV cAg was 20 fmol/L and a negative HCV-RNA qualitative PCR was 1 fmol/L.

Clinical and laboratory measurements

The body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). Subjects fasted overnight before blood samples were obtained. Venous plasma glucose was measured with an automated analyzer, and basal serum insulin was measured using a standard radioimmunoassay. The index of insulin

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resistance and β-cell function was calculated using the fasting value of plasma glucose (we excluded the patients with greater than 130 mg/dL), and the serum insulin level according to the homeostasis model assessment (HOMA) method. HOMA-IR, an insulin resistance marker, is calculated as follows: fasting plasma glucose × fasting insulin/405. HOMA-β, a β-cell function marker, was calculated as follows: 360 × fasting insulin/(fasting plasma glucose-63).[16] White blood cell, red blood cell, platelet, hemoglobin A1c, ALT, aspartate aminotransferase (AST), γ-GTP, total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), free fatty acid (FFA), and ferritin were determined by standard hematometry and laboratory techniques.

Statistical analysis

The data were processed on a personal computer and analyzed using StatView 5.0 (SAS Institute, Cary, NC, USA). Differences between groups were analyzed by Mann–Whitney *U*-test and Pearson χ^2-test. All data in the text and tables are shown as means, unless otherwise indicated. The statistical analysis of the HCV-RNA disappearance rate was by the Kaplan–Meier method with Wilcoxon assay. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Differences of patient characteristics

FIRST, THE PRETREATMENT clinical and laboratory characteristics were compared with All-CHC and All-LDLT patients (Table 1). The BW and BMI in the All-CHC group were higher than that of the All-LDLT group. Therefore, the levels of PEG IFN dose per BW and ribavirin dose per BW were even, but the levels of PEG IFN dose and ribavirin dose in the all LDLT group were lower than in the All-CHC group. The HCV viral load in the all LDLT group was greater than that in the All-CHC group and serotype 1 was the majority in the All-LDLT group. In hematology and laboratory data, the red blood cell count and hemoglobin in the All-LDLT group was lower than that of the All-CHC group, and the FFA level was higher in the All-LDLT group. In the histological examination, fibrosis is more advanced in the All-CHC group than in the All-LDLT group. There was the tendency toward higher levels of fasting plasma glucose and lower levels of HOMA-β in the All-LDLT group than in the All-CHC group. Next, we targeted the serotype 1 and a high HCV titer (ST1H group) above 100 KIU/L by the qualitative PCR method or 300 fmol/L of the cAg assay. These were examined in the same way (Table 2). The ST1H group might have shown the same result as the All group, except the levels of fasting plasma glucose and HOMA-β did not differ with ST1H-CHC and ST1H-LDLT. The mean value of fasting plasma glucose (FPG) was higher than the normal range in the LDLT group. The discontinuance rates of treatment were almost equal, 19 cases (29.7%) and 4 cases (25%) in All-CHC and All-LDLT, respectively. The reasons for discontinuance were adverse effects in All-LDLT patients and the refractory nature of viral response in two All-CHC patients.

The HCV infection in the LDLT group is more obstinate than in the CHC group

The response rate and cure rate of PEG IFN with ribavirin therapy were compared with both groups (Table 2A, All group and B, ST1H group). The HCV response rate to treatment, viral response (VR), was determined by the disappearance of HCV-RNA or by the decline of HCV cAg to less than 1/100 before treatment. The cure rate, sustained viral response rate (SVR), was determined by a negative HCV-RNA by qualitative PCR method at 6 months post-termination of treatment. The VR rate at 8 and 12 weeks, but not at 4 weeks, and the PP-SVR in the LDLT group (Table 3A,B) was worse than that in the CHC group. Non-viral responders, who did not achieve HCV-RNA negativity during the treatment, did not show statistical significance in either SG1H group (Table 3B). As a result, we calculated the prediction of the lack of SVR by non-viral response in the LDLT group. The sensitivity, specificity, positive predictive values and negative predictive value were 1, 0, 0.917 and the acalculia for null viral responders at 24 h, 0.7, 1, 1 and 0.25 at 4 weeks, 0.6, 1, 1 and 0.2 at 8 weeks and 0.6, 1, 1 and 0.2 at 12 weeks, respectively.

The disappearance rate of HCV-RNA was evaluated by the Kaplan–Meier method (Fig. 1 ST1H group). The disappearance rate in the LDLT group was statistically lower than the CHC group. Before 14 weeks after the initiation of treatment, the HCV-RNA disappearance case was not apparent in the ST1H group (Fig. 1).

The decline of HCV load, especially early phase, is blocked in the LDLT group

For the analysis of viral kinetics, we evaluated the decline of the HCV load and the decline rate after treatment with particular emphasis of the early phase of treatment, including D1-W12. In the ST1H group (Fig. 2), the decreased rate on D1 in the LDLT group was

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Table 1 Difference of characteristics between all chronic hepatitis C cases and all living donor liver transplantation cases

Characteristics	All-CHC (n = 64)	All-LDLT (n = 16)	P-value
Age (years)	58 ± 10.8	58.8 ± 4.62	NS
Sex (male : female)	36:28	7:9	NS
Height (m)	1.60 ± 0.098	1.583 ± 0.010	NS
Bodyweight (kg)	61.0 ± 11.0	54.8 ± 8.52	0.025
Body mass index	23.6 ± 2.94	21.8 ± 2.30	0.022
PEG IFN dose (μg)	80.1 ± 18.7	71.9 ± 33.5	0.035
PEG IFN/BW	1.31 ± 0.304	1.35 ± 0.708	NS
Ribavirin dose (mg)	621.9 ± 151.7	525 ± 100	0.030
Ribavirin/BW	10.2 ± 2.23	9.72 ± 2.04	NS
Serotype (1:2)	45:17	15:1	0.081
HCV cAg (fmol/L)	5773 ± 5609	23144 ± 21059	0.001
WBC (/μL)	5006.3 ± 1335	5918.8 ± 2439	NS
RBC (10^4/μL)	445 ± 41.1	350 ± 56.7	< 0.0001
Hemoglobin (g/dL)	13.8 ± 1.06	10.9 ± 1.85	< 0.0001
Platelet (10^4/μL)	16.4 ± 4.48	18.5 ± 10.6	NS
AST (U/L)	62.9 ± 35	64.3 ± 37.2	NS
ALT (U/L)	85 ± 53.0	89.9 ± 57.1	NS
γ-GTP (U/L)	62.1 ± 56.5	138.9 ± 129.1	0.013
Ferritin (ng/dL)	218 ± 216	254 ± 259	NS
TC (mg/dL)	169.8 ± 26.6	167.3 ± 38.8	NS
TG (mg/dL)	105.3 ± 46.8	122.8 ± 44.8	0.069
HDL (mg/dL)	45.2 ± 11.9	46.6 ± 14.9	NS
LDL (mg/dL)	97.3 ± 24.3	88.8 ± 26.7	NS
FFA (mEq/L)	0.492 ± 0.261	0.686 ± 0.299	0.019
FPG (mg/dL)	91.9 ± 15.4	125.1 ± 56.9	0.090
Insulin (mIU/L)	9.16 ± 5.1	8.34 ± 5.16	NS
HOMA-IR	2.08 ± 1.22	1.75 ± 1.42	NS
HOMA-β	135.4 ± 86.2	89.7 ± 86.9	0.075
Fibrosis	1.86 ± 1.18	0.875 ± 0.806	0.004
Activity	1.03 ± 0.48	1.31 ± 0.48	0.067

Data are shown as the means ± standard deviation and values, with statistical analysis calculated by Mann–Whitney U-test for means and Pearson's χ^2-test for values.
Normal values in laboratory tests: ALT (IU/L), 5–40; AST (IU/L), 10–40; γ-GTP (IU/L), < 70 in males, < 30 in females; TC (mg/dL), 150–219; TG (mg/dL), 50–149; FFA (mEq/L), 0.14–0.85; LDL (mg/dL), 70–139; HDL (mg/dL), 40–86 in male, 40–96 in female; hemoglobin (g/dL), 13.5–17.6 in male, 11.3–15.2 in female; WBC (/μL), 3900–9800 in males, 3500–9100 in females; RBC (10^4/μL), 427–570 in males, 376–500 in females; ferritin (mg/dL), 27–320 in males, 3.4–89 in females; platelet (10^4/μL), 13.1–36.2 in males, 13–36.9 in females; insulin (IU/L), 3.06–16.9; FPG (mg/L), 70–109. HOMA-IR, HOMA-β, and BMI are described in the text.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHC, chronic hepatitis C; FFA, free fatty acid; FPG, fasting plasma glucose; HCV cAg, hepatitis C virus core antigen; HDL, high density lipoprotein; HOMA, homeostasis model assessment; LDL, low density lipoprotein; LDLT, living donor liver transplantation; PEG IFN, pegylated interferon; RBC, red blood cell count; TC, total cholesterol; TG, triglyceride; WBC, white blood cell count.

statistically lower than CHC (Fig. 2b) and the viral load of the LDLT group was larger than that in CHC from D0 to W12 (Fig. 2a). The decreased rate at the indicated time without D1 and W12 was not the difference between CHC and LDLT (Fig. 2b). We next analyzed the SG1H-group that matched the pre-treatment HCV cAg titer (Fig. 3). In a similar fashion to Figure 2, the viral load of the matched LDLT group was larger than that of the matched CHC from D1 to W12 (Fig. 3a) and the decreased rate of the matched LDLT group was lower than that of the matched CHC at D1, W2 and W4 (Fig. 3b).

DISCUSSION

IN THE PRESENT prospective study, we compared CHC and LDLT patients treated with PEG IFN and ribavirin for HCV infection. BMI, HCV cAg, red blood

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Table 2 Difference of characteristics of serotype 1 and high virus titer between chronic hepatitis C patients and living donor liver transplantation patients

Characteristics	ST1H-CHC ($n = 42$)	ST1H-LDLT ($n = 15$)	*P*-value
Age (years)	58.5 ± 10.8	58.8 ± 4.78	NS
Sex (male : female)	22:20	6:9	NS
Height (m)	1.60 ± 0.10	1.566 ± 0.081	NS
Bodyweight (kg)	61.8 ± 12.1	53.8 ± 7.69	0.02
Body mass index	24.0 ± 2.78	21.9 ± 2.37	0.012
PEG IFN dose (μg)	81.4 ± 19.5	73.3 ± 34.2	0.052
PEG IFN/BW	1.33 ± 0.269	1.39 ± 0.711	NS
Ribavirin dose (mg)	642.8 ± 150.0	520 ± 101.4	0.011
Ribavirin/BW	10.5 ± 2.13	9.80 ± 2.08	NS
HCV cAg (fmol/L)	6969 ± 5281	24674 ± 20856	0.003
WBC (/μL)	5019.0 ± 1294	6033.8 ± 2479	NS
RBC (10^4/μL)	444 ± 40.1	351 ± 58.6	< 0.0001
Hemoglobin (g/dL)	13.9 ± 1.10	10.8 ± 1.88	< 0.0001
Platelet (10^4/μL)	16.7 ± 4.68	18.9 ± 10.8	NS
AST (U/L)	62.1 ± 31.6	64.2 ± 38.5	NS
ALT (U/L)	84.5 ± 51.8	88.0 ± 58.6	NS
γ-GTP (U/L)	64.0 ± 61.7	113.6 ± 83.1	0.036
Ferritin (ng/dL)	206 ± 164.8	204.5 ± 188.4	NS
TC (mg/dL)	172.6 ± 25.7	165.3 ± 39.2	NS
TG (mg/dL)	108.2 ± 52.2	122.9 ± 46.4	NS
HDL (mg/dL)	46.5 ± 11.9	45.4 ± 14.8	NS
LDL (mg/dL)	97.7 ± 25.4	88.6 ± 27.8	NS
FFA (mEq/L)	0.514 ± 0.251	0.693 ± 0.310	0.049
FPG (mg/dL)	92.4 ± 16.4	123.7 ± 58.6	NS
Insulin (mIU/L)	9.06 ± 5.5	8.34 ± 5.16	NS
HOMA-IR	2.07 ± 1.31	1.86 ± 1.38	NS
HOMA-b	128.0 ± 76.2	95.7 ± 86.5	NS
Fibrosis	1.92 ± 1.19	0.933 ± 0.799	0.008
Activity	1.08 ± 0.474	1.33 ± 0.488	0.098

Data are shown as the means ± standard deviation and values, with statistical analysis calculated by Mann–Whitney *U*-test for means and Pearson's χ^2-test for values.
Normal values in laboratory tests are same as in Table 1.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHC, chronic hepatitis C; FFA, free fatty acid; FPG, fasting plasma glucose; HCV cAg, hepatitis C virus core antigen; HDL, high density lipoprotein; HOMA, homeostasis model assessment; LDL, low density lipoprotein; LDLT, living donor liver transplantation; PEG IFN, pegylated interferon; RBC, red blood cell count; TC, total cholesterol; TG, triglyceride; WBC, white blood cell count.

cell, γ-GTP, FFA and liver fibrosis in the pretreatment clinical characteristics were different in both groups (Tables 1,2). The VR rate of the CHC group was superior to that of the LDLT group, and the SVR by per-protocol analysis was also similar in result to the VR (Table 3). The viral disappearance rate of the CHC group was superior to the LDLT group, regardless of the HCV serotype (Fig. 1). The HCV cAg titer under the treatment in the LDLT group was more than that of the CHC group from D0 to W12 (Figs 2a,3a) and the HCV cAg decrease rate of the LDLT group at the D1 was less than that of the CHC group (Figs 2b,3b). We showed that the reinfected HCV to the graft liver was more refractory than the non-transplanted CHC. The PEG IFN and ribavirin dose per BW was an equal dose in both groups. However, it was difficult to determine the pretreatment predictive factors for the LDLT cases, because only one case showed SVR in the LDLT group. Thus, we considered that the difference of the pretreatment clinical characteristics in both groups might be related to the refractory HCV infection.

The pretreated HCV cAg titer is known to be the principal factor for IFN resistance. For CHC and LDLT patients, a high HCV-RNA titer in the pretreatment sera

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Table 3 Result of pegylated interferon-α-2b plus ribavirin therapy

A. All cases			
Term	All-CHC	All-LDLT	*P*-value
Viral response 4 weeks	40/60 (67%)	5/12 (42%)	NS
Viral response 8 weeks	47/55 (85%)	6/12 (50%)	0.011
Viral response 12 weeks	43/48 (90%)	6/12 (50%)	0.003
Sustained viral response: ITT	20/42 (45%)	2/12 (20%)	0.054
Sustained viral response: PP	20/28 (80%)	2/9 (22%)	0.008
B. Serotype 1 and high virus titer cases			
Term	ST1H-CHC	ST1H-LDLT	*P*-value
Viral response 4 weeks	24/40 (67%)	5/11 (45%)	NS
Viral response 8 weeks	30/36 (83%)	5/11 (45%)	0.012
Viral response 12 weeks	25/29 (86%)	5/11 (45%)	0.008
Sustained viral response: ITT	8/27 (30%)	1/11 (8%)	NS
Sustained viral response: PP	8/15 (53%)	1/9 (11%)	0.029
Non-virological response: ITT	11/27 (41%)	5/11 (45%)	NS
Non-virological response: PP	4/15 (27%)	4/9 (44%)	NS

Data are shown as relevant numbers/target case numbers (percentage of relevant numbers) with statistical analysis using Pearson's χ^2-test for numbers.
CHC, chronic hepatitis C; ITT, intention to treatment analysis; LDLT, living donor liver transplantation; PP, per-protocol analysis.

is associated with non-responder status for IFN treatment.[7,17] In the LDLT condition, the HCV-RNA titer was rapidly increased after immediately decreasing at transplant and the viral load after several weeks post-LDLT exceeded the value of pre-LDLT.[18] The HCV-RNA titer increased rapidly in patients receiving corticosteroids as part of the immunosuppressant regimen.[18,19] We have speculated that the massive amount of HCV, caused by immunosuppressant therapy after the LDLT, was part of the reason for the IFN refractory status. However, comparisons with the pretreated HCV cAg matched groups (Fig. 3) showed the existence of an important factor other than the pretreatment viral load. It will, therefore, be necessary to analyze this problem by evaluating many factors, for example immunosuppressants[10] and regeneration, in the future.

A high level of γ-GTP was also known to be an important factor for IFN treatment.[7,17] Usually, high levels of γ-GTP and FFA have been linked to insulin resistance.[20,21] Therefore, insulin resistance in the liver is assumed in the condition of IFN resistance. However, the LDLT group had the normal range of HOMA-IR,[16] which was lower than that of the CHC group (Tables 1,2). The HCV infection after liver transplantation is associated with insulin resistance.[22] Immunosuppressants, especially corticosteroids, induced insulin resistance.[23] In the present study, the LDLT group had a disturbance of insulin secretion

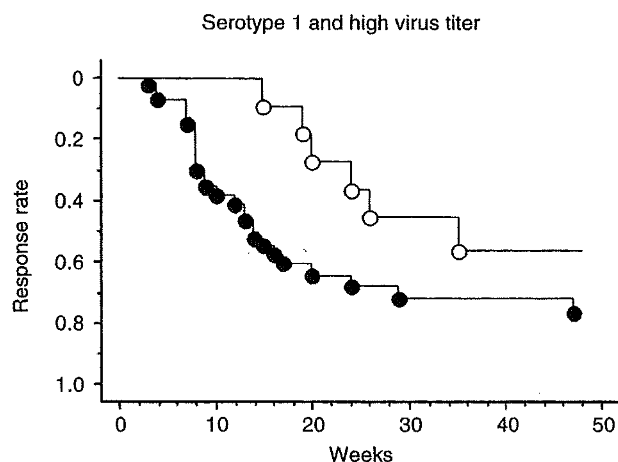

Figure 1 The difference in the hepatitis C ribonucleic acid (HCV-RNA) disappearance rate between the chronic hepatitis C (CHC) group and living donated liver transplantation (LDLT) group during 48 weeks of treatment. HCV-RNA was evaluated by the qualitative PCR method. The disappearance rate was calculated as follows: serum HCV-RNA disappearance case number/all cases in indicated time. The statistical analysis was carried out using the Kaplan–Meier method with the Wilcoxon assay. ST1H group was plotted as the HCV-RNA disappearance line between the white circle of the LDLT group and the black circle of the CHC group. In all cases and the ST1H group, the disappearance rate was statistically significant between the CHC group and the LDLT group ($P < 0.05$).

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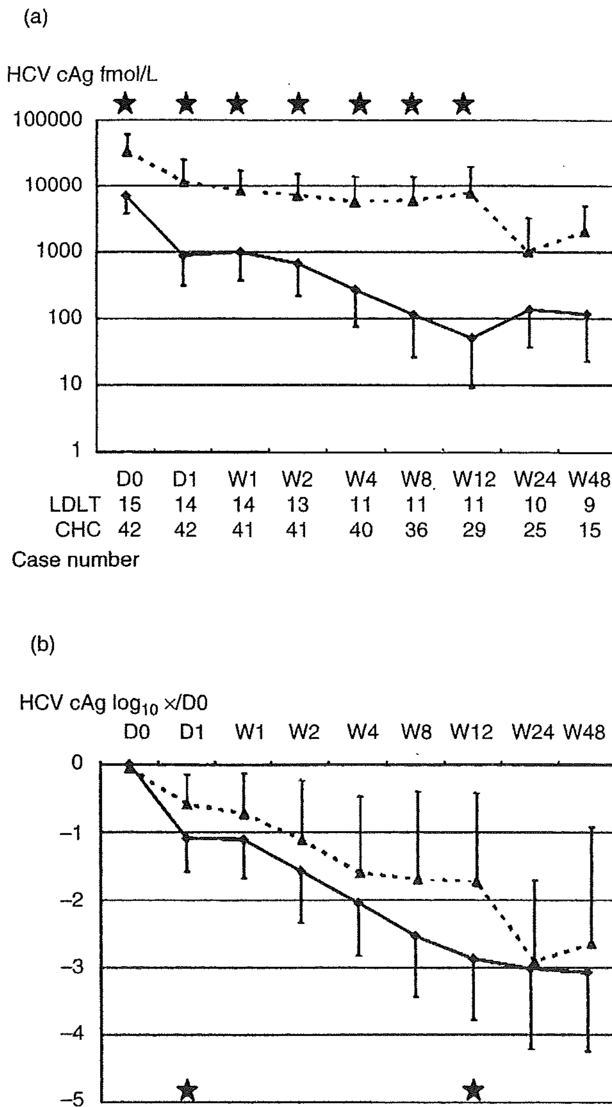

Figure 2 Comparison of viral kinetics between the SG1H-chronic hepatitis C (CHC) group and the SG1H-living donor liver transplantation (LDLT) group during the 48 weeks of treatment. (a) The hepatitis C virus core antigen (HCV cAg) load and (b) reduction rates were plotted by a straight line (SG1H-CHC group), and dotted line (SG1H-LDLT group). The error bar represented the standard deviation. On the *y*-axis, D0 is pretreatment, D1 and WX is time post-treatment day 1 and week X, respectively. The reduction rate was calculated as follows: $\log_{10}$HCV cAg load in indicated time/in D0. HCV cAg titer at the indicated time between SG1H-CHC and SG1H-LDLT were compared. The asterisk mark indicates a significant difference, $P < 0.05$, calculated by Mann–Whitney *U*-test.

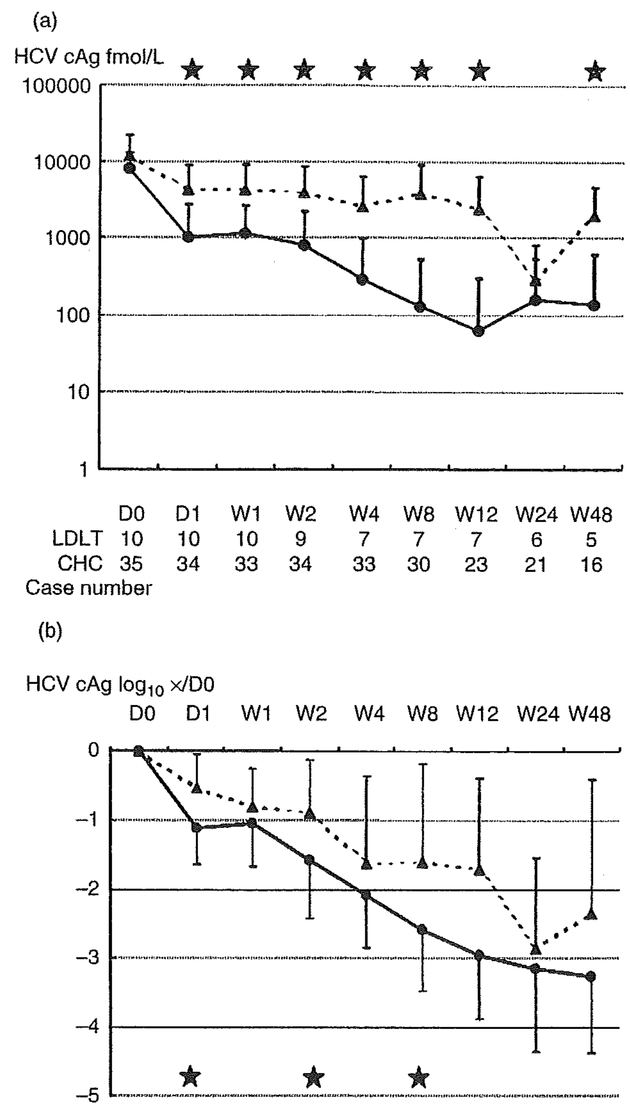

Figure 3 Comparison of viral kinetics between matched pretreatment hepatitis C virus core antigen (HCV cAg) ST1H-chronic hepatitis C (CHC) group and ST1H- living donor liver transplantation (LDLT) group during 48 weeks of treatment. (a) HCV cAg load and (b) reduction rate were plotted by a straight line (matched SG1H-CHC group) and dotted line (matched SG1H-LDLT group). The error bar represents the standard deviation. The asterisk mark is the significant difference, $P < 0.05$, calculated by Mann–Whitney *U*-test.

rather than insulin resistance and high levels of FPG might be caused by the disturbance of insulin secretion. Therefore, further study is necessary to clarify the relationship between the glucose metabolism and the IFN resistance in LDLT patients. The levels of γ-GTP rise at cholestatic conditions. It was reported that the presence

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of a cholestatic profile is associated with an adverse response to IFN treatment in LT.[7] A cholestatic profile provoked the TH2-like lymphocyte response.[19] The authors have previously reported that IL-10, representative of TH2 cytokine, inhibits IFN signaling through an inducible suppressor of cytokine signaling.[24] The high levels of FFA were induced by a catabolic state, such as cirrhosis, and were not fully recovered after LDLT. As a result, the levels of FFA reflected a continuous catabolic state at the beginning of IFN treatment. FFA can induce oxidative stress in various cells,[25,26] and inhibit the IFN induced antiviral gene induction through the inactivation of Jak-1 and Tyk-2.[27] Therefore, we are speculating that high levels of γ-GTP and FFA in the LDLT group have the ability to inhibit IFN signaling as much as in the CHC patients.

We are paying attention to the viral decline of D1/D0 (Figs 2b,3b). The decreased rate of D1 is named as the first phase of HCV decline and is the predictor of SVR.[28,29] The first phase influenced the second phase, which is the decline of HCV after D2.[28] The IFN induced antiviral gene products were considered to be very important for antiviral activity.[11] The expressions of the IFN stimulating genes (ISG) were associated with the early phase of the decline[11] and it was reported that the lack of ISG caused early liver fibrosis in the LT patients with HCV.[30] In the LDLT group, the reduced HCV cAg decreased the rate of D1 and this might be part of the cause of being refractory to IFN. We speculate that an IFN signaling disturbance, related to high levels of γ-GTP and FFA, might have triggered the adverse effect to the HCV cAg decreased rate of D1.

In summary, it became clear that the viral response and SVR is worse in the LDLT group. The first phase of viral decay, the decreased rate of D1/D0, also declined in the LDLT group. High levels of γ-GTP and FFA in the pretreatment sera might also be related to IFN-signaling damage in hepatocytes. At the initiation of pre-emptive therapy, HCV had also been increasing in the graft liver and the catabolic status of energy did not recover for the relatively small size of the graft liver. When beginning treatment for an HCV infection after LT, we should carefully take into account the timing of IFN initiation, in addition to the types of immunosuppressants used.

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Original Article

Efficacy and limitation of bone marrow transplantation in the treatment of acute and subacute liver failure in rats

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Aim: Recent reports have shown that bone marrow cells (BMC) retain the potential to differentiate into hepatocytes. Thus, the BMC have been recognized as an attractive source for liver regenerative medicine. However, it has not been clarified whether BMC transplantation can be used to treat liver damage *in vivo*. In the present study, we explored whether BMC possess therapeutic potential to treat acute and/or subacute liver failure.

Methods: Fulminant hepatic failure (FHF) was induced by 70% hepatectomy with ligation of the right lobe pedicle (24% liver mass), followed by transplantation of BMC into the spleen. Dipeptidyl peptidase IV-positive ($DPPIV^+$) BMC were then transplanted into DPPIV-negative ($DPPIV^-$) recipients following hepatic irradiation (HIR) in which 70% of the liver was resected and the remnant liver irradiated.

Results: There was no benefit of BMC transplantation towards survival in the FHF model. $DPPIV^+$ hepatocytes appeared in the liver tissues of the $DPPIV^-$ HIR model rats, but $DPPIV^+$ hepatocytes replaced less than 13% of the recipient liver.

Conclusion: BMC transplantation may have limitations in the treatment of fulminant or acute liver failure because they do not have sufficient time to develop into functional hepatocytes. Preparative HIR may be beneficial in help to convert the transplanted BMC into host hepatocytes, and provide a survival benefit. Although, However, the precise mechanism warrants further studies.

Key words: bone marrow, fulminant liver failure, irradiation, liver regeneration

INTRODUCTION

WITH AN EVER-INCREASING shortage of donor organs for orthotopic liver transplantation, there is a significant need for alternative therapies for liver disease. Isolated hepatocyte transplantation has been successfully reported in experimental animals and in some clinical human cases.[1–3] However, the procedure of hepatocyte transplantation requires a great number of hepatocytes, and it is still uncertain as to whether or not the transplanted cells can actually engraft in the liver. Recent reports have shown that bone marrow cells (BMC) retain the potential to differentiate into a variety of non-hematopoietic cell lineages,[4–8] including hepatocytes. Thus, BMC have been recognized as an attractive cell source for liver regenerative medicine. For instance, Sakaida *et al.*[9] reported that BMC transplantation exhibited therapeutic potential by reducing liver fibrosis. This therapeutic potential against liver damage is considered to be due to differentiation to mature hepatocytes as well as improvement of intrahepatic micro-conditions. In addition, fusion between BMC and hepatocytes has been reported,[10,11] in which the fusion of host hepatocytes and donor BMC can give rise to mature hepatocytes without *trans-* or dedifferentiation. Such fusion is a new concept, but it is still unknown whether fusion is only a morphological phenomenon or whether it has a significant effect towards regeneration of the liver. On the other hand, previous reports have shown that *in vivo* cell fusion is an unlikely explanation for the "*trans*-differentiation" of bone marrow-derived cells into differentiated phenotypes.[12,13] In any case, investigation of BMC may contribute to the resolution of stem cells or progenitor cells which proliferate to mature hepatocyte, and may result in the promotion of cell transplantation study.

For clinical application, BMC transplantation has an advantage over other cell sources, for example,

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hepatocytes, embryonic stem cells and hepatic stem cells (oval cell or small hepatocytes), because: (i) the extraction technique of BMC has already been established by hematologists through bone marrow transplantation therapy; (ii) autologous transplantation can avoid rejection; and (iii) BMC may have the potential for self replication and aggressive proliferation.

In the present study, we explored whether BMC could have therapeutic potential in the two distinct, well-established, diseased liver models. One was the surgically induced fulminant liver failure model (FHF model) in which liver failure was caused by 68% hepatectomy followed by occlusion of the remnant liver lobes. The other was the hepatic irradiation model (HIR model), in which 68% hepatectomy was performed followed by irradiation of the remnant liver resulting in inhibition of liver regeneration.

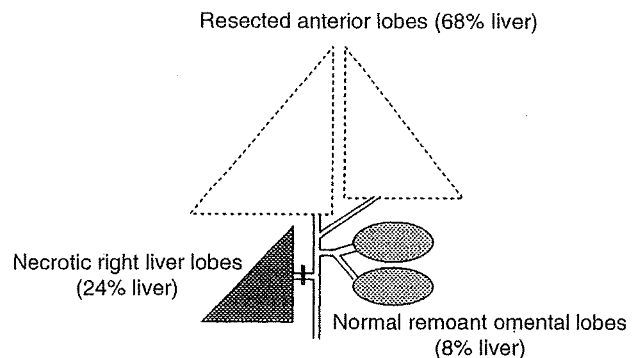

Figure 1 Schematic diagram depicting the technique used to induce the fulminant hepatic failure in this model. Two anterior lobes (68%; median lobe and left anterior lobe) were resected and the right lobes (24%) were rendered necrotic. Only the omental lobes (8%) were left intact.

METHODS

Animals

ADULT MALE SPRAGUE-DAWLEY (SD) rats and DPPIV-positive (DPPIV$^+$) 344 rats were purchased from Japan SLC (Shizuoka, Japan), while DPPIV-negative (DPPIV$^-$) 344 rats were purchased from Charles River Japan (Tokyo, Japan). All animal care and procedures were performed with the approval of the Nagasaki University Institutional Animal Care and Use Committee.

Surgical animal models

Induction of FHF model

Fulminant liver failure was induced as described previously by ourselves.[14,15] Briefly, the abdomen of male SD rats weighing 270–350 g (7–9 weeks old) was entered through a midline incision. The common pedicle to the right lobes of the liver (24% of the liver) was ligated, and the two anterior liver lobes (68% of the liver) were removed using the standard Higgins and Anderson technique.[16] The two omental liver lobes (8% of the liver) were left intact (Fig. 1). All surgical preparations and euthanasia were performed under general (diethyl-ether) anesthesia using sterile surgical technique. At the completion of the surgical procedure and every 12 h after the surgery, each FHF animal received a s.c. bolus of 10 mL of 5% dextrose in normal saline.

Induction of HIR model

The HIR model was generated as described previously.[17] Anesthesia was induced by i.p. injection (0.5 mL/kg pentobarbital). After aseptic preparation, 68% partial hepatectomy was performed through a midline incision. Immediately after partial hepatectomy, the animals were placed in a supine position on a surgical board. A jig with a 5 cm × 7 cm irradiation port was aligned to this platform. Two 1 cm × 2 cm lead shields, each 2 mm in thickness, were wedged under the liver to protect the stomach, kidney, spine and intestines, taking care not to compress the hepatic and aortic vessels. In addition, two lead shields were placed above the chest and the lower abdomen. A Toshiba EXS-300-5 was used (200 kVP, 10 mA, 0.5 mm aluminum, filtration, 43.5 cm SSD; dose rate, 3.2 Gy/min). The abdomen was then closed in two layers.

Bone marrow transplantation

Bone marrow cells were obtained from DPPIV$^+$ rats by flushing from the femurs with Dulbecco's modified Eagle medium (Sigma-Aldrich Japan, Tokyo, Japan) using a 21-G needle. The cells were filtered through a cell strainer (Falcon catalog no. 352350) and centrifuged at 1000 *g* for 5 min at 4°C, as previously described.[4] After washing with phosphate-buffered saline (PBS), pH 7.4, the cell pellet was suspended in 10 mL of lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM ethylene diamine tetra acetate). After centrifugation, cells were washed twice in PBS, and the viability of BMC was confirmed to be more than 80% using Trypan blue dye exclusion.

A small left subcostal incision was made and the spleen was exposed. BMC (2×10^6 cells suspended in 0.5 mL of physiological saline) were injected under the fibrous capsule of the spleen. The numbers of trans-

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planted BMC referred to the numbers of bone marrow and hepatocyte transplantation, as described previously.[4,9,18–20] The abdomen was closed in two layers.

Experimental design

BMC transplantation to FHF model rats

Fulminant liver failure-induced rats underwent intrasplenic injection of BMC (group I; $n = 12$) or normal saline (group II; $n = 12$). Eight rats in each group were monitored until death to determine the survival time, while the other four rats in each group were killed at 12 h after the BMC transplantation for blood analysis. The experiment for the survival rate and the experiments for biochemical blood analysis were separately done.

BMC transplantation to HIR model rats

Two days after induction of HIR, BMC (group A; $n = 15$) or normal saline (group B; $n = 20$) were injected into the spleen. Long-living rats were killed at 1, 3 or 5 months after the operation.

Postoperative evaluation

Blood chemistry

Blood samples were analyzed for total bilirubin (T-Bil) levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the clinical laboratory.

Morphological evaluation (DPPIV staining)

After death of the rats, tissues were rapidly cooled and stored at −80 °C until analysis. To detect DPPIV enzyme expression, cryostat sections (5 µm thick) were made. DPPIV stain was performed as previously described.[21] Briefly, sections were fixed for 5 min in 95% ethanol 5% glacial acetic acid (99:1 v/v) at 0 °C to −10 °C, followed by a 5-min wash in 95% ethanol at 4 °C. Air-dried slides were incubated for 10–20 min at 37 °C in the substrate solution: 2.5 mg Gly-Pro-4-methoxy-b-naphthylamide (Sigma-Aldrich Japan) dissolved in 150 mL of dimethylformamide and mixed with 5 mL of a solution of Fast Blue BB salt (Sigma-Aldrich Japan) in 0.1 M Tris maleate, 0.1 M NaCl, pH 6.5. The slides were rinsed two times in 0.14 M NaCl, incubated for 2 min in 0.1 M $CuSO_4$, and rinsed again in 0.14 M NaCl. The slides were fixed for 10 min in cold 4% paraformaldehyde/ PBS/5 µM $MgCl_2$ and washed in 0.14 M NaCl. The slides were washed in water and counterstained with Harris hematoxylin. Finally, the percentage of $DPPIV^-$ cells were counted using Scion image software (Scion Image Beta 4.03 for Windows XP version; Scion, Baltimore, MD, USA) from three independent rats.

Statistical analyses

Data were analyzed statistically using Mann–Whitney *U*-test and Welch test where appropriate. $P < 0.05$ was considered significant. Data are presented as the mean ± standard deviation.

RESULTS

BMC transplantation to the FHF model rats

Survival time

IN FHF RATS, more than 90% of them died within 48 h (survival time, 33 ± 9 h), but mature hepatocyte transplantation was able to prolong the survival time (73 ± 22 h).[15] However, BMC transplantation did not prolong the survival time. The survival times of group I (30 ± 11 h) versus group II (28 ± 14 h) were not significantly different, and the 48-h survival rate for each group was 12.5% (Fig. 2).

Blood chemistry

The average serum T-Bil levels (group I, 2.93 ± 0.72 mg/dL; group II, 2.82 ± 0.95 mg/dL), AST (group I, 5484 ± 1627 IU/L; group II, 6803 ± 3700 IU/L) and ALT activities (group I, 3008 ± 1531 IU/L; group II, 4123 ± 3868 IU/L) were not significantly different (Fig. 3). As

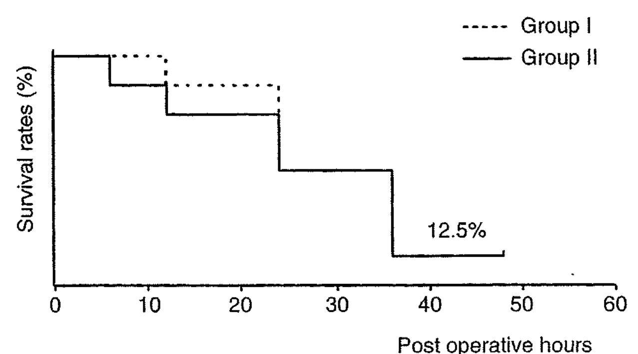

Figure 2 Effect of bone marrow cells (BMC) in the treatment of the fulminant hepatic failure model. Comparison of the survival rate for group I vs group II was not significant (12.5%). The mean survival time was 30 ± 11 and 28 ± 14 h for group I and group II, respectively. Group I, acute liver failure with BMC; group II, acute liver failure without BMC.

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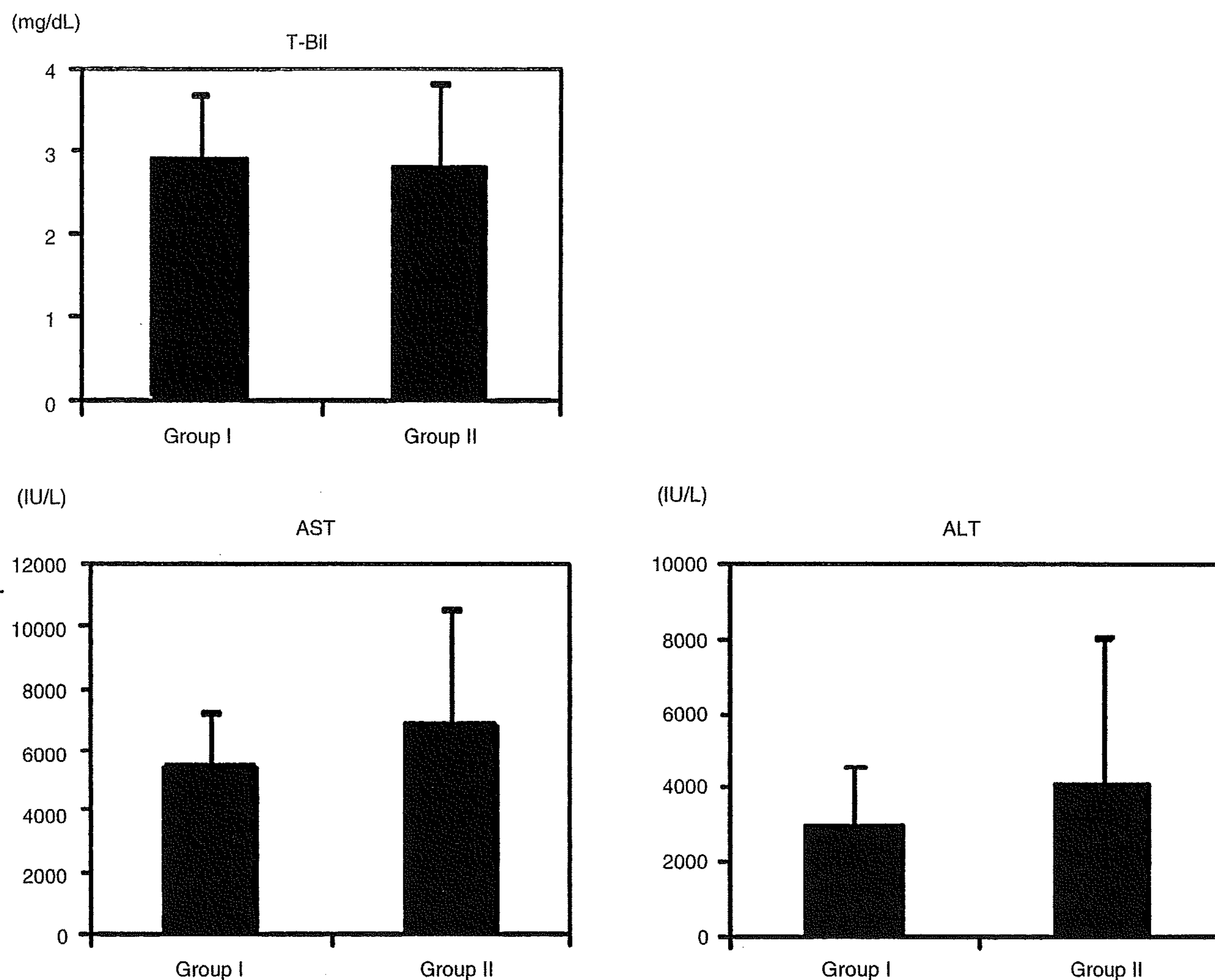

Figure 3 Biochemical changes after bone marrow cell (BMC) treatment in the fulminant hepatic failure model. Between groups I and II, the average serum total bilirubin (T-Bil) levels, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities 12 h after treatment were not significant.

for the HIR model, data from group A was only available 24 h after treatment: T-Bil 0.93 ± 0.49, AST 1007 ± 223 and ALT 457 ± 140.

BMC transplantation to the HIR model rats

Survival rate

Thirteen of 15 rats in group A survived up to 1 month, but 13 of 20 rats in group B did not survive up to 1 month. The survival times of group A (127 ± 109 days) versus group B (35 ± 50 days) were significantly different. The 150-day survival rate of group A (74.2%) was significantly greater than group B (17.1%) (Fig. 4).

DPPIV staining

Morphologically, clusters of DPPIV+ hepatocytes were found in the liver of rats in group A (Fig. 5a) but there were no DPPIV+ cells in the liver in group B. The DPPIV+ hepatocytes in the rats in group A were considered to be bone marrow-derived hepatocytes that replaced a maximum of 13% of the recipient liver (Fig. 5b).

DISCUSSION

WE TESTED WHETHER BMC have the potential to support the diseased liver in two distinct, well-established animal models. One is the surgically

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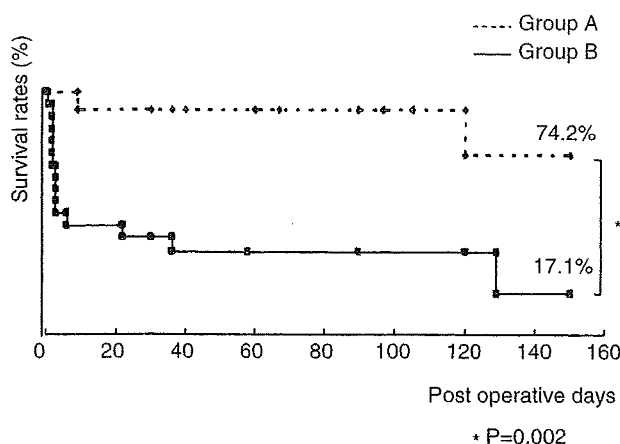

Figure 4 Effect of bone marrow cells in the treatment hepatic irradiation model. The survival rate of group A (74.2%) vs group B (17.1%) was significantly improved ($P = 0.002$).

induced FHF model, in which liver failure is caused by 68% hepatectomy followed by occlusion of the remnant liver lobes. The other is the HIR model, in which 68% hepatectomy is performed followed by irradiation of the remnant liver resulting in fatal liver failure associated with inhibition of liver regeneration. Of note, the HIR model does not emulate human liver disease, but an artificial model to show proliferative advantage to the transplanted cells. Radiation induces critical deterioration in the liver microenvironment that is conducive to the selective proliferation of normal, transplanted hepatocytes as demonstrated previously.[17,20] The precise mechanisms underlying this phenomenon have yet to be elucidated. Radiation is known to inhibit liver growth for extended periods of time; thus, its effect would appear to be similar, at least in principle, to that exerted by retrorsine; blocking that would block of the endogenous hepatocyte cell cycle.

Using these models, we demonstrated that BMC may have the potential to morphologically *trans*-differentiate into hepatocytes in the HIR model. However, our study also demonstrated potential limitations of BMC transplantation in the treatment of fulminant liver failure. In the FHF model, previous studies have demonstrated that mature hepatocyte transplantation can provide survival benefit in liver failure.[15,22] However, a large number of hepatocytes are necessary to adequately treat liver disease. On the other hand, some investigators have suggested that BMC transplantation can be used to treat chronic liver diseases through its regenerative potential.[9] Therefore, we investigated the potential of BMC transplantation to treat FHF in an animal model of acute liver failure induced by the loss of a large amount of hepatocytes followed by lethal systemic inflammation.

As a result, BMC transplantation did not exhibit any survival benefit in rats in this FHF model. This may because BMC require certain conditions and/or adequate time to differentiate into functional hepatocytes. Regarding the numbers of transplanted BMC, we injected 2×10^6 cells under the fibrous capsule of the spleen. In previous reports, the numbers of transplanted cells ranged from 10^6 to 10^7 cells. However, the injection of too many cells can sometimes cause venous thrombosis. Furthermore, our strategy required fewer cells to treat liver damage; namely, 2×10^6 BMC were consid-

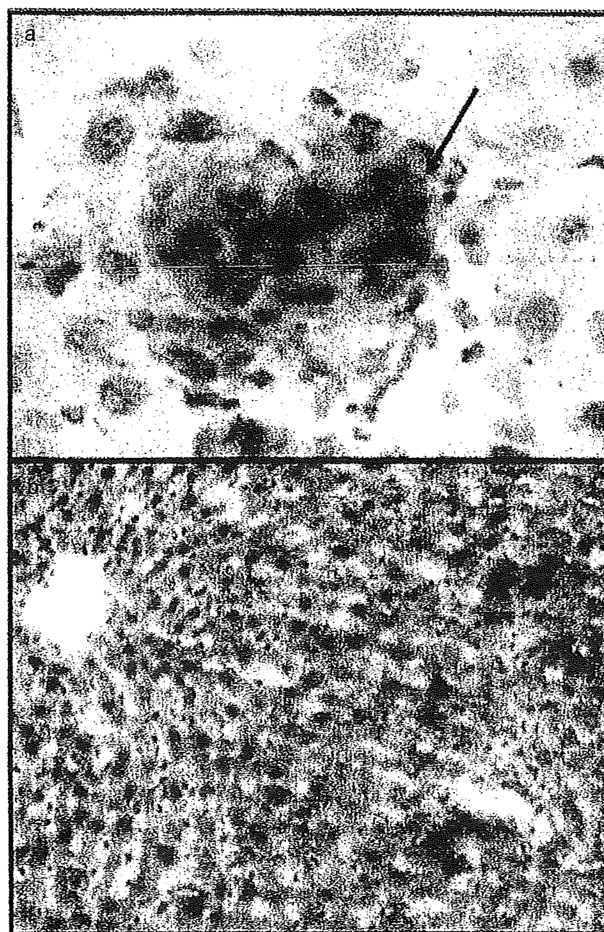

Figure 5 (a) Histological presence of bone marrow-derived hepatocytes. Clusters of dipeptidyl peptidase IV$^+$ hepatocytes were found in the livers of rats from group A (arrow), but no DPPIV$^+$ cells were found in the livers of group B rats. (b) DPPIV$^+$ hepatocytes replaced the recipient liver to a maximum of 13%.

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ered sufficient. Accordingly, while BMC transplantation may be promising, acute liver failure may not be a good target for this treatment strategy.

Similar to previous reports that have indicated the potential of BMC to differentiate into various types of cells, DPPIV staining revealed that several clusters of hepatocytes exhibited the features of BMC derived from transplanted donor cells in the HIR model. It remains unclear whether transplanted BMC were *trans*-differentiated into hepatocytes or were fused with the host hepatocytes. However, the presence of $DPPIV^+$ cells in the HIR model indicates the possibility that these cells are committed stem cells of the liver. This is because the $DPPIV^+$ hepatocytes are morphologically hepatocytes, but their potential may include self-replication and vigorous proliferation. Therefore, we anticipate the therapeutic potential of BMC against acute liver injury. In the present study, total replacement of the liver by BMC was not observed, however, several rats in the BMC transplantation group survived longer than those of the control group. Although the etiology of the survival benefit remains unknown, we speculate that BMC transplantation can contribute not only to the repopulation of hepatocytes but also to the improvement of the internal environment of the liver. For instance, it is hypothesized that BMC can contribute to the suppression or resolution of fibrosis in liver cirrhosis or can activate hepatic mesenchymal cells. The "fibrolysis" effect of BMC transplantation has been demonstrated.[9] Further investigations of the contribution of BMC transplantation to improve the intrahepatic micro-condition are required.

Furthermore, these results suggest that the HIR model itself can be a useful model for cell transplantation in which high-dose irradiation of the liver can injure the host hepatocytes and impart a selective proliferative advantage to the unirradiated donor cells.[21,23] Histologically, extensive loss of hepatocytes occurs in the irradiated liver, and various degrees of micro- and macrovesicular steatosis in the centrizonal areas. Furthermore, preparation of partial hepatectomy induces several chemical growth factors to regenerate the remnant liver. We speculate that this particular condition may lead to selective growth of transplanted cells. Thus, the HIR model could be applied to clinical cases, such as congenital or metabolic liver disease, by transplantation of normal cell sources. Cell transplantation therapy is expected to become a novel and effective therapy against liver diseases, as an alternative to liver transplantation. However, cell transplantation therapy requires a large number of donor cells that are able to survive in the host liver. In general, a sufficient number of mature hepatocytes cannot be provided, but stem cells may solve this problem. Among the currently available stem cell sources, BMC have several advantages, as previously described. To further elucidate this, further studies concerned with the evaluation of pure bone marrow stem cells and the etiology of *trans*-differentiation to hepatocytes are needed. Furthermore, it is still unknown whether "so called" bone marrow-derived hepatocytes are actually *trans*-differentiated from BMC or fused with BMC. We are currently investing the cell fusion phenomenon to determine the contribution of BMC to support liver failure.

In conclusion, BMC transplantation may have limitations in the treatment of these models of fulminant or acute liver failure because they do not have sufficient time to develop into functional hepatocytes. Preparative HIR may be beneficial and help convert the transplanted BMC into host hepatocytes to provide survival benefit, although the precise mechanism warrants further investigation.

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Regeneration of Graft Livers and Limited Contribution of Extrahepatic Cells After Partial Liver Transplantation in Humans

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Abstract *Background* Liver regeneration is still not fully understood. Partial liver transplantation (LT) can provide the opportunity to investigate the mechanisms of liver regeneration, including the contribution of extrahepatic cells to liver regeneration. *Methods* Of 61 patients transplanted with partial liver graft between August 1997 and October 2006, 56 patients were studied, including 49 adults and 7 children. Sequential computed tomography volumetric analysis was performed for volume measurement, while proliferating cell nuclear antigen (PCNA) labeling index was investigated for liver cell proliferation in nonprotocol liver biopsy specimens. In addition, 15 male recipients who had female liver grafts were investigated in order to detect Y chromosomes as extrahepatic cells in nonprotocol liver biopsy specimens. *Results* Graft volume per standard liver volume was markedly increased after adult-to-adult living-donor (LD) LT. In pediatric transplants, there was no volume increase over time. PCNA labeling index was vigorous in adult-to-adult LDLT in the early period after LDLT. No Y chromosome was evident in hepatocytes from female-donor male-recipient grafts during or after liver regeneration. However, in the cases of failing grafts of this type, many Y-chromosome-positive cells were observed in the graft liver. The character of those cells was CD34(−), CK9(−), hepatocyte-specific antigen(−), and CD68(+/−). *Conclusion* In adult-to-adult LDLT, vigorous liver regeneration occurs in the graft liver, demonstrated by not only volumetric but cell kinetic analysis. Involvement of extrahepatic cells in normal liver regeneration seems limited.

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Introduction

The mechanism of liver regeneration is still not fully understood. Although vigorous liver regeneration after living-donor liver transplantation (LDLT) has been reported by us and others [1–3], it has been assessed by imaging studies such as computed axial tomography (CAT) scan, not hepatocyte cell division. In the present study, we took the opportunity to use liver biopsy specimens to verify liver regeneration in partial liver recipients during various periods after LDLT.

In addition, during liver regeneration it has been reported that extrahepatic cells, especially bone marrow (BM)-derived cells, are mobilized and involved [4–6]. However, details regarding how extrahepatic cells are involved and how much they contribute to normal liver regeneration have not been fully elucidated [7–10]. Therefore, we investigated liver biopsy specimens from female-donor male-recipient grafts, in which only XX cells should be present in the graft liver. We used fluorescent in situ hybridization (FISH) to detect Y chromosomes in the liver to identify extrahepatic cells in the liver upon liver regeneration.

Materials and Methods

Patients

Of 61 patients who underwent LDLT between August 1997 and October 2006 at Nagasaki University Hospital, 56 Japanese patients with survival times of more than 3 months

were included for volumetric analysis. For adult recipients, right lobe grafts were transplanted in 40 recipients, while left-side grafts (8 extended left lobe graft, 1 left lobe graft) were performed in 9 recipients. Seven pediatric cases with left lateral lobe graft also underwent volumetric study. Adult patients were defined as those over 16 years old. When liver function test was deranged, total 93 liver biopsies were carried out, consisting of 83 in adult cases and 10 in pediatric cases, and were prepared for proliferative cell nuclear antigen (PCNA) staining. Within these, a total of 24 liver biopsies were performed in 15 recipients on indication from a pool of 19 male recipients (XY) who were transplanted with female livers (XX).

Methods of LDLT

All partial liver grafts were preserved in University of Wisconsin solution and implanted using a piggyback technique. In general, graft selection was based on the results of volumetric studies using CAT scans to obtain ratios of graft volume to standard liver volume of more than 35% in the recipients.

A dual or triple immunosuppressive regimen was used, which included tacrolimus or cyclosporine A, steroid, and mycophenolate mofetil. Patients with compromised renal function were given induction therapy with interleukin-2 antibodies. Biopsy-proven rejections were treated if clinical and laboratory signs mandated steroid bolus treatment. Steroid-resistant rejections were treated with OKT3.

Investigation for Liver Regeneration

Incremental growth of the liver in volume was measured by serial CAT scans using Flexi Trace software (Tree Star, Inc., U.S.A.) at 0, 1–2 weeks, and 3 months after LDLT [1]. In liver biopsy specimens, expression of PCNA (DACO, Carpinteria, CA) was analyzed for intrahepatic proliferation [11].

Four-micrometer liver sections were deparaffinized in xylene and hydrated in graded ethanol. After deparaffinization, rehydration, and heating in 95°C buffer, sections were incubated with each antibody and subsequently with Histofine Simple Stain MAX-PO (MULTI) (Nichirei, Japan). Incubation was performed overnight at 4°C and followed by a wash in three changes of phosphate buffered saline (PBS) for 5 min. For all stainings, the reaction product was developed with the use of 3-diaminobenzidine tetrahydrochloride and H_2O_2. The sections were counterstained with Meyer hematoxylin–eosin.

For hepatocyte staining, the goat anti-human hepatocyte-specific antigen Ab (R&D system, Minneapolis, MN), and 2nd Ab biotinylated rabbit anti-goat Ig (DAKO, Carpinteria, CA) were used. For the staining of CK7 (bile duct marker), CD68 (macrophage marker) and CD34 (hematopoietic cells) were used, respectively, according to the manufacturer's protocol.

Fluorescent In Situ Hybridization (FISH)

FISH was performed in our reference laboratory (SRL, Nagasaki, Japan). Sections from paraffin-embedded biopsied liver tissues were placed on silane-coated glass slides. The slides were deparaffinized immediately in two rinses of 1,000 g/l xylene for 10 min each. Each slide was rehydrated in an ethanol series for 5 min. The slides were then treated with 0.2 mol/l HCl for 20 min, followed by 2 × SSC (0.3 mol/l sodium chloride and 0.03 mol/l sodium citrate) for 20 min at 80°C, treated with 0.05 mg/ml proteinase K in TEN [0.05 mol/l Tris–HCl, pH 7.8, 0.01 mol/l ethylenediamine tetraacetic acid (EDTA), and 0.01 mol/l sodium chloride] for 10 min at 37°C, and placed in 40 g/l formaldehyde in PBS for 10 min. Both FISH probes and target DNA were denatured simultaneously for 10 min at 90°C, and the slides were incubated overnight at 42°C, placed in 2 × SSC for 10 min at 42°C, washed twice in 2 × SSC/500 g/l formaldehyde formamide for 5 min each at 42°C, washed 2 × SSC for 5 min at 42°C, and counterstained in 2 × SSC/0.03 μg/ml 4',6-diamidino-2-phenylindole (DAPI).

Statistical Analysis

For the data, Mann–Whitney *U* test was used. Differences were considered statistically significant for *P*-value less than 0.05.

Results

Liver Volume

Graft volume per standard liver volume at 0, 1, 3, and 6 months after adult-to-adult LDLT was 53.2%, 95.9%, 98.5%, and 101.2% in right lobe grafts and 41.1%, 81.9%, 92.7%, and 102.4% in left-sided grafts, respectively (Fig. 1). Since volume changes in pediatric LDLT were not evident, they are not included in the figure.

DNA Synthesis in the Liver

PCNA labeling index was vigorous in adult-to-adult LDLT in the early period after LDLT, while it was not evident in pediatric LDLT (Fig. 2).

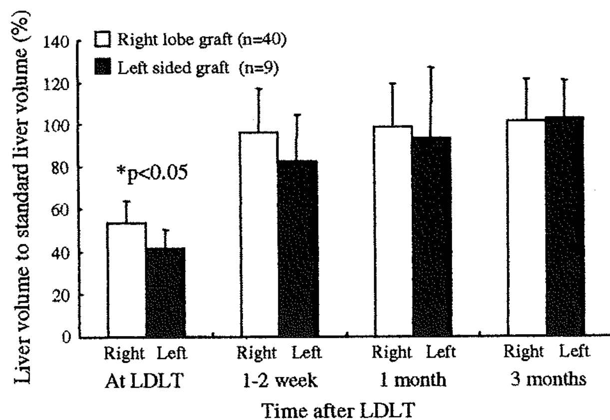


Fig. 1 Liver regeneration of right lobe or left lobe graft liver after adult-to-adult LDLT using volumetric analysis using CAT scan. *LDLT* living-donor liver transplantation, *CAT* computed axial tomography

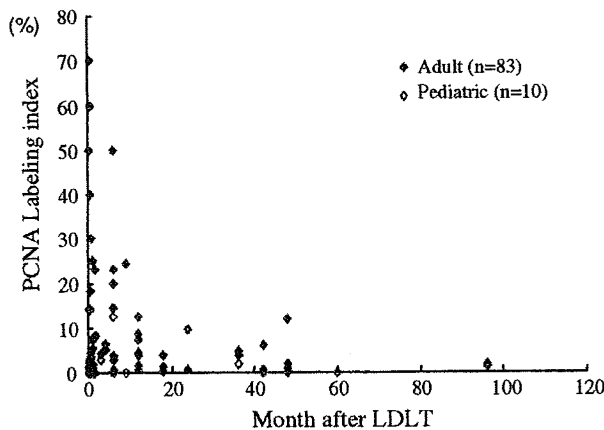


Fig. 2 PCNA labeling index after LDLT using immunohistochemical staining. *PCNA* proliferating cell nuclear antigen, *LDLT* living-donor liver transplantation

FISH and Immunohistochemical Staining for Y-Positive Cells

Y chromosome was not evident in hepatocytes of female-donor male-recipient grafts after normal liver regeneration in adult-to-adult LDLT recipients (Fig. 3, case 1). As seen in this case, when graft livers did not receive any damage and underwent normal liver regeneration, existence of Y-chromosome-positive cells was limited with FISH examination. However, in the case of failing graft, such as in cases 11–13, many Y-chromosome-positive cells were observed in zone 1 of the graft liver (Fig. 3, case 11).

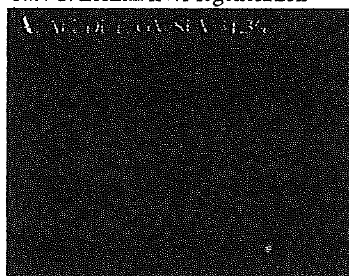
For these cases, immunohistochemical staining was performed in the area with Y chromosomes. CD34(−), CK9(−), hepatocyte Ag(−), and CD68(+/−) were observed using immunohistochemical staining (Fig. 4, case 11). In the case of chronic liver damage (Fig. 5, case 15) after LDLT due to biliary complication, a few Y-positive cells were also detected with nonspecific staining for CD34, CK9, hepatocyte Ag, and CD68. Results of immunohistochemical staining are summarized in Tables 1 and 2.

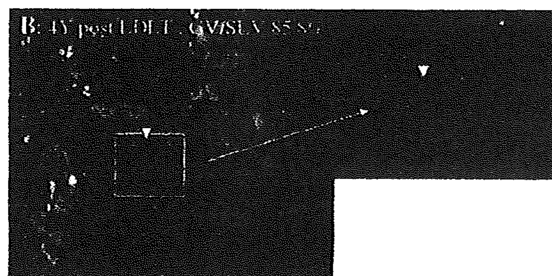
Discussion

In this report, we showed liver regenerative response after partial LT using not only volumetric CAT scan study but also PCNA labeling of biopsy specimens. Previously, we reported vigorous liver regenerative response after partial liver regeneration and investigated liver regenerative growth factors after liver regeneration [11]. Herein, we showed a clear difference in proliferation of graft liver according to recipient body size and blood flow due to the difference in responses when transplanted in adults and children with different standard liver volumes. We did not carry out statistical analysis on PCNA index since it exhibited wide deviation. Liver regeneration remains an unsolved phenomenon, but our results show that it could be related to factors in recipients, as we reported previously [1]. Since protocol biopsy tends to be avoided because of risk of hemorrhage etc., further investigation is needed to assess cell proliferation noninvasively aside from CAT scan. Also since liver biopsy was not done on protocol, rejection or inflammation could have affected the data of PCNA staining. Although it would be interesting to investigate the difference in liver regeneration between patients after liver resection and those after partial liver transplantation, biopsy specimen from patients after liver resection cannot be obtained because of risk of complications. Therefore this also remains for further investigation. Our liver specimens from liver transplant recipients were obtained because of on-demand liver biopsy.

In addition, for combinations of female donor (XX) and male recipient (XY), the Y chromosome was investigated in the biopsy specimen of the female liver (XX) in order to investigate the contribution of extrahepatic cells to liver regeneration. Previously, in an in vivo experiment conducted in 2000, it was reported that hepatocytes could be derived from BM cells [12]. Subsequently, in 2001, Baccarani et al. [13] reported that, in human recipients, replacement of a female liver venous endothelium with male BM showed the possibility of involvement of BM cells in liver rearrangement. Fujii et al. [4] reported that BM cells participated in liver regeneration after hepatectomy, whereas the majority of cells were committed to sinusoidal endothelial cells. Very recently, Conzelmann et al. [5], using their reduced-size LT model, reported that recipient-derived progenitor cells were present and might contribute to liver regeneration in mice. However, in 2005 Di Campli et al. [7] reported no evidence of hematopoietic

Case 1: normal liver regeneration





Case 11: acute cellular rejection · graft failure

Fig. 3 FISH for Y chromosome in liver biopsy specimens. Case 1 showed normal liver regeneration after LDLT. **a** At the time of LDLT, few Y-chromosome-positive cells were seen. **b** With time, although GV/SLV increased, a few Y-chromosome-positive cells were seen only in the sinusoid. **c** Case 11 had severe acute rejection at 1 week after LDLT. **d** In the biopsy specimen, massive accumulation of Y-chromosome-positive cells was seen, mimicking hepatic structure. *FISH* fluorescent in situ hybridization, *GV/SLV* graft volume versus standard liver volume ratio

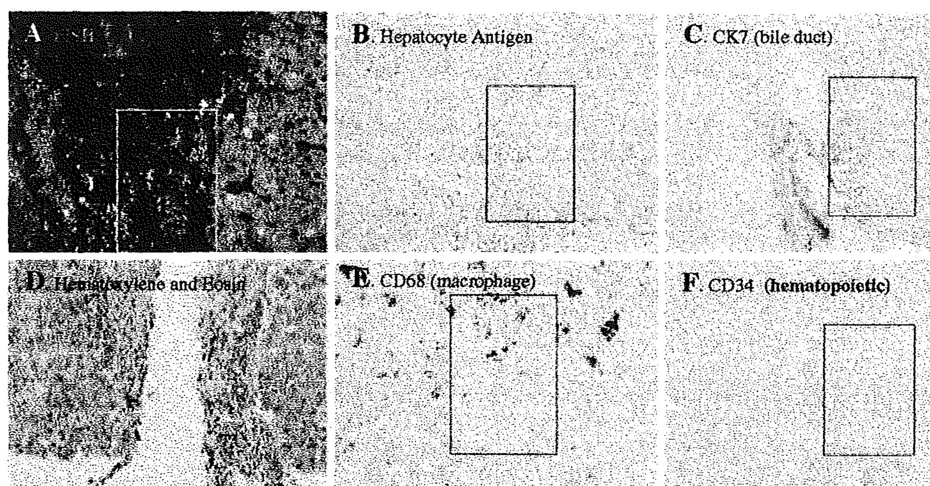


Fig. 4 Immunohistochemical stainings in case 11. Characterization of Y-chromosome-positive cells was attempted in corresponding area. **a** FISH showing Y-chromosome-positive cells (white square), **b** hepatocyte antigen was not positive in the black square, **c** CK7 (cytokeratin 7, bile duct) was not positive in the black square, **d** hematoxylin and eosin staining, **e** CD68 (macrophage) was partially positive in the black square, **f** CD34 (hematopoietic cell) was not positive in the black square

stem cell mobilization in patients who underwent hepatectomy or in patients with acute liver failure. Similarly, in 2006, Moritoki et al. [8], using green fluorescent protein transgenic mice, demonstrated that BM cell transfer seemed not to contribute to the differentiation of cholangiocytes in a chronic cholestasis model. In 2007, Tomiyama [6] reported the limited contribution of cells originating from intact extrahepatic tissue in hepatocyte regeneration in transplanted rat livers. Thus, it is still unknown whether extrahepatic cells such as BM cells could contribute to liver regeneration or liver repair, especially in humans.

In our study, we did not find many Y-chromosome-positive cells after liver transplantation with normal liver regeneration. If extrahepatic cells had been involved and integrated into normal liver regeneration, they should have stayed and been found in the liver biopsied a long time after LDLT. This is indirect evidence that would seem to rule out extrahepatic cell contribution to normal liver regeneration in humans, in contrast to previous reports [12, 13]. On the other hand, when failing livers were biopsied, many Y-chromosome-positive cells were present. Although we could not clearly show the origin of those Y-positive cells, circulating macrophages were candidate sources