retransplantation for liver failure 10–15 years after transplantation [2]. Given this risk, it seems reasonable to offer antiviral therapy to liver transplant recipients. Many reports have noted rates of sustained viral response (SVR) ranging from only 10% to 30% in liver transplant recipients with recurrent HCV treated for 48 weeks [3–10], indicating the need for new treatment regimens with higher SVR rates. Recent reports have indicated that the extension of treatment with peginterferon and ribavirin (PEGIFN/RBV) from 48 to 72 weeks significantly increases the rate of SVR in immunocompetent patients, particularly slow virological responders [11,12], and one report noted that extended treatment for recurrent hepatitis C infection after liver transplantation (LT) was effective [13,14].

There are many predictive factors of successful treatment with the combination of peginterferon and ribavirin in immunocompetent patients, including the viral factors, such as HCV genotype, pretreatment viral load, amino acid (aa) 70 and/or 91 in the HCV core protein, amino acid substitutions in the HCV NS5A region [15–17]. In their multivariate analyses of predictors of SVR, Akuta and colleagues identified substitutions of aa 70 and 91 in the HCV core region (double-wild-type; odds ratio 5.988) as predictive [18], whereas Enomoto identified substitutions of amino acids of the HCV NS5A region (mutant type; odds ratio 5.3) as predictive [15].

With regard to length of treatment, one study reported that an early viral response at 3 months was useful in predicting a lack of response to antiviral therapy in liver transplant recipients with recurrent hepatitis C [19,20]. To our knowledge, however, no study has analyzed viral factors in extended treatment for recurrent hepatitis C infection after liver transplantation.

The aim of the present study was to evaluate the therapeutic efficacy of peginterferon in combination with ribavirin (PEGIFN/RBV) on long-term treatment for recurrent hepatitis C after LDLT, and predictive factors of virological response to this treatment, particularly viral factors. This study is first report of predictive factors associated with virological response in recurrent hepatitis C patients after LDLT, including amino acid substitutions in the core region and NS5A region.

Material and methods

Patients

A total of 53 patients who underwent LDLT due to HCV-related end-stage liver disease from 2000 to January 2009 were enrolled for this retrospective study. Among them, 14 patients died before the start of therapy, 1 refused treatment with antiviral therapy, and 1 did not become positive for HCV RNA after LDLT. Eventually, leaving 37 patients treated with PEGIFN/RBV in our institution. Of these, 10 patients are currently continuing antiviral therapy.

We introduced all patients to IFN therapy in principle. The efficacy of IFN therapy could thus be established in 27 patients (Figure 1).

Antiviral treatment protocol

Patients received 1.5 μg/kg body weight (BW) PEGIFN (Peg-Intron; Schering-Plough, Segrate, Italy) subcutaneously (s.c.) once weekly and 200 mg RBV (Rebetol; Schering-Plough). PEGIFN/RBV was continued for more than 1 year after serum HCV RNA becomes negative. At the end of active treatment, the patients were followed for further 24 weeks without treatment.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local Ethics Committees of all participating centers. Written informed consent was obtained from all participating patients.

Safety assessments

Safety was assessed by clinical and laboratory testing, and by evaluating all adverse events reported at each visit. In accordance with the protocol, growth factors were recommended to encourage optimum patient compliance in relation to predictable hematological side effects such as anemia.

Erythropoietin (EPO; Epogin, Chugai) from 6000 IU/week was used to treat anemia (Hb levels <10 g/dl). RBV was administrated from 200 mg. When Hb increased by more than 10 g/dl, 200 mg per day of RBV was added. The daily dose of RBV was reduced by 200 mg when Hb fell below 10 g/dl, an acute decrease was followed by stabilization of Hb concentration at more than 3 g/dl from baseline, or the appearance of clinical symptoms of anemia (e.g. palpitation, dyspnea on effort, and fatigue) associated with a decrease in Hb of >2 g/dl from baseline. Once the RBV dose was reduced, it was maintained at that level throughout the rest of study if patients complained of anemia-related symptoms of fatigue or pallor. However, RBV was discontinued when Hb fell below 8.5 g/dl or when patients manifested more severe anemia, including orthostatic hypotension. PEGIFN was stopped if significant side effects occurred or if cytopenia persisted (neutrophil count <750/mm^3, platelet count <20,000/mm^3).

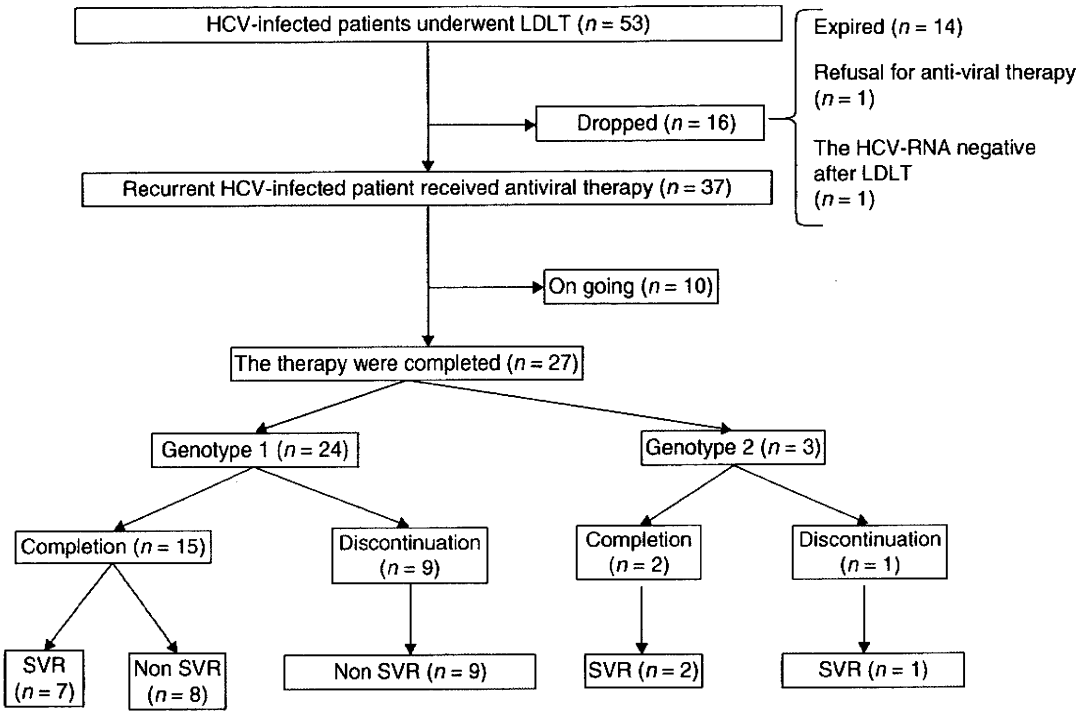

Figure 1. Flow diagram showing the course of HCV-infected patients after living donor liver transplantation. Twenty-seven patients treated with PEGIFN/RBV combination therapy were divided into two groups, namely sustained virological response (SVR) and non-SVR. *n*, number of patients. HCV genotype is shown.

Assessment of efficacy

HCV RNA levels were measured using one of several RT-PCR-based methods (original Amplicor method, high range method, or TaqMan RT-PCR test) at weeks 2 and 4, every 4 weeks of treatment thereafter, and at 24 weeks after the cessation of therapy.

The response was considered to be a SVR after another 6 months of negative serologic results without antiviral treatment. Patients with positive qualitative HCV RNA PCR tests during all examinations were categorized as having a non-virological response. Virological response (VR) was defined as becoming PCR-negative at least once during treatment; early virological response (EVR) as HCV-RNA-positive at 4 weeks after the start of treatment and HCV-RNA-negative at 12 weeks; and late virological response (LVR) as HCV-RNA-negative at more than 13 weeks after the start of treatment.

Analysis of nucleotide sequence of the core and NS5A region

Fifteen patients with genotype 1b completed our protocol. Seven patients achieved SVR whereas eight did not. Using serum obtained before LDLT, we analyzed amino acid (aa) substitutions at aa 70 and aa 91 of the HCV core region (HCV CR) and mutation at the interferon sensitivity-determining region (ISDR) in the nonstructural 5A (NS5A) region of HCV by the direct sequencing method. The core aa 61–110 and NS5A aa 2209–2248 (IFN-sensitive determining region [ISDR]) [15] sequences were determined by direct sequencing using stored serum samples obtained just before therapy. HCV RNA was extracted from serum samples and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Bio Inc., Shiga, Japan). DNA fragments were amplified by PCR using the primers below. Nucleotide sequences of the core region: first-round PCR was performed with primers CC11 (forward, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse, 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and second-round PCR with primers CC9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse), as described by Akuta et al. [16,18,21]. After denaturation at 95°C for 5 min, 35 cycles of amplification were set as follows: denaturation for 30 s at 94°C, annealing of primers for 1.5 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The second PCR was carried out with the same amplification conditions as those used in the first PCR, except that the second PCR primers were used instead of the first PCR primers. Nucleotide sequences of ISDR in NS5A: PCR was performed

with IM11 (forward, 5′-TTC CAC TAC GTG ACG GGC AT-3′) and 5OA2KI (reverse, 5′-CCC GTC CAT GTG TAG GAC AT-3′). After denaturation at 98°C for 30 s, 35 cycles of amplification were set as follows: denaturation for 10 s at 98°C, annealing of primers for 30 s at 66°C, and extension for 15 s at 72°C, followed by final extension at 72°C for 5 min. The amplified PCR products were separated on a 2% agarose gel and purified by GENECLEAN II kit (Q-Bio Gene, Carlsbad, CA). Nucleotide sequences were determined using Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J (Gene Bank accession number; D90208) [22].

Statistical analysis

Variables between the SVR and non-SVR groups were compared using non-parametric tests (Mann–Whitney *U* test, two-tailed test and Fisher's exact probability test). Analyses for efficacy and safety were conducted on an intention-to-treat (ITT) basis, performed on patients who received at least one dose of the study medication.

Predictors of SVR were determined using univariate analyses. All p values <0.05 by two-tailed tests were considered significant. Potential predictive factors associated with SVR included sex, age, body mass index (BMI), viremia level, number of mutations in the ISDR, HCV core region (double mutant/non-double mutant), time from transplantation to therapy, duration of treatment, adherence to PEGIFN treatment, and adherence to RBV and EVR treatment. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

Results

Patients characteristics

Table I shows the baseline characteristics of the 27 patients with recurrent hepatitis C after LT who were treated with PEGIFN/RBV combination therapy. The median age of patients was 56 years, and 17 were male. Median body mass index was 24.3. Most patients were infected with HCV genotype 1 ($n = 24$) and genotype 2 ($n = 3$). Median time for the initiation of antiviral therapy after transplantation was 4 months, and median pretreatment serum HCV RNA levels were 6.6 log IU/ml. Immunosuppressive therapy included tacrolimus in 22 of 27 patients, and cyclosporine in 5 of 27.

Table I. Characteristics of 27 patients with recurrent hepatitis C after living donor liver transplantation.

Age (years)*	56 (29–69)
Gender (male/female)	17/10
Body mass index*	24.3 (14.8–42.2)
Genotype (1/2)	24/3
Viral load at therapy (log IU/ml)*	6.6 (4.9–7.8)
Time from transplantation to therapy (months)*	4 (1–41)
Immunosuppression (tacrolimus/cyclosporine)	22/5

*Values are median (range).

Efficacy and safety assessment

Among 27 patients who were treated with antiviral therapy, 17 were able to complete our protocol (15 patients with genotype 1, 2 patients with genotype 2), whereas 10 patients had to discontinue the protocol (9 patients with genotype 1, 1 patient with genotype 2). SVR rate with PEGIFN/RBV was 37.0% (10/27). By genotype, SVR rate in patients with genotype 1 was 29.2% (7/24) and 100% (3/3) in those with genotype 2 (Figure 1). Most patients with genotype 1b whose HCV RNA reached undetectable level achieved SVR, at 87.5% (7/8), with only one patient not achieving SVR (Table II) (Figure 2).

Ten patients discontinued treatment, due to liver failure owing to the recurrence of HCV in 5 patients, general fatigue in 2, ALT flare due to acute rejection in 1 patient, anemia in 1, and depression in 1 (Figure 1).

Efficacy of long-term interferon therapy for genotype 1b patients

Table II shows details of patients who were treated with PEGIFN/RBV until HCVRNA had reached undetectable levels and were then further treated for at least more than 1 year.

Seven patients achieved SVR by prolonged PEGIFN/RBV for at least 1 year or more. Seven patients were male.

Eight patients had reached undetectable levels of HCV RNA and 7 patients had never reached undetectable levels of HCV RNA. Although 5 of the 8 patients were classified as LVR, 4 patients of these 5 achieved SVR. One male patient aged 69 years (patient no. 5) who had double mutation of aa 70 and aa 91 in the core region and zero substitutions in ISDR achieved SVR after prolongation of therapy (Figure 3). By contrast, another male aged 51 years (patient no. 9) who had double wild aa 70 and aa 91 in the core region and five substitutions in the ISDR did not achieve SVR after prolongation of therapy (Figure 4).

Table II. Details of 15 patients (genotype 1).

Patient no.	Age (years)	Gender	HCV RNA (log IU/ml)	HCV core region (aa 70/aa 91)	Number of mutations in the ISDR	Time from transplantation to therapy (months)	Time to reach undetectable levels of HCV RNA (weeks)	Treatment duration of VR (weeks)	Treatment duration (weeks)	Adherence to PEGIFN (%)	Adherence to RBV (%)	SVR
1	63	Male	6.1	m/m	4	41	3	103	106	35	29	Yes
2	60	Male	5.8	w/w	1	13	10	48	58	100	62	Yes
3	66	Male	6.6	m/m	3	12	12	60	72	80	11	Yes
4	54	Male	6.5	w/w	3	4	16	56	72	77	54	Yes
5	69	Male	6.3	m/m	0	12	21	57	78	42	14	Yes
6	44	Male	6.6	w/m	0	1	28	76	104	88	47	Yes
7	53	Male	6.1	m/w	1	2	54	125	179	57	25	Yes
8	56	Male	6.6	m/w	0	2	27	52	79	66	7	No
9	51	Male	5.9	w/w	5	6	NR	NR	173	80	25	No
10	47	Female	6.6	m/m	1	3	NR	NR	124	63	15	No
11	59	Female	6.6	m/w	1	7	NR	NR	86	100	65	No
12	64	Female	5	m/m	0	3	NR	NR	81	72	25	No
13	58	Female	6.6	m/m	1	3	NR	NR	79	83	63	No
14	65	Female	5.9	w/m	0	3	NR	NR	79	45	19	No
15	56	Male	7.2	m/w	0	3	NR	NR	58	51	26	No

Abbreviations: m = mutant; w = wild; NR = non-virological responder; VR = virological response; SVR = sustained virological response.

Predictive factors of SVR in genotype 1b patients

Among 15 patients who completed our protocol with genotype 1b, Potential predictive factors associated with SVR were analyzed. Variables were follow up, the age, gender, body mass index, duration for the initiation of antiviral therapy after transplantation, pretreatment serum HCV RNA levels, immunosuppressive therapy, the number of mutations in the ISDR, HCV core region (double mutant/non-double mutant) adherence of PEGIFN and adherence of RBV.

There was no significance difference between the SVR and non-SVR groups among the 15 patients with genotype 1b in our study (Table III). EVR rates in the SVR group tend to be higher than that of the non-SVR group, albeit that the difference was not significant ($p = 0.07$) (Table III). Mutation of aa 70 and aa 91 in the core region of the HCV protein and fewer mutations in its ISDR region did not significantly differ between the SVR and non-SVR groups among the 15 patients with genotype 1b in our study.

Although it has been reported that mutation of aa 70 and aa 91 in the core region of the HCV protein is predictive of a non-virological response [17,18], all three patients who had double mutation of aa 70 and aa 91 in the core region achieved SVR in this study.

Moreover, although it has also been reported that fewer mutations in the ISDR region of the HCV protein is predictive of a non-virological response [15], all four patients with 0 or 1 mutation in the ISDR achieved SVR.

Discussion

The optimal duration of therapy for liver transplant recipients with recurrent HCV is unclear. The treatment period for immunocompetent patients in the majority of published studies is 48 weeks. Among immunocompetent patients, the probability of relapse was greater in those responding later [23,24]. Using a mathematical model, Drusano and Preston reported that genotype 1-infected patients require the continuous absence of detectable HCV RNA in serum for 36 weeks to attain 90% probabilities of an SVR (i.e. relapse rate 10%) [25]. It is recently recommended that 72-week IFN treatment, compared to 48-week standard IFN treatment, was effective for the untransplanted patients with chronic hepatitis C whose HCV RNA does not reach undetectable level within 12 weeks [11,12]. It is also well known that patients with recurrent chronic hepatitis after LDLT are unlikely to achieve SVR, compared to immunocompetent

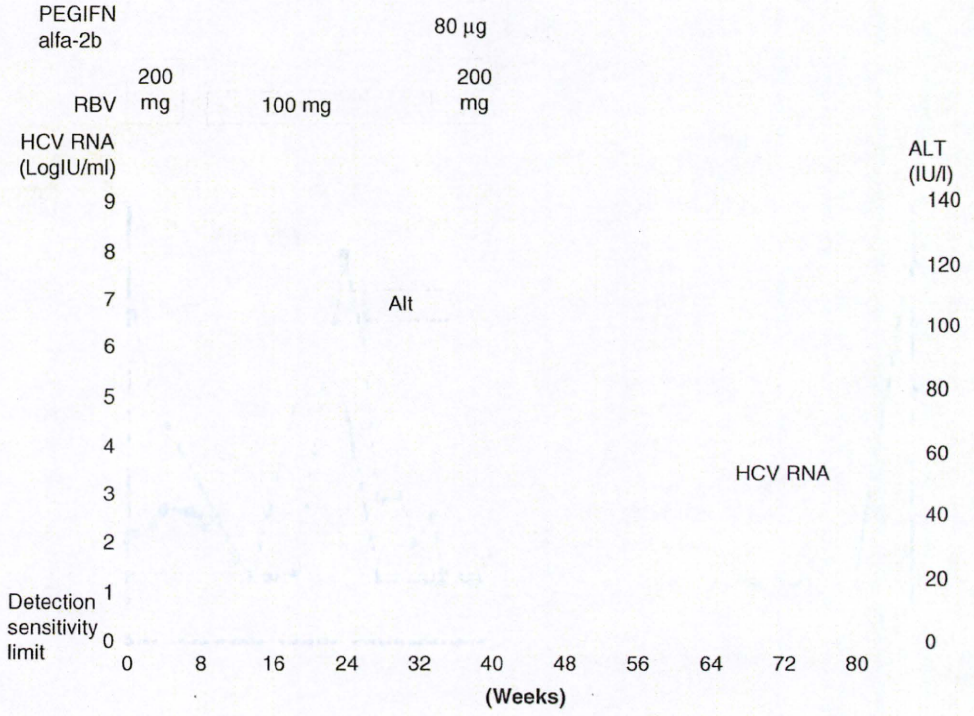

Figure 2. Clinical course in a male patient aged 56 years with genotype 1b, HCV Core 70mutant 91wild, and 0 ISDR mutations. Serum HCV RNA became negative at 27 weeks, after which treatment duration was 52 weeks. However, HCV RNA became positive at 1 week after the cessation of treatment.

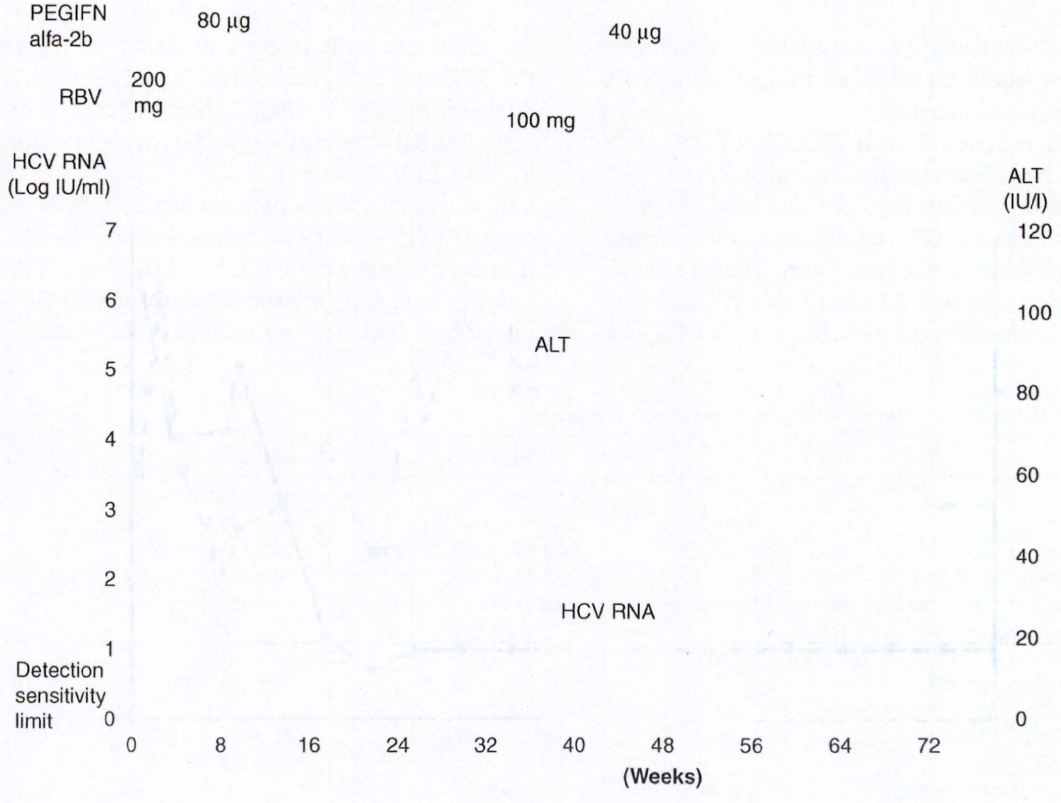

Figure 3. Clinical course in a male patient aged 69 years with genotype 1b, HCV Core 70mutant 91mutant, and 0 ISDR mutations. Virological response occurred at 21 weeks and therapy continued to 78 weeks. Final status was SVR.

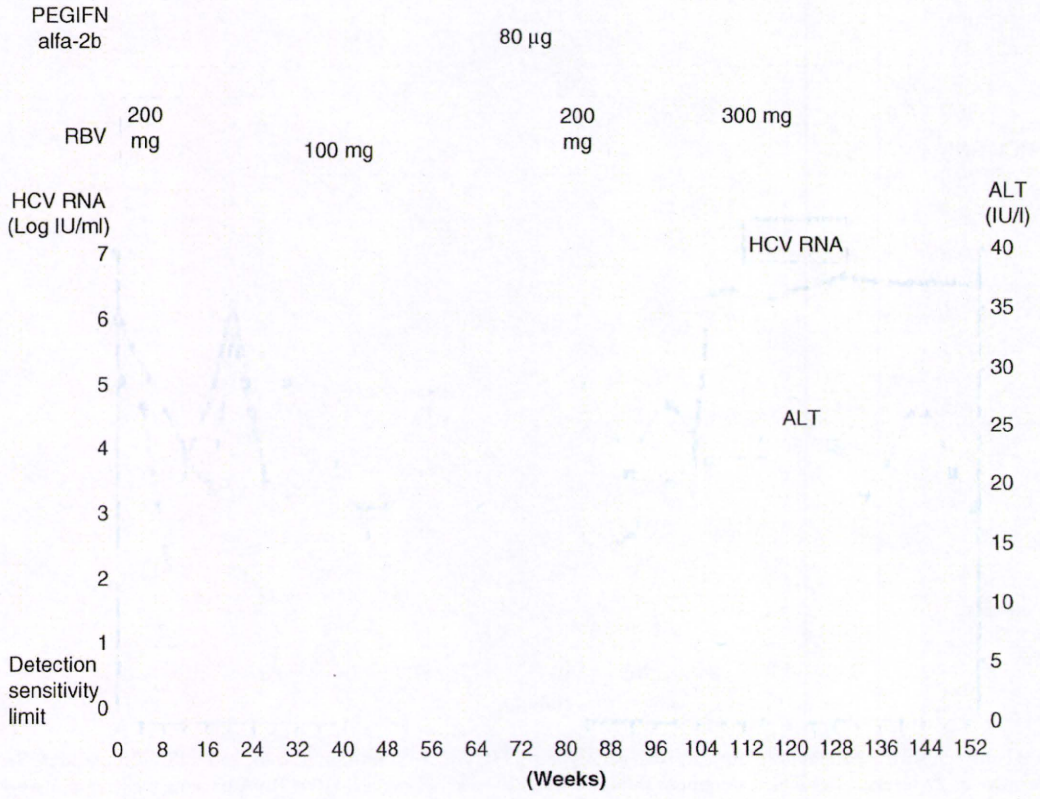

Figure 4. Clinical course in a male patient aged 51 years with genotype 1b, HCV Core 70wild 91wild, and 5 ISDR mutations. He did not develop VR during the dosing period.

untransplanted patients. Consequently, prolonged IFN treatment would be useful and improve the SVR rate for transplanted patients.

We treated recipients with PEGIFN/RBV until HCV RNA had reached undetectable levels and then to continue treatment for at least 1 year. 62.9% of patients (17/27) could complete therapy without severe adverse affects (Figure 1) and relapse rate under this study was 12.5% (Table II), whereas Tamura and Ueda reported a relapse rate of 14% and 3% under the same treatment. As a result, SVR rate was 34% and 50%, respectively [13,14]. With the aid of these results, it might indicate that prolonged PEGIFN/RBV therapy would be useful in eradicating HCV in LDLT patients.

In our study, seven patients achieved SVR by prolonged PEGIFN/RBV for at least 1 year or more. Three of seven patients were EVR and four were LVR.

By contrast, in eight patients who were non-SVR, only one patient had reached undetectable levels of HCV

Table III. Predictive factors associated with SVR in genotype 1b patients.

	SVR (n = 7)	Non-SVR (n = 8)	p-Value
Age (years)*	60 (44–69)	57 (47–65)	0.64
Gender (male/female)	7/0	3/5	0.07
Body mass index*	24.1 (21.4–26.5)	24.2 (18.9–42.2)	0.67
Viral load at therapy (log IU/ml)*	6.3 (5.8–6.6)	6.6 (5.9–7.2)	0.48
Time from transplantation to therapy (months)*	12 (1–41)	3 (3–7)	0.21
Number of mutations in the ISDR (0–1/2–5)	4/3	7/1	0.28
HCV core region (double mutant/non-double mutant)	3/4	3/5	0.6
Duration of treatment (week)*	72 (48–179)	75 (61–133)	0.7
Immunosuppression (tacrolimus/cyclosporine)	6/2	7/1	1
Adherence of PEGIFN (%)*	80 (35.5–100)	71.5 (45.4–100)	0.39
Adherence of RBV (%)*	47.4 (11.2–62.5)	25.5 (15.3–65.9)	0.74
Early virological response (yes/no)	3/4	0/8	0.07

*Values are median (range).

RNA and other seven patients had never reached undetectable levels of HCV RNA. That is, if patients had reached undetectable levels of HCV RNA, they could eradicate HCV RNA in the liver tissue by prolonged IFN therapy for more than 48 weeks after HCV RNA reached undetectable levels. This regimen is similar to that of a recent recommendation that PEGIFN/RBV therapy for 72 weeks is necessary for patients with chronic hepatitis C whose HCV RNA does not reach undetectable levels within 12 weeks.

Recent findings among immunocompetent patients of pretreatment factors that could predict treatment efficacy of 72-week PEGIFN/RBV identified substitution of either or both aa 70 or 91 in the HCV core region, and the number of substitutions in amino acids 2209–2248, the ISDR of NS5A in HCV genotype 1b [26]. By contrast, however, our present results showed that substitution of aa 70 and/or 91 in the HCV core region or the number of ISDR were not predictive of SVR (Table III). All three patients who had double mutation of aa 70 and aa 91 in core region of HCV protein achieved SVR in this study, as did all four patients whose number of mutations in the ISDR was 0 or 1 (Table II).

Recently Fukuhara et al. reported that mutations of the HCV core and NS5A regions of HCV genome were associated with the SVR rates in 50 patients [27]. Although the number of our patients included was less than Fukuhara's, we think that our result is still worth reporting because, in the case of acute hepatitis C, 24-week IFN treatment is enough to eradicate HCV in most cases, suggesting that HCV core mutant and the substitutions of amino acids of the HCV NS5A region are not likely to affect the SVR rate for acute hepatitis C. Since the recurrence of hepatitis C for transplanted patients is another acute hepatitis C, those substitutions might not affect the SVR rate of IFN treatment. Further studies would reveal whether the mutations of the HCV core and NS5A regions of HCV genome were associated with the SVR rates.

Only one patient had HCV relapse after 79 weeks treatment, a male aged 56 years with genotype 1b, HCV Core 70mutant 91wild and number of ISDR mutation 0 (Figure 2). He had a VR at 27 weeks, which lasted for 52 weeks, and continued therapy to 79 weeks. However, he subsequently experienced relapse of HCV. One of many possible reasons was likely low adherence to RBV (7%).

Several reasons may account for the lack of association of HCV core region mutation and number of ISDR mutations with SVR rate. One reason is that it is acute hepatitis after LDLT, which is usually treated as soon as possible: even in those infected with genotype 1, HCV could be eradicated with regular IFN for 24 weeks after acute infection [28–31], meaning that mutation of the core region and NS5A could not be determinants of PEGIFN therapy in LDLT cases. A second reason might be poor adherence to PEGIFN and RBV treatment in patients with LDLT. Among patients who experience severe leucocytopenia, thrombocytopenia and anemia after LDLT, dose reductions in PEGIFN or RBV are therefore inevitable. Therefore, it is reasonable to prolong the duration of PEGIFN/RBV therapy. Taken together, recurrent hepatitis C after LDLT is different from hepatitis C in immunocompetent patients. This might be the reason why any predictive factor but EVR was an only predictive factor of SVR in this study.

There were several limitations in present study. One was that our study is retrospective.

Since it was scheduled that the end point of treatment should be 1 year after serum HCV RNA became negative, it compelled to design the retrospective study as a pilot study. Further prospective study will prove our protocol strongly and help achieving high SVR and low relapse rate. Another limitation was the low number of patients included. Although other institutes also demonstrated good results with similar interferon protocol, as mentioned above [13,14], another study with more number of patients will prove the consistence of our study.

In conclusion, for recurrent hepatitis C after LDLT, our findings indicate that PEGIFN therapy for at least 1 year after HCV RNA reaches undetectable levels might prevent HCV viral relapse. Combination of the new selective inhibitors of HCV, named STAT-C (specifically targeted antiviral therapy for HCV), is expected to further improvements in SVR rates.

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Splenectomy in Chronic Hepatic Disorders: Portal Vein Thrombosis and Improvement of Liver Function

Yuichiro Ushitora[a] Hirotaka Tashiro[a] Shoichi Takahashi[b] Hironobu Amano[a]
Akihiko Oshita[a] Tsuyoshi Kobayashi[a] Kazuaki Chayama[b] Hideki Ohdan[a]

Departments of [a]Gastroenterological Surgery, and [b]Gastroenterology and Hepatology, Hiroshima University Hospital, Hiroshima, Japan

Key Words
Splenectomy · Interferon · Portal vein thrombosis

Abstract
Background: Splenectomy is gaining increasing importance for cirrhotic patients with hypersplenism. However, its safety and efficacy for patients with chronic liver disease remain unclear. ***Methods:*** We retrospectively examined the medical records of 38 consecutive cirrhotic patients who underwent splenectomy or simultaneous hepatectomy and splenectomy for hepatocellular carcinoma. ***Results:*** White blood cell and platelet counts significantly increased 3 months after splenectomy. Serum levels of total bilirubin and prothrombin time significantly improved 1 year after splenectomy. Interferon therapy was administered to 25 patients after splenectomy. A sustained viral response was achieved in 8 patients (42%). The total incidence of portal or splenic vein thrombosis (PSVT) detected by postoperative dynamic computed tomography was 13/38 (34.2%). Multivariate analysis revealed preoperative spleen volume (SV) to be the sole independent predictor of postoperative PSVT. Receiver-operator characteristic curve analysis showed that a cut-off SV of 450 ml corresponded to a sensitivity of 85% and a specificity of 56%. ***Conclusions:*** Splenectomy improved the liver function and facilitated effective interferon therapy in cirrhotic patients with hypersplenism, although preoperative SV was frequently associated with postoperative PSVT.

Splenectomy has been performed as a part of Hassab's operation or esophageal transection for control of variceal hemorrhage [1]. Moreover, splenectomy evidently decreases portal pressure [2] and reverses hypersplenism [3], and it has been concurrently performed in patients with small-for-size liver grafts in the setting of living donor liver transplantation [4–6]. Splenectomy is gaining increasing importance for cirrhotic patients with hypersplenism. Thrombocytopenia is a common complication of liver cirrhosis; because of this complication, initiation of antiviral treatment with interferon (IFN) becomes difficult in cirrhotic patients. Patients with thrombocytopenia often receive inadequate or incomplete IFN therapy because of exacerbation of thrombocyotopenia. Recently, splenectomy has been indicated and performed in cirrhotic patients undergoing treatment for hepatocellular carcinoma (HCC) to improve thrombocytopenia and

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Hirotaka Tashiro
Department of Gastroenterological Surgery
Hiroshima University Hospital
Hiroshima 734-8551 (Japan)
E-Mail htashiro@hiroshima-u.ac.jp

prior to induction of IFN therapy for patients with hepatitis C (HCV) [7, 8]. However, the safety and efficacy of IFN therapy after splenectomy remain unclear.

Liver function parameters have been reported to improve after splenectomy [3, 9, 10]. Chen et al. [10] reported that bilirubin levels were significantly lower on postoperative day 7 in patients who underwent simultaneous hepatectomy and splenectomy than in those who underwent only hepatectomy. Sugawara et al. [3] reported a significant reduction in the serum bilirubin levels 1 week after splenectomy. However, the long-term effects on liver function parameters after splenectomy remain unclear.

Portal vein thrombosis (PVT), which was considered as an uncommon complication, has been reported to be a possible cause of death after splenectomy [11]. However, with the advancements in diagnostic modalities, the incidence of portal or splenic vein thrombosis (PSVT) may apparently be greater than clinically appreciated, and this condition may be asymptomatic [12–15]. Winslow et al. reported that PSVT occurred in 8 (8%) of 101 patients who underwent splenectomy [12]. Sugawara et al. [3] reported that PVT occurred in 3 (7%) of 48 patients who underwent splenectomy. However, recent studies reported the incidence of PSVT to be between 14 and 55% [16–18]. Ikeda et al. [18] reported that PSVT occurred in 12 (55%) patients who underwent laparoscopic splenectomy. Several risk factors contribute to the onset of PSVT. A large spleen is associated with PSVT development after splenectomy [17, 18]. However, the weight of the spleen cannot be precisely measured because of the loss of blood from the spleen during the operation.

The aim of the current study is to determine the long-term effects of splenectomy on hepatic functions, the efficacy of IFN treatment after splenectomy, and the risk factors, including the volume of the spleen for PSVT.

Patients and Methods

Patients

Between January 2003 and December 2008, 38 consecutive patients underwent splenectomy or simultaneous hepatectomy and splenectomy for HCC at Hiroshima University Hospital. In this study, patients who underwent splenectomy for idiopathic thrombocytopenic purpura or after living donor liver transplantation were excluded. Thirteen patients underwent only open splenectomy, and 2 patients underwent laparoscopic splenectomy. Seven patients underwent Hassab's operation. Sixteen patients underwent simultaneous hepatectomy and splenectomy. Splenectomy was performed by laparotomy or hand-assisted laparoscopic splenectomy (HALS). In open surgery, a midline incision was used, and the vessels at the splenic hilum were ligated and divided at the tail of the pancreas. For HALS, patients were placed in the right semilateral position, and a 7-cm skin incision for the hand insertion was made in the left subcostal area. The first trocar (12 mm) was inserted on the left of the umbilicus, where the laparoscope was placed. A CO_2 pneumoperitoneum was then created. Three other trocars were inserted under visual control into the epigastric area, the midclavicular line of the subcostal margin, and the left flank. Splenic attachments were divided using electrocautery, or an ultrasonic dissector. The splenic hilar pedicle was transected with an endoscopic linear vascular stapler. In open surgery, an antithrombotic catheter was inserted via the jejunal vein immediately after laparotomy. The top of the catheter was positioned in the recipient portal vein. A transducer was used to measure the portal vein pressure (PVP) during surgery, and the catheter was removed before closing the abdominal operative wound. Computed tomography (CT) was performed preoperatively and at one week, 1 and 6 months after surgery, or when indicated clinically. Spleen volume (SV) was measured from CT images obtained with a workstation (Virtual Place Advance 300, AZE, Ltd.) [19]. The diagnosis of PSVT was made by a radiologist, and PSVT was classified according to the location of the thrombus. When the thrombus was located solely in the splenic vein, the patient was diagnosed with splenic vein thrombosis (SVT). On the other hand, when the thrombus extended into the trunk of the portal vein, the patient was diagnosed with PVT. PVTs were further classified as intrahepatic and extrahepatic thrombosis. Upon the detection of PSVT with the CT scan, we started anticoagulation therapy that consisted of heparin (10,000 U/day, intravenously) followed by warfarin. The dosages of warfarin were adjusted to achieve an international normalized ratio from 1.5 to 2.0. Until PSVT disappearance was confirmed by CT, the administration of warfarin was continued. Antiviral treatment including pegylated IFNα-2b plus ribavirin was started when platelet counts were increased, and no severe surgical complications developed after splenectomy.

Statistical Analysis

Continuous data are shown as mean ± SEM. The clinical data were compared using the Mann-Whitney U test or Fisher's exact test. The Cox proportional hazards model was used for multivariate analysis. The paired t test was used for comparison of perioperative laboratory data. Receiver-operator characteristic (ROC) curve analysis was performed to determine the optimal cut-off values. Each cut-off value was determined by seeking the most optimal combination of high sensitivity and specificity values, while maintaining the lowest likelihood ratio of a negative test and the highest likelihood ratio of a positive test. p value of less than 0.05 was considered significant. Statistical analysis was performed using the SPSS 16.0 software (SPSS, Chicago, Ill., USA).

Results

Table 1 presents the clinical characteristics of the patients. The patients were 20 men and 18 women (median age, 60.0 years; range, 40–77 years). Thirty-six (94.7%) patients were positive for HCV. The severity of liver disease was evaluated on the basis of the Child-Pugh classification. Of the 38 patients, 27 (71.1%) were classified as Child-

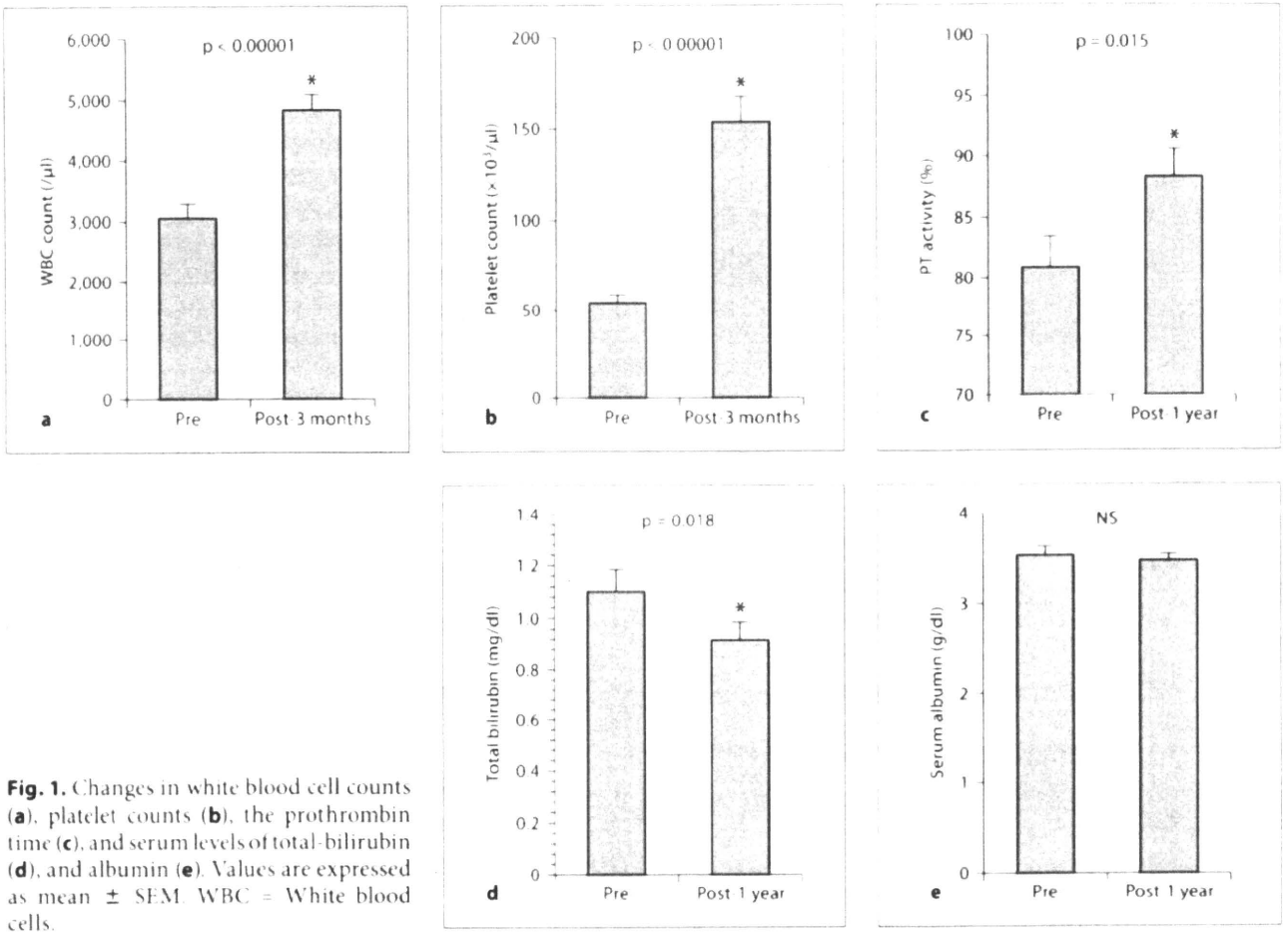

Fig. 1. Changes in white blood cell counts (**a**), platelet counts (**b**), the prothrombin time (**c**), and serum levels of total-bilirubin (**d**), and albumin (**e**). Values are expressed as mean ± SEM. WBC = White blood cells.

Pugh A, and the remaining 11 (28.9%) were classified as Child-Pugh B. Twenty-six (68.4%) patients had previously received IFN therapy, and 19 (50.0%) patients had HCC. Indications for splenectomy included bleeding tendency due to thrombocytopenia (n = 1), difficulties in the induction or continuation of IFN therapy due to thrombocytopenia (n = 31), difficulties in therapies for HCC due to thrombocytopenia (n = 2), endoscopic treatment-resistant esophagogastric varices (n = 8). The changes in the laboratory data are shown in figure 1. Hematological tests conducted before and 3 months after the operation revealed a significant increase in the white blood cell count from 3,055 ± 224 to 4,821 ± 252/μl ($p < 0.00001$), and in the platelet count, from 54.2 ± 4.3 to 153.5 ± 13.5 × 10^3/μl ($p < 0.00001$). Moreover, biochemical tests performed before and 1 year after the operation revealed a significant decrease in the serum levels of total bilirubin from 1.10 ± 0.08 to 0.91 ± 0.07 mg/dl ($p = 0.018$), and prothrombin

Table 1. Clinical characteristics of the patients (n = 38)

Age, years	60 (40–77)
Male, n	20
Female, n	18
Hepatitis HBV, n	1
Hepatitis HCV, n	36
Hepatitis NBNC, n	1
Child-Pugh A, n	27
Child-Pugh B, n	11
White blood cell count, /μl	3,019 ± 224
Hemoglobin, g/dl	12.2 ± 0.29
Platelets, × 103/μl	52.4 ± 4.3
Spleen volumetry, ml	493 ± 45.0
Anamnesis of IFN therapy, n	26
Complicating HCC, n	19

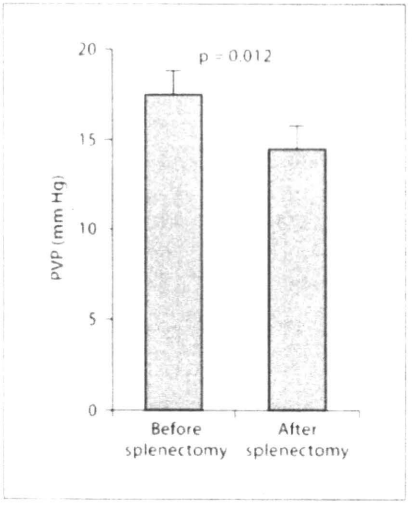

Fig. 2. Changes in portal vein pressure. Values are expressed as mean ± SEM.

Table 2. Comparison of clinical characteristics between patients with or without PSVT after splenectomy

	No PSVT (n = 25)	PSVT (n = 13)	p value
Sex, male/female	16/9	4/9	0.054
HCC, –/+	13/12	6/7	0.73
Preoperative hemoglobin, mg/dl	12.6 ± 0.38	11.5 ± 0.32	0.026
Preoperative platelets, 10^4/mm^3	5.24 ± 0.37	5.25 ± 0.95	0.414
Preoperative PT, %	81.7 ± 2.7	74.0 ± 2.5	0.031
Antithrombin III	60.0 ± 3.4	57.5 ± 4.8	0.410
Child-Pugh, A/B	20/5	7/6	0.096
Splenic volumetry, ml	422 ± 47.7	621 ± 82.9	0.011
Operating time, min	263 ± 15.7	253 ± 24.2	0.678
Blood loss, ml	508 ± 62.9	433 ± 73.8	0.399
Transfusion, –/+	22/3	9/4	0.164

Table 3. Multivariate logistic regression analysis of predictors of PSVT

Variable	p value	OR	95% CI
Sex, female	0.15	3.927	0.609–25.325
Preoperative hemoglobin	0.436	0.773	0.407–1.467
Preoperative PT	0.422	0.96	0.868–1.061
Splenic volumetry	0.036	9.582	1.156–79.415

time (PT) improved significantly from 81 ± 2.5 to 88.8 ± 2.2% (p = 0.015); however, the serum levels of total albumin did not improve significantly. PVP was significantly decreased from 17.5 ± 1.33 to 14.4 ± 1.38 mm Hg immediately after splenectomy (p = 0.012) (fig. 2). Of the 38 patients who underwent splenectomy, 25 received IFN plus ribavirin therapy. The median time between the start of IFN plus ribavirin therapy and splenectomy was 48 days (range 13–977 days). In total, a sustained virological response (SVR) was achieved in 8 (42%) of 19 patients who received IFN therapy. At present, 6 patients continue to receive IFN therapy. Owing to depression and severe diarrhea, IFN therapy was discontinued in 3 patients. No patients who underwent splenectomy discontinued IFN therapy due to thrombocytopenia.

The total incidence of PSVT detected by postoperative dynamic CT (asymptomatic or symptomatic) was 13/38 (34.2%). Specifically, 8 patients exhibited SVT, and 5 exhibited PVT. In 11 of these patients, PSVT was detected for 7–10 days after the operation. In 1 patient, PSVT was detected by dynamic CT approximately 6 months after the operation. In addition, it was detected in another patient approximately 3 months after the operation, and total bilirubin levels in this patient temporarily reached 8 mg/dl. Antiplatelet and anticoagulant drugs were successful in all patients, but PSVT recurred in 2 patients. Univariate analysis (table 2) revealed that the preoperative hemoglobin (Hgb) levels and the preoperative PT were significantly lower in patients with PSVT than in those without PSVT. Moreover, the preoperative SV measured by CT volumetry was significantly higher in patients with PSVT than in those without PSVT. However, multivariate analysis (table 3) revealed SV to be the sole predictor of PSVT in patients who had undergone splenectomy (p = 0.036). The optimal cut-off value was determined by ROC curve analysis (fig. 3). A cut-off SV of 450 ml corresponded to a sensitivity of 85% and specificity of 56%.

Postoperative complications associated with splenectomy developed in 7 patients; superficial surgical site infection (n = 3) and intra-abdominal abscess (n = 2) were treated by percutaneous drainage, and ileus (n = 2) that occurred about 1 year after splenectomy were treated by open surgery.

Discussion

In our study, platelet counts significantly increased after splenectomy, and this result is consistent with those obtained in other studies [3, 7]. Improvements in the liver

function parameters after splenectomy have been reported in the literature. Shimada et al. [9] showed that the Child-Pugh class and ammonia levels of cirrhotic patients improved after splenectomy. Chen et al. [10] reported a significant decrease in the serum bilirubin levels after splenectomy in hepatectomized patients. Sugawara et al. [3] also reported that serum bilirubin levels significantly decreased after splenectomy. However, these assessments were performed in the range from 7 to 40 days after splenectomy. A few studies have reported the mid-term (less than 6 months) effects of splenectomy. Ikegami et al. [20] reported that in 2 cirrhotic patients, liver function parameters, including total bilirubin, serum albumin levels, and PT, improved 3 months after splenectomy. Morihara et al. [8] reported that total bilirubin levels improved 6 months after splenectomy. However, few studies have reported the long-term (more than 6 months) effects of splenectomy. In our study, the serum level of total bilirubin and PT improved significantly 1 year after splenectomy, although the serum levels of albumin did not significantly improve. These results suggest that splenectomy results in long-term improvement in liver function for more than 6 months after surgery. The hemodynamic status of patients with liver cirrhosis is characterized by a systemic hyperdynamic status. Portal hypertension with hyperdynamic splanchnic inflow increases mesenteric permeability, causing leakage of a large amount of fluid into the abdominal cavity. Splenectomy can decrease the inflow into the portal system, resulting in decreased portal pressure [2, 4]. In the current study, PVP was decreased after splenectomy, and this result is consistent with the previous reports. Although the mechanism by which splenectomy improves the liver function is difficult to explain, the improvement in liver function might be associated with a decrease in PVP after splenectomy. Moreover, splenectomy may modify inflammatory mediator generation. In the endotoxin-induced liver injury model, splenectomy prevented liver injury by suppressing Kupffer cell function and tumor necrosis factor [21]. In the warm ischemia-reperfusion model in rats, splenectomy protected against acute multiple organ damage by inhibiting leukocyte infiltration in livers, release of TNF-α, and expression of caspase-3 [22]. Splenectomy promoted the regeneration and healing of livers in cirrhotic rats via inhibition of the production of TGF-β [23]. Splenectomy may improve the liver function through the modification of hemodynamic status and inflammatory mediators. Furthermore, an SVR was achieved in 8 patients (42%) in this study. The effectiveness of the IFN therapy might indirectly contribute to the improvement in liver function.

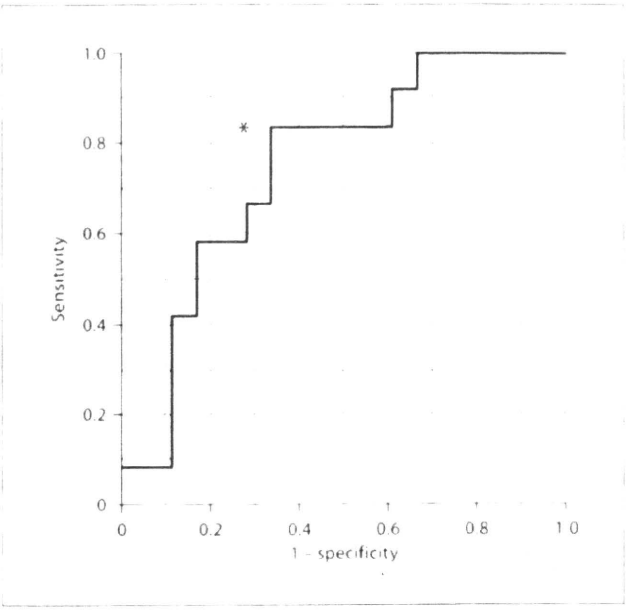

Fig. 3. Receiver-operator characteristics curve of SV for predicting postoperative PSVT in 38 patients who underwent splenectomy; * 450 ml; sensitivity, 85%; specificity, 56%.

Initiation of antiviral therapy with IFN is difficult in patients with thrombocytopenia. Patients with low platelet counts often receive inadequate or incomplete IFN therapy because of the necessary dose reduction or the discontinuation of treatment. Splenectomy is known to improve platelet counts in cirrhotic patients with thrombocytopenia. Recently, some studies have reported that IFN therapy can be safely administered to HCV-positive patients with thrombocytopenia after they have undergone splenectomy. Furthermore, these studies showed that none of the patients required a dose reduction or discontinuation of treatment because of thrombocytopenia, and that the treatment outcomes were similar to those for patients without thrombocytopenia [7, 8]. In the current study, an SVR was achieved in 42% of patients with thrombocytopenia, and IFN therapy did not have to be discontinued because of thrombocytopenia in any of the patients.

In the current study, multivariate analysis revealed that an increase in SV is associated with PSVT after splenectomy. ROC curve analysis revealed that an SV greater than 450 ml is associated with PSVT development after splenectomy. Recent reports have shown that the spleen was significantly heavier in patients with PSVT than in those that did not have PSVT; this finding suggests that

the spleen weight is a possible predictor of postoperative PSVT [17, 18]. However, because of blood loss from the spleen during splenectomy, the weight of the resected spleen is not identical to that of the spleen before the operation. This is the first study to show that the SV recorded before splenectomy is associated with PSVT development after splenectomy; in addition, postoperative PSVT can be predicted by performing preoperative CT volumetry of the spleen. In 11 (84%) of 13 patients who developed PSVT, PSVT was detected 7–10 days after the operation. Postoperative dynamic CT should be planned approximately 1 week after splenectomy for early detection of PSVT. Kawanaka et al. [24] reported that preoperative antithrombin (AT)-III activity was the most important predictive factor for PVT, and that treatment with AT-III concentrates is likely to prevent the development of PSVT. In this study, we have shown that preoperative AT-III activity was not associated with postoperative PSVT. However, as shown in Kawanaka's report, prophylactic administration of AT-III concentrate in the early postoperative phase may prevent postoperative PSVT in case of SV greater than 450 ml, and it might be better to start anticoagulation therapy with a low dose of warfarin 3 or 4 days after the operation, after confirming that postoperative bleeding did not occur.

In conclusion, splenectomy exhibited a long-term positive effect on liver function; furthermore, preoperative SV was associated with postoperative PSVT. IFN therapy did not have to be discontinued because of thrombocytopenia in any of the patients.

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Possibility of Adoptive Immunotherapy With Peripheral Blood-derived $CD3^-CD56^+$ and $CD3^+CD56^+$ Cells for Inducing Antihepatocellular Carcinoma and Antihepatitis C Virus Activity

Marlen Doskali,† Yuka Tanaka,*† Masahiro Ohira,*† Kohei Ishiyama,*† Hirotaka Tashiro,*† Kazuaki Chayama,†‡ and Hideki Ohdan*†*

Summary: We recently showed that interleukin (IL)-2-stimulated $CD56^+$ cells derived from the liver exert vigorous cytotoxicity against hepatocellular carcinoma (HCC) by their binding to the tumor necrosis factor-related apoptosis-inducing ligand expressed on natural killer cells and the corresponding death receptors, and exhibit inhibitory effects on hepatitis C virus (HCV) replication by production of a high level of interferon-γ. These findings prompted us to develop a technique to increase the number of such innate components of cellular immunity from peripheral blood mononuclear cells (PBMCs) so that, they can be easily applied for immunotherapy clinically. We expanded $CD3^-CD56^+$ and $CD3^+CD56^+$ cells ex vivo from PBMCs of human volunteers by using media containing IL-2 and anti-CD3 monoclonal antibody. Among the various culture media used, autoserum supplemented X-VIVO 15 most efficiently supported PBMCs expansion and maintained the viability of the expanded cells (approximately 60-fold expansion after 28-d culture). Cultivation of PBMCs in this medium resulted in the highest proportion of $CD3^-CD56^+$ cells among the propagated lymphocytes (approximately 40% after 28-d culture). An experiment using genomic HCV replicon-containing hepatic cells showed that the $CD3^-CD56^+$ cell-enriched expansion strongly inhibited HCV replicauion when compared with freshly isolated PBMCs. The additional anti-CD3 monoclonal antibody pulse stimulation induced anti-HCV activity even in the $CD3^+CD56^+$ cells among the propagated PBMCs. Further, cytotoxic assay showed that the expansion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells resulted in vigorous cytotoxicity against HCC. In conclusion, $CD56^+$ cells obtained from the PBMCs show anti-HCV activity in addition to anti-HCC activity.

Key Words: adoptive immunity, cytotoxicity, hepatocellular carcinoma, hepatitis C virus, peripheral blood mononuclear cells

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From the *Department of Surgery; ‡Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science; and †Liver Research Project Center, Hiroshima University, Hiroshima, Japan.

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Reprints: Hideki Ohdan, Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan (e-mail: hohdan@hiroshima-u.ac.jp).

Liver failure and hepatocellular carcinoma (HCC) because of chronic hepatitis C infection are the most common indications for liver transplantation (LT). The incidence of both the conditions has been projected to increase further. Recurrent hepatitis C virus (HCV) infection of allografts is universal, occurs immediately after LT, and is associated with accelerated progression to liver cirrhosis, graft loss, and death.[1,2] The infection is thought to reflect suppression of the host-effector immune responses that usually control the HCV replication.[3,4] Further, this immunosuppressive condition is considered to increase the incidence of HCC recurrence after LT.

We recently proposed a novel strategy of adjuvant immunotherapy for preventing HCC recurrence after LT: in this immunotherapy, transplant recipients are intravenously injected with lymphokine-activated killer (LAK) cells including activated $CD3^-CD56^+$ and $CD3^+CD56^+$ cells derived from the liver allografts. Considering that the immunosuppressive regimen currently used after LT reduces the components of adaptive immunity, but effectively maintains the innate components of cellular immunity,[5–7] augmentation of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells that are thought to play a pivotal role in innate immunity, may be a promising immunotherapeutic approach.[8] We confirmed that $CD56^+$ cells derived from the liver allografts treated with interleukin (IL)-2 and anti-CD3 monoclonal antibody (mAb) express a significantly high level of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a critical molecule for tumor cell killing; further, these cells showed high cytotoxicity against HCC, with no such effect on normal cells.[8] After obtaining approval from the ethical committee of our institute, we successfully applied adoptive immunotherapy with liver lymphocytes treated with IL-2 and anti-CD3 mAb to patients with HCC having liver cirrhosis in a phase I trial.[9] Although the long-term benefits of this approach with regard to the control of HCC recurrence after LT remain to be elucidated, the trial provided a unique opportunity to study whether the administration of liver lymphocytes treated with IL-2 and anti-CD3 mAb can also induce an anti-HCV response in transplant recipients with HCV infection. During the first month after LT, the HCV ribonucleic acid (RNA) titers in the sera of the patients who underwent immunotherapy were markedly low compared with those in the sera of the patients who did not undergo immunotherapy; however, such anti-HCV responses were transient. This observation prompted us to develop a technique to increase the number of innate components of cellular immunity from peripheral blood mononuclear cells (PBMCs) so that they can be easily applied for immunotherapy in the clinical setting.

In this study, we attempted to establish a procedure for the ex vivo expansion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells obtained from PBMCs of healthy volunteers.

MATERIALS AND METHODS

Isolation and Cell Culture

PBMCs obtained from healthy volunteers were isolated by gradient centrifugation with Separate-L (Muto Pure Chemicals Co. Ltd, Tokyo, Japan), and suspended in either X-VIVO 15 medium (Lonza, Walkersville, Inc., MD) or RPMI 1640 medium (Gibco Brl, Grand Island, NY). X-VIVO 15 was supplemented with a 50 mmol/L HEPES buffer (Gibco Brl) and used with or without the addition of heat-inactivated 5% autoserum. RPMI 1640 was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sanko Chemical Co. Ltd., Tokyo, Japan), 25 mmol/L HEPES buffer (Gibco Brl), 50 mol/L 2 mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/mL penicillin, and 50 g/mL streptomycin (Gibco Brl). Human recombinant IL-2 (1000 Japanese reference units /mL; Takeda Pharmaceutical, Tokyo, Japan) and 100 ng/mL of anti-CD3 mAb OKT3 (Janssen-Kyowa Co. Ltd., Tokyo, Japan) were added to all culture media, which were maintained at 37°C in a 5% CO_2 incubator during the indicated period. The medium was replaced every 7 days, and cell concentrations were adjusted to 1×10^6 cells/mL. The cell viability was assessed by using the trypan blue dye exclusion test.

Flow Cytometry

Flow cytometric analyses were carried out by using an FACSCalibur dual-laser cytometer (BD Biosciences, San Jose, CA). The following mAbs were used for surface staining of the lymphocytes: flourescein isothiocyanate (FITC)-conjugated anti-CD3 mAb (clone HIT3a; BD Biosciences), PE-conjugated anti-CD56 mAb (clone B159; BD Biosciences), and biotinylated anti-TRAIL (biotin-conjugated anti-TRAIL) mAb (clone RIK-2; eBioscience, Inc., San Diego, CA). The biotinylated mAb was visualized by using allophycocyanin (APC)-streptavidin (BD Biosciences, San Diego, CA). Dead cells identified by light scattering and propidium iodide staining were excluded from the analysis.

Interferon (IFN)-γ production in the lymphocytes was measured by a combination of cell surface and cytoplasmic mAb staining according to the manufacturer instruction. In brief, 4 hours after treatment with leukocyte activation cocktail (BD GolgiPlug, BD Biosciences), the lymphocytes were stained with surface markers anti-CD3-FITC and anti-CD56-APC (BD Biosciences). After washing, the cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences), and washed with 10% Perm/Wash Buffer (BD Biosciences). Subsequently, aliquots were stained with either a mAb against intracellular cytokine anti-IFN-γ-PE or isotype-matched control (BD Biosciences).

Cytotoxicity Assay

HepG2 cells, a hepatoma cell line, were purchased from the Japanese Cancer Research Resources Bank and maintained in 10% RPMI.[10] The cells were labeled with 100 μCi Na_2 (^{51}Cr) O_4 for 60 minutes at 37°C in 5% CO_2 in Dulbecco modified eagle medium (DMEM: Gibco Brl) supplemented with 10% FBS, and then subjected to a cytotoxic assay. The labeled HepG2 cells were adjusted to 1×10^4 cells in 100 μL volumes and were incubated for 4 hours in a total volume of 200 μL with effector cells in 10% DMEM in U-bottomed 96-well tissue culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ). PBMCs from healthy volunteers were used as effectors at effector-target (E:T) ratios of 5:1 to 40:1. As a control, the target cells were incubated in either culture medium alone, to determine spontaneous release, or a mixture of 2% Nonidet P-40 (Nacalai Tesque, Inc., Kyoto, Japan), to define the maximum ^{51}Cr release. The plates were centrifuged at 1000 rpm for 3 minutes to pack the cell layer at the end of the reaction, after which the cell-free supernatants were carefully harvested and their radioactivity was measured with a gamma counter. The percentage of specific ^{51}Cr release was calculated by the following formula:

$$\% \text{ cytotoxicity} = \frac{[(\text{cpm of experimental release} - \text{cpm of spontaneous release})]}{[(\text{cpm of maximum release} - \text{cpm of spontaneous release})]} \times 100.$$

The spontaneous release was < 20% of the maximum release. When indicated, the cytotoxic assay was performed in the presence of 10 μg/mL of anti-TRAIL mAb (N2B2) or, 10 μg/mL of anti-Fas ligand (FasL) mAb (MFL3) (both from BD Biosciences), or 50 nmol/L of Concanamycin A (Wako Pure Chemicals, Osaka, Japan), which inhibited perforin-mediated cytotoxicity. All the assays were performed in triplicate.

Coculture With HCV Replicon Cells

The Huh7/Rep-Feo cell line (HCV replicon cells) was kindly gifted by Dr N. Sakamoto (Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan). An HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally pHCVIbneo-delS).[11] pRep-Feo carries a fusion gene comprising firefly luciferase (*Fluc*) and neomycin phosphotransferase, as described elsewhere.[12,13] After culture in the presence of G418 (Wako Pure Chemical Industries), Huh7/Rep-Feo cell lines stably expressing the replicons were established. For the coculture experiments, transwell tissue culture plates (pore size, 1 μm; Costar, Cambridge, MA) were used. HCV replicon cells (10^5 cells) were incubated in the lower compartment with different numbers of lymphocytes in the upper compartment. DMEM supplemented with 10% FBS was used in this assay. The HCV replicon cells in the lower compartments were collected 48 hours after coculture for luciferase assay. When indicated, anti-CD3 mAb pulse (1 μg/mL) was added 24 hours before harvesting. The luciferase activities were measured with a luminometer (Lumat LB940; Berthold Technologies, Germany) using the Bright-Glo Luciferase Assay System (Promega). When indicated, the assays were performed in the presence of 100 μg/mL anti-human IFN-γ mAb and 100 μg/mL isotype-matched control mAb (R&D Systems, Minneapolis, MN). All the assays were performed in duplicate.

ELISA Assay

Supernatants of propagated PBMCs were collected after 28 days of culture with or without additional stimulation by anti-CD3 mAb pulse (1 day before harvest). IFN-γ levels in the cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using Quantikine kit (R&D systems, Minneapolis, MN), according to the manufacturer instructions. Absorbance was measured at 492 nm using a microplate reader MTP-300 (CORONA Electric, Ibaraki, Japan).

Statistical Analysis

The results were statistically analyzed by using either unpaired or paired Student *t* test or the linear regression analysis when appropriate. *P*-values < 0.05 were considered to be statistically significant.

RESULTS

Ex Vivo Expansion of CD3−CD56+ and CD3+CD56+ cells from PBMCs

Although RPMI 1640 medium supplemented with FBS is widely used to propagate PBMCs in the experimental setting, several studies have shown that X-VIVO 15 medium has been used under serum-free conditions to support the growth of human monocytes, macrophages, lymphocytes, and natural killer (NK) cells.[14–16] X-VIVO 15 contains pharmaceutical grade human albumin, recombinant human insulin, and pasteurized human transferrin; hence, it seems to be favorable in terms of clinical applicability. We compared the capacity of RPMI 1640 and X-VIVO 15 to support the growth of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells from human PBMCs in the presence of IL-2 and anti-CD3 mAb, which are crucial for the expansion of LAK cells.[17–21] PBMCs obtained from 10 human volunteers expanded after 28 days of culture by about 22-fold (range, 8 to 55) in RPMI 1640 containing FBS, 38-fold (range, 10 to 93) in serum free X-VIVO 15, and 62-fold (range 32 to 97) in X-VIVO 15 containing autoserum (Fig. 1A). Autoserum supplemented X-VIVO 15 most efficiently supported cell expansion and also maintained the viability of the expanded cells (Figs. 1A, B).

Further, the cultivation in X-VIVO 15 containing 5% autoserum constantly resulted in the highest proportion of $CD3^-CD56^+$ cells among the propagated lymphocytes (Fig. 1C). Hence, X-VIVO 15 containing autoserum was used for the subsequent experiments. After being cultured for 28 days in the presence of IL-2 and anti-CD3 mAb, propagated PBMCs exclusively consisted of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells (Fig. 2). The proportion of those 2 cell fractions in the propagated PBMCs varied among individuals . The average proportion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells was 41.5% (range, 4.6% to 97.1%) and 51.1% (range, 0.8% to 93.7%), respectively.

Vigorous Cytotoxicity of Propagated PBMCs Against HCC

Cytotoxic assays using freshly isolated and propagated PBMCs as the effectors and the HepG2 cell line as target were performed. As shown in Figure 3, the freshly isolated PBMCs barely mediated potent cytotoxicity against HepG2 cells. Although the ratio of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells highly varied among the propagated PBMCs of each individual after 28 days of culture, all propagated PBMCs uniformly exhibited vigorous cytotoxicity against HCC, regardless of the ratio of these cell types, while those propagated PBMCs showed no cytotoxicity against self-lymphoblasts (data not shown).

Anti-HCC Activity of the Propagated PBMCs By TRAIL

The propagated $CD3^-CD56^+$ and $CD3^+CD56^+$ cells expressed TRAIL on their surface (Fig. 4). To determine the contribution of TRAIL to the anti-HCC activity of the propagated PBMCs, the effect of a neutralizing anti-TRAIL mAb was examined in a cytotoxicity assay using the propagated PBMCs (cultivated for 28 d) as the effector and HepG2 cells as the target. The cytotoxicity of propagated PBMCs against hepatoma cells was partially inhibited by the anti-TRAIL mAb alone and more profoundly inhibited by the combination of anti-TRAIL and anti-FasL mAbs together with Concanamycin A. However, cytotoxicity was detected even in the presence of all those blockers, suggesting that other unknown effectors, in addition to TRAIL, FasL, and perforin, are also involved in hepatoma cell cytotoxicity induced by propagated PBMCs (Fig. 5). These findings indicated that TRAIL expressed on the propagated PBMCs was involved in anti-HCC activity.

Anti-HCV Activity of the Propagated PBMCs

We next analyzed the anti-HCV activity of the propagated PBMCs. Propagated PBMCs strongly inhibited luciferase reporter activity compared with freshly isolated PBMCs (Fig. 6A). The additional anti-CD3 mAb pulse administered 1 day before culturing with HCV replicon-containing cells increased the anti-HCV activity of propagated PBMCs. To identify the cell type expressing the anti-HCV activity, we analyzed the relationship between anti-HCV activity and the ratio of $CD3^-CD56^+$ cells in propagated PBMCs (Fig. 6B). Notably, propagated PBMCs containing more $CD3^-CD56^+$ cells (and, thus, fewer $CD3^+CD56^+$ cells) showed lower luciferase reporter activity, indicating that the anti-HCV activity of $CD3^-CD56^+$ cells was more vigorous than that of $CD3^+CD56^+$ cells. However, this trend was not observed when anti-CD3 mAb pulse was added to the culture medium 1 day before the anti-HCV assay; rather, all propagated PBMCs strongly inhibited luciferase reporter activity regardless of the $CD3^-CD56^+$/$CD3^+CD56^+$ cell ratio. This indicates that the additional anti-CD3 mAb pulse stimulation increased anti-HCV activity of the $CD3^+CD56^+$ cells to levels exhibited by $CD3^-CD56^+$ cells.

We earlier reported that IFN-γ secreted from liver lymphocytes activated by IL-2 and anti-CD3 mAb is responsible for the anti-HCV activity of these cells.[9] Propagated PBMCs also actively produced IFN-γ (Fig. 7A), which likely played a pivotal role in the anti-HCV activity of these cells. IFN-γ expression was significantly higher in $CD3^-CD56^+$ cells than in $CD3^+CD56^+$ cells, consistently with the anti-HCV activity levels of these cells (Fig. 7B). Even after being cultured for 28 days in the presence of IL-2 and anti-CD3 mAb, a considerable number of $CD3^+CD56^+$ cells could be detected by FCM, indicating that CD3 molecules not coated with anti-CD3 mAb were present on the surface of propagated PBMCs. The additional anti-CD3 mAb pulse stimulation 1 day before coculturing propagated PBMCs with HCV replicon-containing cells significantly promoted IFN-γ secretion from $CD3^+CD56^+$ cells (Fig. 8). In addition, blocking IFN-γ with a mAb abrogated the anti-HCV activity of propagated PBMCs cocultured with HCV replicon-containing cells (Fig. 9). Thus, IFN-γ secreted by propagated PBMCs can inhibit HCV virion production probably by suppressing viral RNA and protein synthesis.

DISCUSSION

LAK cells for immunotherapy are conventionally generated after expansion in the presence of IL-2 for a relatively short culture period. The heterogeneous LAK cell population consists of nonmajor histocompatibility

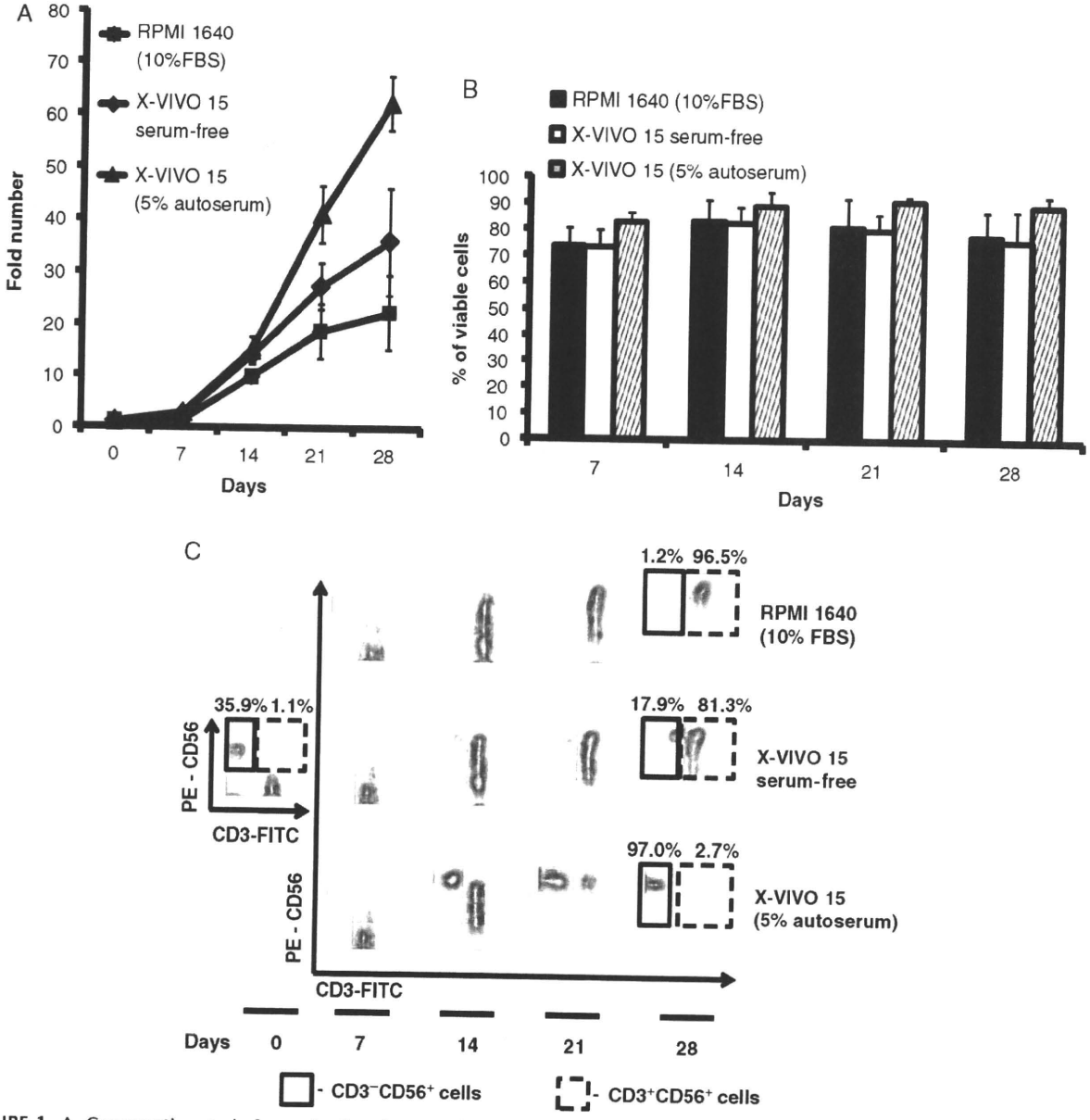

FIGURE 1. A, Comparative study for evaluating the capacity of various medium to propagate PBMCs. PBMCs obtained from healthy volunteers (n = 10) were cultured in X-VIVO 15 supplemented with/without 5% autoserum and RPMI 1640 supplemented with 10% FBS. All cultures were performed in the presence of interleukin-2 (IL-2; 1000 JRU/mL) and anti-CD3 monoclonal antibody (100 ng/mL) during 28 days. B, Viability of the PBMCs propagated in the various media. At 7-day culture, cell viability was assessed by using trypan blue dye exclusion test. The data represent the mean ± SEM (n = 10 individuals). C, Phenotypic characteristics of the PBMCs in various mediums. The representative flow cytometric profiles of the propagated PBMCs from a certain volunteer are shown. The PBMCs were stained with monoclonal antibodies against CD3 and CD56, and analyzed by flow cytometry on day 0 and during culture (on days 7, 14, 21, and 28). FBS indicates fetal bovine serum; PBMCs, peripheral blood mononuclear cells.

complex-restricted $CD3^-CD56^+$ and $CD3^+CD56^+$ cell subsets, both of which contribute to the cytolytic property of LAK cells.[22] The unique $CD3^+CD56^+$ cells are generally referred to as NK-like T cells, because, similar to NK cells, they do not require prior specific sensitization to induce recognition of target cells. Addition of anti-CD3 mAb at the initiation of culture, prolongation of culture duration, and addition of various stimuli at the end of culture are improved methodologies to culture LAK cells and reportedly result in better expansion over the original described method.[23] Such expanded LAK cells have clinically shown modest efficacy against metastatic renal cell carcinoma and melanoma.[24] In this study, X-VIVO 15 medium containing autoserum efficiently supported expansion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells. The $CD3^+CD56^+$ cells in propagated PBMCs did not express invariant T cell receptors, but express conventional T cell receptors-α/β in the preliminary experiments (data not shown), indicating that these cells are different from the invariant natural killer T cells. Further studies are required

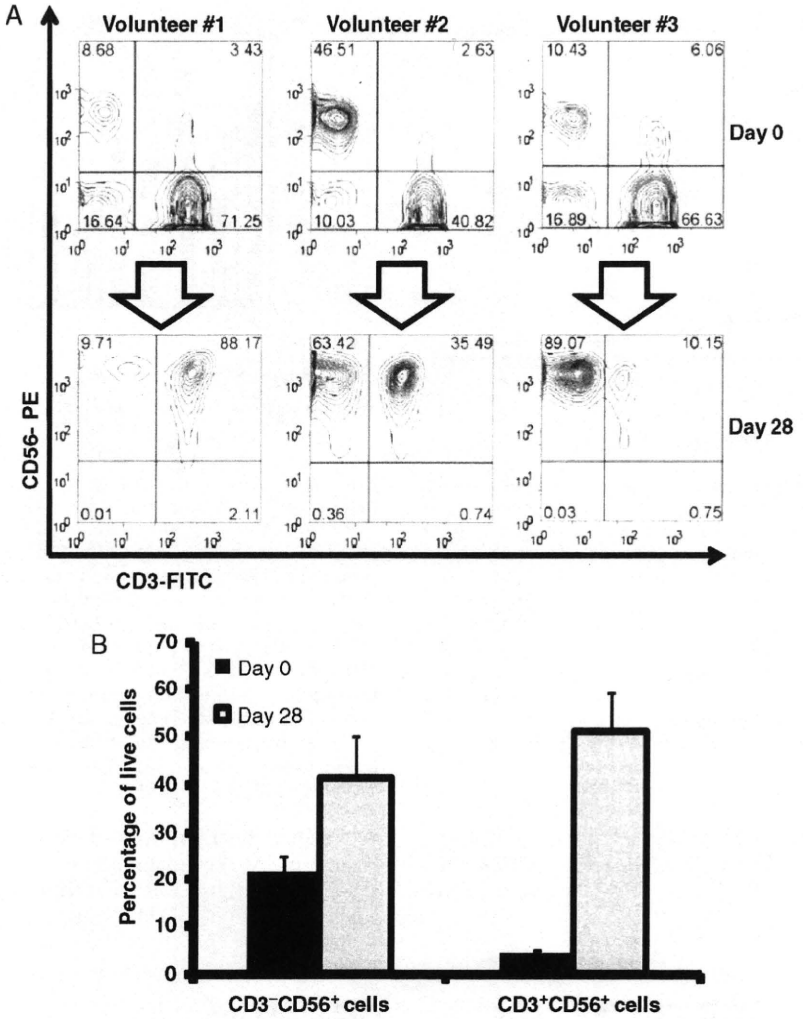

FIGURE 2. The proportion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells among the propagated PBMCs varied among individuals. A, Representative flow cytometric profiles of the propagated PBMCs from 3 different individuals are shown. The PBMCs were cultured in X-VIVO 15 medium supplemented with 5% autoserum in the presence of interleukin-2 and anti-CD3 monoclonal antibody. The harvested cells were stained with monoclonal antibodies against CD3 and CD56, and analyzed on day 0 and 28. B, The proportion of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells among the propagated PBMCs before (day 0) and after (day 28) culture. The data represent mean ± SEM (n = 15 individuals). PBMCs indicates peripheral blood mononuclear cells.

to define the phenotypic and functional properties of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells among the propagated PBMCs.

X-VIVO 15 medium is more efficient in proliferation $CD56^+$ cells than RPMI medium, particularly $CD3^-CD56^+$ cells. To address the possible mechanism underlying this phenomenon, we analyzed the amino acid and cytokine content of X-VIVO 15 and RPMI mediums. X-VIVO 15 contains higher concentrations of branched chain amino acids (valine, leucine, and isoleucine) than RPMI (data not shown). In addition, X-VIVO 15 contains higher concentrations of intercellular adhesion molecule 1 (ICAM)-1. As $CD56^+$ cells express the ICAM-1 ligand lymphocyte function-associated antigen 1 (LFA-1), ICAM-1 and LFA-1 binding might promote NK cell activation.

We recently showed that $CD3^-CD56^+$ and $CD3^+CD56^+$ cells can be extracted from the liver allograft perfusate during transplant surgery, and short culture with IL-2 and anti-CD3 mAb induces the anti-HCV activity and the anti-HCC activity of the NK and NK-like T cells.[9] Short-term (3 days) stimulation with IL-2 significantly upregulates the expression of TRAIL on liver NK cells, but this effect is barely observed on NK cells from PBMCs. Molecular cloning of TRAIL-receptors elucidated that TRAIL binds to at least 4 receptors: 2 are death-inducing receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5), containing cytoplasmic death domains and mediate signal apoptosis; the other 2 are death-inhibitory receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2), lacking a functional death domain and do not mediate apoptosis. However, all have similar affinities and the latter pair may act as decoys.[25,26]

NK cells can destroy many solid tissue-derived malignant cells, such as melanoma, breast cancer, lung cancer, gastric cancer, colon cancer, renal cancer, and ovarian cancer cell lines: this process is mediated primarily by death receptor-ligand interactions.[27] We have found that normal hepatocytes express TRAIL-DR4 and TRAIL-DR5 together with TRAIL-DcR1 and TRAIL-DcR2, but moderately or

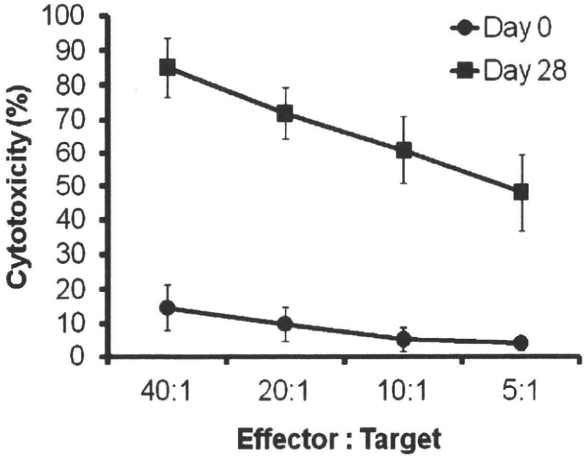

FIGURE 3. The propagated PBMCs showed vigorous cytotoxicity against hepatoma cells. Cytotoxic activities of freshly isolated PBMCs (day 0) and propagated PBMCs after 28 days culture (day 28) against HepG2 cells (a hepatoma cell line) were analyzed by ^{51}Cr release assay. The data represent the mean ± SEM of the percentage of target lysis at effector-target (E:T) ratios of 40:1, 20:1, 10:1, and 5:1 (n = 5 for the freshly isolated PBMCs and n = 10 for the propagated PBMCs). There was statistically significant difference in cytotoxicity against the hepatoma cells between the propagated PBMCs and the freshly isolated PBMCs ($P < 0.05$). The data of each person was determined as average of ^{51}Cr titer in triplicate wells. PBMCs indicates peripheral blood mononuclear cells.

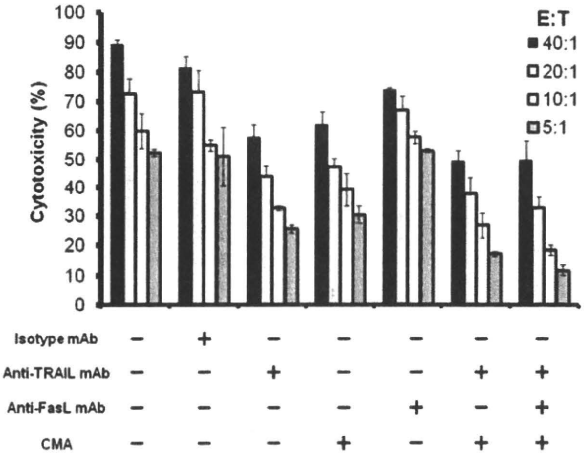

FIGURE 5. The cytotoxicity of the propagated PBMCs against hepatoma cells was partially inhibited by an anti-TRAIL mAb alone and more profoundly inhibited by the combination of the anti-TRAIL mAb and anti-FasL mAb together with CMA. The propagated PBMCs were used as effector cells in assays of cytotoxicity against HepG2 cells (hepatoma cell line) at effector-target (E:T) ratios of (40:1, 20:1, 10:1, and 5:1) in the presence or absence of anti-TRAIL (N2B2) mAb (10 μg/mL), FasL (MFL3) mAb (10 μg/mL), and/or CMA (50 nmol/L). The results shown are the mean ± SD of values from triplicate samples and are representative of 3 similar experiments. CMA indicates Concanamycin A; FasL, Fas ligand; mAb, monoclonal antibody; PBMCs, peripheral blood mononuclear cells; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

poorly differentiated HCCs highly express TRAIL-DR4 and TRAIL-DR5 but did not express TRAIL-DcR1 and TRAIL-DcR2, indicating a susceptibility to TRAIL-expressing NK cell-mediated activity toward HCC.[8] We now showed that long-term (28 d) cultivation in the presence of IL-2 and anti-CD3 mAb significantly upregulated the expression of TRAIL even on $CD56^+$ cells derived from PBMCs and

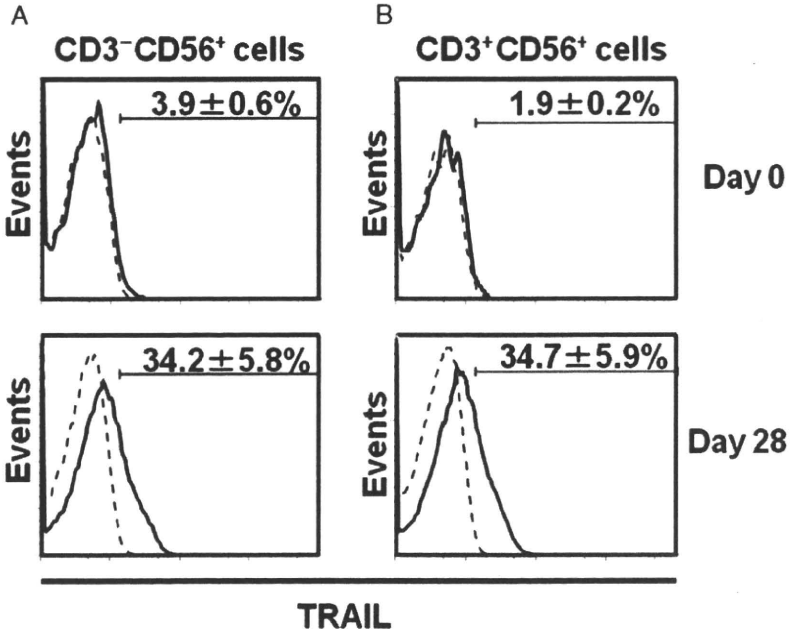

FIGURE 4. The propagated $CD3^-CD56^+$ and $CD3^+CD56^+$ cells expressed the TRAIL on their surface. The histograms represent the log fluorescence intensities obtained on staining for TRAIL after gating on the $CD3^-CD56^+$ (A) and the $CD3^+CD56^+$ (B) cells among freshly isolated PBMCs (day 0) and propagated PBMCs after 28-day culture (day 28). The dotted lines represent negative control staining with isotype-matched monoclonal antibodies. The numbers indicate the percentages of cells positive for TRAIL expression (mean ± SEM, n = 10 individuals). PBMCs indicates peripheral blood mononuclear cells; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.