TABLE 1. Effects of diet on donors

	Nondiet treated (N=87)		Diet treated (N=41)		
		P	Initial consultation	*P*	Postdiet
BMI (kg/m^2)	21.8±0.3	0.0163	23.3±0.6	< 0.0001	21.9±0.4
T. Bil (mg/dL)	0.9±0.0	0.2870	0.8±0.1	0.2556	0.8±0.1
D. Bil (mg/dL)	0.1±0.0	0.3256	0.1±0.1	0.2323	0.1±0.1
AST (IU/L)	18±1	0.0016	22±1	0.1042	20±1
ALT (IU/L)	18±1	0.0007	28±3	0.0128	21±1
γ-GTP (IU/L)	24±2	0.0003	41±6	0.0016	28±4
PT-INR	0.98±0.01	0.1006	0.96±0.01	0.0435	0.98±0.01
Alb (g/dL)	4.8±0.0	0.9389	4.8±0.1	0.0074	4.7±0.1
T-cho (mg/dL)	186±4	0.0002	213±6	0.0004	173±9
TG (mg/dL)	80±5	0.0021	110±9	0.6506	108±13

Continuous variables are expressed as means±standard error.

BMI, body mass index; T. Bil, total bilirubin; D. Bil, direct bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; PT-INR, prothrombin time-international normalized ratio; Alb, albumin; T-cho, total cholesterol; TG, triglyceride.

consultation in the outpatient clinic is examined for his or her potential as a donor after administering a diet treatment. Herein, we refer to these donors as "diet-treated donors." Few studies have analyzed the outcomes of LDLT using diet-treated donors with steatotic livers (*13*).

The aim of this study was to evaluate both safety of the donors and the outcomes of the recipients undergoing LDLT from diet-treated donors.

RESULTS

Effects of Diet on Donors

A total of 87 donors did not receive diet treatment (nondiet-treated donors), and 41 donors were treated with a diet (diet-treated donors). The mean body mass index (BMI) and the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ-GTP), total cholesterol (T-cho), and triglyceride (TG) were significantly higher in diet-treated donors at the initial consultation than in nondiet-treated donors. After the diet, BMI was significantly reduced from 23.3±0.6 to 21.9±0.4 kg/m^2 (P<0.0001) for a median period of 2.9 (range, 0.2–13.6) months, which was limited by the critical status of the recipients. Factors associated with hepatic steatosis, including ALT, γ-GTP, and T-cho levels, also improved with the diet treatment (P=0.0128, 0.0016, and 0.0004, respectively), whereas the level of albumin decreased significantly (P=0.0074) (Table 1).

The results of the preoperative liver biopsy are presented in Table 2. In most of the diet-treated donors, a liver biopsy performed after the diet showed stage 0/1 fibrosis and minimal or mild steatosis. One diet-treated donor had stage 2 perisinusoidal/pericellular fibrosis and a minimal grade of macrovesicular steatosis. No complications associated with liver biopsy were reported.

Preoperative Characteristics of Donors and Recipients

The diet-treated donors were significantly older than the nondiet-treated donors (40.2±1.6 years vs. 35.5±1.4 years, P=0.0484). The mean values of BMI, total bilirubin (T. Bil), AST, ALT, and prothrombin time-international normalized ratio (PT-INR) of the donors measured just before the operation were comparable between the two groups. Although the model for end-stage liver disease (MELD) score was not significantly different between the two groups, it was likely to be higher in the recipients of grafts from nondiet-treated donors than in those of grafts from diet-treated donors (18.1±0.9 vs. 15.2±1.1, P=0.0552) (Table 3). In those of grafts from diet-treated donors, mean MELD score was increased from 13.3 to 15.2 during the diet period.

Surgical Demographics of Donors and Recipients

There were no significant differences between the two groups with respect to graft type and surgical data of donors and recipients, including operative time, blood loss, blood transfusions, graft-to-recipient weight ratio, and cold ischemic time (Table 4).

Donor Postoperative Data

There were no significant differences in perioperative laboratory data on T. Bil, AST, and ALT. Just PT-INRs on postoperative days 1, 2, and 3 were significantly higher in nondiet-treated donors than in diet-treated donors. However, there were no significant differences after postoperative day 5 (Fig. 1). Perioperative complications categorized according to the Clavien's grading system (*14*) showed no

TABLE 2. Results of the liver biopsy

	Stage				
Grade	0	1	2	3	4
Minimal	9	29	1	0	0
Mild	0	2	0	0	0
Moderate	0	0	0	0	0
Severe	0	0	0	0	0

Minimal, ≤10%; mild, 11%–20%; moderate, 21%–30%; severe, >30%.

TABLE 3. Preoperative demographics of donors and recipients

	Nondiet treated (N=87)	Diet treated (N=41)	*p*
Donor			
Age	35.5±1.4	40.2±1.6	0.0484
Gender			
Male	52	27	0.5088
Female	35	14	
Body weight (kg)	59.5±1.1	60.1±1.4	0.7191
BMI (kg/m^2)	21.8±0.3	21.9±0.4	0.7657
Liver function test			
T. Bil (mg/dL)	0.9±0.0	0.8±0.1	0.6782
AST (IU/L)	18±1	20±1	0.1212
ALT (IU/L)	18±1	21±1	0.1088
PT-INR	0.98±0.01	0.98±0.01	0.6924
Relation to the recipient			
Child	50	22	0.2146
Spouse	14	11	
Sibling	11	7	
Parent	8	1	
Others (son in law, niece, and nephew)	4	0	
Recipient			
Age	52.5±1.1	52.8±1.5	0.8715
Gender			
Male	54	26	0.8833
Female	33	15	
Body weight (kg)	63.5±1.3	64.3±2.0	0.7253
Indications			
HCC	37	23	
LC due to HCV	17	5	
FHF	7	1	
LC due to alcohol abuse	4	3	
LC due to HBV	4	2	
Secondary biliary cirrhosis	4	0	
PBC	3	3	
PSC	3	0	
AIH	3	1	
Wilson disease	1	1	
Liver failure posthepatectomy	1	1	
NASH	1	0	
Metastatic liver tumor (insulinoma)	1	0	
Retransplantation	1	0	
Budd-chiari syndrome	0	1	
MELD score	18.1±0.9	15.2±1.1	0.0552
ABO incompatibility			
Identical/compatible	80	35	0.2496
Incompatible	7	6	

Continuous variables are expressed as means±standard error.

BMI, body mass index; T. Bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT-INR, prothrombin time-international normalized ratio; HCC, hepatocellular carcinoma; LC, liver cirrhosis; HCV, hepatitis C virus; FHF, fulminant hepatic failure; HBV, hepatitis B virus; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis; NASH, nonalcoholic steatohepatitis; MELD, Model for End-Stage Liver Disease.

significant differences between the two groups. Perioperative complications in patients with a Clavien grade III or higher included an intraabdominal hematoma in one nondiet-treated donor, biliary leakages in two nondiet-treated donors, and a biliary stenosis in one diet-treated donor. For the right lobe graft, liver regeneration rates on postoperative day 7 were 1.41±0.03 in nondiet-treated donors and 1.44±0.04 in diet-treated donors (P=0.574). For the remaining grafts,

liver regeneration rates were also comparable between the two groups.

Overall Survival in Recipients

There were no significant differences in overall survival between recipients of grafts from nondiet-treated and diet-treated donors. The 1-, 3-, and 5-year survival rates were 79%, 74%, and 70% for recipients of grafts from nondiet-treated donors, whereas the corresponding values were 68%, 68%, and 68% for recipients of grafts from diet-treated donors, respectively (P=0.455).

Biliary Complications in Recipients

Biliary complications in the recipients, including stricture, leakage, and stricture after leakage, showed no statistically significant differences between nondiet-treated and diet-treated donors. The number of patients with biliary diversion was also comparable (data not shown).

DISCUSSION

The condition of both donors and recipients is a critical issue in LDLT. Although safety of donors should be of the highest priority (*15*, *16*), there is considerable controversy with respect to that of extended criteria donors. In particular, it has not been well elucidated if fatty liver affects the donor safety, whereas steatotic liver grafts have been well analyzed and there is still controversy regarding the outcome of recipients (*4–6*).

The incidence of obesity has increased dramatically in developed countries in the last few decades. There has also been a simultaneous rise in the frequency of metabolic syndrome. Nonalcoholic fatty liver disease is characterized by an elevated intrahepatic TG content, with varying degrees of inflammation and fibrosis. A clear differentiation between a simple fatty liver and nonalcoholic fatty liver disease is difficult in the absence of liver biopsy results. Macrovesicular steatosis can lead to inflammation and fibrosis, and the likelihood of graft damage in recipients of a liver graft from a donor with macrovesicular steatosis is high (*17*, *18*). Therefore, the criteria of fatty liver were widened in this study. They were based only on the imaging studies including computed tomography (CT) and/or ultrasound. Oliva et al. (*19*) reported that liver-spleen ratio of less than 1.2 covered all the cases with fatty liver, whereas some authors underlined 1.0 or 1.1 as the cutoff line for fatty liver (*20*, *21*). The authors followed the criteria of Oliva et al. Ruhl and Everhart (*22*) reported that the proportion of elevated ALT activity due to excess weight and obesity (BMI>25 kg/m^2) was 65%. Rinella et al. (*23*) reported a significant correlation between BMI and the degree of macrovesicular steatosis and found that patients with a BMI of less than 25 kg/m^2 did not show macrovesicular steatosis. Moreover, Peng et al. (*24*) reported that patients with a BMI of less than 23 kg/m^2 were likely to display no or mild steatosis. Consequently, the target BMI value in this study was set to 22 kg/m^2. In this study, the results of liver function tests related to hepatic steatosis were significantly improved after the diet treatment. In addition, the histopathological results of the liver biopsies performed after the diet treatment showed less than 20% of macrovesicular steatosis. The main objective of the liver biopsy is to ensure donor safety, which is considered more important than the preservation of graft function (*25*).

TABLE 4. Surgical demographics of donors and recipients

	Nondiet treated (N=87)	Diet treated (N=41)	p
Donor			
Graft type			
Right lobe without MHV	68	29	0.4509
Left lobe with MHV	17	10	
Left lobe without MHV	1	0	
Posterior section	1	2	
Operative time (min)	408±7	409±10	0.9253
Blood loss (mL)	227±15	241±35	0.6772
Allogenic blood transfusion	0	0	
Autologous blood transfusion			
Yes	6	5	0.3183
No	81	36	
Recipient			
Operative time (min)	725±14	752±17	0.2417
Blood loss (mL)	4153±272	4566±612	0.4755
PRBC (U)	7.6±0.8	8.4±1.6	0.6323
FFP (U)	6.6±0.9	5.7±1.2	0.5765
GW (g)	581±14	609±26	0.3109
GRWR (%)	0.94±0.02	0.96±0.05	0.6632
CIT (min)	98±4	100±5	0.7346

Continuous variables are expressed as means±standard error.

MHV, middle hepatic vein; PRBC, packed red blood cell; FFP, fresh-frozen plasma; GW, graft weight; GRWR, graft-to-recipient weight ratio; CIT, cold ischemic time.

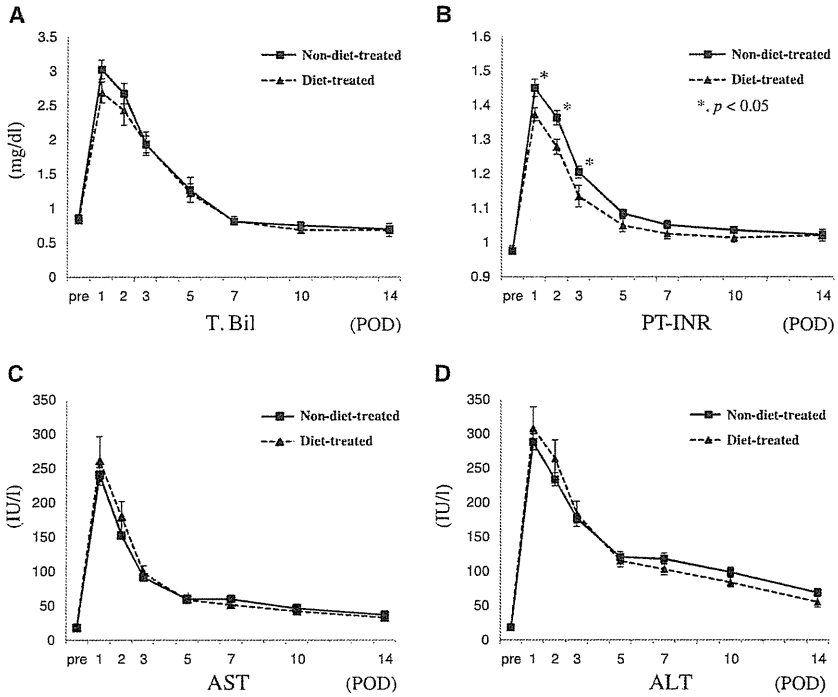

FIGURE 1. Perioperative data on donors. (A) T. Bil. (B) PT-INR. (C) AST. (D) ALT. T. Bil, total bilirubin; PT-INR, prothrombin time-international normalized ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Nakamuta et al. (*10*) reported the effectiveness of a short-term intensive treatment protocol for donors with steatosis. However, the donors in that study were subjected to two invasive liver biopsies. Although a liver biopsy performed before the start of a treatment can be useful to assess the effects of the treatment, it is not necessary for the final decision of inclusion of the donor. In this study, donors were treated with a diet with the target of achieving a BMI of 22 kg/m^2. They were subjected to only one biopsy with the exception of one donor who did not meet the diet goal. In addition, only one candidate was excluded for the safety because repeated liver biopsy revealed the findings of inflammation. The numbers of diet-treated donors in this study are much larger than those reported by Nakamuta et al. The most appropriate method and diet period to ensure successful LDLT are yet to be determined.

With respect to safety of donors, moderate or severe macrovesicular steatosis is generally considered among the exclusion criteria to prevent complications (*25–27*). Consistent with this strategy, postoperative laboratory data, including T. Bil, AST, and ALT levels, and perioperative complications graded according to Clavien's scale were comparable between the nondiet-treated and diet-treated groups. Only PT-INRs on postoperative days 1, 2, and 3 were significantly higher in the nondiet-treated donors than in the diet-treated donors. Although we cannot provide a clear explanation for this difference, an association between the condition of the liver after diet and coagulation disorders is suspected and should be investigated.

The relationship between macrovesicular steatosis and remnant liver regeneration after hepatectomy remains unclear (*27–30*). The present data indicate that steatosis up to mild macrovesicular infiltration does not impair liver regeneration after hepatectomy.

To summarize the results of donors, diet-treated donors are going well, compared with nondiet-treated donor. However, attention should be paid continuously that donor mortality can occur in the high-risk donor candidate.

Although Hayashi et al. (*13*) reported successful results in recipients of grafts from five diet-treated donors, they did not compare the outcome of recipients of grafts from diet-treated donors with that of recipients of grafts from nondiet-treated donors. In the present series, there were no significant differences in overall survival between the two groups, although survival in the nondiet-treated group was slightly better than that in the diet-treated group. Factors including donor age, preoperative MELD score in the recipients, ABO incompatibility, and other factors might affect the overall survival of the recipients. The limited size of the group included in this study makes it difficult to draw firm conclusions with respect to the impact of the use of diet-treated donors on overall survival.

Biliary complications are still considered the Achilles' heel of liver transplantation. Baccarani et al. (*31*) reported that a steatotic graft with more than 25% of macrovesicular infiltration is a risk factor for the development of biliary complications. In our series, there were no significant differences in biliary complications between the two groups, which could be attributed to the strict selection criteria, thus emphasizing that liver biopsy results after the diet treatment should show less than 20% of macrovesicular steatosis with minimal perisinusoidal fibrosis.

In conclusion, the use of diet-treated donors is feasible with respect to safety of the donor and the outcome of the recipient in LDLT when strict selection criteria are used.

MATERIALS AND METHODS

Study Population and Criteria for Diet-Treated Donors

A total of 316 donor candidates came to the initial consultation between April 2003 and March 2010. Of them, 55 candidates were diagnosed as fatty liver on the basis of the results of imaging studies. Hepatic fat deposition was assessed by CT, in which a liver-spleen ratio of less than 1.2 was defined as steatosis (*19, 21*) and/or ultrasonography for the analysis of liver-kidney contrast by an expert hepatologist (*20, 21*). Nine had other suitable candidates, and three were excluded due to diabetes mellitus. One candidate refused the diet program. Finally, 42 candidates received the diet treatment that was an 800 to 1400 kcal/day diet combined with a 100 to 400 kcal/day exercise without drug treatment, targeting a BMI of 22 kg/m^2 for 6 months in the outpatient clinic (*10, 20, 32, 33*). Laboratory data in this group showed the abnormally high level of at least one of the following: ALT, γ-GTP, T-cho, and TG. After these 42 candidates were treated with a diet, all of them underwent a liver biopsy. Candidates showing the absence of moderate/severe steatosis or nonalcoholic steatohepatitis in the liver biopsy specimen and who showed normal liver function and no hyperlipidemia were designated as diet-treated donors. While one donor needed a second liver biopsy after an extended diet-treatment period because the initial biopsy yielded an unsatisfactory result, only one case was excluded with the microscopic findings of inflammation with repeated liver biopsies. The remaining candidates were grouped as nondiet-treated donors. Eighty-seven nondiet-treated donors were compared with 41 diet-treated donors as a control. This study was approved by the Institutional Review Board of Hiroshima University.

Histopathological Evaluation

All liver biopsy specimens were examined by an experienced pathologist. Specimens were categorized by the degree of fibrosis according to Brunt's staging system (*34*) and the degree of macrovesicular steatosis according to the following subgroups: minimal (≤10%), mild (11%–20%), moderate (21%–30%), and severe (>30%) (*5*). Histopathological selection criteria for living donors included a graft with minimal to mild macrovesicular steatosis and/or below grade 2 fibrosis.

Donor Assessment and Surgical Procedure

The selection criteria for donors, including laboratory data and imaging studies, the surgical procedure, and the postoperative management for donor hepatectomy have been described elsewhere (*35*). Recipient surgery has also been described previously (*36*).

Donor Perioperative Complications

Perioperative complications among donors were evaluated using a modified Clavien's grading system (*14*).

Donor Liver Regeneration Rates

Prospective donors were subjected to routine CT on postoperative day 7 after May 2007 to evaluate the remnant liver volume, portal thrombosis, intraabdominal fluid collection, and intrahepatic biliary tract. Of the 128 donors enrolled in this study, 78 underwent CT on postoperative day 7. Regeneration was estimated by calculating the ratio of the actual liver volume at this time point to the original liver volume before the transection. Liver regeneration rate was separately analyzed for right lobe grafts and left lobe/posterior section grafts.

Immunosuppression

Patients were treated with a triple immunosuppression regimen including cyclosporine or tacrolimus in combination with or without steroids and mycophenolate mofetil as described previously (*36*). Methyl prednisolone (1 g/day) was intravenously administered for 3 consecutive days (one or two courses) to treat histologically proven or mixed lymphocytic reaction-proven acute cellular rejection (*37*).

Statistical Analysis

Continuous variables were compared using a paired *t* test, unpaired *t* test, and two-way repeated measures analysis of variance. Categorical variables were compared using the χ^2 test or Fisher's exact test. Survival analysis was performed using the Kaplan-Meier method, and groups were compared with the log-rank test. No patient was lost to follow-up, which was censored at the end of July 2010. Statistical analyses were performed using IBM SPSS Statistics 19 (SPSS Inc., an IBM Company, Chicago, IL). *P* value less than 0.05 was considered statistically significant.

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Hepatology Research

Hepatology Research 2012 doi: 10.1111/j.1872-034X.2012.01059.x

Original Article

Surgical treatment for portosystemic encephalopathy in patients with liver cirrhosis: Occlusion of portosystemic shunt in combination with splenectomy

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Aim: Operative ligation of the portosystemic shunt may control hepatic encephalopathy effectively, but the subsequent increase in portal vein pressure (PVP) leads to high mortality. Splenectomy can decrease inflow into the portal system, resulting in decreased portal pressure.

Methods: We retrospectively examined the effect of splenectomy in combination with shunt closure on portosystemic encephalopathy.

Results: Clinical symptoms of encephalopathy disappeared in all six patients who underwent splenectomy in combination with portosystemic shunt ligation, with the exception of one patient who had relapsing encephalopathy after 6 months. Follow-up computed tomography showed complete obliteration of the portosystemic shunts, except in the one patient with relapsing encephalopathy who underwent balloon-occluded retrograde transvenous obliteration for the remaining splenorenal shunt 8 months after surgery. PVP significantly decreased after splenectomy. PVP did not increase to the baseline PVP value after ligation of the shunts, except in two patients who had elevated PVP after surgery: PVP increased from 18 to 19 mmHg after ligation in one patient and from 18 to 23 mmHg in one patient.

Conclusion: Splenectomy followed by surgical ligation of the portosystemic shunt may be feasible and safe for cirrhotic patients with portosystemic shunts.

Key words: hepatic encephalopathy, portosystemic shunt, splenectomy, surgical ligation

INTRODUCTION

IT IS KNOWN that there are two types of encephalopathy related to liver cirrhosis: portosystemic encephalopathy and end-stage hepatic encephalopathy in severe liver dysfunction. The portosystemic shunt involves blood flow from mainly the supramesenteric vein to the systemic vein, and results in high systemic blood ammonia levels. For hepatic encephalopathy caused by a portosystemic shunt, surgical or interventional radiological closure of the shunt has been reported. Interventional radiology (IVR) represented by balloon-occluded retrograde transvenous obliteration (B-RTO) has been developed as a new therapy for portosystemic encephalopathy.[1–3] Improvement of liver function due to increased portal venous blood flow after B-RTO for gastric varices has been reported.[4,5] However, B-RTO is not expected to provide long-term effects for portosystemic encephalopathy, and it is not necessarily indicated for portosystemic encephalopathy.[6–9] Radiological occlusion of portosystemic shunts is frequently accompanied by ascites or bleeding from collateral vessels due to increased portal vein pressure (PVP).[1]

Operative ligation of the shunt may control encephalopathy effectively, but the formation and rupture of esophageal varices that develop due to the subsequent increase in PVP are associated with high mortality.[10] Simple ligation of the shunt alone is not adopted presently in clinical settings. Splenectomy has been performed as a part of Hassab's operation, or esophageal

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Conflict of interest: The authors have no commercial associations (e.g. consultancies, stock ownership, equity interest or patent/licensing arrangements) that might pose a conflict of interest in connection with the submitted manuscript.

Received 7 March 2012; revision 17 May 2012; accepted 30 May 2012.

transection, to control variceal hemorrhage.[11] Moreover, splenectomy results in decreased portal pressure[12,13] and reversal of hypersplenism,[14] and it has been concurrently performed for patients with small-for-size (SFS) liver grafts in the setting of living-donor liver transplantation (LDLT).[15–17] Therefore, splenectomy in combination with closure of the shunt may efficiently obliterate the portosystemic shunt without increasing PVP.

The aim of the current study is to investigate the feasibility and safety of splenectomy in combination with closure of the shunt for portosystemic encephalopathy in patients with liver cirrhosis.

METHODS

Patients

BETWEEN JANUARY 2003 and September 2011, 60 patients with portal hypertension related to liver disease underwent splenectomy at Hiroshima University Hospital. Among them, six patients underwent splenectomy in combination with closure of portosystemic shunts for hepatic encephalopathy. Table 1 lists the clinical characteristics of the patients. The median age was 62 years (range, 55–73). The cause of liver disease was chronic hepatitis C virus infection in four patients, alcohol abuse in one patient and chronic hepatitis B virus infection in one patient. The Child–Pugh score was 8 in two patients, 9 in three patients and 10 in one patient. Esophageal varices, which were found in four patients, were classified as F2 in one patient and F1 in three patients according to the endoscopic criteria of the Japan Society for Portal Hypertension.[18] According to the classification of consciousness disorders of the Japan Society for Portal Hypertension,[19] four patients had episodes of grade IV encephalopathy (coma), and two of six patients had shown grade II encephalopathy for the last 12 months. In all cases, large portosystemic shunts were confirmed by dynamic computed tomography (CT). One patient had a large left gastric azygos vein and para-umbilical vein shunts, and five patients had large splenic renal shunts. All patients had large spleens. The indications for surgery were as follows: IVR had been performed without success in three cases, and shunt occlusion by IVR was considered difficult in three cases because of huge vessels.

Surgical procedure

Six patients underwent splenectomy followed by closure of portosystemic shunts. During surgery, a midline incision or inverted "L" incision was used, and

Table 1 Patients' characteristics and results

Patient no.	Age	Sex	Etiology	Child–Pugh score	Liver biopsy	Portosystemic shunt	Follow up (months)	Recurrence of encephalopathy	Status	Comment
1	62	F	HCV	8	F3	Splenorenal	36	–	Alive and well	
2	63	M	HCV	8	F4	Splenorenal	30	–	Alive and well	Tx of HCC
3	73	M	HCV	9	F4	Splenorenal	23	–	Alive and well	
4	55	M	Alcohol	10	F4	Splenorenal	22	6 months	Alive and well	B-RTO after surgery
5	62	F	HBV	9	F3	Splenorenal	19	–	Alive and well	
6	62	F	HCV	9	F4	Left gastric azygos and para-umbilical vein	6	–	Alive and well	

B-RTO, balloon-occluded retrograde transvenous obliteration; F3, chronic hepatitis; F4, cirrhosis, HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Tx, treatment.

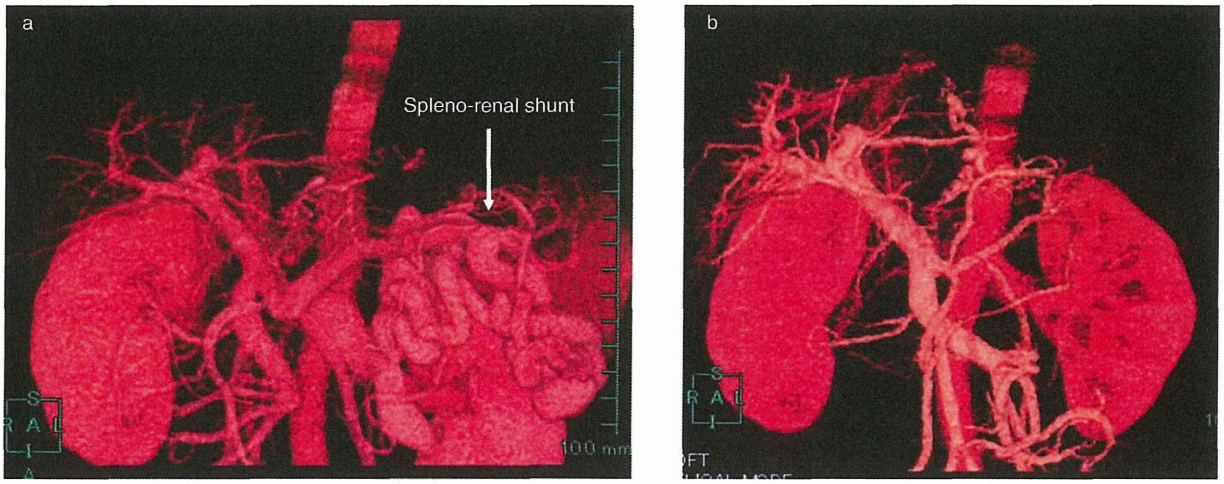

Figure 1 Three-dimensional computed tomography (CT) of the portal vein in case 1. (a) CT before surgery showing huge splenorenal shunts. (b) CT after surgery showing disappearance of a splenorenal shunt.

an antithrombotic catheter was inserted via the jejunal vein immediately after laparotomy. The top of the catheter was positioned in the portal vein. A transducer was used to measure the PVP during surgery, and the catheter was removed before the abdominal operative wound was closed. Splenectomy was performed with ligation and division of the vessels at the splenic hilum. Clamp tests were performed on portosystemic shunts before ligation of the shunts, and portosystemic shunts were ligated if PVP was less than the PVP measured immediately after laparotomy (baseline PVP) or if there was a less than 50% increase in the baseline PVP measured at the clamping test of shunt vessels. Liver biopsy was performed before the abdomen was closed. For follow up, CT was performed preoperatively and at 1 week and 1 and 6 months after surgery, or when indicated clinically. Serum ammonia levels were measured monthly.

Statistical analysis

Student's paired *t*-test was used for comparison of perioperative laboratory data. *P*-values less than 0.05 were considered significant. Statistical analyses were performed using SPSS ver. 16.0 software (SPSS, Chicago, IL, USA).

RESULTS

CLINICAL SYMPTOMS OF encephalopathy disappeared in all six patients within 5 days after surgery. Furthermore, all patients were free from encephalopathy during the median follow up of 23 months (range, 6–36), with the exception of one patient (case 4) who had relapsing encephalopathy and re-elevation of the serum ammonia level after surgery. He underwent B-RTO for the remaining splenorenal shunt, and was alive without encephalopathy at the time of writing this manuscript. The follow-up CT showed complete obliteration of the portosystemic shunts in all patients except the single patient (case 4) who had relapsing encephalopathy (Fig. 1). PVP significantly decreased after splenectomy in all six cases (Fig. 2). Although PVP increased after ligation of the shunts, it increased to the baseline PVP or less in cases 1, 3, 5 and 6 and was only 1 mmHg higher than the baseline PVP in case 2. In case 4, the baseline PVP was 18 mmHg; PVP decreased to

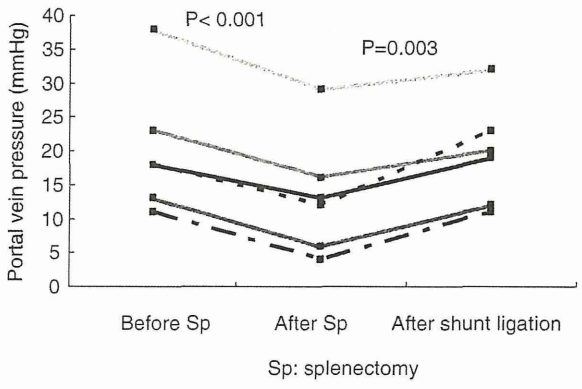

Figure 2 Changes in portal vein pressure during surgery.

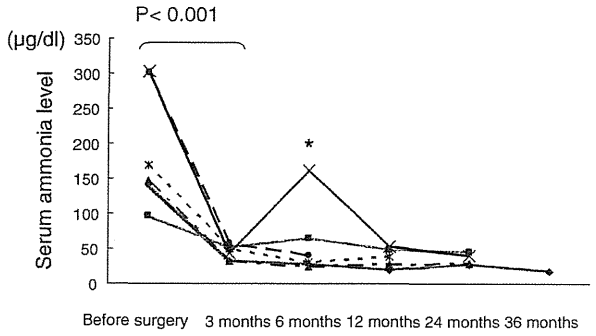

Figure 3 Changes in the serum ammonia level. *One patient (case 4) with relapsing encephalopathy underwent balloon-occluded retrograde transvenous obliteration for the remaining splenorenal shunt 8 months after surgery.

12 mmHg after splenectomy but increased to 29 mmHg at the clamping test of all splenorenal shunts. Thus, several peripheral splenorenal vessels were ligated, and the PVP measured before closing the abdomen eventually increased to 23 mmHg (Fig. 2). The maximum serum ammonia level significantly decreased 3 months after surgery compared with the level before the surgery (Fig. 3). The diameter of the portal vein trunk significantly increased at 1 month after surgery (Fig. 4). Hematological tests conducted before and 3 months after the operation revealed a significant increase in platelet count, from 7.1 ± 0.7 to $20.5 \pm 2.1 \times 10^3/\mu L$ ($P < 0.001$). Examination of liver biopsy specimens showed that four patients had liver cirrhosis and two patients had chronic hepatitis

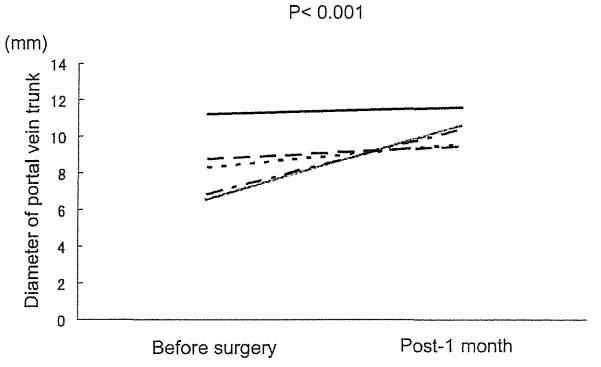

Figure 4 Changes in the diameter of the portal vein trunk before and after surgery. One patient (case 6) with large left gastric azygos vein and para-umbilical vein shunts has been excluded, because the portal flow of the left portal vein decreased due to ligation of the para-umbilical vein.

(Table 1). With regard to the operative characteristics of six patients, the mean operative time was 273 min (range, 180–284), and the mean operative blood loss was 450 mL (range, 180–920). Five patients did not receive operative or perioperative transfusion, whereas one patient received operative transfusion including 4 units of red cell concentrate and 10 units of fresh frozen plasma because of preoperative anemia (hemoglobin level, 7.6 g/dL) and operative blood loss (920 mL with ascites).

Major complications such as development/enlargement of esophagogastric varices were not seen after surgery. However, minor postoperative complications associated with surgery developed in all six patients: three patients developed transient ascites, which was controlled by diuretics medications, and four patients developed splenic vein thrombosis, which was treated by the administration of antithrombin III and warfarin.

DISCUSSION

CHRONIC RECURRENT HEPATIC encephalopathy is often associated with portosystemic shunts in patients with cirrhosis. Encephalopathy of this type is usually treated with lactulose and an oral branched-chain amino acid supplement. In general, IVR including B-RTO may effectively treat portosystemic encephalopathy that is intractable to pharmacotherapy. However, the IVR procedure and the preventive effects of IVR are occasionally limited in a population of patients with portosystemic shunt, because it is not technically feasible to insert numerous coils safely into huge portosystemic shunts.[3] The occlusion of a huge para-umbilical vein shunt is considered difficult by IVR.[8] Percutaneous transhepatic obliteration has the risk of migration of sclerosing agents to the systemic circulation. Several studies have reported poor long-term effects of IVR for portosystemic encephalopathy. Kato *et al.* reported that encephalopathy relapsed in four of six patients who underwent B-RTO for portosystemic encephalopathy between 6 and 30 months after the procedure.[7] Zidi *et al.* showed that long-term improvement was obtained in only one of seven patients who underwent shunt embolization.[6]

Shunt embolization by IVR as well as surgical ligation leads to the subsequent increase in PVP, which may worsen esophageal gastric varices and result in the formation of new portosystemic shunts.[20] Sakurabayashi *et al.* showed that the PVP of two patients with complete shunt occlusion significantly increased from 110 to

220 mmH_2O after shunt embolization.[3] Meanwhile, Yoshida *et al.* presented the benefits of portosystemic shunt obliteration followed by partial splenic embolization (PSE).[21,22] In these studies, PVP tended to increase without significance after obliteration of shunts combined with PSE by IVR, while PVP significantly increased after obliteration of shunts without PSE by IVR.[21] They concluded that PSE can reduce the PVP to a level similar to the PVP before the obliteration of portosystemic shunts and that a new portosystemic shunt is unlikely to develop at lower PVP.

Splenectomy can decrease inflow into the portal system, resulting in a decreased portal pressure.[12,13] In the current study, the PVP decreased after splenectomy, which is consistent with the findings of previous reports. In LDLT settings, the problems of SFS syndrome have become evident, an increased rate of graft loss.[23,24] Although the pathogenesis of SFS graft syndrome seems to be multifocal, an increased sinusoidal pressure in a graft is thought to be the major determining factor. Shimada *et al.* showed that splenectomy decreased portal pressure and improved the outcome of LDLT.[12] Although the mechanism by which splenectomy improves the liver function is unclear, the improved liver function might be associated with a decrease in the PVP after splenectomy. On the other hand, splenectomy may cause the decrease in portal vein flow and rather leads to liver dysfunction. In the current study, portal flow had been partially stolen via the large portosystemic shunts before ligation of the shunts. However, after ligating the portosystemic shunts, the portal flow to the liver increased, as shown in Figure 4, which showed that the diameter of the portal vein trunk increased as measured by CT.

In the current study, splenectomy followed by closure of the portosystemic shunt did not result in an elevation in PVP after portosystemic shunt ligation in all but two patients. Futagawa *et al.* suggested that risk factors for developing liver failure are severity of cirrhosis and a 60% or higher increase in baseline PVP after surgical occlusion of portosystemic shunts.[25] In the current study, we intended to ligate the portosystemic shunts with a less than 50% increase in baseline PVP after surgical occlusion of portosystemic shunts following splenectomy. In fact, 5-mmHg increases in PVP after ligation (increase of ~30% in baseline PVP) were eventually observed in case 4. We did not observe the development of esophageal variceal rupture or postoperative failure, irrespective of transient ascites, in our six cases. Even in the four patients in whom liver cirrhosis was revealed by biopsy, there were no episodes of postoperative liver failure or esophageal variceal rupture. Thus, surgical ligation of the portosystemic shunt following splenectomy may be feasible and safe, as long as the PVP at the clamping test of shunt vessels is not greater than 50% increase in baseline PVP. If clamp test of portosystemic shunts shows that PVP is more than 50% increase of the baseline PVP, some peripheral shunts could be ligated with a less than 50% increase in base line PVP as shown in case 4.

Liver function was classified as Child B in five out of six cases, and the increase in PVP after splenectomy followed by shunt ligation was mild (maximum, 5 mmHg). At present, the threshold at which increase in PVP after portosystemic shunt ligation increases the risk of esophageal variceal bleeding or the development of postoperative hepatic failure is unknown. Because this is a preliminary study, further examination is required to establish the indication, feasibility and effectiveness of this surgical ligation of the portosystemic shunt in combination with splenectomy for patients with hepatic encephalopathy. Furthermore, we should investigate if splenectomy is necessary in patients with normal PVP at the clamp test of portosystemic shunts.

In conclusion, splenectomy followed by surgical ligation of the portosystemic shunt may be feasible and safe for cirrhotic patients with portosystemic shunts who maintain relatively good liver function.

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DOI: 10.3727/096368911X627589

CT-0351Accepted 07/19/2011 for publication in "*Cell Transplantation*"**Clinical-scale isolation of interleukin-2-stimulated liver natural killer cells for treatment of liver transplantation with hepatocellular carcinoma**

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Running title: Clinical-scale natural killer cell therapy

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ABSTRACT

Tumor recurrence is the main limitation of liver transplantation (LT) in patients with hepatocellular carcinoma (HCC) and can be promoted by immunosuppressants. However, there is no prevention or treatment for HCC recurrence after LT. Here, we describe a clinical-scale method for an adoptive immunotherapy approach that uses natural killer (NK) cells derived from deceased donor liver graft perfusate to prevent tumor recurrence after LT. Liver mononuclear cells (LMNCs) that were extracted from deceased donor liver graft perfusate contained a high percentage of NK cells (45.0% ± 4.0%) compared with peripheral blood mononuclear cells (PBMCs) (21.8% ± 5.2%) from the same donor. The CD69 activation marker and the natural cytotoxicity receptors, NKp44 and NKp46, were expressed at high levels in freshly isolated liver NK cells. Furthermore, interleukin (IL)-2-stimulated NK cells showed greater upregulation of activation markers and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is critical for NK cell-mediated antitumor cell death and increased production of interferon. Moreover, IL-2 stimulation induced LMNCs to exhibit a strong cytotoxicity against NK-susceptible K562 target cells compared with PBMCs ($p < 0.01$). Finally, we also showed that the final product contained a very low T cell contamination (0.02×10^6 cells·kg^{-1}), which reduces the risk of graft-versus-host disease (GVHD). Collectively, our results suggest that the adoptive transfer of IL-2-stimulated NK cells from deceased donor liver graft perfusate could be a promising treatment for LT patients with HCC.

Keywords: natural killer cell, immunotherapy, innate immunity, hepatocellular carcinoma, current good manufacturing practice (cGMP),

Hepatocellular carcinoma (HCC) is one of the most common reasons for liver transplantation (LT). In the past decade, the number of LT for patients with HCC has increased since the Milan criteria for HCC have been used for organ allocation in the United States (16,23). However, the rate of recurrence of HCC after LT is 10–20% (21,32). This recurrence remains the most serious issue for LT in patients with HCC. The necessity of using postoperative immunosuppressants in the transplant recipient poses an additional risk for recurrence and hinders the use of cytotoxic chemotherapy drugs (14,23,41,46). However, there is no definitive treatment or prevention for the recurrence of HCC after LT (35,48). Hence, alternative therapies are needed for immunosuppressed HCC patients.

Natural killer (NK) cells are the major components of innate immunity and the first line of defense against invading infectious microbes and neoplastic cells (38). Functional impairment and decreased numbers of NK cells have been identified in HCC or cirrhotic patients (1,5,17). These functional defects in the NK cells might be responsible for the failure of antitumor immune responses after LT with HCC. Since the immunosuppressive regimen that is currently used after LT reduces the adaptive immune components but effectively maintains the innate components of cellular immunity (12,13,24), augmentation of the NK cell response may be a promising immunotherapeutic approach (28).

Recently, we characterized the phenotypical and functional properties of liver NK cells extracted from living donor liver graft perfusate (17). We have also proposed a novel strategy of adjuvant immunotherapy to prevent tumor recurrence after LT. This immunotherapy involves intravenously injecting LT recipients with activated donor liver allograft-derived NK cells. This immunotherapy has been successfully performed in 14 living donor LT recipients at Hiroshima University, Japan (27). Some research groups have shown that deceased donor

liver graft contains a unique subset of NK cells (18,25,26). However, the function and characteristics of liver NK cells that are derived from deceased donors and processed for clinical immunotherapy are not well known. Here, we demonstrated for the first time the phenotypical and functional properties of liver NK cells that were extracted from deceased donor liver graft perfusate under current good manufacturing practice (cGMP) conditions.

PATIENTS AND METHODS

Collection of Samples

Fourteen donors who underwent organ recovery for LT were involved in this study. The donors included 11 men and 3 women aged 20-71 years (mean age ± SD, 43.4 ± 17.6 years). Informed consent was obtained from each donor, and the study protocol was approved by the Ethics Committee at the University of Miami. Standard testing for infectious disease, including assays for the detection of hepatitis B and C and human immunodeficiency virus (anti-HCV, anti-HIV, anti-HBcore, and HBsAg), was performed. A donor who tested positive for any of the infectious disease markers listed above was excluded from this study. Peripheral blood (40 mL) was collected from the organ donors. Subsequently, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (GE Healthcare, Sweden) density-gradient centrifugation and resuspended in X-VIVO 15 medium (LONZA, Walkersville, MD) supplemented with 100 $\mu g \cdot mL^{-1}$ of gentamycin (APP Pharmaceuticals, Schaumburg, IL), 10% human AB serum (Valley Biomedical, Winchester, VA) and 10 $U \cdot mL^{-1}$ of sodium heparin (APP Pharmaceuticals, Schaumburg, IL) (culture medium). During organ recovery, the aorta was clamped and the liver flushed in situ with up to 4 L of University of Wisconsin (UW) solution to remove blood from the vasculature. After organ recovery, the liver was placed in a bag and perfused through the portal vein with an additional 2 L of UW solution at the back table. This perfusate was collected from the vena cava and used to study

liver mononuclear cells (LMNCs). The perfusate was retrieved in our cGMP cell processing facility (4,9,37). Since the UW solution has a high viscosity (45), the perfusate was centrifuged at 2800 × *g* for 30 min at 4 °C in order to ensure adequate centrifugation. The cell pellet was then subjected to Ficoll-Hypaque density-gradient centrifugation. A cell viability of 90% was ensured by trypan blue exclusion prior to all assays.

Cell culture

LMNCs and PBMCs were cultured with 1000 $U \cdot mL^{-1}$ of human recombinant interleukin (IL)-2 (Proleukin, Novartis, Emeryville, CA) in culture medium at 37 °C in an atmosphere supplemented with 5% CO_2. Anti-CD3 monoclonal antibody (mAb) (Orthoclone OKT3, Ortho Biotech, Raritan, NJ) was added to the culture medium (100 $\mu g \cdot mL^{-1}$) 1 day prior to cell harvesting. After 4 days of culture, the cells were harvested for further analysis. Testing for lot release included cell counts, viability, Gram stain, and endotoxin. Cell counts and viability were performed using the trypan blue dye exclusion method. Test samples were stained with trypan blue and then microscopically examined with a hematocytometer. A minimum of 1×10^7 cells with a cell viability of>80% was required to release the NK-cell product for infusion. The Gram staining was performed at the Clinical Microbiology Laboratory (Jackson Memorial Hospital, Miami, FL) by using standard methods, with the lot release criterion of "no organisms seen." Endotoxin testing by the Limulus Amebocyte Lysate assay was performed on the final product by using the Endosafe-PTS (portable test system; Charles River, Wilmington, MA). An endotoxin value of not more than 5 $EU \cdot kg^{-1}$ was used for lot release. Although not included as a lot release criterion, the final product was tested for sterility by collecting specimens for aerobic, anaerobic, and fungal cultures and inoculating them in vials filled with Soybean-Casein Digest broth and Fluid Thioglycollate media (BD Bactec, Becton Dickinson, Sparks, MD). The specimens were cultured for 14

days at 37 °C. Mycoplasma testing was performed using the VenorGeM Mycoplasma Detection Kit (Sigma-Aldrich, St. Louis, MD).

Flow cytometry

All flow cytometry (FCM) analyses were performed on a FACSCalibur cytometer or LSR II Flow Cytometer (BD Biosciences, San Jose, CA). For phenotyping of the surface markers, the leukocytes were stained with the following monoclonal antibodies (mAbs): fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and anti-CD15 (BD Biosciences), goat anti-mouse IgG and anti-CD56 (BioLegend, San Diego, CA); phycoerythrin (PE)-conjugated anti-CD16, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), NKp44, NKp46, CD69, CD94, CD25, CD14, CD19, and CD7; allophycocyanin (APC)-conjugated anti-CD56 (B159) and CD11b (BD Biosciences); APC-eFluor 780-conjugated anti-CD3; eFluor 625-conjugated anti-CD15; biotin–conjugated anti-CD4; Peridinin Chlorophyll Protein Complex (PerCP)-eFluor 710-conjugated anti-CD11c (eBioscience, San Diego, CA); Qdot565-conjugated anti-CD8; Qdot655-conjugated anti-CD19; Alexa Fluor 568-conjugated streptavidin; and Alexa Fluor 700-conjugated anti-CD14 (Invitrogen, Carlsbad, CA). Dead cells were excluded by light scatter and 7-aminoactinomycin D (7-AAD) or 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) staining. Cytokine production of lymphocytes was measured by a combination of cell surface and cytoplasmic mAb staining according to the manufacturer's instructions. Briefly, 4 h after treatment with Leukocyte Activation Cocktail (BD GolgiPlug, BD Biosciences), the lymphocytes were stained with anti-CD3-FITC and anti-CD56-APC surface markers (BD Bioscience). After washing, the cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and washed with Perm/Wash Buffer (BD Biosciences). Subsequently, aliquots were stained with either a mAb against intracellular cytokines; anti-interferon (IFN)-γ-PE, anti-tumor necrosis factor (TNF)-α-PE, or anti-IL-2-PE (BD Biosciences).

Cell Targets

K562, a human chronic myelogenous leukemia cell line (ATCC #CCl-243), was cultured in DMEM medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Mediatech, Inc., Manassas, VA), 100 $U \cdot mL^{-1}$ penicllin, and 100 $\mu g \cdot mL^{-1}$ streptomycin (Invitrogen) (complete medium) at 37 °C in 5% CO_2. Target cells were harvested during the logarithmic phase of growth, washed in PBS, and counted using trypan blue staining prior to use.

Cytotoxicity assay

The cell cytotoxicity assay was performed by FCM as described previously (20). Briefly, target cells were labeled with 0.1 μM Carboxyfluorescein diacetate (CFDA) SE Cell Tracer Kit (Invitrogen) for 5 min at 37 °C in 5% CO_2. The labeled cells were washed twice in PBS, resuspended in complete medium, and counted using trypan blue staining. The effector cells were co-incubated at various effector / target ratios of target cells for 1 h at 37 °C in 5% CO_2. As a control, either target cells or effector cells were incubated alone in a complete medium to measure spontaneous cell death: 7-AAD was added to every tube. The data were analyzed using the Flowjo software (Tree Star, Inc. Ashland, OR). The cytotoxic activity was calculated as a percentage by using the following formula: % cytotoxicity = [(% experimental 7-AAD^+ dead targets) − (% spontaneous 7-AAD^+ dead targets)]/[(100 − (% spontaneous 7-AAD^+ dead targets)] × 100.

Statistical analysis

For comparison between 2 groups, the Student's *t*-test (two-tailed) was performed. For

comparison of more than 2 groups, one-way ANOVA followed by the Student-Newman-Keuls *post hoc* analysis was performed. A p value < 0.05 was considered statistically significant. Values are expressed as the mean ± SEM.