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 2013; **43**: 249–254 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.

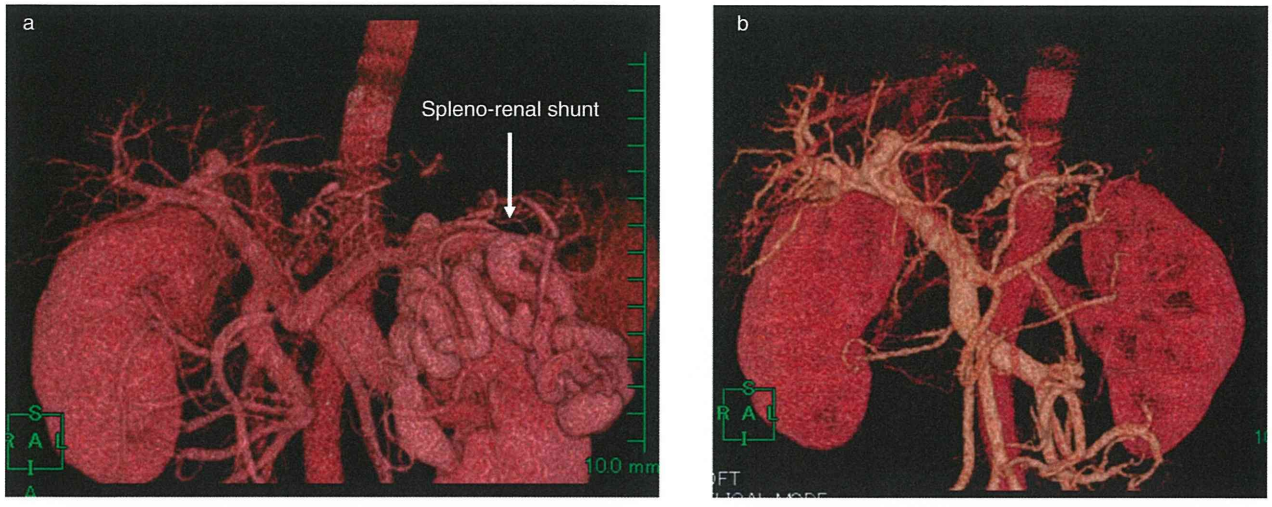

Figure 1 Three-dimensional computed tomography (CT) of the portal vein in case 1. (a) CT before surgery showing huge splenorenal shunts. (b) CT after surgery showing disappearance of a splenorenal shunt.

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

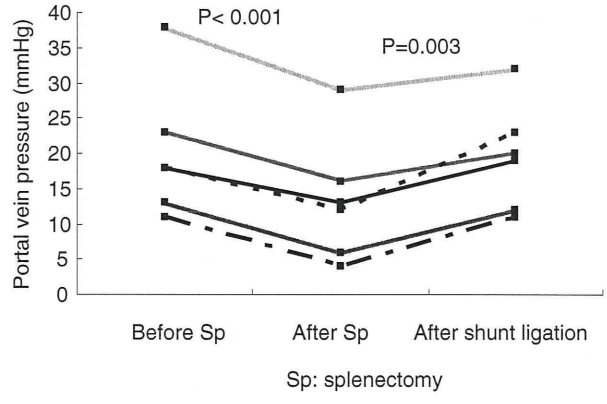

Figure 2 Changes in portal vein pressure during surgery.

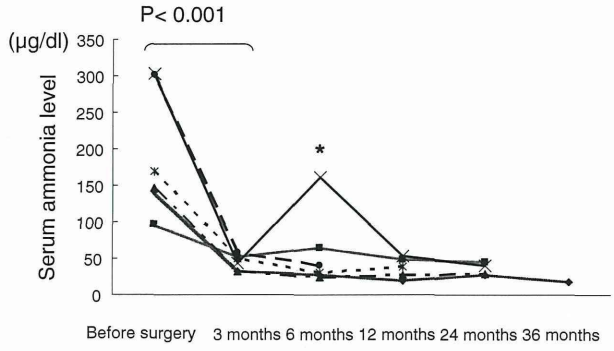

Figure 3 Changes in the serum ammonia level. *One patient (case 4) with relapsing encephalopathy underwent balloon-occluded retrograde transvenous obliteration for the remaining splenorenal shunt 8 months after surgery.

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

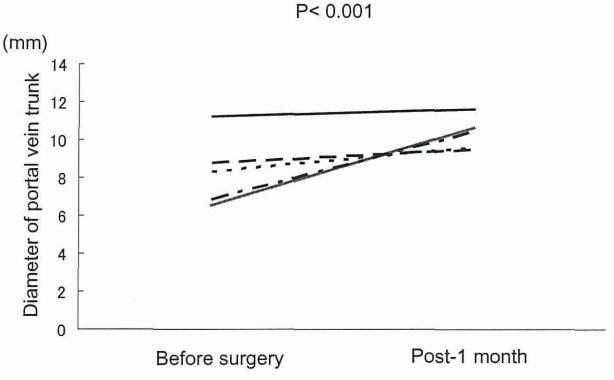

Figure 4 Changes in the diameter of the portal vein trunk before and after surgery. One patient (case 6) with large left gastric azygos vein and para-umbilical vein shunts has been excluded, because the portal flow of the left portal vein decreased due to ligation of the para-umbilical vein.

(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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Cell Transplantation, Vol. 21, pp. 1397–1406, 2012

0963-6897/12 $90.00 + .00
DOI: http://dx.doi.org/10.3727/096368911X627589
E-ISSN 1555-3892
www.cognizantcommunication.com

Clinical-Scale Isolation of Interleukin-2-Stimulated Liver Natural Killer Cells for Treatment of Liver Transplantation With Hepatocellular Carcinoma

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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-2 (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.

Key words: Natural killer cell; Immunotherapy; Innate immunity; Hepatocellular carcinoma; Current good manufacturing practice (cGMP)

INTRODUCTION

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 US (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

Received December 15, 2010; final acceptance July 19, 2011. Online prepub date: March 16, 2012.
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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 μg/ml of gentamycin (APP Pharmaceuticals, Schaumburg, IL), 10% human AB serum (Valley Biomedical, Winchester, VA), and 10 U/ml 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 $2,800 \times g$ 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/ml of human recombinant interleukin-2 (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 (1 μg/ml) 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/kg 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-γ (IFN-γ)-PE, antitumor necrosis factor-α (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/ml penicllin, and 100 μg/ml 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 coincubated 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 two groups, the Student's *t*-test (two-tailed) was performed. For comparison of more than two groups, one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis was performed. A value of $p < 0.05$ was considered statistically significant. Values are expressed as the mean ± SEM.

RESULTS

Deceased Donor LMNCs Contain a Large Population of NK and NT Cells

As an initial step, we compared the characteristics between LMNCs and PBMCs derived from deceased donors to determine whether liver NK cells could be used for clinical immunotherapy. To characterize the donor liver and peripheral NK cells, we collected liver graft perfusate and peripheral blood during regular organ procurement. The liver graft perfusate contained a large number of mononuclear cells ($1.2 \pm 0.2 \times 10^9$ cells), with a viability of 90 ± 3%. The phenotype of these cells was markedly different from that of matched donor PBMCs (Table 1). The proportions of CD3$^-$CD56$^+$ NK and CD3$^+$CD56$^+$ natural killer-like T (NT) cells in the LMNCs were significantly higher than those in the

Table 1. Immunophenotypical Comparison of NK Cells in Liver Perfusate and Peripheral Blood

	LMNC	PBMC	*p*-Value
CD3$^-$CD56$^+$ NK	45.0 ± 4.0	21.8 ± 5.2	0.001
CD3$^+$CD56$^+$ NT	16.0 ± 1.8	3.3 ± 1.3	0.00001
CD3$^+$CD56$^-$ T	22.8 ± 2.4	37.0 ± 4.7	0.014
CD3$^+$CD4$^+$ T	7.1 ± 0.8	20.7 ± 3.3	0.002
CD3$^+$CD8$^+$ T	28.5 ± 2.8	17.4 ± 3.4	0.017
CD19$^+$ B	3.8 ± 1.3	9.8 ± 3.0	0.085
CD14$^+$ mono	16.7 ± 7.5	18.2 ± 12.5	0.909
CD15$^+$ Gran	13.6 ± 6.2	17.0 ± 13.3	0.806
	Liver NK Cells	PB NK Cells	
CD16$^+$	79.3 ± 2.4	96.8 ± 1.2	0.000001
TRAIL$^+$	5.1 ± 1.0	2.8 ± 0.9	0.089
NKp44$^+$	3.8 ± 1.1	0.2 ± 0.1	0.011
NKp46$^+$	96.8 ± 0.9	90.1 ± 3.7	0.045
CD69$^+$	75.0 ± 6.3	9.6 ± 3.0	0.00002
CD94$^+$	88.1 ± 10.3	90.6 ± 6.5	0.828
CD25$^+$	1.7 ± 1.1	1.1 ± 0.7	0.618

The values indicate the percentage of cell types after Ficoll density-gradient centrifugation (mean ± SEM, $n = 4–14$). Statistical analysis was performed using Student's *t*-test. LMNC, liver mononuclear cells; PBMC, peripheral blood mononuclear cells; NK, natural killer cells; NT, natural killer-like T cells; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; mono, monocyte; Gran, granulocyte.

PBMCs. In contrast, the LMNCs possessed a smaller number of T cells and B cells than did the PBMCs. There was no significant difference in the number of monocytes or granulocytes. Phenotypical flow cytometry analysis of other surface markers was then performed in a comparative analysis between liver and blood NK cells (Table 1). The CD69 early activation marker was expressed on the majority (75.0%) of liver NK cells, whereas the same subset in the PBMCs showed a significantly lower frequency of expression (9.6%). When the expression of the nonmajor histocompatibility complex class I-specific-activating NK cell receptors (natural cytotoxicity receptors; NKp44 and NKp46) was examined in both liver and peripheral blood, nearly all NK cells (>90%) expressed NKp46. In agreement with Vitale et al. (40), NKp44 was not detectable in peripheral blood NK cells, while a mean of 3.8% of liver NK cells expressed NKp44. These results indicate a physiological activation status for liver NK cells. The percentage of NK cells expressing CD16, an NK cell lysis receptor (22), was higher in PBMCs than in LMNCs. Both the liver and peripheral blood NK cells expressed the C-type lectin receptor CD94. This molecule binds human leukocyte antigen (HLA)-E loaded with leader peptides from major histocompatibility complex (MHC) class I molecules (10).

Next, we analyzed the response of NK cells in LMNCs and PBMCs after IL-2 stimulation. TRAIL is a type II transmembrane protein that belongs to the TNF family, which preferentially induces apoptotic cell death in a wide variety of tumor cells but not in most normal cells (30,43,44). We previously reported that in vitro IL-2 stimulation upregulated the expression of TRAIL and induced strong cytotoxicity for liver NK cells extracted from living donor liver graft perfusate (17). As shown in Figure 1, freshly isolated liver NK cells and peripheral blood NK cells barely expressed TRAIL. Stimulation with IL-2 significantly upregulated the expression of TRAIL in liver NK cells, but this effect was barely observed for peripheral blood NK cells. IL-2 stimulation also resulted in an increased expression of the activation molecule NKp44 and maintained the expression of the inhibitory receptor CD94. These results indicate that cultivated NK cells have a compensatory mechanism to protect the self-MHC class I-expressing cells from NK cell-mediated cell death.

Characteristics of the Liver NK Cell-Enriched Product

For determining whether NK cells from deceased donor liver graft perfusate could be processed using cGMP-compliant components, the LMNC cultivation was analyzed. At the start of the culture (preculture), the mean percentage of NK cells was 45.0% (range: 21.2–76.2%), whereas T cells constituted 22.8% (range: 6.6–35.2%). After processing, NK cells were enriched to 52.0 ± 5.0%. The viability of the enriched NK cells, as determined by trypan blue staining, remained >90% during the process. No microbial contamination was detected in the final product or in the culture medium. In addition, the cell processing resulted in a significant reduction of T cells in the final product. The percentage of $CD3^+CD56^-$ T cells decreased to 0.6 ± 0.2% (0.18×10^5 cells/kg). Other $CD56^+$ components of the final product included NT cells (0.2 ± 0.1%). Next, we further examined the phenotype of the $CD56^-$ fraction of the final product. After IL-2 stimulation, the phenotype of the final product was assessed using another T-cell marker, CD7, with or without the addition of OKT3. As shown in Table 2, the final product contained $CD7^+$ T cells at 24.7%. Goat anti-mouse IgG antibody detection of OKT3 (isotype: mouse IgG) on T cells showed that 14.4% of the final product was bound with OKT3. After administration of the final product to the recipient, these T cells would be depleted by several mechanisms, including T-cell opsonization and clearance by mononuclear phagocytic cells, and complement-mediated cell lysis (6,7,39). The remainder of the T cells (10.3% of the final product) is involved in CD3 internalization or modulation, which induce T-cell dysfunction (6,36). Other components of the final product are shown in Table 2.

For phenotypically characterizing the NK cells in the final product relative to those in the starting material, a detailed flow cytometry analysis was undertaken. As shown in Figure 2, freshly isolated liver NK cells barely expressed TRAIL, NKp44, and CD25 (IL-2αR) and produced little cytokines. The cell processing significantly upregulated the expression of TRAIL and NKp44 in liver NK cells, but these changes were not seen in peripheral blood NK cells. The expression of CD69 and CD25 in liver NK cells also increased, but not significantly. In contrast, NKp46 expression significantly decreased after the cell processing. The activating receptors are defined by their ability to directly mediate the killing of the targets. Nevertheless, recent findings have demonstrated that the activation of some of the NK-triggering receptors requires the synergistic stimulation of more than one receptor (3). Our results are compatible with this theory. Intracellular staining flow cytometry showed that IL-2 stimulation induced significant cytokine production [IFN-γ and TNF-α (5.8–37.0% and 4.1–59.2%, respectively, $n = 4$, $p < 0.01$)] in liver NK cells (Fig. 2). These results are similar to those of studies of living donor liver graft perfusate (17,27). Next, NK cell cytotoxicity assays using LMNCs and PBMCs isolated from the deceased donor as effectors and K562 as targets were performed. Cytotoxicity against the standard NK cell target K562 was markedly elevated using

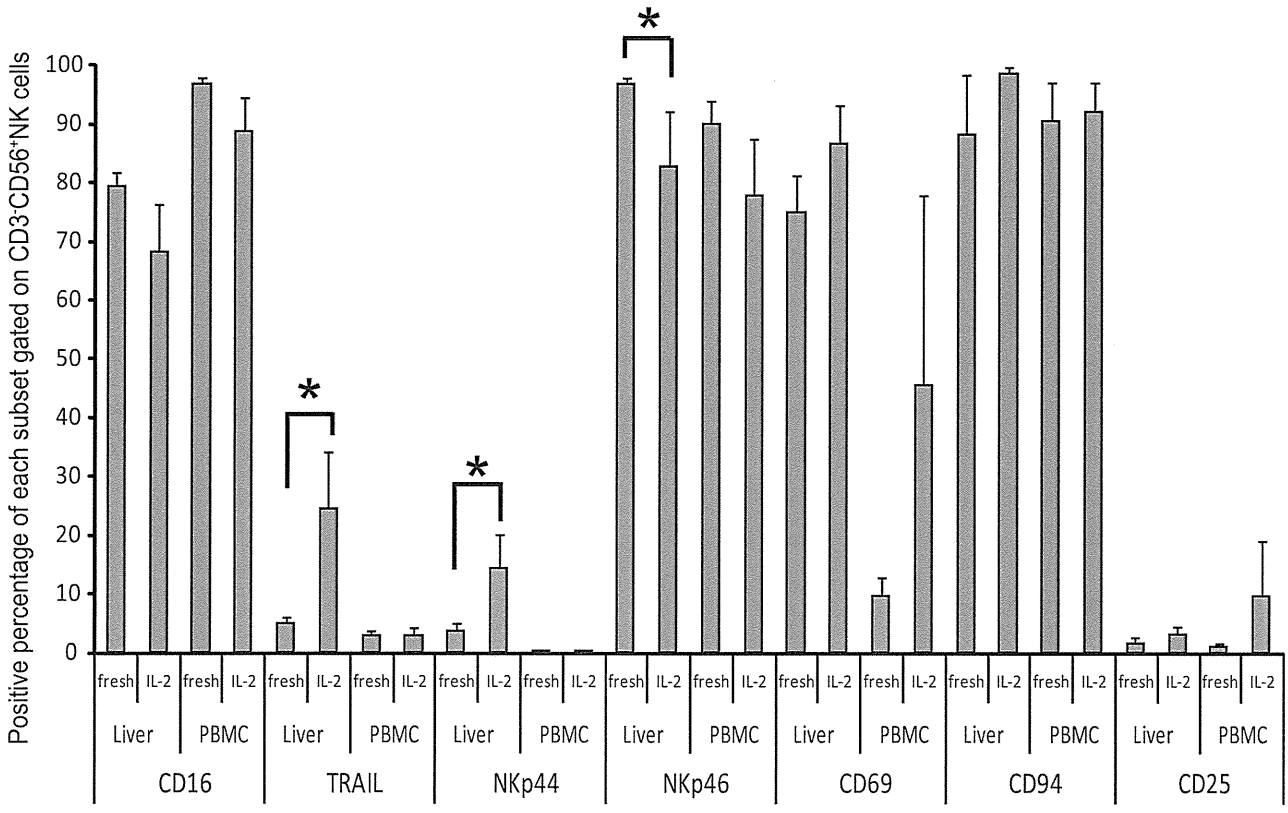

Figure 1. Comparison of surface marker expression patterns in response to interleukin-2 (IL-2) stimulation in liver perfusate and blood natural killer (NK) cells. Flow cytometry (FCM) analysis of freshly isolated or cultured with IL-2 (1,000 U/ml) liver mononuclear cells (LMNCs) and peripheral blood mononuclear cells (PBMCs) obtained from deceased donor after staining with anti-CD3 and anti-CD56 mAbs. The numbers indicate the mean percentage of each positive subset on electronically gated $CD3^-CD56^-$ NK cells (mean ± SEM, $n = 4–14$). Statistical analyses were performed using Student's t-test (*$p < 0.01$ vs. fresh control).

Table 2. Phenotypical Characteristics of the $CD56^-$ Fraction of the Final Product From the Liver Perfusate

	% of the $CD56^-$ Fraction	% of the Final Product
Goat anti-mouse IgG antibody	—	14.4 ± 5.6
$CD56^-$	—	43.4 ± 11.8
$CD7^+$	56.8 ± 14.5	24.7*
$CD4^+$	10.5 ± 3.9	4.6*
$CD8^+$	27.5 ± 7.7	12.0*
$CD19^+$	27.1 ± 11.0	11.8*
$CD14^+$	0.4 ± 0.1	0.2*
$CD15^+$	2.6 ± 0.3	1.1*
$CD11b^+$	4.6 ± 3.2	2.0*
$CD11c^+$	4.6 ± 1.8	2.0*

The values indicate the percentage of each marker (mean ± SEM, $n = 5$).
*The percentage of the final product was calculated as follows: % of $CD56^-$ fraction × $CD56^-$ percentage (43.4)/100.

effector cells from the final products relative to those from precultured LMNCs and PBMCs (Fig. 3). At a 20:1 effector/target cell ratio, 56.3% of the K562 targets were killed on average by the final products, whereas precultured LMNCs and pre- and post-PBMCs killed only 11.8%, 2.5%, and 23.8% of K562 targets, respectively. We also tested the difference between with and without addition of OKT3 after IL-2 stimulation. The addition of OKT3 did not significantly enhance the NK cytotoxicity of either PBMCs or LMNCs.

DISCUSSION

In this study, we demonstrated the phenotypical and functional properties of NK cells extracted from deceased donor liver graft perfusate under cGMP conditions. Methods for processing allogeneic NK cell products for human use on a clinical scale are limited to FDA-approved selection facilities and devices. The cGMP facility at University of Miami has published methods for processing different products (4,9). Lot release testing is described in the Patients and Methods

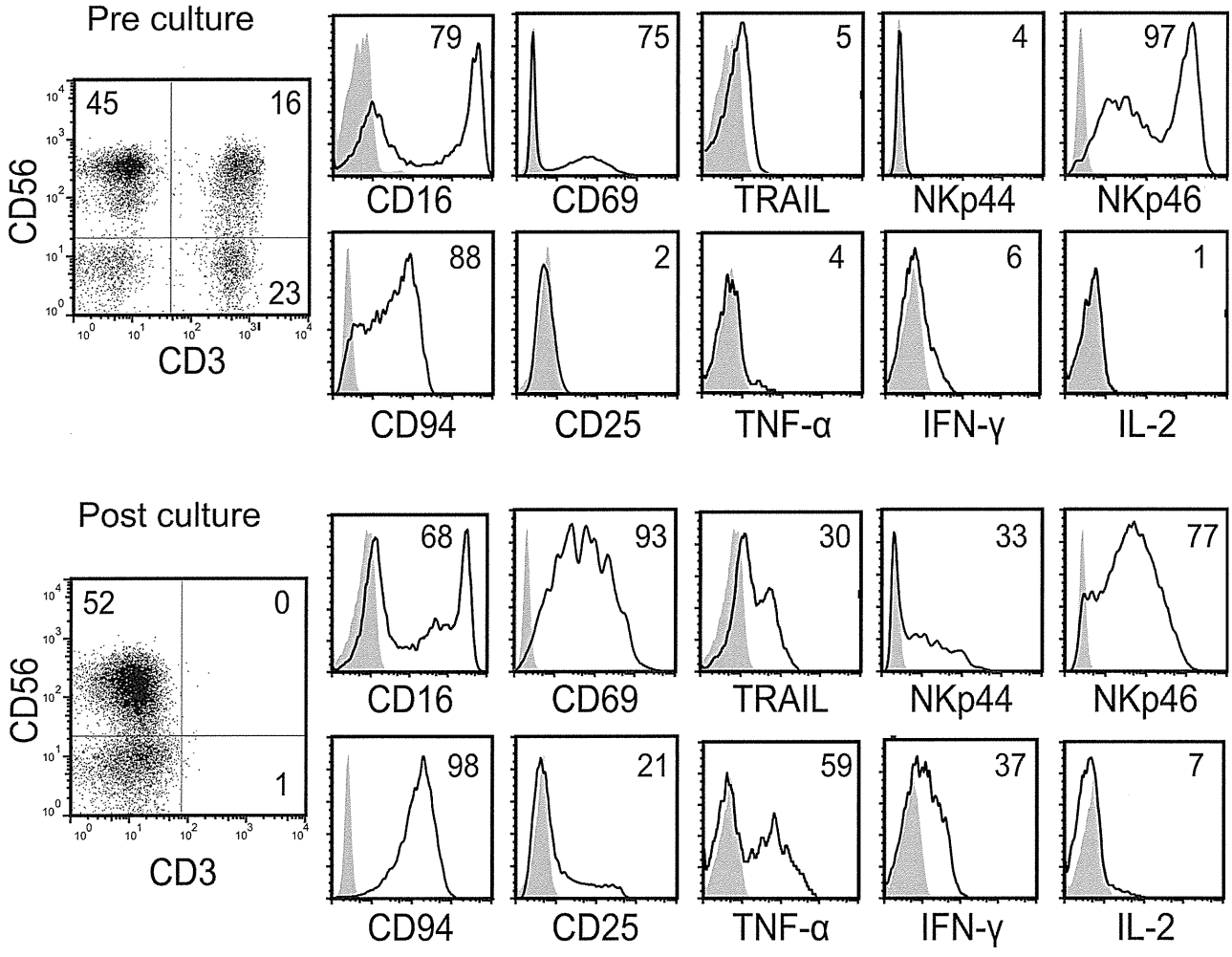

Figure 2. Liver NK cells inductively express significant levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and cytokines after cell processing. FCM analysis of LMNCs obtained from deceased donor liver graft perfusate before the culture (upper panel) and after the culture (lower panel), after staining with mAbs against CD3 and CD56. Lymphocytes were gated by forward and side scatter. The FCM dot plot profiles are representative of 14 independent experiments. The percentages of $CD3^+CD56^-$ (T), $CD3^-CD56^+$ (NK), and $CD3^+CD56^+$ (NT) cells are indicated at each quadrant. Histograms show the logarithmic fluorescence intensities obtained on staining for each surface marker or intracellular protein after gating on the $CD3^-CD56^+$ NK cells. Shaded regions indicate negative control staining with isotype-matched mAbs. The numbers indicate the mean percentages of positive cells in each group ($n = 4–14$). The histogram profiles are representative of 14 independent experiments.

section and is as dictated by the FDA guidelines for cellular products. First, LMNCs were shown to contain a large number of NK and NT cells, with both cell types possessing characteristics different from those of PBMCs. Second, in vitro stimulation with IL-2 induced liver NK cells to strongly upregulate activation markers, cytotoxicity, and cytokine production and to maintain the expression of inhibitory receptors. These results were compatible with those for living donor liver graft perfusate (17). Finally, we confirmed that the final product met the lot release criteria and contained low T cell numbers, thereby reducing the possibility of GVHD in a recipient.

This study demonstrated that deceased donor liver graft perfusate contained an average of $1.2 \pm 0.2 \times 10^9$ mononuclear cells and 5.3×10^8 NK cells, whereas living donor liver graft perfusate, which contained $9.1 \pm 0.8 \times 10^8$ mononuclear cells (Ohdan H et al., Hiroshima University, Japan, unpublished data). However, this number is several fold higher than the numbers calculated in previous studies on deceased donor liver perfusate (2,18,25). This discrepancy may be due to the fact that we collected the liver perfusate at the time of organ procurement while others did so just before liver transplantation.

NK cells can destroy many solid tissue-derived malignant cells through death receptor–ligand interactions

(42). Previously, we found that normal hepatocytes express TRAIL-DR4 and TRAIL-DR5 together with TRAIL-DcR1 and TRAIL-DcR2, but that moderately or poorly differentiated HCCs highly express TRAIL-DR4 and TRAIL-DR5 but do not express TRAIL-DcR1 and TRAIL-DcR2, which indicates a susceptibility to TRAIL-expressing NK cell-mediated activity toward HCC (17,28). We have now shown that IL-2 stimulation significantly increases the expression of TRAIL in liver NK cells that are extracted from deceased donor liver graft perfusate (Fig. 1). Functionally, we also have shown that IL-2-activated liver NK cells were highly cytotoxic against tumors compared with PBMCs (Fig. 3). In addition to having an antineoplastic effect, NK cells are important components of the innate immune response due to their ability to lyse virus-infected cells and to recruit cells involved in adaptive immune responses. IFN-γ is a known host mediator that shapes the tumor phenotypes in a broader process known as "immunoediting" (19). Mice that lack either IFN-γ or its functional receptor are more susceptible to both viral and bacterial infections, indicating that IFN-γ plays an important role in antiviral and antibacterial responses (33,47). It is possible that these liver NK cells can prevent the replication of viruses including hepatitis C virus through an IFN-γ-dependent mechanism. Further studies are required to address this possibility.

The induction of GVHD is a major risk factor associated with the use of lymphocyte infusions from unrelated or haploidentical family donors (8,15). For clinical-scale experiments, OKT3 was added to the culture media 1 day prior to cell harvesting. The administration of OKT3-coated T cells in vivo has been shown to result in the opsonization and subsequent trapping or lympholysis of cells by the reticuloendothelial system (6,7,39). This method has been performed for clinical NK therapy in Japan, with no GVHD cases reported (27). Our final product from the cadaveric donor liver

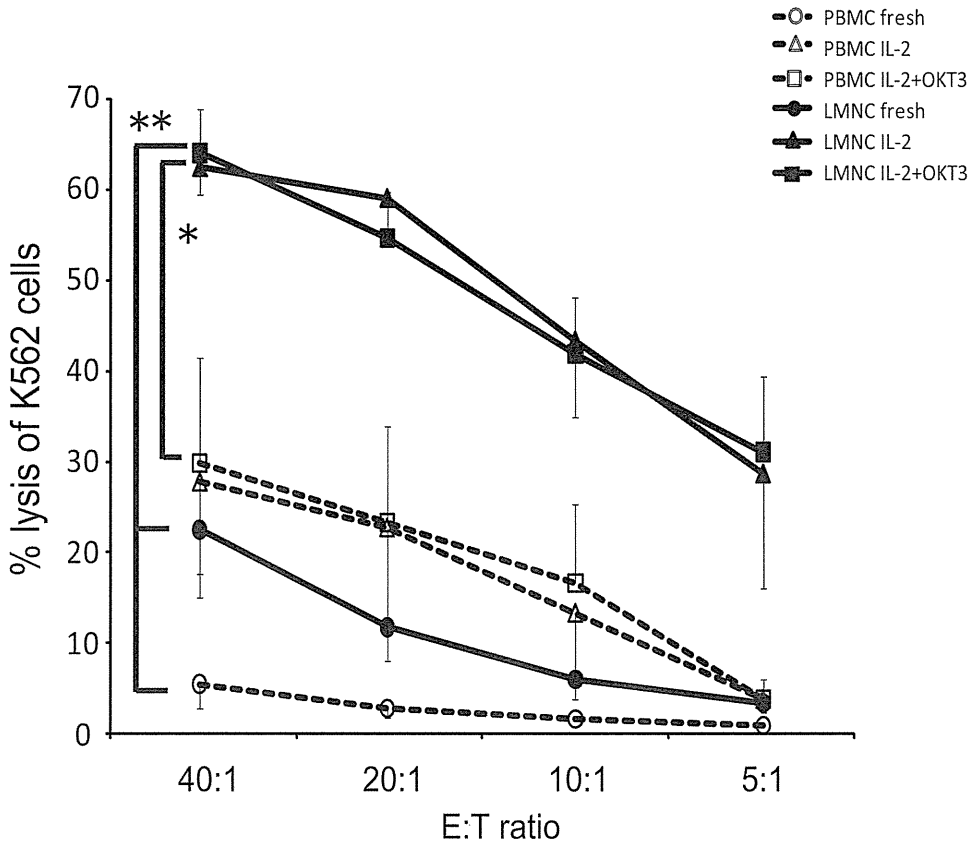

Figure 3. LMNC final products show strong cytotoxicity against NK-susceptible target cells. The NK cytotoxic activities of freshly isolated (circle) and IL-2 stimulated (triangle) LMNCs (black) and PBMCs (white) with or without anti-CD3 monoclonal antibody (OKT3; square) against K562 target cells were analyzed by a FCM-based cytotoxic assay. All data are expressed as the mean ± SEM ($n = 5$). Statistical analysis was performed using one-way ANOVA followed by Student-Newman-Keuls post hoc analysis (*$p < 0.01$ vs. PBMC fresh and LMNC fresh, **$p < 0.01$ vs. PBMC IL-2 and PBMC IL-2 + OKT3).

perfusate contained 0.02×10^6 $CD3^+CD56^-$ T cells/kg. There are some clinical studies regarding T-cell contamination. For example, Miller et al. reported that the final T-cell dose was 0.18×10^6 cells/kg and that GVHD did not occur after haploidentical NK cell infusion (24). Schulze et al. reported that T-cell contamination was 0.01×10^6 cells/kg in allogeneic stem cell transplantation and that no GVHD occurred (34). Frohn et al. performed allogeneic NK cell infusion for renal cell carcinoma. The T cell contamination was 1.0% (0–7%) in their study (11). Passweg et al. defined the upper limit of acceptable T cell contamination as 0.1×10^6/kg BW for allo-NK cell infusion in stem cell transplantation. No patients developed clinical signs of GVHD after NK cell infusion (29). Compared with other clinical studies, our final product contains an acceptable level of T-cell contamination. NK cells exert alloreactivity after mismatched haploidentical transplantations due to an incompatibility between killer cell inhibitor receptors of donor NK cells and the recipient HLA type C (31). However, there is no known evidence of NK-mediated GVHD in humans.

In conclusion, liver NK cells derived from deceased donor liver graft perfusate inductively expressed TRAIL and secreted IFN-γ. IL-2-stimulated liver NK cells showed strong cytotoxicity against NK-susceptible K562 targets. Hence, these cells are potentially useful for the immunotherapy of LT recipients with HCC. This study is the first attempt to apply cadaveric donor liver NK cells to clinical cell transplantation. Our results will have a positive effect on adoptive immunotherapy using liver NK cells. However, further clinical studies are needed to elucidate the role played by donor liver NK cell infusions in the treatment of HCC patients after LT.

ACKNOWLEDGMENTS: This work was supported by a grant from the Florida Department of Health and the Bankhead-Coley Cancer Research Program (1BG-08). The authors declare no conflicts of interest.

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Breast Cancer Res Treat (2012) 134:139–155
DOI 10.1007/s10549-011-1944-x

PRECLINICAL STUDY

Mechanistic analysis of the antitumor efficacy of human natural killer cells against breast cancer cells

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Received: 1 July 2011 / Accepted: 26 December 2011 / Published online: 20 January 2012

Abstract We investigated the role of human natural killer (NK) cells in the peripheral blood (PB) and liver in controlling breast cancer. The proportion of NK cells among liver mononuclear cells was significantly higher than among PB mononuclear cells. Liver NK cells inductively expressed higher levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) than PB NK cells in response to interleukin-2 (IL-2). Liver NK cells displayed higher cytotoxicity against various breast cancer cell lines (MDA-MB231, MDA-MB453, MDA-MB468, and MCF-7) after IL-2 stimulation than did PB NK cells. Anti-HER2 monoclonal antibody (mAb) promoted the cytotoxicity of both the types of NK cells toward HER2-expressing cell lines. All breast cancer cell lines highly expressed death-inducing TRAIL receptors, death receptor 4, but did not express death-inhibitory receptors (DcR1 and DcR2). Both PB and liver NK cell-induced cytotoxicity was inhibited partially by anti-TRAIL mAb and more profoundly by the combination of anti-TRAIL mAb and concanamycin A, indicating that TRAIL and perforin are involved. IL-2-stimulated liver and PB NK cells exhibited upregulated expression of CXCR3, which bind to the chemokines CXCL9, CXCL10, and CXCL11 secreted by breast cancer cells. We also found that IFN-γ promoted the production of CXCL10 from breast cancer cells. The results of this study show that IFN-γ secreted from NK cells likely promotes the production of CXCL10 from breast cancer cells, which in turn accelerates the migration of CXCR3-expressing NK cells into the tumor site. These findings suggest the possibility of a therapeutic approach by either activation of endogenous PB and liver NK cells or adoptive transfer of in vitro-activated autologous NK cells.

Keywords NK cells · TNF-related apoptosis-inducing ligand (TRAIL) · TRAIL-receptors · ADCC · Chemokine · Breast cancer

Abbreviations

NK	Natural killer
TRAIL	TNF-related apoptosis-inducing ligand
PBMC	Peripheral blood mononuclear cell
LMNC	Liver mononuclear cell
mAbs	Monoclonal antibodies

Introduction

Natural killer (NK) cells, the frontline defense in cellular immunity, exert an effector function on neoplastic cells, modified cells, and invading infectious microbes without the necessity for priming [1, 2]. Although, NK cells might

Electronic supplementary material The online version of this article (doi:10.1007/s10549-011-1944-x) contains supplementary material, which is available to authorized users.

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play an important role in prevention of both early and metastatic cancer, the role of NK cell activity in controlling breast cancer is still controversial and few studies have addressed whether enhancing this activity is of clinical benefit to breast cancer patients.

A variety of mechanisms are involved in controlling neoplastic cells by NK cells, one of which is the direct release of cytolytic granules that contain perforin, granzymes, and granulysin by exocytosis to kill target cells (i.e., the granule exocytosis pathway) [3, 4]. Most mature human NK cells in peripheral blood (PB) constitutively express granzyme B and perforin, and have basal cytotoxicity against NK-sensitive targets. Cytokine exposure with interleukin (IL)-2 or IL-15 is known to increase the baseline granzyme B and perforin abundance and cytotoxic activity of NK cells, and also converts basal NK cytotoxicity to lymphokine-activated killing. Another mechanism is mediated by death-inducing ligands such as Fas ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Fas, a TNF family protein, is expressed on breast cancer cell membranes [5, 6], suggesting that activation of the Fas/FasL pathway induces apoptosis mediated by caspase activation. An additional mechanism is involved when HER2-overexpressing breast cancer cells are targeted because differential levels of HER2 expression in normal versus HER2-overexpressing tumor cells, together with the clear involvement of HER2 in tumor progression, make HER2 an ideal target for therapeutic approaches. NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) is thought to contribute to the therapeutic effects of monoclonal antibodies (mAbs) specifically directed against the extracellular domain of HER2 (trastuzumab).

NK cells are abundant in the liver in contrast to their relatively small percentage in the peripheral lymphatics and other lymphatic organs in rodents [7–9] and humans [10]; however, the underlying reason for the anatomically biased distribution of NK cells has not been elucidated. In addition, liver NK cells have been shown to mediate higher cytotoxic activity against tumor cells than PB NK cells do in rodents [7–9, 11]. However, these functional differences between PB and liver NK cells have not been extensively investigated in humans because of the limited availability of appropriate samples. In this study, we extracted NK cells from allograft liver perfusates in clinical liver transplantation and examined the quantitative and qualitative cytotoxic functions of those liver NK cells targeting various breast cancer cell lines in comparison with PB NK cells. Through the experiments, we attempted to define whether PB NK cells can recognize and kill breast cancer cells, and whether liver resident NK cells can hinder metastasis of breast cancer to the liver, to assess the potential therapeutic use of NK cells, i.e., by either activation of endogenous NK cells or adoptive transfer of in vitro-activated autologous NK cells. As the therapeutic efficacy of endogenous or exogenous NK cells likely depends on their migration and accumulation at tumor metastasis sites, we further analyzed the expression of receptors and ligands for chemokines secreted from breast cancer cells on PB and liver NK cells.

Materials and methods

Isolation of liver and PB lymphocytes

Liver mononuclear cells (LMNCs) were obtained from liver perfusates in clinical living donor liver transplantation as previously described [10]. LMNCs were isolated by gradient centrifugation with Separate-L (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). PB mononuclear cells (PBMCs) were also isolated by gradient centrifugation with Separate-L from heparinized PB from healthy volunteers and liver transplant donors. LMNCs and PBMCs were suspended in DMEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sanko Chemical Co., Tokyo, Japan), 25 mmol/l HEPES buffer (Gibco), 50 μmol/l 2-mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco) (10% DMEM). The ethics committee at Hiroshima University Hospital approved this study.

Cell culture

LMNCs and PBMCs were cultured with human recombinant IL-2 (100 Japanese reference U/ml; Takeda, Tokyo, Japan) in DMEM at 37°C in a 5% CO_2 incubator. Cells were harvested for further analyses after 5 days in culture. Cell viability was assessed by the dye-exclusion test.

Isolation of NK cells

LMNCs and PBMCs were separated into a $CD3^{-}CD56^{+}$ NK cell fraction and a non-NK cell fraction (T cells, NKT cells, B cells, and monocytes/macrophages) by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), using the human NK cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated fractions was assessed by FCM, and only preparations with purities >90% were used for functional studies.

Cell lines

The human breast cancer cell lines were obtained as follows: MDA-MB-231 and MDA-MB-468 were from ATCC