Ann Surg Oncol (2012) 19:2888–2896
DOI 10.1245/s10434-012-2338-y

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Prognostic Significance of Antithrombin III Levels for Outcomes in Patients with Hepatocellular Carcinoma After Curative Hepatectomy

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ABSTRACT

Background. Although several studies have shown that serum antithrombin III (ATIII) has anti-inflammatory effects, the prognostic value of ATIII in HCC is unknown. We investigated the influence of preoperative ATIII levels on the outcome of patients who underwent hepatectomy for hepatocellular carcinoma (HCC).

Methods. Data from 440 patients (314 patients with ATIII ≥70 % and 126 patients with ATIII <70 %) who underwent curative hepatectomy for HCC were retrospectively collected and analyzed. To overcome bias due to the different distribution of covariates for the 2 groups, propensity score matching was performed on the patients, and outcomes were compared.

Results. The propensity score analysis revealed that 65 patients with ATIII of ≥70 % (group 1) and 65 patients with ATIII of <70 % (group 2) had the same preoperative and operative characteristics (excluding the ATIII level). The overall survival rate and the disease-free survival rate was significantly higher in group 1 than in group 2 ($P = 0.005$ and 0.011, respectively). Multivariate analysis showed that ATIII was a significant favorable factor for overall survival and disease-free survival of patients with HCC after curative hepatectomy.

Conclusions. The prognosis of patients with HCC was found to be associated with preoperative antithrombin III levels. ATIII may be useful for predicting outcomes of patients with HCC after curative hepatectomy.

Electronic supplementary material The online version of this article (doi:10.1245/s10434-012-2338-y) contains supplementary material, which is available to authorized users.

First Received: 19 November 2011;
Published Online: 31 March 2012

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Hepatic resection is a well-accepted therapy for hepatocellular carcinoma (HCC), but many patients develop cancer recurrence, with the cumulative 5-year HCC recurrence rate being over 60 %.[1,2] A high incidence of tumor recurrence after hepatic resection remains a major drawback. The risk factors for prognosis after resection of HCC have been extensively studied.

Antithrombin III (ATIII) is a heparin-binding protein and a major inhibitor of coagulation proteases, primarily thrombin and factor Xa.[3] ATIII has been reported to efficiently inhibit tumor angiogenesis in a mouse model.[4,5] In clinical settings, decreased plasma ATIII levels have been described in a variety of different cancers, including lung, colon, ovary, and prostate cancers.[6–8] However, few data are available on the impact of ATIII on outcomes of patients with HCC who underwent hepatectomy.

In this study, we aimed to investigate the effect of ATIII on survival and HCC recurrence in patients who underwent curative hepatic resection by both methods of one-to-one match study using propensity score and multivariate analysis.

METHODS

Between the years 2000 and 2008, a total of 440 patients with HCC underwent curative hepatectomy as an initial treatment at the Department of Gastroenterological Surgery, Hiroshima University Hospital, Hiroshima, Japan. The study was approved by the concerned institutional

review boards. Written informed consent was obtained from all patients. The patients were categorized into 2 groups on the basis of their preoperative ATIII level: ≥70 % (group 1, $n = 314$), and <70 % (group 2, $n = 126$).

The type of hepatectomy selected was based on liver function and tumor extent.[9,10] Liver function was assessed by the Child-Pugh classification and the indocyanine green retention rate at 15 minutes (ICG-R15).[11] If the liver function was sufficient, anatomic resection (segmentectomy, sectionectomy, or hemihepatectomy) was performed.[12,13] In patients with insufficient hepatic reserve, limited resection was performed. For example, right hemihepatectomy could be tolerated if the ICG-R15 was in the normal range. One-third of the liver parenchyma could be resected for patients with ICG-R15 of 10–19 %, segmentectomy was possible for patients with ICG-R15 of 20–29 %, and limited resection was possible for patients with ICG-R15 of ≥30 %.[10] Hepatectomy was performed using procedures described by Itamoto et al.[9] Postoperative follow-up included liver function tests, serum alfa-fetoprotein (AFP), hepatic ultrasonography on a 3-month basis, and computed tomographic scans every 6 months. Follow-ups were performed in outpatient clinics or by the patients' general practitioner. Patients with intrahepatic recurrence were managed with ablative therapies such as radiofrequency ablation and percutaneous ethanol injection therapy, transarterial chemoembolization (TACE), or surgery, including living donor liver transplantation. Data were updated until June 2011 and survival was computed from the date of the initial surgery.

Definitions

Normal ATIII is defined as a level of ≥70 % in this study, because it has been shown that anti-thrombin activity of heparin was significantly decreased in the serum ATIII level-dependent manner when plasma ATIII levels were <70 %.[14,15] Major hepatectomy was defined as the resection of 3 or more Couinaud segments. Curative hepatectomy was defined as the removal of all recognizable tumors. All postoperative complications were reviewed for at least 30 days after surgery. The complications were graded according to the method described by Clavien et al.[16] Complications were considered morbid if they were of grade IIIA or greater. Postoperative mortality was defined as any death that occurred within 30 days of surgery.

Receiver-Operating Characteristic (ROC) Analysis

ROC curve analysis was performed to determine the optimal cutoff values for subsequent analyses. Each cutoff value was determined by seeking the most optimal combination of high sensitivity and specificity values, while maintaining the lowest likelihood ratio of a negative test and the highest likelihood ratio of a positive test.

Statistical Analysis

For continuous variables, parametric analyses were performed using Student's t test, and Mann–Whitney U test was used for non-parametric analyses. Categorical variables and postoperative courses were compared using χ^2 tests with Yates correction. The Kaplan–Meier method was used for analyses of overall survival and disease-free survival, whereas comparisons between groups were performed using the log-rank test. For factors determined to be significant for overall and disease-free survival using univariate analysis, we performed multivariate analyses using the Cox proportional hazards model. An initial Cox proportional hazards model was applied to the entire study population to identify poor prognostic predictors. To overcome bias due to the different distribution of covariates among patients from the 2 groups, a one-to-one match was created using propensity score analysis.[17,18] The propensity score represents the probability of each individual patient being assigned to a particular condition in a study given a set of known covariates. Propensity scores are used to reduce selection bias by equating groups on the basis of these covariates and are used to adjust for selection bias in observational studies through matching. Variables entered in the propensity model were age, sex, anti-hepatitis C virus (HCV) antibody, and liver function test including total bilirubin, prothrombin time, ICG-R15 %, albumin, and Child–Pugh classification. Tumor size, number of tumor, vascular invasion, and AFP were used as tumor factors. Operative bleeding, operative time, transfusion, and type of hepatectomy were used as operative factors. The model was then used to obtain a one-to-one match by using the nearest-neighbor matching method.[19,20] Once the matched groups were obtained, overall and disease-free survival analyses were performed within each matched subgroup to assess the influence of preoperative ATIII level on prognosis after adjusting the confounding factors. A difference was considered significant if the P value was <0.05. Statistical analyses were performed using the SPSS statistical software version 16 (Chicago, Illinois, USA).

RESULTS

ROC Curve Analysis for Cutoff Value of ATIII

The optimal cutoff values of ATIII for survival and recurrence were determined by ROC curve analysis, respectively. A cutoff value of survival was 72 % of ATIII

with a sensitivity of 47 % and specificity of 72 %. A cutoff value of recurrence was 69 % of ATIII with a sensitivity of 36 % and specificity of 81 %. ATIII value of 70 % has been chosen as a cutoff level in this study, since normal ATIII is defined as a level of ≥70 % (Supplementary Figs. 1 and 2).

Clinicopathological Characteristics and Postoperative Course of the Entire Study Group

Differences between the characteristics of patients in the 2 groups are shown in Table 1. Specifically, patients in group 1 had higher prothrombin time (PT) activity, lower serum bilirubin, lower ICG-R15, lower proportion of patients with Child–Pugh class B, greater maximum tumor diameter, and higher frequency of microvascular invasion. The level of preoperative ATIII in group 1 was significantly higher than that in group 2 (88.4 vs. 59.6 %; $P < 0.001$).

In the entire study population, the overall survival rate of patients in group 1 was significantly higher than that of patients in group 2 ($P < 0.001$): in group 1, the 3- and 5-year overall survival rates were 85.0 and 75.8 %, respectively, whereas in group 2, they were 77.1 and 53.1 %, respectively (Fig. 1a). Furthermore, the disease-free survival rate of patients in group 1 was significantly higher than that of patients in group 2 ($P < 0.001$): the 1-, 2-, and 3-year disease-free survival rates were 75.3, 60.1, and 48.1 %, respectively, in group 1 and 63.5, 43.6, and 27.4 %, respectively, in group 2 (Fig. 1b). Postoperative complications did not differ between the 2 groups (Table 2). Table 2 shows the patterns of cancer recurrence and the treatment details of the recurrences in both groups. The overall recurrence rate was also significantly lower in group 1 than in group 2 ($P < 0.001$): 53.5 versus 70.6 %. Regarding treatment for HCC recurrence, the proportion of patients in whom repeat hepatectomy was selected for treatment in group 1 tended to be higher than that in group 2 ($P = 0.07$). Furthermore, the proportion of patients in whom living donor liver transplantation was selected for treatment in group 1 was significantly lower than that in group 2 ($P = 0.038$).

Results after Propensity Score Match

The characteristics of propensity score-matched patients are shown in Table 1. Sixty-five of the 314 patients with preoperative ATIII levels ≥70 % were matched with 65 of the 126 patients with preoperative ATIII levels <70 % after covariate adjustment. Therefore, 249 patients in group 1 and 61 patients in group 2 were excluded because their propensity scores could not be matched. The study group of 130

TABLE 1 Baseline characteristics and operative data on patients who underwent hepatectomy

Characteristic	Whole study series			Propensity matched series		
	ATIII ≥ 70 % ($n = 314$)	ATIII < 70 % ($n = 126$)	P	ATIII ≥ 70 % ($n = 65$)	ATIII < 70 % ($n = 65$)	P
ATIII (U/ml)	88.4 ± 13.2	59.6 ± 8.5		82.5 ± 11.2	62.3 ± 6.0	
Age (years)	65.2 ± 10.3	64.6 ± 9.4	0.578	65.2 ± 8.2	64.5 ± 10.1	0.685
Sex (M/F)	238/76	85/41	0.074	44/21	42/23	0.711
Anti-HCV antibody positive	185 (59.2 %)	93 (70.8 %)	0.004	47 (72.3 %)	45 (69.2 %)	0.699
Prothrombin time (%)	90.7 ± 16.1	79.1 ± 15.1	<0.001	85.6 ± 14.1	83.8 ± 11.6	0.424
T-Bil (mg/dl)	0.78 ± 0.31	0.91 ± 0.34	<0.001	0.82 ± 0.29	0.83 ± 0.29	0.831
Albumin (g/dl)	4.00 ± 0.42	3.50 ± 0.45	<0.001	3.72 ± 0.36	3.72 ± 0.33	0.939
ICG-R15 (%)	14.8 ± 8.5	23.6 ± 9.7	<0.001	18.4 ± 10.6	18.5 ± 6.8	0.958
Child–Pugh grade, A/B	303/11	96/30	<0.001	60/5	62/3	0.465
Extent of hepatic resection, major/minor	54/260	13/113	0.069	9/56	8/57	0.794
Operation time (min)	292.8 ± 101.8	283.5 ± 105.1	0.394	277.0 ± 84.3	273.9 ± 76.5	0.585
Blood loss (ml)	380.8 ± 478.4	466.8 ± 633.9	0.123	357.5 ± 415.1	357.8 ± 257.8	0.498
Transfusion	18 (5.7 %)	13 (10.3 %)	0.089	2 (3 %)	2 (3 %)	1
AFP (ng/ml)	4568.3 ± 34234	856.3 ± 3546	0.225	884.7 ± 4657	410.8 ± 994.4	0.788
No. of tumors	1.75 ± 1.88	1.62 ± 0.92	0.44	1.65 ± 1.46	1.63 ± 0.91	0.529
Maximum tumor diameter (mm)	39.7 ± 31.4	32.4 ± 27.7	0.024	33.1 ± 23.3	34.0 ± 24.4	0.407
Vascular invasion	94 (29.9 %)	27 (21.4 %)	0.071	16 (24.6 %)	17 (26.1 %)	0.84

Data are reported for whole study and for the matched study population after propensity score analysis. Continuous variables are expressed as mean ± standard deviation

ATIII anti-thrombin III, *HCV* hepatitis C virus, *T-Bil* total bilirubin, *ICG-R15* indocyanine green retention rate at 15 min, *AFP* alfa-fetoprotein

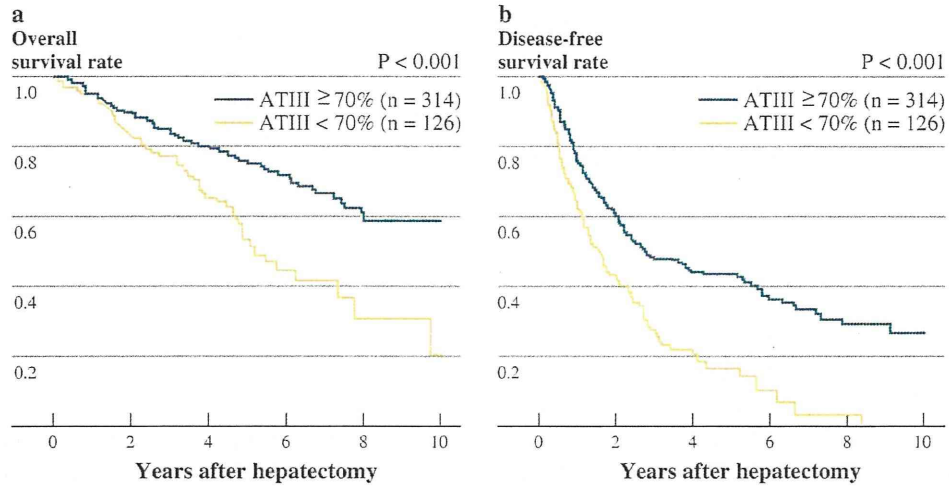

FIG. 1 Outcomes of the entire study population of 440 patients who underwent liver resection for HCC by stratified with the level of ATIII. **a** Kaplan–Meier curves for the overall survival rate after hepatectomy. Overall survival rates of HCC patients with serum ATIII level of more than 70 IU/ml (group 1, $n = 314$) at 3 and 5 years (85.0 and 75.8 %, respectively) were significantly lower than those of the serum ATIII level of <70 IU/ml (group 2, $n = 126$) at 3 and 5 years (77.1 and 53.1 %, respectively) ($P < 0.001$). **b** Kaplan–Meier curves for the disease-free survival rate after hepatectomy. Disease-free survival rates of group 1 at 1, 2, and 3 years (75.3, 60.1, and 48.1 %) were significantly lower than those of the group 2 at 1, 2, and 3 years (63.5, 43.6, and 27.4 %) ($P < 0.001$)

patients was well matched. In particular, all covariates that significantly affected overall survival in the entire study group were equally distributed over the 2 matched groups. Matched patients in groups 1 and 2 had similar anti-HCV antibody positivity (72.3 vs. 69.2 %; $P = 0.699$), PT activity (85.6 vs. 83.8 %; $P = 0.424$), ICG-R15 (18.4 vs. 18.5 %; $P = 0.958$), serum AFP levels (884.7 vs. 410.8 ng/ml; $P = 0.778$), maximum tumor diameter (33.1 vs. 34.0 mm; $P = 0.407$), number of tumors (1.65 vs. 1.63; $P = 0.529$), and microvascular invasion (24.6 vs. 26.1 %; $P = 0.840$). Other clinical variables and tumor characteristics were also similar in both groups. The preoperative ATIII level of patients in group 1 was significantly higher than that of patients in group 2 (82.5 vs. 62.3 %; $P < 0.001$). The postoperative course of the matched study groups is shown in Table 2. Postoperative complications did not differ between the 2 groups. The mean follow-up period ± standard deviation of groups 1 and 2 was 37.8 ± 36.2 and 34.3 ± 31.0 months, respectively. The overall survival rate of patients in group 1 was significantly higher than that of patients in group 2 ($P = 0.005$): in group 1, the 3-, and 5-year overall survival rates were 92.5 and 83.4 % respectively, whereas in group 2, they were 75.8 and 57.1 %, respectively (Fig. 2a). Furthermore, the disease-free survival rate of patients in group 1 was significantly higher than that of patients in group 2 ($P = 0.012$): the 1-, 2-, and 3-year disease-free survival rates were 74.3, 52.6, and 37.0 %, respectively, in group 1 and 55.6, 43.0, and 29.1 %, respectively, in group 2 (Fig. 2b).

Table 2 shows the patterns of cancer recurrence and the treatment details of the recurrences in both groups. The overall recurrence rate in group 1 tended to be lower than that of group 2 (58.5 vs. 73.8 %; $P = 0.064$). Regarding treatment for HCC recurrence, the proportion of patients in whom repeat hepatectomy was selected for treatment in group 1 tended to be higher than that in group 2 ($P = 0.093$). Furthermore, the proportion of patients in whom TACE was selected for treatment in group 1 was significantly lower than that in group 2 ($P = 0.041$).

Table 3 shows the results from the univariate and multivariate analyses of prognostic factors for overall survival in the whole study. Factors found to be significant in the univariate analysis were PT activity, serum ATIII level, serum albumin level, Child–Pugh grade, extension of hepatectomy, operation time, transfusion, serum AFP level, multiple tumors, tumor size, and microscopic vascular invasion. Multivariate analysis revealed that PT activity, serum ATIII level, serum AFP level, multiple tumors, and microscopic vascular invasion were the independent prognostic factors of overall survival. Table 4 shows the results from the univariate and multivariate analyses of prognostic factors for disease-free survival in the whole study. Factors found to be significant in the univariate analysis include HCV antibody, PT activity, serum ATIII level, serum total bilirubin level, serum albumin level, ICG-R15, Child–Pugh grade, operation time, serum AFP level, multiple tumors, and microscopic vascular invasion. Multivariate analysis revealed that PT activity, serum

TABLE 2 Follow-up data including postoperative complications after curative hepatectomy

Characteristic	Whole study series			Propensity matched series		
	ATIII (≥70 U/ml) (n = 314)	ATIII (<70 U/ml) (n = 126)	P	ATIII (≥70 U/ml) (n = 65)	ATIII (<70 U/ml) (n = 65)	P
Mean follow-up duration (years)	4.09 ± 2.87	3.55 ± 2.30		4.3 ± 2.56	3.46 ± 2.21	
Operative complications						
Clavien–Dindo grade[a]			0.3			1
IIIa	12 (3.8 %)	6 (4.8 %)		2	2	
IIIb	4 (1.3 %)	2 (1.6 %)		0	0	
IVa	0	2 (1.6 %)		0	0	
IVb	2 (0.6 %)	0		0	0	
V	1 (0.3 %)	1 (0.8 %)		0	0	
90-day mortality	10 (3.2 %)	5 (4.0 %)	0.68	2 (3.1 %)	4 (6.2 %)	0.4
Overall recurrence	168 (53.5 %)	89 (70.6 %)	<0.001	38 (58.5 %)	48 (73.8 %)	0.064
First recurrence time (years)	1.83 ± 1.76	1.59 ± 1.57	0.28	1.63 ± 1.40	1.43 ± 1.46	0.52
Recurrence pattern[a]						
Intrahepatic	130 (41.4 %)	81 (64.3 %)	<0.001	34 (52.3 %)	44 (67.7 %)	0.073
Single	58 (18.5 %)	40 (31.7 %)	0.002	14 (21.5 %)	23 (35.4 %)	0.08
Multiple	72 (22.9 %)	41 (32.5 %)	0.037	20 (30.8 %)	21 (32.3 %)	0.85
Extrahepatic	38 (12.1 %)	8 (6.3 %)	0.075	4 (6.2 %)	4 (6.2 %)	1
Main treatment for first recurrence[b]						
Hepatectomy	43 (25.6 %)	14 (15.7 %)	0.07	16 (42.1 %)	12 (25.0 %)	0.093
RFA	28 (16.7 %)	20 (22.5 %)	0.256	7(18.4 %)	10 (20.8 %)	0.78
PEI	7 (4.2 %)	3 (3.4 %)	0.754	4 (10.5 %)	3 (6.3 %)	0.471
TACE	55 (32.7 %)	36 (40.4 %)	0.546	6 (15.8 %)	17 (35.4 %)	0.041
LDLT	2 (1.2 %)	5 (5.6 %)	0.038	1 (2.6 %)	1 (2.1 %)	0.867
Other	22 (13.1 %)	5 (5.6 %)	0.063	1 (2.6 %)	3 (6.3 %)	0.429
No treatment	11 (6.5 %)	6 (6.7 %)	0.953	0	2 (4.2 %)	0.203

ATIII serum antithrombin III, *RFA* radiofrequency ablation, *PEI* percutaneous ethanol injection, *TACE* transcatheter arterial chemoembolization, *LDLT* living donor liver transplantation

[a] Data are expressed as the number of patients (percentage of total patients)

[b] Data are expressed as the number of patients (percentage of patients with recurrence)

ATIII level, serum AFP level, multiple tumors, and microscopic vascular invasion were the independent prognostic factors of overall survival.

DISCUSSION

To our knowledge, this is the first study that investigates the influence of ATIII on HCC patients using propensity score analysis. The present study demonstrated that when other prognostic variables were appropriately adjusted for, overall and disease-free survival after hepatectomy was significantly prolonged in HCC patients with high preoperative levels of ATIII. Therefore, a low preoperative level of ATIII may be considered a risk factor for tumor recurrence and prognosis. The results of this study are in agreement with certain studies, which showed that a decrease in plasma ATIII levels was a risk factor for tumor recurrence and prognosis in patients with several cancers including lung, colon, ovary, and prostate cancers.[6–8]

The serpin ATIII controls a number of important coagulation enzymes, including factor Xa and thrombin, with the aid of its cofactor, heparin. Heparin activates antithrombin by inducing conformational changes in the protein that specifically enhances binding. While the classical function of ATIII is of an anticoagulant regulator of blood clotting proteinases such as thrombin, recent studies demonstrate its ability to attenuate inflammatory responses by inhibiting cytokines and other inflammatory mediators found within serum and tissue.[3] ATIII has also been reported to suppress the invasion and metastasis of several cancers. Recent studies by Kurata et al.[21] indicate that ATIII prevented hepatic ischemia/reperfusion-induced metastasis of colon cancer cells in a rat model by blocking tumor necrosis factor alpha production. Macrophage inhibitory factor (MIF) has been known to be associated

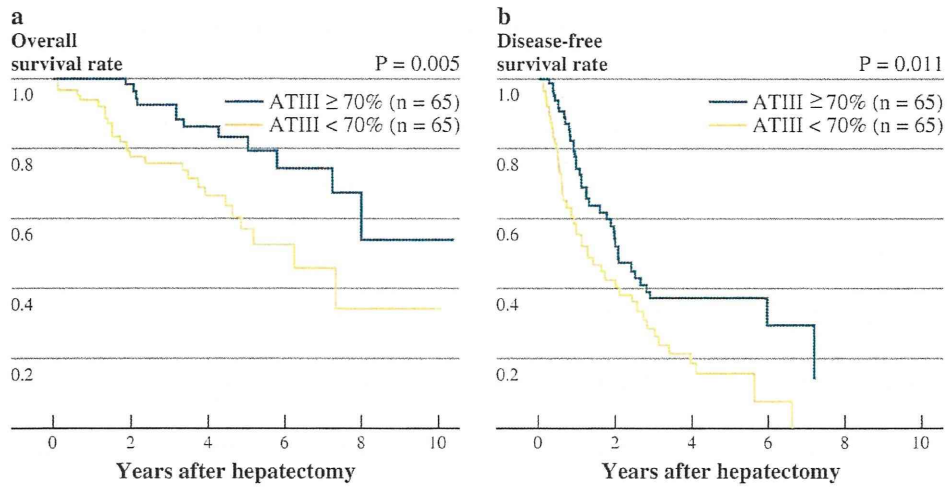

FIG. 2 Outcomes of the matched study population of 130 patients who received liver resection for HCC by stratified with the level of ATIII. **a** Kaplan–Meier curves for the overall survival rate after hepatectomy. Overall survival rates of HCC patients with serum ATIII level of >70 IU/ml (group 1, $n = 65$) at 3 and 5 years (92.5 and 83.4 %, respectively) were significantly lower than those of the serum ATIII level of <70 IU/ml (group 2, $n = 65$) at 3 and 5 years (75.8 and 57.1 %, respectively) ($P = 0.005$). **b** Kaplan–Meier curves for the disease-free survival rate after hepatectomy. Disease-free survival rates of group 1 at 1, 2, and 3 years (74.3, 52.6, and 37.0 %) were significantly lower than those of the group 2 at 1, 2, and 3 years (55.6, 43.0, and 29.1 %) ($P = 0.012$)

with some cancer cell proliferation and invasion. ATIII has been identified as an endogenous MIF-binding protein by forming ATIII–MIF complexes, which reduces MIF biological activity.[22] Recent evidence has shown that thrombin contributes to a more malignant phenotype in vivo by activating tumor-platelet adhesion, tumor adhesion to the subendothelial matrix, tumor implantation, tumor growth, and tumor-associated angiogenesis.[23–25] Kaufmann et al.[26] have shown that some HCC cell lines express a thrombin receptor, proteinase-activated receptor (PAR), and a thrombin-induced increase in HCC cell migration by mediating PAR. Rullier et al.[27] have shown that PAR-1 positive tumor cells are found in HCC. These results suggest that ATIII can suppress proliferation and migration of HCC cells by inhibiting thrombin-induced tumor growth and angiogenesis. It has been also shown that the expression of osteopontin is increased significantly in HCC, and is closely associated with poor prognosis, early recurrence, and metastasis.[28,29] Thrombin cleaves osteopontin into 2 fragments of approximately equivalent size. Osteopontin fragments generated by thrombin cleavage enhance proliferation and adhesion of HCC cells through the activation of integrin β-focal adhesion kinase signaling.[30] Thrombin has been shown to contribute to tumor progression in both a coagulation-dependent and coagulation-independent manner.[31] Further basic and clinical studies are needed to elucidate the antitumor mechanisms of ATIII.

In this study, repeat hepatectomy rather than TACE was selected as a recurrence treatment in more patients with normal level of ATIII, while more patients with decreased level of ATIII underwent TACE rather than hepatectomy for recurrence. This result was thought to be due to high occurrence of early recurrence within 1 year of surgery in patients with decreased level of ATIII. Many cases of recurrence within 1 year after primary hepatectomy are thought to be intrahepatic metastasis from the primary HCC, and survival rate in patients with early recurrence showed worse outcome.[32,33] In our series, most patients who had early recurrence within 1 year of primary hepatectomy were unlikely to receive repeat hepatectomy. Portolani et al.[2] have reported that curative treatment including surgery, percutaneous ethanol injection, and radiofrequency ablation, was feasible in 29.3 % in the early recurrence, while it was 67.6 % in the late recurrence: the proportion of patients who underwent curative treatment for HCC recurrence was significantly higher in the late recurrence than in the early recurrence ($P < 0.05$).

In this study, we have chosen the cutoff for ATIII based on the lower limit of normal level. The optimal cutoff values of ATIII for survival and recurrence determined by ROC curve analysis were 69 and 72 %, respectively. These results indicate that the cutoff value of ATIII with 70 % is valid in this study, and these results are consistent with that normal ATIII is defined as a level of ≥70 %.

Before matching by using the propensity score, the clinical characteristics of the entire study population that can strongly influence outcomes differed significantly between the 2 groups. The proportion of patients with better liver function was higher in group 1 than in group 2, and the proportion of patients with advanced HCC also

TABLE 3 Univariate and multivariate analysis of predictive variables of overall survival in the whole study

Variable	Univariate analysis		Multivariate analysis		
	5-year survival rate (%)	*P*	Hazard ratio	95 % CI	*P*
Age					
≥70 ($n = 167$); <70 ($n = 273$)	68.2; 69.7	0.959			
Gender					
Male ($n = 323$); female ($n = 117$)	69.9; 68.9	0.646			
Anti-HCV antibody					
Positive ($n = 279$); negative ($n = 161$)	68.5; 70.1	0.357			
Prothrombin time (%)					
≥80 ($n = 307$); <80 ($n = 133$)	77.0; 56.7	<0.001	1.62	1.069–2.457	0.023
ATIII (%)					
≥70 ($n = 314$); <70 ($n = 126$)	75.8; 53.1	<0.001	1.596	1.012–2.516	0.044
T-Bil (mg/dl)					
≤1.0 ($n = 307$); >1.0 ($n = 133$)	72.1; 63.0	0.196			
Albumin (g/dl)					
≥4.0 ($n = 332$); <4.0 ($n = 108$)	71.7; 61.7	0.048	1.034	0.621–1.719	0.898
ICG-R15 (%)					
>15 ($n = 213$); ≥15 ($n = 227$)	73.2; 65.6	0.093	1.088	0.729–1.623	0.681
Child–Pugh grade					
A ($n = 399$); B ($n = 41$)	71.9/50.8	0.001	0.792	0.418–1.501	0.475
Extend of hepatic resection					
Major ($n = 373$); minor ($n = 67$)	70.5; 61.2	0.023	0.751	0.449–1.283	0.237
Operation time (h)					
≥6 ($n = 280$); >6 ($n = 180$)	73.2; 62.5	0.008	0.844	0.553–1.289	0.433
Blood loss (ml)					
<1,000 ($n = 34$); ≥1,000 ($n = 406$)	69.9; 60.2	0.065	1.3	0.607–3.402	0.414
Transfusion					
No ($n = 409$); yes ($n = 31$)	71.2; 40.0	<0.001	0.641	0.293–1.296	0.122
AFP (ng/ml)					
≤100 ($n = 313$); >100 ($n = 127$)	76.2; 53.2	<0.001	0.571	0.417–0.924	0.004
No. of tumor					
Single ($n = 286$); multiple ($n = 154$)	77.0; 54.7	<0.001	0.532	0.386–0.824	0.001
Tumor size (5 cm)					
≥5 ($n = 365$); <5 ($n = 75$)	70.8; 62.3	0.033	0.917	0.505–1.664	0.776
Vascular invasion					
No ($n = 319$); yes ($n = 121$)	76.1; 51.8	<0.001	2.05	1.303–2.901	0.0001

HCV hepatitis C virus, *ATIII* anti–thrombin III, *T-Bil* total bilirubin, *ICG-R15* indocyanine green retention rate at 15 min, *AFP* alfa-fetoprotein

tended to be higher in the group 1 than in the group 2. To overcome bias due to the different distribution of the severity of liver function impairment between the 2 groups, a one-to-one match was created using propensity score analysis. After matching by propensity score, prognostic variables were appropriately handled, and there was no significant difference in prognostic factors excluding ATIII between the 2 matched groups. This study had a limitation related to the small sample size after propensity score matching. Two hundred forty-nine patients in group 1 and 61 patients in group 2 were excluded by propensity score matching, because their propensity scores could not be matched. Thus, further examination with a larger number of patients may be necessary.

Multivariate analysis agreed with that in previous publications, showing that vascular invasion, multiple tumors, and tumor marker such as AFP were independent prognostic factors associated with overall and disease-free survival rates. These results were compatible with previous reports.[34,35] Regarding with liver function, PT activity and

TABLE 4 Univariate and multivariate analysis of predictive variables of disease-free survival in the whole study

Variable	Univariate analysis		Multivariate analysis		
	5-year survival rate (%)	*P*	Hazard ratio	95 % CI	*P*
Age					
≥70 (n = 167); <70 (n = 273)	36.4; 39.7	0.377			
Gender					
Male (n = 323); female (n = 117)	36.6; 39.2	0.357			
Anti-HCV antibody					
Positive (n = 279); negative (n = 161)	34.4; 45.9	0.0164	0.728	0.482–1.101	0.133
Prothrombin time (%)					
≥80 (n = 307); <80 (n = 133)	47.5; 20.5	<0.001	1.621	1.074–2.447	0.021
ATIII (%)					
≥70 (n = 314); <70 (n = 126)	45.7; 20.0	<0.001	1.596	1.012–2.516	0.044
T-Bil (mg/dl)					
≤1.0 (n = 307); >1.0 (n = 133)	43.1; 29.1	0.023	1.009	0.677–1.505	0.965
Albumin (g/dl)					
≥4.0 (n = 332); <4.0 (n = 108)	42.3; 27.4	<0.001	1.043	0.630–1.727	0.871
ICG-R15 (%)					
>15 (n = 213); ≥15 (n = 227)	46.9; 30.3	<0.001	1.088	0.729–1.623	0.681
Child–Pugh grade					
A (n = 399); B (n = 41)	40.6; 21.3	0.034	0.518	0.438–1.517	0.518
Extent of hepatic resection					
Major (n = 373); minor (n = 67)	37.5; 43.6	0.349			
Operation time (h)					
≥6 (n = 280); >6 (n = 180)	40.1; 35.8	0.018	0.84	0.559–1.261	0.4
Blood loss (ml)					
<1000 (n = 34); ≥1000 (n = 406)	40.6; 38.4	0.276			
Transfusion					
No (n = 409); yes (n = 31)	41.0; 38.4	0.262			
AFP (ng/ml)					
≤100 (n = 313); >100 (n = 127)	39.4; 27.0	0.004	0.568	0.386–0.836	0.004
No. of tumors					
Single (n = 286); multiple (n = 154)	43.9; 29.1	<0.001	0.555	0.379–0.811	0.002
Tumor size (5 cm)					
≥5 (n = 365); <5 (n = 75)	37.7; 48.0	0.372			
Vascular invasion					
No (n = 319); yes (n = 121)	76.1; 39.2; 28.751.8	0.004	2.031	1.368–3.015	0.0001

HCV hepatitis C virus, *ATIII* anti–thrombin III, *T-Bil* total bilirubin, *ICG-R15* indocyanine green retention rate at 15 minutes, *AFP* alfa-fetoprotein

ATIII level were significant factors in multivariate analysis. Operative variables such as extension of hepatectomy, blood loss, and transfusion, were associated with poor outcomes in univariate analyses, but these factors were not significant factors in the multivariate analysis.

In conclusion, one-to-one matching study using propensity scores and multivariate analysis showed that the ATIII level was associated with favorable outcomes in HCC patients after curative hepatectomy. ATIII may be useful for predicting outcomes of patients with HCC after curative hepatectomy.

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Safety and Feasibility of Diet-Treated Donors With Steatotic Livers at the Initial Consultation for Living-Donor Liver Transplantation

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Background. The purpose of this study was to evaluate both safety of diet-treated donors and the feasibility of their use for living-donor liver transplantation (LDLT).

Methods. A total of 128 living donors were enrolled in this study between April 2003 and March 2010. Of them, 41 were diagnosed with hepatic steatosis at the initial consultation. Donor selection was based on the findings of liver biopsy accompanied with normalization of liver function tests after diet treatment consisting of an 800 to 1400 kcal/day diet and a 100 to 400 kcal/day exercise without drug treatment, targeting body mass index of 22 kg/m^2.

Results. Body mass index of diet-treated donors was significantly reduced with diet from 23.3±0.6 to 21.9±0.4 kg/m^2 (P<0.0001). Liver function tests associated with fatty liver, including alanine aminotransferase, gamma-glutamyl transpeptidase, and total cholesterol levels, also improved with diet (P=0.0128, 0.0016, and 0.0004, respectively). The liver biopsy results of most of these donors showed stage 0/1 fibrosis and minimal/mild steatosis after the diet therapy. Surgical outcomes, including postoperative liver function tests, perioperative complications, and liver regeneration rates, did not significantly differ between nondiet-treated and diet-treated donors. Surgical outcomes and the overall survival did not significantly differ between recipients of grafts from nondiet-treated and diet-treated donors.

Conclusion. The use of diet-treated donors for living-donor liver transplantation is feasible with respect to donor safety and the outcome of the recipient when strict selection criteria are used.

Keywords: Diet, Steatosis, Living donor liver transplantation, Biopsy, Fatty liver.

(*Transplantation* 2012;93: 1024–1030)

Liver transplantation is the only treatment option for patients with end-stage liver disease. However, the shortage of organs remains a serious problem, and annual death rates per 1000 patient-years at risk is 113.6 while on the waiting list (United Network for Organ Sharing at www.unos.org. accessed in May 2009). Many liver transplantation centers have been forced to modify their criteria for acceptable donors to increase the donor pool. A modified extended criteria donor has been applied to deceased-donor liver transplantation (DDLT), including older donors, donors with prolonged ischemia, donation after cardiac death, those with liver infected with certain viruses, obese donors, and those with steatotic (fatty) livers (*1*).

Implantation of donor livers with severe fatty infiltration is frequently associated with a high incidence of severe ischemic damage, resulting in primary dysfunction and/or primary nonfunction after DDLT (*2–6*).

Meanwhile, living-donor liver transplantation (LDLT) has been accepted and established as an alternative to DDLT (*7*) since it was first successfully performed in 1989 (*8*). Soejima et al. (*9*) described the feasibility of using a steatotic graft even in LDLT with respect to primary nonfunction and reported the effectiveness of short-term treatments consisting of a protein-rich diet, exercise, and bezafibrate for 2 to 8 weeks for donors with a fatty liver (*10*). The obvious advantages of LDLT are the reduction in the mortality of patients on the transplant waiting list and the provision of sufficient preparation time, which is a great merit in scheduling the transplantation (*11, 12*). In our institute, a candidate of living donors with a fatty liver at the initial

This work was supported by Grants-in-Aid for Scientific Research No. 21791289 from the Japan Society for the Promotion of Science.
The authors declare no conflicts of interest.
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Received 19 April 2011. Revision requested 10 May 2011.
Accepted 23 January 2012.

ISSN: 0041-1337/12/9310-1024
DOI: 10.1097/TP.0b013e31824c9e25

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.

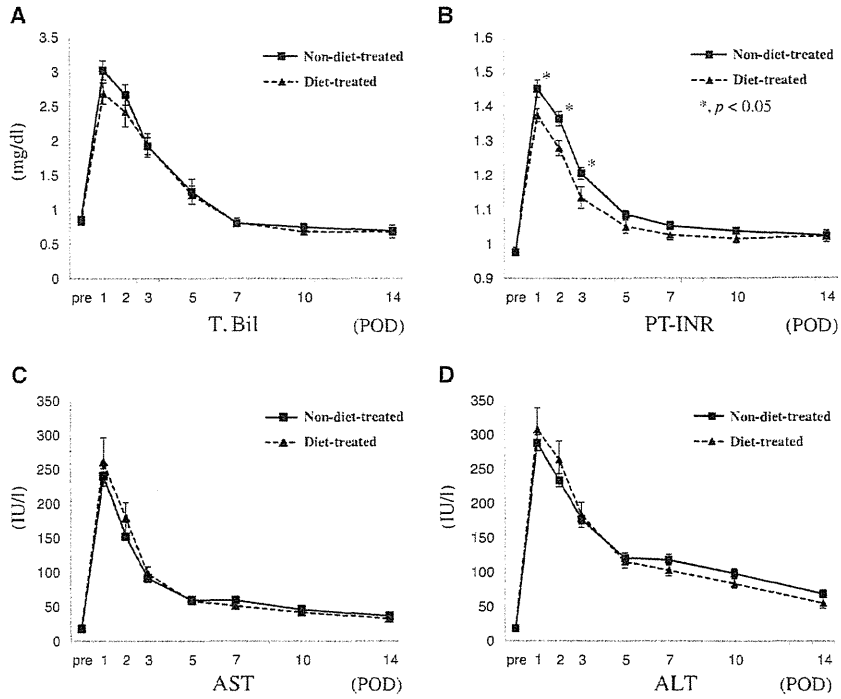

FIGURE 1. Perioperative data on donors. (A) T. Bil. (B) PT-INR. (C) AST. (D) ALT. T. Bil, total bilirubin; PT-INR, prothrombin time-international normalized ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

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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Cell Transplantation, Vol. 21, pp. 1397–1406, 2012
Printed in the USA.

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