Table 2 Operative and postoperative characteristics of the 2 groups of patients with HCC

Variable	Diet group (n = 26)	Exercise group (n = 25)	*P*
Surgical procedure (limited/anatomic)	16/10	14/11	.6879
Operating time (min)	326 ± 115	338 ± 152	.7404
Operative blood loss (mL)	986 ± 1,129	1,219 ± 1,767	.5811
Blood transfusion (yes/no)	3/23	4/21	.6435
Tumor size (cm)	4.10 ± 2.29	5.53 ± 5.33	.2230
Number of tumors	1.42 ± .70	1.40 ± .65	.9034
Histology (good/moderate/poor)	6/18/2	5/20/0	.3367
Microscopic capsule formation (yes/no)	21/5	22/3	.5466
Microscopic vascular invasion (yes/no)	14/12	12/13	.6763
Microscopic surgical margin (yes/no)	2/24	2/23	.9674
Associated liver disease (normal/fibrosis or hepatitis/cirrhosis)	3/14/9	5/11/9	.6568
Tumor stage (I or II/III or IV)	21/5	19/6	.6789
Morbidity (yes/no)	3/23	2/23	.6710
Mortality (yes/no)	0/26	0/25	
Postoperative hospital stay (d)	17.5 ± 11.3	13.7 ± 4.0	.1200

Data are expressed as mean ± SD or as numbers of patients.

changes in whole body fat-free mass ("muscle mass") in either group. Laboratory tests revealed that the platelet count at 6 months was significantly higher in the high-frequency group than in the standard group. Both fasting serum insulin and HOMA-IR showed significant decreases in the high-frequency group compared with the standard group. The branched-chain amino acid/tyrosine ratio was also significantly increased in the high-frequency group.

Comments

Rest has traditionally been considered essential for patients with chronic liver disease, because hard exercise causes a decrease in hepatic blood flow. However, attention has recently been paid to the negative effect of reduced muscle mass due to lack of exercise in patients with chronic liver disease.[32] In fact, when patients with liver disease performed exercise, there was no clinically important deterioration in hepatic function, and a slight improvement was actually observed.[33,34] It is known that physical activity reduces the risk for breast cancer and colon cancer and may also reduce the risk for several other types of cancer.[35–39] Regular exercise plays an important role in helping maintain a healthy body weight, while excess weight increases the circulating levels of estrogens, androgens, and insulin, all of which are associated with cell proliferation and tumor growth.[40] Physical activity may reduce the risk for cancer by decreasing circulating levels of insulin and insulin-like growth factors and by improving energy metabolism. Physical activity also helps prevent the occurrence of type 2 diabetes, which is associated with an increased risk for cancer of the colon, pancreatic cancer, and possibly other tumors.[41–44]

Table 3 Physiologic parameters of the 2 groups of patients with HCC

Variable	Whole body	Trunk	Waist	Hip
Body mass at 6 mo (% of baseline)				
Diet group	100 ± 4	99 ± 7	102 ± 8	99 ± 8
Exercise group	95 ± 5	95 ± 5	93 ± 7	96 ± 3
P	.0375*	.1119	.0028*	.0894
Fat mass at 6 mo (% of baseline)				
Diet group	97 ± 18	96 ± 18	99 ± 18	96 ± 17
Exercise group	86 ± 14	84 ± 15	82 ± 22	89 ± 14
P	.0685	.0595	.0365*	.2268
Fat-free mass at 6 mo (% of baseline)				
Diet group	100 ± 6	101 ± 7	105 ± 9	101 ± 9
Exercise group	101 ± 6	102 ± 9	102 ± 15	101 ± 7
P	.6492	.7422	.4822	.9479
Bone mineral density at 6 mo (% of baseline)				
Diet group	98 ± 3	95 ± 8	103 ± 12	92 ± 26
Exercise group	98 ± 3	97 ± 9	100 ± 17	98 ± 5
P	.9923	.6309	.6009	.4119

*Statistically significant ($P < .05$).

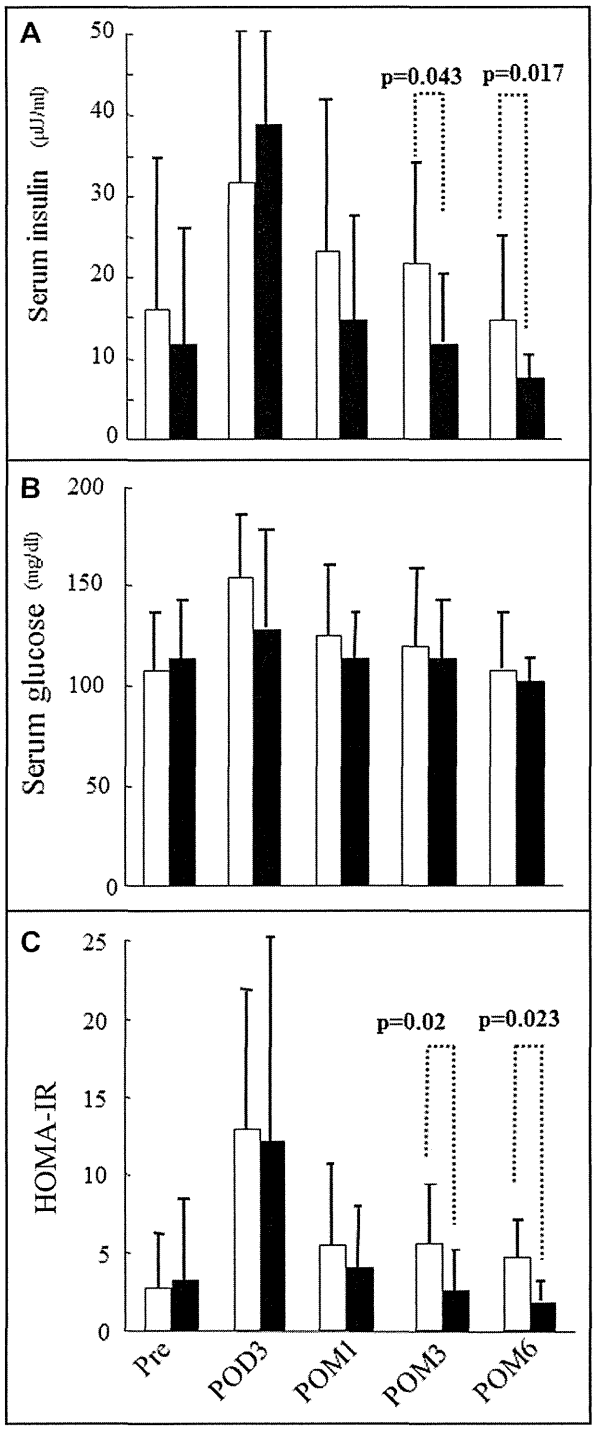

Figure 3 Effect of exercise on insulin resistance in patients with HCC with hepatic impairment. (A) Serum free insulin, (B) serum glucose, and (C) HOMA-IR in the diet group (white bars) and the exercise group (black bars). POD = postoperative day; POM = postoperative month.

However, there have been few reports about the effects of long-term exercise, and there have been no studies of perioperative exercise in patients with chronic hepatitis or cirrhosis undergoing hepatectomy for HCC. In the present study, exercise was started at approximately 1 month preoperatively, resumed approximately 1 week after surgery, and continued for 6 months at an intensity based on the AT of each patient. Each patient performed >3 60-minute exercise sessions weekly (mainly on a bicycle ergometer during hospitalization and by walking after discharge), and also received dietary guidance at 1, 3, and 6 months. Exercises mainly targeted the lower body, while upper-body muscle training was started from 2 months postoperatively to avoid wound pain. We hypothesized that exercise at the AT would reduce visceral fat and prevent loss of muscle mass. We found that the whole body mass and the fat mass at the waist both decreased significantly in the exercise group, while the skeletal muscle mass showed little change in either group (Table 3). No significant differences were detected between the 2 groups with respect to laboratory data such as lipids or rapid turnover proteins. None of the patients showed an increase of transaminases with exercise, and there were no clinical problems in the patients who performed exercise >3 times a week. At 3 and 6 months postoperatively, both serum insulin and the insulin resistance index showed significant improvement in the exercise group (Fig. 3).

It was recently reported that hepatic impairment, particularly cirrhosis, leads to secondary insulin resistance and hyperinsulinemia, which can then promote carcinogenesis. Management of insulin resistance is thus a critical issue for patients with chronic liver disease both to protect liver function and to prevent hepatocarcinogenesis. Alterations in glucose metabolism also affect fat metabolism, which may result in the excess production of lipid peroxide and reactive oxygen species,[45,46] which in turn damages hepatocytes,[17] leading to possible development of HCC.[46] However, it is difficult to manage insulin resistance associated with chronic liver disease because restriction of calorie intake conflicts with the need to overcome malnutrition arising from hepatocellular damage. A reduction in body weight with exercise has been reported to be advantageous in obese patients with chronic liver disease.[33] The present study had a short postoperative observation period, so no differences in the recurrence of HCC or of mortality were detected between the 2 groups (data not shown), but longer follow-up is necessary to confirm this finding.

When we divided the exercise group into standard and high-frequency subgroups on the basis of the weekly number of exercise sessions, AT Vo_2 and peak Vo_2 were significantly higher in the high-frequency group after 6 months. In addition, whole body mass and fat mass were significantly lower in the high-frequency group compared with the standard group at 6 months. Furthermore, the platelet count and branched-chain amino acid/tyrosine ratio were increased and the serum insulin/insulin resistance index ratio was significantly improved in the high-frequency group.

Table 4 Comparison of exercise tests, DEXA parameters, and laboratory data between the standard and high-frequency exercise subgroups

Variable	Standard (n = 11)	High frequency (n = 14)	*P*
Exercise parameters			
AT Vo_2 at 6 mo (% of baseline)	102 ± 14	115 ± 18	.0379*
Peak Vo_2 at 6 mo (% of baseline)	103 ± 12	118 ± 11	.0015*
DEXA parameters			
Whole body mass at 6 mo (% of baseline)	97 ± 4	93 ± 6	.0314*
Whole body fat mass at 6 mo (% of baseline)	98 ± 16	80 ± 15	.0075*
Whole body fat-free mass at 6 mo (% of baseline)	98 ± 6	103 ± 5	.0628
Laboratory data			
Albumin (g/dL)			
Pre	3.79 ± .52	3.98 ± .37	.3077
Post	4.09 ± .38	4.13 ± .31	.8055
Platelet count ($\times 10^4/\mu L$)			
Pre	18.8 ± 10.8	17.6 ± 4.9	.7362
Post	11.3 ± 2.5	14.8 ± 4.6	.0288*
ALT (U/L)			
Pre	56 ± 22	45 ± 37	.3898
Post	35 ± 33	31 ± 27	.7529
Insulin (μU/mL)			
Pre	9.1 ± 4.5	8.5 ± 5.8	.8099
Post	10.7 ± 4.6	5.8 ± 2.8	.0193*
Glucose (mg/dL)			
Pre	111 ± 16	117 ± 24	.5079
Post	101 ± 20	102 ± 10	.8991
HOMA-IR			
Pre	2.43 ± 1.12	2.59 ± 2.21	.8488
Post	2.71 ± 1.23	1.47 ± .68	.0232*
BCAA			
Pre	438 ± 102	462 ± 58	.4958
Post	474 ± 114	604 ± 214	.0960
AAA			
Pre	91 ± 19	80 ± 16	.1888
Post	102 ± 26	87 ± 13	.1184
BTR			
Pre	5.08 ± .84	5.43 ± .90	.4263
Post	4.78 ± 1.02	6.92 ± 2.20	.0091*

Data are expressed as mean ± SD.

AAA = aromatic amino acids; ALT = alanine aminotransferase; BCAA = branched-chain amino acids; BTR = branched-chain amino acid/tyrosine ratio; DEXA = dual-energy x-ray absorptiometric; HOMA-IR = homeostasis model of assessment of insulin resistance; Vo_2 = oxygen consumption.

*Statistically significant ($P < .05$).

One limitation of the present study was the selection of patients. The patients taking part in the study were less sick than many patients who might undergo this protocol, and patients with more advanced disease may not respond as well.

In conclusion, perioperative and postoperative exercise for patients with HCC with hepatic impairment led to weight loss (because of a decrease in fat mass) and improvement in insulin resistance but had no effect on skeletal muscle mass. Maintenance of postoperative physical strength and earlier resumption of daily activities could be possible by intensifying perioperative and postoperative exercise. We recommended that patients continue to exercise for 6 months after surgery. However, because some failed to continue to exercise long term for various reasons, patients should be followed up carefully after hepatectomy and encouraged to perform continuous long-term exercise.

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Clinicopathological Features of Recurrence in Patients After 10-year Disease-free Survival Following Curative Hepatic Resection of Hepatocellular Carcinoma

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Abstract
Background The present study aimed to clarify the clinicopathologic features of long-term disease-fee survival after resection of hepatocellular carcinoma (HCC).
Methods This retrospective study identified 940 patients who underwent curative resection of HCC between 1991 and 2000 at five university hospitals. Seventy-four patients with 10 years of recurrence-free survival were identified and followed up. They were divided into two groups, 60 recurrence-free and 14 with recurrence after a 10-year recurrence-free period.
Results Overall survival rates of recurrence and non-recurrence groups were 68 and 91 % at 16 years, and 34 and 91 % at 20 years ($p = 0.02$), respectively. There were five (36 %), and two deaths (3 %), respectively, after 10 recurrence-free years. A second resection for recurrence was performed in four patients (29 %), and mean survival was 15.3 years after the first hepatectomy. Although three patients in the non-recurrence group (5 %) developed esophageal and/or gastric varices, seven patients in the recurrence group (50 %) developed varices during 10 years ($p < 0.0001$). In multivariate analysis, preoperative and 10-year platelet count was identified as a favorable independent factor for maintained recurrence-free survival after a 10-year recurrence-free period following curative hepatic resection of HCC.
Conclusions Recurrence of HCC may occur even after a 10-year recurrence-free period. Long-term follow-up after resection of HCC is important, and should be life-long. Patients with higher preoperative and 10-year platelet counts are more likely to have long-term survival after resection. A low platelet count, related to the degree of liver fibrosis, is a risk factor for recurrence and survival of HCC after curative resection.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide [1]. Although the incidence of HCC is highest in Asia and Africa, recent studies have shown that the incidence and mortality rates of HCC are also rising in North America and Europe [2, 3]. There has been an increase in the number of reports of nonsurgical therapeutic options for small HCC, such as percutaneous ethanol injection therapy [4], microwave coagulation therapy [5], and percutaneous radiofrequency ablation (RFA) [6], but there is ongoing controversy regarding the best method to treat small tumors. In Japan, liver transplantation is not a practical option for most HCC patients, because the national health insurance scheme only covers transplantation for patients with decompensated cirrhosis whose

tumors fit the Milan criteria. Resection is therefore generally the first-line treatment for patients with small tumors and underlying chronic liver disease, but the long-term survival rate after potentially curative resection of HCC is still unsatisfactory because of the high rate of recurrence [7]. To improve prognosis, it is important to prevent the recurrence of HCC after its initial resection, but standard therapy for intrahepatic metastasis has not yet been developed. Therefore, the number of reports on long-term survivors after the initial hepatectomy remains small, especially on the basis of long-term (>10 years) observation of patients, with or without recurrence [8–12]. Furthermore, there are few reports regarding the characteristics of patients who survive without recurrence for at least 10 years after curative liver resection. In the present study, we retrospectively analyzed patients with long-term survival and compared them with patients who had recurrence of HCC after a 10-year disease-free period, in order to gain insight into the demography and biological behavior of HCC, and to identify the prognostic factors associated with survival.

Materials and methods

Subjects

Between 1991 and 2000, a total of 940 patients with HCC underwent curative resection (defined as macroscopic removal of all tumors) at five participating university hospitals. Thirty two patients died in the hospital and the remaining 908 were followed up as outpatients. Seventy-four patients (8 %) with 10-year recurrence-free survival after initial hepatectomy were included in this study. The median and mean duration of follow-up for the maximum time or until death was 12.6 and 13.3 years (range: 10.0–21.0 years). The median age of the patients at the initial hepatectomy was 59 years (range: 26–77 years). The 74 patients were stratified into a recurrence group and a recurrence-free group.

Clinicopathologic variables and surgery

Before surgery, each patient underwent conventional liver function tests, measurement of the indocyanine green retention rate at 15 min (ICGR15), and measurement of serum type IV collagen 7S. Hepatitis virus screening was done by measurement of hepatitis B surface antigen (HBsAg) and hepatitis C virus antibody (HCVAb). The levels of alpha-fetoprotein (AFP) and protein induced by vitamin K absence/antagonism-II (PIVKA-II) were also measured in all patients. Surgical procedures were classified according to the Brisbane terminology proposed by Strasberg et al. [13]. Accordingly, anatomic resection was defined as resection of the tumor together with the related portal vein branches and the corresponding hepatic territory, and was classified as hemihepatectomy (resection of half of the liver), extended hemihepatectomy (hemihepatectomy plus removal of additional contiguous segments), sectionectomy (resection of two Couinaud subsegments [14]), or segmentectomy (resection of one Couinaud subsegment). All of the non-anatomically designated procedures were classified as limited resection, and were performed in patients with peripheral or central tumors and moderate liver dysfunction [15]. Patients with peripheral tumors and those with extrahepatic growths were treated by partial hepatectomy because this procedure achieved a sufficient surgical margin. In contrast, central tumors located near the hepatic hilum or major vessels were only treated by enucleation because it was too difficult and/or dangerous to remove enough liver tissue to obtain an adequate margin. A senior pathologist at each of the five participating university hospitals reviewed each specimen for histologic confirmation of the diagnosis. The width of the surgical margin was measured as the distance from the tumor edge to the resection line. The tumor stage was defined according to the TNM classification [16]. Histologic staging of fibrosis and grading of necroinflammation in the underlying liver was performed using the Knodell histologic activity index (HAI) [17] at the initial operation and at the second operation for those requiring repeat hepatectomy.

Follow-up

Perioperative/postoperative complications and deaths were recorded to determine the morbidity and mortality of hepatectomy. All surviving patients were followed up after discharge, with physical examination, liver function tests, ultrasonography, computed tomography (CT), or magnetic resonance imaging being performed at least every 3 months to check for intrahepatic recurrence, with chest radiographs obtained to detect pulmonary metastasis. A chest CT was performed if a chest radiograph showed any abnormalities. Bone metastases were diagnosed by bone scintigraphy.

When recurrence of HCC was detected from changes in tumor markers or from imaging findings, recurrence limited to the remnant liver was treated by transarterial chemoembolization (TACE), lipiodolization, second resection, or percutaneous local ablative therapy such as radiofrequency ablation (RFA). After detection of extrahepatic metastases, active treatment was performed in patients with a good hepatic functional reserve (Child-Pugh class A or B) and good performance status (0 or 1), while other patients were only given radiation therapy for bone metastases to relieve

symptoms. Surgical resection was performed in patients with a solitary extrahepatic metastasis and no intrahepatic recurrence. In addition, all patients underwent endoscopy to evaluate the severity of esophageal and/or gastric varices before surgery and at 10 years after surgery.

Prognostic factors

We performed univariate and multivariate analysis of 28 clinicopathologic operative factors to identify independent variables related to the postoperative recurrence of HCC in patients with 10-year recurrence-free survival after curative hepatic resection. The factors investigated were gender, presence or absence of hepatitis C virus (HCV), and liver function (including albumin, alanine aminotransferase [ALT], and platelet count). The tumor factors studied were alpha fetoprotein (AFP), vitamin K absence or antagonist II (PIVKA-II), and histologic features (including tumor diameter, differentiation, vascular invasion, and associated liver disease). The operative factors that we assessed were operative blood loss, perioperative blood transfusion, and surgical procedure. All of the variables that were significant according to univariate analysis were then examined with a Cox proportional hazards model to identify variables that were independently associated with recurrence.

We also performed univariate and multivariate analysis of six clinical factors to identify independent variables related to postoperative recurrence of HCC more than 10 years after curative hepatic resection. The factors investigated were 10-year liver function (including albumin, aspartate aminotransferase [AST], ALT, and platelet count). The tumor factors studied were AFP and PIVKA-II.

Statistical analysis

Continuous variables are presented as mean ± standard deviation. The significance of differences between the recurrence-free and recurrence groups was assessed by the Chi square test or the Mann–Whitney *U*-test, as appropriate. The Kaplan–Meier method was used to calculate the recurrence rate and overall survival rate as of December 2010, and the significance of differences in survival was estimated with the generalized log-rank test. The Cox regression model (stepwise method) was used for multivariate analysis. In all analyses, $p < 0.05$ was considered to indicate statistical significance.

Results

Preoperative and postoperative characteristics

Table 1 summarizes the preoperative characteristics of recurrence-free and recurrence groups. No difference was detected between the two groups with respect to gender, age, HBsAg, HCVAb, Child-Pugh class, alcohol abuse, preoperative TACE, ICGR15, and serum albumin, total bilirubin, prothrombin time, AST, ALT, AFP, or PIVKA-II. Postoperative characteristics at 10 years after surgery for each group are listed in Table 1. No difference was

Table 1 Preoperative and 10-year clinical characteristics of hepatocellular carcinoma (HCC) recurrence and non-recurrence groups

Variable	Non-recurrence group ($n = 60$)	Recurrence group ($n = 14$)	p value
Gender, male/female	47/13	13/1	0.21
Age, years	58.4 ± 9.8	58.9 ± 8.8	0.85
HBsAg (±)	16/44	3/11	0.69
HCVAb (±)	23/37	3/11	0.23
Child-Pugh class: A/B	57/3	13/1	0.75
Alcohol abuse (±)	19/41	5/9	0.77
Preoperative TACE (±)	36/24	9/5	0.77
ICGR15, %	11.9 ± 5.6	14.8 ± 5.9	0.16
Albumin, g/dL	4.1 ± 0.3	3.9 ± 0.3	0.08
Albumin (10 years), g/dL	4.1 ± 0.3	3.9 ± 0.4	0.10
Total bilirubin, mg/dL	0.79 ± 0.38	0.80 ± 0.24	0.91
Total bilirubin (10 years), mg/dL	0.75 ± 0.20	0.90 ± 0.60	0.81
Prothrombin time, %	100 ± 18	105 ± 28	0.54
Prothrombin time (10 years), %	102 ± 14	99 ± 19	0.90
Platelet count ($\times 10^4/\mu L$)	19.2 ± 7.3	13.3 ± 3.4	0.02
Platelet count (10 years) ($\times 10^4/\mu L$)	15.5 ± 4.1	12.1 ± 4.2	0.03
AST, IU/L	48 ± 44	38 ± 11	0.48
AST (10 years), IU/L	41 ± 26	55 ± 35	0.23
ALT, IU/L	57 ± 56	46 ± 26	0.56
ALT (10 years), IU/L	39 ± 27	63 ± 50	0.08
Type IV collagen 7S (10 years), ng/ml)[a]	4.93 ± 0.84	7.58 ± 0.79	0.002
AFP, ng/ml	311 ± 1198	918 ± 1646	0.12
AFP (10 years), ng/mL	9.3 ± 11.7	56.1 ± 104.1	0.04
PIVKA-II (mAU/mL)	601 ± 1626	38 ± 44	0.40
PIVKA-II (10 years), mAU/mL	25 ± 29	47 ± 61	0.21

Data presented as mean ± standard deviation (SD) or the number of patients. *HBsAg* hepatitis B surface antigen; *HCVAb* hepatitis C virus antibody; *TACE* transcatheter arterial chemoembolization; *ICGR15* indocyanine green retention rate at 15 min; *AST* aspartate aminotransferase; *ALT* alanine aminotransferase; *AFP* α-fetoprotein; *PIVKA-II* protein induced by vitamin K absence/antagonism-II

[a] Indicated data were not available for all patients. The number of patients with the measurement was 34 and 6 in the non-recurrence and recurrence groups, respectively

Table 2 Changes in gastric and/or esophageal varices after 10 years of recurrence-free survival following curative hepatic resection

	Recurrence group ($n = 14$)				Non-recurrence group ($n = 60$)			
10 years after surgery: Before surgery	F0	F1	F2	F3	F0	F1	F2	F3
F0	7 (50 %)	5 (36 %)	0	0	54 (90 %)	3 (5 %)	0	0
F1	0	0	1 (7 %)	0	0	3 (5 %)	0	0
F2	0	0	0	1 (7 %)	0	0	0	0

detected between the two groups with respect to serum albumin, total bilirubin, prothrombin time, AST, ALT, and PIVKA-II. Patients in the non-recurrence group had a significantly higher platelet count preoperatively and at 10 years after surgery compared with those in the recurrence group. Patients in the non-recurrence group had significantly lower levels of type IV collagen 7S at 10 years after surgery compared with those in the recurrence group. Patients in the recurrence group had higher AFP levels at 10 years after surgery compared with those in the non-recurrence group. Table 2 shows the changes in esophageal and/or gastric varices after 10 years of recurrence-free survival following hepatic resection. Seven patients in the recurrence group (50 %) developed varices during the 10 years, while only three patients in the non-recurrence group (5 %) developed varices ($p < 0.0001$).

Surgical results and pathologic classification

The operating time, blood loss, blood transfusion requirement, procedures, and complications attributable to surgery did not differ significantly between the two groups. The pathologic features of each group are presented in Table 3. The number of tumors, tumor diameter, histology, associated liver disease, incidence of positive microscopic capsule formation, surgical margins, vascular invasion, and TNM stage did not differ significantly between the two groups.

Factors that influence recurrence of HCC after curative hepatic resection

Table 4 shows univariate and multivariate analyses of potential operative risk factors associated with recurrence of HCC in patients after a 10-year recurrence-free period following curative resection. Variables significantly associated with recurrence in the univariate and multivariate analyses were preoperative albumin $\leq$4.0 g/dL (odds ratio = 17.86; 95 % confidence interval (CI) = 1.64–42.0; $p = 0.02$), and preoperative platelet count $\leq 15 \times 10^4$/mL (odds ratio = 37.48; 95 % CI = 2.25–52.0; $p = 0.01$).

Table 5 shows univariate and multivariate analyses of potential 10-year risk factors associated with recurrence of HCC in patients after 10 years of recurrence-free survival following curative hepatic resection. Univariate and multivariate analyses showed that only platelet count $\leq 15 \times 10^4$/mL at 10 years after surgery (odds ratio = 12.92;

Table 3 Intraoperative and postoperative characteristics of the two groups

Variable	Non-recurrence group ($n = 60$)	Recurrence group ($n = 14$)	p value
Operating time, min	261 ± 77	284 ± 87	0.38
Operative blood loss, mL	1,095 ± 1129	1,508 ± 1,545	0.26
Blood transfusion (±)	18/42	4/10	0.92
Surgical procedure (Anatomic/limited resection)	32/28	5/9	0.24
Number of tumors (single/multiple)	55/5	13/1	0.88
Tumor size, cm	4.48 ± 3.98	3.16 ± 1.65	0.23
Histology (good/moderate/poor/necrosis)	11/30/15/4	3/5/3/3	0.70
Microscopic capsule formation (±)	50/10	13/1	0.37
Surgical margin (±)	8/52	0/14	0.60
Microscopic vascular invasion (±)	16/44	1/13	0.15
Associated liver disease (normal/fibrosis or hepatitis/cirrhosis)	21/31/8	3/8/3	0.55
TNM stage (I or II/III or IV)	48/12	12/2	0.62
Morbidity (±)	8/52	2/12	0.93

Data presented as mean ± SD or the number of patients

Table 4 Preoperative and operative risk factors predicting HCC recurrence in patients after 10 years of recurrence-free survival following curative hepatic resection

Variable	Univariate analysis			Multivariate analysis		
	Odds ratio	95 % CI	*p* value	Odds ratio	95 % CI	*p* value
Male gender	3.60	0.43–20.1	0.24	–	–	–
HCV Ab	0.58	0.14–2.48	0.46	–	–	–
Albumin ≤4.0 g/dL	8.64	1.01–44.62	0.049	17.86	1.64–42.0	0.02
Platelet ≤15 × 10^4/mL	10.75	1.25–60.11	0.03	37.48	2.25–52.0	0.01
ALT >41 IU/L	2.50	0.55–11.11	0.24	–	–	–
AFP >10 ng/ml	1.33	0.41–4.31	0.63	–	–	–
PIVKA-II >40 mAU/mL	6.25	0.68–30.0	0.10	–	–	–
Operative blood loss >800 mL	3.85	0.15–14.67	0.26	–	–	–
Blood transfusion	3.90	0.85–17.82	0.08	9.56	0.68–33.61	0.09
Limited resection	2.51	0.74–8.48	0.14	–	–	–
Tumor size >3.4 cm	3.23	0.92–11.11	0.07	6.67	0.51–50.0	0.15
Vascular invasion	4.95	0.60–11.67	0.14	–	–	–
Poorly differentiated	0.90	0.22–3.71	0.88	–	–	–
Cirrhosis	0.67	0.13–3.39	0.63	–	–	–

CI confidence interval

Table 5 Risk factors at 10 years predicting HCC recurrence in patients after 10 years of recurrence-free survival following curative hepatic resection

Variable	Univariate analysis			Multivariate analysis		
	Odds ratio	95 % CI	*p* value	Odds ratio	95 % CI	*p* value
Albumin ≤4.0 g/dL	4.50	0.89–22.75	0.07	7.69	0.70–43.33	0.10
Platelet ≤15 × 10^4/mL	6.06	1.11–33.33	0.04	12.92	1.08–63.91	0.04
AST >46 IU/L	1.46	0.34–6.35	0.62	–	–	–
ALT >47 IU/L	1.79	0.40–7.91	0.45	–	–	–
AFP >23 ng/mL	8.57	0.76–26.54	0.08	15.22	0.49–68.99	0.12
PIVKA-II >48 mAU/mL	7.29	0.64–32.64	0.11	–	–	–

95 % CI = 1.08–63.91; $p = 0.04$) was an independent predictor of recurrence of HCC.

In the non-recurrence group, the platelet count decreased at 1 year after curative hepatic resection, but there was no further marked decrease thereafter (Fig. 1). On the other hand, the platelet count in the recurrence group gradually decreased at 5 years after surgery. Patients in the non-recurrence group had a significantly higher platelet count preoperatively and at 7 and 10 years after surgery compared with those in the recurrence group.

Recurrence and survival

Figure 2 shows the recurrence status and therapeutic modalities used for disease recurrence in the 14 patients who had recurrence after a 10-year disease-free period.

Intrahepatic disease recurrence with fewer than three nodules and intrahepatic disease recurrence with more than three nodules were observed in 11 patients (79 %) and three patients (21 %), respectively. None of the patients were found to have an extrahepatic recurrence as the primary recurrence. The median and mean disease-free survival in the 14 patients with HCC recurrence was 11.0 and 11.6 years (range, 10.2–15.7 years), respectively, after the first hepatectomy (Fig. 3). The therapeutic modalities used included repeat hepatectomy in four patients (29 %), RFA in two patients (14 %), TACE in seven patients (50 %), and best supportive care in one patient (7 %). The HAI score in the underlying liver of these four patients changed from 1.0 ± 1.2 at first hepatectomy to 7.8 ± 1.3 at second resection ($p = 0.02$).

In the recurrence group, there were five deaths (36 %), four of which could be attributed to recurrence of HCC.

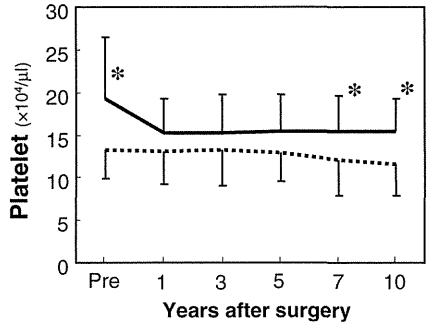

Fig. 1 Platelet count of recurrence and non-recurrence groups after hepatic resection. Data are shown as the mean ± SD. *$p < .05$ versus the recurrence group at the corresponding time

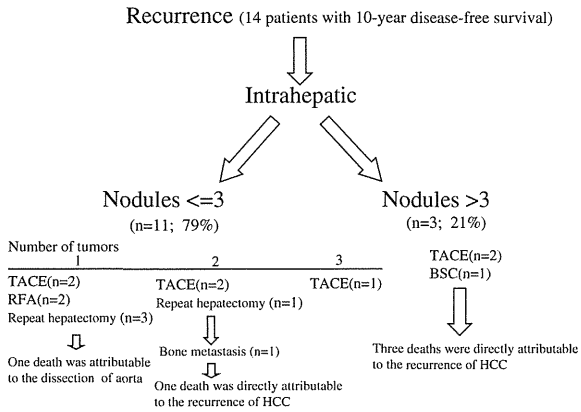

Fig. 2 Hepatocellular carcinoma (HCC) recurrence status and therapeutic modalities for treatment of recurrence in the 14 10-year disease-free survivors in whom late disease recurrence was detected. *RFA* percutaneous radiofrequency ablation; *BSC* best supportive care

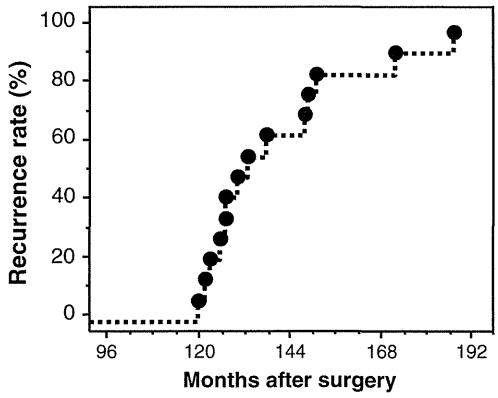

Fig. 3 Recurrence rate in patients with recurrence of HCC. The median and mean disease-free survival in the 14 patients with late disease recurrence was 11.0 and 11.6 years (range: 10.2–15.7 years) after the first hepatectomy

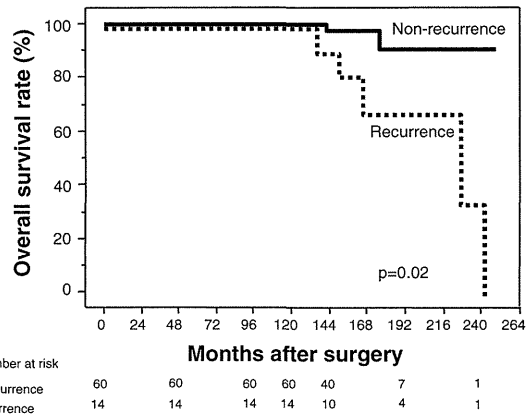

Fig. 4 Comparison of overall survival after hepatectomy in patients with or without recurrence of HCC. The survival rate of the recurrence group (*dotted line*) was significantly poorer than that of the non-recurrence group (*unbroken thin line*; $p = 0.02$). The number of patients at risk is shown below the graph

A second resection was performed in four patients, none of whom died, and median and mean survival was 15.0 and 15.3 years (range, 10.9–20.2 years), respectively, after the first hepatectomy. In the non-recurrence group, two of the 60 patients (3 %) died, with one death attributable to lung cancer at 11.9 years and the other to bile duct cancer at 14.7 years, respectively, after surgery. The overall survival rates of the recurrence and non-recurrence groups were 91 and 97 % at 12 years, 68 and 91 % at 16 years, and 34 and 91 % at 20 years, respectively (Fig. 4). There were significant differences ($p = 0.02$) in survival between the two groups.

Discussion

The recurrence rate of HCC after surgical resection is high, ranging from 50 to 100 % after 5 years in high-volume specialty centers worldwide [18–21]. Recently, close postoperative follow-up to detect recurrent HCC at an early stage, and various management strategies for recurrent HCC (including repeat resection, local ablative therapy, and/or TACE) have contributed to prolonged survival after initial hepatectomy [22–24]. Numerous studies to date have reported cumulative 5-year survival rates of approximately 40–50 %, based largely on relatively short-term follow-up [18, 22, 23, 25–27], and only a few reports of long-term follow-up of more than 10 years have been reported to date. Shimada et al. [11] reported 105 10-year survivors (21.8 %), including 42 disease-free survivors (8.7 %), among 578 patients. Fukuda et al. [12] reported that 29 of 250 patients survived for more than 10 years after initial hepatectomy, and 9 of those patients survived without

cancer recurrence. To our knowledge, there has been no detailed analysis of a large series of HCC patients with more than 10 years disease-free survival after curative hepatic resection. In the current series, 74 patients (8 %) survived for 10 years without disease recurrence after the initial hepatectomy. Fourteen of these patients (19 %) had later disease recurrence and received treatment with one or more treatment modalities. Generally, surgeons think that if there is no recurrence of cancer for 10 years after the initial operation, it should be thought that there will be little likelihood of recurrence of cancer long afterward. Should clinical follow-up then be terminated? From the results of this study, we believe that clinical follow-up after resection of HCC is very important and should continue for the remainder of the patient's life. Intrahepatic recurrence has been suggested to arise from three causes [28]: (1) the continued growth of residual tumor after incomplete excision may cause recurrence near the cut margin and may be responsible for early recurrence; (2) a metachronous and unrecognized synchronous multifocal primary tumor may cause recurrence far from the original site of the lesion and may be responsible for a late recurrence; and (3) a new primary tumor may develop. Because the median disease-free interval between the first operation and recurrence was 11.0 years in this series, and based on the higher incidence of dysplasia in patients with cirrhosis, it might be possible that there was new development of the cancer in these patients.

In multivariate analysis, the preoperative and 10-year platelet counts were identified as favorable independent factors for survival after 10 years of recurrence-free survival after curative hepatic resection of HCC. The platelet count is a simple test, and results can be determined easily by a routine laboratory procedure. In the present study, there was a significantly higher incidence of esophageal and/or gastric varices and serum type IV collagen 7S at 10 years after surgery in the recurrence group. In four patients with repeat hepatectomy for recurrent HCC, histological fibrosis of the underlying liver at the second operation was more advanced than at the first operation. Type IV collagen 7S is known to be useful for quantitative evaluation of liver fibrosis, and is employed for indirect testing of serum samples [17]. A low platelet count at 10 years after surgery, with a higher incidence of varices, higher levels of type IV collagen 7S, and more advanced fibrosis in patients with repeat hepatectomy, reflects the severity of portal hypertension, and indicates development of liver fibrosis in the recurrence group. Moriyama et al. [29] reported that monitoring of platelet counts is useful for determining the development of liver fibrosis in HCV-associated chronic liver diseases and for the determination of a highly carcinogenic state in the liver after interferon therapy. The importance of fibrosis in hepatocarcinogenesis has been described in patients with chronic viral hepatitis [30–32]. The annual incidence of development of HCC increased with the progression of liver fibrosis during long-term follow-up in patients with hepatitis B and C [31, 32]. Fibrosis of the liver develops through repeated necroinflammation and regeneration in the liver of patients with chronic hepatitis, and eventually progresses to liver cirrhosis [33]. Because this process requires vigorous mitosis of the hepatocytes in response to cell destruction, increased fibrosis is associated with a large amount of mitotic activity, which may allow accumulation of genetic transformations [34, 35].

The mechanisms involved in thrombocytopenia, which is observed in patients with chronic hepatitis, have not been fully elucidated. Increased sequestration and destruction of platelets in the enlarged spleen, which is an important mechanism involved in liver cirrhosis and portal hypertension, may not contribute to thrombocytopenia in chronic hepatitis C [36]. Possible mechanisms for thrombocytopenia have been proposed in chronic hepatitis, including decreased production of liver-derived thrombopoietin [37, 38], and increased destruction of platelets by antiplatelet antibodies [39]. Recent accumulated evidence indicates that the thrombocytopenia is caused by an autoimmune mechanism as a result of HCV infection. Nagamine et al. [40] found HCV-RNA in the platelets obtained from HCV-positive patients, indicating the possibility that an association of HCV/anti-HCV antibody immune complexes with the platelet surface can result in platelet antibody immunoglobulin) G (IgG) expression. They also demonstrated that platelet-associated IgG levels increased with the degree of histological progression in patients with chronic hepatitis C. In any case, thrombocytopenia is related to hepatocarcinogenesis through the development of liver fibrosis in patients with chronic hepatitis. It seems likely that a low platelet count is a risk factor for carcinogenesis from chronic hepatitis and for recurrence and survival of HCC after treatment, including liver resection [41–44]. The platelet count may be useful as a marker of late recurrence in HCC patients who survived in the long term.

Our first choice of treatment for recurrence of HCC is principally repeat hepatectomy because this procedure has been accepted as the most effective treatment [45]. However, repeat hepatectomy is limited to patients with resectable intrahepatic recurrence and well-preserved liver function. Our study indicated that nonsurgical treatments, such as RFA or TACE, could contribute to prolongation of survival when repeat hepatectomy is not indicated. Therefore, it is important to select appropriate treatment according to the pattern of recurrence, location of the tumor, and preserved liver function.

In conclusion, our data demonstrated that for patients with higher preoperative and 10-year platelet counts who

underwent curative resection of HCC, long-term survival after resection could be expected. However, because tumor recurrence is common even after 10 years, postoperative follow-up is important and should continue for the remainder of the patient's life. The platelet count is a useful, inexpensive, and convenient marker of late recurrence (later than 10 years following curative resection) of HCC. Finally, aggressive therapy for recurrence, including a second resection when necessary, is recommended.

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Hepatology Research

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Original Article

Interleukin-1β induces tumor necrosis factor-α secretion from rat hepatocytes

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Aim: Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine involved in various inflammatory diseases. The only production of TNF-α in the liver is thought to be from hepatic macrophages known as Kupffer cells, predominantly in response to bacterial lipopolysaccharide (LPS).

Methods: Primary cultured rat hepatocytes were used to analyze TNF-α expression in response to the pro-inflammatory cytokine, interleukin-1β (IL-1β). Livers of rats subjected to LPS-induced endotoxemia were analyzed.

Results: Immunocytochemistry and enzyme-linked immunosorbent assays demonstrated that IL-1β-treated rat hepatocytes secreted TNF-α, and RNA analyses indicated that TNF-α mRNA was induced specifically by IL-1β. Northern blot analysis showed that not only mRNA, but also a natural antisense transcript (asRNA), was transcribed from the rat *Tnf* gene in IL-1β-treated hepatocytes. TNF-α was detected in the hepatocytes of LPS-treated rats. Both TNF-α mRNA and asRNA were expressed in the hepatocytes of LPS-treated rats, human hepatocellular carcinoma and human monocyte/macrophage cells. To disrupt the interaction between TNF-α asRNA and TNF-α mRNA, sense oligonucleotides corresponding to TNF-α mRNA were introduced into rat hepatocytes resulting in significantly increased levels of TNF-α mRNA. One of these sense oligonucleotides increased a half-life of TNF-α mRNA, suggesting that the TNF-α asRNA may reduce the stability of TNF-α mRNA.

Conclusion: IL-1β-stimulated rat hepatocytes are a newly identified source of TNF-α in the liver. TNF-α mRNA and asRNA are expressed in rats and humans, and the TNF-α asRNA reduces the stability of the TNF-α mRNA. Hepatocytes and TNF-α asRNA may be therapeutic targets to regulate levels of TNF-α mRNA.

Key words: antisense transcript, cancer, gene expression, inflammation, mRNA stability

INTRODUCTION

TUMOR NECROSIS FACTOR-α (TNF-α), which was first identified as a cytokine leading to necrosis of tumors, is produced by macrophages stimulated by lipopolysaccharide (LPS), an endotoxin component that is derived from Gram-negative bacteria.[1,2] The production of TNF-α and other cytokines, such as interleukin (IL)-1β, IL-6 and interferon (IFN)-γ, is part of the acute-phase inflammatory response to activate endothelial cells and leukocytes.[1,2] TNF-α can be involved in both cell death and cell proliferation. Under pathophysiological conditions, TNF-α plays a pivotal role as a pro-inflammatory cytokine. Dysregulation of TNF-α levels is observed in inflammatory diseases, such as chronic rheumatoid arthritis, sepsis, cachexia in cancer patients,

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osteoporosis and viral hepatitis.[2–5] In rat models of fulminant hepatic failure, induced by treatment with D-galactosamine and LPS or by treatment with LPS after partial hepatectomy, the levels of TNF-α and inducible nitric oxide synthase (iNOS) rapidly increase in the liver.[6,7]

Tumor necrosis factor-α is primarily produced as a type II membrane protein, proteolytic cleavage of which produces a soluble homotrimer consisting of 17-kDa proteins (soluble TNF-α).[1] TNF-α exerts its biological functions via interaction with TNF-α receptors, which are present on various types of cells, including hepatocytes. The TNF-α signal is transduced to the transcription factor, nuclear factor κB (NF-κB), to activate various genes involved in cell death and proliferation, inflammation and cancer.[8] Macrophages and monocytes are the primary sources of TNF-α, although other cells, including lymphocytes, endothelial cells, fibroblasts and neurons, also produce TNF-α. Kupffer cells, macrophages that reside in the liver, have been thought to be the main source of TNF-α secretion from the liver.[9] Although hepatocytes are the most abundant cells in the liver (~60–80%),[10] they have not been shown to produce TNF-α except when chronically infected with hepatitis C virus.[11] In our previous study on rat hepatocytes, we noticed that TNF-α was prominently induced by IL-1β. Thus, we investigated the ability of rat hepatocytes to produce TNF-α. We also examined the *in vivo* expression of the *Tnf* gene in the livers of rats subjected to LPS-induced endotoxemia and also *TNF* expression in human cancer tissues.

Levels of TNF-α are strictly regulated by several gene expression mechanisms. TNF-α mRNA is induced in response to stimuli and then rapidly degraded. First, NF-κB transcriptionally regulates the induction of TNF-α mRNA. Second, stability of the mRNA is regulated by *cis*-control elements located in the 3′-untranslated region (3′UTR): a cluster of AU-rich elements (AREs),[12] the 2-aminopurine-responsive element (APRE),[13] and the constitutive decay element (CDE).[14] ARE motifs are frequently found in the 3′UTR of inducible genes, such as iNOS, inflammatory cytokines and chemokines.[15,16] Third, RNA-binding proteins modulate the stability of TNF-α mRNA by binding to the AU-rich elements (ARE) in the 3′UTR; human homolog R of embryonic lethal-abnormal visual protein (HuR) stabilizes the mRNA,[17,18] and tristetraprolin (TTP), destabilizes the mRNA.[19] Finally, a miRNA miR16 binds to another ARE motif and indirectly interacts with TTP.[20]

Recently, genome-wide transcriptome analyses have reported the presence of many natural antisense transcripts (asRNA).[21] We found that in IL-1β-treated hepatocytes, asRNA are transcribed from the *iNOS* gene, as well as from many other inducible genes, including inflammatory cytokine and chemokine genes.[16,18,22] Furthermore, these asRNA modulate mRNA stability by interacting with the 3′UTR of the corresponding mRNA.[16,18] This unique mechanism of controlling mRNA stability is assumed to be a general post-transcriptional regulatory mechanism.[23,24] Therefore, we predicted and demonstrated the existence of a TNF-α asRNA in hepatocytes and then investigated whether TNF-α asRNA regulates the stability of TNF-α mRNA.

METHODS

Preparation of primary cultures of rat hepatocytes

HEPATOCYTES WERE ISOLATED from the livers of male Wistar rats (Charles River Laboratories Japan, Yokohama, Japan) by collagenase perfusion, as previously described.[25] Briefly, dispersed cells were centrifuged, and the pellet was resuspended in Williams' E medium (Sigma-Aldrich, St Louis, MO, USA) and seeded at 1.2×10^6 cells/dish. The resultant hepatocytes were incubated at 37°C overnight and treated with 1 nM rat IL-1β (PeproTech, Rocky Hill, NJ, USA). The hepatocytes were determined to consist of at least 98% hepatocytes by microscopic observation (data not shown). The animal experiments were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus, and the Animal Care and Use Committee of the Kansai Medical University Animal Center.

THP-1 cells

The human monocyte/macrophage cell line THP-1 (RBRC-RCB1189; Cell Bank, RIKEN BioResource Center, Tsukuba, Japan) was cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) at 37°C. *Escherichia coli* O111:B4 LPS (Wako Pure Chemicals Industries, Osaka, Japan) was added to the medium and incubated for 6 h for RNA extraction.

Experimental models of endotoxemia

Male Wistar rats (150–300 g) were subjected to 70% hepatectomy.[7] LPS in saline (1.5 mg/kg bodyweight) was injected into the penile vein 48 h after the operation. The liver was excised at 1 and 3 h after the injection for RNA extraction. To isolate hepatocytes from a partially hepatectomized rat, the remnant liver was per-

fused by collagenase[25] 1 h after the LPS injection, and hepatocytes were isolated and seeded into dishes. After 4 h incubation in the absence of LPS and IL-1β, total RNA was extracted and analyzed. To detect TNF-α immunoreactivity in the liver, male Sprague–Dawley rats (250–300 g; Charles River Laboratories Japan) were anesthetized with isoflurane, and LPS (50 μg/kg bodyweight) and D-galactosamine (GalN; 500 mg/kg bodyweight) were injected into the penile vein.[6] Liver specimens were taken 1 and 3 h after LPS/GalN treatment for immunohistochemical analysis.

Detection of TNF-α protein

Cultured cells were treated with IL-1β or LPS, and the conditioned medium was analyzed by an enzyme-linked immunosorbent assay (ELISA) using TNF-α Quantikine ELISA Kits (R&D Systems, Minneapolis, MN, USA).

Immunocytochemistry

Hepatocytes treated with IL-1β or LPS were immunolabeled, as previously described.[26] A rabbit antirat TNF-α immunoglobulin G (IgG) and a tetramethylrhodamine isothiocyanate (TRITC)-conjugated porcine antirabbit IgG (Dakopatts, Glostrup, Denmark) were used at 1:400 and 1:40 dilutions, respectively. Visualization was performed with a Fluoview FV300 confocal laser scanning microscope system (Olympus, Tokyo, Japan). TRITC and Nomarski differential interference contrast microscopy images were obtained sequentially through separate channels and merged electronically with computer software.

Immunohistochemistry

Liver specimens taken 3 h after LPS treatment were fixed in 4% formaldehyde and embedded in paraffin. Sections (3–5 μm) were deparaffinized, autoclaved, blocked and incubated with a rabbit antihuman TNF-α IgG (H-156; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 23°C for 2 h. Immunoreactivity was detected with a Histofine Simple Stain Rat MAX-PO kit (Nichirei Bioscience, Tokyo, Japan), which includes polymers with horse radish peroxidase (HRP) and a goat antirabbit IgG, and 3,3′-diaminobenzidine-tetrachloride (DAB) as a substrate. Alternatively, blocked sections were incubated with a HRP-conjugated sheep antirat albumin IgG (Bethyl Laboratories, Montgomery, TX, USA) and stained with DAB. The resultant sections were counterstained with hematoxylin and examined under a Research Biological Digital Microscope BA210EINT (Shimadzu Rika, Tokyo, Japan).

Northern blot analysis

RNA was resolved by agarose gels, blotted onto Nytran N membrane (Whatman, Brentford, UK), and hybridized with a sense or antisense TNF-α probe at 60°C overnight.[18] To prepare a single-stranded sense probe, four copies of the *Tnf* gene fragment (nucleotides 832–993) were cloned to a pMNT vector[27] and *in vitro* transcribed with SP6 RNA polymerase (Takara Bio, Otsu, Japan) and [α-^{32}P]CTP (111 TBq/mM; PerkinElmer, Waltham, MI, USA). Similarly, to prepare single-stranded antisense probe, four copies of the *Tnf* gene fragment (nucleotides 632–831) were cloned and transcribed with T7 RNA polymerase (Takara Bio) and [α-^{32}P]CTP. Membranes were washed at 50°C and visualized by autoradiography.

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was prepared from culture cells using Sepasol I Super G (Nacalai Tesque, Kyoto, Japan). Human cancer tissues from surgical specimens and the livers of partially hepatectomized rats were dissected and soaked in RNA-later (Applied Biosystems, Austin, TX, USA), and total RNA was prepared with RNAquaous kits (Applied Biosystems) and TURBO DNA-free kits (Applied Biosystems). cDNA was produced by strand-specific RT using an oligo(dT) primer for mRNA or a sense primer for asRNA.[18] Step-down PCR[18,28] was performed with paired primers (Table 1), and the products were resolved by agarose gel electrophoresis. mRNA levels were estimated in triplicate by real-time PCR with SYBR Green I and the Thermal Cycler Dice Real Time System (Takara Bio).[18] The values obtained were normalized to elongation factor-1α (EF) mRNA. The amplified DNA was confirmed by sequencing. These sequence data have been submitted to the DNA Data Bank of Japan/European Bioinformatics Institute/GenBank databases under accession numbers AB553578, AB553579, AB671197 and AB675034. All the procedures involving human subjects and materials were approved by the Ethics Review Board of Kansai Medical University and the Ethics Review Board of Ritsumeikan University, Biwako-Kusatsu Campus. Written informed consent was obtained from each subject.

Prediction of secondary structure and design of sense oligonucleotides

Secondary structures were predicted using the mfold program.[29] The widely conserved regions designated as domains A to E in the 3′UTR of the TNF-α mRNA each

Table 1 Primers used for strand-specific RT–PCR in this study

Transcript	Sequence (5′→3′)	RT/PCR†	Direction	cDNA (bp)‡
Rat:				
TNF-α mRNA	TCCCAACAAGGAGGAGAAGTTCC	PCR	Forward	275
	GGCAGCCTTGTCCCTTGAAGAGA	PCR	Reverse	
TNF-α asRNA	GCGACTGGCGTGTTCATCCGTTC	RT	Forward	
	ATTTGGTGACCAGGCTGTCGCTA	PCR	Forward	307
	GCAAAGGTAATTAGGCTTAGGCTC	PCR	Reverse	
Human:				
TNF-α mRNA	CAGAGGGAAGAGTTCCCCAGGGA	PCR	Forward	249
	CTTGAAGAGGACCTGGGAGTAGA	PCR	Reverse	
TNF-α asRNA	ACGAACATCCAACCTTCCCA	RT	Forward	
	TGAATGTATTTATTTGGGAGACCGG	PCR	Forward	274
	TCGCCACTGAATAGTAGGGCGAT	PCR	Reverse	
Rat and human:				
EF mRNA	TCTGGTTGGAATGGTGACAACATGC	PCR	Forward	335
	CCAGGAAGAGCTTCACTCAAAGCTT	PCR	Reverse	

†Gene-specific sense primers used for reverse transcription (RT) to synthesize cDNA to the antisense transcript (asRNA). An oligo(dT) primer used to synthesize cDNA for each mRNA. Primer pairs used for polymerase chain reaction (PCR).
‡The size of the cDNA fragment amplified by each pair of PCR primers is shown in base pairs (bp).
asRNA, antisense RNA; EF, elongation factor 1α; TNF-α, tumor necrosis factor-α.

include at least one stem-loop structure. Oligonucleotides blocked by phosphorothioate bonds (GeneDesign, Ibaraki, Japan) were designed according to a published method.[16,18] The sequences SeA1 to SeE1, which correspond to domains A to E, respectively, are as follows (an asterisk indicates a phosphorothioate bond): SeA1, A*G*A*TGTCTCAGGCCTCC*C*T*T; SeB1, G*G*A*ACCCCCTATATTTA*T*A*A; SeB2, A*T*T*TGCTTATGAATGTA*T*T*T; SeC1, A*A*C*ATGTTTTCTGTGAA*A*A*C; SeD1, A*A*C*AAGATATTTATCTA*A*C*C; SeE1, A*C*T*GAACCTCTGCTCCC*C*A*C; and random oligonucleotides (negative control), N*N*N*NNNNNNNNNNNNN*N*N*N.

Transfection of hepatocytes

Duplicate samples of hepatocytes (3.0×10^5 cells/dish) were transfected with oligonucleotide (1.5 μg) using MATra A Reagent (1.5 μL; IBA, Göttingen, Germany).[18] The cells were cultured overnight and then treated with IL-1β.

Statistical analysis

The results in the figures are representative of at least three independent experiments yielding similar findings. Values are represented as the means ± standard deviations (SD). Differences were analyzed by Student's *t*-test. Statistical significance was set at $P < 0.05$ and $P < 0.01$.

RESULTS

Hepatocytes secrete TNF-α in response to IL-1β

WE FIRST EXAMINED TNF-α secretion in primary cultures of rat hepatocytes. The cells were treated with IL-1β or LPS, and the level of TNF-α in the medium was measured. As shown in Figure 1(a), TNF-α levels were significantly increased in the presence of IL-1β. In contrast, when LPS was added, TNF-α levels did not increase. Other substances, such as TNF-α itself, IFN-γ and IL-6 did not induce TNF-α secretion (Fig. 1a). These results suggest that the hepatocytes secreted TNF-α specifically in response to IL-1β.

Next, we directly examined TNF-α production in hepatocytes by immunocytochemistry using an anti-TNF-α antibody (Fig. 1b). When IL-1β was added, TNF-α protein was detected (panel D), and the merged image revealed that the TNF-α signal was localized in the cytoplasm (F). Rat hepatocytes are very large and often binuclear[30] and no cell types apart from hepatocytes were stained. In contrast, no TNF-α signal was observed in hepatocytes treated with LPS (G-I), although TNF-α signals were localized in small, elongated cells (most likely Kupffer cells). These data confirmed that the TNF-α protein was exclusively localized in the cytoplasm of IL-1β-stimulated hepatocytes.

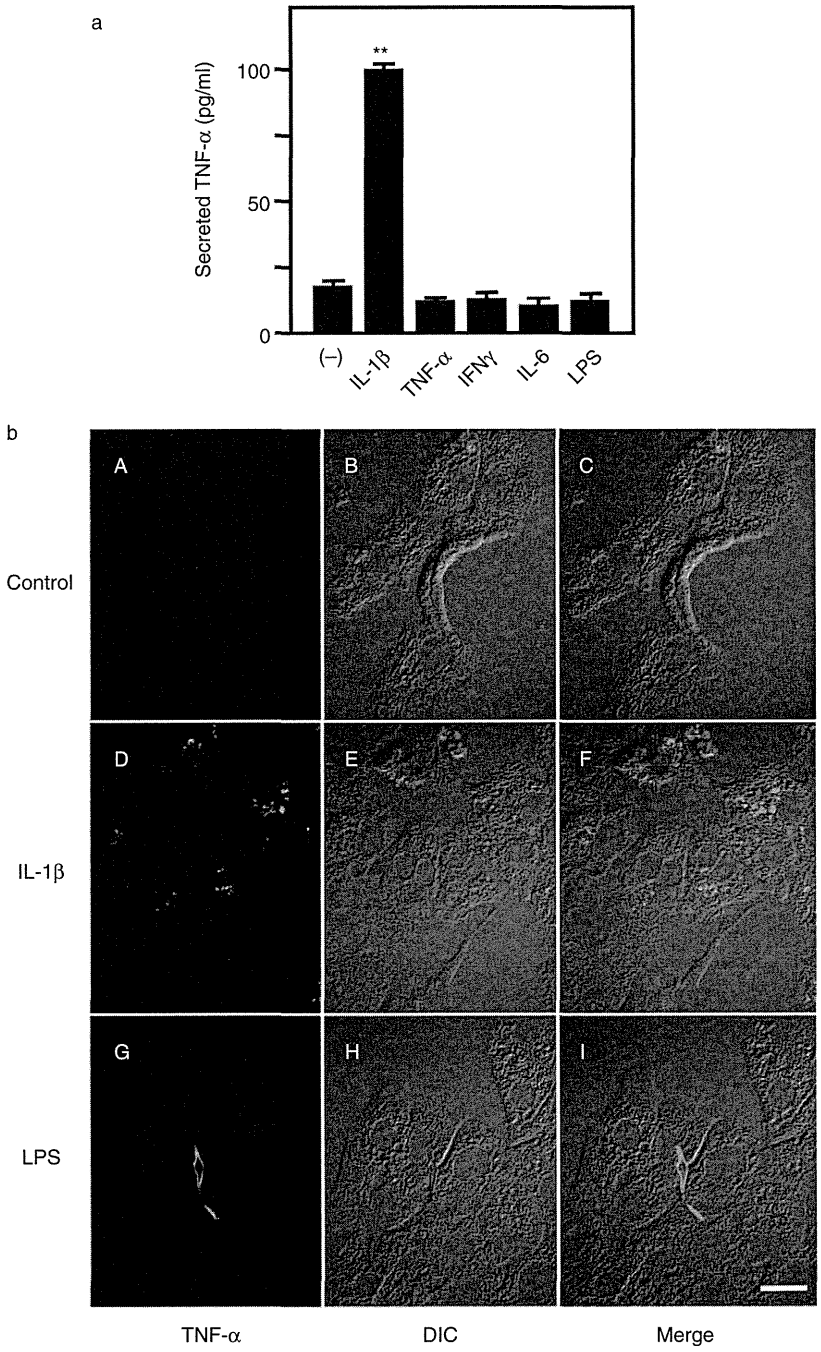

Figure 1 Rat hepatocytes secrete TNF-α in response to IL-1β. (a) Rat hepatocytes secrete TNF-α. Hepatocytes were incubated in the presence of IL-1β (1 nM), TNF-α (1 nM), IFN-γ (500 U/mL), IL-6 (1 nM) or LPS (0.5 μg/mL) for 6 h. The TNF-α secreted into the medium was measured by ELISA (mean ± SD). ** $P < 0.01$ versus untreated control (–). (b) IL-1β-treated hepatocytes produce TNF-α. Rat hepatocytes were incubated in the absence (controls; panels A–C) or presence of IL-1β (D–F) or LPS (G–I) for 6 h to monitor TNF-α expression. The cells were fixed and stained for TNF-α by immunofluorescence (A, D, G). The corresponding differential interference contrast (DIC) microscopy images are shown (B, E, H). The TNF-α signals and DIC images were merged (C, F, I). Hepatocytes, which are large and often binuclear (C, F, I), and small, elongated Kupffer cells (C, F, I) were observed. No signals were obtained when the cells were treated with the secondary antibody alone (A). Scale bar, 10 μm. ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; SD, standard deviation; TNF-α, tumor necrosis factor-α.

Induction of TNF-α in hepatocytes

We then characterized *Tnf* expression in the hepatocytes. As the concentration of IL-1β increased, the secretion of TNF-α into the medium increased in a dose-dependent manner (Fig. 2a). Real-time PCR showed that the level of TNF-α mRNA also increased in a dose-dependent manner (Fig. 2b). We then examined the time course of

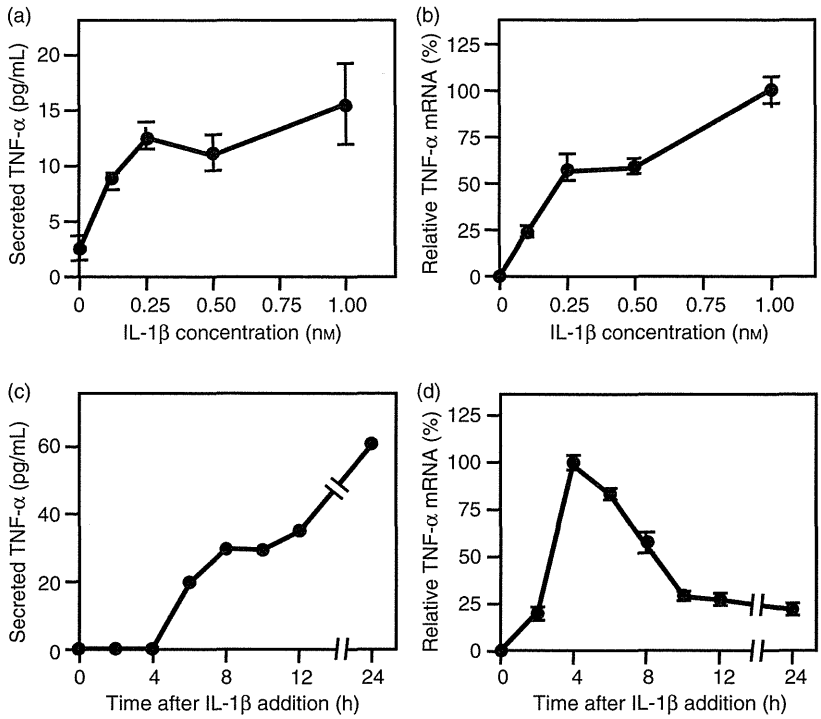

Figure 2 Expression of TNF-α in IL-1β-treated hepatocytes. (a) Dose-dependent TNF-α secretion from rat hepatocytes. Hepatocytes were treated with various concentrations of IL-1β for 6 h, and the TNF-α levels in the medium (mean ± SD) were measured by ELISA. (b) Dose-dependent expression of TNF-α mRNA. Hepatocytes were treated with various concentrations of IL-1β for 4 h, and total RNA was prepared. The levels of TNF-α and EF mRNA were measured by RT and real-time PCR. The relative amounts of TNF-α mRNA normalized to EF mRNA (internal control) are expressed as the mean ± SD, shown as a percentage. The normalized value at 1 nM was set at 100%. (c) The time course of TNF-α secretion by hepatocytes. Hepatocytes were treated with 1 nM IL-1β, and the TNF-α levels in the medium were measured by ELISA. (d) The time course of TNF-α mRNA expression. Hepatocytes were treated with 1 nM IL-1β, and total RNA was subjected to real-time RT–PCR. The relative amounts of TNF-α mRNA normalized to EF mRNA are expressed as the mean ± SD, shown as a percentage. The normalized value at 4 h was set at 100%. EF, elongation factor 1α; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; PCR, polymerase chain reaction; RT, reverse transcription; SD, standard deviation; TNF-α, tumor necrosis factor-α.

TNF-α induction in IL-1β-treated hepatocytes. The level of TNF-α protein in the medium increased markedly, starting 6 h after IL-1β addition (Fig. 2c), whereas the level of TNF-α mRNA increased 2 h after IL-1β addition, peaked at 4 h, and decreased thereafter (Fig. 2d). These data suggest that the induction of the *Tnf* gene in response to IL-1β occurs with rapid synthesis and degradation of TNF-α mRNA.

TNF-α asRNA is expressed in rat hepatocytes

We have previously shown that asRNA are transcribed from several inducible genes in rat hepatocytes;[16,18] therefore, we speculated that an asRNA complementary to the 3′UTR of TNF-α mRNA might be also transcribed. To test this hypothesis, we performed northern blot analysis with a TNF-α sense probe (Fig. 3a). This probe corresponded to an asRNA-specific region in the 3′UTR of TNF-α mRNA (Fig. 3b). A 2.5-kb TNF-α asRNA was detected in hepatocyte poly(A)$^+$ RNA (isolated from cells treated with IL-1β) but not in poly(A)$^-$ RNA.

To measure the levels of TNF-α asRNA, we designed a set of primers corresponding to the 3′UTR to detect TNF-α asRNA by strand-specific RT–PCR (Fig. 3b). When a sense primer to TNF-α mRNA (i.e. a primer in the forward direction) was used for RT, only cDNA encoding TNF-α asRNA would be amplified by PCR. The

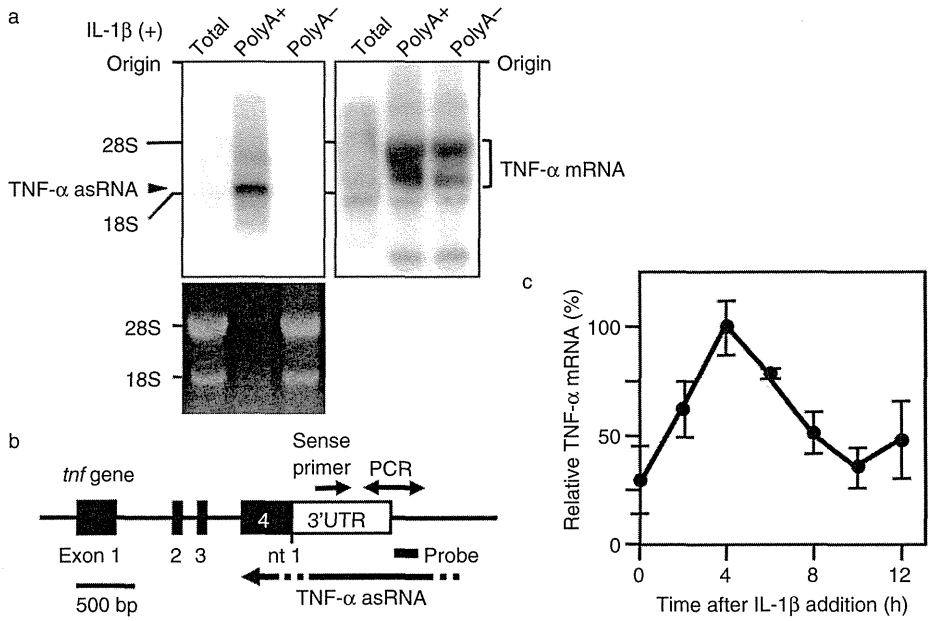

Figure 3 TNF-α asRNA expressed in rat hepatocytes. (a) Northern blot analysis of hepatocyte RNA. Total and poly(A)$^-$ RNA (10 μg/lane) and poly(A)$^+$ RNA (2.5 μg/lane) from IL-1β-treated hepatocytes were analyzed. Agarose gel stained by ethidium bromide is shown beneath the autoradiogram. The arrowhead indicates TNF-α asRNA detected by the sense probe (bold line in b), which corresponded to a region in the 3'UTR. The bracket indicates TNF-α mRNA detected by the antisense probe. 28S and 18S show positions of rRNA bands. (b) Structure of the rat *Tnf* gene. TNF-α asRNA (a large arrow) includes the region corresponding to the 3'UTR of TNF-α mRNA. RT was strand-specifically primed by a sense primer, and the resultant cDNA was amplified by a pair of PCR primers (two-headed arrow). The sense probe to detect the TNF-α asRNA in northern blot analysis is indicated by a bold line. (c) The expression pattern of TNF-α asRNA in rat hepatocytes. At each time point during the incubation with IL-1β, total RNA was analyzed by real-time RT–PCR. The expression levels of TNF-α asRNA are expressed as a percentage (mean ± SD). 3'UTR, 3'-untranslated region; asRNA, antisense RNA; EF, elongation factor 1α; IL, interleukin; nt, nucleotide; PCR, polymerase chain reaction; RT, reverse transcription; SD, standard deviation; TNF-α, tumor necrosis factor-α.

strand-specific RT and real-time PCR showed the induction of TNF-α asRNA with a peak at 4 h (Fig. 3c).

Rat TNF-α asRNA is expressed *in vivo*

To investigate the *in vivo* expression of TNF-α asRNA, we analyzed rats subjected to LPS-induced endotoxemia. The rats were partially hepatectomized, and LPS was administrated 48 h after the operation. As shown in Figure 4(a), experimental endotoxemia induced both TNF-α mRNA and asRNA in the liver. The levels of TNF-α mRNA and asRNA peaked 1 h after the LPS injection, which correlates with the observation that the TNF-α protein levels in the liver reached a maximum 1 h after LPS administration to partially hepatectomized rats.[7] To confirm that hepatocytes expressed TNF-α asRNA, hepatocytes were separated from the remnant liver of the rat 1 h after LPS treatment, seeded into dishes, and incubated for 4 h in the absence of LPS and IL-1β. RT–PCR analyses revealed that both TNF-α mRNA and asRNA was expressed in these hepatocytes (Fig. 4b), suggesting that expression of the *Tnf* gene was induced in hepatocytes from the liver of LPS-treated rats.

Furthermore, we performed immunohistochemical analyses of the livers from LPS-treated rats. Rats were administrated LPS and GalN, which are another popular model of experimental endotoxemia,[6] and the livers were immunostained by an anti-TNF-α antibody (Fig. 4c). TNF-α immunoreactivity of the liver specimen taken 3 h after LPS/GalN injection (panels A, B) was higher than that taken at 1 h (C) and located in the large cells, namely, hepatocytes (B). Hepatocytes were identified by albumin immunoreactivity (D). These results confirm the *in vivo* expression of TNF-α protein in hepatocytes of LPS-treated rats.